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TWO-DIMENSIONAL REACTION THIN-LAYER CHROMATOGRAPHY IN THE ANALYSIS OF PHOSPHATIDE PLASMALOGENS

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SUMMARY

A two-dimensional reaction thin-layer chromatography procedure on microsamples of mixtures of alkenyl acyl and diacyl phosphatides has been described by which it is possible to determine simultaneously their plasmalogen content, the fatty acid composition of individual analogs and the fatty aldehyde composition of the alkenyl acyl analog.

INTRODUCTION

In nature the phosphatide plasmalogens are always accompanied by their diester analogs[1]. The separation of these analogs in native form from one another by either chemical means[2] or chromatographic techniques[1] reportedly has failed because of the slight structural difference between them.

Although methods for obtaining pure phosphatide plasmalogens by either selective enzymatic[4,7] or chemical hydrolysis[5,6] are available, the resulting compounds do not represent the native plasmalogens in composition, because some hydrolysis of the plasmalogens always occurs during these treatments. Hence methods to determine the plasmalogen content[4,8–13] and the fatty acid and aldehyde (only in plasmalogens) composition[14] of these analogs in a mixture were sought.

Recently[1,15] we developed a preparative reaction thin-layer chromatographic method for determining simultaneously the plasmalogen content and the fatty acid and aldehyde composition of mixtures of alkenyl acyl (plasmalogen) and diacyl ethanolamine phosphatides from hog tissues. The main reason for working on a preparative scale was to devise a method to obtain enough material of methyl esters and aldehydes for structural analysis. However, the methyl ester composition determined by this procedure represented the mixture of alkenyl acyl and diacyl ethanolamine phosphatides rather than the individual analogs.

In the present communication we describe a two-dimensional reaction thin-layer chromatographic procedure by which we determined not only the plasmalogen content of micro samples of a mixture of alkenyl acyl and diacyl ethanolamine phosphatides from beef heart but also the fatty acid composition of the individual analogs and the fatty aldehyde composition of the alkenyl acyl analogs.

EXPERIMENTAL

Preparation of thin-layer plates

Glass plates (20 cm × 20 cm) were coated with a well stirred aqueous suspension of Silica Gel G (30 g in 60 ml water) using a thin-layer applicator (Desaga, Heidelberg). This produced a layer that was approximately 250 μ in thickness. The plates, after air-drying at room temperature for 15–30 min, were activated in an air-oven at 110° for 1 h.

Solvents

All the solvents used were of reagent grade quality. Absolute methanol was used for preparing 12% methanolic potassium hydroxide solution.

Preparation of sample

A mixture of alkenyl acyl and diacyl ethanolamine phosphatides from beef heart lipids was obtained by preparative thin-layer chromatography (TLC)[16] on Silica Gel G plates using chloroform–methanol–concentrated ammonia (70:30:5) as the developing solvent. The isolated sample was shown to be essentially devoid of contaminants by TLC using the above cited system; and its infrared absorption spectrum, except for an additional absorption at 6.0 μ, showed all of the absorptions typical of a sample of diacyl ethanolamine phosphatide[17].

Two-dimensional thin-layer chromatography

A mixture of alkenyl acyl and diacyl ethanolamine phosphatides (250 to 1000 μg) was spotted as a chloroform solution at A, the lower left hand corner of a thin-layer plate. The same amount was also spotted at B, the lower right hand corner of the same thin-layer plate (Fig. 1). After removing the chloroform from these spots with a stream of dry nitrogen, the phosphatide spots were exposed to fumes of concentrated hydrochloric acid for 2.5 min (Fig. 2). The excess hydrochloric acid was removed with a stream of dry nitrogen and the spots were exposed to ammonia fumes for 2 min. The excess ammonia from the spots was removed with a stream of dry nitrogen and then the plate was developed to a height of 14 cm in a TLC chamber containing chloroform–methanol–concentrated ammonia (70:30:5) as the developing solvent. The plate was removed from the TLC chamber and dried with a stream of dry nitrogen. A strip, 4 cm wide at the right side of the plate (Fig. 1), was sprayed with a 5% solution of iodine in chloroform which exhibited the positions of the liberated aldehyde (c), (spot almost at the solvent front) and the lyso-(2-acyl) (a) and diacyl ethanolamine phosphatides (b), with approximate R_F values 0.20 and 0.45, respectively. The position corresponding to the aldehydes (c) liberated by the ethanolamine phosphatides at A was scraped off with a razor blade and after extracting with diethyl ether, the aldehydes were analyzed by GLC[16]. A strip 4 cm wide at the left side of the plate was then sprayed with a solution of 12% potassium hydroxide in absolute methanol for methanolysis of the phosphatide[18] and then dried with a stream of dry nitrogen in a TLC chamber containing calcium chloride for 10 min. A solution of an internal standard—methyl heneicosanoate—was spotted at the spots corresponding to lyso and diacyl ethanolamine phosphatides on the left hand strip. The amount of internal standard used for the lyso derivative was always half of that used for the diacyl analog for two reasons:

(1) the alkenyl analog and the diacyl were present in approximate equal amounts and (2) the former should always give 1 ml of fatty acid per mole of the analog while the latter should give 2 moles of fatty acids per mole of the analog. As reference standard, methyl linoleate was spotted at the upper left hand corner of the plate (C) and after turning the plate through 90° was developed (in the second dimension) with toluene as the solvent. When the solvent rose to a height of 14 cm, the plate was removed, dried

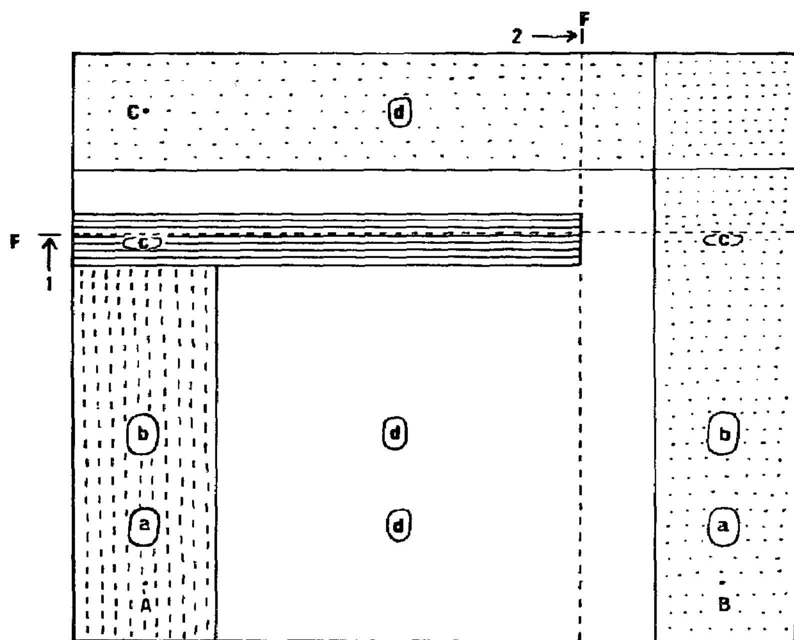

Fig. 1. Two-dimensional thin-layer chromatogram of alkenyl acyl and diacyl ethanolamine phosphatides. A, B = Spots containing 250 μg of a mixture of alkenyl acyl and diacyl ethanolamine phosphatides; C = methyl linoleate as reference standard; $F \uparrow_{1}$ = solvent front for the development in the first dimension; $F \uparrow_{2}$ = solvent front for the development in the second dimension; a = 2-acyl ethanolamine phosphatide (lyso compound); b = diacyl ethanolamine phosphatide; c = aldehyde liberated from alkenyl acyl ethanolamine phosphatide; d = methyl esters, reference, and those from 2-acyl and diacyl ethanolamine phosphatides containing internal standards. [dotted pattern] Area sprayed with a 5% iodine solution in chloroform. [dashed pattern], Area sprayed with a 12% potassium hydroxide solution in absolute methanol. [lined pattern], Zone of Silica Gel G scraped off for recovering the liberated aldehydes from alkenyl acyl ethanolamine phosphatide.

with a stream of dry nitrogen and then a strip 4 cm wide on the left side of the plate (in the second dimension) was sprayed with 5% iodine solution in chloroform. Thus, having located the position of the reference methyl ester (d) and the reference lyso-(2-acyl) (a) and diacyl ethanolamine phosphatides (b), the methyl esters (containing internal standards) liberated by the lyso-(2-acyl) and diacyl ethanolamine phosphatides could be located, scraped off and extracted for GLC analysis[16].

Phosphorus estimations were carried out as described by RHEE AND DUGAN[19] using perchloric acid for the digestion.

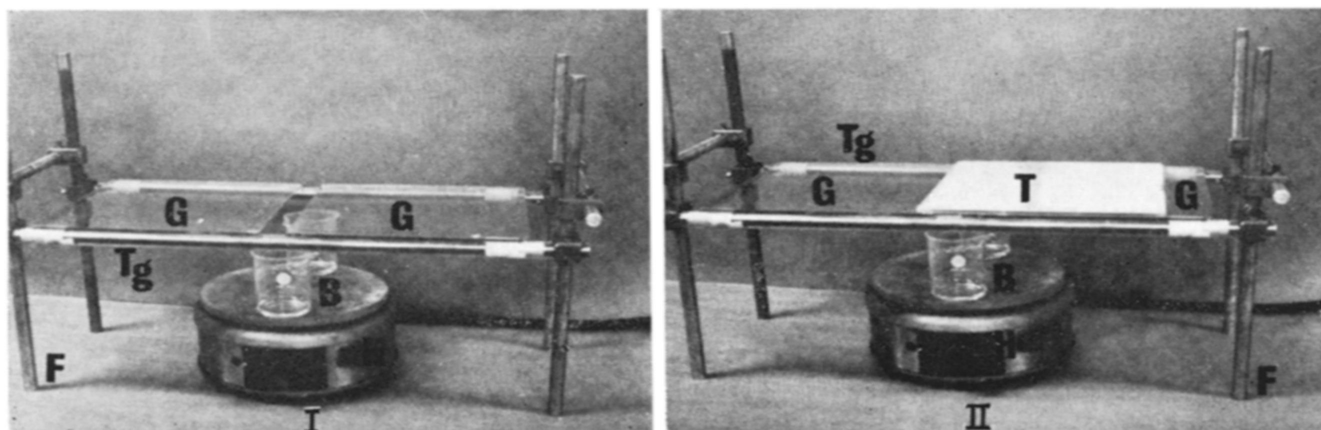

Fig. 2. Set-up for two-dimensional thin-layer chromatography of alkenyl acyl and diacyl ethanolamine phosphatides. Part I shows the set-up before acid treatment of the ethanolamine phosphatide, part II the set-up when the acid treatment was actually in progress. G = Glass plates used for supporting another glass plate (T) coated with a layer of Silica Gel G; F = aluminum frame; Tg = glass tube slipped over an aluminum rod in the frame F, to prevent aluminum reacting with acid fumes; B = beakers containing boiling concentrated hydrochloric acid; H = hot plate (on); T = a glass plate coated with a thin layer (250 μ) of Silica Gel G. The thin-layer plate 'T' containing the spotted ethanolamine phosphatides was laid on the two glass plates G in such a manner that the spots of ethanolamine phosphatides were directly exposed to the fumes of hydrochloric acid while the rest of the plate was prevented from coming into contact with the acid fumes.

RESULTS

The analysis of the methyl esters obtained from alkenyl acyl and diacyl ethanolamine phosphatides and the analysis of fatty aldehydes obtained from alkenyl acyl ethanolamine phosphatides for two different sample sizes are given in Table I. The samples show good agreement in their composition.

TABLE I

FATTY ACID AND FATTY ALDEHYDE COMPOSITION OF ALKENYL ACYL AND DIACYL ETHANOLAMINE PHOSPHATIDES FROM BEEF HEART

Carbon number	*Alkenyl acyl ethanolamine phosphatides*				*Diacyl ethanolamine phosphatides*	
	Percent of total fatty aldehydes		*Percent of total fatty acids*		*Percent of total fatty acids*	
	Sample size 500 µg	*Sample size 1000 µg*	*Sample size 500 µg*	*Sample size 1000 µg*	*Sample size 500 µg*	*Sample size 1000 µg*
C16:0	29.8	29.2	0.4	0.3	1.7	2.0
C17:Br	3.3	3.2	—	—		
C17:0	3.3	3.7	—	—		
C18:0	42.6	43.3	1.2	1.5	49.9	48.3
C18:1	10.9	11.7	1.5	2.3	5.0	5.0
C18:2	6.6	6.6	62.0	60.4	8.7	9.7
Unidentified	3.6	2.4	—	—	—	—
C20:3	—	—	3.7	4.6	—	—
C20:4	—	—	31.2	30.8	34.7	35.0

TABLE II

PLASMALOGEN CONTENT OF ETHANOLAMINE PHOSPHATIDES FROM BEEF HEART

Fatty acid internal standard method	*Phosphorus determination method*	*Weighing method*
49.9 (Average of two determinations each, on two sample sizes)	50.8 (Average of four determinations)	48.6 (Average of three determinations)

The plasmalogen content of the sample is given in Table II and has been calculated by three different methods: (1) from the amount of internal fatty acid methyl ester standard present in the fatty acid methyl esters obtained from alkenyl acyl and diacyl ethanolamine phosphatides[20], (2) from the phosphorus content of 2-acyl and diacyl ethanolamine phosphatides[13], and (3) from the amounts of dimethyl acetals and fatty acid methyl esters obtained by cold methanolysis of 100 mg sample as reported earlier[16]. All the three methods show good agreement.

DISCUSSION

The acid hydrolysis of alkenyl acyl ethanolamine phosphatide and the alkaline methanolysis of 2-acyl and diacyl ethanolamine phosphatide on TLC plates was shown to be quantitative[15]. A modification of that technique[15], as described in this communication, not only made it possible to determine separately the fatty acid composition of alkenyl acyl and diacyl ethanolamine phosphatides, but also the plasmalogen content of the sample either by phosphorus determination of the glyceryl phosphoryl ethanolamine residues released by the alkenyl acyl and diacyl analogs or by use of a methyl ester internal standard. This method could also be used successfully on the mixtures of choline phosphatide analogs.

The technique described by OWENS[13] in which aqueous mercuric chloride solution was used to split the vinyl ether linkage of the plasmalogens instead of hydrochloric acid fumes as described here, could also be used to achieve the same end. However, the use of *aqueous* spray reagent for hydrolysis purposes necessitated longer reactivation time for the plates and more rigorous conditions.

In our earlier work[15] we used methanolic hydrochloric acid reagent to hydrolyze the vinyl ether bond but in the present study fumes from concentrated hydrochloric acid were found to be equally effective.

This technique thus provides a simple method for characterization of the alkenyl acyl phosphatides and their accompanying diacyl analogs which is as effective as the specific iodination reaction technique[21].

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