Journal of Chromatography, 487 (1989) 239–245
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4524

SEPARATION OF THE MOLECULAR SPECIES OF DIACYL AND ALKENYLACYL SUBCLASSES OF THE METHYL ESTER OF DINITROPHENYLETHANOLAMINE GLYCEROPHOSPHOLIPID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received August 1st, 1988; revised manuscript received September 30th, 1988)

SUMMARY

A high-performance liquid chromatographic (HPLC) procedure for the separation of the molecular species of alkenylacyl and diacyl subclasses after the derivatization of ethanolamine glycerophospholipids (EGP) to the methyl ester of the dinitrophenylated lipids is described. Methyl esters of dinitrophenylethanolamine glycerophospholipids were first separated into alkenylacyl, alkylacyl and diacyl subclasses by thin-layer chromatography, then each subclass was separated into individual molecular species by reversed-phase HPLC. By converting the EGP into UV-absorbing derivatives, it proved possible to utilize the specificity of spectrophotometric detection for the determination of the individual molecular species. Alkenylacyl and diacyl derivatives were resolved into fifteen to twenty different species in a single HPLC run The method should facilitate studies on the metabolism of the polar head group of both molecular species of alkenylacyl and diacyl glycerophosphoethanolamine in a variety of tissues using radioactive precursors.

INTRODUCTION[a]

Alkenylacyl glycerophosphoethanolamine (GPE) (plasmalogen) is widely distributed in various kinds of tissues and cells such as brain, skeletal muscle, heart, testis, macrophages and platelets [1]. These vinyl ether-linked phosphoglycerides contain larger amounts of polyunsaturated fatty acids at the 2-position, such as 20:4, 22:5 and 22:6, than the corresponding diacyl subclass [2–4]. It has been

[a]Abbreviations. EGP, ethanolamine glycerophospholipid; GPE, glycerophosphoethanolamine; Me-DNP-EGP, methyl ester of dinitrophenylethanolamine glycerophospholipid; fatty acids are denoted by the numbers of carbon atoms and double bonds, thus 18:1 represents oleic acid; the molecular species of phosphoglycerides are denoted by the fatty acid at the 1-position (left) and the fatty acid at the 2-position (right) of the glycerol moiety, thus 16:0–20:4 represents the 1-palmitoyl-2-arachidonoyl species The term 'radyl' is used to indicate an acyl, alkyl or alkenyl group.

0378-4347/89/$03.50

suggested that alkenylacyl GPE may play an important role as an intracellular storage form of polyunsaturated fatty acids [5]. These ether-linked phosphoglycerides could provide 20:4, which is a precursor of prostaglandin or leukotriene [6,7]. Several in vivo and in vitro experiments have indicated that the metabolism of the molecular species of alkenylacyl GPE-containing polyunsaturated fatty acids is different from that of the corresponding diacyl GPE [8,9]. These facts suggest that a study on the metabolism of the individual molecular species of plasmalogen would be important in clarifying how polyunsaturated fatty acids could be preferentially retained in alkenylacyl GPE. However, little attention has been paid to the molecular species of alkenylacyl GPE in view of the difficulties involved in their isolation.

In previous studies, we developed an HPLC method for the separation of the molecular species of ether-linked and diacyl subclasses after derivatization to 1,2-diradyl-3-acetylglycerol [10] or to dimethylphosphatidate [11]. Suitable non-polar derivatives of phosphoglycerides can be separated into their subclasses and molecular species by high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) far more effectively than the native lipids themselves. Other HPLC methods have been developed by other groups for the determination of the molecular species of 1,2-diradyl-3-benzoyl derivatives [12] and dinitrobenzoyl derivatives [13]. These methods give excellent resolutions of the molecular species of phosphoglycerides, although the polar head group of phosphoglycerides has to be removed. For the investigation of lipid metabolism, a commonly used technique is measurement of a labelled precursor for the de novo pathway, such as inorganic phosphate or ethanolamine. Renkonen [14] succeeded in separating five or six different molecular species of the methyl esters of dinitrophenylethanolamine glycerophospholipids (Me-DNP-EGP) by argentation TLC. HPLC separation of the molecular species of lipids is advantageous in comparison with argentation TLC, as the separation of the molecular species by reversed-phase HPLC is based not only on the unsaturation but also on the lengths of the fatty chains.

This paper describes the utilization of reversed-phase HPLC as an efficient method for the separation of the methyl ester species of DNP derivatives of alkenylacyl and diacyl GPE without the need for elimination of polar head groups.

EXPERIMENTAL

Materials and chemicals

2,4-Dinitrofluorobenzene was obtained from Aldrich (Milwaukee, WI, U.S.A.). Silica gel thin-layer plates and a LiChrosorb RP-18 column were purchased from Merck (Darmstadt, F.R.G.). A DEGS column and 17:0 methyl ester were obtained from Gasukuro Kogyo (Tokyo, Japan). HPLC-grade organic solvents were purchased from Wako (Osaka, Japan).

Sample preparation

Lipids were extracted from fresh porcine heart and bovine red blood cells by the method of Bligh and Dyer [15]. Ethanolamine glycerophospholipids (EGP)

were isolated by diethylaminoethylcellulose (DEAE-cellulose) and subsequent silicic acid column chromatography [16]. The purity of EGP was checked by TLC.

EGP were dinitrophenylated and methylated according to the procedure of Renkonen [14]. The EGP (0.1–2.0 mg) were dissolved in 2 ml of benzene, 50 μl of triethylamine and 5 μl of 2,4-dinitrofluorobenzene. The reaction mixture was left to stand at room temperature for 2 h and then evaporated to dryness. Dinitrophenylated EGP were extracted by the Bligh and Dyer method and the phosphate groups of DNP-EGP were methylated with diazomethane for 10 min at room temperature. After the reaction, excess of reagent was evaporated. The methyl esters of the dinitrophenyl-EGP (Me-DNP-EGP) were fractionated into alkenylacyl, alkylacyl and diacyl subclasses by silica gel TLC (silica gel G, Kieselgel 60) developed by seven successive ascending runs with *n*-hexane–chloroform (3:7) as described previously [14]. The yellow bands of individual subclasses, which were visible to the eye, were scraped off and extracted with chloroform–methanol (1:2).

HPLC procedures

The Me-DNP-EGP subclasses were dissolved in methanol for separation of the molecular species by HPLC on a Model 6A instrument (Shimadzu, Kyoto, Japan) with a reversed-phase column (LiChrosorb RP-18, 25 cm$\times$0.4 cm I.D.). Samples were eluted with an isocratic mobile phase (acetonitrile–isopropanol–methanol–water, 85:4:3:2) at a flow-rate of 1 ml/min. Each peak was detected by measuring the UV absorbance at 235 nm and integration of the peak area was performed with a Model C-R3A integrator (Shimadzu). Eluates from the column were collected and transmethylated with 0.5 *M* sodium methoxide solution for the identification of fatty acyl moieties of separated molecular species by gas chromatographic (GC) analysis using a 15% DEGS columns (60–50 mesh). The amount of each molecular species was determined after preparing the fatty acid methyl ester by GC with 17:0 methyl ester as the internal standard. To determine the alkenyl group composition, the alkenyl analogues were treated with hydrogen chloride gas and the liberated aldehydes were analysed by GC [17]. The preparation of 1,2-diradyl-3-acetylglycerol from EGP with phospholipase C (*Bacillus cereus*) and separation of their subclasses by TLC were performed as reported previously [16].

RESULTS AND DISCUSSION

The Me-DNP-EGP were prepared by the introduction of DNP into the amino group of EGP and subsequent methylation with diazomethane. The yield of Me-DNP-EGP was $75 \pm 3.2\%$, as determined by radioactivity measurement of ^{32}P-labelled EGP and quantitation of inorganic phosphate. The conversion of EGP to less polar derivatives allowed the separation of alkenylacyl, alkylacyl and diacyl subclasses by TLC. Separation of subclasses by TLC was difficult, but it was successfully accomplished by multiple development with less polar solvents, such as the mixture of chloroform and *n*-hexane described previously [14] (Fig. 1).

Fig. 1. Separation of alkenylacyl, alkylacyl and diacyl subclasses of EGP as methyl dinitrophenylated derivatives by TLC with seven successive ascending runs with *n*-hexane–chloroform (3:7). The plates were sprayed with 50% sulphuric acid and heated at 125°C for 30 min. (A) Mixture of EGP from bovine erythrocytes and porcine heart, (B) EGP from bovine erythrocytes; (C) EGP from porcine heart

EPG from heart tissues and erythrocytes are useful standards for alkenylacyl and alkylacyl GPE, respectively. All three kinds of subclasses were well separated by TLC with multiple development. To determine the accuracy of the present methods for the separation of the alkenylacyl and diacyl subclasses, those obtained from Me-DNP-EGP derivatives were compared with the data obtained by another previously established method (Table I). The fatty acid composition of alkenylacyl and diacyl subclasses obtained from Me-DNP-EGP was also in good agreement with the fatty acid composition of those obtained from 1,2-diradyl-3-acetylglycerols.

Me-DNP-EGP were separated into their respective molecular species by reversed-phase HPLC. A mixture of acetonitrile, isopropanol and water was successfully used as the mobile phase for the separation of individual molecular species within 45 min (Fig. 2). The alkenylacyl and diacyl derivatives from EGP were fractionated into twelve separate peaks by reversed-phase HPLC. On GC analysis of individual peaks, fifteen or sixteen different molecular species composed of alkenylacyl and diacyl GPE from heart tissues were detected. The number of molecular species separated by HPLC was much higher than that obtained by conventional techniques such as argentation TLC. The 16:0–18:1 species were clearly separated from the 18:0–18:2 species under the present HPLC conditions, but both species of 1,2-diradyl-3-acetylglycerol [10], 1,2-diradyl-3-benzoylglycerol [12] and dimethylphosphatidate [11] proved impossible to resolve. Some molecular species were not completely separated. The combination of reversed-

TABLE I

COMPARISON OF THE FATTY ACIDS OF THE ALKENYLACYL AND DIACYL ANALOGUES AFTER DERIVATIZATION OF METHYL DINITROPHENYL-EGP (Me-DNP-EGP) AND 1,2-DIRADYL-3-ACETYLGLYCEROL

Values are expressed as mole percentages (mean ± S.D., $n = 5$).

Species	Alkenylacyl		Diacyl	
	Me-DNP-EGP	Diradylacetyl- glycerol	Me-DNP-EGP	Diradylacetyl- glycerol
16:0	1 7±0.2	1.4±0.3	6.8±0.6	7.2±1.0
18:0	0.3±0.1	0.3±0.1	35.6±2.7	33.5±2.2
18:1	4.5±0.3	4.4±0.2	4.9±0 1	4.9±0.1
18:2	37.9±1.8	35.1±2.0	20.0±1.7	22.0±0.8
20:3	3.3±0.7	3.0±0.3	1.5±0.2	1.6±0.2
20:4	33.2±2.2	35.6±1.9	24.5±0.8	24.4±0.6
20:5	6.2±1.1	5.5±1.3	0.4±0.1	0.3±0.1
22:5	5.0±1.2	6.5±1.4	1.9±0.3	2.4±0.3
22:6	8.9±0.3	9.1±0.8	4.6±0.2	4.5±0.1

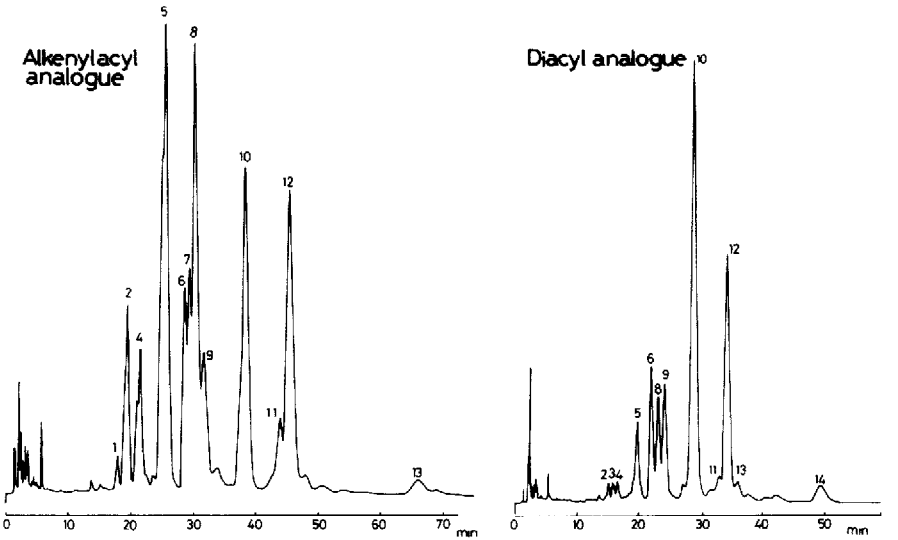

Fig. 2. HPLC separation of the molecular species of methyl dinitrophenylated derivatives of alkenylacyl and diacyl GPE. Me-DNP-EGP were dissolved in 20 μl of methanol and then injected and chromatographed on a LiChrosorb RP-18 column at a flow-rate of 1.0 ml/min. The mobile phase was acetonitrile–isopropanol–methanol–water (85:4:3:2). Each peak was monitored at 235 nm. The peak numbers correspond to those in Table II.

phase HPLC with argentation TLC could facilitate the complete separation of individual molecular species.

The results of the determination of individual molecular species of alkenylacyl and diacyl GPE from porcine heart are presented in Table II. Both subclasses contained almost identical molecular species; however, their quantitative distri-

TABLE II

DISTRIBUTION OF THE MOLECULAR SPECIES OF ALKENYLACYL AND DIACYL ANALOGUES OF ETHANOLAMINE GLYCEROPHOSPHOLIPIDS FROM PORCINE HEART

Values are expressed as mole percentages (mean ± S.D., $n = 3$).

Peak No.[a]	Molecular species	Alkenylacyl (%)	Diacyl (%)
1	18:1-20:5	0.8±0.6	0.7±0.1
2	16:0-20:5	3.7±0.2	0.8±0.1
3	18:2-18:2	–	0.9±0.1
4	16:0-22:6	6.8±0.8	1.0±0.2
5[b]	16:0-20:4	20.5±0.9	5.8±0.3
5	16:0-22:5	4.2±0.5	–
6	18:0-20:5	3.1±0.3	–
7	18:1-18:2	3.3±0.6	8.4±0.3
8	16:0-18:2	18.2±2.9	6.1±0.2
9	18:0-22:6	3.6±0.2	7.3±0.5
10[b]	18:0-20:4	15.4±0.4	38.7±1.4
10	18:0-22:5	2.6±0.1	–
11	16.0-18:1	2.9±0.1	1.8±0.4
12	18:0-18:2	12.9±3.1	24.7±1.5
13	18:0-20:3	–	2.4±0.3
14	18:0-18:1	1.5±0.1	2.4±0.2

[a]Peak numbers correspond to the peaks in Fig. 2

[b]For alkenylacyl analogues, peaks 5 and 10 include two partially resolved peaks.

butions were different for the two subclasses. The alkenylacyl GPE consisted mainly of the 16:0–18:2 and 16:0–20:4 species, whereas the predominant molecular species in the diacyl subclass was 18:0–20:4, which comprised about 40% of the total. The fatty acid compositions of the alkenylacyl and diacyl derivatives calculated from the compositions of the molecular species, shown in Table II, were almost identical with those of the original unfractionated samples, shown in Table I. This result indicates that no selective loss of molecular species occurred after passage through the reversed-phase column.

A good relationship between the amounts of molecular species and the absorbance observed at 235 nm, in terms of peak area, was obtained. Peak areas were proportional to the amounts of Me-DNP-EGP and a linearity was maintained from at least 5 to 150 nmol of Me-DNP-EGP species. The area per picomole of Me-DNP-EGP was 330±40 μV s, which is approximately the same as that obtained for dinitrobenzoyldiacylglycerol (260 μV s) [13]. The limit of detection was ca. 100 pmol.

CONCLUSION

The HPLC method described allowed the separation of the molecular species of EGP derivatives with quantitative detection and without any destruction of their structure. The method is therefore useful for studies of the metabolism of

the polar head groups of individual molecular species of EGP, especially alkenylacyl GPE, for which little information is currently available, and should provide new insights into the formation of alkenylacyl GPE in various mammalian tissues. In addition, the method can be applied to the separation of molecular species of serine glycerophospholipids.

ACKNOWLEDGEMENTS

We acknowledge the technical assistance of Hiroyuki Asano and Fumiko Ogasawara. This work was supported in part by a Grant-in-Aid (62580135) from the Ministry of Education, Science and Culture of Japan.

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