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On-line two-dimensional capillary strong anion exchange/reversed phase liquid chromatography–tandem mass spectrometry for comprehensive lipid analysis

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ABSTRACT

An on-line two-dimensional liquid chromatography method was developed for comprehensive lipid profiling by coupling strong anion exchange (SAX) and nanoflow reversed-phase liquid chromatography (nRPLC) prior to electrospray ionization-tandem mass spectrometry (2D-SAX/nRPLC–ESI-MS/MS). Lipids can be classified into four different types according to the electrical propensities of the lipids: anionic, weak anionic, neutral polar, and special lipids. In 2D-SAX/nRPLC, various lipids can be fractionated in the first dimension (SAX: 5 μm to 100 Å, 5.0 cm × 75 μm i.d.) by step elution (methanol and salt solution), followed by the molecular separation of lipids in the second dimension (RP: 3 μm to 100 Å, 7.0 cm × 75 μm i.d.) with binary gradient LC. Since the elution of lipids from SAX can be achieved with a very small volume of eluent delivered from an autosampler, it can be simply implemented with an LC–ESI-MS instrument for full automation, and the salt step elution, including the two-step injection procedure, can be used for the selective analysis of the desired lipid fraction. For nRPLC–ESI-MS/MS run in either positive or negative ion mode, a common ionization modifier (0.05% ammonium hydroxide with 5 mM ammonium formate) was introduced into the binary mobile phase solutions so that 2D-LC–MS could be operated in both ion modes without changing the mobile phase solutions. The developed on-line 2D-SAX/nRPLC–ESI-MS/MS was evaluated with 22 different standard lipids for the optimization of the salt step elution and was applied to a healthy human plasma lipid extract, resulting in the identification of a total of 303 plasma lipids, including 14 different classes.

1. Introduction

Lipids are mostly water-insoluble biological molecules having a wide variety of polarities and are major components of biological membranes. Lipids play important roles in energy storage and signal transduction between cells, and are involved in human diseases such as diabetes, atherosclerosis, Alzheimer's disease, and cancer [1–3]. Lipids can be classified into eight categories: fatty acyls (FA), glycerophospholipids (GP or phospholipids), glycerolipids (GL), sphingolipids (SP), sterol lipids (ST), prenol lipids, saccharolipids, and polyketides [4,5]. Since each group of lipids contains a different combination of acyl chains, the simultaneous analysis of the entire lipidome is a complicated task. High-performance liquid chromatography (HPLC) in combination with electrospray ionization-mass spectrometry (ESI-MS) has been commonly used as a sensitive method for lipid analysis. While normal-phase liquid chromatography (NPLC) utilizing stationary phases such as silica [6–8], amine [9], and diol [10,11], including hydrophilic interaction chromatography [12,13], is an effective technique for resolving different lipid classes or subclasses (i.e., different head groups of phospholipids), it is incapable of separating lipids by individual molecular species or of estimating the relative abundances of individual components. Reversed-phase liquid chromatography (RPLC) is widely used for lipid analysis [14–17] and is effective for identifying low-concentration lipid species, since individual lipid species can be separated by the hydrophobic interactions between the alkyl chains (i.e., C18) on the stationary phase and the lipid fatty acyl chains. Recent reports show the profiling of more than 500 lipids from human plasma sample using several analytical methods including GC–MS and LC–MS/MS for each lipid category separately [18] and the identification of 444 lipids from rat plasma using ultrahigh performance LC–MS (UPLC–MS) with enhanced resolution and speed [19]. Nanoflow RPLC–ESI-MS/MS has been powerfully employed for characterizing phospholipids (PLs) from

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human plasma and urine samples with limit of detection (LOD) at low fmol/μL levels [20–23]. However, the comprehensive separation and characterization of lipids is still demanding due to the complexity of the lipids.

To overcome this limitation, multidimensional separation methods that are either off-line or on-line have been investigated. Strong cation exchange (SCX) chromatography [24,25] and NPLC [26,27] were used to fractionate different lipid classes in an off-line mode and the collected fractions were analyzed by RPLC in the second dimension. With anion exchange HPLC, phosphatidylinositide levels of cells were specifically analyzed with conductivity detection [28]. On-line two-dimensional liquid chromatography (2D-LC) approaches with silver ion HPLC coupled with RPLC (Ag-LC/RPLC) [29,30] or with a combination of NPLC and RPLC have been made [31,32]. Ag-LC/RPLC is useful to differentiate the different degrees of unsaturation in acyl chains by Ag ion interactions with double bonds, but is too specific for TG profiles and not sufficient to analyze the complicated lipid mixtures. On-line 2D-NPLC/RPLC was developed by introducing a solvent evaporation interface between NP and RP columns to remove the organic mobile phase used in the NPLC process [31]. While 2D-NPLC/RPLC–ESI-MS/MS with a solvent evaporation interface expanded the capability of phospholipid identification to a great extent, it has some limitations when applied to a wide variety of lipid classes, since earlier work focused on 12 subclasses of phospholipids. Besides the need for a vacuum pump, the detection limit of this method was relatively high (55–65 fmol) [32].

In this study, an on-line 2D capillary strong anion exchange/nanoflow RPLC (2D-SAX/nRPLC) separation with ESI-MS/MS has been developed to simultaneously analyze 22 lipid classes including four categories of lipids (GP, GL, SP, and ST). Since lipids can be classified into four different types according to their electrical propensities, namely anionic, weak anionic, neutral polar, and special lipids [33], anionic lipids can be differentiated from neutral lipids by SAX resin. However, SAX has not previously been fully integrated into the 2D-LC separation of lipid classes with the exception of an anion exchanger cartridge being used to purify gangliosides from tissues [34]. In case of using SCX for lipid separation, lipid affinity to SCX resin is so weak that selective separation of different lipid classes cannot be made. In this study, an on-line 2D-SAX/nRPLC is assembled by coupling a capillary SAX column (5 μm to 100 Å SAX resins in 5.0 cm × 75 μm i.d.) with a capillary RP analytical column (3 μm to 100 Å C18 resins in 7.0 cm × 75 μm i.d.) using two six-way valves for a full automation. Ionic solutions of different concentrations can be delivered for step elution (methanol or salt solution) from the autosampler to the SAX column, and lipid fractions of different electric propensities desorbed from the SAX column are loaded into a short C18 trap prior to being transferred to the analytical column. Then, typical binary gradient nRPLC separation of each lipid fraction followed by ESI-MS/MS analysis can be achieved as reported in several recent studies. The configuration of on-line capillary 2D-SAX/nRPLC is analogous to that of the dual-purpose sample trap for on-line SCX/nRPLC–MS/MS system for peptides [35]. While in the dual trap (SCX/RP) system, organic mobile phases for gradient RPLC elution after each salt step elution pass through SCX trap throughout 2D-separations, the current 2D-SAX-RPLC configuration utilizes a separate SAX column prior to a RP trap so that RPLC mobile phases can bypass the SAX column during gradient elution, preventing the unwanted release of bound lipids from SAX. To carry out a sequential nRPLC–MS run in both positive and negative ion modes depending on the type of lipids eluted from the SAX column by each salt step fractionation, a common ionization modifier for both ion modes was first introduced by investigating the ionization effect of modifiers so that nLC–ESI-MS/MS runs could be carried out without changing the mobile phase solution containing a different ionization modifier. Evaluations of 2D-SAX/nRPLC–ESI-MS/MS were made with 22 standard lipids of different classes to optimize the salt step concentrations of ammonium acetate solution for consecutive RPLC separations and to determine the limits of detection (LOD) and recovery rates. The developed method was applied to a human plasma lipid extract sample.

2. Materials and methods

2.1. Materials

All lipid standards used for the optimization of SAX/nRPLC–ESI-MS/MS were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Twenty-two standard lipids represented each lipid class, including four different categories (GP, GL, SP, and ST), and they are listed by their polar and ionic natures in Table 1 along with the LOD and recovery values obtained with SAX/nRPLC–ESI-MS/MS, which will be discussed later. All solvents [CH_3CN, isopropanol, CH_3OH, H_2O, $CHCl_3$, and methyl *tert*-butyl ether (MTBE)] were HPLC grade. Lipid standards were first dissolved in solvent A [6:3:1 (v/v) $CH_3OH/CHCl_3/H_2O$] and then diluted with CH_3OH for nLC–ESI-MS/MS. Ammonium hydroxide (AH) and ammonium formate (AF) from Sigma Aldrich (St. Louis, MO, USA) were used as ionization modifiers to be added to mobile phase solutions. Capillary tubes with 20, 50, and 75 μm i.d. (360 μm o.d. for all) were purchased from Polymicro Technology, LLC (Phoenix, AZ, USA).

2.2. Lipid extraction from plasma sample

A human plasma sample was obtained from a healthy male volunteer (age 26). The extraction of lipids from the plasma sample followed the modified Folch method with MTBE/CH_3OH reported in an earlier work [36]. Briefly, 0.3 mL of CH_3OH was added to 0.1 mL of the plasma sample and after a short vortexing, the mixture was placed in an ice bath for 10 min. Then, 1.0 mL of MTBE was added to the mixture and it was vortexed for an hour. Next, 0.25 mL of MS-grade water was added to the mixture, which was then vortexed at room temperature for 10 min. After the mixture was centrifuged at 5000 rpm for 10 min, the upper organic layer was transferred to a different centrifuge tube; then, 0.3 mL of methanol was added to the remaining aqueous layer and agitated in a shaker for 10 min. The mixture was sonicated with a tip for 2 min and centrifuged to collect the portion in the supernatant. The previously removed organic fraction was added to the collected supernatant and then the mixture was dried in a vacuum centrifuge. The dried lipid extracts were dissolved in solvent A, diluted to a total volume of 500 μL, and stored. For SAX/nRPLC–ESI-MS/MS analysis, the final lipid solution in storage was diluted to a concentration of 5 μg/μL in CH_3OH.

2.3. Capillary SAX/RPLC–ESI-MS/MS

The capillary SAX column, a C18 trapping column, and capillary RPLC columns were prepared in our laboratory. The capillary SAX column was packed with a methanol slurry of 5-μm 100-Å Nucleosil SB from Macherey-Nagel Co. (Düren, Germany) to 5.0 cm in a capillary tube (75 μm i.d.) ended with a sol-gel frit (2 mm in length) under a constant pressure of He (1000 psi). The sol–gel frit was simply formed by baking at 100 °C for 2 h after immersing the capillary end very briefly in a solution of 1:4 (v/v) formamide/potassium silicate. The C18 trapping column was similarly packed with RP resins [5-μm 100-Å from Michrom Bioresources Inc. (Auburn, CA, USA)] for 1.0 cm in a frit-ended capillary (75 μm i.d.). The analytical column was packed with a methanol slurry of RP resins [3-μm 100-Å Watchers ODS-P from Isu Industry Co. (Seocho, South Korea)] in a 7.0 cm long pulled tip capillary, of which one end of the capillary