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Note

High-performance liquid chromatography of diglyceride *p*-nitrobenzoates

An approach to molecular analysis of phospholipids

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As part of a program of structural studies of the outer membrane of Gram negative bacteria[1], it became necessary to devise a method for characterisation of the molecular species of phospholipids present.

Although phosphatidyl ethanolamine and phosphatidyl serine have been studied as the 4-biphenylcarbonyl derivatives[2], other reported separations of phospholipids by high-performance liquid chromatography (HPLC) have employed the unmodified lipids[3–5]. This has necessitated ultraviolet detection at short wavelengths[3,4] or insensitive refractive index detection[5].

The most complete structural studies of phospholipids have involved degradation to 1,2-diglycerides by means of phospholipase C from *Bacillus cereus*. These diglycerides are fractionated, on the basis of degree of unsaturation, using silver nitrate-impregnated silica gel plates[6,7]. The positional distribution of the fatty acids in the fractions so obtained is then determined by means of a lipase[8]. The individual combinations of fatty acyl substituents cannot, in general, be determined by this method.

We now report HPLC separation of the *p*-nitrobenzoates of mono- and diglycerides which enables complete molecular analysis of phospholipids.

EXPERIMENTAL

Materials

Diglycerides were purchased from Nu Chek Prep (Elysian, MN, U.S.A.), *p*-nitrobenzoyl chloride and 4-dimethylaminopyridine from Aldrich (Milwaukee, WI, U.S.A.), bovine serum albumin (A grade) from Calbiochem (San Diego, CA, U.S.A.), phosphatidyl choline from Applied Science Labs. (State College, PA, U.S.A.), phospholipase C from *B. cereus* (E.C. 3.1.4.3) and lipase from *Rhizopus arrhizus* (E.C. 3.1.1.3) from Boehringer (Mannheim, G.F.R.) and Unichrom HPLC solvents from Ajax Chemicals (Sydney, Australia).

High-performance liquid chromatography

Two LDC Constametric pumps (Models I and IIG) with a dynamic gradient mixer were operated in conjunction with an LDC Gradient Master. A Rheodyne

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Model 7120 syringe-loading injector was used with a 20-μl loop and detection was by means of an LDC UV III monitor operating at 254 nm. A 25-cm Brownlee RP-18 column (10 μm) was used. All samples were dissolved in acetonitrile for injection. All chromatographic manipulations were carried out at 25 $\pm$ 1°C.

Calculation of effective average chain length (EACL)

Retention times (t_R) are expressed relative to that of 1,2-dipalmitin-3-*p*-nitrobenzoate. For the series of saturated 1,2-diglyceride derivatives, a plot of log (relative t_R) *vs.* average fatty acid chain length is linear with equation:

$$\log (\text{rel. } t_R) = 0.145 \text{ (chain length)} - 2.31.$$

Substitution into this expression permits calculation of EACL values.

Preparation of diglyceride p-nitrobenzoates

Diglyceride (1 mg or less) was dissolved in dry pyridine (1 ml) containing 4-dimethylaminopyridine (1 mg), *p*-nitrobenzoyl chloride (4 mg) added and the mixture heated in a sealed tube at 60°C for 2 h. One drop of water was then added and the solution heated for a further 5 min before evaporation to dryness in a stream of nitrogen. The mixture was then partitioned between benzene and water (each 2 ml) and the organic layer washed in turn with equal volumes of 0.1 *M* hydrochloric acid, 0.1 *M* sodium hydroxide, water and saturated sodium chloride solution. Evaporation of the benzene gave the diglyceride nitrobenzoate, which was immediately dissolved in acetonitrile for storage.

Bromination of diglyceride p-nitrobenzoates

Diglyceride *p*-nitrobenzoate, dissolved in chloroform (0.5 ml), was treated with sufficient 5% (w/v) solution of bromine in chloroform to give a persistent colour. After 5 min at room temperature, the sample was carefully evaporated to dryness in a stream of dry nitrogen.

Treatment of phospholipid with phospholipase C

Phospholipid (0.1–2 mg) was dispersed in water (0.2 ml) by sonication before addition of 0.1 *M* triethanolamine HCl buffer, pH 8.0 (0.2 ml). Phospholipase C suspension (3 μl) was added and the mixture incubated at 37°C for 1 h. The mixture was diluted with water (1 ml) and the diglyceride extracted into benzene (2 ml).

Lipase treatment of diglyceride nitrobenzoate

Diglyceride nitrobenzoate (2 mg or less) was dispersed by means of sonication in 0.2 *M* Tris–HCl buffer, pH 8.0 (1 ml) containing bovine serum albumin (4 mg) and 5 m*M* calcium chloride. Lipase suspension (3 μl) was added and the mixture incubated at 37°C for 1 h. After extraction with benzene (2 ml) the organic layer was evaporated in a stream of nitrogen.

Isomerisation of monoglyceride p-nitrobenzoate

2-Palmitoyl-3-*p*-nitrobenzoyl-*sn*-glycerol (approx. 100 μg) was dispersed in 0.2 *M* Tris–HCl buffer, pH 8.0 (1 ml) and incubated overnight at 37°C. The resultant 1-palmitoyl-3-*p*-nitrobenzoyl-*sn*-glycerol was extracted with benzene (2 ml).

RESULTS AND DISCUSSION

Introduction of a *p*-nitrobenzoate chromophore into a diglyceride by acylation of the free hydroxyl group permits sensitive ultraviolet detection at 254 nm. Acylation in the presence of 4-dimethylaminopyridine[10] proceeds more rapidly than acyl migration. Only trace amounts of the nitrobenzoates of the 1,3-diglycerides were detected after derivatisation of the 1,2-isomers.

Chromatography of a mixture of diglyceride *p*-nitrobenzoates shows good separation and peak shape (Fig. 1a). For convenience, a flow-rate of 1 ml/min was normally used. Significant improvement in chromatographic performance is, however, obtained using a flow-rate of 0.5 ml/min.

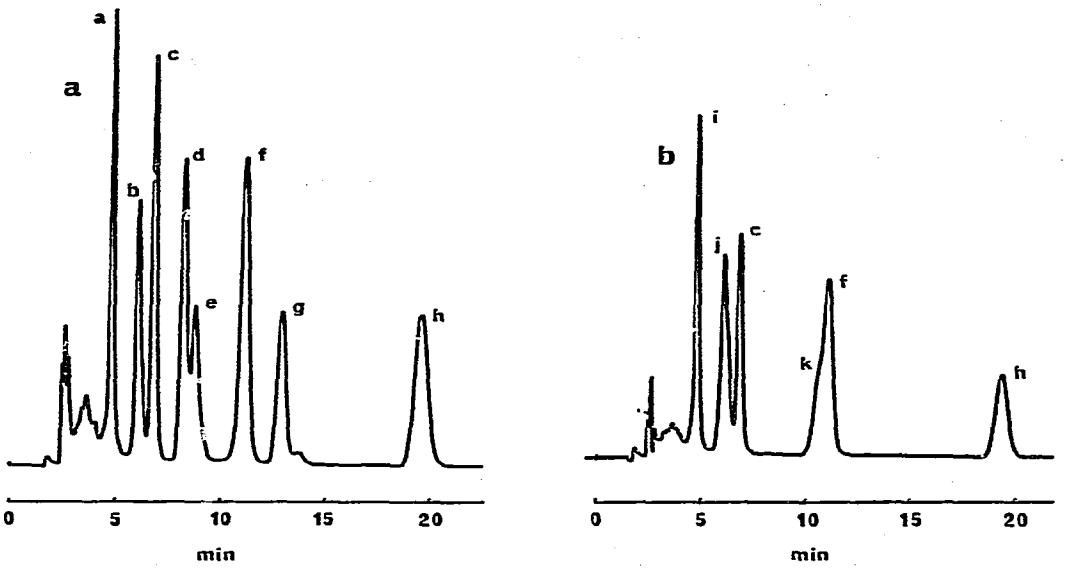

Fig. 1. Separation of diglyceride *p*-nitrobenzoates by HPLC. Operating conditions: column, 25 cm RP-18 (10 μm); mobile phase, 2-propanol–acetonitrile (35:65); column temperature, 25 ± 1°C; flow-rate 1 ml/min. Peaks: a = 1,2-di-12:0; b = 1,2-di-16:1(9); c = 1,2-di-14:0; d = 1,3-di-18:1(11); e = 1,2-di-18:1(11); f = 1,2-di-16:0; g = 1-18:0-2-18:1; h = 1,2-di-18:0; i = a + bromo-b; j = bromo-d + bromo-e; k = bromo-g.

The retention behaviour of the diglyceride derivatives is conveniently expressed in terms of the EACL of the fatty acids present (see Experimental). As expected from studies of fatty acid esters[11,12] and phosphatidyl choline[5], unsaturated glycerides have smaller EACL values than the corresponding saturated species (Table I). The 1,2- and 1,3-isomers are well resolved; in each case, the 1,3-diglyceride derivative elutes before the 1,2-isomer (Table I).

Relative peak areas relate directly to the molar ratios of the diglycerides present. Analysis can conveniently be carried out at the microgram level: 0.6 μg (approx. 1 nmol) of 1,2-dipalmitin, as the *p*-nitrobenzoate, gives a full-scale peak at a detector setting of 0.032 a.u.f.s.

TABLE I

HPLC PROPERTIES OF DIGLYCERIDE *p*-NITROBENZOATES ON RP-18

Fatty acyl substituents	*Retention time relative to 1,2-di-16:0*		*Effective average chain length*
	1,2-Isomer	*1,3-Isomer*	
di-12:0	0.27		12.0
		0.25	11.78
di-14:0	0.52		14.0
		0.47	13.67
di-16:0	1.00		16.00
		0.89	15.58
di-18-0	2.00		18.00
		1.67	17.47
di-14:1(9)	—		—
		0.24	11.66
di:16:1(9)	0.43		13.40
		0.40	13.19
di-18:1(11)	0.75		15.07
		0.70	14.86
di-18:2(9, 12)	0.21		11.26
			—
1-18:0-2-18:1(9)	1.23	—	16.55

It is useful to have a simple criterion for judging the presence and degree of unsaturation in each molecular species. Reaction of bromine with unsaturated diglyceride *p*-nitrobenzoates produces bromodiglyceride derivatives which elute even earlier than the unsaturated species (Fig. 1b). The decrease of the EACL values of the brominated species relative to the corresponding unsaturated materials depends on the degree of unsaturation (Table II). The magnitude of this decrease is approximately linear with respect to the number of double bonds present.

TABLE II

EFFECT OF BROMINATION ON HPLC PROPERTIES OF UNSATURATED 1,2-DIGLYCERIDE-*p*-NITROBENZOATES

Substituents before bromination	*Relative retention time of brominated derivatives**	*Effective average chain length (EACL)*	*Bromination shift, Δ EACL*
1-18:0-2-18:1(9)	0.96	15.80	−0.75
di-16:1(9)	0.27	12.01	−1.39
di-18:1(11)	0.43	13.40	−1.67
di-18:2(9,12)	0.11	9.32	−1.94

* Relative to 1,2-di-16:0 *p*-nitrobenzoate.

Treatment of a 1,2-diglyceride nitrobenzoate with lipase leads cleanly to the 2-acyl-3-*p*-nitrobenzoyl-*sn*-glycerol without competing release of *p*-nitrobenzoic acid. The monoglyceride derivatives are readily resolved by HPLC, permitting determination of the 2-acyl substituents (Fig. 2). The slightly alkaline buffer used for the

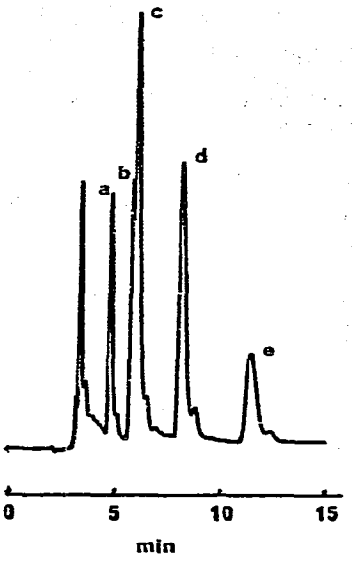

Fig. 2. Separation of 2-acyl-3-*p*-nitrobenzoyl-*sn*-glycerols by HPLC. Eluting solvent, acetonitrile. For other operating conditions, see legend to Fig. 1. Peaks: a = 2-12:0; b = 2-16:1(9); c = 2-14:0; d = 2-16:0; e = 2-18:0.

incubation leads to slow isomerisation of the products to the more stable 1-acyl-3-*p*-nitrobenzoyl-*sn*-glycerols. This is evidenced by the small peaks on the trailing edge of the main peaks (Fig. 2). Complete isomerisation can be achieved by incubation of the 2-acyl derivatives in buffer at pH 8.0.

1,3-Diglyceride-*p*-nitrobenzoates are converted by lipase to 2-*p*-nitrobenzoyl-glycerol with little accumulation of the intermediate monoglyceride derivatives.

Because of the sensitivity and good chromatographic performance in this system, it is possible to isolate a peak fraction from a heavy injection of diglyceride derivatives, treat it with lipase and re-inject to identify the monoglyceride product. We therefore have available an extension of existing methods for molecular analysis of diglycerides and phospholipids which permits characterisation of discrete molecular species.

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