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Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoid metabolites in human samples☆

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ABSTRACT

Over the past decade, the number of known eicosanoids has expanded immensely and we have now developed an ultra-performance liquid chromatography–electrospray ionization triple quadrupole mass spectrometric (UPLC-QTRAP/MS/MS) method to monitor and quantify numerous eicosanoids. The UPLC-QTRAP/MS/MS approach utilizes scheduled multiple reaction monitoring (MRM) to optimize sensitivity, number of metabolites that can be analyzed and the time requirement of the analysis. A total of 184 eicosanoids including 26 deuterated internal standards can be separated and monitored in a single 5 min UPLC run. To demonstrate a practical application, human plasma samples were analyzed following solid-phase extraction (SPE) and the recovery rate and matrix effects were determined for the 26 deuterated internal standards added to the plasma. The method was validated and shown to be sensitive with the limit of quantitation at pg levels for most compounds, accurate with recovery rates of 70–120%, and precise with a CV < 30 for all compounds. Also, the method showed a linear response over a range spanning several orders of magnitude. In a QC human plasma sample, we identified and rigorously quantified over 120 eicosanoids.

1. Introduction

Eicosanoids comprise a class of bioactive lipids derived from a unique group of polyunsaturated essential fatty acids that mediate a wide variety of important physiological functions [1]. They exert complex control over many physiological processes, including inflammation [2]. Also, many eicosanoids are associated with chronic disease conditions including heart disease [3], cancer [4], and arthritis [5].

Arachidonic acid (AA) and related polyunsaturated fatty acids serve as the metabolic precursors for eicosanoid synthesis. Biologically, these molecules are generally stored in the *sn*-2 position of the glycerol backbone of membrane phospholipids. To be used for biosynthesis, the arachidonic acid must first be released from phospholipids via phospholipase A_2 (PLA_2) [6] and is then acted on by enzymes of the cyclooxygenase pathway (COX) or the lipoxygenase pathway (LOX) to form prostaglandins (PG) and thromboxanes (TX) or leukotrienes (LT), respectively. The COX site incorporates molecular oxygen at the 11- and 15-carbons on arachidonic acid to form PGG_2, followed by a peroxidase activity that reduces the peroxide to a hydroxyl to form PGH_2. PGH_2 is an intermediate for a number of different bioactive products through the action of PG synthases that can form a number of important signaling molecules, including PGI_2, TXA, PGE_2, PGD_2, and PGF_{2a}. Alternatively, the LOX pathway produces LTs including 5-HETE, 15-HETE, LTA_4, LTB_4, LTC_4, etc., which constitute a family of biologically active molecules formed in response to immunological and non-immunological stimuli. For example, 5-LOX produces 5-hydroperoxy-eicosatetraenoic acid (5-HpETE) by incorporating one molecular oxygen at the C-5 position of arachidonic acid that can be reduced to 5-HETE, or undergo a catalytic rearrangement in the 5-LOX active site to form LTA_4. An additional set of enzymes catalyzes the stereospecific rearrangement of LTA_4 to produce LTB_4, LTC_4, and LTD_4.

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This complete enzymatic system produces hundreds of eicosanoids derived from AA and related polyunsaturated fatty acids with very similar structures, chemistries, and physical properties [7], which makes the analysis of eicosanoids a challenging task, especially in biological samples. The concentration of eicosanoids in plasma or serum is the lowest among all endogenous lipid metabolites [8,9]. However, under certain conditions, the plasma level of eicosanoids may change considerably and thus, eicosanoids may serve as a useful readout reflecting disease progression [10]. As a result, current research is focused on developing fast, sensitive, and reliable methods that accurately profile and quantify eicosanoid biomarkers [11,12].

In the past, eicosanoids were mainly analyzed by enzyme-linked immunosorbent assays (EIA) [13,14], gas chromatography–mass spectrometry (GC–MS) [15], and liquid chromatography–mass spectrometry (LC–MS) [16]. The drawback of EIA is a lack of specificity and the ability to determine multiple analytes in a single set of analyses. GC–MS provides greater sensitivity and selectivity for eicosanoid analysis, but requires chemical derivatization steps that limit its application. The rapid progress of liquid-chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) has facilitated the use of this technology for accurate monitoring of eicosanoid metabolites in biological samples [7]. Previous reports include liquid–liquid extraction for the determination of PGE_2 and LTB_4 in plasma using LC–MS [17], the analysis of a limited number of PGs and LTs in cell culture media by LC–MS [18], an on-line two-dimensional reversed-phase LC–MS for the simultaneous determination of PGE_2, PGF_{2a}, and 13,4-dihydro-15-keto PGF_{2a} [19], and an LC–MS method for the simultaneous determination of 23 eicosanoids [20]. We previously developed a widely used and comprehensive high-throughput lipidomic analysis of eicosanoids using LC–MS to monitor 141 unique species in a single 22 min analysis [21] and applied it to investigate the human plasma lipidome [8,10].

Subsequently, a UPLC–MS platform that enables profiling of 122 eicosanoids from human whole blood [22] was developed using an LC–MS/MS approach similar to our previous protocol [7,21]. More recently published protocols include a targeted HPLC–MS/MS analysis platform for 100 oxylipins and 36 oxylipins was detected from 250 μL human plasma in 26 min [23], an LC–MS/MS method for rapid and concomitant quantification of 26 PUFA metabolites from Caco-2 cells [24], an LC–MS/MS for the simultaneous analysis of arachidonic acid and 32 related metabolites in 1 mL human plasma [25], an online HPLC–MS/MS analyzed more than 20 different oxidized fatty acids and their precursors from 200 μL plasma or urine [26], a fast LC–MS/MS method including on-line SPE and LIT fragment confirmation for the profiling of 7 PUFAs and 94 oxidized metabolites from 200 μL plasma [27]. However, these methods all need much larger biological samples and/or longer analysis times to generally detect and quantify fewer lipid species. These constraints render the application of these methods to large studies and with often limited material impractical.

In order to address the need for large-scale high-throughput analysis of eicosanoids in small quantities of human plasma and tissue samples, we now report the development of a method utilizing an ultra-high-performance liquid chromatography-QTRAP MS/MS (UPLC–MS/MS) for monitoring 184 eicosanoids in a 5 min run. The new methodology is validated by identification and quantitation of eicosanoids in 20 μL human plasma.

2. Experimental

2.1. Reagents

All eicosanoids and deuterated internal standards were purchased from Cayman Chemical. Optima LC–MS grade acetonitrile (ACN), methanol (MeOH), and water were obtained from Fisher Scientific. Isopropanol (IPA) was purchased from Sigma–Aldrich. Formic acid (FA) was obtained from EMD Technologies. Dulbecco's phosphate-buffered saline (DPBS) was obtained from Corning Life Science.

2.2. Sample preparation

2.2.1. Preparation of primary standard and internal standard solutions

For the preparation of calibration curves, stock solutions were prepared in ethanol that contained all eicosanoid standards, each at a concentration of 0.25 ng/μL. Working standard solutions for all eicosanoids were prepared by serial dilution of the stock solutions to create the necessary concentrations. A solution containing 26 internal (deuterated) eicosanoid standards was prepared at 0.01 ng/μL in ethanol. All solutions were stored at −80 °C until use.

2.2.2. Extraction of metabolites from plasma and tissue

Aliquots of 20 μL control plasma (Human Source Plasma, Gemini Bio-Products) were diluted to 1 mL with phosphate salt buffer spiked with 100 μL of the internal standard solution. The eicosanoids were extracted using Strata-X reversed-phase SPE columns (8B-S100-UBJ, Phenomenex). Columns were washed with 3 mL of MeOH and then equilibrated with 3 mL of H_2O. After loading the sample, the columns were washed with 10% MeOH to remove impurities, and the metabolites were then eluted with 1 mL of MeOH and stored at −80 °C to prevent metabolite degradation. Prior to analysis, the eluant was dried under vacuum and redissolved in 50 μL of the UPLC solvent A (water/acetonitrile/acetic acid (60:40:0.02; v/v/v)) for UPLC/MS/MS analysis.

For the analysis of tissue samples, the wet weight is accurately determined and at least 2 mg of tissue (wet weight) is homogenized in 10% methanol. The eicosanoids are extracted from homogenates by SPE following the identical purification protocol as for the plasma samples.

2.3. UPLC–MS/MS

An Acquity UPLC system (Waters Corp.) was used. Reversed-phase separation was performed on an Acquity UPLC BEH shield RP18 column (2.1 × 100 mm; 1.7 μm; Waters). The mobile phase consisted of (A) ACN/water/acetic acid (60/40/0.02, v/v) and (B) ACN/IPA (50/50, v/v). Gradient elution was carried out for 5 min at a flow rate of 0.5 mL/min. Gradient conditions were as follows: 0–4.0 min, 0.1–55% B; 4.0–4.5 min, 55–99% B; 4.5–5.0 min, 99% B; A 10 μL aliquot of each sample was injected onto the column. The column temperature was kept at 40 °C. All samples were kept at 4 °C throughout the analysis.

Mass spectrometry was performed on an ABI/Sciex 6500 QTRAP hybrid, triple quadrupole, linear ion trap mass spectrometer equipped with a Turbo V ion source. Eicosanoids were detected in negative electrospray ion mode. Curtain gas (CUR), nebulizer gas (GS1), and turbo-gas (GS2) were set at 10 psi, 30 psi, and 30 psi, respectively. The electrospray voltage was −4.5 kV, and the turbo ion spray source temperature was 525 °C.

Eicosanoids were analyzed using scheduled multiple reaction monitoring (MRM). Mass spectrometer parameters including the declustering potentials and collision energies were optimized for each analyte. Nitrogen was employed as the collision gas. Data acquisitions were performed using Analyst 1.6.2 software (Applied Biosystems). Multiquant software (Applied Biosystems) was used to quantify all metabolites.