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A highly efficient, high-throughput lipidomics platform for the quantitative detection of eicosanoids in human whole blood

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ABSTRACT

We have developed an ultra-performance liquid chromatography–multiple reaction monitoring/mass spectrometry (UPLC–MRM/MS)-based, high-content, high-throughput platform that enables simultaneous profiling of multiple lipids produced ex vivo in human whole blood (HWB) on treatment with calcium ionophore and its modulation with pharmacological agents. HWB samples were processed in a 96-well plate format compatible with high-throughput sample processing instrumentation. We employed a scheduled MRM (sMRM) method, with a triple–quadrupole mass spectrometer coupled to a UPLC system, to measure absolute amounts of 122 distinct eicosanoids using deuterated internal standards. In a 6.5-min run, we resolved and detected with high sensitivity (lower limit of quantification in the range of 0.4–460 pg) all targeted analytes from a very small HWB sample (2.5 μl). Approximately 90% of the analytes exhibited a dynamic range exceeding 1000. We also developed a tailored software package that dramatically sped up the overall data quantification and analysis process with superior consistency and accuracy. Matrix effects from HWB and precision of the calibration curve were evaluated using this newly developed automation tool. This platform was successfully applied to the global quantification of changes on all 122 eicosanoids in HWB samples from healthy donors in response to calcium ionophore stimulation.

Arachidonic acid (AA)[2] produced from phospholipids by cytosolic phospholipase A_2 ($cPLA_2$) is a gateway metabolic intermediate for the production of multiple bioactive lipids [1]. Those lipids, named eicosanoids, have been the subject of intense research over recent years due to their signaling functions in virtually every organ system and physiological processes [2]. In particular, leukotrienes and prostaglandins, produced via the 5-lipoxygenase (5-LO) and cyclooxygenase (COX) pathways, respectively, play major roles in the initiation and maintenance of inflammatory responses leading to multiple diseases [3], including asthma and atopic reactions as well as cardiovascular disease and cancer [4,5]. Multiple therapeutic agents ranging from preclinical research to clinical assets specifically target gene products required for leukotriene and prostaglandin biosynthesis and signaling, demonstrating the critical importance of the eicosanome in the modulation of proinflammatory and autoimmune responses [6].

The first committed step in leukotriene biosynthesis consists in the sequential conversion of AA to 5-(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HpETE) and then leukotriene A_4 (LTA_4) [7–10] by the interaction of 5-LO with the 5-LO-activating protein (FLAP). LTA_4 is then hydrolyzed to LTB_4 by the LTA_4 hydrolase, or conjugated with reduced glutathione via the concerted action of LTC_4 synthase and FLAP, to generate the cysteinyl leukotriene (CysLT) LTC_4, which is then exported from producing cells and converted by the action of extracellular peptidases to LTD_4 and the terminal product LTE_4 [11]. Both LTB_4 and CysLTs act as potent chemoattractants and leukocyte-activating mediators and are well-established biomarkers for the monitoring of the pharmacological

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[2] *Abbreviations used:* AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; 5-LO, 5-lipoxygenase; COX, cyclooxygenase; 5-HpETE, 5-(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LTA_4, leukotriene A_4; FLAP, 5-LO-activating protein; CysLT, cysteinyl leukotriene; LC, liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; HWB, human whole blood; IS, internal standard; SPE, solid phase extraction; UPLC, ultra-performance liquid chromatography; sMRM, scheduled multiple reaction monitoring; DP, declustering potential; CE, collision energy; RE, relative error; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; HPLC, high-performance liquid chromatography; TIC, total ion chromatogram; XIC, extracted ion chromatogram.

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activity of drug candidates for immune disease indications [12–14]. Eicosanoid biosynthesis and associated autocrine and paracrine signaling are tightly controlled processes. Inhibition of prostaglandin and/or leukotriene biosynthetic pathways results in compensatory changes in alternative biosynthetic pathways (also known as "shunting") [15,16].

Frustratingly, analytical methods at hand allow for the detection of only a handful of analytes derived from AA, precluding complete assessment of global changes in prostanoid pathways and pharmacological on- and off-target activities. In an effort to address this lack of appropriate methods to explore eicosanome pharmacology, we have developed a high-throughput liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based platform to globally profile 122 lipids covering the majority of eicosanoids in the AA metabolism network. Previously, Dumlao et al. [17] described an LC/MS (mass spectrometry) method to quantify eicosanoids in cell cultures and tissue samples. Unfortunately, the throughput of that assay does not address the need for large-scale analysis, which is required in clinical and preclinical applications where a sensitive, accurate, and fast-turnaround methodology for the determination of these lipids in human whole blood (HWB) is of essence. In the current article, we describe a validated platform for both experimental data acquisition and their subsequent analysis, representing a major improvement in the application of LC/MS-based lipidomics to pharmacology and drug discovery.

Materials and methods

Reagents and materials

In total, 132 AA-derived eicosanoids and 28 deuterium-labeled internal standards (ISs) (see Supplementary Table 1 in supplementary material; due to the limitation of the chromatographic separation, only 122 can be distinctively quantified) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Calcium ionophore A23187 was purchased from Sigma–Aldrich (St. Louis, MO, USA). RPMI-1640 medium was purchased from Thermo Scientific (Rockford, IL, USA). HWB samples were obtained from the Normal Blood Donor Service at the Scripps Research Institute (La Jolla, CA, USA).

Sample preparation

HWB samples were diluted 1:1 with RPMI-1640 medium (+25 mM Hepes and L-glutamine) and dispensed into 96-well round-bottom plates at 200 μl/well. Calcium ionophore A23187 (final concentration 30 μM) was added, followed by incubation at 37 °C for 30 min. Plates were centrifuged at 1300 rpm for 10 min, and supernatants were transferred into 2-ml 96-well deep plates. Isotope-labeled ISs (1 ng each) were added to supernatants immediately, and the mixtures were diluted with 10% MeOH in H_2O to 1 ml, followed by solid phase extraction (SPE) using Strata-X 33-μm polymeric reversed phase 96-well plates (60 mg/well, Phenomenex, Torrance, CA, USA) pretreated with MeOH and equilibrated with H_2O. After sample application, extraction plates were washed with 10% MeOH. The lipids were then eluted with 1 ml of MeOH, followed by SpeedVac (GenVac) drying. Dried samples were stored at −80 °C until analysis.

Preparation of standards for calibration curves

The combined stock solution of standards (132 lipids) was prepared by adding 10 μg of each lipid to a glass tube, bringing the total volume to 10 ml with MeOH to obtain a final concentration of 1 μg/ml for each component. The stock solution was then serially diluted with MeOH in half-log steps from 300 to 0.1 ng/ml. The combined stock solution of ISs was prepared by adding 5 μg of each lipid to a siliconized glass tube, bringing the total volume to 100 ml with MeOH to obtain a final concentration of 50 ng/ml for each standard. A 20-μl aliquot of this stock solution was added to each 200 μl of HWB sample as well as to 40 μl of each serial dilution of the standard stock solution. Standards were extracted via SPE and dried exactly as described for HWB samples. Stock solutions were stored at −80 °C until analysis.

Chromatography conditions

Both HWB samples and standards were resuspended in mobile phase A (see below) mixed 1:1 with MeOH. A 5-μl sample aliquot was injected into an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). Lipids were separated on a Kinetex C18 column (150 × 2.1 mm, 1.7 μm particle size, Phenomenex) using a flow rate of 400 μl/min at 50 °C. Lipids were eluted using mobile phases consisting of H_2O/acetonitrile/acetic acid (70:30:0.1, v/v/v, mobile phase A) and isopropanol/acetonitrile/acetic acid (50:50:0.02, v/v/v, mobile phase B). The gradient program employed was as follows (given as percentages of mobile phase A): 0 to 0.24 min, 100%; 0.6 min, 70%; 2.75 min, 55%; 3.25 min, 40%; 4.5 min, 25%; 4.62 min, 5%; 5.2 min, 5%; 5.3 min, 100%; 6.5 min, 100%. The gradient was linear between each stated time point.

MS conditions

MS detection was performed using an AB SCIEX API 4000 system (AB SCIEX, Foster City, CA, USA) equipped with a Turbo IonSpray source, and data acquisition software was Analyst version 1.5. Quantification was performed via scheduled multiple reaction monitoring (sMRM) in negative ion mode employing the following parameters: −4500 V ion spray voltage, 20 psi curtain gas, 30 psi ion source gas 1 (GS1), 30 psi ion source gas 2 (GS2), 6 psi collision gas (CAD), −10 V entrance potential (EP), −10 V collision cell exit potential (CXP), 600 °C, and 20-s MRM detection window. The optimized MRM fragmentation transitions and parameters for declustering potential (DP) and collision energy (CE) for each lipid are listed in Supplementary Table 1. In addition, the lipids are sorted by their pathways, derived fatty acids, and branch enzymes to emphasize their structural similarities and retention time overlaps.

Quantification and validation

Quantification was performed using a stable isotope dilution technique where the signature transition of each lipid versus IS was monitored on a 9-point calibration curve. This method was validated using an in-house developed R-based software package.

Table 1
List of lipid molecules with concentrations below the LLOD in blank HWB.

PGD_2	2,3-dinor-11β $PGF_{2\alpha}$	7-HDoHE
PGA_2	8,9-DHET	20oh LTB_4
LTB_4	8-HEPE	15d-PGJ_2
TXB_3	11-HETE	12-HEPE
12 epi LTB_4	17-HETE	18-HEPE
PD1	15-HETγE	15d-PGD_2
Tetranor 12-HETE	Bicyclo PGE_2	6t,12epi LTB_4
13-HOTγEg	12oxo-LTB_4	15k $PGF_{1\alpha}$
11-HEPE	8,15-diHETE	20cooh LTB_4
5-oxoETE	6t LTB_4	6k PGE_1
17,18-EpETE	6k $PGF_{1\alpha}$	Δ17 6k-$PGF_{1\alpha}$
Dihomo $PGF_{2\alpha}$		