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Fast high performance liquid chromatography analysis in lipidomics: Separation of radiolabelled fatty acids and phosphatidylcholine molecular species using a monolithic C_{18} silica column

J.-F. Merlin [1], J. Gresti [1], S. Bellenger, M. Narce *

EA 2422 Lipides et Nutrition, Université de Bourgogne, Dijon, France

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Abstract

HPLC procedures using conventional C_{18} columns are usually used to separate simple and complex lipid mixtures but these methods of separation remain often laborious and very slow. Here, monolithic columns were successfully applied to separate lipids – radiolabelled fatty acid mixtures and individual phosphatidylcholine (PC) molecular species. For that, isocratic elution was performed using two Chromolith™ Performance RP-18e columns connected in series. Detection was achieved by online measurement of radioactivity for radiolabelled fatty acids and by UV absorbance at 205 nm for PC molecular species. The performances of such silica rods were compared to conventional reverse-phase silica columns. Monolithic stationary phase separated radiolabelled fatty acids and PC molecular species two times and four times faster, respectively. In each analysis, monolithic columns allowed better separation efficiency per unit of time, with lower inlet pressure. The main advantages of this method for lipid separation are that, under isocratic conditions, it is simpler and much faster, while remaining accurate and selective when compared to conventional methods. Therefore, monolithic columns may represent a powerful tool for the near future in the field of lipidomics.

Keywords: HPLC; Fatty acid; Desaturation; Phosphatidylcholine molecular species; Monolithic column; Lipidomics

1. Introduction

Lipids are essential molecules in the structure of biological membranes (phospholipids), signalling processes (eicosanoids derived from polyunsaturated fatty acids) or energy reserves (triacylglycerols). Among phospholipids, lecithins – known as phosphatidylcholines (PC) – are rich in polyunsaturated fatty acids (PUFA) mainly esterified at the sn-2 position and therefore contribute to membrane fluidity. A large number of fatty acid combinations or rearrangements are possible on the sn-1 and sn-2 positions of the PC, influencing its biological functions. Due to these numerous possibilities, PC include a great deal of different molecular species which are usually difficult to separate. Thus, improving the separation of PC molecular species remains of great interest in lipid biochemistry and in pathophysiological investigations [1,2].

In animal cells such as hepatocytes, the main PUFA are biosynthesized from their dietary precursors. We previously described a RP-HPLC-based method to separate biological radiolabelled unsaturated fatty acids produced by desaturation from their substrates [3]. This method is the most commonly used to assess desaturase activities [4–7], and has been further improved by the use of online radioactivity detection. It requires conventional C_{18} particle-packed column [3,7], which are usually used to separate simple lipids [3,8,9] and also complex lipids [10–12].

The chromatographic separation of PC molecular species based on the method developed by Patton et al. [13] also requires conventional C_{18} particle-packed column. This method requires a long resolution time, a large level of solvent consumption or a complex gradient solvent [14]. Thus, more recently, McHowat et al. described a gradient elution RP-HPLC method separating all PC molecular species in 110 min [15]. Indeed, due to the large

Abbreviations: ACN, acetonitrile; ChCl3, chloroform; MeOH, methanol; PC, phosphatidylcholine; RP-HPLC, reverse-phase liquid chromatography; D6D, delta-6 desaturase; SCD1, stearoyl CoA desaturase

* Corresponding author at: Lipides et Nutrition, Faculté des Sciences Gabriel, Université de Bourgogne, 6 boulevard Gabriel, 21000 Dijon, France. Tel.: +33 380396317; fax: +33 380396330.
E-mail address: michel.narce@u-bourgogne.fr (M. Narce).

[1] These authors have contributed equally to this work and must be considered as first authors.

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number of different fatty acid molecular species on its structure, the lipid class of PC remains difficult to separate.

A new type of stationary phase offers perspectives in different areas of HPLC inducing major changes in column technology: continuous bed or monolithic column. Liao and coworkers were the first to use a compressed polyacrylamide gel to separate proteins [16]. Today, two main types of continuous bed coexist, the organic polymer-based monolithic columns generally prepared by radical polymerization and inorganic silica-based monolithic columns prepared by a sol–gel method [17,18]. These columns can be further octadecylsilyated to C_{18} phases by an on-column reaction. Monolithic silica columns are characterized by large-sized through-pores (generally 2 μm) which result in low flow resistance and small-sized mesopores (up to 10 nm) which permit high efficiency separation [19]. Thus, increasing the flow rate is rendered possible without high column backpressure. In addition, this new generation of column offers the possibility of saving analysis time and solvent, compared to conventional particle-packed columns [20].

Whereas fast separations of biopolymers such as proteins, polynucleotides and smaller biomolecules with monolithic columns have been widely reported [21–24], the separation of simple or complex lipid mixtures using monolithic columns has been poorly studied, despite their well-known chromatographic properties. Recently, the identification (in about 70 min) of a lyso-PC from Bronchoalveolar Lavage Fluid has been reported using a Chromolith CapRod RP18e monolithic silica capillary column [25]. This result suggests that monolithic columns could be suitable to separate classes from a phospholipid mixture.

The aim of this work was to investigate whether monolithic columns can be applied to the separation of lipids – radiolabelled fatty acids mixture and individual PC molecular species – and whether it can improve it, by measuring the performance of such silica rods in comparison to conventional reverse-phase silica columns.

2. Materials and methods

2.1. Chemicals

Radiolabelled fatty acids and liquid scintillation cocktail (Ultima-Flo™ AP) were purchased from PerkinElmer Life Sciences Inc. (Wellesley, MA). Authentic phosphatidylcholines used as standards and choline chloride were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade solvents were purchased from Carlo Erba (Milano, Italy). Water was purified to 15 MΩ cm on a Elix S (Millipore, Molsheim, France).

2.2. HPLC apparatus

The HPLC experiments were performed on a Varian Prostar system with a solvent delivery module 230 (Varian Inc., Palo Alto, CA). Column temperature was regulated with a Croco-Cil column heater (Cluzeau Info Labo, Ste Foy la Grande, France). Detection was performed with a Varian UV-VIS detector model 310 (Varian Inc., Palo Alto, CA). Radiolabelled fatty acid methyl esters were detected online by a radioisotope detector (Packard Flow Scintillation Analyzer, PerkinElmer Life Sciences Inc., Wellesley, MA). The data were analyzed with a Varian Star chromatography workstation software version 5.5 (Varian Inc., Palo Alto, CA).

2.3. Chromatography

All separations have been performed at a temperature of 25 °C using two different stationary phases: first, one conventional LichroCART Superspher™ RP-18 (250 mm × 4 mm i.d., 4 μm particles) then two Chromolith™ Performance RP-18e (100 mm × 4.6 mm i.d.) connected in series both purchased from Merck KGaA (Darmstadt, Germany). Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, Cotati, CA) with a 20 μl loop.

2.3.1. Radiolabelled fatty acids

The mixture of radiolabelled fatty acids was transmethylated (BF_3-methanol, Sigma–Aldrich Co.) according to Slover and Lanza [26]. Fatty acid methyl esters were separated isocratically using acetonitrile and water ACN/H_2O 97/3 (v/v) as a mobile phase. When using the Superspher™ column, the flow rate was 1.5 ml/min with an inlet pressure of 100 bar. When using two Chromolith™ columns in series, the flow rate was 2 ml/min with an inlet pressure of 30 bar. Fatty acids were identified by comparison with retention times from ^{14}C-labelled fatty acid standards.

2.3.2. PC molecular species

Total lipids were extracted with $CHCl_3$/MeOH 2/1 (v/v) from rat liver according to the Folch method [27]. Phospholipids were first separated by TLC, using $CHCl_3$/MeOH/H_2O 65/25/4 (v/v/v) as solvent system. Lipid components were identified after spraying the plates with 7,12-dichlorofluoresceine dye in ethanol and visualized under ultraviolet light. PC were immediately scraped off the plates and extracted in the presence of $CHCl_3$/MeOH/H_2O 5/5/1 (v/v/v). The chloroform layer was taken and stored at −20 °C. Prior to HPLC injections, samples were dried under nitrogen and dissolved in 200 μl of MeOH/ACN 10/1 (v/v). Fifty to 100 nmol of PC were injected per run.

PC molecular species were first separated according to the HPLC method originally developed by Patton et al. [13] and modified as follows: a Superspher™ column was eluted with a mixture of acetonitrile, methanol, water (ACN/MeOH/H_2O 20/79/3 (v/v/v)) and 20 mM choline chloride. The mobile phase was pumped isocratically at a flow rate of 1 ml/min and an inlet pressure of 70 bar. Detection was achieved at a wavelength of 205 nm.

PC molecular species were then separated by using two Chromolith™ columns in series. The solvent ratio was ACN/MeOH/H_2O 33/64.5/2 (v/v/v) at a flow rate of 2 ml/min and an inlet pressure of 30 bar.

Individual species of PC were characterized according to the method of McHowat et al. [28] and identified by coelution with the corresponding standards.