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Journal of Chromatography B, 660 (1994) 380–385

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatographic method for determination of the metabolism of polyunsaturated molecular species of phosphatidylserine labeled in the polar group

Biradavolu Seenaiah, John S. Ellingson*

Department of Pathology and Cell Biology, Jefferson Medical College of Thomas Jefferson University, Philadelphia, PA 19107, USA

First received 17 March 1994; revised manuscript received 27 June 1994

Abstract

A reversed-phase HPLC method to monitor the incorporation of radiolabeled precursors into the polar group of individual polyunsaturated molecular species of phosphatidylserine (PS) is presented. PS labeled in the polar group was decarboxylated and subsequently converted to trinitrophenyl-phosphatidylethanolamine (Tnp-PE), which was separated into its molecular species by reversed-phase HPLC within 90 min, using a gradient of acetonitrile–methanol and ammonium acetate. A major feature of the method is the complete resolution of the stearoyl species, 18:0/20:4 and 18:0/22:6, at ambient temperature. By determining the amount of radioactivity incorporated into each fraction, the metabolism of individual molecular species of PS, and also of PE, labeled in the polar group can be investigated.

1. Introduction

Glycerophospholipids exist as different classes, each possessing a different polar group. Each class consists of a heterogeneous mixture of molecular species with defined arrangements of fatty groups on the *sn*-1 and the *sn*-2 positions of the glycerol moiety, indicating a highly regulated metabolism of the individual molecular species. Metabolic studies indicated that specific pools of phospholipids are functionally compartmentalized according to their fatty acid composition (molecular species), age (newly vs. previously synthesized) and source of the polar group (modification vs. de novo synthesis) [1–8]. Consequently, to precisely define the mechanisms used in the synthesis and intracellular transfer of phospholipid molecules among subcellular membranes, methods to investigate the metabolism of individual molecular species are required.

The synthesis of PS in mammalian cells is usually monitored by measuring the incorporation of radiolabeled serine into the polar group of the lipid by following the base exchange of L-serine with either choline or ethanolamine moieties of phosphatidylcholine (PC) or phosphatidylethanolamine (PE), respectively [9]. Consequently, investigations of PS molecular species metabolism require a method to separate the intact lipid still containing the radiolabeled polar group. Analytical HPLC methods have been developed to separate the molecular

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00308-R

species of intact phospholipids [10–13], and the molecular species of PE and PS derivatized with 2,4,6-trinitrobenzenesulfonic acid (TNBS) or 1-fluoro-2,4-dinitrobenzene have been analyzed by HPLC at a temperature of 40° [13]. However, usually no resolution of 18:0/22:6 and 18:0/20:4 into separate fractions, i.e. detector response returning to baseline for more than 30 s, is achieved by reversed-phase HPLC. This posed a special problem for our investigations on the synthesis of PS species, because these two stearoyl polyunsaturated molecular species comprise 68 mol% of the total rat liver PS [10]. Consequently, to assure that the radioactivity incorporated into each species could be accurately determined, we developed an HPLC procedure to completely resolve the two polyunsaturated stearoyl species.

Recently PS metabolism has received considerable attention since PS has been found to participate in signal-transduction events by activating protein kinase C [14]. In plasma membranes it is localized mainly on the cytoplasmic surface [15], where its anionic head group affects the membrane surface properties and its polyunsaturated acyl chains influence the hydrophobic environment of the bilayer. PS plays a unique metabolic role since it serves as a precursor for specific pools of PE and PC, which have molecular species compositions and functions different from those synthesized de novo from CDP-intermediates [1–5]. Therefore, the HPLC procedure described in this study for detection of the incorporation of radioactive precursors into the PS molecular species provides a valuable tool for other investigators, who are elucidating the role of the individual molecular species of PS destined for any of its various functions.

2. Experimental

2.1. Materials

Standard phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL, USA). 2,4,6-Trinitrobenzenesulfonic acid was from Pierce Chemical Co. (Rockford, IL, USA). [^{3}H]Serine was from DuPont-New England Nuclear (Boston, MA, USA). HPLC-grade solvents were from Fisher Scientific (Pittsburgh, PA, USA).

2.2. General procedures

To minimize oxidation of the polyunsaturated PS, all buffers, extracting solvents and TLC chambers were flushed with nitrogen prior to use, and all enzyme assays were conducted in tubes with PTFE-lined screw-caps under a nitrogen atmosphere.

2.3. Preparation of [^{3}H]serine-labeled PS

Rat liver microsomes were isolated and incubated for 20 min with [^{3}H]serine (37.5 μCi/μmol) as previously described [16], except that no Triton X100 or hydroxylamine was included to maintain the structural integrity of the microsomes. The reaction was stopped with 2 ml of chloroform–methanol (2:1, v/v), and washed by the Folch procedure [17]. The labeled PS was isolated by TLC in a solvent system of methylacetate–*n*-propanol–chloroform–methanol–0.25% aqueous KCl (25:25:25:10:9, v/v) or by two-dimensional TLC [18]. The silicic acid containing the PS was scraped from the plate and extracted with chloroform–methanol–0.1 *M* HCl (2:1:0.1, v/v), and centrifuged at 500 *g* for 10 min. This extraction procedure was repeated twice. The combined supernatants were washed once with 0.2 volume of water and evaporated. The PS was dissolved in a few milliliters of chloroform.

2.4. Decarboxylation of PS

The ^{3}H-labeled PS was decarboxylated to [^{3}H]PE by a 30-min incubation with a purified mitochondrial fraction as described by Vance and Vance [16], after which an additional 0.3 mg of mitochondrial protein was added and the incubation continued for another hour. The lipids were extracted as described for the PS synthase reaction. The mitochondrial PS-decarboxylase converted all the PS to PE as determined by TLC

and detection of PS by iodine staining and reaction with ninhydrin and phosphomolybdate spray reagents as previously described [18].

2.5. Preparation of trinitrophenyl-PE

Standard PE or the lipids extracted from the decarboxylation assay system (containing [^{3}H]serine-derived [^{3}H]PE) were treated with 2,4,6-trinitrobenzenesulfonic acid (TNBS), and the trinitrophenyl-PE (Tnp-PE) product was purified and isolated by TLC as described by Hullin et al. [13], a procedure in which the conversion of PE to Tnp-PE is complete.

2.6. Analysis of the Tnp-PE molecular species by reversed-phase HPLC

Seventy to eighty nmol of the Tnp-[^{3}H]PE isolated from the decarboxylation assay were dissolved in methanol, filtered through a 0.45-μm fluoropolymer ACRO-L13 disposable filter assembly (Gelman Sciences, Ann Arbor, MI, USA) and injected onto a Beckman Ultrasphere ODS HPLC column (250 × 4.6 mm I.D. packed with 5-μm octadecyl silica particles) protected with a 5 × 4.6 mm I.D. Ultrasphere ODS guard column. The molecular species were separated by modifying the procedure of Hullin et al. [13]: a new gradient procedure was used at ambient temperature, rather than at 40°C. The flow-rate was 1 ml/min. The initial mobile phase consisting of 8% solvent A [10 m*M* ammonium acetate, pH 5.0] and 92% solvent B [acetonitrile–methanol (1:1, v/v)] was maintained for 5 min, followed by a 30-min linear decrease of solvent A to 6%; this composition was maintained for 30 min, followed by a 20-min linear decrease of solvent A to 3%; this composition was maintained for 10 min. The column was reequilibrated by 10-min linear increase of solvent A to the original 8%, and that composition was maintained for a minimum of 30 min before application of the next sample. The Tnp-PE eluted from the column was detected by UV absorption at 338 nm. The eluted fractions were evaporated, and the radioactivity in each fraction was determined by liquid scintillation spectrophotometry. The chromatographic system used was a Perkin-Elmer Series 4 HPLC instrument equipped with a Perkin-Elmer LC 95 UV spectrophotometer and a Model 3600 data station. The HPLC-grade solvents were filtered through a 0.45-μm FH hydrophobic Millipore filter (Millipore, New Bedford, MA, USA) and flushed with helium gas.

2.7. Identification of molecular species by gas chromatography

The Tnp-PE prepared from rat liver microsomal PE was separated into its molecular species by HPLC, and subsequently the species of Tnp-PE in each fraction were converted to their fatty acid methyl esters [19]. The molecular species composition of each fraction was identified from the fatty acid composition determined by capillary gas chromatography [20].

3. Results and discussion

The elution of the Tnp-PE species is shown in Fig. 1 and the identification of the fractions is presented in Table 1. The four major polyunsaturated species 16:0/22:6 (fraction 1), 16:0/20:4 (fraction 2), 18:0/22:6 (fraction 7), and 18:0/20:4 (fraction 8) had retention times of 36, 39, 51, and 57 min respectively, and were completely resolved within 90 min at ambient temperature. The detector response returned to the

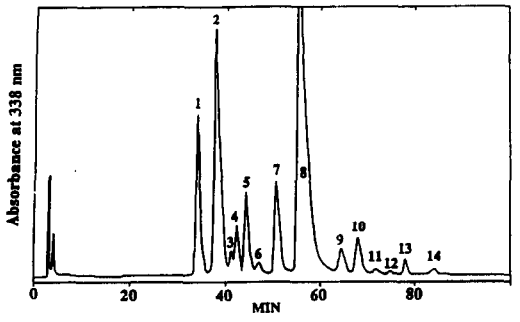

Fig. 1. Separation of molecular species of Tnp-PE by reversed-phase HPLC. Seventy-five nmol of Tnp-PE were separated as described in the text and detected at 238 nm (full scale is 0.1 absorbance units). The composition of the fractions is identified in Table 1.

Table 1
The molecular species of Tnp-PE derivatives and their relative retention times

Peak number[a]	Molecular species[b]	RRT[c] (mean ± S.D., $n = 5$)
1	16:0/22:6	1.00
2	16:0/20:4	1.11 ± 0.02
3	18:1/20:4	1.22 ± 0.01
4	16:0/18:2	1.26 ± 0.01
5	16:0/22:5	1.31 ± 0.02
7	18:0/22:6	1.50 ± 0.02
8	18:0/20:4	1.64 ± 0.05
9	18:0/18:2	1.91 ± 0.02
10	18:0/22:5	2.01 ± 0.02
13	20:0/22:6[d]	2.31 ± 0.03
14	20:0/20:4[d]	2.50 ± 0.02

The molecular species of Tnp-PE prepared from rat liver microsomal PE were separated and analyzed as described in detail in the text. Results are presented as the average of five chromatographic analyses ± S.D.

[a] Peak numbers correspond to those in Fig. 1, and the very minor peaks 6, 11, and 12 were not identified.

[b] The molecular species are listed as the fatty acid in the *sn*-1 position followed by the one in the *sn*-2 position.

[c] RRT is the relative retention time, and for the calculation the retention time of 16:0/22:6 was used as the reference time.

[d] Species tentatively identified by relative retention times.

baseline for 1 min between the 18:0/22:6 and 18:0/20:4 fractions, which is an essential requirement of any chromatographic procedure used to investigate the metabolism of PS molecular species, because PS from most tissues contains significant amounts of both species [21]. The palmitoyl species, 16:0/22:6 and 16:0/20:4, were also completely resolved as the detector response returned to baseline for at least 30 s between these two fractions, thereby allowing comparison of the metabolism of these two compounds and that of the corresponding stearoyl species. Another polyunsaturated stearoyl species, 18:0/22:5 (fraction 10), was also resolved from the other species and its palmitoyl counterpart, 16:0/22:5 (fraction 5). The diunsaturated stearoyl species 18:0/18:2 (fraction 9) was also resolved from the polyunsaturated species and from its palmitoyl counterpart, 16:0/18:2 (fraction 4). Thus the metabolism of the stearoyl polyunsaturated species can be compared to that of the diunsaturated species.

Despite the fact that this HPLC procedure uses a gradient elution, the lines for each fatty acid in the *sn*-2 position were relatively parallel in a plot of the relative retention times vs. the effective carbon number of the fatty acid in the *sn*-1 position (data not shown). Consequently, we used this plot to tentatively identify two late-eluting peaks as 20:0/22:6 (fraction 13) and 20:0/20:4 (fraction 14).

In our initial studies of the synthesis of PS in rat liver microsomes, we used the established procedure of Patton et al. [10] to determine which molecular species of PS had incorporated labeled serine. With that procedure and the procedure described here we observed that 68–70% of the radioactive serine was incorporated into 18:0/22:6 and 18:0/20:4, showing that the derivatization steps used in our procedure did not alter the distribution of the label compared to the HPLC procedure using intact, non-derivatized species of PS. When we used the procedure described by Patton et al. the resolution of 18:0/22:6 and 18:0/20:4 was not always complete, and the percent of radioactivity detected in 18:0/22:6 varied from 10 to 25%. Variable results were often obtained with repeated analyses of the same sample. The reason for this variation can be deduced from the results presented in Table 2 showing that the majority of the [^{3}H]serine (56.2%) was incorporated into 18:0/20:4, with only a relatively low amount (11.8%) being incorporated into 18:0/22:6. Thus when a small amount of the highly labeled 18:0/20:4 species was not resolved from the 18:0/22:6 species, a large variation in the percentage of label was found in the smaller 18:0/22:6 peak.

Because numerous attempts to completely resolve the Tnp-PS species were unsuccessful, we decided to decarboxylate the PS to PE, since we could easily resolve the Tnp-PE species. The data in Table 2 show that although several isolation procedures and chemical modifications of PS were used, a consistent sample of labeled molecular species of PS was produced and no variable oxidation of the polyunsaturated fatty acids occurred. The solvent eluting between the

Table 2
Synthesis of molecular species of phosphatidylserine in rat liver microsomes

Molecular species	Percentage of total cpm
16:0/22:6	4.6 ± 0.8
16:0/20:4	5.9 ± 1.0
16:0/18:2	1.4 ± 1.2
16:0/22:5	1.9 ± 0.3
18:0/22:6	11.8 ± 0.4
18:0/20:4	56.2 ± 1.3
18:0/18:2	4.7 ± 0.5
18:0/22:5	5.4 ± 0.3
18:0/18:1	2.4 ± 0.3
20:0/22:6[a]	1.7 ± 0.3
20:0/20:4[a]	1.6 ± 0.3

The incorporation of [^{3}H]serine into PS was conducted for 20 min at 37°C in a reaction mixture volume of 0.2 ml containing 10 m*M* $CaCl_2$, 25 m*M* HEPES (pH 7.4), 0.4 m*M* [^{3}H]serine (37.5 μCi/μmol) and 200 μg of microsomal protein. Molecular species were separated by HPLC and detected at 335 nm with a Perkin-Elmer LC 95 UV-Vis spectrophotometer as described in the text. The solvent front routinely contained 3–5% of the counts. Values are the average % of the total [^{3}H]serine incorporated into HPLC-separated molecular species ± S.D. from five separate analyses of PS synthesized in 5 different preparations of liver microsomes.
[a] Species tentatively identified by relative retention times.

two species contained little or no radioactivity above the background level, clearly demonstrating that the larger 18:0/20:4 peak did not overlap with the smaller 18:0/22:6 fraction.

The fact that PS contains predominantly one molecular species posed a special problem for the analysis of PS species by the previously reported analytical procedures used to separate intact PS species. When 60–80 nmol of rat liver microsomal PS were placed on the column, the minor species were difficult to detect. If the amount of the sample is increased to obtain a higher amount of radioactivity in the minor species and consequently a larger detector response, the 18:0/22:6 peak often partially overlapped the 18:0/20:4 peak. Hence, another important feature of the method presented here is that the detector responses for the minor PS species, 16:0/22:6 (fraction 1) and 16:0/20:4 (fraction 2) were large and easily observed. This was brought about by decarboxylation of the PS using mitochondria as a source of PS decarboxylase, followed by extraction of the lipids including mitochondrial PE, which contained significant amounts of 16:0/22:6 and 16:0/20:4 [21]. When the total lipid extract was reacted with TNBS, the mitochondrial PE provided the palmitoyl marker Tnp-PE species. One disadvantage of this procedure is that the molecular species of PS can not be quantitated, since they were converted to PE in the mitochondria. However, other procedures are available to determine the molecular species composition of PS using reversed-phase HPLC of Tnp-PS [13] or diacylglycerolbenzoates prepared by phospholipase C hydrolysis of PS [22].

Although we designed this procedure to monitor the incorporation of labeled precursors into the polar moiety of PS, it can also be used to measure the incorporation of precursors into the hydrophobic groups. It could also be used to conduct double labeling studies in which both the headgroup and the hydrophobic groups were labeled with different isotopes. This HPLC procedure can also be used to conduct metabolic studies on the incorporation of the precursor ethanolamine into specific molecular species of PE. In that case the decarboxylation step is not necessary. We have shown that the HPLC method presented here is a valuable tool to examine the metabolic and functional compartmentalization of molecular species of the highly polyunsaturated PS by observing that stearoyl-polyunsaturated molecular species of PC and PE are selectively used for PS synthesis [23].

Acknowledgement

This study was supported by USPHS Grants AA07215, AA07186 and AA07463.

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