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THE DETERMINATION OF THE FATTY ACID COMPOSITION OF SERUM LIPIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY; AND A COMPARISON WITH COLUMN CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of fatty acid patterns of individual phospholipids (*viz.* lecithin, lysolecithin and sphingomyelin) and neutral lipids (*viz.* sterol esters, free fatty acids and triglycerides) of serum by gas–liquid chromatography, after their separation by thin-layer chromatography. Replicate determinations indicate that the method gives highly reproducible results, and there is no evidence of loss of unsaturated fatty acids by oxidation.

In a comparison between thin-layer and column chromatographic methods, values for the fatty acid composition of cholesterol esters, triglycerides and free fatty acids were in good agreement. With phospholipids agreement for values of lecithin and lysolecithin was moderately good but with sphingomyelin agreement was poor.

The thin-layer method has the advantage of being much faster and requiring smaller quantities of lipid for analysis.

INTRODUCTION

Separation of the major classes of lipids by column chromatography has been successfully achieved using silicic acid. Subsequently the fractions could be analysed for their fatty acid content using gas–liquid chromatography. With phospholipids complete separation could not be achieved, but by using differential hydrolysis it was possible to obtain the fatty acid patterns of the individual fractions.

The disadvantages of column chromatography are well known. The amount of sample has often to be quite large and the procedure is time-consuming and expensive. With the introduction of thin-layer chromatography, a method was available for the separation of small quantities of lipids in a short time. In principle there seemed no reason why such separated lipids could not be analysed for their fatty acid patterns by gas–liquid chromatography. One possible disadvantage might be that a larger surface of lipid would be exposed to air and that oxidation of the fatty acids might occur. Before applying such a method it was therefore felt necessary to determine if such oxidation would occur and whether values obtained were reproducible and comparable with those obtained using column chromatography.

METHODS AND MATERIALS

Reagents and glassware

In view of the small amount of material which may be handled by the thin-layer chromatography method it is very important to prevent contamination of the samples of methyl esters with material from solvents and from glassware. All of the glassware used for the preparation of methyl esters is kept separate from routine laboratory glassware and is washed after use with chromic acid cleaning solution (30 ml saturated sodium dichromate in 1 litre of concentrated sulphuric acid). All reagents are analytical grade and except for sulphuric acid, are redistilled from acid-washed glassware. It is desirable to do routine checks on contamination by running a blank with a series of samples.

The preparation of extracts

Extraction of tissue is carried out by using the technique of FOLCH *et al.*[1]. For serum, 20 ml of serum is added to 200 ml of chloroform–methanol (2:1, v/v) and allowed to stand, with occasional shaking, at room temperature for 0.5 h. After filtration 0.2 volume of sodium chloride (0.7 %, w/v) are added to the extract and the mixture allowed to stand overnight at 4° or alternatively centrifuged at 4°. When the two phases have separated the upper aqueous phase is discarded and the lower chloroform phase evaporated to dryness *in vacuo* at 40° on a rotary evaporator. Residual water is removed by repeated addition of small amounts of methanol and subsequent evaporation on the rotary evaporator. In order to avoid oxidation, the rotary evaporator is always vented to pure nitrogen and the extract is transferred from the evaporator flask to a storage vial with a small quantity of chloroform–methanol (2:1, v/v), containing hydroquinone (10 mg/100 ml) as antioxidant, and stored under nitrogen at −20°. After evaporation to dryness some material, presumably denatured lipoprotein, fails to redissolve in the chloroform–methanol; this is washed with a small volume of solvent in order to extract traces of lipid.

Preparation of thin-layer plates

Thin-layer plates are prepared with the Desaga applicator using silicic acid by the method of VOGEL *et al.*[2]. Mallinckrodt silicic acid for chromatography (100 mesh) is sieved to give a material of 200 mesh, the coarse material being discarded. This fine material is extracted overnight in a Soxlet with chloroform–methanol (2:1, v/v) in order to remove contaminating lipid. 30 g of the dried extracted silicic acid are ground in a mortar with 600 mg of dental plaster of Paris, 50 ml of water are added and the mixture slurried. The slurry is applied quickly to the plates before the plaster sets. The plates are allowed to dry in air for a few minutes and then activated by heating at 100° for 2 h. Plates which have been stored in a desiccator are found to be satisfactory.

Processing of the chromatograms

In order to obtain sufficient material for preparation of methyl esters for gas–liquid chromatography, approx. 5 mg of phospholipid or neutral lipid must be applied to the plate for separation. With 300-μ thick plates, this amount of material (obtainable from approx. 5 ml serum) may be loaded on to the plate as a streak approx.

10 cm wide. A spot of serum extract and a spot of a marker compound are also applied to the plate in order to aid identification of components. The extract of 5 ml of serum made up in 0.2 ml of solvent gives approximately 3% solution of phospholipids. This is a convenient volume for streaking across the plate by means of a 1-ml tuberculin syringe. For phospholipid separations, a convenient marker solution is a 3% solution of egg-yolk phosphatides in chloroform–methanol (2:1, v/v) prepared by the method of HANAHAN *et al.*[3]. A convenient marker for the neutral lipids contains the following components (with a final concentration of each of 20 mg/ml): cholesterol, 1,2- or 1,3-diglyceride, stearic acid, glyceryl tristearate, methyl stearate and cholesterol. In order to prevent lateral movement of the solvent on the plate, lines are drawn along the length of the plate to enclose lanes as follows: two lanes 3 cm wide for the markers and three lanes each 5 cm wide for the sample streak. A line is drawn across the top of the plate in order to stop the solvent front and the samples are put on to the plate at the origin 2 cm from the bottom as follows: Lane 1, 5 μl of a 3% (v/v) solution of marker; Lane 2, 5 μl of a 3% (v/v) solution of the serum extract; Lane 3, a streak of serum extract.

The chromatograms are developed by ascending solvent in tanks lined by absorbent paper. For phospholipid separation the chromatogram is developed in chloroform–methanol–water (80:25:2, v/v). For separation of neutral lipids, a

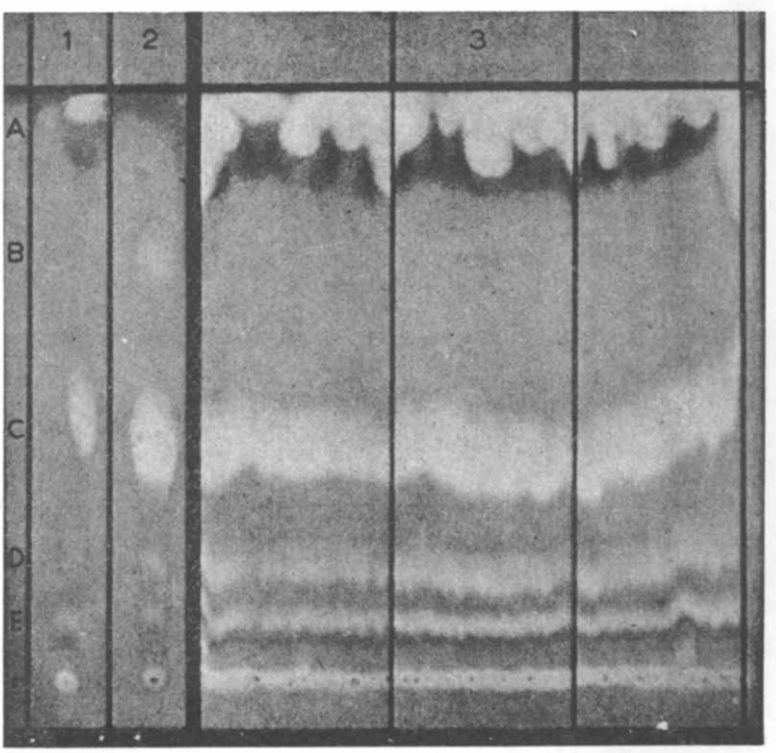

Fig. 1. Phospholipids separated by thin-layer chromatography using chloroform–methanol–water (80:25:3 v/v). Column 1, 5 μl 3% (v/v) serum lipids; Column 2, 5 μl 3% (v/v) egg-yolk phospholipids; Column 3, streak of serum lipids. Components: A, neutral lipids B, cephalin; C, lecithin; D, sphingomyelin; E, lysolecithin; F, origin.

mixture of light petroleum (b.p. 40–60°), diethyl ether and acetic acid is used. For optimum separation of the cholesterol esters from triglycerides, light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (90:10:1, v/v) is used, whilst for optimum separation of the 1,2-diglycerides, 1,3-diglycerides and monoglycerides, light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (60:40:1, v/v) is used.

When the chromatogram has been developed, it is removed from the tank, allowed to dry in air for 5 min, and then sprayed with a 0.2 % solution of 2′,7′-dichlorofluorescein in ethanol. Using ultraviolet light, the position of the lipids may be located by the yellow fluorescence (see Figs. 1 and 2). The areas of silicic acid containing the lipid can be removed by scraping with a spatula. This material is used in the preparation of the methyl esters of the fatty acids as below.

Methylation of the separated components

The lipids are transmethylated in the presence of silicic acid from the plate by refluxing with 10 % (w/v) sulphuric acid in dry methanol for 1 h at 80° except for sphingomyelin which is refluxed for 16 h. A crystal of hydroquinone, as an antioxidant, and carborundum chips, to prevent bumping, are added to each sample. After refluxing, 20 ml of water is added to the sample and the methyl esters of the

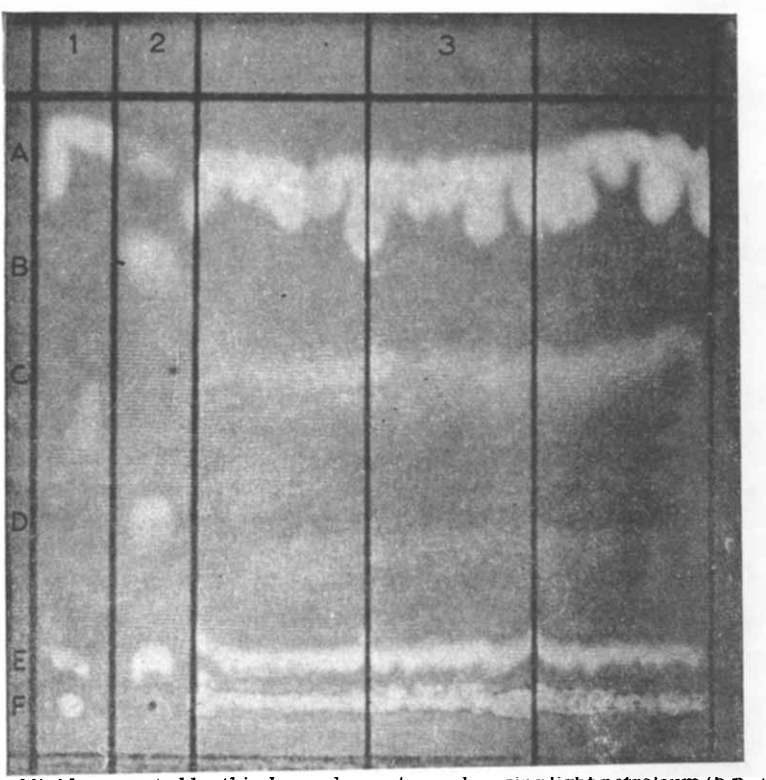

Fig. 2. Neutral lipids separated by thin-layer chromatography using light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (90:10:1, v/v). Column 1, 5 μl 2 % (v/v) solution of serum lipids; Column 2, 5 μl 2 % (v/v) marker solution; Column 3, streak of serum lipids. Components: A, cholesterol esters; B, methyl esters of fatty acids; C, triglycerides; D, free fatty acids; E, cholesterol, diglycerides and monoglycerides; F, origin.

fatty acids are extracted twice with 10 ml of light petroleum (b.p. 40–60°). The extracts are washed with water and dried over a mixture of anhydrous sodium sulphate–sodium bicarbonate (4:1, w/w). The extract is evaporated *in vacuo* to a small volume and stored under nitrogen at —20° until required for gas–liquid chromatography.

Gas–liquid chromatography

The Pye argon chromatograph with columns of 10 % polyethylene glycol adipate on Celite at 190° is used for the identification of the methyl esters of the fatty acids. Because of the small sample size, the following technique is used for the introduction of a sample to the column. The sample is made up in a small volume of light petroleum (b.p. 40–60°) and an appropriate amount is put on to a sample holder which consists of a thin-walled steel tube, 3 mm diameter and 5 mm long packed with glass yarn. The solvent is allowed to evaporate and the holder containing the methyl esters is dropped on to the column. This technique avoids the appearance on the chromatogram of a large solvent peak and therefore permits easier measurement of the early peaks. The gas flow is started 1 min after the introduction of the holder in order to allow for the DANIEL's effect[4].

The amounts of methyl esters are expressed as a percentage of the total after measurement of the peak areas by multiplication of the peak height and the peak width at half the peak height.

Separation of lipids by column chromatography

The above method was compared with the standard separation procedure using column chromatography. The neutral lipids and phospholipids of serum were separated by the method of BOTTCHER *et al.*[5]. The phospholipids were further separated according to BOTTCHER AND VAN GENT[6] by a stepwise elution on silicic acid involving the following solvents[7]: (1) 100 ml chloroform, neutral lipids eluted. (2) 200 ml methanol–chloroform (1:4, v/v), cephalins eluted. (3) 350 ml methanol–chloroform (3:2, v/v), lecithin and sphingomyelin eluted. (4) 100 ml methanol–chloroform–water (80:16:4, v/v), sphingomyelin and lysolecithin eluted. The fractions were taken almost to dryness in a rotary evaporator at 40° and differential hydrolysis was applied before each fraction was methylated. Gas–liquid chromatography was carried out as described above except that the methyl esters were applied to the top of the column using capillary pipettes.

Specimens examined

Pig serum: Specimens of serum were obtained from three pigs which had been fed on three different dietary levels of linoleic acid (0.1, 0.5, 0.9 % of dietary calories) from weaning until slaughter at 200 lbs live weight. Full details of these have already been published elsewhere[8].

Standard compounds

Cholesterol, stearic acid, glyceryl tristearate, methyl stearate, and cholesteryl palmitate were commercial samples (B.B.H., Poole (Great Britain)). The 1,3- and 1,2-triglycerides were synthetic materials (Dr. CHAPMAN, University Chemical Laboratory, Cambridge). Sphingomyelin (Lights, Colnbrook (Great Britain)) gave a

single spot on thin-layer chromatography and was stated by the manufacturers to be pure as judged by its infrared spectrum.

RESULTS AND DISCUSSION

Reproducibility of method

The reproducibility of the method described above has been tested by replicate analysis of one sample. Two aliquots of pig serum (20 ml) were extracted as described and the fatty acid patterns of the neutral lipids and phospholipids determined after separation on different plates run under identical conditions. Table I shows the mean and standard error of the mean of four separate determinations. The results indicate the method has a very good reproducibility. The chief error is in the gas–liquid chromatographic method which in our hands has a consistent error of 5 % for any fatty acid. Table II shows the results of four analyses by gas–liquid chromatography of a homogeneous sample of methyl esters prepared from pure sphingomyelin. Many recently published papers contain no reference to the accuracy of the gas–liquid

TABLE I

THE FATTY ACID COMPOSITION OF PIG-SERUM LIPIDS SEPARATED IN QUADRUPLICATE BY THIN-LAYER CHROMATOGRAPHY AND ESTIMATED BY GAS–LIQUID CHROMATOGRAPHY

*Acids**	*Sterol esters***	*Triglycerides*	*Free fatty acids*	*Lecithin*	*Lysolecithin*	*Sphingomyelin*
16:0	8.4 ± 0.3	14.9 ± 0.6	30.3 ± 1.0	21.1 ± 1.1	34.8 ± 0.9	30.0 ± 1.9
16:1	5.4 ± 0.6	4.6 ± 0.1	4.6 ± 0.7	1.4 ± 0.1	2.7 ± 0.3	2.0 ± 0.4
18:0	1.2 ± 0.2	4.2 ± 0.8	12.2 ± 0.8	28.2 ± 2.9	26.3 ± 0.8	11.4 ± 0.6
18:1	53.4 ± 0.8	60.8 ± 1.4	31.2 ± 0.7	26.1 ± 1.1	17.7 ± 1.1	4.8 ± 0.6
18:2	25.1 ± 0.6	2.9 ± 0.1	2.3 ± 0.2	8.1 ± 0.7	3.0 ± 0.3	1.8 ± 0.2
20:0	Trace	0	0.7	0	0.3	8.3 ± 0.1
20:3	1.3 ± 0.3	1.9 ± 0.1	4.2 ± 0.6	5.2 ± 0.2	2.2 ± 0.2	2.8 ± 0.4
20:4	2.4 ± 0.1	Trace	1.1 ± 0.2	5.2 ± 0.3	2.9 ± 0.4	2.6 ± 0.4
22:0	0	0	0	0	0	5.8 ± 1.0
23:0	0	0	0	0	0	5.7 ± 1.3
24:0	0	0	0	Trace	0	7.9 ± 1.6
24:1	0	0	0	Trace	0	7.3 ± 0.3

* The figure before colon denotes chain length; that after it the number of double bonds.
** Three determinations only.

TABLE II

FATTY ACID PATTERNS OF PURE SPHINGOMYELIN*

	Acids											
	16:0	16:1	17:0	17:1	18:0	19:0	20:0	21:0	22:0	23:0	24:0	24:1
Mean (%)	2.2	0.3	0.3	0.2	37.9	0.3	1.1	0.3	6.8	6.8	22.5	21.4
Standard error of the mean	± 0.1	—	—	—	± 0.5	—	—	—	± 0.2	± 0.2	± 0.2	± 0.6

* Mean of four determinations.

TABLE III

FATTY ACID COMPOSITION OF STEROL ESTERS AND TRIGLYCERIDES FROM THREE DIFFERENT SAMPLES OF PIG SERA OBTAINED BY THE COLUMN METHOD AND THIN-LAYER METHOD

C, column method; T, thin-layer method.

Acids	*Sterolesters*						*Triglycerides*					
	Pig 1		*Pig 4*		*Pig 5*		*Pig 1*		*Pig 4*		*Pig 5*	
	C	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*
16:0	7.8	8.5	8.4	8.4	8.4	6.8	14.4	17.2	14.2	14.9	15.9	19.9
16:1	6.1	8.2	3.8	5.4	2.7	2.3	4.5	5.7	4.5	4.6	3.2	5.4
18:0	2.3	1.4	2.5	1.2	1.8	1.6	5.6	3.7	6.0	4.2	6.1	7.2
18:1	58.3	58.2	51.8	53.4	50.0	49.6	57.8	62.2	61.4	60.8	58.1	51.9
18:2	15.6	13.7	25.3	25.1	29.9	27.6	2.6	Trace*	4.5	2.9	6.2	4.2

* Trace.

chromatography method employed and the expression of results to one decimal place would seem to be optimistic. Experience has shown that greater errors can be introduced if the following points are not observed. (a) Peaks of reasonable width and height must be presented on the chromatogram by suitable choice of chart paper speed and sensitivity. (b) Chromatograms with asymmetric peaks and peaks off the paper, which can only be measured by extrapolation must be rejected.

Comparison of the column and thin-layer method

A comparison of the percentage of fatty acid composition of the neutral lipids (Table III) and phospholipids (Table IV) of three pig-serum samples was made, using the thin-layer method and the column method of BOTTCHER *et al.*[5,6]. The agreement for neutral lipids is excellent, and fair for lecithin and lysolecithin. In the case of sphingomyelin, the thin-layer method gives a consistently higher value for the percentage of palmitic acid. The different figures obtained between the two methods may be due to the fact that the sphingomyelin isolated in the column separation is further purified by alkaline hydrolysis, whilst that isolated by the plate separation may be a mixture of alkali stable and labile materials which run together under the conditions of the thin-layer separation. Further characterisation by the thin-layer technique is being attempted and recent studies on pure sphingomyelin have shown the composition of methyl esters obtained by the transmethylation procedure described above is varied and the conditions for methylation may be quite critical. Furthermore, samples of methyl esters from sphingomyelin show a gradual quantitative conversion of 24:1 to 24:0 fatty acids during storage at —20° under nitrogen. These are problems, however, which are associated with the preparation of methyl esters in the absence of silicic acid and are quite independent of the thin-layer method for separation.

The accuracy of the column separation method is unknown since only one determination of the fatty acid pattern is available. Clean separation of the phospholipids by the column method is very difficult and contamination of one fraction by another is possible. In contrast, separation of phospholipids by the thin-layer method is excellent. This may explain why the agreement in the case of phospholipid fatty

TABLE IV

FATTY ACID COMPOSITION OF THE PHOSPHOLIPIDS FROM THREE DIFFERENT SAMPLES OF PIG SERA, OBTAINED BY THE COLUMN METHOD AND THIN-LAYER METHOD

C, column method; T, thin-layer method.

Acids	*Lecithin*						*Lysolecithin*						*Sphingomyelin*					
	Pig 1		*Pig 4*		*Pig 5*		*Pig 1*		*Pig 4*		*Pig 5*		*Pig 1*		*Pig 4*		*Pig 5*	
	C	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*	*C*	*T*
16:0	16.8	16.7	17.0	21.1	18.6	18.1	31.9	23.0	32.7	34.8	30.7	44.2	17.6	37.7	14.9	30.0	28.5	35.7
16:1	2.1	2.1	1.3	1.4	0.9	1.4	3.2	1.1	2.9	2.7	3.8	1.9	2.4	0	0.9	2.0	0	0.7
18:0	21.7	17.8	24.7	28.2	18.5	18.8	24.5	25.8	29.2	26.3	25.8	25.5	10.4	12.6	9.0	11.4	12.2	13.9
18:1	32.2	35.4	28.6	26.1	28.4	37.3	26.7	31.6	24.0	17.7	22.7	16.7	8.0	6.1	3.3	4.8	5.5	9.9
18:2	6.6	7.1	9.3	8.1	11.2	9.7	4.6	4.9	3.1	3.0	3.6	2.8	1.6	2.4	1.2	1.8	1.6	3.2
20:3 + 21:0	8.9	9.8	5.6	5.2	3.9	2.5	3.8	7.2	1.9	2.2	0.9	1.4	2.0	2.3	1.3	2.8	1.5	1.0
20:4	3.4	3.5	6.3	5.2	9.7	8.1	1.0	2.2	1.8	2.9	1.7	1.7	1.0	1.0	0.7	2.6	1.4	2.1
23:0	0.2	0.3	0.2	0	0	0	0	0	0	0	0	0	8.2	4.0	11.9	5.7	5.2	3.6
24:0	0	1.2	0	0	0	0	0	0	0	0	0	0	17.2	11.5	20.4	7.9	7.8	4.5
24:1	1.3	0.5	1.0	0	1.9	0	0	0	0	0	0	0	12.2	10.1	11.5	7.3	13.3	9.4

acid patterns is only fair. Where the column separation of two lipids is sharp as is the case with sterol esters and triglycerides, the values obtained by the column and thin-layer methods are in good agreement.

Applications

The method has been used successfully for materials other than serum *e.g.* lymph nodes, aortae, and lipomas. Where small amounts of material are available, the neutral lipid area can be eluted from the silicic acid of the plate used in separating phospholipids. This material is then applied to a second plate and the neutral lipids separated. By this means the fatty acid composition of the individual phospholipids and neutral lipids from as little as 5 mg of lipid may be determined.

CONCLUSIONS

The advantages of the thin-layer method for separation of lipids is well known and much saving of time and materials can be achieved by its use. Furthermore, the amounts of sample required are small and the method may have important applications in the field of atherosclerosis where the availability of lipids is limited, such as is the case in the normal arterial wall.

No evidence was obtained that the conditions of separation led to oxidation of the poly unsaturated fatty acids since replicate values for linoleic and arachidonic acid compare closely with those obtained after column separation. Indeed the quickness of separation would seem to recommend it, since the time in which oxidation can occur is limited.

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