Separation of alkenylacyl, alkylacyl, and diacyl analogues and their molecular species by high performance liquid chromatography

Yasuhito Nakagawa[1] **and Lloyd A. Horrocks**[2]

Department of Physiological Chemistry, The Ohio State University, Columbus, OH 43210

Abstract High performance liquid chromatography methods were established for separation of alkenylacyl, alkylacyl, and diacyl acetylglycerols derived from ethanolamine glycerophospholipids (EGP) and for separation of the individual molecular species from each of the separated classes. The EGP were isolated from bovine brain, hydrolyzed with phospholipase C, and acetylated with acetic anhydride. The three classes of diradylacetylglycerols were separated quantitatively on a μPorasil silica column. Individual classes were further fractionated on a Zorbax ODS reverse phase column. By gas–liquid chromatographic quantitation of each peak, 29–33 different molecular species were identified within each class. For alkenylacyl-GPE, the major species were 18:1-18:1, 21.8%, and 16:0-18:1, 14.8%. Polyenoic fatty acids predominated at the 2-position of diacyl-GPE. The major species were 18:0-22:6 (n-3), 25.5%, and 18:0-20:4 (n-6), 15.8%. Three species of alkylacyl-GPE, 18:0-22:6 (n-3), 16:0-22:4 (n-6), and 18:0-22:4 (n-6), each accounted for 10%.—**Nakagawa, Y., and L. A. Horrocks.** Separation of alkenylacyl, alkylacyl, and diacyl analogues and their molecular species by high performance liquid chromatography. *J. Lipid Res.* 1983. **24:** 1268–1275.

Supplementary key words ethanolamine glycerophospholipids • bovine brain • phospholipase C • plasmalogen • ether lipids

Ether phospholipids are important components of central nervous system membranes (1), blood cells (2, 3), and many other vertebrate tissues (4); however, little is known about the composition and structure of the molecular species of these lipids. This is because methods for the separation of alkenylacyl, alkylacyl, and diacyl compounds derived from glycerophospholipids and for the separation of individual molecular species of these classes have not been developed sufficiently. The TLC methods presently used have insufficient resolution, poor reproducibility, and require too much time.

HPLC on reverse-phase columns is more effective for the separation of molecular species than argentation TLC because the separation of individual molecular species by HPLC is based not only on the degree of unsaturation of fatty acids, but also on the difference of the carbon chain length. During recent years, a variety of molecular species of phosphoglycerides have been separated by reverse-phase HPLC (5–10). Smith and Jungalwala (5) separated molecular species of choline phosphoglycerides from egg, and bovine and porcine liver by reverse-phase HPLC on a Nucleosil-5-C_{18} column and detected 20–25 different molecular species. Another HPLC method was developed by Patton, Fasulo, and Robins (6) for the separation of choline, ethanolamine, serine, and inositol glycerophospholipids of rat liver using a C_{18}-reverse-phase column. They found that 30–35 different molecular species could be routinely identified and reproducibly quantitated. HPLC is a very useful technique for detailed analysis of molecular species of glycerophospholipids; however, these previous works were carried on without distinguishing between alkenylacyl, alkylacyl, and diacyl classes. In one study, Curstedt (7) described the separation of alkenylacyl, alkylacyl, and diacyl analogues and then the partial separation of molecular species of analogues of ether glycerophospholipids from the diradylacetylglycerols derived from beef brain and rat liver choline glycerophospholipids. Very long times were required for the analyses because detection was by collection of fractions and assay by GLC of fatty acid methyl esters.

This study demonstrates the utilization of HPLC to provide a rapid and efficient method for separating the alkenylacyl, alkylacyl, and diacyl analogues derived

Abbreviations: EGP, ethanolamine glycerophospholipid; GPE, glycerophosphoethanolamine; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; RRT, relative retention time. The fatty acids are identified by the number of carbon atoms and double bonds. Thus, 18:1 represents oleic acid which contains 18 carbon atoms and one double bond. The molecular species of glycerophospholipids are identified by the fatty chains at 1-position (left-hand side) and the fatty acids at the 2-position (right-hand side) positions of the glycerol moiety. Thus, 16:0-18:1 represents the 1-palmitoyl, 2-oleoyl molecular species of a glycerophospholipid.

[1] Present address: Faculty of Pharmaceutical Science, Teikyo University, Sagamiko, Tsukuigun, Kanagawa 199-01, Japan.

[2] To whom correspondence should be addressed.

from bovine brain ethanolamine glycerophospholipids by normal phase HPLC. In addition, we have developed reverse-phase HPLC procedures that separate most of the molecular species of alkenylacyl, alkylacyl, and diacyl analogues. We have identified 29–33 molecular species in each class.

MATERIALS AND METHODS

Lipids were extracted from fresh bovine brain by the method of Bligh and Dyer (11) and ethanolamine glycerophospholipids were isolated by diethylaminoethyl cellulose (DEAE-cellulose) column and subsequent silicic acid column chromatography (12). The isolated ethanolamine glycerophospholipids gave a single spot on TLC. Alkenylacyl, alkylacyl, and diacyl analogues were prepared from ethanolamine glycerophospholipids as described previously (13). Purified ethanolamine glycerophospholipids (1–3 mg) were incubated for 6 hr at room temperature with 1 mg of crude phospholipase C obtained from *Bacillus thuringiensis* (IMA12077); bacterial incubation conditions and the crude enzyme preparation were as previously described for *B. cereus* phospholipase C (14). The completeness of the reaction (lack of glycerophospholipids at the origin) was checked by TLC using petroleum ether–diethyl ether–acetic acid (60:40:1 (by vol) as solvent. The mixture of diradylglycerols was acetylated with acetic anhydride and pyridine at 37°C for 3 hr. The solution was taken to dryness and dissolved in hexane for the separation of alkenylacyl, alkylacyl, and diacyl analogues by HPLC.

The HPLC separations were performed with two solvent delivering systems (Model 100, Altex Scientific Co. Berkeley, CA), injection system (Model 210, Altex), a column block heater (Jones Chromatography Co., Columbus, OH), and a variable wavelength detector (LC-75, Perkin-Elmer, Norwalk, CT) which was operated at 205 nm. A microprocessor (Model 420, Altex) was used to control the pumps and recorder (Model BD-40, Kipp and Zonen, Delft, Holland). The acetylated lipids (1–50 μmol) dissolved in 20 μl of hexane were separated into alkenylacyl, alkylacyl, and diacyl classes by chromatography on a 3.9 mm × 30 cm μPorasil column (Waters Associates, Inc., Milford, MA). The solvent system was cyclopentane–hexane–methyl-t-butyl ether–acetic acid 73:24:3:0.03 (by vol) pumped at a flow rate of 2 ml/min at 36°C. After collection of each peak of the acetylated lipids, molecular species were separated by reverse-phase HPLC on a 4.6 mm × 25 cm Zorbax ODS column (DuPont Co., Wilmington, DE). Alkenylacyl and alkylacyl analogues were eluted with acetonitrile–2-propanol–methyl-t-butyl ether–water 63:28:7:2 (by vol). For the separation of molecular species of the diacyl analogue, the solvent system was acetonitrile–2-propanol–methyl-t-butyl ether–water 72:18:8:2 (by vol). Flow rates were 0.5 ml/min and the column temperature was 33°C.

Fractions from the column were collected for the identification and quantitation of each peak by GLC analysis. Fatty acids were analyzed at 195°C using a 428 gas chromatograph (Packard, Downers Grove, IL) equipped with a glass column (6 ft × ⅛ inch) packed with 10% Alltech CS-10 on Chromosorb W (Alltech Associates, Deerfield, IL), after transmethylation with 0.5 N NaOH in methanol for 30 min at room temperature. To determine the alkenyl group compositions, the alkenylacyl analogues were treated with HCl gas and the liberated aldehydes were analyzed at 170°C with a column (6 ft × ⅛ inch) packed with 10% Apiezon L on Anakrom AS (Analabs, Inc., North Haven, CT) according to the method of Ferrell, Radloff, and Radloff (15). Bistrimethylsilyl derivatives were prepared from the deacylated alkylacyl analogue for the determination of the alkyl group composition at the 1-position (13). The alkylacyl analogues collected from the column were hydrolyzed with 0.5 N NaOH containing 90% methanol at 37°C for 90 min. The resulting alkylglycerols were purified by TLC on silica gel G developed with petroleum ether–diethyl ether–acetic acid 30:70:1 (by vol). The alkylglycerols were reacted with hexamethyldisilazane and trimethylchlorosilane in pyridine for 5 min to make bistrimethylsilyl derivatives which were analyzed at 275°C using a column packed with 10% Apiezon L on Anakrom AS. The quantities of alkenylacyl, alkylacyl, and diacyl analogues were determined by GLC of the fatty acid methyl esters; 17:0 was used as an internal standard. Integration of peak areas was performed with software and an A/D interface from Nelson Analytical (Cupertino, CA) on an HP-85 desk-top computer (Hewlett-Packard, Palo Alto, CA).

HPLC-grade organic solvents were purchased from EM Science (Gibbstown, NJ) except methyl-t-butyl ether which was purchased from Burdick & Jackson Labs. (Muskegon, MI). Distilled water was purified using a Milli-Q system plus an Organex-Q cartridge (Millipore Co., Bedford, MA). Any remaining organic materials were removed with an Organic-pure water purifier (Barnstead, Boston, MA). Aqueous and organic solutions were filtered with 0.22 μm GS and 0.5 μm FH, respectively, (Millipore Co.) just prior to use.

RESULTS

Separation of alkenylacyl, alkylacyl, and diacyl analogues with normal-phase HPLC

Binary mixtures of hexane with 2-propanol, methanol, or acetonitrile were not able to separate the mixture

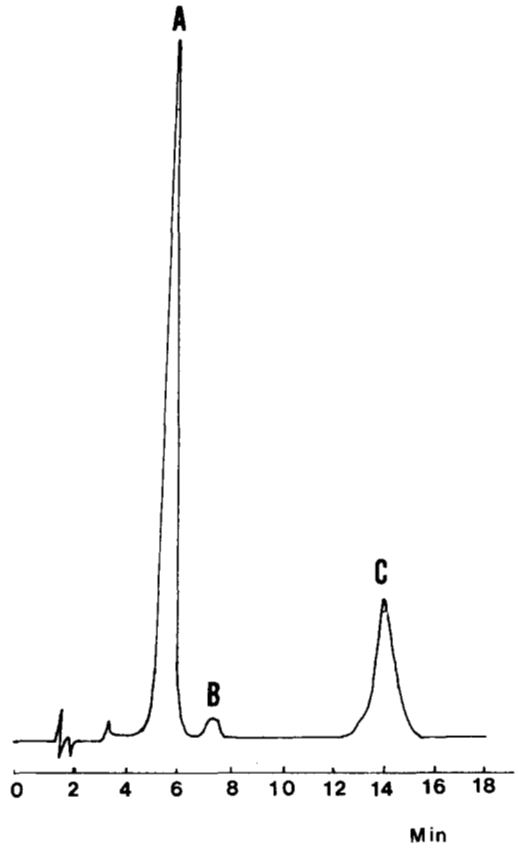

Fig. 1. Separation of alkenylacyl (A), alkylacyl (B), and diacyl (C) analogues by normal-phase HPLC on a μPorasil column. The solvent mixture was cyclopentane–hexane–methyl-t-butyl ether–acetic acid 73:24:3:0.8 (by vol) at a flow rate of 2 ml/min. Column temperature was 36°C. The sample (0.2 μmol) was dissolved in 20 μl of hexane and injected. Detection was by absorption at 205 nm. The full-scale absorbance was 0.64.

of alkenylacyl, alkylacyl, and diacyl analogues with normal-phase HPLC. An isocratic mobile phase containing cyclopentane–hexane–methyl-t-butyl ether–acetic acid was successful for the baseline separation of alkenylacyl, alkylacyl, and diacyl analogues within 16 min (**Fig. 1**). Broadening of peaks due to differences in chain length and degree of unsaturation within each class was minimal with these conditions. The isomeric 1,3-diacyl-2-acetylglycerol could be separated from 1,2-diacyl-3-acetylglycerol. The retention time of the former compound was 10 min with these conditions. Collected fractions were identified by TLC using known standards of 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol, and 1,2-diacyl-3-acetylglycerol as described previously (13). Recoveries were essentially complete for HPLC on the μPorasil silica column with an average of 99.5 ± 1.5 (SD) % as determined by GLC. The relative amounts of alkenylacyl, alkylacyl, and diacyl analogues in bovine brain ethanolamine glycerophospholipids separated with HPLC were 68.8 ± 0.4%, 3.5 ± 0.9%, and 27.7 ± 0.6%, respectively. The results with TLC separation were alkenylacyl, 66.7 ± 3.2%; alkylacyl, 5.2 ± 2.9%; and diacyl, 28.1 ± 2.1%. Analysis by normal-phase HPLC of analogues separated by TLC showed the presence of some impurities particularly in the 1-alkyl-2-acyl-3-acetylglycerol fraction. No cross-contamination of fractions separated by HPLC could be detected by rechromatography by normal-phase HPLC. Thus, the small differences between HPLC and TLC values are due to cross-contamination of TLC fractions. No significant differences in the fatty acid compositions of alkenylacyl, alkylacyl, and diacyl analogues were

TABLE 1. Comparison of fatty acid compositions of alkenylacyl, alkylacyl, and diacyl analogues after normal-phase HPLC, TLC, and reverse-phase HPLC[a]

	Alkenylacyl			Alkylacyl			Diacyl		
	HPLC	TLC	RPHPLC	HPLC	TLC	RPHPLC	HPLC	TLC	RPHPLC
16:0	2.6 ± 1.2	3.2 ± 1.1	1.9 ± 0.4	5.2 ± 1.9	5.2 ± 0.3	3.6 ± 0.3	7.6 ± 0.8	7.0 ± 1.0	6.6 ± 0.4
18:0	0.9 ± 0.9	0.7 ± 0.4		0.6 ± 0.5	1.0 ± 0.6		31.6 ± 1.2	29.6 ± 2.9	32.6 ± 0.9
18:1	40.5 ± 3.3	38.8 ± 2.4	38.0 ± 3.2	15.7 ± 2.8	16.5 ± 1.8	16.9 ± 0.4	21.8 ± 1.9	23.6 ± 1.8	21.6 ± 0.7
18:2				1.8 ± 0.6	1.7 ± 0.2	1.2 ± 0.2			
20:1	9.3 ± 0.7	9.1 ± 0.3	6.0 ± 0.9	15.7 ± 1.8	15.0 ± 0.9	14.3 ± 0.7	2.6 ± 0.9	3.0 ± 0.7	1.4 ± 0.2
20:3	1.7 ± 1.0	2.5 ± 0.8	2.6 ± 0.7	2.5 ± 1.0	2.3 ± 0.2	2.1 ± 0.1	0.9 ± 0.2	1.4 ± 0.4	2.8 ± 0.4
20:4	11.2 ± 0.8	11.8 ± 0.5	12.4 ± 0.2	7.1 ± 1.7	8.3 ± 1.7	4.9 ± 0.5	9.5 ± 1.1	9.4 ± 0.2	10.1 ± 0.4
22:4	13.6 ± 0.4	14.1 ± 0.9	16.1 ± 1.4	23.3 ± 2.4	21.5 ± 2.9	25.5 ± 1.2	4.7 ± 0.3	5.3 ± 0.7	5.9 ± 0.6
22:5	2.2 ± 1.0	2.6 ± 1.2	2.9 ± 0.2	4.7 ± 1.2	6.1 ± 1.8	5.1 ± 0.4	2.1 ± 0.2	2.8 ± 0.5	2.5 ± 0.2
22:6	13.1 ± 0.7	13.4 ± 0.3	16.3 ± 0.1	19.0 ± 1.6	17.4 ± 3.0	22.0 ± 1.2	16.9 ± 2.5	16.5 ± 0.8	16.5 ± 0.9
X[b]	4.6 ± 0.2	4.4 ± 0.4	3.9 ± 0.9	3.9 ± 0.5	4.0 ± 0.2	4.3 ± 0.3	1.0 ± 0.2	1.4 ± 0.2	0.7 ± 0.4

[a] Values are expressed as the mean percentage (mol%) ±SD, n = 5 for normal-phase HPLC; n = 3 for TLC; and n = 3 for reverse-phase HPLC. The latter was calculated from the composition of molecular species reported in Table 2.

[b] X is an unknown fatty acid. The methyl ester of the unidentified fatty acid has the same retention time as 20:5 (n-6) methyl ester with GLC but has 22 carbon atoms. On reverse-phase HPLC (23), it is eluted just before 22:3 (n-3). This fatty acid was not further characterized.

found by GLC for HPLC separation as compared with TLC separation (**Table 1**). Also, the fatty acid compositions of alkenylacyl and diacyl analogues were in good agreement with previous results (16).

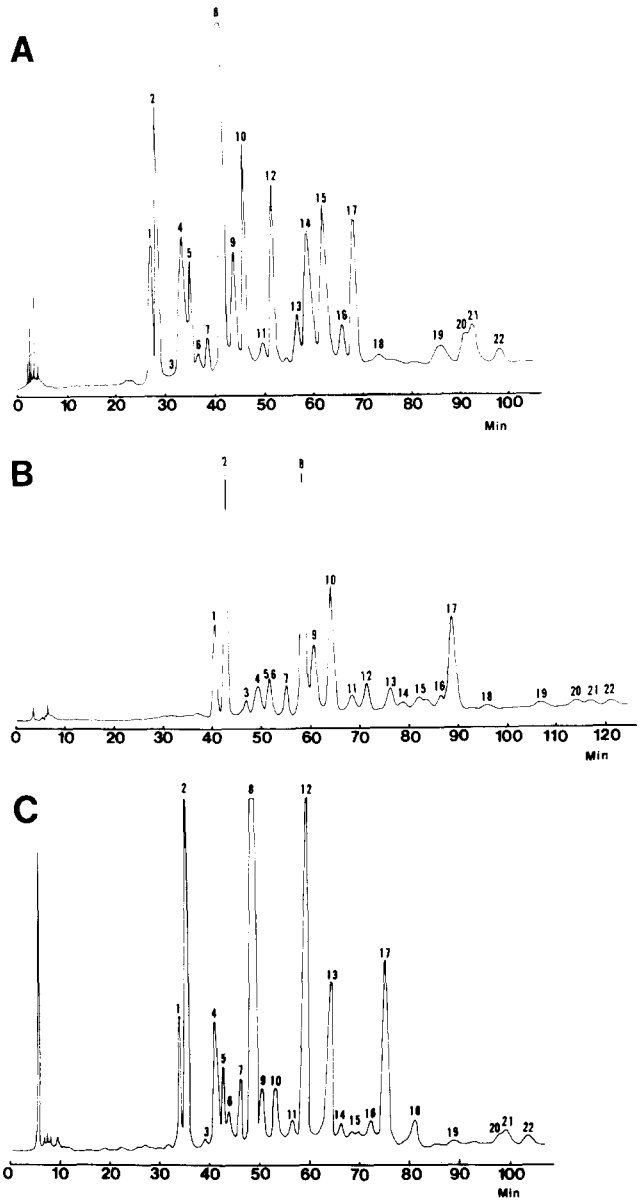

Fig. 2. HPLC separation of molecular species of alkenylacyl (A), alkylacyl (B), and diacyl (C) analogues. Alkenylacyl (1.1 μmol), alkylacyl (0.6 μmol), and diacyl (0.9 μmol) analogues were dissolved in 20 μl of acetonitrile, injected, and chromatographed on a Zorbax ODS reverse-phase column at a flow rate of 0.5 ml/min. The solvent mixtures were acetonitrile–2-propanol–methyl-t-butyl ether–water 63:28:7:2 for the separation of alkenylacyl and alkylacyl analogues and 72:18:8:2 for the separation of diacyl analogues. Column temperature was 33°C. Detection was by absorption at 205 nm with full-scale absorbance of 2.56. The molecular species were identified and quantitated by GLC analysis as described in Materials and Methods. The peak numbers correspond to those in Table 2.

TABLE 2. Distribution of molecular species of alkenylacyl, akylacyl, and diacyl analogues of bovine brain[a]

Peak Number[b]	Probable Molecular Species[c]	Alkenylacyl	Alkylacyl	Diacyl
1	18:1-22:6 (n-3)	2.7 ± 0.4	3.0 ± 0.2	1.7 ± 0.2
2	16:0-22:6 (n-3)	4.7 ± 0.3	8.8 ± 0.6	5.5 ± 0.4
3	18:1-22:5 (n-3)	0.1 ± 0.7	0.3 ± 0.1	0.1 ± 0.1
4	18:1-20:4 (n-6)	4.7 ± 0.4	1.1 ± 0.1	2.9 ± 0.3
	16:0-22:5 (n-3)	0.3 ± 0.1	0.7 ± 0.1	0.1 ± 0.1
5	16:0-20:4 (n-6)	2.8 ± 0.1	1.6 ± 0.2	1.1 ± 0.2
6	18:1-22:5 (n-6)	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
7	16:0-22:5 (n-6)	0.9 ± 0.1	1.4 ± 0.1	0.8 ± 0.1
8	18:0-22:6 (n-3)	8.7 ± 1.1	10.2 ± 0.6	25.5 ± 0.5
	18:1-20:3 (n-6)	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
	18:1-18:2 (n-6)		0.3 ± 0.1	
9	18:1-22:4 (n-6)	4.0 ± 0.9	5.1 ± 0.3	1.3 ± 0.1
	16:0-20:3 (n-6)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
	16:0-18:2 (n-6)		0.5 ± 0.2	
10	16:0-22:4 (n-6)	7.3 ± 0.6	10.1 ± 0.7	1.2 ± 0.2
	18:1-20:3 (n-9)	0.6 ± 0.3	0.4 ± 0.1	0.6 ± 0.1
11	18:0-22:5 (n-3)	0.3 ± 0.1	0.8 ± 0.2	0.3 ± 0.1
	16:0-20:3 (n-9)	0.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
12	18:0-20:4 (n-6)	4.8 ± 0.2	2.2 ± 0.3	15.8 ± 0.5
13	18:0-22:5 (n-6)	0.8 ± 0.1	1.3 ± 0.1	3.0 ± 0.3
14	18:1-18:1	21.8 ± 0.2	5.5 ± 0.2	10.1 ± 0.8
15[d]	16:0-18:1	12.9 ± 1.1	6.8 ± 0.2	5.0 ± 0.9
	16:0-16:0	1.9 ± 0.3	3.6 ± 0.2	
	18:1-X		1.6 ± 0.1	0.3 ± 0.1
	18:0-18:2 (n-6)		0.4 ± 0.1	
16	18:0-20:3 (n-6)	0.3 ± 0.1	0.3 ± 0.1	0.9 ± 0.2
	16:0-X	1.5 ± 0.3	1.7 ± 0.1	0.3 ± 0.1
17	18:0-22:4 (n-6)	4.7 ± 0.2	10.3 ± 0.6	8.6 ± 0.9
18	18:0-20:3 (n-9)	0.6 ± 0.2	0.3 ± 0.1	1.6 ± 0.2
19	18:1-20:1	3.4 ± 0.2	5.7 ± 0.2	2.2 ± 0.1
20	16:0-20:1	3.0 ± 0.6	8.6 ± 0.3	0.6 ± 0.1
21	18:0-18:1	3.7 ± 0.6	4.5 ± 0.1	8.5 ± 0.5
22	18:0-X	0.7 ± 0.4	1.0 ± 0.1	0.8 ± 0.2
Recovery		98.3 ± 2.1	94.5 ± 3.6	97.6 ± 6.7

[a] Values are means ± SD; n = 3 for alkenylacyl; n = 6 for alkylacyl; and n = 4 for diacyl.
[b] Peak numbers correspond to the numbers of peaks in Fig. 2.
[c] For diacyl analogues, it is assumed that the less saturated acyl group is at the 1-position.
[d] For alkylacyl and diacyl analogues, peak 15 includes two partially resolved peaks. The molecular species 18:1-16:0 is probably also present in this mixture.

Separation of alkenylacyl, alkylacyl, and diacyl analogues into molecular species with reverse-phase HPLC

Alkenylacyl, alkylacyl, and diacyl analogues were each resolved into 22 separate peaks by reverse-phase HPLC (**Fig. 2**). We detected 29–33 different molecular species of alkenylacyl, alkylacyl, and diacyl analogues (**Table 2**). The retention time of each molecular species was longer with increasing chain length, and shorter with increasing degree of unsaturation of fatty chains (**Fig. 3**). With the present HPLC system, some molecular species with the same number of carbon atoms and double bonds could be separated, such as 18:0-22:6

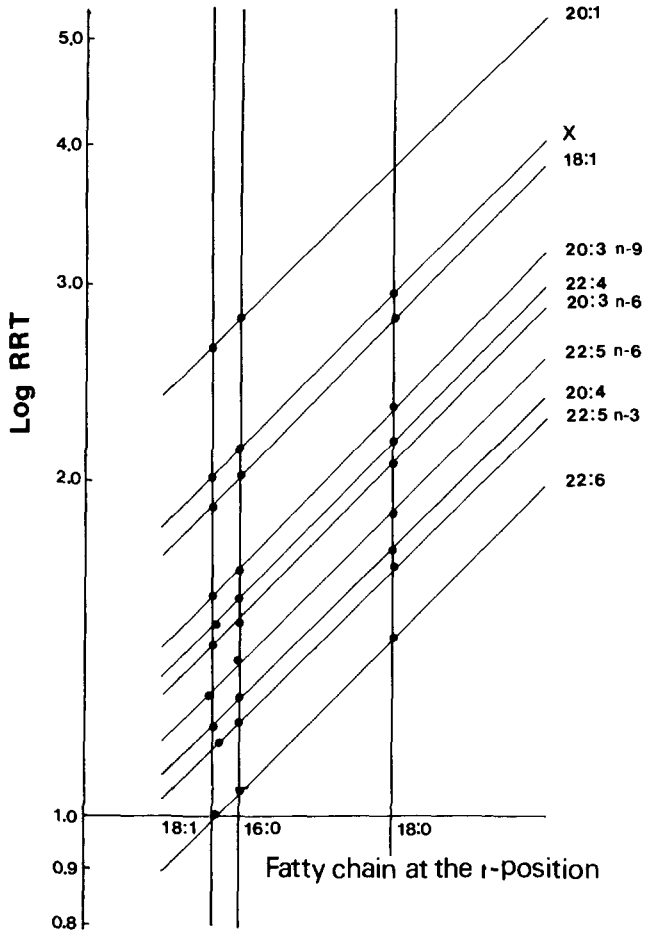

Fig. 3. Relationships between fatty acid compositions of molecular species of alkenylacyl and alkylacyl analogues and the retention time. Relative retention times (RRT) were calculated by dividing the retention time of each molecular species by the retention time of 18:1-22:6 molecular species (6). RRT values for molecular species containing 16:0 and 18:0 at the 1-position were plotted and lines were drawn to connect the points for molecular species having the same fatty acid at the 2-position. RRT values for molecular species containing 18:1 at the 1-position were plotted on the parallel lines that have the same fatty acid at the 2-position. A vertical line was drawn through these points parallel to the ordinant.

from 18:1-22:5 and 18:0-18:2 from 18:1-18:1. Furthermore, molecular species containing 22:5 (n-3) eluted earlier and were resolved from similar molecular species containing 22:5 (n-6). A similar resolution was observed for the molecular species containing 20:3 (n-6) and (n-9). In addition, 18:0-20:4 was separated from 16:0-22:4.

All three classes of ethanolamine glycerophospholipids contain nearly the same molecular species, but the quantitative distributions of molecular species are clearly different for each class (Table 2). The 18:1-18:1 and 16:1-18:1 molecular species account for 22 and 13%, respectively, of the alkenylacyl analogue. On the other hand, only 4% of the 18:0-18:1 molecular species was found in the alkenylacyl analogue. The major molecular species in the diacyl analogue are combinations of highly polyunsaturated fatty acids at the 2-position with stearate at the 1-position, such as 18:0-22:6, 26%; 18:0-22:4, 9%; and 18:0-20:4, 16%. No molecular species of the alkylacyl analogue, in contrast to the other two analogues, account for more than 10% of the total, which is the proportion of the 18:0-22:6, 18:0-22:4, and 16:0-22:4 molecular species. The recoveries of alkenylacyl, alkylacyl, and diacyl analogues after the separation of molecular species with HPLC were greater than 94% as calculated from the fatty acid determinations by GLC (Table 2).

The fatty acid compositions of alkenylacyl, alkylacyl, and diacyl analogues calculated from the molecular species composition in Table 2 are approximately identical to those in the original unfractionated samples (Table 1). The lower percentage of 20:1 in alkenylacyl analogues is probably due to nonrecovery of the 18:0-20:1 molecular species in the present analysis. Saturated molecular species other than 16:0-16:0 may have escaped detection because their absorbancies are relatively low at 205 nm. If desired, such species can be collected and assayed by GLC.

Distinct differences were found in the proportions in which the unsaturated fatty acids at the 2-position are combined with the fatty chains at the 1-position (**Fig. 4**). In the alkenylacyl analogue, 16:0 and 18:1 fatty chains at the 1-position were combined primarily with 18:1 at the 2-position (40–50%). In contrast, the molecular species with 18:0 alkenyl groups at the 1-position contained little 18:1 fatty acid but significant amounts of 22:6 and 20:4 fatty acids. In the diacyl analogue, 16:0 fatty acid at the 1-position is paired mainly with 22:6 and 18:1 fatty acids, which accounted for nearly 75% of the total molecular species with 16:0 fatty acid at the 1-position. The 18:0 fatty acid at the 1-position is preferentially combined with 22:6 and 20:4, which constituted about 40% and 25%, respectively. Nearly 50% of total fatty acids at the 2-position are 18:1 when 18:1 fatty acid is at the 1-position, in contrast to the low proportion of 18:0-18:1 molecular species. The distribution of fatty acids at the 2-position in molecular species of the alkylacyl analogue is similar to that in the alkenylacyl and diacyl analogues. The 18:0 alkyl groups are paired mainly with 22:6, and 18:1 alkyl groups are preferentially associated with 18:1 at the 2-position. The most plentiful fatty acid at the 2-position of alkylacyl analogue, 22:4, was not combined preferentially with any alkyl group at the 1-position.

DISCUSSION

Intact alkenylacyl, alkylacyl, and diacyl classes of ethanolamine glycerophospholipids have not been sepa-

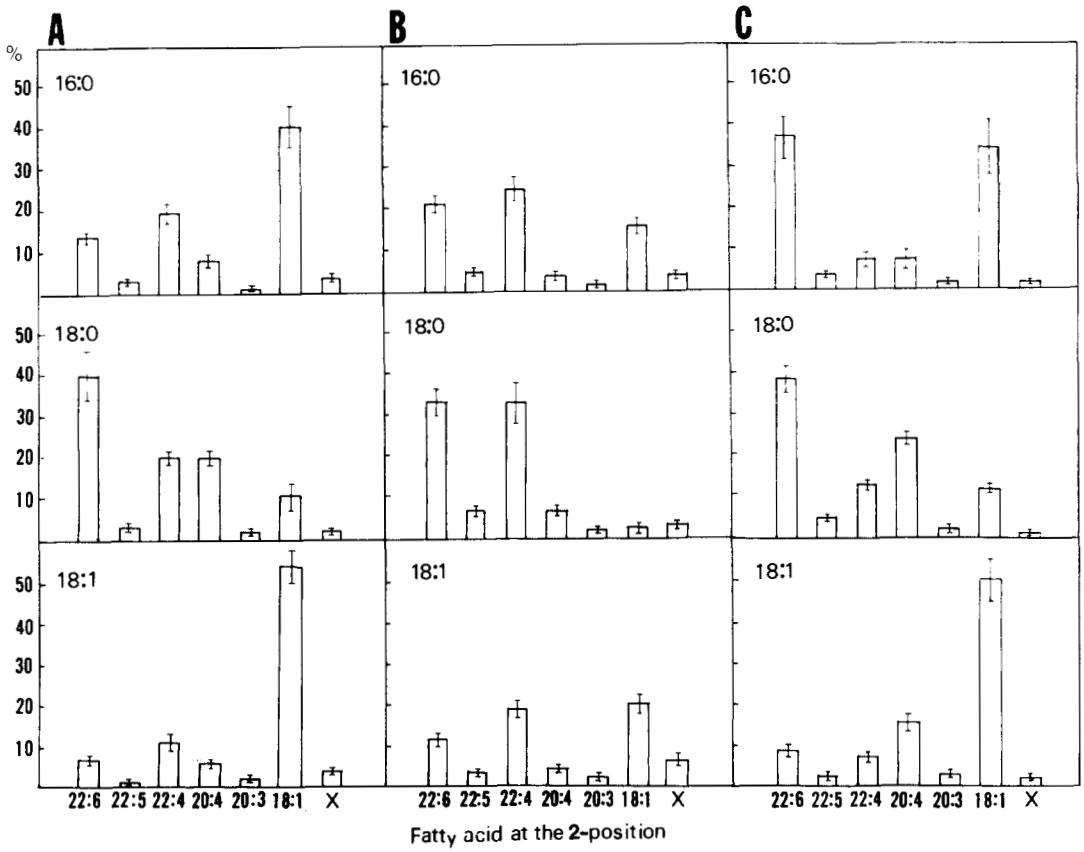

Fig. 4. Distribution of fatty acids at the 2-position of alkenylacyl (A), alkylacyl (B), and diacyl (C) analogues containing 16:0, 18:0, and 18:1 fatty chains at the 1-position. The distribution was calculated from the percent distribution of molecular species in Table 2. The percentage for a molecular species with a particular fatty acid at the 2-position was divided by the total percentage for all molecular species containing the same fatty chain at the 1-position. Numbers in inset show the side chain at the 1-position.

rated by HPLC. However, after removal of the polar head group by phospholipase C and acetylation with acetic anhydride, the resulting 1-alkenyl-2-acyl-, 1-alkyl-2-acyl-, and 1,2-diacyl-3-acetylglycerols can be separated by HPLC or TLC methods (13). The uniqueness of the present method is the solvent mixture of cyclopentane–hexane–methyl-t-butyl ether–acetic acid 73:24:3:0.8 (by vol). A cyclic hydrocarbon is required in the solvent mixture for resolution of alkenylacyl, alkylacyl, and diacyl analogues in a short time. The method of Curstedt (7) is also effective for the complete separation of the alkenylacyl, alkylacyl, and diacyl glycerols, but it has several disadvantages. First, the inclusion of an aromatic solvent in their solvent system precludes the use of absorbance at 205 nm to monitor the separation. Although the absorbance cannot be used directly for quantitation, it is sensitive, convenient, and nondestructive. Second, the total separation time for the previous method was about 15 hr. This time is too long to detect small amounts of lipids. Our procedure separates all three compounds completely within 16 min and is highly reproducible. The recovery of alkenylacyl, alkylacyl, and diacyl analogues after chromatography was quantitative and the fatty acid composition of the lipid classes was not changed by HPLC. Since it is necessary or very desirable to know the fatty acid composition of the separated lipids, quantitation was by GLC.

The separation of molecular species of alkenylacyl, alkylacyl, and diacyl analogues by reverse phase HPLC was also developed. Each class was resolved into 22 separate peaks which allowed identification of a total of 91 different probable molecular species of ethanolamine glycerophospholipids. The retention times of molecular species of lipids were dependent on the chemical linkage at the 1-position of the glycerol backbone. Diacyl analogues were eluted from the reverse-phase column considerably faster than the corresponding alkenylacyl and alkylacyl analogues having an ether-linkage at the 1-position. The difference in retention times was so great that a lower concentration of 2-propanol was required for the diacyl analogue than for ether-linkage analogues. Within each class, the order of elution of the individual molecular species was the same and dependent on the nature of hydrocarbon side chains.

Molecular species are eluted from the reverse-phase column in order of increasing degree of unsaturation and decreasing chain length (5). Thus, the retention time decreased as the number of double bonds in the fatty acid increased, e.g., the series 18:0-22:4, 18:0-22:5, and 18:0-22:6, and also increased with increased chain length of the fatty acid, e.g., 18:0-20:4 and 18:0-22:4. Furthermore, we observed that some molecular species having the same number of carbon atoms and double bonds could be separated by reverse-phase HPLC. The position of the double bond influences the separation of molecular species. For example, 18:0-22:5 (n-3) eluted before 18:0-22:5 (n-6) and 18:1-18:1 was eluted before 18:0-18:2. Separations are also influenced by the position of carbon atoms in isomeric molecular species. For example, 18:0-20:4 and 16:0-22:4 are well resolved. Since the introduction of a double bond or additional carbon atoms into the side chain at the 1-position has a greater effect on the capacity factor than the same change in the fatty acid at the 2-position, the side chain at the 1-position has a greater interaction with the stationary phase of the column than does the fatty acid at the 2-position.

The distributions of individual molecular species in the alkenylacyl and alkylacyl analogues were clearly different. By the accepted pathway of alkenylacyl ethanolamine glycerophospholipid biosynthesis, they might be expected to possess a composition of molecular species identical to the direct precursor, the alkylacyl ethanolamine glycerophospholipids (4, 17). The different composition may result from a different pathway of alkenylacyl biosynthesis (4, 18) or from specificity of enzymes for specific molecular species of alkylacyl ethanolamine glycerophospholipid for desaturation or for turnover of fatty acids at the 2-position.

The distribution of fatty acids at the 2-position was distinctly different for molecular species containing 16:0, 18:0, or 18:1 side chains at the 1-position of alkenylacyl, alkylacyl, and diacyl analogues. The characteristic localization of 18:1 at the 2-position with 16:0 at the 1-position was reported previously (7, 19–22). Yeung and Kuksis (19) determined the mass distribution of individual molecular species of diacyl and alkenyl acyl ethanolamine glycerophospholipids of dog kidney by using enzymic treatment and TLC methods. Monoene species of diacyl compounds contained mainly 16:0-18:1 molecular species (about 90%) and only 10% 18:0-18:1 molecular species. The content of 16:0-18:1 in monoene species of alkenylacyl analogues was also about four times as much as that of 18:0-18:1 molecular species. The major monoene component of ethanolamine glycerophospholipids of rat liver was 16:0-18:1 molecular species (20).

The methodology reported in this paper will be useful for the investigation of the composition and metabolism of individual molecular species of glycerophospholipids and will provide new insights into the formation and degradation of different molecular species of glycerophospholipids in various tissues. The mild conditions and quantitative recoveries make the methods particularly well-suited for the study of polyunsaturated fatty acid metabolism. For example, if labeled arachidonate is the precursor, the metabolism of 16:0-20:4, 18:0-20:4, 18:1-20:4, and corresponding 22:4 molecular species can all be studied separately.

This study was supported in part by Research Grants NS-08291 and NS-10165 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received 4 February 1983 and in revised form 26 May 1983.

REFERENCES

1. Sun, G. Y., and A. Y. Sun. 1972. Phospholipids and acyl groups of synaptosomal and myelin membranes isolated from the cerebral cortex of squirrel monkey. *Biochim. Biophys. Acta.* **280:** 306–315.
2. Rogier, S. V. 1980. Gas chromatographic determination of the fatty acid pattern of red cell membrane plasmalogens in healthy children. *J. Chromatogr.* **182:** 27–33.
3. Sugiura, T., Y. Masuzawa, and K. Waku. 1980. Alkenyl and alkyl ether phospholipids in pig mesenteric lymph node lymphocytes. *Lipids.* **15:** 475–478.
4. Horrocks, L. A. 1972. Content, composition and metabolism of mammalian and avian lipids that contain ether groups. *In* Ether Lipids; Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 177–272.
5. Smith, M., and F. B. Jungalwala. 1981. Reversed-phase high performance liquid chromatography of phosphatidylcholine: a simple method for determining relative hydrophobic interaction of various molecular species. *J. Lipid Res.* **22:** 697–704.
6. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high-performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.
7. Curstedt, T. 1977. Analysis of molecular species of ether analogues of phosphatidylcholines from biological samples. *Biochim. Biophys. Acta.* **489:** 79–88.
8. Jungalwala, F. B., V. Hayssen, J. M. Pasquini, and R. H. McCluer. 1979. Separation of molecular species of sphingomyelin by reversed-phase high-performance liquid chromatography. *J. Lipid Res.* **20:** 579–587.
9. Hsieh, J. Y. K., D. K. Welch, and J. G. Turcotte. 1981. General method for the analysis of phosphatidylcholines by high-performance liquid chromatography. *J. Chromatogr.* **208:** 398–403.
10. Hsieh, J. Y. K., D. K. Welch, and J. G. Turcotte. 1981. High pressure liquid chromatographic separation of molecular species of phosphatidic acid dimethyl esters derived from phosphatidylcholine. *Lipids.* **16:** 761–763.
11. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of

total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
12. Waku, K., H. Ito, T. Bito, and Y. Nakazawa. 1974. Fatty chain of acyl, alkenyl and alkyl phosphoglycerides of rabbit sarcoplasmic reticulum. *J. Biochem.* **75:** 1307–1312.
13. Nakagawa, Y., K. Waku, and Y. Ishima. 1982. Changes in the composition of fatty chains of diacyl, alkylacyl and alkenylacyl ethanolamine and choline phosphoglycerides during the development of chick heart ventricular cells. *Biochim. Biophys. Acta.* **712:** 667–676.
14. Ikezawa, H., M. Yamanegi, R. Taguchi, T. Miyashita, and T. Ohyabu. 1976. Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. *Biochim. Biophys. Acta.* **450:** 154–164.
15. Ferrell, W. J., D. M. Radloff, and J. E. Radloff. 1970. Method for hydrolysis and isolation of plasmalogen-bound fatty aldehydes. *Anal. Biochem.* **37:** 227–235.
16. Sun, G. Y., and L. A. Horrocks. 1969. Acyl and alk-1-enyl group compositions of the alk-1′-enyl acyl and diacyl glycerophosphoryl ethanolamines of mouse and ox brain. *J. Lipid Res.* **10:** 153–157.
17. Horrocks, L. A., and M. Sharma. 1982. Plasmalogens and O-alkyl glycerophospholipids. *In* New Comprehensive Biochemistry. Vol. 4, Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Biomedical Press, Amsterdam. 51–93.
18. Gunawan, J., and H. Debuch. 1977. On the biosynthesis of plasmalogens during myelination in the rat. Incorporation of radioactivity from 1-[^{3}H]alkyl-2-[1-^{14}C]-octadecenoyl-*sn*-glycero-3-phosphoethanolamine. *Hoppe-Seyler's Z. Physiol. Chem.* **358:** 537–543.
19. Yeung, S. K. F., and A. Kuksis. 1974. Molecular species of ethanolamine phosphatides of dog and pig kidney. *Can. J. Biochem.* **52:** 830–837.
20. Holub, B. J., and A. Kuksis. 1971. Structural and metabolic interrelationships among glycerophosphatides of rat liver in vivo. *Can. J. Biochem.* **49:** 1347–1356.
21. Trewhella, M. A., and F. D. Collins. 1969. The relative turnover of individual molecular species of phospholipids. *Lipids.* **4:** 304–307.
22. Marai, L., and A. Kuksis. 1973. Comparative study of molecular species of glycerolipids in sarcotubular membranes of skeletal muscle of rabbit, rat, chicken and man. *Can. J. Biochem.* **51:** 1365–1379.
23. Aveldaño, M. I., M. VanRollins, and L. A. Horrocks. 1983. Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography. *J. Lipid Res.* **24:** 83–93.