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Sensitive analysis of phospholipid molecular species by high-performance liquid chromatography using fluorescent naproxen derivatives of diacylglycerols

A. RASTEGAR and A. PELLETIER
Centre de Neurochimie du CNRS, Antenne de Cronenbourg, BP20CR, 67073 Strasbourg Cedex (France)
G. DUPORTAIL
Laboratoire de Physique, Faculté de Pharmacie, CNRS UA491, BP24, 67401 Illkirch Cedex (France)
and
L. FREYSZ and C. LERAY
Centre de Neurochimie du CNRS, Antenne de Cronenbourg, BP20CR, 67037 Strasbourg Cedex (France)
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ABSTRACT

A sensitive high-performance liquid chromatographic (HPLC) method for the separation and determination of diacylglycerophospholipid and diacylglycerol (DAG) molecular species has been developed. Phospholipids are hydrolysed with phospholipase C and the resulting DAGs are reacted with naproxen chloride in the presence of 4-dimethylaminopyridine. The naproxen–DAGs were purified by thin-layer chromatography on silica gel G plates. Molecular species were separated using reversed-phase HPLC with isocratic elution and determined by measuring the absorbance at 230 nm or fluorescence at 352 nm (excitation at 332 nm). The method was applied to the determination of diacylglycerophosphoethanolamine in rat cerebrum and cerebellum. The molar absorption coefficient of the naproxen derivatives was 53 000 $lmol^{-1}$ cm^{-1} at 230 nm, permitting the generation of linear concentration-dependent determinations down to less than 10 pmol. A ten-fold increase in sensitivity was obtained with a fluorescence detection system owing to the fluorescent properties of the proposed adduct.

INTRODUCTION

The accurate separation and determination of molecular species of glycerophospholipids and diacylglycerols (DAGs) is difficult owing to the lack of chromogenic moieties in such molecules. The separation of intact phospholipid molecular species has been proposed [1–4], but without sufficient resolution and sensitivity. Further, the determination of the eluted molecular species is complex owing to the absorption properties which depend on the unsaturation of the individual molecules. The use of post-column fluorescence detection is attractive because it allows the determination of intact phospholipid species in the nanomole range [5]. Unfortunately, none of these techniques permits the resolution of phospholipid subclasses (alk-1-enylacyl, alkylacyl and diacyl types).

Several studies have been reported in which UV-absorbing derivatives of dira-

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dylglycerols[a] prepared from phospholipids after phospholipase C hydrolysis were used [6–9]. Recent improvements in the detection sensitivity have been reported in coupling diacylglycerol moieties with fluorescent compounds [10,11].

This paper describes a method for the determination of the diacylglycerol moieties by high-performance liquid chromatography (HPLC) in the picomole range after their derivatization with naproxen chloride. The technique has been utilized for the analysis of the diacylglycerophosphoethanolamine (PE) composition of rat brain cerebrum.

EXPERIMENTAL

Chemicals

Naproxen (6-methoxy-α-methyl-2-naphthaleneacetic acid) and 4-dimethylaminopyridine were obtained from Aldrich. Oxalyl chloride was obtained from Janssen Chimica and methanol, chloroform, acetonitrile (HPLC grade), 2-propanol (HPLC grade), diethyl ether, light petroleum (b.p. 35–60°C) and hexane were obtained from SDS (Peypin, France). Phospholipase C type XIII (from *Bacillus cereus*), 1,2-dipalmitoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, 1-stearoyl 2-arachidonoyl-*sn*-glycerol were obtained from Sigma (St. Louis, MO, U.S.A.) and pyridine and silica gel 60 thin-layer chromatographic (TLC) plates from Merck (Darmstadt, F.R.G.).

Synthesis of naproxen chloride

Naproxen (500 mg) was dissolved in 50 ml chloroform, 0.3 ml of oxalyl chloride was slowly added and the resulting solution was refluxed for 1 h. The solvent was evaporated to dryness in a rotary evaporator under reduced pressure at 40°C and the residue was kept overnight over potassium hydroxide under vacuum. The powdered naproxen chloride was used without further purification. All the procedures were carried out under reduced light.

Preparation of diacylglycerols from phospholipids

Rat brain cerebrum and cerebellum from adult animals were extracted according to Folch *et al.* [12]. The phospholipids were purified by silicic acid column chromatography [13], spotted on boric acid-impregnated silica gel plates (LK5; Whatman, Clifton, NJ, U.S.A.) and separated using chloroform–ethanol–water–triethanolamine (30:35:7:35, v/v) as solvent [13]. The PE-containing spot was made visible by spraying the TLC plates with 0.01% primuline dye in acetone–water (4:1, v/v) and viewing under UV light. The phospholipid was eluted with 2 × 2 ml of chloroform–methanol–water (5:5:1, v/v/v) and, after evaporation of the solvent, PE was dissolved in chloroform–methanol (2:1, v/v). An aliquot was sonicated in a sonic water-bath (Branson) for 10 s in 1 ml of sodium phosphate buffer (50 m*M*, pH 7.4) containing 30 m*M* sodium borate. After addition of 1.5 ml of diethyl ether and 30 U of phospholipase C, the capped tubes were shaken vigorously for 45 min at room temperature. The ether phase and one wash with diethyl ether were combined and the solution was evaporated to dryness under a stream of nitrogen at room temperature and rapidly derivatized.

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[a] The term "radyl" is used to denote an acyl, alkyl or alkenyl group.

Derivatization of diglycerides

Up to 1 mg of dried diradylglycerols prepared from phospholipids as described above, 5 mg of naproxen chloride and 10 mg of 4-dimethylaminopyridine were kept under vacuum for 20 min at room temperature. The mixture was dissolved in 1 ml of dry pyridine and heated in a sealed vial at 80°C for 15 min. A 2-ml volume of 0.1% $NaHCO_3$ was added and the derivatized diradylglycerols were extracted twice with 2 ml of hexane. After evaporation of the solvent, the residue was dissolved in a convenient volume of dichloromethane.

Chromatographic analyses

Derivatized diradylglycerols were separated by TLC on silica gel 60 plates developed with light petroleum–diethyl ether (75:25, v/v). The alk-1-enylacyl, alkylacyl and diacyl subclasses were made visible under UV light as brillant spots after spraying with primuline. The naproxen derivates migrated with $R_F \approx 0.82$, 0,75, 0.67 for the alk-1-enylacyl, alkylacyl and diacyl subclasses, respectively, as determined with appropriate standards. The silica gel band containing the diacyl derivatives was scraped off and extracted twice with 2 ml of acetonitrile.

The molecular species were separated by HPLC using a Waters Model 510 pump and either a C_{18} reversed-phase column (150 × 4 mm I.D.) (Resolve, 5 μm; Waters Assoc.) or a 125 × 4 mm I.D. LiChroCART (Lichrospher 100RP-18, 5 μm; Merck–Clevenot, France) at room temperature. The solvent was acetonitrile–2-propanol (95:5, v/v) pumped at a flow-rate of 2 ml min^{-1}. The separated components were measured either with a UV spectrophotometer (Uvidec-100-V; Spectra-Physics) at 230 nm or a scanning fluorescence detector (Waters Model 470, excitation at 332 nm, emission at 352 nm). Peak-area percentages and retention times were obtained with a Spectra-Physics SP4290 recorder–integrator.

The identification of individual molecular species was obtained by gas chromatographic analysis of methyl esters prepared from resolved peaks, by calibration with various diacylglycerol species from commercial sources and by developing a relative retention time–carbon number plot as described previously [14,15]. The fatty acids were analysed from the collected peaks containing not less than 1 nmol of each species.

Fatty acid methyl esters were prepared [16] and analysed with a Perkin-Elmer Sigma 1 instrument fitted with a capillary column (bonded fused silica, 50 m × 0.32 mm I.D., Superox; Alltech), a flame ionization detector and a Sigma 10 data system. The separation was carried out at 190°C with helium as carrier gas. Aliquots of 2 μl of sample were injected in the splitless mode and the methyl esters were identified using appropriate standards.

Spectroscopic analyses

UV spectra were obtained using a Kontron recording spectrophotometer (Uvikon 820) and the fluorescence spectra with an SLM 48000 spectrofluorimeter. Fluorescence lifetimes were determined with a laboratory-built instrument (Ortec components), which is based on the single photoelectron technique.

RESULTS AND DISCUSSION

Naproxen chloride has recently been proposed as a fluorescent reagent for the chiral derivatization of optically active amines and alcohols [17]. This substituted naphthylacetic acid showing a strong intrinsic fluorescence and ultraviolet absorbance seems well suited for the determination of diglycerides after its coupling with the alcohol function.

In order to determine the optimum conditions for the derivatization reaction with 0.1–0.5 mg of diradylglycerols, the effects of excess of reagent, catalyst, reaction time and temperature were determined. A diradylglycerol:reagent:catalyst ratio of 1:5:10 (w/w) was found to be optimum when the reaction proceeded for 15 min at 80°C. Under these conditions, 75 ± 3% of diacylglycerols were converted (deter-

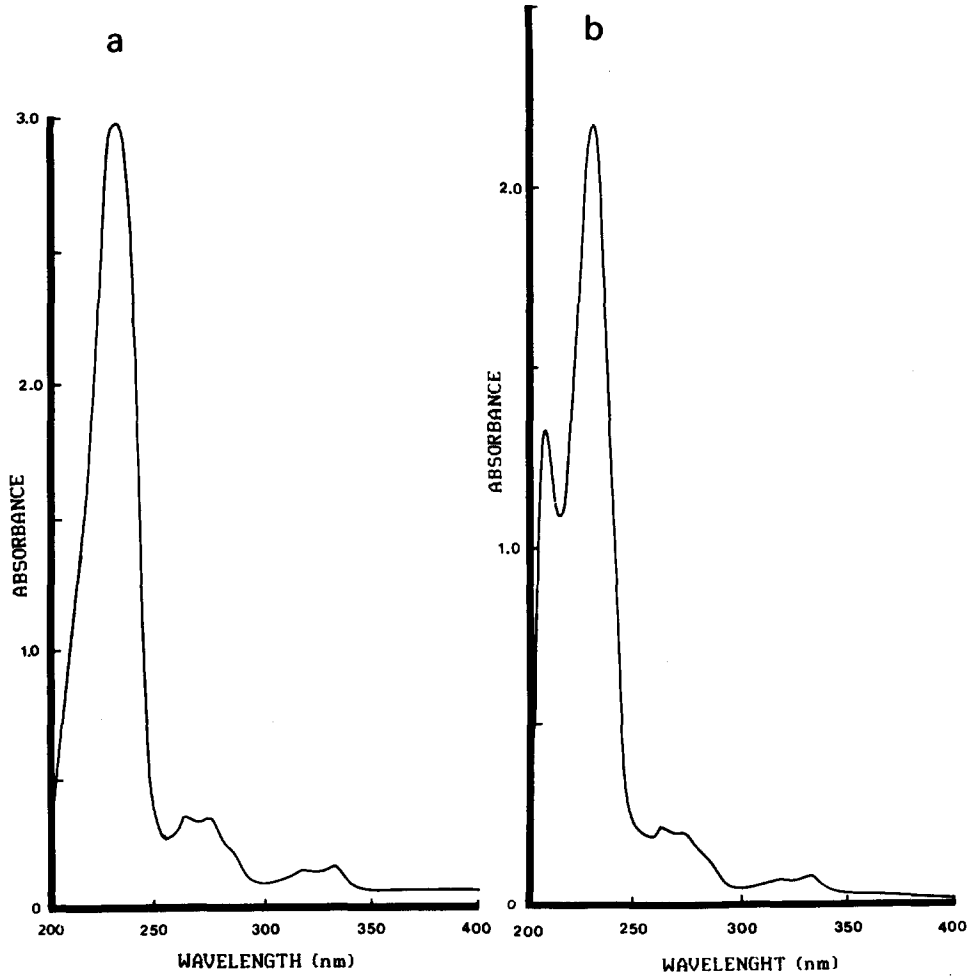


Fig. 1. Absorption spectra of (a) a methanolic solution (50 μM) of naproxen chloride and (b) a methanolic solution (40 μM) of naproxen–diacylglycerols derived from rat cerebrum phosphatidylethanolamine.

mined by fatty acid analysis) and no acyl migration occurred. With UV detection a high sensitivity was observed as the molar absorption coefficient of naproxen–dioleoylglycerol was *ca.* 53 000 l mol^{-1} cm^{-1} at 230 nm, the detection limit being about 10 pmol. Naproxen chloride had an absorption spectrum (Fig. 1a) similar to the lipid derivatives (Fig. 1b) but with a higher molar absorption coefficient at 230 nm (*ca.* 57 000 l mol^{-1} cm^{-1}). Further, HPLC separations of the naproxen diacylglycerols obtained from rat cerebrum diacylphosphatidylethanolamine (Table I) gave similar patterns with detection at 230 and 240 nm, indicating that the quantification is not affected by the degree of fatty acid unsaturation and is directly related to the amount of each molecular species present in the mixture.

In contrast to unsubstituted naphthalene, the absorption spectrum of naproxen chloride displays an additional band near 330 nm, allowing the excitation of the derivatized molecules in this region. This characterization avoids interferences from several fluorescent impurities. The spectral properties of naproxen chloride and the naproxen–diacylglycerol complex displayed the same exitation maximum (in methanol) at 332 nm and the same fluorescence maximum at 352 nm (Fig. 2), without any effect of the degree of acyl chain unsaturation. This was confirmed using various diacylglycerol molecular species with different degrees of unsaturation either collected at the HPLC column outlet or derivatized from diacylglycerol from commercial sources (namely 1,2-dipalmitoylglycerol, 1,2-dioleoylglycerol and 1-stearoyl-2-arachidonoylglycerol). The determined fluorescence quantum yield was 0.35 ± 0.02 in methanol for naproxen chloride and for the three derivatives from the above commercial diacylglycerols. This determination was performed using β-naphthol in cyclohex-

TABLE I

DISTRIBUTION OF MOLECULAR SPECIES IN DIACYLPHOSPHATIDYLETHANOLAMINE FROM RAT CEREBRUM

Peak No.[a]	Molecular species[b]	Composition (%)
1	16:1/20:4*n*6	0.14
2	16:1/22:5*n*6	0.19
3	18:1/22:6*n*3	1.43
4	16:0/22:6*n*3	5.30
5	18:1/20:4*n*6	5.12
6	16:0/20:4*n*6	5.15
7	18:1/18:2*n*6	1.70
8	18:0/22:6*n*3	8.77
9	16:0/22:4*n*6	1.91
10	18:0/22:5*n*3	1.03
11	18:0/20:4*n*6	32.56
12	18:1/18:1*n*9	7.65
13	16:0/18:1*n*9	9.86
14	18:0/20:3*n*6	0.41
15	16:0/16:0	6.47
16	16:0/20:1*n*9	0.30
17	18:0/18:1*n*9	12.40

[a] Peak numbers correspond to those shown in Fig. 3a.

[b] The molecular species are identified by the fatty acid at the 1-position (left) and the fatty acid at the 2-position (right) of the glycerol moiety. The fatty acids are identified by the number of carbon atoms and double bonds.

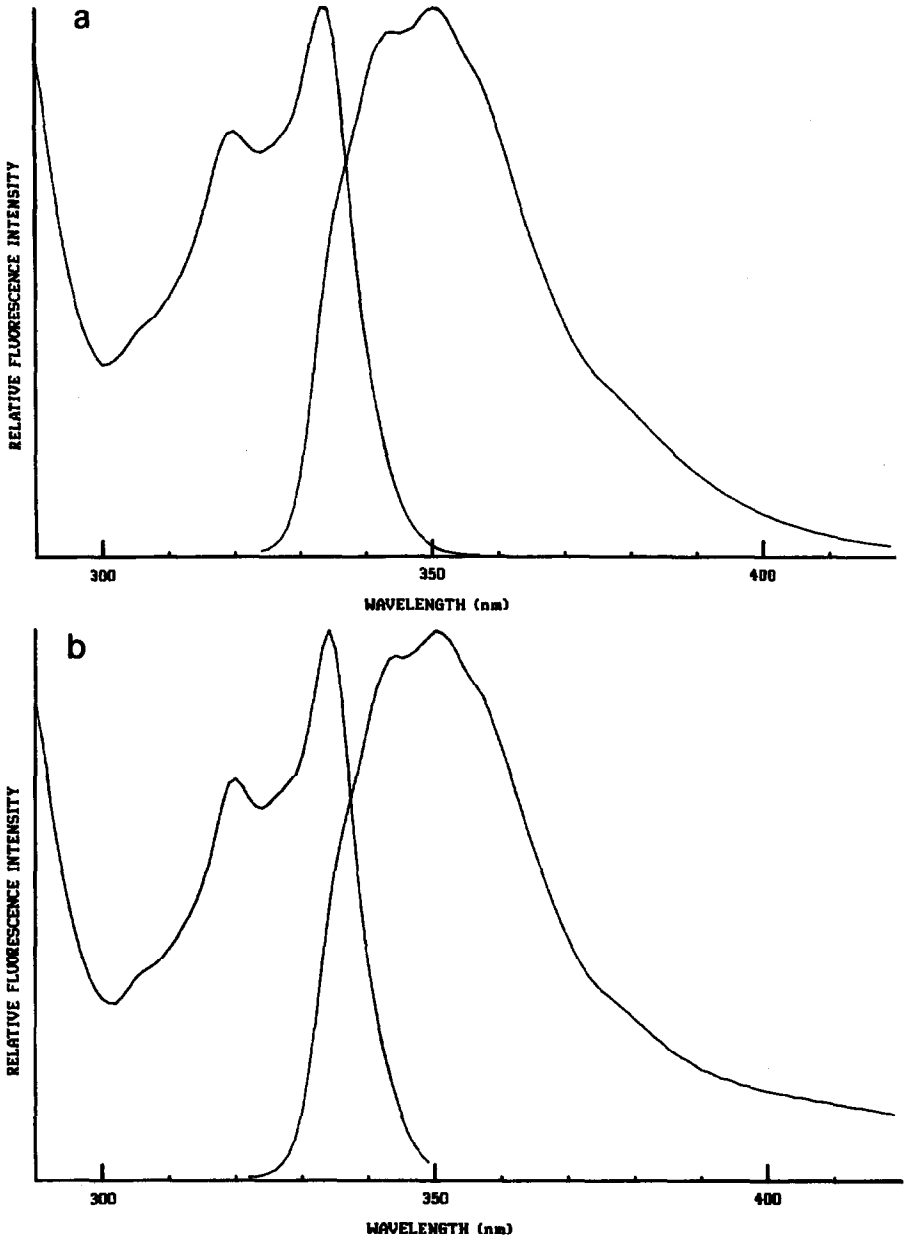
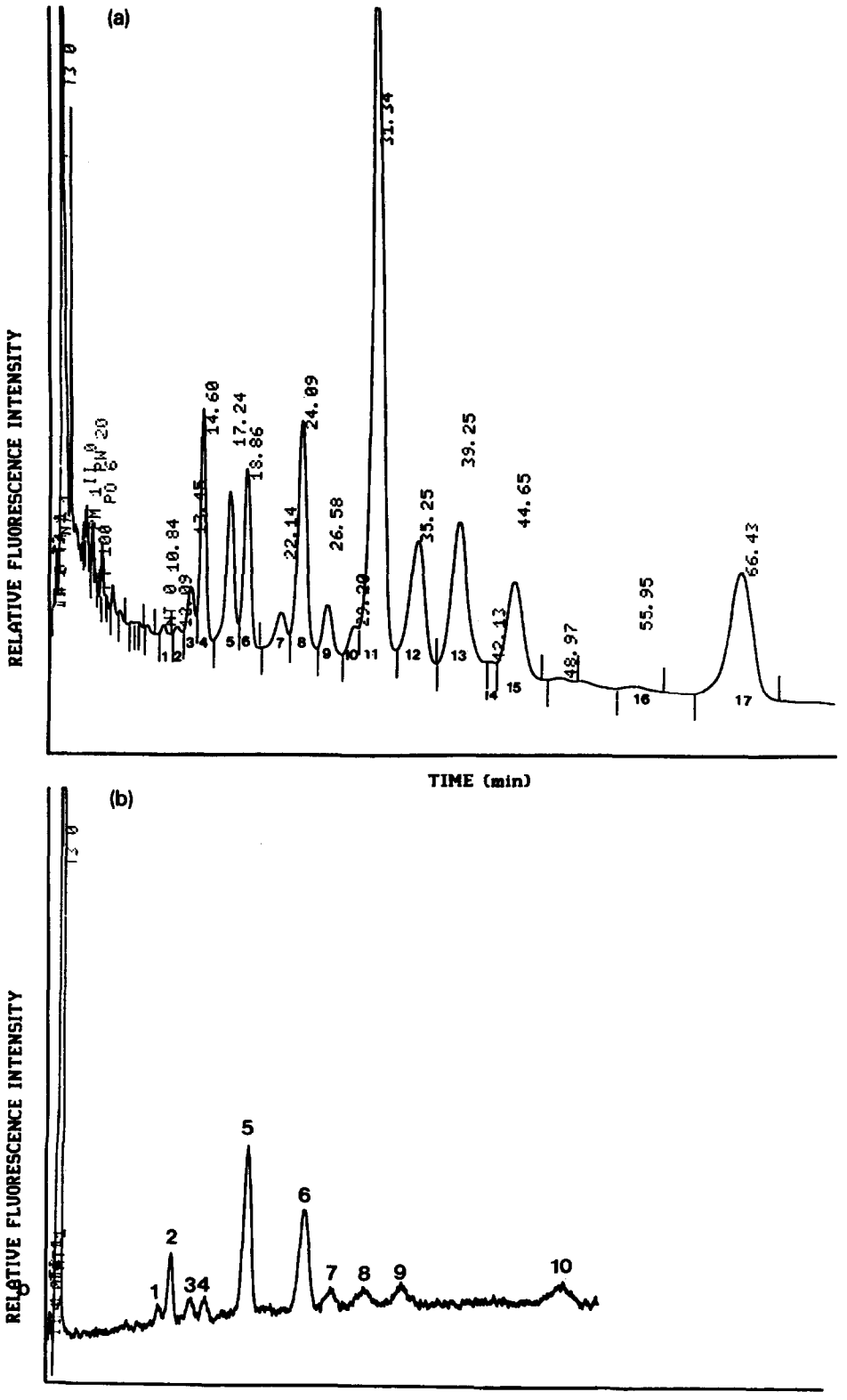


Fig. 2. Excitation spectra (left, $\lambda_{em.}$ = 360 nm) and emission spectra (right, $\lambda_{exc.}$ = 320 nm) of (a) a methanolic solution (50 μM) of naproxen chloride and (b) a methanolic solution (40 μM) of naproxen–diacylglycerols derived from rat cerebrum phosphatidylethanolamine.

Fig. 3. High-performance liquid chromatograms of (a) an acetonitrile solution (32 μM) and (b) acetonitrile → solution (2 μM) of naproxen–diacylglycerols derived from rat cerebrum phosphatidylethanolamine. Operating conditions: column, 12.5 cm LiChrospher 100 RP-18 (5 μm); mobile phase, acetonitrile–2-propanol (95:5); flow-rate, 2 ml min^{-1} at room temperature. The sample [(a) 1.6 nmol and (b) 100 pmol of diacylglycerols] was injected with a 50-μl loop. Fluorescence detector: excitation at 332 nm, emission at 352 nm. For identity of peaks in (a) see Table I. Peaks in (b): (1) 2.3 pmol 18:1/22:6; (2) 8.6 pmol 16:0/22:6; (3) 3.0 pmol 18:1/20:4; (4) 3.0 pmol 16:0/20:4; (5) 31.3 pmol 18:0/22:6; (6) 24.6 pmol 18:0/20:4; (7) 5.0 pmol 18:1/18:1; (8) 5.5 pmol 16:0/18:1; (9) 5.0 pmol 16:0/16:0; (10) 8.6 pmol 18:0/18:1.


(a)
RELATIVE FLUORESCENCE INTENSITY
TIME (min)
10.84
13.45
14.60
17.24
18.86
22.14
24.09
26.58
29.20
31.34
35.25
39.25
42.13
44.65
48.97
55.95
66.43
(b)
RELATIVE FLUORESCENCE INTENSITY
1
2
34
5
6
7
8
9
10

ane (fluorescence quantum yield $\varphi = 0.32$) as a standard [18]. The constancy of the fluorescence quantum yield was confirmed by that of the fluorescence lifetime ($\tau = 7.6 \pm 0.1$ ns) for each molecular species. With such a quantum yield, the fluorescence efficiency can be considered to be good and allows quantitative determinations, as it does not depend on the degree of unsaturation of the fatty acids.

Fig. 3a depicts a chromatogram of naproxen–diacylglycerols synthesized from rat cortex diacyl glycerophosphoethanolamine and the distribution of the main species is listed in Table I. Identical molecular species distributions were obtained after dinitrobenzoyl derivatization and UV detection [19], but with a ten times lower sensitivity. No differences were detected in the species distribution and yield when widely different amounts of DAGs (from 10 to 1000 μg) were processed. The linearity of the response was verified with fluorescence detection and ranged from 15 pmol to 10 nmol of a single molecular species, the detection limit being at least 1 pmol (Fig. 3b). The achievement of a greater sensitivity is dependent on the reduction of the background noise by using purer and hence more expensive solvents.

In conclusion, these studies have shown that naproxen is a useful reagent for the determination of DAG molecular species. The proposed method is very sensitive to picomole levels of these lipids and can be extended as for other derivatization procedures to all diradylglycerols. The formation of stable fluorescent derivatives is facile, using a readily available compound whose activation to the acyl chloride is easy to perform. When compared with other chromophores or fluorophores, the naproxen adduct is at least ten times more sensitive and allows the excellent resolution of molecular species on C_{18} columns with very simple isocratic HPLC equipment. Preliminary experiments have indicated that the procedure is sufficiently sensitive for the determination of the molecular species distribution of diacyl PE and diacylglycerophosphocholine purified from cultured neurons by our TLC procedure [13]. It may also be possible to elucidate the changes occurring in the distribution of molecular species of free diacylglycerols and the different phospholipids in small biological samples such as biopsies, cultured cells and membrane preparations.

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