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Original Research Article

Validation of a tandem mass spectrometry method using combined extraction of 37 oxylipins and 14 endocannabinoid-related compounds including prostamides from biological matrices

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ABSTRACT

There is a clinical need for more relevant coverage of bioactive lipids using smaller sample volumes. Therefore, we have validated a tandem mass spectrometry method for combined solid phase extraction of 37 compounds in the oxylipin (OxL) and 14 in the endocannabinoid (eCB) metabolome, as well as prostamides. The limits of quantification (LOQ) for compounds in the eCB metabolome were in the range 0.5–1000 fg on column, intraday accuracy and precision ranges (%) were 83–125 and 0.3–17, respectively, and interday accuracy and precision ranges (%) were 80–119 and 1.2–20, respectively, dependent upon the compound and the concentration studied. Corresponding values for OxL were 0.5 fg–4.2 pg on column (LOQ), 85–115% (inter- and intraday accuracy) and <5% (precision). The combined extraction method was successfully applied to tissues, cell extracts, human plasma and milk samples. A deeper study of levels in elk, pig and cow brain, as well as cow heart and liver revealed tissue and species-specific elevation of eicosanoids: arachidonate diols, 20-HETE and 12(S)-HEPE (cow liver), LTB_4 (cow brain), and monohydroxy metabolites (HETEs), epoxides and 5-oxo-ETE in elk brain, which might be caused by factors of stress and/or post-mortem reactions in the tissues.

1. Introduction

Oxylipins (OxL) and endocannabinoids (eCB) are families of bioactive lipids derived from a number of polyunsaturated fatty acids such as ω6 arachidonic acid (20:4n6) and ω3 α-linoleic acid (18:3n3). Oxylipins are biosynthesized mainly via three enzymatic pathways ([1], Fig. S1, Supporting Information): cyclooxygenase (COX, e.g. prostaglandins and thromboxanes), lipoxygenase (LOX, e.g. leukotrienes), and cytochrome P450 (CYP, e.g. epoxides and downstream diols synthesized by soluble epoxide hydrolase), as well as by non-enzymatic oxidation. The most well studied endogenous eCB, 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl ethanolamine (AEA, anandamide), are able to bind to and activate cannabinoid receptors (CB_1 and CB_2) [2]. Several other fatty acid amides and glycerol esters in the eCB metabolome have been reported to activate CB receptors or to act as entourage compounds [3].

Bioactive lipids in the OxL and eCB metabolomes are distributed in very different levels across tissues, biofluids and species [3,4]. A variety of physiological processes have been associated with the modulation of these levels, for instance inflammation, pain and appetite [5–7]. Interrelations between the OxL and eCB metabolomes have recently attracted attention, not only with regard to their involvement in common physiological processes, but also in terms of their participation in similar enzymatic pathways [8]. Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are the main catabolic enzymes for eCB, but studies have revealed a key role of COX-2 activity (especially if FAAH is inhibited) in the presence of anandamide to generate prostamides (prostaglandin ethanolamides) [9,10]. Therefore, a deeper understanding of the eCB, OxL and COX-2 interplay requires a broader lipidomic profiling of the OxL and eCB metabolomes with the addition of prostamides in the panel of analytes.

Liquid–liquid extraction (LLE) [11] and solid phase extraction (SPE) [12] are the most widely used techniques to isolate eCB and OxL from biological matrices. Although for specific applications LLE has proven to be superior to SPE, in general SPE shows higher sensitivity and smaller sample volume are therefore required [13,14]. There are a few common SPE protocols [15–18] based on the cartridges type such as sorbent chemistry, particle size, solvents used for sample equilibration, washing and elution. The ideal situation is to utilize a protocol resulting in high extraction efficiency and

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minimal matrix interference, which is a difficult task when comprehensive methods are used with a large panel of analytes with similar chemical properties. Ostermann et al. [14] have made a thorough comparison of six published SPE protocols (including the protocol we follow in our lab – Waters OASIS-HBL-EA) and one LLE method for free oxylipin detection in human plasma. They found the Waters cartridge SepPak to be the best compromise between recovery of internal standards, free oxylipin extraction from samples and ion suppression effect. Moreover, the OASIS-HBL-EA cartridge facilitated good results with exception of ion suppression for the linoleate epoxide (9,10-EpOME-d_4) and 9-HODE-d_4. However, this kind of comparison is largely affected by the system and methodology used (eluents, gradient and ion source parameters) and cannot automatically be transferred to another setting.

A broad range of techniques have been employed for the separation, detection and quantification of bioactive lipids, for instance enzyme-linked immunosorbent assays [19], gas chromatography (GC) coupled to mass spectrometry (MS) [20], liquid chromatography (LC) coupled to ultraviolet (UV) detector, MS, or to tandem mass spectrometry (MS/MS) [3,15,16]. Currently, LC–MS/MS is the most powerful technique given its sensitivity and specificity. Several papers have recently been published within the field of bioactive lipid profiling [16,18,21–23]. Formerly, analytical methods were focused on compounds produced through a specific pathway (i.e. prostanoids), but the high interrelations of lipid metabolites with different origins sharing the same enzymatic cascade has prompted the development of more comprehensive analytical platforms covering a larger number of bioactive lipids. Nevertheless, it is highly challenging to develop comprehensive methods for simultaneous quantification of OxL and eCB. Besides the specifications related to each compound family, such as chemical similarities (there are a great number of isomers within each class) and chemical instability (especially for eCB [3]), also biologically low levels combined with large concentration ranges of different compounds present a challenge. Furthermore, OxL are more easily ionized in negative mode due to the free carboxylic acid moiety, while eCB and prostamides are more sensitive in positive mode. This constitutes a major drawback of one single LC–MS/MS run, since it requires switching ionization mode (negative/positive) leading to sensitivity loss [8]. However, it was successfully used to quantify 42 compounds in the OxL and eCB metabolomes in a rat model of osteoarthritis [8].

Our previous methods for profiling the OxL [24] and eCB [25] metabolome separately provided a starting point for further advancement to include a broader range of metabolites, e.g. prostamides, and to increase the sensitivity, with the goal of obtaining a more clinically relevant coverage of the bioactive lipids using smaller sample volumes. To that end, we have developed a method for high-throughput (one single extraction step) quantification of 14 compounds in the eCB metabolome (including two prostamides) and 37 compounds in the OxL metabolome. The method was validated and successfully applied to a variety of biological matrices such as tissues, cells, plasma and milk. Furthermore, tissue- and species-specific elevation of certain eicosanoids was revealed in a study of brain, liver and heart levels in cow, pig and elk.

2. Material and methods

2.1. Chemical and reagents

The following native and deuterated standards were purchased from Cayman Chemicals (Ann Arbor, MI, USA): AEA, 2-AG, PEA, OEA, DEA, NAGly, EPEA, DHEA, POEA, LEA, SEA, 2-LG, PGF_{2a}-EA, PGE_2-EA, $PGF_{2\alpha}$-EA-d_4, PGE_2-EA-d_4, AEA-d_8, 2-AG-d_8, OEA-d_4, $PGF_{2\alpha}$, PGE_2, TXB_2, PGD_2, 5(6)-EET, 8(9)-EET, 11(12)-EET, 14(15)-EET, 5,6-DHET, 8,9-DHET, 11,12-DHET, 14,15-DHET, 9(10)-EpOME, 12(13)-EpOME, 9(10)-DiHOME, 12(13)-DiHOME, 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 20-HETE, 9-HODE, 13-HODE, 15(S)-HETrE, 12-HEPE, 17-HDoHE, 5-oxo-ETE, 12-oxo-ETE, 15-oxo-ETE, 13-oxo-ODE, LTB_4, Resolvin D2, Resolvin D1, 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA), 12(13)-DiHOME-d_4, 12(13)-EpOME-d_4, 9-HODE-d_4, PGE_2-d_4 and TXB_2-d_4. 9,10,13-TriHOME and 9,12,13-TriHOME were obtained from Larodan (Sweden, Malmö). Acetonitrile (ACN) and methanol (MeOH) were from Merck (Darmstadt, Germany). Isopropanol was from VWR PROLABO (Fontenay-sous-Bois, France). Acetic acid was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Butylhydroxytoluene (BHT) was from Cayman Chemical and ethylenediaminetetraacetic acid (EDTA) from Fluka Analytical, Sigma–Aldrich (Buchs, Switzerland). Glycerol was from Fischer Scientific (Loughborough, UK). All solvents and chemicals were of HPLC grade or higher. Water was purified by a Milli-Q Gradient system (Millipore, Milford, MA, USA).

2.2. Preparation of native standards and deuterated internal standard curves

Analytical quantification standards were used as ready-made standard stock solutions or as solutions prepared from solid substances and stored at −80 °C. 2-AG and 2-LG were prepared and stored in ACN and the other standards were prepared and stored in ethanol. Final stock solution concentrations of native standards were: 250 μg/mL (2-AG, 2-LG, POEA and LEA), 125 μg/mL (OEA, PEA, AEA, DHEA, NAGly, DEA and EPEA), 83 μg/mL (SEA), 1 μg/mL ($PGF_{2\alpha}$-EA and PGE_2-EA) and 10 μg/mL (all OxL native standards). Stock solutions of internal standards (IS) were prepared to a final concentration of 40 μg/mL (2-AG-d_8), 10 μg/mL (AEA-d_8, OEA-d_4, $PGF_{2\alpha}$-EA-d_4, PGE_2-EA-d_4, 12(13)-EpOME-d_4, and 12(13)-DiHOME-d_4) and 5 μg/mL (9(S)-HODE-d_4, PGE_2-d_4 and TXB_2-d_4).

Stock solutions of eCB-related compounds were prepared fresh on a monthly basis and stored at −80 °C, while the same was done for OxL every 6 months. The stock solutions were further diluted in methanol at 10 different calibration levels (Tables S1 and S2, Supporting Information) and prepared fresh before analysis. To mimic the extraction of the endogenous compounds, deuterated compounds were used as IS and added to samples before extraction. For each native compound, a suitable IS was selected based on structural similarities (Table S3, Supporting Information), and consequently similar retention time. Recovery rates of each IS were calculated by adding the recovery standard CUDA in the last step to the LC vial (Table S4, Supporting Information).

2.3. Sample preparation

A variety of biological samples were included for validation purposes of the developed method. Tissue samples from offal after livestock slaughter performed by the same proficient person for all animals (independent from research) were collected from domestic (bovine and porcine) and wild (elk) animals. The elk (injured before slaughter) and cow were approximately 18 months old, while pigs were approximately 8 months old at the time of death. Sample collection was performed immediately for the pigs, approximately 8 h post-mortem for the cow, and 60 h post-mortem for the elk. All samples were frozen and stored at −80 °C until analysis. For all frozen tissue samples ($N = 3$ for each type), the weight (127 ± 8 mg for cow heart; 322 ± 9 mg for cow liver; 177 ± 16 mg for pig brain; 121 ± 4 mg for elk brain and 80.5 ± 3 mg for cow brain) was accurately measured before grounding in 1 mL methanol, containing 10 μL antioxidant