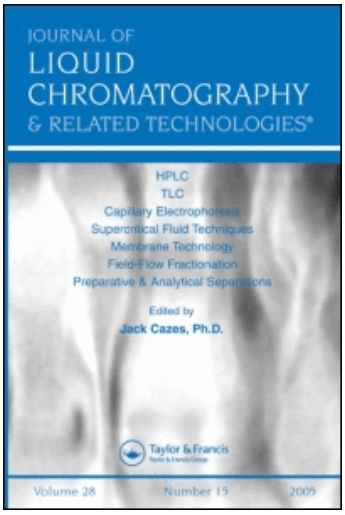

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QUANTITATIVE ANALYSIS OF ALKYLACYL, ALKENYLACYL, AND DIACYL TYPES OF DIGLYCERIDES OBTAINED FROM GLYCEROPHOSPHOLIPIDS

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ABSTRACT

The 1-alkyl-2-acyl, 1-alk-1'-enyl-2-acyl, and 1,2-diacyl-sn-glycerol moieties of choline and ethanolamine glycerophospholipids were converted to the dinitrobenzoyl derivatives. These compounds were separated by high performance liquid chromatography and quantitated by their absorbance at 235 nm. Peak areas were proportional to the amount injected. Separations were optimal for the derivatives from 5 to 30 nmol of the glycerophospholipids. The compositions of ethanolamine glycerophospholipids from rat brain and of choline and ethanolamine glycerophospholipids from human platelets agreed well with previous results obtained with much longer procedures.

INTRODUCTION

The ether-linked glycerophospholipids with alkyl or alk-1-enyl groups instead of an acyl group at the sn-1 position of glycerol have important biological functions as precursors of eicosanoids and platelet activating factor (1, 2). Studies of their composition and metabolism require methods for the separation of the three types of intact glycerophospholipids or of the diradylglycerols (radyl can be acyl, alkenyl, or alkyl) derived from them. Methods for the separation of the intact glycerophospholipids are not available. However, the diradylglycerols obtained by hydrolysis with phospholipase C can be derivatized and then separated by high performance liquid chromatography (3). From 1-50 μmol of the acetyl derivatives were separated into classes on a silica column and each class was then used for the isolation of molecular species. Because the acetyl derivatives were detected by the absorption of the double bonds at 205 nm, only unsaturated molecular species were detected easily and quantitation was by gas-liquid chromatography of the fatty acid methyl ester derivatives. Banzoate derivatives of diradylglycerols were used for the separation of molecular species by Blank et al. (4). These derivatives, including those of saturated compounds, are quantitated by absorbance at 230 nm. Dinitrobenzoate derivatives are generally more useful than benzoate derivatives because the byproducts are easier to remove. While this study was in progress, Kito et al. (5) reported the preparation and separation of dinitrobenzoyl derivatives of diacylglycerols.

MATERIALS AND METHODS

Ratfish liver oil was obtained from Western Chemical Industries, Vancouver, Washington. Soybean lecithin was from Sigma, St. Louis, MO. 3,5-Dinitrobenzoyl chloride and dimethylaminopyridine were from Fluka, Buchs, Switzerland,

pancreatic lipase was from Nutritional Biochemicals, Cleveland, OH, and phospholipase C, Bacillus cereus, was from Boehringer, Mannheim, F.R.G. Organic solvents from Carlo Erba, Milan, Italy were distilled with glass apparatus. Commercial grade pyridine was dried by distillation from KOH, refluxed over p-toluenesulfonyl chloride, then redistilled.

Sample of diradylglycerols were taken to dryness in glass-stoppered tubes, then dissolved in 1 ml of dry pyridine containing 8 μmol 4-dimethylaminopyridine and 43 μmol dinitrobenzoyl chloride. The mixture was heated under nitrogen at 60°C for 2 h. One drop of water was then added, the mixture was again heated for 5 min then the solvent was removed with a rotary vacuum evaporator. The residue was dissolved in 2 ml of chloroform which was washed five times with 2 ml of 0.1% NaHCO3. After removal of the solvent, the residue was dissolved in a convenient volume of hexane (HPLC grade). This procedure is similar to that of Batley et al. (6) for p-nitrobenzoates of diacylglycerols.

A Perkin-Elmer Model 3B HPLC system equipped with a Rheodyne 7125 injector, 50 μl, and a 4.5 x 250 mm Spherisorb type S5W column with a 4.5 x 50 mm Viosil Si60 guard column was used for separations. Peaks were detected at 235 nm with a Perkin-Elmer LC85 variable wavelength detector equipped with Autocontrol and the areas were integrated by a Perkin-Elmer Sigma 10 system. The fraction collector was a 2211 Superrak (LKB). Isocratic elution was with cyclohexane and hexane, 3:1 v/v, containing 5% methyl t-butyl ether and 0.1% 2-propanol at a flow rate of 1 ml/min.

The alkylacylglycerol standard was prepared from ratfish liver oil, a material predominately composed of alkyldiacylglycerols. A mixture of 0.6 ml of the oil, 10.7 ml 1M Tris-HCl, pH 8.0, 0.5 ml 45% $CaCl_2$, 0.2 ml 1% sodium deoxycholate, and 80 mg pancreatic lipase was stirred for 45 min at 40°C. After extraction and washing, the alkylacylglycerols

were purified on a silicic acid column eluted with a gradient of benzene and chloroform.

Ethanolamine glycerophospholipids were isolated from 10 adult rat brains. After extraction, a phospholipid fraction was obtained from a silicic acid column. An alumina column was then used to separate the ethanolamine glycerophospholipids. The composition of this fraction was determined by the two-dimensional TLC and hydrolysis method of *Horrocks and Sun* (7). The remainder of this fraction was used for the preparation of diradylglycerols according to Blank et al. (8). A portion was dissolved in 2 ml of diethyl ether and mixed with 0.7 ml of 50 mM phosphate buffer, pH 7.1. After addition of 2 mg phospholipase C, the mixture was stirred for 3 h. The ether phase and 2 washes with diethyl ether were combined and taken to dryness. These diradylglycerols were derivatized as described above.

Human platelets were prepared by the method of Mustard et al. (9). Choline and ethanolamine glycerophospholipids were obtained by HPLC of the lipid extract by the method of Dugan et al. (10), then hydrolyzed with phospholipase C and derivatized as described above.

RESULTS

The completeness of the reaction was tested with the diacylglycerols from soybean lecithin. With quantities from 1.83 nmol to 3.66 µmol, no underivatized diacylglycerols were visible after TLC separation on silica gel with petroleum ether, diethyl ether, and acetic acid, 80:20:1 by vol. On HPLC, the area of the dinitrobenzoyldiacylglycerol peak at 21.0 min was proportional to the amount of starting diacylglcyerols from 3.6 to 36 nmol with a correlation coefficient of 0.993. The standards of derivatized alkylacylglycerols and alkenylacylglycerols were eluted at 9.1 and 10.0 min respectively. Cholesterol does not interfere

because its derivative eluted at 4.2 min. The relative retention times of the derivatized diradylglycerols varied somewhat according to their source (Table 1).

Two separate experiments were done with ethanolamine glycerophospholipids from rat brain. The amounts used were 0.75, 1.5, 3.0, 9.0, and 12.0 nmol for the first experiment and 0.8, 1.6, 3.2, 4.8, 6.4, 8.0, 12.8, and 16.0 nmol for the second. From 1 to 4 HPLC separations were made of the dinitrobenzoyl-diradylglycerols from each sample (Fig. 1). The correlation coefficients for peak areas were 0.997 for the alkenylacyl-glycerols, 0.996 for the alkylacylglycerols, and 0.998 for the diacylglycerols. The composition obtained by this method agreed well with that obtained by TLC and phosphorus assay and with literature values (Table 2). The results from one assay of the composition of the choline and ethanolamine glycerophospholipids from human platelets are also reported in Table 2.

DISCUSSION

A relatively rapid and quantitative procedure has been described for the analysis of types of glycerophospholipid classes. The previous method with HPLC separation of acetylated diradylglycerols requires quantitation by assay of acyl groups by gas-liquid chromatography (3). Other methods with derivatives of diradylglycerols utilize separation by thin-layer chromatography followed by scraping of the spots, elution, and measurement by spectrophotometry (4,14,15). Previous analyses of glycerophospholipid types have involved differential hydrolysis with cleavage of alkenyl groups by acid and hydrolysis of acyl groups by alkali (7,11,12). If either hydrolysis is incomplete, the values for the alkylacyl or alkenylacyl types may be high. Mueller et al. (13) treated the glycerophospholipid with HCl for 5 min in order

TABLE 1.

Retention Times of Derivatized Diradylglycerols Relative to Alkenylacylglycerols.

Source	Alkylacyl	Diacyl
Standards	0.91	2.09
Platelet ChoGpl	0.95	2.28
Platelet EtnGpl	0.88	2.20
Rat brain EtnGpl	0.90	2.07

Abbreviations: Cho, choline; Etn, ethanolamine; Gpl, glycerophospholipids.

TABLE 2
Composition of Glycerophospholipids from Rat Brain and Human Platelets.

Source	Alkylacyl	Alkenylacyl	Diacyl
	percent		
Rat brain EtnGpl, HPLC	4.5	46.6	48.9
Rat brain EtnGpl, TLC	8.7	44.0	47.0
Rat brain EtnGpl, (11)	7.9	50.0	42.1
Rat brain EtnGpl, (12)	4.2	53.0	42.8
Platelet ChoGpl, HPLC	5.5	6.6	87.9
Platelet ChoGpl, (13)	9.7	8.8	81.8
Platelet ChoGpl, (14)	4.5	1.4	94.1
Platelet EtnGpl, HPLC	0.1	43.5	56.4
Platelet EtnGpl, (13)	3.5	60.4	36.1
Platelet EtnGpl, (14)	1.7	45.3	53.0

Abbreviations as in Table 1.

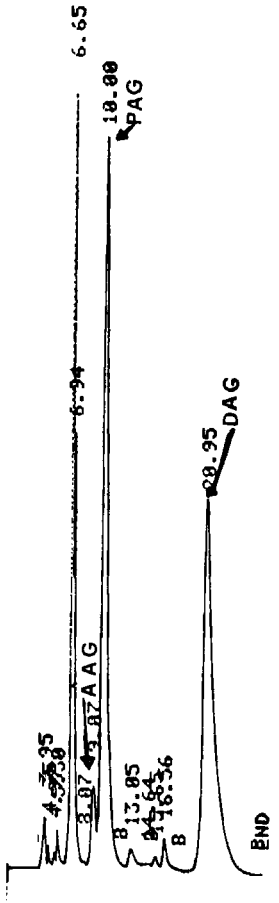

FIGURE 1. Separation of dinitrobenzoyl derivatives of alkylacylglycerols (AAG), alkenylacylglycerols (PAG), and diacylglycerols (DAG) prepared from EtnGpl from rat brain.

to cleave the alkenyl groups from the plasmalogens. With times greater than 1 min some hydrolysis of acyl groups is possible. This could explain the higher values they reported for the plasmalogens. After phospholipase C hydrolysis, they separated the diacylglycerols from the alkylacylglycerols by preparative thin-layer chromatography. Nakagawa and Horrocks (3) found that a similar preparation of alkylacylglycerols contained some diacylglycerols when analyzed by HPLC although the separation by thin-layer chromatography appeared complete. Natarajan et al. (14) reduced the glycerophospholipids with $LiAlH_4$ and assayed the products by gas-liquid chromatography of suitable derivatives. They reported 1.8% of the total phospholipids as lysocholine glycerophospholipids. These probably arose from inadvertent hydrolysis of choline plasmalogens. If they are included in the choline glycerophospholipid composition, the percentage of choline plasmalogens is 5.6, a value in good agreement with our value of 6.6%.

Known standards are necessary for the identification of the peaks for diradylglycerol derivatives from HPLC separations. With the acetates, the order of elution was alkenylacylglycerols, then alkylacylglycerols. This order was reversed with the dinitrobenzoates. The presence of a cyclic hydrocarbon in the eluting solvent is necessary for resolution of the diradyl derivatives. Cyclohexane was used in this study whereas cyclopentane was used with the acetates (3). A different brand of silica column was also used in the present study. The latter is the most likely cause of the reversal of elution order.

The present procedure for the quantitative separation of the diradylglycerol derivatives should be useful for the determination of specific radioactivities for metabolic studies. The separated diradylglycerols can also be used for the separation of molecular species by reverse-phase HPLC.

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