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High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines ☆

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ABSTRACT

Fatty acid-derived eicosanoids and N-acylethanolamines (NAE) are important bioactive lipid mediators involved in numerous biological processes including cell signaling and disease progression. To facilitate research on these lipid mediators, we have developed a targeted high-throughput mass spectrometric based methodology to monitor and quantitate both eicosanoids and NAEs, and can be analyzed separately or together in series. Each methodology utilizes scheduled multiple reaction monitoring (sMRM) pairs in conjunction with a 25 min reverse-phase HPLC separation. The eicosanoid methodology monitors 141 unique metabolites and quantitative amounts can be determined for over 100 of these metabolites against standards. The analysis covers eicosanoids generated from cycloxygenase, lipoxygenase, cytochrome P450 enzymes, and those generated from non-enzymatic pathways. The NAE analysis monitors 36 metabolites and quantitative amounts can be determined for 33 of these metabolites against standards. The NAE method contains metabolites derived from saturated fatty acids, unsaturated fatty acids, and eicosanoids. The lower limit of detection for eicosanoids ranges from 0.1 pg to 1 pg, while NAEs ranges from 0.1 pg to 1000 pg. The rationale and design of the methodology is discussed. This article is part of a Special Issue entitled Lipodomics and Imaging Mass Spectrometry.

1. Introduction

Eicosanoids and N-acylethanolamines (NAEs) are very important bioactive lipid molecules that signal numerous physiological processes [1,2]. Comprehensive metabolomic tools to study these lipids and their biological involvement have been challenging since lipids represent a very diverse group of molecules comprised of many different classes and subclasses [3]. Additionally, each subclass has many distinct chemical features making it very difficult to monitor every type of lipid in a single analysis. LIPID Metabolites And Pathways Strategy (LIPID MAPS), an NIH-funded consortium was created to develop the infrastructure required including the specific methodology reported herein, an extensive lipid database, and to develop lipid standards (http://www.lipidmaps.org).

Eicosanoids and NAEs comprise two classes of important bioactive lipid signaling molecules that act through binding to their cognate receptors. Eicosanoids and NAEs play a key role in the innate immune system modulating inflammation, cellular recruitment, pain signaling, blood pressure response, and fever [1,2]. Additionally, many of these lipid metabolites have been implicated in a wide range of complex disease pathologies including cancer [4,5], atherosclerosis [6], rheumatoid arthritis [7], cystic fibrosis [8] and neurodegeneration [9].

Eicosanoids represent a large diverse group of lipids, in part, due to nonspecific synthases that can utilize different polyunsaturated fatty acids (PUFA) as substrates. The complexity of the eicosanoids is further complicated because these molecules can then act as substrates for other synthases, either through an intracellular or transcellular mechanism [2,10]. They are derived from polyunsaturated fatty acids (typically arachidonic acid) located at the sn-2 position of membrane glycerophospholipids liberated by enzymes with phospholipase A_2 (PLA_2) activity [11,12]. Group IVA $cPLA_2$ has been thought to be the main phospholipase responsible for the fatty acyls liberated from membrane phospholipids, while a recent report suggest that MAGL is the main lipase responsible for this activity in brain [13,14]. These free fatty acyls serve as substrates for cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes [1,2,15,16]. Most eicosanoid studies have just focused on prostaglandin E_2 (PGE_2) and the role it plays in inflammatory responses. Since many eicosanoids display redundant signaling properties, efforts have been made to study these mediators collectively.

NAEs represent a class of endogenous bioactive signaling lipids composed of a fatty acyl conjugated to ethanolamine through the amide bond [17,18]. The arachidonoyl species, anandamide (AEA),

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has received the most attention due to its anti-inflammatory action, nearly all endogenous fatty acyl species have been detected as ethanolamides *in vivo* [19]. A number of different pathways have been implicated in NAE formation, but the specific enzymes generating synaptic AEA formation remains unclear. To date, three main enzymatic routes for the formation of AEA from n-acyl phosphatidylethanolamines (NAPE) have been identified: (1) hydrolysis by a NAPE-specific phospholipase D (NAPE-PLD), (2) sequential phospholipase A/B activity of ABHD4, followed by a metal-dependent phosphodiesterase, and (3) sequential phospholipase C (PLC) and phosphatase activity. Inactivation of NAE signaling occurs primarily through their hydrolysis to form the free fatty acids and ethanolamine. Fatty acid acyl hydrolase (FAAH) has been identified as the primary means of AEA metabolism through a well-characterized serine hydrolase mechanism. Predominately, FAAH associates with intracellular membranes such as the endoplasmic reticulum and the Golgi apparatus. A great number of small molecules have been developed as selective inhibitors of FAAH [20–23], which has been a hotly pursued pharmacological target.

The quantitation of eicosanoids has been a challenging task due to the number of chemically different yet structurally similar metabolites (over 100). In the past, enzyme-linked immunosorbent assays (EIA) were employed to monitor a single eicosanoid species [24,25]. Due to the lack of commercially available antibodies and their non-specificity, this approach was severely limited. Additionally, the EIA approach lacked the robustness to perform large scale analyses. The technological advancements in mass spectrometry and its application to monitor eicosanoids have led to a robust foundation for eicosanoid research. Using gas chromatography mass spectrometry (GC/MS) allowed for many eicosanoids to be monitored simultaneously, however, a lone chemical derivatization agent was not suitable for all eicosanoids to be monitored in a single analysis [26]. Also, GC/MS was not suited for monitoring every type of eicosanoid species [27]. Electrospray-ionization tandem mass spectrometry (ESI-MS/MS), which does not require a prior derivatization step, has become a staple in eicosanoid biology since it was first employed by Margalit and colleagues for simultaneously monitoring 14 different eicosanoid species in a single analysis [28]. The number of distinct eicosanoids that can be monitored in a single analysis has been steadily increasing as more pure standards have become commercially available, as well as improvements in mass spectrometer hardware and data analysis software.

Previously, we reported on the use of GC/MS to analyze free fatty acids [29] and LC/MS/MS to analyze eicosanoids [30]. Our initial eicosanoid methodology was capable of monitoring 60 unique species in a single 16 min analysis. Since our initial report, significant improvements have been made which more than double the number of eicosanoids monitored (141) and quantified (100). Additionally, we have applied this technique toward a separate methodology capable of monitoring 36 NAE metabolites. Here, we present the design and rationale behind our high-throughput LC/MS/MS methodologies for monitoring and quantitating eicosanoid and NAE metabolites, and highlight the improvements made over our previous technique. Also, we provide an example of the application of our methodology.

2. Materials and methods

2.1. Sample preparation

The same sample preparation is used when analyzing eicosanoids or NAEs. All samples were resuspended in 1.0 ml of 10% methanol water (v/v). Tissue samples were subjected to sonication for 6 s to break up any connective tissue. Samples were spiked with 50 μL of a 50 pg/μL (2.5 ng total) deuterated internal standard solution. Lipid metabolites were extracted using Strata-X 33 u polymerized solid reverse phase extraction columns (Phenomenex, CA; cat # 8B-S100-UBJ) as indicated by manufacturer's directions. Briefly, columns were washed with 3.5 mL of 100% methanol, followed by 3.5 mL of water before samples were extracted. Samples were washed with 3.5 mL of 10% methanol to remove non-specific binding metabolites. Lipids were eluted into 1.0 mL of methanol and stored at −80C ° before being analyzed to prevent metabolite degradation.

2.2. High performance liquid chromatography (HPLC)

Both eicosanoid and NAE samples are subjected to the same treatment for HPLC analysis, although different buffer systems are employed. Extracted samples in 100% methanol are lyophilized to dryness using a Speed-Vac concentrator (Savant, model # SC110-120), and resuspended in 90 μL of their respective solvent A. For the eicosanoids methodology, solvent A_{EICOS} consists of water–acetonitrile–acetic acid (70:30:0.02; v/v/v), while solvent B_{EICOS} consists of acetonitrile–isopropyl alcohol (50:50, v/v). For the NAE methodology, solvent A_{NAE} consists of water–acetonitrile–acetic acid ((70:30:0.1; v/v/v) + 1 g/L ammonium acetate) and solvent B_{NAE} consists of acetonitrile–isopropyl alcohol–acetic acid ((45:45:10; v/v/v) + 1 g/L ammonium acetate). Samples can be subjected to each methodology alone or analyzed together in series. After a sample has been analyzed for eicosanoids, the presence of NAEs can be determined with the addition of 10 μL of a water–acetonitrile–acetic acid (70:30:0.1; v/v/v) + 5 g/L ammonium acetate solution that makes the sample suitable to be analyzed in positive-ion mode.

An aliquot of 40 μL of sample injected on the HPLC system was the standard amount routinely analyzed. Eicosanoids were separated on a Synergi reverse-phase C18 column (2.1 × 250 mm; Phenomenex, CA) and NAEs were separated on a Luna reverse-phase C8 column (2.1 mm · 250 mm, Phenomenex, CA). Both sample types use a flow rate of 300 μL/min at 50 °C. The gradient program used to separate

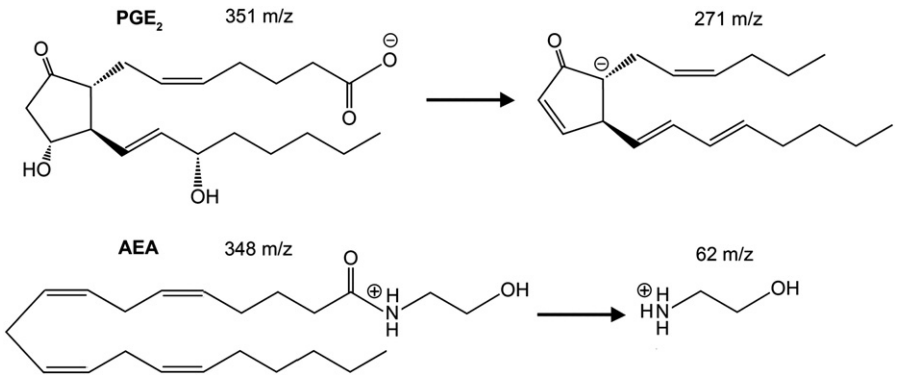

Fig. 1. The structures of the parent and daughter ions for PGE_2 and AEA used in this method.