Journal of Chromatography A, 1450 (2016) 76–85

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Retention behavior of lipids in reversed-phase ultrahigh-performance liquid chromatography–electrospray ionization mass spectrometry

Magdaléna Ovčačíková, Miroslav Lísa, Eva Cífková, Michal Holčapek*

University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry, Studentská 573, 53210 Pardubice, Czech Republic

ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 10 April 2016
Accepted 29 April 2016
Available online 3 May 2016

Keywords:
Lipids
Lipidomics
Ultrahigh-performance liquid chromatography
Mass spectrometry
Human plasma
Retention behavior

ABSTRACT

Reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) method using two 15 cm sub–2 μm particles octadecylsilica gel columns is developed with the goal to separate and unambiguously identify a large number of lipid species in biological samples. The identification is performed by the coupling with high-resolution tandem mass spectrometry (MS/MS) using quadrupole – time-of-flight (QTOF) instrument. Electrospray ionization (ESI) full scan and tandem mass spectra are measured in both polarity modes with the mass accuracy better than 5 ppm, which provides a high confidence of lipid identification. Over 400 lipid species covering 14 polar and nonpolar lipid classes from 5 lipid categories are identified in total lipid extracts of human plasma, human urine and porcine brain. The general dependences of relative retention times on relative carbon number or relative double bond number are constructed and fit with the second degree polynomial regression. The regular retention patterns in homologous lipid series provide additional identification point for UHPLC/MS lipidomic analysis, which increases the confidence of lipid identification. The reprocessing of previously published data by our and other groups measured in the RP mode and ultrahigh-performance supercritical fluid chromatography on the silica column shows more generic applicability of the polynomial regression for the description of retention behavior and the prediction of retention times. The novelty of this work is the characterization of general trends in the retention behavior of lipids within logical series with constant fatty acyl length or double bond number, which may be used as an additional criterion to increase the confidence of lipid identification.

1. Introduction

Lipids fulfill multiple essential roles within all eukaryotic cells in living organisms [1]. Living cells contain thousands of different lipid molecules that fall into eight lipid categories according to LIPID MAPS classification, namely fatty acyls, glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids, saccharolipids and polyketides [1–5] containing many classes and subclasses. The dysregulation of the lipid metabolism contributes to numerous serious human diseases, such as obesity, diabetes, cardiovascular diseases and cancer. Therefore, they are investigated as possible biomarkers of these diseases [6–9].

Lipidomic analysis starts with the liquid – liquid lipid extraction from biological materials using organic solvents. The most frequently used extraction procedures are based on chloroform – methanol – water systems according to Folch et al. [10] or Bligh and Dyer [11], or the extraction using methyl *tert*-butyl ether solvent instead of chloroform [12]. Gas chromatography–mass spectrometry is an established approach for fatty acyl profiling [13]. Various analytical strategies are used in the lipidomic analysis using nontargeted and targeted lipidomic approaches [14–18]. Another possible division of lipidomic approaches is according to used analytical methodology. Shotgun lipidomics using triple quadrupole instruments and characteristic precursor ion and neutral loss scans [19–21] is well established approach for the fast quantitation of lipid molecular species from extracts of biological samples without a chromatographic separation. The second approach is the use of liquid chromatography–mass spectrometry (LC/MS) coupling, where various chromatographic modes can be selected depending on the required type of separation, such as reversed-phase (RP) LC [22–25], normal-phase (NP) LC [26,27], hydrophilic interaction liquid chromatography (HILIC) [14,15], silver-ion LC [13,28,29] and chiral LC [30,31]. The RP separation mode coupled with MS is widely used in a comprehensive lipidomic analysis to identify individual molecular species in different biological samples [22,32–34], where lipids are separated according to the length of fatty acyl chains and

* Corresponding author.
E-mail address: Michal.Holcapek@upce.cz (M. Holčapek).

http://dx.doi.org/10.1016/j.chroma.2016.04.082
0021-9673/

the number and position of double bonds (DB) [22]. In the RP mode, mobile phases are typically composed of mixture of water containing volatile buffers and polar organic solvents, such as methanol, acetonitrile and 2-propanol. RP mode provides intra- and interclass separation of lipid species, especially in ultrahigh-performance liquid chromatography (UHPLC) configuration [22], but on the other hand the quantitation is more demanding, because the lipid class internal standards do not coelute with analytes unlike the lipid class separation in HILIC or NP modes. NP-LC is particularly suitable for the separation of nonpolar lipid classes, where individual nonpolar lipid classes are separated based on their polarity [27]. HILIC separation allows the lipid class separation, where individual lipid classes are separated according to their polarity and electrostatic interactions [14,15]. HILIC and RP modes have relatively good complementarity of retention mechanisms, therefore various modes of their 2D-LC coupling have been already applied for the lipidomic analysis [35–37]. The HILIC-like separation can be also achieved in ultrahigh-performance supercritical fluid chromatography (UHPSFC) on silica columns, but with shorter analysis time and more efficient separation [38]. The silver-ion LC is a special chromatographic mode based on the formation of weak reversible complexes of silver ions with π electrons of DB, which enables the resolution of triacylglycerols (TG) and diacylglycerols (DG) isomers differing in the number, positions and geometry of DB [13,28,29]. The most demanding separation task is a chiral resolution, which has been applied to TG enantiomers [30,31].

The main goal of our work is the study of the retention behavior of individual lipids in RP-UHPLC to describe general dependences of retention times on the carbon number (CN) and the DB number. For this purpose, RP-UHPLC method with two C18 columns in series is optimized and coupled to high-resolution MS/MS to unambiguously identify the large number of lipids. The retention data are collected for lipid extracts of human plasma, human urine and porcine brain samples. Individual lipid species are identified based on accurate *m/z* values of their molecular adducts and characteristic fragment ions in their MS/MS spectra measured in positive- and negative-ion modes. Relative dependences of retention times on the CN or the DB number are fitted with the second degree polynomial regressions.

2. Material and methods

2.1. Chemicals and standards

Acetonitrile, 2-propanol, methanol, (all LC/MS gradient grade), hexane (HPLC grade), chloroform (HPLC grade, stabilized by 0.5–1% ethanol), ammonium acetate, sodium chloride, sodium methoxide, standards of cholest-5-en-3ß-yl octadecanoate [cholesteryl ester (CE) 18:1] and 3ß-hydroxy-5-cholestene [cholesterol (Chol)] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). Standards of polar lipid classes containing C18:1(9Z) fatty acyl(s), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), ceramide (Cer), and sphingomyelin (SM d18:1/12:0), ceramide (Cer d18:1/12:0), cholesteryl (d7) ester (Chol d7 16:0) and oleic acid-d9 (FA d9 18:1) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Nonpolar lipid standards of TG 18:1/18:1/18:1, TG 19:1/19:1/19:1 and DG 18:1/18:1 were purchased from Nu-Chek Prep (Elysian, MN, USA). The lipid nomenclature follows the shorthand notation for lipid structures published by Liebisch et al. [39] and the LIPID MAPS [2] classification system. Samples of human plasma and urine were obtained from healthy volunteers from the research team. Porcine brain was obtained from the local store.

2.2. Sample preparation

Blood was collected to heparin-lithium tubes and ultracentrifuged to obtain plasma. The total lipid extracts of human plasma, human urine and porcine brain tissue were prepared according to Folch procedure [10] using the chloroform – methanol – water solvent system with minor modifications [14,15]. Human plasma (50 μL) was homogenized with 3 mL of the chloroform – methanol (2:1, v/v) mixture, while porcine brain tissue (50 mg) and human urine (2 mL) were homogenized with 6 mL of the chloroform-methanol mixture (2:1, v/v) in the ultrasonic bath at 40 °C for 10 min. Then, deionized water (600 μL for human plasma and 1200 μL for porcine brain) was added (no additional water for human urine), and the mixture was centrifuged at 3000 rpm for 3 min under ambient conditions. The chloroform (bottom) layer containing lipids was collected, evaporated by a gentle stream of nitrogen and redissolved in 1 mL of the chloroform – 2-propanol (1:1, v/v) mixture for the RP-UHPLC/ESI-MS analysis.

2.3. RP-UHPLC conditions

Experiments were performed with an Agilent 1290 Infinity series (Agilent Technologies, Santa Clara, CA, USA). Two identical Acquity UPLC BEH C_{18} columns (150 mm × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA) were coupled in series and used for the separation of total lipid extracts under the following conditions. Flow rate 180 μL/min, injection volume 2 μL, column temperature 40 °C, mobile phase gradient 0 min – 21.5% of solvent A and 78.5% of solvent B, 160 min – 100% of solvent B, where solvent A was 5 mmol/L aqueous ammonium acetate and solvent B was the mixture of 99.5% of acetonitrile – 2-propanol (1:2, v/v) and 0.5% water, the concentration of ammonium acetate in solvent B was also 5 mmol/L. The system backpressure reached 1000 bar during the gradient analysis.

2.4. ESI-MS conditions

The hybrid QTOF mass spectrometer (micrOTOF-Q, Bruker Daltonics, Bremen, Germany) with an ESI source was used as the detector under the following conditions: capillary voltage 4.5 kV, nebulizing gas pressure 1.0 bar, drying gas flow rate 8 L/min and drying gas temperature 200 °C. ESI mass spectra were measured in the range of *m/z* 50–1500 in positive- and negative-ion modes. Argon as the collision gas at the collision energy of 20–25 eV was used for MS/MS experiments. MS/MS spectra are recorded in both polarity modes using the data independent mode for all ions exceeding the instrumental intensity threshold of 10^4. The external calibration of the mass scale was performed with sodium formate clusters before individual measurements together with the internal recalibration using the most abundant known lipids. The data were acquired using the DataAnalysis software (Bruker Daltonics).

3. Results and discussion

3.1. RP-UHPLC separation of lipids

The goal of our RP-UHPLC analysis is the identification of the large number of lipid species, which is then used for the study of the retention behavior of individual lipids in logical series with the constant number of carbon atoms or DB. For this purpose, we have selected the coupling of two 15 cm C18 columns with sub–2 μm particles (150 mm × 2.1 mm, 1.7 μm) and aqueous ammonium acetate – acetonitrile – 2-propanol gradient, which