Journal of Chromatography A, 1351 (2014) 37–45

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Elucidation of phosphatidylcholine isomers using two dimensional liquid chromatography coupled in-line with ozonolysis mass spectrometry

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ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 14 March 2014
Accepted 20 April 2014
Available online 14 May 2014

Keywords:
Phosphatidylcholine isomers
Two dimensional liquid chromatography
Mass spectrometry
Ozonolysis
Double bond location

ABSTRACT

In this study, two dimensional (2D) liquid chromatography (LC) was coupled in-line with ozonolysis-mass spectrometry (O_3-MS) for the elucidation of phosphatidylcholine (PC) structures in phospholipid extracts. In O_3-MS, PC molecules carried by mobile phase pass through a length of gas-permeable tubing surrounded by ozone vapor resulting in oxidative cleavage of double bonds. The characteristic aldehyde products are then directly analyzed by eletrospray ionization (ESI)-MS, allowing assignment of the double bond positions within the PC fatty acyl chains. The on-line 2D-LC method employs hydrophilic interaction chromatography as the first dimension to separate phospholipid classes and reversed phase chromatography in the second dimension to further separate PC molecular species. Thus, by combing the separation power of 2D-LC with in-line O_3-MS (2D-LC/O_3-MS), PC species in complex mixtures can be identified including the detailed structure of their two fatty acid chains. When the 2D-LC/O_3-MS method was applied to a rat liver phospholipid extract, 19 PC molecular species were identified with fatty acid isomers unambiguously assigned.

1. Introduction

Phospholipids (PL) are important structural and functional components of cell membranes [1]. Phosphatidylcholine (PC), which is comprised of the characteristic choline head group and fatty acyl chains at *sn*-1 and *sn*-2 position of the glycerol backbone, is the most abundant PL class in eukaryotic cell membranes. The chain length, number and position of double bonds along the fatty acyl chains of PC can greatly influence the structural and dynamic properties of membranes. For example, membrane fluidity is at a maximum when the double bond is located in the middle of the fatty acyl chain [2]. Despite the importance of double bond locations to the function of phospholipids, it remains a challenge in lipidomic studies to differentiate all of the isobaric species that may exist. Strategies to differentiate between phospholipid isomers with different head groups have been described [3] but assumptions are often made in order to assign substituent fatty acid isomers.

The use of electrospray ionization-mass spectrometry (ESI-MS) not only achieves sensitive detection of PL [4], but also combines with the high separating power of liquid chromatography (LC) so that identification of PL classes and even PL molecular species can be achieved [5–7]. Normal phase LC (NPLC) retains analytes based on their polar interaction with a silica stationary phase. Thus, PL are separated into classes, depending on the nature of their head group, such that the more polar PL class has a longer retention time (t_R) in NPLC. Chloroform, methanol, hexane and 2-propanol are typical mobile phases used for NPLC separations of PL classes but in addition, electrospray compatible aqueous buffers such as ammonium hydroxide and ammonium formate are also added in the mobile phase to improve peak shape and response [6,8,9]. Reverse phase LC (RPLC) has been mainly used for the separation of molecular species within a single PL class, since the hydrophobicity of acyl chains depends on the chain length and the number of double bonds. Both isocratic [10–12] and gradient elutions [13] on C18 columns have been used for the separation of PC molecular species in food and biological samples. When the retention orders of PC molecular species were established under different separation conditions, most species retained the same relative elution order, which could be helpful for the identification of unknown PC isomers. However, differences in PC elution orders were found between literature reports, probably due to differences in mobile phase composition [10–12]. Furthermore, despite achieving good separations between PC species, many PC assignments remain ambiguous especially for those identified by LC methods not compatible with MS. Recently,

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http://dx.doi.org/10.1016/j.chroma.2014.04.069
0021-9673/

two ultra high performance LC columns were connected in series which resulted in an improved one dimensional separation of lipid molecular species in blood plasma [14].

Due to the natural diversity present within PL classes and molecular species, no single technique can provide adequate separation for all PL molecules. Hence, the idea of combing the NPLC separation of PL classes and the RPLC separation of molecular species was proposed in order to achieve a nearly complete separation of all PL molecules in complex biological samples [15]. Houjou et al. used NPLC to separate PL extracts into classes that were separately collected then analyzed by C30 RPLC/ESI-MS for molecular species identification [16]. This off-line two dimensional (2D) LC/MS experiment did reveal the diversity of PL species present in rat liver, but had some disadvantages due to the need for fraction collection, potentially leading to sample loss and oxidation. The first application of on-line NPLC × RPLC/MS analysis for PL profiling was realized by a solvent-evaporating interface [17]. In this system, the mobile phase from the NPLC first dimension was evaporated by a vacuum pump at the interface; then the mobile phase from RPLC passed through the interface and transferred the fraction into the second dimension C18 column for further molecular species separation. In this way, the problem of immiscibility between the mobile phases from NPLC and RPLC was solved, and the components of interest were also enriched at the interface. Another solution to the problem of solvent incompatibility between NPLC and RPLC is the use of hydrophilic interaction chromatography (HILIC). Unlike NPLC that uses high proportions of organic solvents, a partially aqueous mobile phase with a polar organic solvent such as acetonitrile can be used on a HILIC column. A stop-flow 2D-LC/MS method for PL analysis was recently developed in which 23 fractions separated by HILIC were collected and injected onto a C18 column for species separation via a 100 μL sample loop [18]. Further improvement of the stop-flow 2D-LC method was made by using an intermediate column to trap the components coming from the HILIC column; these components were then eluted from the trap using a make-up flow [19].

Even though MS and tandem MS (MS/MS) have emerged as a powerful tool for the structural elucidation of PL, it still fails to provide some key aspects of PL structure information such as the position of double bonds in the fatty acyl chains that are critical to certain biological function. The assignment of the specific fatty acyl chains still relies heavily on the natural abundance of the fatty acids that are present in the PC fraction, as determined by gas chromatography coupled to flame ionization detector (GC-FID) following PC hydrolysis and methylation [20–22]. However, this is not a definite identification of the fatty acyl substituents of a particular PC molecular species. In order to confirm the identification of unsaturated fatty acids where many positional isomers are possible, GC coupled to electron ionization mass spectrometry (GC/EI-MS) analysis was performed on dimethyloxazolines (DMOX) derivatives of fatty acids from PC [8,23]. In another approach, multiple-stage MS (MS^n) analysis of lithiated adducts of PL was performed on a linear ion-trap mass spectrometer. The product ions arising from MS^n scans of $[M+Li]^+$ and $[M-H+2Li]^+$ ions were used for assignment of the polar head group and fatty acyl substituents, as well as for the localization of double bonds along the chains [24].

Ozone-induced dissociation (OzID-MS) is another approach for the determination of double bond locations that has been shown to be applicable to PL [25]. During OzID-MS analysis, ozone vapor was introduced into an ion trap MS for in situ ozonolysis, and the ozonolysis products with characteristic *m*/*z* were used for localization of double bonds in PL. However, this may not be directly compatible with LC and introduction of ozone into the mass spectrometer is needed. In order to have an ozonolysis method compatible with LC separations of complex lipid extracts, we have developed a method utilizing a type of gas-permeable, liquid-impermeable Teflon tubing as an in-line ozonolysis device (O_3-MS) [26]. In the O_3-MS experiment, a length of the semi-permeable tube passes through a glass chamber filled with ozone. The ozone penetrates the tubing walls and reacts with unsaturated lipids in the sample within the mobile phase flowing along the tube. The characteristic ozonolysis products that result from the oxidative cleavage of double bonds are then detected in real-time by MS. With an LC column placed prior to the in-line ozonolysis device, each eluting compound can undergo ozonolysis at the site of any unsaturations. Hence, double bond positions from complex lipid mixtures can be determined, as we have demonstrated for fatty acid methyl ester mixtures [26,27]. Potentially, this approach could also be applied to lipid extracts for the identification of PL molecular species.

In this study, we will further develop in-line O_3-MS method for the unambiguous assignment of double bond positions in both fatty acyl chains of PC molecules. On-line 2D-LC will be developed for the separation of PC molecular species, and the 2D-LC will also be coupled to in-line O_3-MS for the detailed structural determination of PC molecules in the mixture. In addition, 2D-LC/O_3-MS approach will be applied to the rat liver PL extract for the identification of PC molecular species.

2. Experimental

2.1. Nomenclature

We adopted as much as possible the nomenclature and abbreviation conventions from Fahy et al. [28]. Diacyl-phosphatidylcholine is described by the PC(n:j/s:t) nomenclature, where n is the number of carbon atoms and j is the number of double bonds in one substituent; s is the number of carbons, and t is the number of double bonds in the other substituent. They only indicate the composition of these two chains; the order does not necessary represent their *sn*-1 or *sn*-2 position, except for the PC standards. In this study, it is advantageous to locate the double bond by counting from the terminal methyl group, thus the "*n*-" terminology is used. For example, 1-octadecanoyl-2-(9Z,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine is expressed as PC(18:0/18:2 (*n*-6,9)).

2.2. Materials

HPLC grade water, tetrahydrofuran (THF), chloroform, methanol, acetonitrile (ACN), isopropanol (IPA) were purchased from Fisher Scientific Company (Ottawa, ON, Canada). HPLC grade ammonium formate and formic acid were obtained from Sigma (St. Louis, MO, USA). All the standards PC(18:0/22:6(*n*-3,6,9,12,15,18)), PC(18:0/20:4(*n*-6,9,12,15)), PC(18:1(*n*-9)/18:1(*n*-9)), PC(18:0/18:2(*n*-6,9)), PC(18:1(*n*-12)/18:1(*n*-12)) and PC(18:0/18:1(*n*-9)) were purchased from Avanti polar lipids, Inc. (Alabaster, AL, USA). Each standard solution was prepared in methanol at a concentration of 200 μg/mL. The Teflon AF-2400 tubing (0.020″ OD, 0.010″ ID) was purchased from Biogeneral Inc. (San Diego, CA, USA).

2.3. Extraction of PL from rat liver

Rat livers from suckled rats were collected and ground in liquid nitrogen. The ground rat liver was used as the sample for PL extraction according to a modified Bligh and Dyer method developed by Xiong et al. [29]. In brief, 100 mg of samples were mixed with 2 mL of extraction solvent (chloroform/methanol/water, 1:2:0.8) and homogenized at 10,000 rpm for 5 min with a Polytron PT1300 D homogenizer (Kinematica AG, Switzerland) and then centrifuged at 3000 rpm for 5 min. The extraction procedure was repeated three times and all of the supernatants were combined in a 10 mL volumetric flask and made up to volume with methanol. The extract