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A stable method for routine analysis of oxylipins from dried blood spots using ultra-high performance liquid chromatography–tandem mass spectrometry

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SUMMARY

Oxylipins are biologically important lipid mediators that are derived enzymatically from polyunsaturated fatty acids (PUFA) and have a major role in regulating inflammatory processes. The currently available methods for measuring oxylipins from human biological samples have limitations, which restricts their use in large studies. We have developed a novel method for measuring 21 oxylipins from dried blood spot (DBS) using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) and stable isotope dilution analysis. Our new method is reproducible and precise and enables the high throughput analysis and quantitation of bioactive oxylipins in small volumes of blood. In the future, this new method can be readily applied to measure oxylipins in large studies.

Abstract

Oxylipins are downstream lipid mediators enzymatically-produced from polyunsaturated fatty acids (PUFA) that are implicated as the biological effectors of these fatty acids. Recently reported methods for the quantitation of oxylipins require complex extraction procedures. In this study, we report the development and validation of a novel system for the quantitation of 21 individual oxylipins from a dried blood spot (DBS) using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) and stable isotope dilution analysis. Linearity and precision of the method were determined and the stabilities of the 12 most abundant oxylipins were tested during 2 months of storage at room temperature, after being spiked into blood and prepared as DBS on PUFAcoat™ paper. Responses were linear across the concentration range analysed for all oxylipins (r^2 values ranged from 0.953 to 0.998). Intra–day and inter–day variations were ≤16% for all oxylipins. Recovery of oxylipins from the DBS ranged from 80 – 115%. The 12 spiked oxylipins were stable for 2 months when stored as DBS at room temperature. Our method is reproducible and precise, and provides the opportunity to accurately quantitate these oxylipins in a small sample volume.

Abbreviations: BHT, 3,5–di–*tert*–4–butylhydroxytoluene; CXP, cell exit potential; CV, coefficient variation; CE, collision energy; DP, declustering potential; 9,10–diHOME, 9,10–dihydroxyoctadecenoic acid; DBS, dried blood spots; 16(17)–EpDPA, 16,17–epoxydocosapentaenoic acid; 19(20)–EpDPA, 19,20–epoxydocosapentaenoic acid; 5(6)–EET, 5,6–epoxyeicosatrienoic acid; 8(9)–EET, 8,9–epoxyeicosatrienoic acid; 11(12)–EET, 11,(12)–epoxyeicosatrienoic acid; 14(15)–EpETE, 14,15–epoxyeicosatetraenoic acid; 9(10)–EpOME, 9(10)–epoxyoctadecaenoic acid; 4–HDHA, 4–hydroxydocosahexaenoic acid; 5–HETE, 5–hydroxyeicosatetraenoic acid; 8–HETE, 8–hydroxyeicosatetraenoic acid; 9–HETE, 9–hydroxyeicosatetraenoic acid; 11–HETE, 11–hydroxyeicosatetraenoic acid; 12–HETE, 12–hydroxyeicosatetraenoic acid; 15–HETE, 15–hydroxyeicosatetraenoic acid; 13–HODE, 13–hydroxyoctadecadienoic acid; 9*S*–HOTre, 9*S*–hydroxyoctadecatrienoic acid; LTB_4, leukotriene B_4; LOD, limit of detection; LOQ, limit of quantitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXA_4, lipoxin A_4; MRM, multiple reaction monitoring; 9–oxoODE, 9–oxooctadecadienoic acid; 13–oxoODE, 13–oxooctadecadienoic acid; PUFA, polyunsaturated fatty acids; SEM, standard error of mean; UHPLC–MS/MS, ultra-high performance liquid chromatography–tandem mass spectrometry

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1. Introduction

Oxylipins derived from the enzymatic oxidation of polyunsaturated fatty acids (PUFA) are potent downstream lipid mediators that play a major role in regulation of inflammatory and immune processes in the body [1,2]. Oxylipins are produced via three enzymatic pathways (cyclooxygenase, lipoxygenase, and cytochrome P450) or via non–enzymatic autoxidation [3]. Recent evidence suggests that increased levels of some oxylipins, particularly the pro–inflammatory oxylipins derived from omega–6 PUFA, are associated with diseases including diabetes [4], kidney disease [5], rheumatoid arthritis [6] and atherosclerosis [7]. Conversely, oxylipins derived from omega–3 PUFA have been reported to supress inflammation and contribute to the resolution of immune responses [8,9]. However, understanding of this complex system and the roles of the respective oxylipins in human health remains limited, largely due to the current lack of cost–effective and reliable methods for their quantitation.

The measurement of oxylipins in biological fluids is complicated by both their low abundance and inherent instability. In addition, many of the oxylipins, particularly those derived from the same parent fatty acids, have very similar structures but different bioactivities, thus need to be separated and quantified individually to ascertain their specific role [10]. The concentrations of oxylipins in biological samples have previously been measured using radioimmunoassay [11] and enzyme–linked immunosorbent assay [12]. However, these methods are impractical and not cost-effective due to their inability to quantify more than one oxylipin per assay. Furthermore, the stability of the parent free fatty acids and the oxylipins themselves during sample extraction and subsequent analytical steps in the process is questionable.

More recently, approaches have been developed for the quantitation of oxylipins by GC–MS [13,14] and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [15–18]. These have the advantage of enabling the simultaneous measurement of multiple oxylipins from the same sample. However, current methods require large sample volumes and labour–intensive sample preparation approaches, including solid–phase extraction and chemical derivatisation, which make these methods impractical for large population studies. There is also the potential for oxidation of compounds to occur during the long sample preparation processes.

Methods that enable the accurate and reliable measurement of a suite of oxylipins in small volumes of blood are still required, so that oxylipin profiles can be efficiently analysed in large clinical trials and related to clinical outcomes. Dried blood spot (DBS) technology provides a convenient blood sampling approach that is well–established, predominantly for neonatal screening but also for other clinical chemistry applications, especially in conjunction with mass spectrometry analyses [19]. A particular advantage of this approach is that only minimal blood volumes are required and drying spots on paper helps preserve analytes, even during storage at room temperature [20]. Indeed, a modified DBS system, called PUFAcoat™ paper, has been shown to be effective at inhibiting degradation of fatty acids for at least 2 months when stored at room temperature [21].

The current study reports the development of a robust, high–throughput method using stable isotope dilution analysis for the simultaneous measurement by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) of omega–3 and omega–6 PUFA–derived oxylipins from DBS samples extracted from PUFAcoat™ paper.

2. Materials and methods

2.1. Standards and reagents

LC–MS grade methanol and acetonitrile were purchased from Merck (VIC, Australia). A.C.S grade formic acid and analytical standard grade 3,5–di–*tert*–4–butylhydroxytoluene (BHT) were obtained from Sigma–Aldrich (NSW, Australia). Analytical standards (≥98% purity), 9*S*–hydroxyoctadecatrienoic acid (9*S*–HOTre), 14,15–epoxyeicosatetraenoic acid (14(15)–EpETE), 4–hydroxydocosahexaenoic acid (4–HDHA), 16,17–epoxydocosapentaenoic acid (16(17)–EpDPA), 19,20–epoxydocosapentaenoic acid (19(20)–EpDPA), 13–hydroxyoctadecadienoic acid (13–HODE), 9–oxooctadecadienoic acid (9–oxoODE), 13–oxooctadecadienoic acid (13–oxoODE), 9,10–dihydroxyoctadecenoic acid (9, 10–diHOME), 9(10)–epoxyoctadecaenoic acid (9(10)–EpOME), 5–hydroxyicosatetraenoic acid (5–HETE), 8–hydroxyeicosatetraenoic acid (8–HETE), 9–hydroxyeicosatetraenoic acid (9–HETE), 11–hydroxyeicosatetraenoic acid (11–HETE), 12–hydroxyeicosatetraenoic acid (12–HETE), 15–hydroxyeicosatetraenoic acid (15–HETE), 5,6–epoxyeicosatrienoic acid (5(6)–EET), 8,9–epoxyeicosatrienoic acid (8(9)–EET), 11,(12)–epoxyeicosatrienoic acid (11(12)–EET), leukotriene B_4 (LTB_4), lipoxin A_4 (LXA_4) and deuterated internal standards (≥99% purity) d_4–13–HODE, d_4–LTB_4 and d_8–12–HETE, were purchased from Cayman Chemical Company (Michigan, USA).

2.2. Subjects and sampling

Ethical approval for the collection of venous and capillary blood for this study was obtained from The University of Adelaide Human Research Ethics Committee (H–2016–088). All participants provided written informed consent prior to blood collection. For each participant, 40 μL of venous blood was spotted onto PUFAcoat paper™ (Xerion Limited, VIC, Australia) and air–dried at room temperature for 3 h in the dark. Oxylipin levels in whole blood extracted from DBS were determined by UHPLC–MS/MS.

2.3. UHPLC–MS/MS method for the assessment of oxylipins from DBS

2.3.1. DBS sample extraction

Lipid extraction was conducted according to a previously detailed method [22]. Briefly, a 6 mm disc was taken from each DBS and placed in a 96–well plate. Extraction solvent (150 μL of 80% aqueous methanol) containing deuterated internal standard mix (0.01 ng/μL of d_4–13–HODE, d_4–LTB_4 and d_8–12–HETE, stock prepared in methanol) was added to each well, and the plate was covered and gently shaken on a plate shaker for 30 min at room temperature. The extract from each well was transferred to a fresh well in a new plate, sealed and analysed by UHPLC–MS/MS.

2.3.2. Instrumentation

Analyses were conducted with an Agilent 1290 Infinity LC system (Agilent Technologies, VIC, Australia) equipped with a binary pump and thermostated autosampler held at 4 °C, connected to a 5500 triple quadrupole mass spectrometer (AB Sciex, VIC, Australia), using electrospray ionisation in negative mode. The mass spectrometer had the same conditions as described previously [22] but with parameters optimised for each analyte using infusion experiments (Table 1).

2.4. UHPLC–MS/MS method validation

2.4.1. Preparation of standard curves for validation and batch analysis

Solutions were prepared with methanol containing 0.005% BHT. A stock solution of mixed oxylipin standards (consisting of 9*S*–HOTre, 14(15)–EpETE, 4–HDHA, 16(17)–EpDPA, 19(20)–EpDPA, 13–HODE, 9–oxoODE, 13–oxoODE, 9,10–diHOME, 5–HETE, 8–HETE, 9–HETE, 11–HETE, 12–HETE, 15–HETE, 5(6)–EET, 8(9)–EET, 11(12)–EET, LTB_4 and LXA_4,) was diluted volumetrically to prepare five working solutions, which were further diluted to obtain nine calibration standards. All prepared standards were stored at −80 °C in glass vials with screw caps (Adelab Scientific, SA, Australia) sealed with parafilm (Bemis Flexible Packaging, QLD, Australia).

Each calibration standard solution (10 μL) was spiked into 490 μL of