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Note

Separation and quantitation of phospholipid subclasses as their diradylglycerobenzoate derivatives by normal-phase high-performance liquid chromatography

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The ability to separate and quantify subclasses and molecular species within subclasses of a specific phospholipid are important in lipid research. One approach to these analyses has been to replace the phosphobase group of the glycerophosphatides with a less polar moiety. Methods have been described for replacement of the phosphobase group with nitrobenzoate[1], dinitrobenzoate[2], benzoate[3], and acetate[4] before analysis by high-performance liquid chromatography (HPLC). Derivatives containing the phenyl group offer the advantage of direct on-line quantitation by UV detection during HPLC analyses. Only two of these methods[3,4] described chromatographic separation of the diacyl-, alkylacyl-, and alk-1-enylacylglycero subclasses before the analysis of molecular species by reverse-phase HPLC. Subclasses of the acetate derivatives can be separated by either thin-layer chromatography (TLC)[5] or HPLC[4], whereas only a TLC method has been described for the separation of subclasses of the benzoate derivatives[3]. In this paper we describe a normal-phase HPLC method for separation and quantitation of diacyl-, alkylacyl-, and alk-1-enylacylglycerobenzoate subclasses that can be used as an alternative approach to our previous TLC method for separation of these subclasses.

MATERIALS AND METHODS

Preparation of diradylglycerobenzoate standards for HPLC analyses

1,2-Diradyl-*sn*-glycero-3-phosphoethanolamine from beef brain (Sigma, St. Louis, MO, U.S.A.), 1,2-[1-^{14}C]dipalmitoyl-*sn*-glycero-3-phosphocholine (New England Nuclear, Boston, MA, U.S.A.), and the total phospholipid fraction from Ehrlich ascites cells were each hydrolyzed with phospholipase C (*Bacillus cereus*, Sigma)[6]. The resulting diradylglycerols were benzoylated with benzoic anhydride as previously described[3,7] except that 1 ml of concentrated ammonium hydroxyde (29% ammonia, Mallinckrodt, St. Louis, MO, U.S.A.) at room temperature, instead of sodium hydroxide at 0°C, was used to terminate the benzoylation reaction in order to insure complete removal of excess benzoic anhydride. Benzoate derivatives prepared from the beef brain ethanolamine phosphoglycerides served as the source for isolation of the 1-alk-1′-enyl-2-acyl-*sn*-glycero-3-benzoate (alk-1-enylacylglycerobenzoate) and

the 1,2-diacyl-*sn*-glycero-3-benzoate (diacylglycerobenzoate) subclasses, whereas the 1-alkyl-2-acyl-*sn*-glycero-3-benzoate (alkylacylglycerobenzoate) subclass was isolated from the derivatives of Ehrlich ascites cell phospholipids. The diradylglycerobenzoate subclasses were separated by TLC on 250-μm thick layers of silica gel G developed in benzene–hexane–diethyl ether (50:45:4)[3]. Developed plates were sprayed with a 50% aqueous solution of ethanol containing 0.1% (w/v) 2,7-dichlorofluorescein, and were viewed under UV light to locate the separated subclasses; each subclass was extracted as previously described[3]. The amounts of each subclass were determined by measurement of optical densities in cyclohexane at 230 nm (molar absorptivity $1.3 \cdot 10^4$). Standard mixtures containing various amounts of each subclass were prepared for analyses by HPLC.

Quantitative analyses of subclasses in beef brain ethanolamine phosphoglycerides

Beef brain ethanolamine phosphoglycerides were hydrolyzed with phospholipase C and benzoate derivatives of the diradylglycerols were prepared as described in the previous paragraph, but in this instance the subclasses were separated by normal-phase HPLC instead of TLC. For HPLC analyses, we first removed the 4-dimethylaminopyridine catalyst by application of a chloroform solution of the unfractionated benzoylation products to a small glass column (disposable Pasteur pipette) packed to a height of approximately 2 cm with silicic acid (Unisil, Clarkson, Williamsport, PA, U.S.A.). The diradylglycerobenzoates were eluted with 11–12 ml of chloroform and the 4-dimethylaminopyridine was retained by the column. Average percent recovery of 1,2-[1-^{14}C]dipalmitoyl-*sn*-glycero-3-benzoate from the short silicic acid column was 97.3 ($\pm$ 1.3 S.D., $n = 3$). After evaporation of the chloroform, the eluted diradylglycerobenzoates were dissolved in cyclohexane for analysis by normal-phase HPLC.

Beef brain ethanolamine phosphoglycerides were also analyzed for their content of the alk-1-enylacyl subclass by exposure of the total lipid extract to hydrochloric acid followed by TLC separation of the lysophosphatidylethanolamine (represents the alk-1-enylacyl subclass) from the unreacted phospholipids (the diacyl plus alkylacyl subclasses)[8]. Phosphorus was determined by the method of Rouser *et al.*[9]

Normal-phase HPLC separation and quantitation of subclasses of diradylglycerobenzoates

A Beckman Model 324 M dual-pump HPLC system fitted with an Altex 250 $\times$ 4.6 mm I.D. analytical Ultrasphere-Si (5 μm) column was used to separate the benzoate derivatives. Detection of the benzoates was performed at 230 nm with a Hitachi Model 100-40 variable-wavelength spectrophotometer connected to a Shimadzu C-RIA data processor for integration of HPLC peak areas. An isocratic solvent system of cyclohexane–diethyl ether–glacial acetic acid (97:3:0.07) at a flow-rate of 1 ml/min was used for the normal-phase HPLC separations.

RESULTS AND DISCUSSION

Initial attempts to separate the three diradylglycerobenzoate subclasses by HPLC were made using various amounts of diethyl ether in either hexane or cyclohexane. Although the subclasses were well separated, the HPLC peak areas obtained

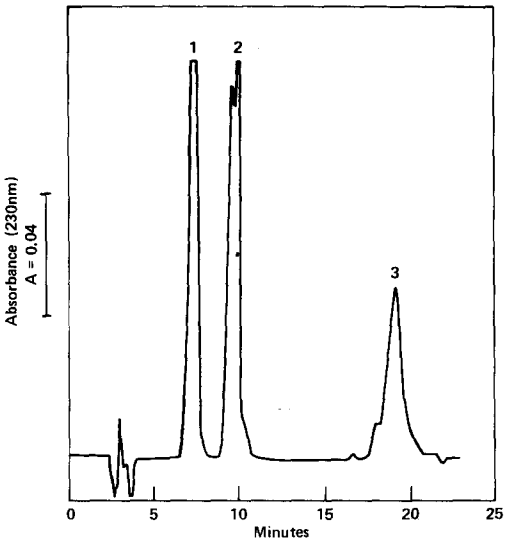

Fig. 1. Normal-phase HPLC separation of a standard mixture of diradylglycerobenzoates (mixture 1, Table I). Peaks: 1 = alk-1-enylacylglycerobenzoates; 2 = alkylacylglycerobenzoates; 3 = diacylglycerobenzoates. As an indication of the sensitivity of this technique, peak 1 represents 7.7 nmol.

from injections of known amounts of the alk-1-enylacylglycerobenzoates indicated that recoveries were as low as 40%. No attempts were made to determine the reason for the low HPLC detection response of the alk-1-enylacyl subclass, because we found that including a small amount of acetic acid (0.07%) in the solvents eliminated this problem. Cyclohexane, instead of hexane, was chosen as the primary solvent component because it gave less separation of molecular species within the subclasses (which provided less problem with peak integration) and gave slightly better resolution between the alk-1-enylacyl and alkylacyl subclasses than hexane. An example of the normal-phase HPLC separation of the three diradylglycerobenzoate subclasses is shown in Fig. 1. The separation and elution sequence of the three subclasses are similar to those previously shown for diradylglyceroacetates[4]; however, because benzoate derivatives give direct on-line quantitation, there is no need for a secondary method of quantitation as required for the acetate derivatives.

Results from triplicate analyses of three different standard mixtures of diradylglycerobenzoates by normal-phase HPLC are given in Table I. Based on these results, the normal-phase HPLC method demonstrates both good accuracy and reproducibility for quantitative analysis of diradylglycerol subclasses. Furthermore, the distribution of molecular species within each individual sublcass, as determined by reversed-phase HPLC[3], was unchanged after separation and collection from normal-phase HPLC (Table II). Therefore, the normal-phase HPLC method described in this report should also be useful as a preparative tool since selective losses of individual molecular species do not occur under these conditions. Average recovery of 1,2-[1-^{14}C]dipalmitoylglycerobenzoate from normal-phase HPLC was 94.3% ($\pm$3.0 S.D., n = 3).

TABLE I

QUANTITATIVE ANALYSIS OF STANDARD MIXTURES OF DIRADYLGLYCEROBENZOATE SUBCLASSES BY NORMAL-PHASE HPLC

Values for HPLC represent average mole % ± S.D. (n = 3); known values are also expressed as mole %.

Subclass	*Mixture 1*		*Mixture 2*		*Mixture 3*	
	HPLC	*Known*	*HPLC*	*Known*	*HPLC*	*Known*
Alk-1-enylacyl	38.4 ± 0.4	39.6	36.2 ± 0.7	37.4	15.0 ± 0.2	15.4
Alkylacyl	37.1 ± 0.4	36.5	17.8 ± 0.3	17.3	29.2 ± 0.6	28.5
Diacyl	24.5 ± 0.8	23.9	46.0 ± 1.0	45.3	55.8 ± 0.8	56.0

A comparison was made between the normal-phase HPLC method and a conventional method based on hydrochloric acid exposure of lipids coupled with TLC, and phosphorus determination[8] for quantitation of beef brain ethanolamine phosphoglyceride subclasses. The content of the alk-1-enylacyl and alkylacyl plus diacyl

TABLE II

COMPARISON OF MOLECULAR SPECIES IN DIRADYLGLYCEROBENZOATE SUBCLASSES BEFORE AND AFTER ISOLATION BY NORMAL-PHASE HPLC

All values represent mole %. A standard mixture of alk-1-enylacyl-(56 nmol), alkylacyl-(52 nmol), and diacyl-(68 nmol) glycerobenzoates was separated and collected using the normal-phase HPLC system. The isolated subclasses were analyzed by reversed-phase HPLC for molecular species composition before and after normal-phase HPLC fractionation into the subclasses. Conditions for the reversed-phase HPLC analyses and the identification of the species associated with the HPLC peak numbers are the same as previously reported[3]. N.D. means not detected.

HPLC peak number	*Alk-1-enylacyl*		*Alkylacyl*		*Diacyl*	
	Before	*After*	*Before*	*After*	*Before*	*After*
1	0.9	0.9	2.3	2.2	0.7	0.7
2	2.0	1.8	5.7	5.6	3.8	3.8
3	3.1	2.7	3.6	3.7	1.9	1.9
4	2.0	1.8	4.8	4.8	1.4	1.4
5	0.7	0.6	0.8	0.9	0.9	0.9
6a+6b	12.0	11.9	11.9	11.9	24.0	23.9
7	6.3	6.3	19.6	19.4	2.5	2.4
8	4.6	4.9	3.8	3.0	13.7	13.7
9	2.3	2.5	0.5	1.5	4.9	4.9
10	18.8	18.9	4.6	4.1	9.0	9.0
11	20.0	20.1	23.1	22.7	15.0	15.0
12+13	0.7	0.6	5.7	5.5	1.7	1.7
14	1.8	1.8	0.4	0.3	0.9	0.8
15	4.3	4.7	0.2	0.2	2.5	2.4
16	12.6	12.7	4.3	4.4	13.8	13.7
17	0.2	0.1	2.1	2.1	<0.1	<0.1
18	0.8	0.6	N.D.	N.D.	<0.1	<0.1
19	0.8	0.9	N.D.	N.D.	<0.1	<0.1
20	3.4	3.3	N.D.	N.D.	2.0	2.1

TABLE III

QUANTITATIVE ANALYSIS OF SUBCLASSES IN ETHANOLAMINE GLYCEROPHOSPHATIDES FROM BEEF BRAIN: COMPARISON OF HPLC AND TLC METHODS

Values represent the average mole % ± S.D. for both the normal-phase HPLC method (n = 3) and for the TLC method (n = 9).

Subclass	*HPLC*	*TLC**
Alk-1-enylacyl	66.7 ± 0.9	65.6 ± 1.0
Alkylacyl	3.5 ± 0.5	34.3 ± 1.0
Diacyl	29.8 ± 1.0	

* Values for TLC are based on phosphorus analysis after pretreatment of the sample with hydrochloric acid as described in the Materials and methods section.

subclasses in beef brain ethanolamine phosphoglycerides was found to be virtually indentical by both methods (Table III). In addition, the percentage of the alkylacyl subclass found by HPLC (3.5, Table III) is identical to that reported by Nakagawa and Horrocks[4] for beef brain ethanolamine phosphoglycerides.

This new HPLC method can be used to replace our previously described preparative TLC procedure[3] for the separation and isolation of the three diradylglycerobenzoate subclasses. The time needed for HPLC analysis of these subclasses in a single sample is about the same as that of the TLC method; however, HPLC offers the advantages of providing direct on-line quantitation during the separation of subclasses and it is more sensitive (can easily detect a subclass containing as little as 1 μg) than the visualization of TLC chromatograms under UV light.

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