Short Communication

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Reaction thin-layer chromatography in the analysis of mixtures of alkenyl acyl- and diacyl-phosphatides

Recently KAUFMANN, RADWAN AND AHMAD[1] reported quantitative alkaline methanolysis of monoacyl-(lyso-) and diacyl-phosphatides directly on thin-layer plates. By this technique they calculated the phosphatide content of mixed lipids by multiplying the weight of fatty acid methyl esters obtained from phosphatides by appropriate factors. These values agreed well with those obtained by phosphorus determinations. However, they obtained low values for tissue lipids that contained substantial amounts of either alkenyl acyl-phosphatides, or sphingolipids, or both. The explanation for this discrepancy was the resistance of the vinyl ether linkage of the plasmalogens (alkenyl acyl-phosphatides) or the acid-amide linkage of the sphingolipids to alkaline methanolysis.

In the present communication we describe a suitable modification of the method of KAUFMANN *et al.*[1] by which the alkenyl content, and the fatty acid and aldehyde composition of mixtures of alkenyl acyl- and diacyl-ethanolamine phosphatides may be determined.

Experimental

A mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides from hog brain and hog spinal cord were prepared by the method of FOLCH[2] and purified by preparative thin-layer chromatography (TLC) on Silica Gel G plates using chloroform–methanol–concentrated ammonia (70:30:5) as the developing solvent. The Silica Gel G plates were prepared as described previously[3].

Reaction thin-layer chromatography (*plate method*) was carried out as follows: 1 ml of solution (50 mg/ml) of a mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides in chloroform was deposited as a 16 cm long strip, 2 cm above the lower edge of the plate and parallel to it. Margins of 2 cm were left at both ends of the strip for reference standards. The strip was sprayed with a solution containing 12 % hydrochloric acid in methanol, and the plate was left undisturbed in an atmosphere of nitrogen for 2 min. An aldehyde reference (50 μg of palmitaldehyde) was spotted at the left end of the strip and then the entire strip was exposed to a stream of dry nitrogen to remove the residual hydrochloric acid and methanol. The plate was developed in toluene until the solvent front reached the top of the plate. The plate was removed, and, after drying with a stream of nitrogen, the strip containing monoacyl-(lyso-) and diacyl-ethanolamine phosphatides was subjected to a spray of solution containing 12 % potassium hydroxide in methanol and left undisturbed in an atmosphere of nitrogen for 5 min. A reference methyl ester standard (50 μg of methyl linoleate) was spotted at the right end of the strip, and the entire strip exposed to a stream of dry nitrogen to remove the residual methanol from the plate. The plate was

then redeveloped in the same direction with toluene until the solvent front reached half the height of the plate. The plate was dried with a stream of nitrogen and only the two 2 cm strips containing the reference standards were sprayed with 0.2 % solution of 2,7-dichlorofluorescein. The location of the reference palmitaldehyde (R_F 0.5) and methyl linoleate (R_F 0.23) was determined under ultraviolet light. The unsprayed portions corresponding to the aldehydes and methyl esters liberated from the mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides were scraped off into separate containers and quantitatively extracted with diethyl ether. After distilling off the diethyl ether, the residual aldehydes and methyl esters were weighed, and from these weights the alkenyl content of the mixture was calculated using the following formula:

$$\text{Percent alkenyl} = \frac{2(\text{amount of DMA, calculated from weight of aldehyde})}{(\text{calculated amount of DMA} + \text{amount of ME})} \times 100$$

Methanolysis of the phosphatide mixture was carried out at —30° (*cold method*) as described previously[4]. Up to 100 mg of a mixture of alkenyl acyl- and diacyl-ethanolamine phosphatides was dissolved in 20 ml of diethyl ether contained in a 125 ml Erlenmeyer flask. This flask was immersed in a dry ice–acetone bath maintained at —30°. While the contents of the flask were being agitated with a magnetic stirrer, 2 ml of 100 % sulfuric acid was added and the stirring continued for 10 min during which time the temperature of the bath rose to 0°. The bath temperature was lowered again to —30°, 15 ml of absolute methanol added and the stirring continued for 10 min. The reaction mixture was then carefully neutralized with 20 ml of 35 % potassium hydroxide in methanol, followed by continuous swirling until the jelly-like mixture turned into a white milky solution. After 10 min, the contents of the flask were transferred to a separatory funnel containing 200 ml of cold distilled water, and the Erlenmeyer flask was rinsed twice with 20 ml of diethyl ether. The products of methanolysis were extracted with 150 ml of diethyl ether, and the ether extract was washed free of alkali with distilled water and dried over anhydrous sodium sulfate. The mixture of methyl esters and dimethyl acetals resulting from cold methanolysis was separated quantitatively by preparative TLC on Silica Gel G using toluene as the developing solvent, and the two fractions, after scraping off, were extracted and weighed. The alkenyl content of the mixture was calculated as follows:

$$\text{Percent alkenyl} = \frac{2(\text{amount of DMA})}{(\text{amount of DMA} + \text{amount of ME})} \times 100$$

The gas–liquid chromatography (GLC) analysis of aldehydes and methyl esters obtained by *plate method* and dimethyl acetals and methyl esters obtained by *cold method* were made with a Beckman GC-2A instrument equipped with a hydrogen flame ionization detector containing an aluminum column (6 ft. long, 1/8 in. outer diameter) packed with Gas Chrom P, 80–100 mesh impregnated with 20 % ethylene glycol succinate and 2 % phosphoric acid. The temperature of the column was 160° and helium flow rate was 30 ml/min. Peaks of the aldehydes, dimethyl acetals and methyl esters were identified by comparing their retention times with authentic and synthetic standards. The chain lengths of unsaturated dimethyl acetals, methyl esters and aldehydes (after converting them to dimethyl acetals) were verified by GLC after hydrogenation. The peak areas were quantitated by triangulation.

TABLE I

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG SPINAL CORD (SAMPLE 1)

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	—	—	—	—
C16:0	7.0	6.9	29.1	28.7
C16:1	0.8	0.7	—	—
C17:0	—	—	—	—
Unidentified	—	—	—	—
Unidentified	—	—	—	—
C18:0	9.0	8.8	25.5	25.3
C18:1	59.1	58.6	45.4	46.0
C18:2	—	—	—	—
C18:3	—	—	—	—
C19:0	Traces	Traces	—	—
C20:0	0.3	0.5	—	—
C20:1	19.0	19.2	—	—
C20:4	3.4	3.5	—	—
C22:1	1.4	1.8	—	—

TABLE II

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG SPINAL CORD (SAMPLE 2)

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	—	—	—	—
C16:0	5.3	5.4	33.6	33.9
C16:1	0.6	0.7	—	—
C17:0	—	—	—	—
Unidentified	—	—	—	—
Unidentified	—	—	—	—
C18:0	9.3	9.2	23.4	23.2
C18:1	56.7	55.9	43.0	42.9
C18:2	—	—	—	—
C18:3	—	—	—	—
C19:0	—	—	—	—
C20:0	0.8	0.7	—	—
C20:1	21.1	20.8	—	—
C20:4	2.1	2.5	—	—
C22:1	4.1	4.8	—	—

TABLE III

FATTY ACID AND ALDEHYDE COMPOSITION OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES FROM HOG BRAIN

Carbon number	*Percent of total fatty acids*		*Percent of total fatty aldehydes*	
	Plate method	*Cold method*	*Plate method*	*Cold method*
C15:0	0.8	0.9	—	—
C16:0	7.4	7.6	33.4	33.1
C16:1	1.3	1.7	—	—
C17:0	Traces	Traces	—	—
Unidentified	1.2	1.6	—	—
Unidentified	Traces	Traces	—	—
C18:0	21.2	21.0	27.8	28.3
C18:1	49.8	49.2	37.7	37.2
C18:2	1.1	0.6	1.1	1.4
C18:3	1.7	1.7	—	—
C19:0	—	—	—	—
C20:0	—	—	—	—
C20:1	10.6	10.7	—	—
C20:4	4.9	5.0	—	—
C22:1	—	—	—	—

TABLE IV

THE PERCENT ALKENYL CONTENT OF A MIXTURE OF ALKENYL ACYL- AND DIACYL-ETHANOLAMINE PHOSPHATIDES

Hog spinal cord (Sample 1)		*Hog spinal cord (Sample 2)*		*Hog brain*	
Plate method	*Cold method*	*Plate method*	*Cold method*	*Plate method*	*Cold method*
92.2	90.4	91.9	89.6	Not determined	

Results

The analyses reported in Tables I–IV by the *plate method* (reaction thin-layer chromatography) and the *cold method*, for three different mixtures of alkenyl acyl- and diacyl-phosphatides from hog tissues, indicate good agreement for their fatty acid and aldehyde composition as well as for their alkenyl content. Thus the *plate method* described above is a quick alternative for the analysis of mixtures of alkenyl acyl- and diacyl-phosphatides.

Discussion

This modification of the method of KAUFMANN *et al*[1] has resulted in a useful technique for characterizing mixtures of alkenyl acyl- and diacyl-phosphatides. The hydrolysis of vinyl ether bonds present in phosphatides either by methanolic hydrochloric acid as described here or aqueous mercuric chloride solutions[5] on TLC plates can partly overcome the difficulties previously encountered by KAUFMANN *et al.*[1] in

analyzing phosphatide mixtures containing alkenyl acyl-phosphatides. The hydrolysis of acid-amide bonds of sphingolipids on thin-layer plates still remains as a problem.

The hydrolysis of vinyl ether bond by hydrochloric acid solution in methanol is more advantageous than by aqueous mercuric chloride solution[5] because the reactivation of plates after using aqueous mercuric chloride solution spray required appreciably longer time and more rigorous conditions[5].

Reaction thin-layer chromatography, as described in this communication, was used on a preparative TLC scale and in this way sufficient amounts of methyl esters and aldehydes can be obtained for their structural analysis. When only small amounts of mixtures of alkenyl acyl- and diacyl-phosphatides are available, the same procedure can still be used to determine their fatty acid and aldehyde composition. The alkenyl content of the mixture can then be determined by incorporating internal standards of an aldehyde and a methyl ester into the mixture which were not originally present.

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