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A comprehensive quantification method for eicosanoids and related compounds by using liquid chromatography/mass spectrometry with high speed continuous ionization polarity switching

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ABSTRACT

Fatty acids and related metabolites, comprising several hundreds of molecular species, are an important target in disease metabolomics, as they are involved in various mammalian pathologies and physiologies. Selected reaction monitoring (SRM) analysis, which is capable of monitoring hundreds of compounds in a single run, has been widely used for comprehensive quantification. However, it is difficult to monitor a large number of compounds with different ionization polarity, as polarity switching requires a sub-second period per cycle in classical mass spectrometers. In the present study, we developed and evaluated a comprehensive quantification method for eicosanoids and related compounds by using LC/MS with high-speed continuous ionization polarity switching. The new method employs a fast (30 ms/cycle) continuous ionization polarity switching, and differentiates 137 targets either by chromatography or by SRM transition. Polarity switching did not affect the lower limits of quantification, which ranged similarly from 0.5 to 200 pg on column. Lipid extracts from mouse tissues were analyzed by this method, and 65 targets were quantitatively detected in the brain, including 6 compounds analyzed in the positive ion mode. We demonstrated that a fast continuous ionization polarity switching enables the quantification of a wide variety of lipid mediator species without compromising the sensitivity and reliability.

1. Introduction

Fatty acids are one of the essential components of mammalian organisms. They are esterified as building blocks in various lipids such as neutral lipids (fats), membrane phospholipids, and cholesteryl esters. Fatty acids in the triglycerides are mobilized by lipases, and they serve as energy source in the tissues through β-oxidation. Fatty acids in membrane phospholipids affect their physicochemical properties, and those esterified in cholesterol are critical for cholesterol homeostasis. In addition to these housekeeping roles, a number of fatty acids and related metabolites are known to function as signaling molecules that mediate a variety of physiological and pathological processes through their binding to receptors on target cells.

Eicosanoids such as prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and hydroxyeicosatetraenoic acids (HETE) are a class of lipid mediators synthesized from dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA), and are known to play various pathological and physiological roles *in vivo* [1,2]. To date, relations between eicosanoid production and diseases have been extensively studied using disease models in animals or samples obtained from humans, providing insights into their roles in pathogenesis [3–5]. Recent studies have shed light on the physiological functions of the compounds that were recognized as byproducts or inactive metabolites, such as 12-hydroxy-heptadecatrienoic acid (12-HHT) or 15-keto-PGE_2 [6,7]. Non-enzymatic oxidation by endogenous reactive oxygen species converts AA to isoprostanes such as 8-*iso*-$PGF_{2\alpha}$, known as biomarkers of oxidative stress [8,9]. There is an increasing interest in the physiological or pathophysiological functions of omega-3 fatty acids such as EPA, docosahexaenoic acid (DHA), and α-linolenic acid as well as their metabolites

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such as resolvins and protectins [10]. Omega-6 linoleic acid causes hydroxy octadecadienoic acids (HODEs), non-eicosanoid lipid mediator associated with atherosclerosis [11,12]. The biological functions of arachidonoyl ethanolamide (AEA) and its metabolites such as PG-ethanolamides are also of interest [13]. More than 500 fatty acid related metabolites have been listed in the Lipid MAPS database (http://www.lipidmaps.org/), warranting a method capable of monitoring several hundreds of lipid metabolites, in order to understand the pathogenic mechanisms associated with these metabolites, as well as facilitate lipid biomarker discovery.

Selected reaction monitoring (SRM) is a selective and sensitive detection mode available in triple quadrupole mass spectrometers (TQ–MS), which detects target compound(s) by monitoring a precursor-to-product 'transition' of target ions. We have previously reported the simultaneous monitoring method for 13 eicosanoids and platelet activating factor by using the column-switching liquid chromatography (LC)/TQ–MS system [14]. Isoprostanes have also been adopted as targets for comprehensive profiling in the evaluation of stress markers [15,16]. Recent studies describe methods that analyze around 100 eicosanoid species in a single chromatographic analysis [17–21].

Although SRM has the potential to monitor several hundreds of compounds simultaneously, chromatographic separation is required for compounds that are not differentiated by SRM transitions. In SRM analysis, there are some trade-offs between the number of targets and the sensitivity, as mentioned in some reports [18,20]. Recent MS systems offer (so called) segmented- or scheduled-SRM to minimize such trade-offs, where each target compound is monitored in a narrow chromatographic time window set around the compound's retention time, minimizing the SRM transitions run simultaneously at a certain time point.

Ionization polarity switching during the analysis is a common function of a mass spectrometer for detecting the compounds that are ionized in different polarity, and has been available over the last decade. Most recent instruments are equipped with a fast polarity switching capability of several tens of milliseconds (ms), making it possible to simultaneously monitor compounds that are ionized in different polarity. A quantification of 135 primary metabolites, including sugars and amino acids with continuous polarity switching, has been reported previously [22]. However, in the lipid field, the benefits of the fast polarity switching have not been fully demonstrated. Dumlao et al. reported a method that detects 36 ethanolamides by positive electrospray ionization (ESI) and 141 eicosanoids by negative ESI in a separate run [19]. Recently, the application of positive ESI for SRM quantification of cysteinyl-LTs (*cys*-LTs) in combination with negative ESI for LTB_4 and for 6-*trans* LTB_4 in a single chromatographic analysis was reported [23]; however, this method does not use continuous polarity switching, since the retention times of these targets were quite different.

Here, we report a comprehensive quantitative method for lipid mediators and its biological application by using a high-speed TQ–MS with fast (15 ms) continuous ionization polarity-switching system.

2. Materials and methods

2.1. Chemicals

All lipid standards, including 151 target compounds and 12 deuterium-labeled compounds, that were used as internal standards were purchased from Cayman Chemical (Ann Arbor, MI), and were dissolved in methanol and stored at −80 °C. LC/MS-grade acetonitrile, HPLC-grade formic acid, HPLC-grade methanol, and ethanol were purchased from WAKO (Osaka, Japan). Ultrapure water was prepared by using the milli-Q system (Millipore, Billerica, MA).

2.2. LC/MS conditions

The LC/MS system consisted of two LC-30AD pumps, an SIL-30AC auto-sampler, a CTO-20A column oven, a CBM-20A system controller, and a triple quadrupole mass spectrometer LCMS-8040 (Shimadzu, Kyoto, Japan). A reversed-phase column (Kinetex C8, 2.1 × 150 mm, 2.6 μm, Phenomenex, Torrance, CA) was used for chromatographic separation. For mobile phases A and B, 0.1% formic acid in water and acetonitrile were used, respectively. The flow rate was 0.4 mL/min. The sample cooler and column oven temperatures were set at 5 °C and 40 °C, respectively. The gradient of mobile phase B concentration was programmed as 10% (0 min) – 25% (5 min) – 35% (10 min) – 75% (20 min) – 95% (20.1 min) – 95% (28 min) – 10% (28.1 min) – 10% (30 min). Methanol was used as a sample solvent. Sample injection volume was 5 μL. Fifteen micro-liters of water was co-injected for improvement of chromatographic peak leading and splitting, which was observed for several compounds. The auto-sampler was programmed to aspirate 5 μL of sample, 1 μL of air, and 15 μL of water in that order, and they were injected directly through the sampling needle so that water entered the column first. Chromatographic retention times (T_R) set in the method are shown in Table 1. Peak resolution (R) was calculated by using the following formula: $1.18 \times (T_{R2} - T_{R1})/(W_{H1} + W_{H2})$, where W_H is full-width at half-maximum (FWHM) of the peak. The parameters for the mass spectrometer were set as follows: nitrogen gas used as nebulizer- and drying gas were set at 3 L/min and 15 L/min, respectively. Argon gas (purity, >99.9995%) was used for collision-induced dissociation (CID). Heat block and desolvation line temperatures were set at 400 °C and 250 °C, respectively.

2.3. SRM conditions

In the method with continuous ionization polarity switching (polarity-switching method), the MS instrument was programmed to perform SRM with 1 ms pause time (dead time between SRM transitions) and 10 ms dwell time (ion monitoring time). Each SRM transition was programmed to monitor a 2 min-window (expected chromatographic retention time ± 1 min). The ionization polarity-switching time was 15 ms (30 ms/cycle). A method without ionization polarity switching (negative-only method) was prepared by replacing all 21 positive SRM transitions in the polarity-switching method with the same number of dummy transitions in the negative ion mode (Supplementary Table S1). Two additional methods, normal- and short-dwell time methods, were used to evaluate the effect of dwell time (Supplementary Table S2); normal dwell time method was programmed to perform 45 SRM transitions simultaneously with 10 ms dwell time, and short dwell time method was programmed to perform the same number of SRM transitions with 1 ms dwell time. Dummy transitions were inserted in the short dwell time method, so that both methods had identical total cycle time of 525 ms, including the time required for switching the ionization polarity.

2.4. Lower limit of quantification (LLOQ) and linear dynamic range

An internal standard method was used for quantification. A mixture of 12 internal standards (IS mixture) that contained 0.5 ng/μL each of the tetranor-PGEM-d6, TXB_2-d4, PGE_2-d4, PGD_2-d4, LTC_4-d5, LTB_4-d4 and 15-HETE-d8, 1.0 ng/μL of 5-HETE-d8, 0.25 ng/μL of oleoylethanolamide (OEA)-d4, and 5.0 ng/μL each of EPA-d5, DHA-d5, and AA-d8 was prepared. A standard mixture containing