Quantitative analysis of retinal glycerolipid molecular species acetylated by acetolysis

Hong-Gwan Choe,* Rex D. Wiegand, and Robert E. Anderson*

Program in Neuroscience and Cullen Eye Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030*

Summary A method for the quantitative analysis of molecular species of 1,2-diacylglycerol acetates (1,2-DGAC) containing polyunsaturated fatty acids is described. Phosphatidylethanolamine (PE) isolated from frog retina was used to test the method. PE was converted to 1,2-DGAC by acetolysis. The molecular species of the 1,2-DGAC were resolved by reverse-phase high performance liquid chromatography (HPLC), detected by UV absorption spectroscopy at 210 nm, and identified by gas–liquid chromatography (GLC) of the fatty acid methyl esters (FAME). Molar response curves were generated for each DGAC molecular species that eluted as a single entity from HPLC by determining the moles of fatty acids in the molecular species collected and the response (peak area unit) of the UV detector. Each molecular species response curve was linear from about 10 pmoles to 4–8 nmoles, allowing the slope of each curve to be used as a molar absorptivity. This method provides a means for quantification of most of the molecular species of all glycerolipid classes.—**Choe, H-G., R. D. Wiegand, and R. E. Anderson.** Quantitative analysis of retinal glycerolipid molecular species acetylated by acetolysis. *J. Lipid Res.* 1989. **30:** 454–457.

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HPLC has played an important role in the study of the metabolism of glycerolipid molecular species (1). Although some intact phospholipids can be separated into molecular species and quantified (2), the molecular species of phospholipids, in general, can best be resolved by HPLC after removal of the polar head groups and appropriate derivatization (3–5). For example, the diacylglycerols (DG) prepared from phospholipids by phospholipase C treatment can be benzoylated to produce DG benzoates (DGBZ), which can be fractionated into molecular species by HPLC. Each DGBZ molecular species can be quantified by its absorption at 230 nm using the molar absorptivity of the benzoate derivatives (4). However, some phospholipids, such as phosphatidylinositol (PI) and phosphatidic acid (PA), are not readily hydrolyzed by commonly available phospholipase C, so another derivatization procedure must be used. Acetolysis has been shown to directly convert glycerophospholipids into DG acetates (DGAC) (6). The molecular species of DGAC can be detected by measuring the UV absorbance of the double bonds of fatty acids. However, UV detection has not been applied for quantitative analysis, because individual peaks from the HPLC have different molar absorbance responses due to the different number of double bonds, and disaturated molecular species give very low response.

In this report, we present a method for quantification of molecular species of phospholipids containing high contents of polyunsaturated fatty acids that is based on acetolysis followed by HPLC separation and UV absorption spectroscopy. The molar absorptivities of DGAC molecular species were determined individually, to allow conversion of the detector responses (peak areas) to the amounts of molecular species. PE extracted from frog retina was used to verify the method.

MATERIALS AND METHODS

Lipid extraction and isolation

Frog retinas were dissected unded dim red light and placed immediately into chloroform–methanol–conc. HCl 100:100:1 (by vol); ~20 mg retina (wet weight)/ml. Lipids were extracted from the retinal membranes over-

Abbreviations: DG, diacylglycerol; DGAC, diacylglycerol acetates; DGBZ, diacylglycerol benzoates; FAME, fatty acid methyl esters; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; PE, PC, and PI, phosphatidylethanolamine, -choline, and -inositol, respectively; PA, phosphatidic acid; PLC, phospholipase C.

night at −20°C and partitioned into chloroform by the addition of 0.2 volume of 0.2 N HCl. The aqueous phase was discarded and the chloroform layer was washed twice with 0.6 volume of chloroform–methanol–0.2 N HCl 3:48:47 (by vol). The chloroform phase was evaporated under a stream of nitrogen and the lipid was dissolved in chloroform–methanol 2:1 (by vol). The lipid extract was resolved into phospholipid classes by thin-layer chromatography (TLC) as described previously (7). Lipid spots were located by spraying the plate with 0.05% (w/v) 2,7-dichlorofluorescein in aqueous methanol (75%) and viewing under UV. The lipids were extracted from the silica gel with two washes of chloroform–methanol–conc. HCl 100:100:1 (by vol). The lipid extract was separated into two phases by adding 0.2 N HCl. After removing aqueous phase, the chloroform phase was washed as described above.

Acetolysis

Acetolysis of the phospholipid class was carried out as described by Renkonen (6). The lipid extract containing ~300 nmol of PE was evaporated to dryness under a stream of nitrogen, and 2 ml of acetolysis mixture (acetic acid–acetic anhydride 3:2, by vol) was added to start the reaction. The mixture was sealed in a Teflon-lined screw-capped test tube and heated overnight at 145°C. After incubation, the tube was cooled to room temperature and 2 ml of water was added. The reaction products were extracted from the aqueous suspension of the acetolysis mixture three times with 2 ml hexane and applied to a KG silica gel plate (10 × 20 cm, 250 μm, Whatman). 1,2-DGAC were isolated by developing the plate in hexane–ethyl ether 70:30 (by vol); the plate was visualized under UV light as described above. The 1,2-DGAC fraction was scraped from the plate into 2 ml ethanol and mixed vigorously. Two ml water was added, and the suspension was extracted three times with 2 ml hexane and concentrated for subsequent analyses.

Phospholipase C treatment and acetylation

Phospholipids were hydrolyzed with phospholipase C and the resulting diglycerides were acetylated with acetic anhydride in the presence of pyridine as described previously (8).

Reverse-phase HPLC and GLC

The 1,2-DGAC was separated into molecular species by reverse-phase HPLC. A Microsorb C18 column (5 μm, 250 × 4.6 mm, Rainin) was used for separation with the elution solvent system, acetonitrile–isopropanol 70:30 (by vol). The 1,2-DGAC was dried under a flow of nitrogen and the residue was dissolved in 40–60 μl of the elution solvent and injected on the column. The flow rate was 0.5 ml/min and the molecular species were detected by monitoring the absorbance at 210 nm. Each peak was collected for identification and quantification of its species by GLC analysis of the fatty acid methyl ester (FAME) in the presence of an internal standard (heneicosanoic acid, 21:0), as previously described (8).

RESULTS AND DISCUSSION

PE was converted to corresponding 1,2-DGAC by one of two different procedures: acetolysis, under conditions described by Renkonen (6), or enzymatic conversion of PE to DG with phospholipase C and subsequent acetylation with acetic anhydride (8). FAME were prepared from the original PE and from the two 1,2-DGAC preparations and analyzed by GLC. The fatty acid compositions of the three samples were similar (**Fig. 1**), indicating that neither derivatization procedure caused selective changes. In addition, the HPLC profiles of the two DGACs prepared by the two different procedures were also similar (**Fig. 2**). Thus, acetolysis does not selectively alter the fatty acid or molecular species composition of the product 1,2-DGAC, although the acetolysis reaction is carried out at 145°C. Renkonen (6) reported no intermolecular acyl migration in the acetolysis procedure. We verified this by carrying out acetolysis on a mixture of 18:0–18:0 PA and 18:1–18:1 PA, and showing by HPLC that no 18:0–18:1 DGAC was formed during acetolysis (data not shown). In contrast, intramolecular acyl migration, to some small extent, has been reported to occur during acetolysis (9, 10). Similarly, we observed very small amounts of 1,3-DGAC (≤5% of the DGAC) that migrated higher on TLC than

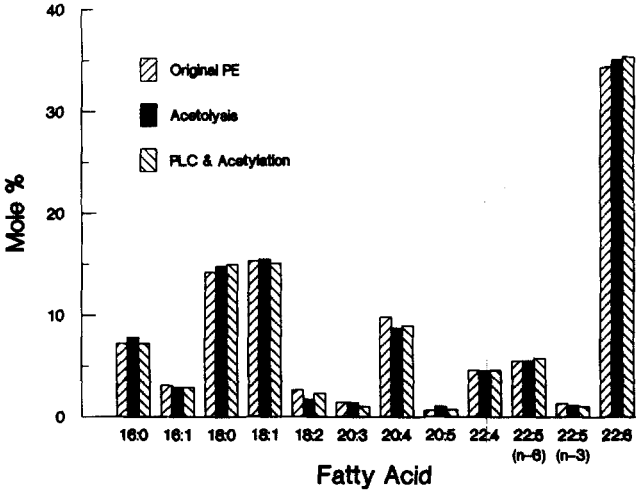

Fig. 1. Comparison of the fatty acid compositions of original PE and 1,2-DGACs prepared by acetolysis or phospholipase C treatment followed by acetylation. PE was prepared from whole retina lipid extracts, and 1,2-DGAC were separated by TLC after acetolysis or acetylation of the PE. Quantitative analysis of fatty acids was accomplished by GLC using known amounts of fatty acids as internal standards (heneicosanoic acid, 21:0). Values are the average of duplicates. Two separate experiments gave similar results.

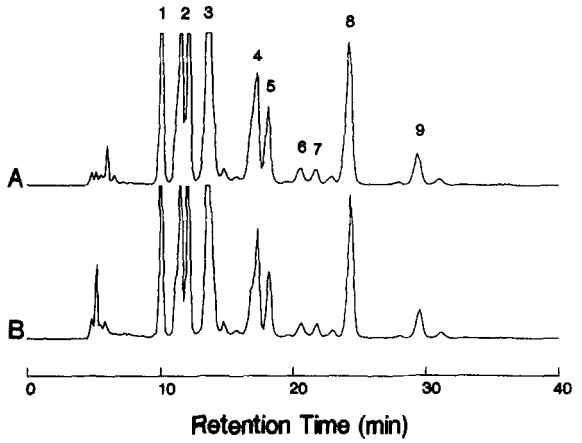

Fig. 2. Comparison of 1,2-DGAC HPLC profiles from retina PE produced by two different procedures: acetolysis (A) and phospholipase C treatment followed by acetylation (B). Both samples were dissolved in 50 μl acetonitrile–isopropanol 70:30, injected on the column, and eluted at 0.5 ml/min flow rate. The molecular species were detected at 210 nm.

did 1,2-DGAC. A larger amount of an unidentified product was found to migrate above the DGACs on TLC; this product was also observed by Renkonen (6). Overall, acetolysis yielded 50–55% recovery of 1,2-DGAC, whereas phospholipase C followed by acetylation yielded 80–85% recovery of 1,2-DGAC. Although the yields of 1,2-DGAC by these two procedures were different, the product of either procedure was representative of the molecular species of the original phospholipid (Figs. 1 and 2).

Nine major peaks from the reverse-phase HPLC profile were collected (Fig. 2A) and the fatty acids comprising the molecular species of each peak were identified by GLC (**Table 1**). It is well known that reverse-phase HPLC resolves the DGAC with respect to length of the carbon chain and the degree of unsaturation. The retention time of molecular species increases with increasing chain length and decreasing number of double bonds. Accordingly, the fatty acids found in each peak were paired to assign a molecular species. Table 1 shows the molecular species composition of each peak. Although the position of the fatty acids was not determined in this study, the sequence of fatty acids in the 1,2-DGAC was assigned according to the positional distribution analysis of PE reported by Wiegand and Anderson (11). Five peaks were found to be composed of a single molecular species that accounted for more than 90% of total fatty acids of each peak.

The relationship between the amounts of each molecular species (determined by GLC analysis) and corresponding peak areas which represent UV absorption of the species at 210 nm is shown in **Fig. 3.** All response curves were linear in the ranges from about 10 pmol to 4–8 nmol of molecular species, so the slopes of each curve were used as molar absorptivities. The molar absorptivity

TABLE 1. Percent distribution of molecular species in each peak

Peak Number	Molecular Species[a]	Percent Distribution[b]	
1	22:6-22:6	96%	(98%)
2	22:5 (n-3)-22:6 20:4-22:6 22:5 (n-6)-22:6		
3	22:4-22:6 18:2-22:6 16:1-22:6		
4	18:1-22:6	95%	(95%)
5	16:0-22:6	95%	(90%)
6	18:1-20:4 16:0-22:5 (n-3)	86% 8%	(89%) (ND)
7	16:0-20:4 18:1-22:5 (n-6)	85% 12%	(76%) (7%)
8	18:0-22:6	92%	(89%)
9	18:0-20:4	98%	(98%)

Each peak from HPLC (as shown in Fig. 2) was collected and subjected to methylation for GLC analysis.

[a]The first number denotes the carbon chain length and the second denotes the number of double bonds in the aliphatic chain.

[b]Values in parentheses are from a second experiment; ND, not detectable.

of each molecular specie showed close correlation with the total number of double bonds (**Fig. 4**). The molar absorptivity of 18:0–20:4 was the lowest among those examined for PE from retina, and was about the same as for DGBZ measured at 230 nm (12). Thus, we conclude that acetolysis is as sensitive as benzoylation for those molecular species containing at least four double bonds. Since the UV absorption is mainly due to the double bonds, it is difficult to quantify small amounts of molecular species containing

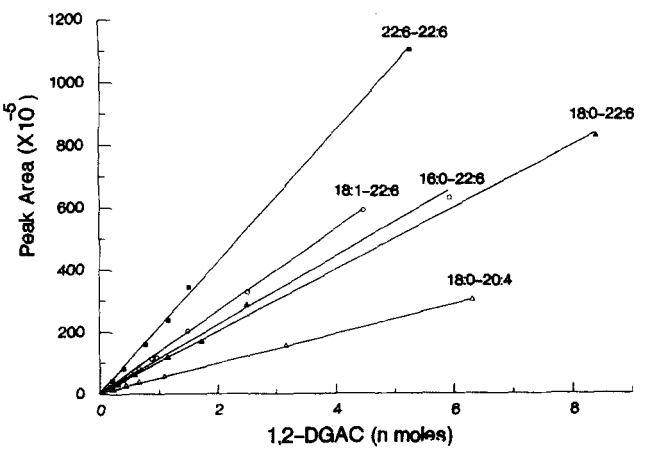

Fig. 3. Proportionality between the amount of each 1,2-DGAC molecular species and its peak area. The amounts of 1,2-DGAC molecular species in stock solutions prepared from retinal PE were determined by GLC analysis three times and averaged. Serial dilutions were made of the 1,2-DGAC stock and injected onto the column.

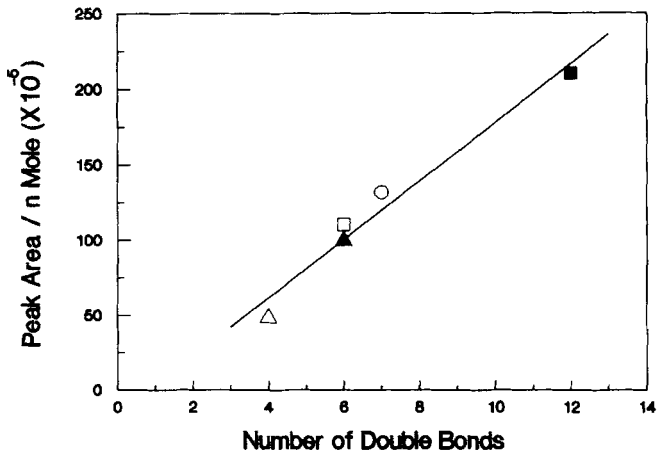

Fig. 4. Relationship between the molar absorptivities for each molecular species and the total number of double bonds. The symbols represent the same molecular species shown in Fig. 3.

few double bonds. This is an important consideration when phospholipids containing high levels of the saturated fatty acids are examined, such as PC from lung. However, this cannot be a drawback in dealing with tissues that have a high content of polyunsaturated molecular species.

In the present study, it was not possible to quantify a mixture of species eluting as a single peak, since each molecular species has a different molar absorptivity. It may be possible to resolve the other dipolyunsaturated species by manipulating the solvent proportions, although this was not attempted.

What is the advantage of acetolysis in quantifying molecular species over other derivatization procedures such as phospholipase C followed by benzoylation (4)? First, acetolysis is a one-step procedure. This may be important when nanomolar amounts of phospholipids are to be analyzed and multiple transfers can result in loss of glycerolipid. Second, acetolysis can be applied to all glycerolipid classes. Only some specific phospholipase C preparations can hydrolyze PI and none hydrolyze PA. The disadvantages of using acetolysis for the quantitative analysis of molecular species are that different molar absorptivities must be determined for each molecular species and that saturated molecular species cannot be measured.

Currently, we are using the acetolysis procedure to investigate the biosynthesis and turnover of phospholipid molecular species in retinal photoreceptor membranes. Following the incubation of a retina with a radioactive precursor such as glycerol, glycerolipids can be isolated, acetylated, fractionated by HPLC, quantified, and collected, and specific radioactivity of molecular species can be determined. Since every phospholipid can be acetylated by this simple procedure, we have a precise way to study the metabolism of selected molecular species of all glycerolipid classes.

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