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Note

Thin-layer and high-performance liquid chromatographic separation of glycerolipid subclasses as benzoates

Derivatives of ether and ester analogues of phosphatidylcholine, phosphatidylethanolamine and platelet activating factor

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Methods for separation and quantitation of glycerolipid subclasses containing alk-1-enyl, alkyl and acyl groups are important tools in lipid research. Many of these methods are based on the chromatographic separation of derivatives that are prepared from the original lipid class. Although extensive use has been made of acetate derivatives[1], groups that have a reasonably high molar absorptivity such as nitrobenzoates[2], dinitrobenzoates[3], dinitrophenylurethanes[4] and benzoates[5] are better suited for direct on-line quantitation by high-performance liquid chromatography (HPLC). Three of these derivatives have been used for the HPLC separation of the subclasses of long-chain diradylglycerols[1,5,6], derived from phospholipids via phospholipase C hydrolysis. In this paper the HPLC methodology for the separation of diradylglycerol benzoates[5] is extended to include several other lipids. Data on the relative migration of these benzoate derivatives during thin-layer chromatography (TLC) are also included. This information should be particularly valuable for investigators in the lipid mediator field who are interested in the analytical identification of radiolabeled lipids that migrate in the same general area as 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) during thin-layer chromatography (TLC).

MATERIALS AND METHODS

1-Alk-1′-enyl-*sn*-glycerol(1-alk-1′-enyl-Gro) and 1-alkyl-Gro were obtained as previously described[7]. 1-Acyl-Gro (monoolein) was purchased from Sigma (St. Louis, MO, U.S.A.). 1-Alk-1′-enyl-2-acetyl-Gro and 1-alkyl-2-acetyl-Gro were prepared by acetylation of the corresponding 1-radyl-Gro followed by treatment of the diacetates with pancreatic lipase and isolation of both the 1-radyl-2-acetyl-Gro and 1-radyl-3-acetyl-Gro by TLC as described[8], except that the radylacetyl-Gro were

extracted from the TLC plates with diethyl ether instead of hexane. 1-Acyl(palmitoyl)-2-acetyl-Gro was prepared by acetylation of palmitoyllysoglycerophosphocholine (Sigma) and subsequent hydrolysis of this product by phospholipase C (ref. 9). Sources of the long-chain diradyl-Gro subclasses were described previously[5]. A preparation of ceramides from the sphingomyelin of bovine brain was purchased from Sigma.

Benzoylation of the compounds was accomplished as previously described[5,7] except that 1 ml of concentrated ammonium hydroxide at room temperature, instead of 0.1 *M* sodium hydroxide at 0°C, was used to remove excess benzoic anhydride and benzoic acid at the termination of the reactions. Benzoate derivatives were separated on silica G (EM Science, Gibbstown, NJ, U.S.A.)-coated (250 μm) TLC plates in chromatographic tanks lined with filter paper using a solvent system of benzene–hexane–diethyl ether (50:45:5, v/v/v). TLC R_F values were measured after charring the developed plates with sulfuric acid spray and heat (180°C, 30 min). All benzoate derivatives were purified by TLC[10] and redissolved in cyclohexane before analysis by normal-phase HPLC. Except for the hardware (an isocratic, single-pump HPLC system purchased from Rainin, Woburn, MA, U.S.A.) normal-phase HPLC was carried out exactly as described previously[5]. The mobile phase consisted of cyclohexane–diethyl ether–glacial acetic acid (97:3:0.07, v/v/v) at a flow-rate of 1 ml/min. Separations were made on an Altex 250 × 4.6 mm I.D. column packed with Ultrasphere-Si (5 μm; Beckman, Norcross, GA, U.S.A.) and the benzoate derivatives were detected by measuring the absorbance at 230 nm.

RESULTS AND DISCUSSION

Normally a difference in TLC R_F values of about 0.05 is needed to achieve a separation of two compounds when the distance from the origin to the solvent front is 14–15 cm. The TLC solvent system described separated the benzoate derivatives of the alk-1-enyl, alkyl and acyl subclasses within each class of glycerolipid (Table I). If 1-radylglycerodibenzoates are excluded, the TLC system is capable of separating the

TABLE I

TLC MOBILITIES OF BENZOATE DERIVATIVES OF VARIOUS LIPID CLASSES

Benzoylated compounds	*TLC R_F values*[a]
(A) 1-Alk-1′-enyl-2-acyl-Gro	0.54 ± 0.02
(B) 1-Alkyl-2-acyl-Gro	0.46 ± 0.02
(C) 1,2-Diacyl-Gro	0.35 ± 0.01
(D) 1-Alk-1′-enyl-2-acetyl-Gro	0.30 ± 0.02
(E) 1-Alkyl-2-acetyl-Gro	0.22 ± 0.01
(F) 1-Acyl-2-acetyl-Gro	0.16 ± 0.01
(G) 1-Alk-1′-enyl-Gro	0.42 ± 0.02
(H) 1-Alkyl-Gro	0.36 ± 0.02
(I) 1-Acyl-Gro	0.26 ± 0.01
(J) Ceramide	0.07 ± 0.00

[a] Average TLC R_F values ± S.D. were calculated from plates ($n = 3$ different days) that were chromatographed as described in Materials and Methods.

remaining six subclasses of glycerolipid benzoate derivatives and the benzoate derivatives of ceramides. We have found the benzoate derivatives to be particularly important in discerning the amount of radiolabeled acetate incorporated into sphingomyelin (which has a TLC R_F similar to PAF) relative to the amount actually incorporated into PAF by intact cells.

Separation of several benzoate derivatives by normal-phase HPLC is shown in Fig. 1. As expected, the HPLC resolution of these derivatives is better than with TLC. In fact, as indicated by the multiple peaks within some subclasses, there is even a partial resolution of molecular species. The only major overlap of peaks occurred with the benzoates of 1-alk-1′-enyl-Gro and 1-alkyl-2-acyl-Gro (peaks B and G in Fig. 1). If there is doubt about the presence of these two components in a sample, the peak can be collected from HPLC, treated with acid to hydrolyze the labile vinyl ether linkage, and then rerun the sample on HPLC. Any peak found at this elution time, after the acid treatment, should be representative of the 1-alkyl-2-acyl-Gro subclasses. The benzoate derivative of cholesterol, which could be a contaminant in the TLC isolation of diglycerides from a sample of total lipids, elutes from this normal-phase HPLC system 4 to 4.5 min after injection (not shown in Fig. 1) and therefore, would not interfere.

1-Radyl-2-acetyl-*sn*-glycero-3-phosphocholine and 1-radyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine can contain 1-alk-1′-enyl, 1-alkyl, and/or 1-acyl groups at the *sn*-1 position of glycerol[11,12]. Therefore, normal-phase HPLC separation[5,13] of these subclasses, after phospholipase C hydrolysis and benzoylation, provides another method for analysis of these subclasses of PAF analogues (see peaks D, E, and F in Fig. 1); this technique is particularly helpful in experiments that have utilized radiolabeled acetate as a measure of PAF production. Samples suspected of containing the PAF analogues should be benzoylated immediately after phospholipase C hydrolysis to minimize isomerization of acetate from the *sn*-2 position to the *sn*-3 position. If isomerization of the acetate has occurred, the 1-radyl-2-benzoyl-3-acetyl-Gro derivatives would elute at about 19, 25 and 55 min for the alk-1-enyl, alkyl, and acyl subclasses, respectively, in the HPLC system described (peaks not shown in Fig. 1). As an example of sensitivity using our instrumentation, 0.2 nmol of 1-alkyl-2-

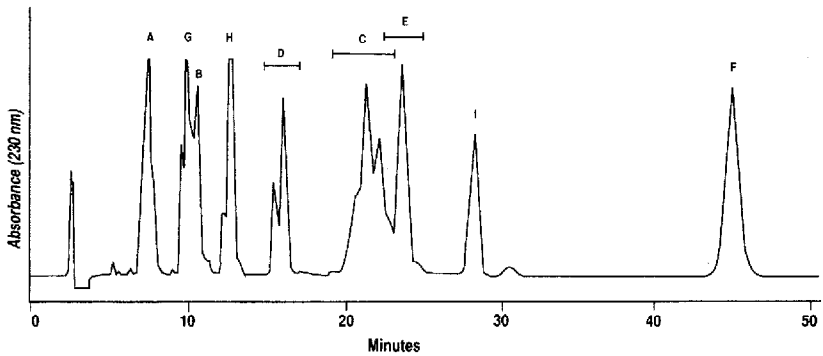

Fig. 1. Normal-phase HPLC separation of various glycerolipid subclasses as their benzoate derivatives was carried out as described in Materials and Methods. Letters for peak identification refer to Table I. Detection at 0.020 a.u.f.s.; peak A represents 1.2 nmol.

acetyl-3-benzoyl-Gro yields an HPLC peak with twenty-fold greater height than any baseline fluctuations.

The chromatographic techniques described here (TLC and HPLC of benzoate derivatives) have proven extremely useful in our investigations of the alkyl-, alk-1-enyl- and acyl subclasses of choline and ethanolamine glycerophosphatides and diradylglycerols. The information in this report should assist others in interpreting data obtained with the phospholipase C/benzoylation methodology[5].

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