Journal of Chromatography, 381 (1986) 225—231
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3216

IMPROVED PROCEDURE FOR THE SEPARATION OF THE MOLECULAR SPECIES OF DIMETHYLPHOSPHATIDATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 19th, 1986; revised manuscript received April 21st, 1986)

SUMMARY

We describe a rapid and efficient high-performance liquid chromatographic (HPLC) procedure for the separation of the molecular species of dimethylphosphatidic acid derived from phosphatidylcholine, which naturally occurs in mammalian tissues. The separation was accomplished on a reversed-phase column after derivatization of phosphatidylcholine to dimethylphosphatidate by means of phospholipase D and subsequent diazomethane treatments. Separation of the major molecular species of dimethylphosphatidate by reversed-phase HPLC was achieved within 40 min. Dimethylphosphatidate from rat liver phosphatidylcholine was resolved into twelve separate peaks Thirteen different molecular species in rat liver phosphatidylcholine were identified by gas chromatographic determination This improved method is applicable to studies on the metabolism of the phosphate group of the molecular species of phospholipid of a variety of tissues using a radioactive precursor.

INTRODUCTION

It is well known that all types of phospholipids are composed of populations of well defined molecular species, which are present in characteristic proportions in various mammalian tissues [1]. The different molecular species of phospholipids exhibit striking differences in their physicochemical properties, and the knowledge of the composition of the molecular species of phospholipids in membranes is especially important for determining the physical properties and physiological conditions of the membranes [1]. Furthermore, some molecular species of phospholipids were revealed to have specific functions, such as a platelet-activating factor, which is a potent chemical mediator (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) [2], and a lung sur-

0378-4347/86/$03.50

factant (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) [3]. These facts indicate that investigation of membrane phospholipids at the molecular species level is important for determining the exact roles of phospholipids in mammalian tissues. Although there have been many reports on phospholipids concerning their composition in membranes, their biological activities and metabolism, little attention has been paid to the molecular species of phospholipids, since molecular species with a single structure are difficult to isolate.

High-performance liquid chromatography (HPLC) on a reversed-phase column is more effective for the separation of molecular species than argentation thin-layer chromatography (TLC), which is a commonly used technique [4]. During recent years, HPLC has been increasingly applied to the separation of the individual molecular species of phospholipids. Recently, we developed an HPLC system for the separation of the molecular species of 1,2-diradyl-3-acetylglycerol derived from phospholipids [5]. A similar method was used for the analysis of a lung surfactant [6]. Another HPLC method was developed by Blank et al. [7] for the quantitative analysis of 1,2-diradyl-3-benzoyl derivatives. These HPLC methods show excellent resolution of the molecular species compared to those for the separation of intact phospholipids [8, 9]; however, in these previous works, the polar head group of phospholipids had to be removed. In biological research, it is common practice to measure the radioactivity in a phospholipid fraction following the administration of a labelled precursor for the de novo pathway, such as phosphate or choline. The HPLC method for the separation of molecular species having polar head groups allows further elucidation of the regulatory mechanism for the synthesis or degradation of individual molecular species of phospholipids in mammalian tissues.

Renkonen [10] succeeded in spearating five to seven different molecular species of dimethylphosphatidate by argentation TLC. In one study, Hsieh et al. [11] applied this method to the separation of egg phosphatidylcholine by reversed-phase HPLC; however, the separation of individual molecular species was not sufficient for those phospholipids present in mammalian tissues that contain highly polyunsaturated fatty acids, such as 20:4*.

In the present study, we developed an improved HPLC method for the separation of the molecular species of dimethylphosphatidate derived from phosphatidylcholine of rat liver.

EXPERIMENTAL

Lipids were extracted from fresh livers of Wistar rats (150 g) (Sankyo Lab. Service, Tokyo, Japan) by the method of Bligh and Dyer [12]. Phosphatidylcholine was isolated by diethylaminoethyl cellulose (DEAE-cellulose) and subsequent silicic acid column chromatography [13]. The purity of the phosphatidylcholine was checked by TLC. Phosphatidylcholine was hydrolysed

*Fatty acids are denoted by the number of carbon atoms and double bonds; thus, 20:4 represents arachidonic acid, which contains twenty carbons and four double bonds. The molecular species of phospholipids are denoted by the fatty acid at the 1-position (left-hand side) and the fatty acid at the 2-position (right-hand side) of the glycerol moiety; thus, 16:0-20:4 represents the 1-palmitoyl-2-arachidonoyl molecular species.

with cabbage phospholipase D. The procedures were essentially the same as those previously reported by Renkonen [10]. Phosphatidylcholine (150 μg of inorganic phosphorus) was emulsified in 8 ml of a 0.1 *M* acetate buffer (pH 5.5) and 1.5 ml of 1 *M* calcium chloride solution, and then 0.1 mg of cabbage phospholipase D (P-L Biochemical, Milwaukee, WI, U.S.A.) and 4 ml of diethyl ether were added. The mixture was stirred at room temperature for 20 h, after which the reaction was stopped by the addition of 3 ml of 0.5 *M* EDTA; then phosphatidate was extracted with chloroform–methanol mixtures. The extracted phosphatidate was dissolved in 2 ml of chloroform–methanol–water (127:63:10) and then 0.5 ml of 0.1 *M* hydrochloric acid was added. After rapid mixing, the upper phase was removed. The lower phase was evaporated to dryness and then dissolved in 0.5 ml of diethyl ether. To prepare dimethylphosphatidate, phosphatidate was methylated with diazomethane in 1 ml of diethyl ether. After 30 min, the excess reagent was removed under a stream of nitrogen.

The dimethylphosphatidate was dissolved in 20 μl of methanol for separation of the molecular species by HPLC (Model 655, Hitachi, Tokyo, Japan) on a reversed-phase column (LiChrosorb RP-18, 25 × 0.4 cm I.D., particle size 5 μm, Merck). Samples were eluted with an isocratic mobile phase (acetonitrile–isopropanol–methanol–water, 50:27:18:5) at the flow-rate of 1.5 ml/min. Each peak was detected by the ultraviolet absorbance at 205 nm. Eluates from the column were collected and then transmethylated with 0.5 *M* sodium methoxide for determination of the molecular species in each peak by gas chromatographic (GC) analysis [13]. The quantity of each molecular species was determined, after preparing fatty acid methyl esters, by GC with 17:0 methyl ester as the internal standard.

Determination of phosphate in phospholipid was performed by the method of Rouser et al. [14].

The preparation of 1,2-diacyl-3-acetylglycerol from phosphatidylcholine with phospholipase C and separation of the molecular species by HPLC were performed according to the methods previously reported [5, 15].

HPLC-grade organic solvents and water were purchased from Wako (Osaka, Japan).

RESULTS AND DISCUSSION

Dimethylphosphatidate was prepared by hydrolysis of phosphatidylcholine with phospholipase D and subsequent methylation with diazomethane. The yield of dimethylphosphatidate was 81.0 ± 1.5%, as determined on phosphorus measurement and on quantitation by fatty acid analysis. The fatty acid composition of the parent phosphatidylcholine was compared to those of phosphatidate and dimethylphosphatidate originitating from the parent phosphatidylcholine, as shown in Table I. No selective loss of esterified fatty acids in phosphatidylcholine occurred as a result of the phospholipase D and subsequent diazomethane treatments, since significant differences in the fatty acid compositions were not found among phosphatidylcholine, phosphatidate and dimethylphosphatidate.

Dimethylphosphatidate was separated into molecular species by reversed-

TABLE I

FATTY ACID COMPOSITION OF PHOSPHATIDATE AND DIMETHYLPHOSPHATIDATE DERIVED FROM PHOSPHATIDYLCHOLINE OF RAT LIVER

Phosphatidate and dimethylphosphatidate originated from the same phosphatidylcholine.

Fatty acid	Composition (mean ± S.D., n = 6) (weight %)		
	Phosphatidylcholine	Phosphatidate	Dimethylphosphatidate
16:0	23.4 ± 1.5	23.8 ± 1.0	23.1 ± 1.0
18:0	19.7 ± 0.7	19.2 ± 1.0	18.1 ± 0.5
18:1	9.1 ± 0.4	8.9 ± 0.3	9.2 ± 0.2
18:2	18.9 ± 0.5	19.1 ± 0.6	20.5 ± 0.7
20:4	24.9 ± 1.3	24.1 ± 2.1	25.3 ± 0.9
22:6	4.1 ± 0.3	4.0 ± 0 1	3.7 ± 0 2

phase HPLC. The binary mixtures of methanol with water used in the previous work did not allow satisfactory separation of the individual molecular species. A mixture of acetonitrile, isopropanol, methanol and water was successfully used as the mobile phase for the separation of individual molecular species of dimethylphosphatidate, especially the molecular species containing highly polyunsaturated fatty acids, within 40 min. Reversed-phase HPLC separation of the molecular species of dimethylphosphatidate derived from phosphatidylcholine of rat liver is illustrated in Fig. 1. Dimethylphosphatidate was resolved into twelve separate peaks with the present HPLC system. We deter-

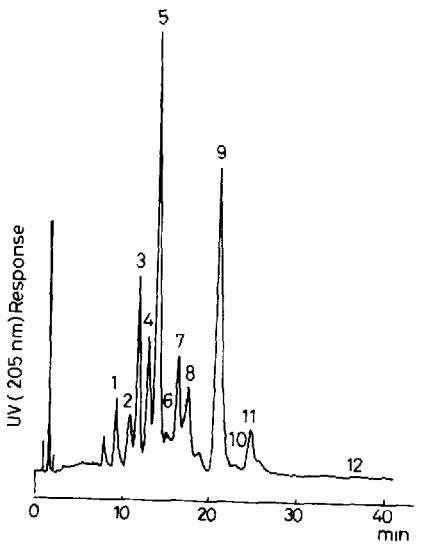

Fig. 1. HPLC separation of the molecular species of dimethylphosphatidate derived from phosphatidylcholine of rat liver. Dimethylphosphatidate (0.8 μmol) was dissolved in 20 μl of methanol, and then injected and chromatographed on a LiChrosorb RP-18 column at a flow-rate of 1.5 ml/min. The mobile phase was acetonitrile—isopropanol—methanol—water (50:27:18:5). Each peak was monitored at 205 nm, and collected for identification and quantitation by GC. The peak numbers correspond to those in Table II.

mined thirteen different molecular species by GC analysis of fatty acids (Table II). The retention time of the molecular species increased with increasing chain length and decreased with increasing degree of unsaturation of fatty acids.

The amounts of the molecular species separated by HPLC were determined by GC after the transmethylation of fatty acids of the molecular species, since the absorption of 205 nm is dependent upon the number of double bonds in the molecular species. The results of quantitation of individual molecular species of dimethylphosphatidate derived from rat liver are presented in Table III. The predominant molecular species of phosphatidylcholine contained 20:4 at the 2-position, such as the 16:0-20:4 (20% of the total) and 18:0-20:4 (17%) species. The total of these arachidonoyl molecular species accounted for approximately 45% of the total phosphatidylcholine in rat liver. The high proportion of arachidonoyl molecular species of phosphatidylcholine in rat liver is in good agreement with the earlier finding using a combination of argentation and high-temperature GC [16]. A different and established method for the separation of the molecular species of phosphatidylcholine [5] was used for comparison with the results of the present method. Table III also shows the composition of the molecular species of phosphatidylcholine in rat liver after derivatization to 1,2-diacyl-3-acetylglycerol. The results obtained using different derivatization methods show good agreement with each other.

A method for predicting the retention times of the molecular species in an unknown sample would be useful, since dimethylphosphatidate was separated into multiple peaks by reversed-phase HPLC. In a previous study, we successfully predicted the retention times of the individual molecular species of 1,2-diradyl-3-acetylglycerol using the method of plotting the logarithm of the relative retention time (RRT) of each molecular species [5], developed by

TABLE II

RETENTION TIMES OF THE MOLECULAR SPECIES OF DIMETHYLPHOSPHATIDATE SEPARATED BY REVERSED-PHASE HPLC

Peak* No.	Molecular species	Retention time (min)	RRT**
1	18:2-20:4	9.4	1.0
2	18:1-22:6	11.0	1.2
2	18:2-18:2		
3	16:0-22:6	11.9	1.3
4	18:1-20:4	13 1	1.4
5	16:0-20:4	14.1	1.5
6	18:1-18:2	15.4	1.6
7	16:0-18:2	16.6	1.8
8	18:0-22.6	17.8	1.9
9	18:0-20:4	21.2	2.3
10	16:0-18·1	23.0	2 5
11	18:0-18:2	25.0	2.7
12	18:0-18.1	36.0	3.8

*Peak numbers correspond to those in Fig. 1.

**RRT (relative retention time) was calculated by dividing the retention time of each molecular species by the retention time of the 18:2-20·4 species.

TABLE III

COMPOSITION OF THE MOLECULAR SPECIES OF DIMETHYLPHOSPHATIDATE DERIVED FROM PHOSPHATIDYLCHOLINE OF RAT LIVER

The composition of the molecular species of phosphatidylcholine of rat liver was determined after derivatization to dimethylphosphatidate or 1,2-diacyl-3-acetylglycerol. Values are means ± S.D. (n = 6 for dimethylphosphatidate and n = 3 for 1,2-diacyl-3-acetylglycerol).

Molecular species	Composition (%)	
	Dimethylphosphatidate	1,2-Diacyl-3-acetylglycerol
18:1-22:6	0.7 ± 0.1	0.7 ± 0.1
18:0-22:6	2.1 ± 0.4	1.4 ± 0.1
16:0-22:6	4.8 ± 0.8	4.0 ± 0.3
18:2-20:4	1.9 ± 0.3	2.0 ± 0.2
18:1-20:4	6.3 ± 1.0	5.8 ± 0.5
18:0-20:4	17.3 ± 2.6	19.8 ± 0.6
16:0-20:4	20.0 ± 1.7	21.3 ± 1.1
18:2-18:2	1.4 ± 0.1	1.4 ± 0.2
18.1-18:2	3.7 ± 0.4	3.3 ± 0.2
18:0-18:2	16.4 ± 1.6	16.0 ± 2.0
16:0-18.2	16.9 ± 2.5	16.1 ± 1.5
18:0-18:1	1.4 ± 0.3	1.1 ± 0.2
16:0-18:1	7.0 ± 0.3	7.6 ± 0.2

Patton et al. [9]. A graphical representation of the RRT values is shown in Fig. 2. A series of oblique parallel lines was obtained when lines were drawn that connected the points of molecular species having the same fatty acid at the 2-position. The order of elution of individual molecular species of dimethylphosphatidate was constant and entirely dependent on the fatty acyl moiety composition. We could predict the retention time of the 18:1-18:1 species (21.6 min) from this graph.

There have been several reports on the different synthetic rates for the molecular species of phosphatidylcholine in the de novo pathway, which may

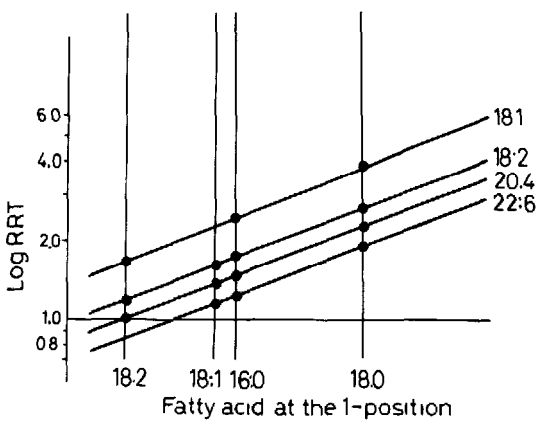

Fig. 2. Relationship between the fatty acid compositions of molecular species of dimethylphosphatidate and the retention times. All RRT values were plotted according to previous reports [5, 9].

be due to the different selectivity of cholinephosphotransferase for the synthesis of each molecular species of phosphatidylcholine. Kanoh [17] found that the dienoic molecular species of phosphatidylcholine was the most active in incorporating [^{32}P]phosphate, [^{14}C]glycerol and [^{14}C]choline in rat liver. Waku and Nakazawa [18] reported that the saturated molecular species of phosphatidylcholine, prelabelled with labelled glycerol, show a fast turnover in mouse Ehrlich ascites tumour cells. However, in these works, the molecular species were only resolved into five to seven species by argentation TLC on the basis of the degree of unsaturation. More precise separation of the molecular species of phospholipids is important for elucidation of the regulatory mechanism for the synthesis of the molecular species of phospholipids in mammalian tissues. Being efficient for the separation of the molecular species of phosphatidylcholine, the HPLC procedure described in this report is very suitable for this application and will provide new information as to the formation and degradation of different molecular species of phosphatidylcholine in various tissues. For example, the metabolisms of polar head groups, fatty acyl moieties and glycerol back-bones of the individual molecular species can be compared, if [^{32}P]phosphate, [^{14}C]glycerol and [^{3}H]fatty acid are used as precursors. Phosphatidylcholine labelled with these precursors were separated by the present method and the radioactivities of eluted peaks were counted for as long as necessary, permitting work with low specific activity.

ACKNOWLEDGEMENTS

We wish to acknowledge the technical assistance of Ayako Shibata. This work was supported in part by a Grant-in-Aid (60580140) from the Ministry of Education, Science and Culture of Japan.

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