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TWO-DIMENSIONAL REACTION THIN-LAYER CHROMATOGRAPHY IN THE ANALYSIS OF MIXTURES OF ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES

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SUMMARY

Micro-samples of mixtures of alkenyl acyl-, alkyl acyl- and diacyl choline phosphatides, after their conversion into three families of diglyceride acetates by subjecting them to phospholipase C action followed by acetylation, were analyzed by a two-dimensional reaction thin-layer chromatographic procedure which permitted a quantitative determination of the three analogs, their individual fatty acid compositions and fatty aldehyde composition of the alkenyl acyl analog.

INTRODUCTION

Because alkenyl acyl-[1] and alkyl acyl phosphatides[2] accompany the diacyl phosphatides[3] ubiquitously in nature, the problem of their separation from one another has plagued researchers[4]. Even now, a quantitative separation of these analogs from one another in *Native Form* has not been described. However, their quantitative conversion to diglyceride acetates by enzymatic cleavage with phospholipase C followed by acetylation with pyridine and acetic anhydride[5] has enabled us today at least to separate them quantitatively in a non-polar derivatized form from one another. Thus the lipophilic components of the individual phosphatide analogs can be characterized by their suitable derivatization followed by gas chromatographic analysis[6]. However, the present available methods require larger quantities of samples, more time and more chemical steps before the final characterization of the individual phosphatide analogs is achieved. This paper describes a two-dimensional reaction thin-layer chromatographic procedure by which a microsample of a mixture of three families of diglyceride acetates derived from alkenyl acyl-, alkyl acyl-, and diacyl choline phosphatides has been analyzed for the contents of individual analogs, the fatty acid compositions of individual analogs, and the fatty aldehyde composition of the alkency acyl analog.

EXPERIMENTAL

The isolation of choline phosphatides from beef heart[7], preparation of Silica

Gel G plates[8], and the gas–liquid chromatographic analysis of fatty acid methyl esters and fatty aldehydes[9] was carried out as described previously[7–9]. A 2 *N*-sodium methoxide reagent in absolute methanol[10] was prepared by dissolving 4.6 g of clean metallic sodium in 100 ml of absolute methanol. All solvents used were of reagent grade quality.

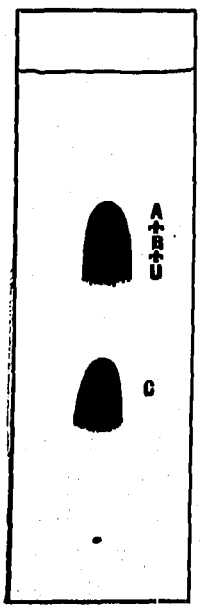

Fig. 1. Thin-layer chromatography of diglyceride acetates derived from choline phosphatide analogs of beef heart. Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: Skelly F–diethyl ether (88:12). Spray reagent: Aqueous sulfuric acid (50%), plate charred at 160° for 10 min. Spots identification: (A) = Alkenyl acyl glyceryl acetate; (B) = alkyl acyl glyceryl acetate; (U) = unknown compound; (C) = diacyl glyceryl acetate.

The quantitative conversion of choline phosphatides from beef heart to diglyceride acetates (alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates) was carried out by enzymatic cleavage of the phosphatides with phospholipase C followed by acetylation with pyridine and acetic anhydride[5].

The separation of alkenyl acyl- (A) and alkyl acyl glyceryl acetates (B) with an unknown compound (U) as a group from the accompanying diacyl glyceryl acetates (C) was achieved on layers of Silica Gel G with Skelly F–diethyl ether (88:12) as the developing solvent (Fig. 1). The same system was useful in separating alkyl acyl glyceryl acetate (B), diacyl glyceryl acetates (C), 2-acyl, 3-acetyl glycerol (A′), free aldehyde (D), (the latter two products are formed during acid hydrolysis of alkenyl acyl glyceryl acetate) and an unknown compound (U) from one another (Fig. 2).

Two-dimensional thin-layer chromatography was carried out as follows: A microsample (250–1000 μg) of a mixture of diglyceride acetates derived from beef heart choline phosphatides was spotted as a chloroform-solution at A′, the lower left hand corner of a thin-layer plate (Fig. 3). The same amount was also spotted at the lower right hand corner of the same thin-layer plate. After removing the chloroform from these spots with a stream of dry nitrogen, the spots were exposed to fumes of concentrated hydrochloric acid for 2.5 min[8]. The excess of hydrochloric acid was removed from the plate by blowing with a stream of dry nitrogen and the plate was then developed with Skelly F–diethyl ether (88:12) to a height of 14 cm. The plate was

removed from the solvent and dried with a stream of dry nitrogen. A strip 4 cm wide at the right side of the plate (reference strip) was sprayed with a 5 % solution of iodine in chloroform which exhibited the positions of the liberated aldehydes (D), alkyl acyl glyceryl acetate (B), diacyl glyceryl acetate (C) and 2-acyl, 3-acetyl glycerol (A′) and the unknown compound (U). The position corresponding to the aldehydes (D) liberated from diglyceride acetates that were spotted at the lower left hand corner of the plate was scraped off with a razor blade, extracted with diethyl ether, and analyzed by gas chromatography[8]. A strip 4 cm wide at the left side of the plate was then spray-

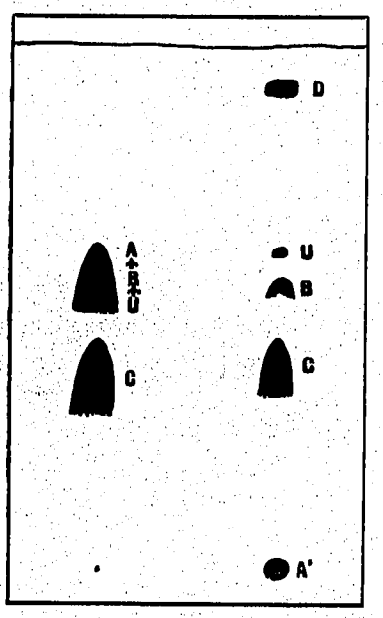

Fig. 2. Thin-layer chromatographic separation of diglyceride acetates derived from beef heart choline phosphatides (left) and their acid hydrolysis products (right). Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: Skelly F–diethyl ether (88:12). Spray reagent: Aqueous sulfuric acid (50 %). Plate charred at 160° for 10 min. Spots identification: (D) = Fatty aldehydes; (U) = Unknown compound; (B) = akyl acyl glyceryl acetate; (C) = diacyl glyceryl acetate; (A′) = 2-Acyl, 3-acetyl glycerol.

ed with a solution of 2 *N* sodium methoxide in absolute methanol which almost instantaneously interesterified the fatty acid esters of alkyl acyl glyceryl acetates, diacyl glyceryl acetates and 2-acyl, 3-acetyl glycerol. The sprayed strip was dried with a stream of dry nitrogen for 10 min, after which appropriate quantities of an internal standard (methyl heneicosanoate) were spotted at locations corresponding to alkyl acyl glyceryl acetate, diacyl glyceryl acetate and 2-acyl, 3-acyl glycerol in the left hand strip. As a reference standard, methyl linoleate was spotted at the upper left hand corner of the plate (E). The plate was then turned counterclockwise through 90° and developed with toluene.

When the solvent front rose to a height of 14 cm, the plate was removed, dried with a stream of dry nitrogen and then a strip 4 cm wide on the left side of the plate (second dimension) was sprayed with 5 % iodine solution in chloroform. Thus having located the position of the reference methyl ester (ML_R) and reference alkyl acyl glyceryl acetate (B), diacyl glyceryl acetate (C) and 2-acyl, 3-acetyl glycerol (A′), the methyl esters (together with their internal standards) liberated by the latter com-

pounds from the left hand strip (first dimension) were located, scraped off, extracted with diethyl ether and analyzed by gas chromatography.

RESULTS AND DISCUSSION

The gas chromatographic analysis of methyl esters obtained from alkenyl acyl-, alkyl acyl- and diacyl phosphatides and the aldehydes obtained from alkenyl acyl phosphatides by the previous method[8] and the present method are given in Table I. The previous method did not separate the alkyl acyl- and diacyl phosphatides from

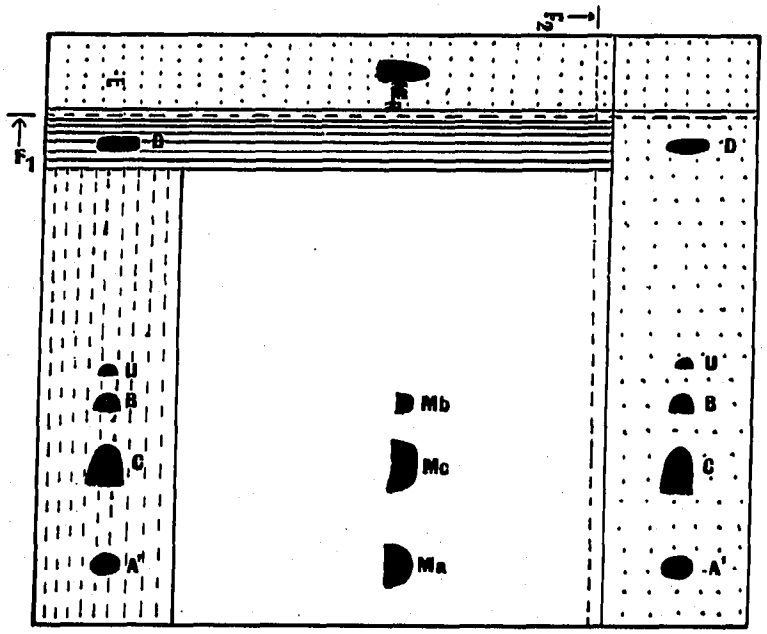

Fig. 3. Two-dimensional thin-layer chromatographic analysis of mixtures of alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates. Adsorbent: Silica Gel G. Activation: 1 h at 110°. Solvent system: 1st dimension, Skelly F–diethyl ether (88:12); 2nd dimension, toluene. Spray reagent: Aqueous sulfuric acid (50%), plate charred at 160° for 10 min. Spots identification: (A′) = The location of original samples where the 2-acyl, 3-acetyl glycerol remains held after acid treatment of the diglyceride acetates followed by development with Skelly F–diethyl ether (88:12); (C) = diacyl glyceryl acetate; (B) = alkyl acyl glyceryl acetate; (U) unknown compound; (D) = aldehyde; (E) = location for spotting reference methyl linoleate. Abbreviations: Mb, Mc and Ma, fatty acid methyl esters derived from alkyl acyl-, diacyl- and 2-acyl glyceryl acetate, respectively; ML_R = reference methyl linoleate; F_1 = first solvent front; F_2 = second solvent front. ▒▒▒ = Zone of Silica Gel G scraped off for recovering the liberated aldehydes from alkenyl acyl glyceryl acetate; :::: = area sprayed with 5% iodine solution in chloroform; ||||||| = area sprayed with a 2 *N* solution of sodium methoxide in absolute methanol.

one another because of their stability toward fumes of concentrated hydrochloric acid under the experimental conditions. In the present method, the diglyceride acetates derived from diacyl phosphatides are separated as a class from the diglyceride acetates derived from alkenyl acyl- and alkyl acyl phosphatides which move together in the investigated system. But because of the susceptibility of alkenyl acyl glyceryl acetate alone to fumes of concentrated hydrochloric acid, these two could be separated from one another and also from diglyceride acetates derived from diacyl phosphatides. Thus the present method gives an added advantage over the previous method[8]. In this method also a prior isolation of individual phosphatide analogs as

diglyceride acetates is avoided, thus improving a method devised in another laboratory[6].

The results in Table I demonstrate a good agreement in the fatty acid composition of the alkenyl acyl phosphatides by the previous method[8] as well as by the present method. The same is true in the case of diacyl phosphatides because only

TABLE I

COMPOSITION OF LIPOPHILIC COMPONENTS OF ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDES FROM BEEF HEART

	Fatty acid composition of alkenyl acyl choline phosphatides (% of total fatty acids)		*Fatty acid composition of alkyl acyl choline phosphatides (% of total fatty acids)*		*Fatty acid composition of diacyl choline phosphatides (% of total fatty acids)*		*Fatty aldehyde composition of alkenyl acyl choline phosphatides (% of total fatty aldehydes)*	
	Previous method[8]	*Present method*	*Previous method*[8]	*Present method*	*Previous method*[8] *(includes the values of alkyl acyl analogs)*	*Present method*	*Previous method*[8]	*Present method*
C14:0	—	—	[a]	—	—	—	2.1	2.1
C15:0	—	—		—	—	—	2.1	3.5
C15:?	—	—		2.1	—	—	2.2	2.0
C16:0	1.6	1.8		4.7	32.4	33.3	64.9	65.0
C16:1	tr.	0.3		0.5	1.8	2.1	2.7	3.3
C17:?	—	—		—	—	—	5.4	5.0
C17:0	—	—		1.7	—	0.7	2.6	3.1
C18:0	1.2	0.9		3.7	10.8	12.2	13.5	12.2
C18:1	12.7	11.8		13.7	18.5	18.3	4.5	3.8
C18:2	58.5	59.3		50.0	30.2	27.1		
C18:3	1.2	1.3		2.6 (18:3 ?)	—	0.8		
C20:3	8.6	8.7		4.8	2.8	2.0		
C20:4	16.2	15.9		9.9	3.5	3.5		
C20:5				6.3	—	—		
C22:5				—				
C22:?				tr.				

[a] These cannot be determined separately. The values included with diacyl analogs.

4.25 % of alkyl acyl choline phosphatide occur in the original sample.

The figures in Table II show good agreement in the plasmalogen content of the original sample as determined by the previous method as well as by the present method. The slightly higher value obtained for the content of diacyl phosphatide by the previous method is explained by the inability of that method to distinguish between the diacyl- and alkyl acyl phosphatides. The content of the alkyl acyl phosphatide as determined by the fatty acid internal standard method (4.25) in the present studies is higher than that obtained by subtracting the diacyl values obtained by the two methods ($0.5(47.3 + 46.9) - 44.0 = 3.1$). This difference is understandable because of the low content of alkyl acyl phosphatides as well as the approximations used in these calculations.

The present technique of separating the diglyceride acetates derived from alkenyl acyl phosphatides from the diglyceride acetates derived from alkyl acyl phosphatides has advantages over the double development technique described by RENKONEN[7]. The present method requires only one development for separating these

analogs from one another and also liberates the aldehyde from the alkenyl acyl analog which also is separated from the rest of the compounds in the mixture. This separation aids easy characterization of the homologs and vinylogs of aldehydes.

In the previous method[8] from this laboratory the phosphatides were interesterified with the methanolic KOH reagent of KAUFMANN *et al.*[11]. This reagent could not

TABLE II

ALKENYL ACYL-, ALKYL ACYL- AND DIACYL CHOLINE PHOSPHATIDE CONTENTS OF BEEF HEART CHOLINE PHOSPHATIDE

Alkenyl acyl choline phosphatide content of beef heart choline phosphatide (%)			*Alkyl acyl choline phosphatide content of beef heart choline phosphatide* (%)		*Diacyl choline phosphatide content of beef heart choline phosphatide* (%)		
Previous method[8]		*Present method*	*Previous method*[8]	*Present method*	*Previous method*[8]		*Present method*
Fatty acid internal standard method	*Phosphorous determination method*	*Fatty acid internal standard method*		*Fatty acid internal standard method*	*Fatty acid internal standard method*	*Phosphorous determination method*	*Fatty acid internal standard method*
52.7	53.1	51.7	[a]	4.25	47.3[b]	46.9[b]	44.0

[a] This cannot be determined by this method. It is included in the value of diacyl analog.
[b] Includes the value of alkyl acyl analogs.

be used in the present method because of its inability to interesterify the triglyceride fatty acids. The recently reported method of OETTE AND DOSS[10] in which a 2 *N* solution of sodium methoxide in absolute methanol was found to interesterify directly the fatty acids of triglycerides on thin-layer plates was successfully used as described for interesterifying the fatty acids of alkenyl acyl-, alkyl acyl- and diacyl glyceryl acetates. The present method reported here fails only to convert microquantities of residual glyceryl ethers on the plate to suitable derivatives for gas chromatographic analysis of their molecular species, and as only about 20 μg of residual glyceryl ethers can be derived from 1000 μg of diglyceride acetates from the plate, their conversion to suitable derivatives by techniques other than reaction chromatography may very likely introduce too many uncertain factors to make the method dependable.

The availability of the unknown compound (U) in very small amounts precluded its further investigation.

Although the new method has been used in analyzing mixtures of analogs of choline phosphatides, its application to other phosphatides (ethanolamine-, serine- and inositol phosphatides) is possible, since they all can be converted to diglyceride acetates.

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NOTE ADDED IN PROOF

The unknown compound (U) has been tentatively identified as dialkyl glyceryl acetate on the basis of its chromatographic characteristics and non-liberation of long-chain fatty acid methyl esters or aldehydes on the TLC plate: This indicates indirectly the presence of dialkyl choline phosphatides in the original choline phosphatides of beef-heart.

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