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NOVEL QUANTITATIVE METHOD FOR DETERMINATION OF MOLECULAR SPECIES OF PHOSPHOLIPIDS AND DIGLYCERIDES

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SUMMARY

A novel method is described for the quantitative analysis of subclasses (alk-1-enylacyl, alkylacyl, and diacyl types) and molecular species within each subclass of glycerophosphatides. Diradylglycerols from phospholipase C hydrolysis of the phospholipids are converted to benzoate derivatives, the benzoates are separated into their respective subclasses by thin-layer chromatography, and quantitated by measuring absorbance at 230 nm. Molecular species within individual subclasses are separated using a combination of argentation thin-layer chromatography and reversed-phase high-performance liquid chromatography with direct, on-line quantitation at 230 nm. We applied the method to the analysis of ethanolamine phosphatides from beef brain and were able to quantitate the three diradylglycerol subclasses (alk-1-enylacyl, alkylacyl, and diacyl types) as well as *ca.* 29 molecular species within each of these subclasses. This new quantitative approach for the analysis of specific molecular species of glycerolipids should be applicable to studies involving a variety of biologically important lipids, such as phosphatidylcholine, phosphatidylinositol, platelet activating factor, plasmalogens, and neutral type glycerolipids including diacylglycerols.

INTRODUCTION

Methodology to determine quantitatively the distribution of individual molecular species within specific phospholipid classes offers a valuable tool in lipid research. Older methods to determine molecular species, *e.g.*, argentation thin-layer chromatography (TLC) combined with gas–liquid chromatography (GLC) analysis of diradylglycerol derivatives prepared from phospholipids after phospholipase C treatment, were not widely used because of their limited ability to resolve many molecular species considering the extensive time and effort required for these analyses. Recently, high-performance liquid chromatography (HPLC) with reversed-phase columns has been utilized for the molecular species analyses of phospholipids[1–12]. Successful methods for the separation of molecular species by reversed-phase HPLC have been reported for intact phospholipids[1–4,7,10,11] and derivatives of intact phospholipids[6,8,9] as well as derivatives of diradylglycerols produced by phospholipase C hydrolysis of

the phospholipids[5,12]. Except for lipid derivatives with a chromophoric group[5,6], quantitation of the separated lipid components required collection of each fraction for phosphorus determinations and/or GLC of the methyl esters of fatty acids formed from the acyl groups. So far only one of the HPLC methods[12] (acetate derivatives of diradylglycerols) has provided an analysis of the three subclasses (alk-1-enylacyl, alkylacyl, and diacyl types) that occur in phospholipids; however, since HPLC detection of the diradylglyceroacetates[12] is based on absorbance of double bonds at 205 nm, completely saturated species are not detected and quantitation of the others requires collection, methylation, and GLC analyses of all HPLC separated components.

Recently we described a reversed-phase HPLC method for the quantitative analysis of alk-1-enyl- and alkyl-glycerols as their dibenzoate derivatives[13]. Utilization of the chromophoric benzoate groups has the advantage that the components separated by HPLC can be quantitated directly on-line from the HPLC detector response (230 nm) rather than requiring collection of fractions for subsequent phosphorus and/or GLC analyses. In the present work we have utilized the benzoate derivative of diradylglycerols to develop a quantitative reversed-phase HPLC method for analysis of diacyl-, alkylacyl-, and alk-1-enylacyl-glycerols derived from specific phospholipid classes after phospholipase C hydrolysis. The method is also applicable for the direct analysis of diglycerides, a lipid class that has a significant role in phosphatidylinositol metabolism and protein kinase C activation.

EXPERIMENTAL

Materials

Ethanolamine phosphoglycerides from beef brain, phospholipase C from *Bacillus cereus*, fluorescent zinc silicate, benzoic anhydride, and 4-dimethylaminopyridine were purchased from Sigma (St. Louis, MO, U.S.A.). 1,2-Dipalmitin, 1,2-diolein, and 1,2-dilinolein were supplied by NU-CHEK Prep (Elysian, MN, U.S.A.). Chloroform (containing 1% ethanol v/v), acetonitrile, 2-propanol, methanol, and hexane were HPLC grade from Burdick & Jackson (Muskegon, MI, U.S.A.). Diethyl ether and benzene were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). *rac*-Hexadecyl-2-octadecenoyl-*sn*-glycerophosphocholine was purchased from R. Berchtold, Biochemisches Labor, Bern, Switzerland.

[^{14}C(U)]Linoleic acid (0.9 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (87.4 Ci/mmol), purchased from New England Nuclear (Boston, MA, U.S.A.), were added to confluent monolayers of L-M cells (2μCi/75 cm^2 flask, 4 flasks/compound) and after incubation for 4 h at 37°C the cellular lipids were extracted by the method of Bligh and Dyer[14]. Radiolabeled phosphatidylcholine was isolated from the L-M cell lipids by TLC on 250 μm layers of silica gel H developed in chloroform–methanol–glacial acetic acid–water (50:25:8:3).

Methods

Up to 10 mg of phospholipid were hydrolyzed with 34 units of phospholipase C for 4 h at room temperature according to the method of Mavis *et al.*[15]; after evaporation of the diethyl ether with a stream of nitrogen, the diradylglycerols were extracted[14] from the hydrolysate. Benzoate drivatives of the diradylglycerols were

prepared as previously described[13]. Up to 2 mg of diradylglycerols were dissolved in 0.3 ml of benzene containing 10 mg of benzoic anhydride and 4 mg of 4-dimethylaminopyridine and allowed to stand for 1 h. The samples were placed in an ice bath and 2 ml of 0.1 *N* sodium hydroxide were added slowly. The diradylglycerobenzoates produced in this reaction mixture were extracted three times with hexane. When the amount of diradylglycerols was increased to 10 mg, the benzoylation reagents were increased four-fold.

Diradylglycerol benzoates were separated into subclasses (alk-1-enylacyl, alkylacyl, and diacyl types) by TLC using silica gel G plates prepared with 1 g of fluorescent zinc silicate per 50 g of silica gel G. After development in a solvent system of benzene–hexane–diethyl ether (50:45:4) the three lipid subclasses were easily visualized under UV light as dark spots against a fluorescent background. The benzoates were well separated with relative R_Fs of *ca.* 0.56, *ca.* 0.45, and *ca.* 0.33 for the alk-1-enylacyl, alkylacyl, and diacyl subclasses, respectively. The silica gel band containing each subclass of diradylglycerobenzoates was scraped into 2 ml of ethanol and mixed vigorously; 2 ml of water were added and the suspension extracted three times with hexane. After evaporation of the hexane, an appropriate volume of ethanol was added (depending on the amount of lipid) and the absorbance measured at 230 nm to determine the amount of each subclass of diradylglycerobenzoates present.

Separation of molecular species within each subclass of diradylglycerobenzoates was accomplished with a Beckman Model 324 M dual pump HPLC system fitted with a 45 × 4.6-mm pre-column and a 250 × 4.6-mm analytical column; both columns were packed with 5-μm Ultrasphere-ODS. Separated components were quantitatively measured at 230 nm with a Beckman Model C-RIA recorder-integrator. Samples were injected in the same solvent (20 μl) used for elution. Molecular species within the diacyl-, alk-1-enylacyl-, and alkylacyl-glycerobenzoates were separated by isocratic elution with acetonitrile–2-propanol (v/v) in ratios of 70:30, 65:35, and 63:37, respectively. Column flow-rates were 1 ml/min for all analyses.

Benzoate derivatives of each subclass (diacyl, alkylacyl, and alk-1-enylacyl types) of the diradylglycerols were also fractionated by argentation TLC. These plates were prepared by slurrying silica gel G in a 10% silver nitrate solution before spreading the layers. Diacyl- and alkylacyl-glycerobenzoates were resolved by argentation TLC with 3.5% ethanol in chloroform, whereas 5% ethanol in chloroform was used to separate the alk-1-enylacyl derivatives (percentage values for ethanol include the 1% ethanol perservative in the stock chloroform). Developed plates were sprayed with 0.05% 2,7-dichlorofluorescein in ethanol and viewed under UV light to locate the separated lipid fractions (five areas) that were then extracted by the same technique used for recovery of the diradylglycerobenzoate subclasses separated on silica gel G plates. GLC analysis revealed that the five lipid bands detected on the argentation silica gel layers contained in order of decreasing R_F, 0–2, 3, 4, 5, and 6 or more double bonds per molecule.

GLC with a Tracor Model 560 chromatograph fitted with a 6 ft. × 1/8 in. column packed with 10% EGSS-X on 100–120 mesh Gas Chrom P resolved the methyl esters of fatty acids and dimethyl acetals of fatty aldehydes obtained by acid catalyzed methanolysis of the diradylglycerobenzoate peaks collected from HPLC. The column oven temperature was 185°C, and components were detected with a hydrogen flame detector. Identification of peaks resolved by GLC was made by com-

paring their retention times with those of standards in known mixtures analyzed under identical conditions. Composition of the alkyl chains in the samples was determined by HPLC, after methanolysis and subsequent formation of the dibenzoates of the alkylglycerols, as previously described[13].

RESULTS AND DISCUSSION

A summary of the methodology used to determine quantitatively the molecular species present in a phospholipid class is outlined in the flow-chart depicted in Fig. 1. Based on the analysis of the products obtained after phospholipase C treatment of the radiolabeled phosphatidylcholines from L-M cells and the ethanolamine phospholipids from beef brain, the yield of diradylglycerols by this procedure was 98.6 ± 0.8% ($n=3$). The quantitative nature of the benzoylation reaction, as previously shown[13], was also verified since there was no evidence of unreacted diradylglycerols after TLC analysis of the benzoylation reaction products. Molar absorptivities measured in acetonitrile–2-propanol (3:2) at 230 nm for the benzoates of dipamitoyl-, dioleoyl-, dilinoleoyl-, and hexadecyloleoyl-glycerols were essentially the same giving an average value of 13,175 ± 670 (S.D.), which agrees with the published value for alcohol benzoates[16]. TLC recoveries for the benzoates of dipalmitoyl- and hexadecyloleoyl-glycerols were 95.7 ± 3.2% ($n=3$) and 96.0 ± 1.8% ($n=4$), respectively.

Analysis of beef brain ethanolamine phospholipids by TLC and phosphorus determinations[17] after exposure to hydrochloric acid[18] gave an average value of 63.2

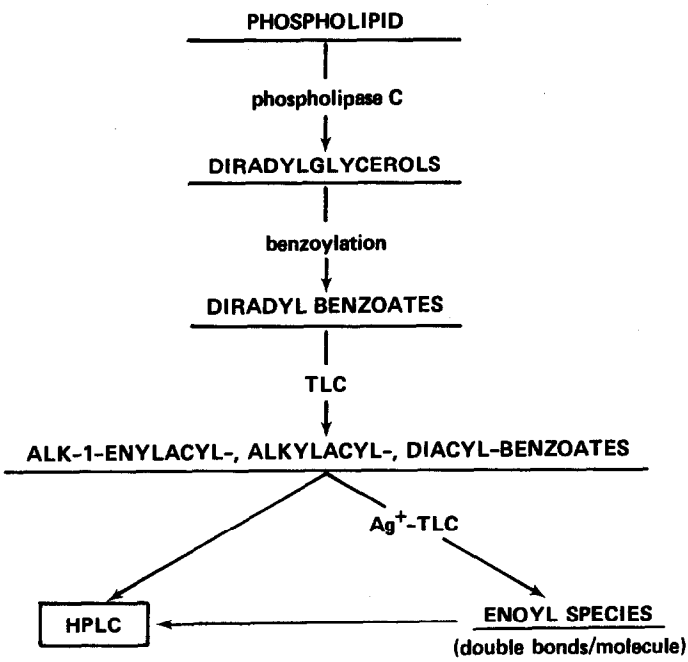

Fig. 1. Flow chart of the methods used to quantitate the three subclasses (alk-1-enylacyl, alkylacyl, and diacyl types) and the distribution of molecular species within each subclass of phospholipid as their diradylglycerobenzoates.

± 2.3% ($n=4$) for the content of the alk-1-enylacyl (plasmalogen) subclass; the remainder (36.8%) consisted of both the alkylacyl and diacyl subclasses. Distribution of the three subclasses in the beef brain ethanolamine phospholipids as determined by TLC separation of the diradylglycerobenzoates (Fig. 1) and the subsequent measurement of absorbance at 230 nm was 63.5 ± 1.2%, 5.9 ± 1.2%, and 30.6 ± 1.3% ($n=6$) for the alk-1-enylacyl, alkylacyl, and diacyl subclasses, respectively. Alternatively, since a direct linear correlation exists between the amount of diradylglycerobenzoates injected into the HPLC column and the total integration units obtained from the C-RIA recorder (correlation coefficient = 0.985, $n=5$), the total amount of each subclass separated by TLC can be quantitated from the total HPLC integration units.

Reversed-phase HPLC separations of the molecular species comprising each subclass isolated from beef brain ethanolamine phospholipids are illustrated in Fig. 2 (A, B, and C). Both argentation TLC of the diradylglycerobenzoates and GLC analysis (after acidic methanolysis) of the fractions collected during HPLC were used to identify and determine the molecular distribution within each HPLC fraction that contained more than one species. After argentation TLC of each subclass only one or two HPLC peaks contained more than one molecular species of diradylglycerobenzoates (*e.g.*, Fig. 2 peak 16, 16:0–20:1 plus 18:0–18:1); aliphatic moieties (after acid methanolysis) in these HPLC fractions were analyzed by GLC to quantify each molecular species.

Identification and quantitation of the molecular species in each diradylglycerol subclass of beef brain ethanolamine phospholipid are given in Table I. Since the amount of 18:2 was low in all three diradylglycerol subclasses of beef brain ethanolamine phosphatides, the elution times of the 18:1–18:2, 16:0–18:2, and 18:0–18:2 molecular species were verified by collection of 0.2-ml fractions during the HPLC elution of ^{14}C-18:2 labeled diradylglycerobenzoates prepared from the labeled choline phosphatides obtained from L-M cells. The order of elution of the diradyl benzoates is very similar to those previously shown for the acetate derivatives of diradylglycerols[12].

Although the absolute amounts of each molecular species differ (Table I) from those reported for the acetate derivatives[12] the relative distributions are very similar. The beef brain ethanolamine phospholipids used in our experiments were also lower in polyunsaturated fatty acids than reported by Nakagawa and Horrocks[12]; these quantitative differences are likely explained by the different sources of the ethanolamine phospholipids used for the analyses. The preferential pairing of aliphatic moieties (*e.g.*, 18:0 with 20:4) reported earlier[12] for the three subclasses of beef brain ethanolamine phospholipids was also verified in our work.

An independent check for quantitation of our method was made by comparing the distribution of individual aliphatic side chains determined by GLC with those calculated from the HPLC data in Table I. These comparisons, shown in Table II, are in good agreement with each other and confirm the identification and quantitative measurement of the diradylglycerol molecular species by our HPLC method.

Because of the multiplicity of peaks obtained by HPLC analysis of the diradylglycerol benzoates, a method to predict retention times of molecular species in an unknown sample would be useful. Since the technique developed by Patton *et al.*[10] of graphing the logarithm of the relative retention times (RRT) of each molec-

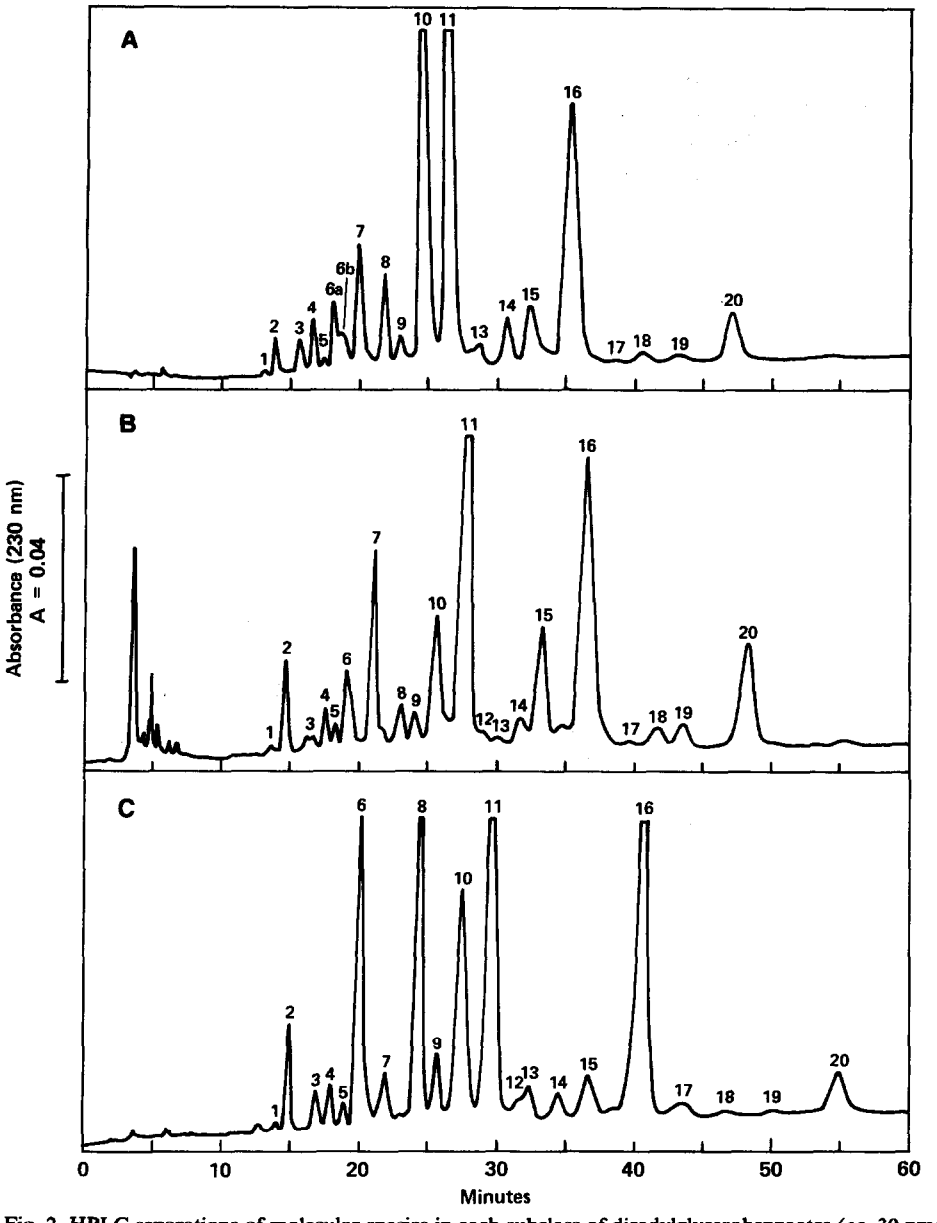

Fig. 2. HPLC separations of molecular species in each subclass of diradylglycerobenzoates (*ca.* 30 nmol) prepared from beef brain ethanolamine phosphoglycerides. A = alk-1-enylacylglycerobenzoates, B = alkylacylglycerobenzoates, and C = diacylglycerobenzoates.

ular species of intact phospholipid was also used to successfully predict the HPLC elution times of diradylglyceroacetates[12], we applied this technique to analyze the elution profiles shown in Fig. 2. Because the 16:0–18:1 molecular species should be present in most samples and is commercially available in both diacyl and alkylacyl

TABLE I

QUANTITATIVE ANALYSES OF MOLECULAR SPECIES IN SUBCLASSES OF BEEF BRAIN ETHANOLAMINE PHOSPHOGLYCERIDES AS THEIR DIRADYLGLYCEROBENZOATES

*Peak number**	*Molecular species***	*Alk-1-enylacyl****	*Alkylacyl*§*	*Diacyl****
1	18:1–22:6	0.2 ± 0.1	0.3	0.3 ± 0.1
2	16:0–22:6	0.7 ± 0.1	2.7	2.5 ± 0.1
3	18:1–20:4	1.0 ± 0.1	0.9	1.2 ± 0.1
4	16:0–20:4	1.2 ± 0.1	1.2	1.1 ± 0.1
5	16:0–22:5	0.2 ± 0.1	0.7	0.6 ± 0.1
6a	18:0–22:6	2.3 ± 0.4	2.7	9.6 ± 0.4
6b	18:1–18:2	–	–	0.4 ± 0.2
	18:1–20:3 (*n*-6)	0.3 ± 0.2	–	0.5 ± 0.1
	18:1–22:4	0.8 ± 0.2	1.1	0.5 ± 0.1
7	16:0–22:4	2.8 ± 0.1	6.1	1.6 ± 0.3
	16:0–20:3 (*n*-6)	1.2 ± 0.2	1.3	0.4 ± 0.1
	16:0–18:2	–	0.5	–
8	18:0–20:4	2.6 ± 0.1	1.0	11.9 ± 0.2
	16:0–20:3 (*n*-9)	0.6 ± 0.1	0.8	0.2 ± 0.1
9	18:0–22:5	1.1 ± 0.1	1.1	2.5 ± 0.1
10	18:1–18:1	21.8 ± 0.6	6.7	12.7 ± 0.3
11	16:0–18:1	26.3 ± 1.4	16.1	14.5 ± 0.8
	18:0–22:4	2.2 ± 0.1	4.3	4.0 ± 0.1
	18:0–20:3 (*n*-6)	2.5 ± 0.6	1.0	1.0 ± 0.1
	18:0–18:2	–	–	–
12	16:0–16:0	0.7 ± 0.2	0.9	1.0 ± 0.4
13	18:0–20:3 (*n*-9)	0.6 ± 0.2	0.3	1.3 ± 0.2
14	17:0–18:1	2.4 ± 0.1	1.8	1.1 ± 0.2
15	18:1–20:1	4.4 ± 0.6	7.3	2.5 ± 0.4
16	18:0–18:1	12.5 ± 0.5	18.2	22.3 ± 0.6
	16:0–20:1	6.6 ± 0.2	16.6	1.6 ± 0.1
17	16:0–18:0	–	–	0.9 ± 0.1
18	17:0–20:1	0.7 ± 0.1	1.4	–
19	18:1–22:1	0.5 ± 0.1	1.5	–
20	18:0–20:1	3.6 ± 0.2	8.0	3.0 ± 0.2

* Peak numbers correspond to those shown in the HPLC tracings of Fig. 2.

** The first number denotes length of the carbon chain and the second the number of double bonds in the chain (vinyl ether double bonds in the alk-1-enylacyl subclass are not included).

*** Values represent the average mole % ± S.D. ($n=3$).

§ Because of the limited material in this subclass, values represent mole % based on a single analysis of a pooled sample from three separate preparations.

forms, we selected this species as the basis for calculating the RRT of the other molecular species of lipids (*e.g.*, RRT of 16:0–18:1 = 1.00). A graphical representation of RRTs for molecular species of the alk-1-enylacylglycerobenzoates is shown in Fig. 3. Similar patterns were also obtained for the diacyl and alkylacyl species (data not shown). The graphical representation of RRTs in Fig. 3 demonstrates that the molecular species of diradylglycerobenzoates, as previously shown for intact phospholipids[10] and acetate derivatives of diradylglycerols[12], are eluted during HPLC in a predictable sequence.

Since both 16:0 and 18:1 ether moieties were found in the 16:0–18:1 HPLC

TABLE II

COMPOSITION OF ALIPHATIC MOIETIES IN SUBCLASSES OF BEEF BRAIN ETHANOLAMINE PHOSPHOGLYCERIDES

*Aliphatic moiety**	*Alk-1-enylacyl***		*Alkylacyl****		*Diacyl***	
	HPLC§	*GLC*	*HPLC*	*GLC*	*HPLC*	*GLC*
16:0	20.4 ± 0.4	19.5 ± 0.4	23.8	24.6	12.8 ± 0.7	10.7 ± 0.4
17:0	1.5 ± 0.1	2.1 ± 0.4	1.6	1.2	0.6 ± 0.1	1.0 ± 0.3
18:0	14.1 ± 0.4	12.4 ± 0.3	13.3	14.4	28.5 ± 0.4	27.6 ± 0.4
18:1	46.0 ± 1.3	44.8 ± 0.9	25.8	25.1	34.3 ± 0.4	33.9 ± 0.3
20:1	7.5 ± 0.5	8.2 ± 0.4	16.7	13.7	3.5 ± 0.3	4.3 ± 0.3
20:3	2.7 ± 0.3	1.2 ± 0.1	1.7	1.8	1.7 ± 0.2	1.6 ± 0.1
20:4	2.4 ± 0.1	3.7 ± 0.2	1.6	1.6	7.1 ± 0.2	7.2 ± 0.1
22:4	2.9 ± 0.1	3.9 ± 0.1	5.7	5.9	3.0 ± 0.2	3.1 ± 0.1
22:5	0.5 ± 0.3	tr.	0.9	1.4	1.6 ± 0.1	1.7 ± 0.1
22:6	1.6 ± 0.2	1.7 ± 0.1	2.8	2.5	6.2 ± 0.3	5.6 ± 0.2
Total	99.6	97.5	93.9	92.2	99.3	96.7

* Only aliphatic groups that were greater than 1% in at least one subclass are included, and no distinction is made between ether and ester aliphatic groups.

** Values represent the average mole % ± S.D. ($n=3$).

*** Because of the limited material in this subclass, values represent mole % based on a single analysis of a pooled sample from three separate preparations.

§ Values for HPLC were calculated from the data in Table I.

peak of the alk-1-enylacyl and alkylacyl subclasses (16:0 was predominant), it appears that the reversed-phase column does not separate diradylglycerobenzoates that differ in the location of the aliphatic groups at the *sn*-1 *vs.* *sn*-2 position of glycerol. However, since the diacylglycerobenzoates can be hydrolyzed by pancreatic lipase (data not shown), the position of the acyl groups (*sn*-1 *vs.* *sn*-2) in HPLC separated components should be discernable by using this enzymic treatment.

Our investigation shows that benzoate derivatives of diradylglycerols (derived from phospholipids treated with phospholipase C), can be quantitatively measured by their absorbance at 230 nm. This method utilizes a simple TLC system for separation and direct quantitation of the benzoates of the three subclasses (diacyl, alkylacyl, and alk-1-enylacyl types) that can occur in glycerophosphatides. Use of argentation TLC in conjunction with reversed-phase HPLC avoids one of the disadvantages encountered in analysis of molecular species (*i.e.*, the number of HPLC peaks that contain more than one species is minimized). Using argentation TLC instead of GLC for identification and quantitation of HPLC peaks also allows the purified, intact diradylglycerobenzoates to be collected from HPLC for subsequent analyses such as lipase hydrolysis of *sn*-1 acyl groups and distribution of radiolabels that may have been incorporated. However, depending on the composition of aliphatic chains in particular phospholipids and/or the specific information desired, the argentation TLC step may not be needed in all applications of the methodology described in this report. Our method offers an improved approach for quantitation, sensitivity of detection, and fewer steps than previously available for the analysis of subclasses of phospholipids or diradylglycerols and specific molecular species associated with each subclass.

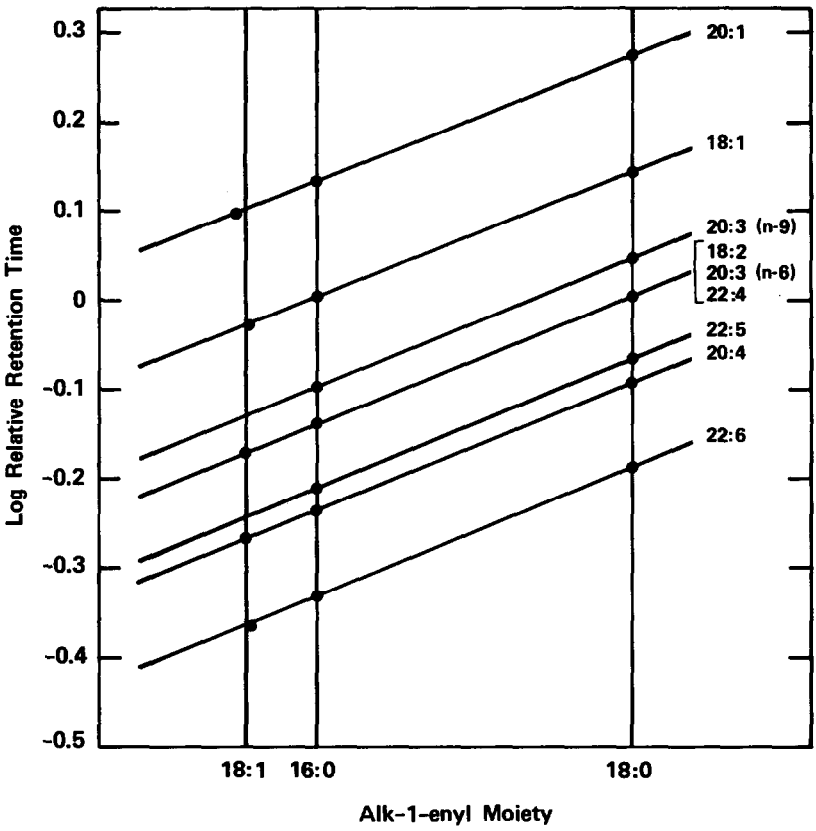

Fig. 3. Log RRT graph of the alk-1-enylacylglycerobenzoates shown in Fig. 2A. All RRTs were calculated relative to the 16:0–18:1 molecular species (Fig. 2A, peak 11) and plotted as described by Patton *et al.*[10] and Nakagawa and Horrocks[12].

ACKNOWLEDGEMENTS

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