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Note

Separation of alkylacyl and diacyl glycerophospholipids and their molecular species as naphthylurethanes by high-performance liquid chromatography

H. RABE* and G. REICHMANN

Institute of Pathological and Clinical Biochemistry, Charité Hospital, Humboldt University, Schumannstrasse 20-21, 1040 Berlin (G.D.R.)

Y. NAKAGAWA

Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa (Japan)

and

B. RÜSTOW and D. KUNZE

Institute of Pathological and Clinical Biochemistry, Charité Hospital, Humboldt University, Berlin (G D.R)

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During the past few years different methods have been developed for the species analysis of glycerophospholipids by high-performance liquid chromatography [1–8]. Many biological materials can be investigated by these methods, but an exact analysis is possible only in materials consisting only of the diacyl type of glycerophospholipids. Ether-linked glycerophospholipids are widely distributed in various tissues and cells, such as the brain, heart, kidney, lymphocytes, erythrocytes and platelets. The proportion of these ether compounds may be up to 70%, in ethanolamine glycerophospholipids (EGP) of alveolar macrophages, for example [9].

The separation of the ether lipids from the diacyl type and their further analysis may be important for two reasons. First, for investigations of the species pattern of microsomal diacylglycerols and phospholipids in regard to de novo synthesis it is necessary to analyse the pure diacyl phospholipids because ether lipids have different metabolic pathways. Secondly, the proportion of

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ether compounds and their species pattern may also be of some interest. Although the biological significance of ether-linked glycerophospholipids is not yet fully clarified, alkylacyl glycerophospholipids are of increasing importance. They are thought to be precursors of the platelet activating factor (PAF), and also seem to play a role in tumour growth.

Recently we described a method for the derivatization and separation of diacylglycerols (endogenous or derived from glycerophospholipids by the action of phospholipase C) by the use of α-naphthyl isocyanate [4,5]. The resulting naphthylurethanes can be detected either by UV detection at 290 nm or by measuring their fluorescence. The fluorescence detection method is very sensitive and the detection limit is ca. 5 pmol for a single molecular species. In this report we describe the use of the naphthyl derivatives for normal-phase high-performance liquid chromatographic (HPLC) separation of alkylacyl and diacyl subclasses with subsequent species analysis by reversed-phase HPLC.

EXPERIMENTAL

Materials

Dimethylformamide (DMF) was purified by distillation with octadecyl isocyanate. α-Naphthyl isocyanate was purified by distillation in vacuo. 1,2-Dimyristin, 1,2-dipalmitin, 1,2-distearin and cholesterol were supplied by Serva (Heidelberg, F.R.G.). Phospholipase C (*Bacillus cereus* and *Clostridium welchii*) were from Sigma (St. Louis, MO, U.S.A.) 1,4-Diazabicyclooctane and precoated silicagel 60 plates were purchased from Merck (Darmstadt, F.R.G.). Silprep was from Applied Science Labs. (State College, PA, U.S.A.). Hexane and tetrahydrofuran were of HPLC grade. All other solvents were of reagent grade and distilled before use.

Methods

The lipids of bovine erythrocytes were extracted by a method modified from that of previous reports [10,11]. Briefly, to washed erythrocytes sodium dodecyl sulphate and methanol were added. Phase separation was achieved by addition of 3 *M* KCl and chloroform. The lower phase was washed first with methanol–0.6 *M* HCl–1 m*M* Na_2HPO_4 (3:48:47) then with methanol–0.6 *M* HCl (3:48) and chloroform–methanol–0.01 *M* HCl (3:48:47). To hydrolyse alkenylacyl analogues, lipid extracts were placed as a spot on thin-layer plates and treated with 25 μl of trichloracetic acid (TCA) reagent (a 1:1 mixture of 2% TCA and 8% HCl) [12]. This was allowed to react for 10 min at ambient temperature before drying with warm air.

Phospholipids were then separated on precoated silicagel 60 plates by two-dimensional thin-layer chromatography (TLC) with chloroform–methanol–concentrated ammonia (130:50:10) in the first dimension and chloroform–acetone–methanol–acetic acid–water (60:80:20:20:10) in the second dimen-

sion. Ethanolamine glycerophospholipids were converted into diradylglycerols by phospholipase C hydrolysis [13]. Diradylglycerols were then derivatized to naphthylurethanes as previously described [5]. Normally, we dissolved 10–100 nmol of the diradylglycerol sample in 100 μl DMF. A 10-μl volume of α-naphthyl isocyanate, yielding at least 500-fold molar excess of the reagent, and 10 μl of 1,4-diazabicyclo[2.2.2]octane (0.1 *M* in DMF) were added. The stoppered vial was heated at 85°C for 30 min. The excess reagent was destroyed by addition of 300 μl of methanol, and the reaction mixture was centrifuged to obtain a clear supernatant, which was evaporated to a final volume of 100 μl. For further separation into subclasses, byproducts formed during the derivatization procedure have to be removed using a short RP18 column. The purified diradylglycerolurethanes were then separated into alkylacyl and diacyl subclasses by normal-phase HPLC on an Si 100 column. The diacyl and alkylacyl fractions were collected for species separation on an RP18 column.

Chromatographic conditions

The HPLC separations were carried out on a Hewlett-Packard 1084B high-performance liquid chromatograph equipped with a variable-wavelength detector set at 290 nm. For fluorescence detection a Fluorichrom (Varian, Los Altos, CA, U.S.A.) was used (excitation 280 nm, emission 360 nm). The solvent system for the separations on the RP18 columns was methanol (solvent A) and methanol–water (80:20) (solvent B). For the purification run, a short (100 mm×4.6 mm I.D.) column packed with LiChrosorb RP18 (10 μm) (Merck) was used. Elution started at 70% B and a linear gradient was run to 55% B in 25 min. Elution was then completed using 100% solvent A. For the separation of alkylacyl and diacyl subclasses the chromatographic column (250×4 mm I.D.) was filled with LiChrosorb Si 100 (5 μm). The solvent system was hexane–tetrahydrofuran (95:5) pumped at a flow-rate of 1 ml/min at room temperature. Separation of molecular species of individual subclasses was achieved using an RP18 (5 μm) LiChrosorb column (200 mm×4.6 mm I.D.). Diacyl species were separated with a linear solvent gradient running from 70% B to 4% B between 0 and 120 min. For the alkylacyl species the solvent gradient ranged from 50% B to 4% B in the same time. If not destroyed by the procedure described, alkenylacyl species were eluted under the same conditions but they could not be separated clearly from the alkylacyl species. In all experiments the flow-rate was 1 ml/min and the oven temperature was set at 60°C.

For gas chromatographic (GC) analysis of the separated peaks (fatty acid methyl ester and bis-trimethylsilyl derivatives of alkylglycerols), a Varian 2100 instrument with a flame ionization detector and two data systems (CDS 101 and Shimadzu C-R3A) were used. A coiled glass column (1.8 m×2 mm I.D.) was packed with 10% EGSS-X on Gas-Chrom Q (100–120 mesh). The sepa-

ration was carried out with a temperature programme from 140 °C to 190 °C at 1 °C/min. The carrier gas was nitrogen (flow-rate 20 ml/min).

Identification

Identification of the diacyl molecular species was made by transmethylation with boron trifluoride–methanol, and subsequent analysis of the fatty acid methyl ester by GC and by calculation of relative retention times as described previously [5]. Separated peaks from the alkylacyl subgroup were hydrolysed with 0.5 *M* NaOH in 90% methanol at 37 °C for 90 min. After neutralization, alkylglycerols and fatty acids were separated by TLC with light petroleum–diethyl ether–acetic acid (30:70:1). Fatty acids were analysed as their methyl esters and alkylglycerols as their bis-trimethylsilyl derivatives.

RESULTS AND DISCUSSION

Reversed-phase HPLC of different glycerolipid derivatives has now become an important tool for the analysis of molecular species. Despite its high efficiency, the simultaneous analysis of the diacyl subgroup and their ether analogues is not possible. The preceding separation of the ether and diacyl subclasses is possible by TLC [14,15] and HPLC [8,16–19] methods, but only a few of these separations [8,14,17,19] are connected with a subsequent species analysis. As shown in Fig. 1, ether analogues can be separated from the diacyl type of glycerophospholipids after derivatization with α-naphthyl isocyanate by normal-phase HPLC, allowing the direct quantitation of the subclasses by comparison of the peak areas. Different from our work on derivatization with subsequent species analysis it was necessary to separate the diradylglycerol-

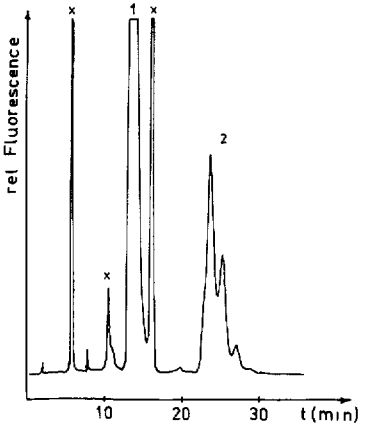

Fig. 1. Separation of naphthylurethanes from alkylacyl- (peak 1) and diacylglycerols (peak 2) prepared from EGP from bovine erythrocytes. For experimental conditions see *Methods*. The peaks marked with × are unknown components containing small amounts of or no fatty acids.

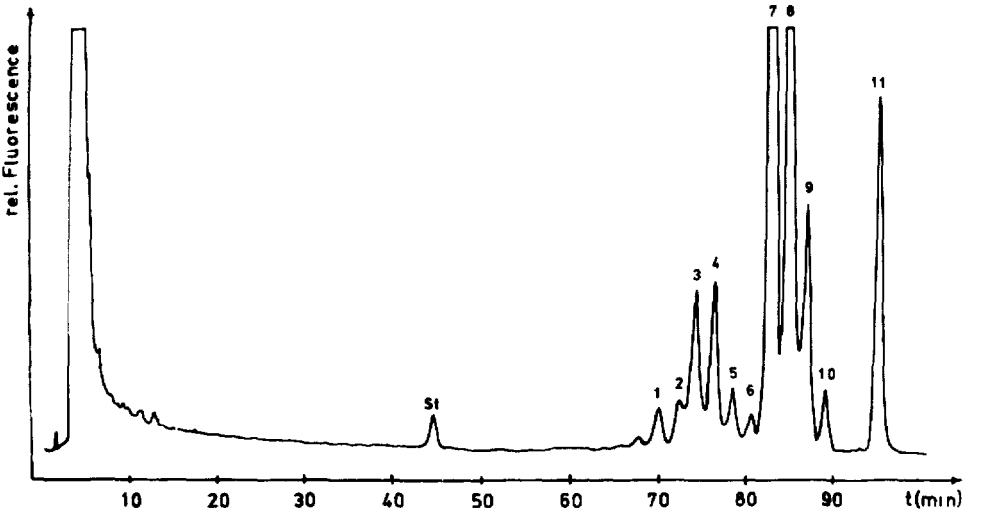

Fig. 2. Species separation of alkylacylglycerol naphthylurethanes from EGP from bovine erythrocytes. For peak identification see Table II. For separation conditions see *Methods*.

urethanes from other reaction products. This was done by HPLC on a short RP18 column as described in Experimental. Under the conditions used there is incomplete resolution of the alkenylacyl and alkylacyl types of ether lipid. For the analysis of the species pattern of alkylacylglycerols the acidic hydrolysis of alkenylacyl species is necessary. Recently Touchstone et al. [12] described a method for in situ reaction of alkenylacyl glycerophospholipids on thin-layer plates with 2% TCA–8% HCl (1:1). In contrast to other methods for destroying plasmalogens there is little or no effect on saturated or unsaturated diacyl analogues. Because the hydrolysis is done on thin-layer plates the best time for this is before the two-dimensional phospholipid separation.

Two problems can be solved by the method described here. First, diacyl glycerophospholipids can be purified from their ether analogues. This is necessary to obtain a precise diacyl species pattern for the subsequent species analysis by reversed-phase HPLC. Secondly, the relative proportion of alkylacylglycerols can be determined directly by comparing the peak areas of the alkylacyl and diacyl subgroups. It is then possible to investigate the species pattern of these compounds also (see Fig. 2). This may be of interest for investigations into PAF and their precursors.

Although the separation of all three subclasses of diradylglycerol naphthylurethanes is possible by TLC with benzene–hexane–diethyl ether, we preferred to use HPLC for the separation of subclasses for several reasons. First, the fractions obtained by normal-phase HPLC are purer than those obtained by TLC [8]. Secondly, it is difficult to analyse materials with a low percentage of

TABLE I

RELATIVE PROPORTIONS OF MOLECULAR SPECIES IN DIACYL EGP OF BOVINE ERYTHROCYTES

Molecular species[a]	Composition ($n=3$) (%)
18:2/20:4	4.9±0.54
18:2/18:2	2.8±0.10
16:0/20:4 (95)	3.4±0.15
16:0/22:6 (5)	
16:0/18:2 (80)	5.1±0.21
14:0/22:2 (20)	
18:1/18:2 (86)	15.8±2.04
18:1/20:4 (10)	
16:0/22:5 (4)	
16:0/20:3 (40)	3.0±0.40
18:0/20:5 (60)	
17:0/18:2	1.6±0.12
16:0/18:1 (82)	14.9±0.45
18:0/16:1 (18)	
18:1/18:1 (51)	28.2±1.75
18:0/20:4 (40)	
18:0/22:6 (9)	
18:0/18:2 (89)	9.3±0.86
18:0/22:5 (11)	
18:0/20:3	1.6±0.30
18:0/16:0	0.8±0.46
18:0/18:1	8.7±0.55

[a]GC composition of peaks containing more than one species is given in parentheses.

ether analogues because of problems with the detection and quantification of these minor components. Usually the plates have to be overloaded, which also causes imcomplete resolution of the subgroups. Since our method of derivatization with naphthyl isocyanate is very sensitive, small amounts of samples or materials with a small proportion of ether compounds can be analysed with respect to their species composition.

For the introduction of our method we used EGP from bovine erythrocytes. This material contains sufficient of the alkyl subgroup. Our results are in good agreement with the work of Hanahan et al. [20], who found that the proportion of ether compounds was ca. 75%. We estimated the proportion to be 73.9+2.15% ($n=3$). GC of the unfractionated sample shows that the most abundant alcohol component was the $C_{16\ 0}$ with 45.9%, followed by 18:1 with 28.8% and 18:0 with 16.4%. The alcohols 16:1, 17:0 and 17:1 were detected as minor components.

The results of the species separation of the diacyl and alkylacyl subclasses are shown in Tables I and II, respectively.

The individual species of the alkylacyl fraction containing 18:1 as the fatty

TABLE II

RELATIVE PROPORTIONS OF MOLECULAR SPECIES IN ALKYLACYL EGP OF BOVINE ERYTHROCYTES

Peak No.	Molecular species[a]	Composition ($n=3$) (%)
1	16:0/16:1	1.13±0.29
2	16.1/18:1	1.37±0.52
	18:1/20:4	
3	16:0/18·2	6.3 ±0.5
4	18:1/18:2	6.9 ±0.99
5	17:1/18:1	1.7 ±0.53
6	17:0/18:2	0.8 ±0.34
	16:0/16:0	
	16:1/18:0	
7	16:0/18:1	33.8 ±1.8
8	18:1/18:1	23.26±4.82
9	17:1/18:0	10.5 ±1.96
	18:0/18:2	
10	17:0/18:2	1.83±0.24
11	18·0/18:1	12.08±1.06

[a]The first number denotes the length of the alkyl carbon chain and the second number the length of the fatty acid chain.

acid component accounted for more than 70% of the total. The most prevalent species in the alkylacyl fraction was 16/18:1 followed by 18:1/18:1 and 18/18:1. In the species pattern of the diacyl subclass the most striking feature is the high percentage of species containing two unsaturated fatty acids.

In conclusion, our method of group separation with subsequent species analysis using fluorescent naphthylurethane derivatives can be used for detecting changes in the proportion of alkylacyl and diacyl subclasses and in individual molecular species associated with each subclass.

REFERENCES

1 F.B. Jungalwala, J. E. Evans and R.H. McCluer, J. Lipid Res., 25 (1984) 738–749.
2 M. Smith and F.B. Jungalwala, J. Lipid Res., 22 (1981) 697–704.
3 G.M. Patton, J.M. Fasulo and S.J. Robins, J. Lipid Res., 23 (1982) 190–196.
4 J. Krüger, H. Rabe, G. Reichmann and B. Rüstow, J. Chromatogr., 307 (1984) 387–392.
5 B. Rüstow, H. Rabe and D. Kunze, in A. Kuksis (Editor), Chromatography of Lipids in Biomedical Research and Clinical Diagnosis, Elsevier, Amsterdam, 1986, Ch. 7.
6 M. Batley, N.H. Packer and J.W. Redmond, J. Chromatogr., 198 (1980) 520–525.
7 H. Takamura, H. Narita, R. Urade and M. Kito, Lipids, 21 (1986) 356–361.
8 Y. Nakagawa and L.A. Horrocks, J. Lipid Res., 24 (1983) 1268–1275.
9 Y. Nakagawa, T. Sugiura and K. Waku, Biochim. Biophys. Acta, 833 (1985) 323–329.
10 D. Maretzki, M. Kostic, B. Reimann, E. Schwarzer and S.M. Rapoport, Biomed. Biochim. Acta, 10 (1986) 1227–1236.

11 R.I. Grove, D. Fitzpatrick and S.D. Schimmel, Lipids, 16 (1981) 691–693.
12 J.C. Touchstone, K.A. Snyder and S.S. Levin, J. Liq. Chromatogr., 7 (1984) 2725–2733.
13 O. Renkonen, J. Amer. Oil Chem. Soc., 42 (1965) 298–306.
14 M.L. Blank, M. Robinson, V. Fitzgerald and F. Snyder, J. Chromatogr., 298 (1984) 473–482.
15 T. Sugiura, Y. Masuzawa and K. Waku, Lipids, 15 (1980) 475–478.
16 T. Curstedt, Biochim. Biophys. Acta, 489 (1977) 79–88.
17 Y. Nakagawa, K. Fujishima and K. Waku, Anal. Biochem., 157 (1986) 172–178.
18 E. Francescangeli, S. Porcellati, L.A. Horrocks and G. Goracci, J. Liq. Chromatogr., 10 (1987) 2799–2808.
19 P.E. Haroldsen and R.C. Murphy, Biomed. Mass Spectrom., 14 (1987) 573–578.
20 D.J. Hanahan, J. Ekholm and C.M. Jackson, Biochemistry, 2 (1963) 630–641.