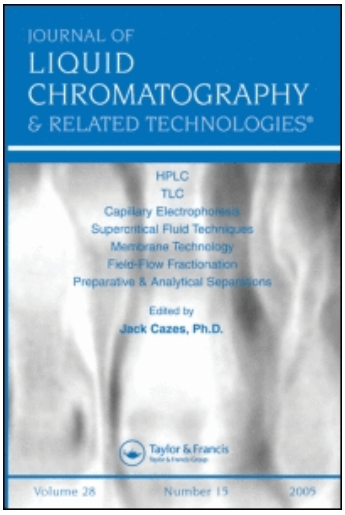

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Analysis of Plasmalogens by In Situ Reaction on Thin Layer Chromatograms

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INTRODUCTION

An "in situ" hydrolysis method that attacks only the enol ether double bond of plasmalogen is described. Alkyl and unsaturated phospholipids are not affected. Treatment of the plasmalogen on thin layers of silica gel with solutions of trichloroacetic acid in dilute hydrochloric acid result in more complete hydrolysis of the vinyl-ether linkage. After development of the chromatogram differential quantitation using copper sulfate charring (1) and densitometry will give the amount of vinyl-ether lipid present in the sample. The results of the method with ethanolamine plasmalogen are presented.

Plasmalogens are the alk-1-enyl glycero-ether classes of phospholipids. They are widely distributed in nature in animal and anaerobic bacterial cells. Almost 20% of the phospholipids in the central nervous system of the human adult are plasmalogens. The myelin sheath phospholipids are 30% plasmalogen.

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The plasmalogens are in highest concentration in the nervous tissue and in striated muscle of the heart (32%). Adipose tissue contains a 1:4 ratio, plasmalogen to phospholipid. These proportions vary within each tissue class and from species to species. Ethanolamine plasmalogen varies from a low of 23% in the retina of the frog to 75% of the phospholipid in the sciatic nerve of the rat. It is found in high concentrations in seminal fluid (2). Methods for the identification and quantitation of the vinyl-ether linked lipids particularly the plasmalogens have been reported and widely used. These have been compiled by Kates (3) and reviewed by Horrocks and Sharma (4). These methods usually depend on the susceptibility of the enol ether bond to electrophyllic attack. Treatment of alk-1-enyl-glycerolipids with fumes from hydrochlorice acid produced aldehydes (5). Hack and Ferrans (6) hydrolyzed the vinyl part of the plasmalogens on thin layer chromatograms with mercuric chloride, followed by Schiff's reagent to detect aldehydes formed. However, we have found that the mercuric chloride method resulted in reaction with some unsaturated phospholipids to give several reaction products. Dinitrophenylhydrazine was used to derivatize the aldehyde released by acid hydrolysis. The hydrochloric acid and phenylhydrazine are made up in a single solution (7). Goldfine et al., have used 90% acetic acid to hydrolyze plasmenyl-ethanolamine (8). A conparision of the present method with some of the conventional methods indicated that evaluation of the methodology of phospholipid determination is needed. The present method was developed in order to avoid time consuming hydrolysis followed by gas chromatography or other analytical procedures and enable quantitation by densitometry.

MATERIALS AND METHOD

The phospholipids used in this study are synthetic except the plasmalogens which were obtained from natural sources as indicated. Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine plasmalogens were obtained from Avanti Biochemicals (Birmingham, AL) as were

the synthetic materials. A sample of ethanolamine plasmalogen was obtained from Supelco (Bellefonte, PA) through the courtesy of Dr. Lloyd Whiting. A mixture of plasmalogens from beef heart was obtained from Dr. Howard Goldfine (University of Pennsylvania). Solutions were dissolved in chloroform or benzene at 0.5 ug/ul concentration.

Whatman LK-5 (Clifton, NJ) 20x20 cm silica gel layers, 250u thick with a double thick preadsorbant area were used. The plates were scored into 10 mm lanes with a Schoeffel scoring device, then washed overnight by continuous development in a 1:1 $CHCl_3$: MEOH solution. The plates were allowed to completely dry and stored in a Camag Trockengestell until used. Glass distilled solvents were EM Sciences chromatographic grade. The mobile phase was chloroform:ethanol:triethylamine:water, (30:34:30:8). Trichloroacetic acid (TCA), dinitrophenylhydrazine, cupric sulfate, mercuric chloride and Schiff's reagent were of reagent grade. The cupric sulfate solution was prepared as a 10% (w/v) solution in 8% v/v phosphoric acid. The TCA reagent was a solution of 2% TCA-8% HCl (1:1). The TCA solution must be made fresh.

Samples (1-2 ug) were applied in duplicate as a streak across the middle of the preadsorbent area. Alternate lanes were used on each of two plates. One lane of each pair or plate was treated with 25 ul of the TCA reagent over the still wet sample. This was allowed to react for 10 minutes at ambient temperature before drying with forced warm air from a hair dryer at low temperature setting. The chromatogram was then predeveloped twice to the preadsorbent/sorbent interface with $CHCl_3$-MEOH, (1:1). This procedure extracted the sample and deposited it as a narrow band at the juncture. Following development, the solvent was evaporated completely from the plate which was then developed in an unlined chromatagraphy tank with the mobile phase until the solvent front reached 2 cm from the top of the plate.

The chromatogram was allowed to dry at ambient temperature, then placed

on a warm surface to remove residual solvent and finally dried in an oven at 180°C for two minutes. When cool, the chromatograms were sprayed until saturated with the 10% cupric sulfate in 8% phosphoric acid (w/v). The plate was kept at ambient temperature for 5 minutes then placed in a 120°C oven for 5 minutes to remove a major portion of the water. Charring was performed at 170°C for 10 minutes in an oven.

The chromatograms were scanned on a Kontes Model 800 densitometer in a double beam mode using the 440 nm filter. The densitometer was interphased with a Hewlett Packard 3385A integrator recorder. Quantitation was performed by interpolation on calibration curves prepared from standards of each compound. By differentiation between the treated and untreated lanes the plasmalogen can be quantitated since the area of the plasmalogen zones decreased in the case of the treated lane.

Mercuric chloride solution 0.05 M (50 ul) was applied to the sample in the pre-adsorbent region of the plate by a method modified from Hack and Ferrans (6) followed by chromatographic development.

The method using simultaneous cleavage with acid and hydrazone formation was that of Shipski and Barclay (7). A solution of 0.4% dinitrophenylhydrazine in 2 N HCl was sprayed on the developed chromatogram. This was heated in an oven at 110 ° for a few minutes. Yellow zones denoted the formation of the hydrazone of the released aldehyde. The method for hydrolysis of plasmalogens using fumes from concentrated hydrocloric acid was that of Horrocks (8).

RESULTS AND DISCUSSION

The trichloroacetic acid -hydrochloric reagent appears to react specifically with the vinyl ether linkage since saturated and unsaturated

TABLE 1

Hydrolysis of Phospholipids

Phospholipid	TCA/HCl	$HgCl_2$	HCl Fumes
Dileoyl PC	NR	3 products	Destroyed
Dilinolenyl PC	NR	Destroyed	Destroyed
Dipalmitoyl PC	NR	NR	NR
Dimyristyl PC	NR	NR	NR
Dipalmitoyl PEA	NR	NR	NR
1-palmitoyl-2-oleoyl PS	NR	NR	Reaction
PC (brain)	several products	several products	several products
PEA (heart)	several products	several products	several products

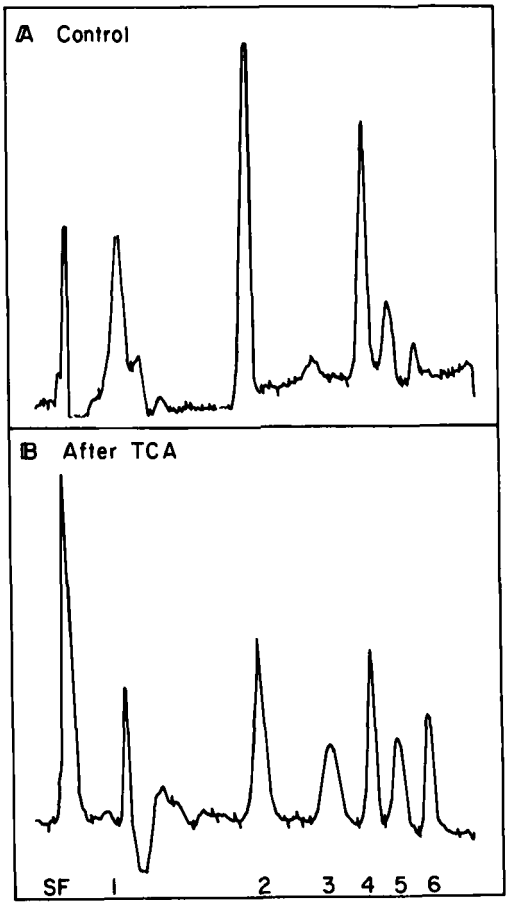
PEA = Phosphatidyl ethanolamine

PC = Phosphatidylcholine

PS = Phosphatidyl serine

NR = No Reaction

analogues were not affected. Table 1 shows the effect of hydrolyzing phospholipids obtained from the various sources. The plasmalogens that are available commerically are natural rather than synthesized preparations and therefore are a mixture of plasmalogen and a related phospholipid, e.g. phosphatidyl choline is 30% plasmalogen, 70% phosphatidyl choline. The results for the beef heart extract, which has a mixture of phosphatidyl choline plasmalogen and phosphatidyl ethanolamine plasmalogen are shown in Figure 1. The decrease in the area of peaks and the appearance of peaks at a



Legend Figure 1

Result of Treatment of Beef Heart "Plasmalogens" with the Trichloroacetic-Hydrochloric acid Reagent

SF. solvent front

1. Phosphatidylglycerol area
2. Phosphatidylethanolamine
3. lysophosphatidylethanolamine
4. phosphatidylcholine
5. sphingomyelin
6. lysophosphatidylcholine

new position indicates the presence of plasmalogens in this sample since the peaks in the untreated chromatogram are not present in the chromatogram obtained after TCA/HCl treatment. The new peaks had R_f corresponding to the reference lysophospholipid. The method has been applied to seminal fluid and the presence of plasmalogen readily indicated. Further evidence of the specificity of the TCA/HCl method is seen when it was compared with the $HgCl_2$ method of Owens (6) on synthetic phospholipid standards. The TCA/HCl or the $HgCl_2$ was applied directly to the samples. There was no reaction for any of the samples exposed to the TCA/HCl. However when the $HgCl_2$ method was used, three new products could be seen on the chromatogram of dioleoyl phosphatidyl choline and there was complete destruction of the dilinolenyl phosphatidyl choline. Since fatty acid side chains of phospholipids could be any one or a mixture of these the $HgCl_2$ would not be useful since it appeared to attack any unsaturated linkage.

The acid cleavage with hydrazone formation method of Skipski et al.(7) could not be evaluated by densitometry since the resulting background was too great and non-uniform. The method utilizing fumes from hydrocholoric (5) acids was not reproducible and low yields were obtained.

The presence of plasmalogens and unsaturated phospholipids in seminal fluid is generally known (9, 10, 11). The methods used for their assay are tedious and include two dimensional chromatography as well as hydrolysis and gas chromatography. Plasmalogen was readily shown by the present method.

Results of a comparison of methods are listed in Table 2. These methods were applied to ethanolamine plasmalogen furnished by Dr. Lloyd Whiting. This is a relatively pure preparation. The present method resulted in a 90.3% hydrolysis of the sample. TCA alone gave only 68% and the 5% HCl alone was only 72% effective. When the chromatogram was placed above fuming HCl for 5

TABLE 2

Table II Hydrolysis of Ethanolamine Plasmalogen*

Method	Number of Experiments	% Hydrolyzed**
Present Method	6	90***
2% TCA	5	68
5% HCl	6	72
HCl fumes	9	20

* Supplied by Dr. Lloyd Whiting, Supelco Inc.

** Represented as percentage of total sample hydrolyzed, the true amount of plasmalogen present not being known. The literature gives a value of 85% for this preparation.

*** Standard error of the mean 4.89

minutes, there was 20% hydrolysis or complete destruction of the sample and the results were not reproducible.

The hydrolysis of the vinyl ether to an aldehyde was verified with Schiff reagent using a modification of the procedure described by Hack and Ferrans (6). Following development and drying, the chromatogram was sprayed lightly with Schiff's reagent. After the color development the plate was sprayed with dilute sodium bisuefite solution. The adehyde reacting zone remained unchanged while the other zones and background were bleached.

The method described provides reproducible results with a minimum amount of manipulation for the in situ reaction, followed by separation and direct densitometric quantitation of plasmalogens.

The addition of this method to the previously reported methods (1) for the differentiation of the saturated and unsaturated fatty acid side chains permits in situ analysis of phospholipids. In this way, a direct densitometric method is provided for saturated, unsaturated and the vinyl ether analogues of the various classes of phospholipids.

References

1. J. C Touchstone, S. S. Levin, M.F. Dobbins and P. C. Beers. J. Liquid Chromatogr. 6, 179 (1983).

2. A. Darin-Bennett, A. Poulos, and J. G.White Andrologico 8, 37 (1976).

3. M. Kates Techniques in Lipidology, North Holland/American Elsvier, 1972.

4. L. A. Horrocks and M. Sharma, in J. N. Hawthorne and G.B. Ansell eds. Phospholipids, Elsvier Biochemical Press, 1982, p. 51.

5. L. A. Horrocks, J. Lipid Res., 9, 469 (1968).

6. M.H. Hack and V.J. Ferrans, Z. Physiol. Chem., 315, 157 (1959).

7. V.P. Skipsky and M. Barclay, Methods in Enzymology 14, 530 (1969).

8. H. Goldfine, N. C. Johnson and M. C. Phillips, Biochemistry 20, 2908 (1981).

9. A. Darin-Bennett, I. G. White and D. O. Hoskins, J. Reprod. Fert. 49, 119 (1977).

10. R. W. Evans and B. P. Setchell, J. Reprod. Fert. 57, 189 (1979).

11. D. P. Sclivonchik, P. C. Schmid, V. Natarajan and H. H. O. Schmid, Biochem. Biophys. Acta 618, 242 (1980).