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Oxidized fatty acid analysis by charge-switch derivatization, selected reaction monitoring, and accurate mass quantitation

Xinping Liu [a], Sung Ho Moon [a], David J. Mancuso [a], Christopher M. Jenkins [a], Shaoping Guan [a], Harold F. Sims [a], Richard W. Gross [a,b,c,*]

[a] Department of Medicine, Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA
[b] Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110, USA
[c] Department of Chemistry, Washington University, St. Louis, MO 63130, USA

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ABSTRACT

A highly sensitive, specific, and robust method for the analysis of oxidized metabolites of linoleic acid (LA), arachidonic acid (AA), and docosahexaenoic acid (DHA) was developed using charge-switch derivatization, liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI MS/MS) with selected reaction monitoring (SRM) and quantitation by high mass accuracy analysis of product ions, thereby minimizing interferences from contaminating ions. Charge-switch derivatization of LA, AA, and DHA metabolites with *N*-(4-aminomethylphenyl)-pyridinium resulted in a 10- to 30-fold increase in ionization efficiency. Improved quantitation was accompanied by decreased false positive interferences through accurate mass measurements of diagnostic product ions during SRM transitions by ratiometric comparisons with stable isotope internal standards. The limits of quantitation were between 0.05 and 6.0 pg, with a dynamic range of 3 to 4 orders of magnitude (correlation coefficient $r^2 > 0.99$). This approach was used to quantitate the levels of representative fatty acid metabolites from wild-type (WT) and iPLA$_2\gamma^{-/-}$ mouse liver identifying the role of iPLA$_2\gamma$ in hepatic lipid second messenger production. Collectively, these results demonstrate the utility of high mass accuracy product ion analysis in conjunction with charge-switch derivatization for the highly specific quantitation of diminutive amounts of LA, AA, and DHA metabolites in biologic systems.

Polyunsaturated fatty acids such as arachidonic acid (AA,[1] 20:4n-6), docosahexaenoic acid (DHA, 22:6n-3), and linoleic acid (LA, 18:2n-6) are essential components of mammalian membranes that serve multiple roles in cellular function [1–5]. Typically, these polyunsaturated fatty acids are stored in an inactive form esterified to membrane phospholipids and are released during cellular activation by a wide variety of phospholipases to facilitate cellular responses to external perturbations [6–10]. The precise complement of lipid second messengers in each cell type is determined, in large part, by the integrated array of cell type-specific phospholipases

* Corresponding author at: Department of Medicine, Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA. Fax: +1 314 362 1402.
E-mail address: rgross@wustl.edu (R.W. Gross).

[1] *Abbreviations used:* AA, arachidonic acid; DHA, docosahexaenoic acid; LA, linoleic acid; LC, liquid chromatography; ESI, electrospray ionization; SRM, selected reaction monitoring; AMPP, *N*-(4-aminomethylphenyl)-pyridinium; S/N, signal/noise; WT, wild-type; TXB$_2$, thromboxane B$_2$; 6keto-PGF$_{1\alpha}$, 6-keto prostaglandin F$_{1\alpha}$; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$; PGF$_{1\alpha}$, prostaglandin F$_{1\alpha}$; PGE$_2$, prostaglandin E$_2$; PGE$_1$, prostaglandin E$_1$; PGD$_2$, prostaglandin D$_2$; LTB$_4$, leukotriene B$_4$; 5-HETE, 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 8-HETE, 8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 20-HETE, 20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid; 5,6-EET, 5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid; 8,9-EET, 8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid; 11,12-EET, 11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid; 14,15-EET, 14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid; 9-HODE, 9-hydroxy-10E,12Z-octadecadienoic acid; 13-HODE, 13-hydroxy-9Z,11E-octadecadienoic acid; 9-OxoODE, 9-oxo-10E,12Z-octadecadienoic acid; 13-OxoODE, 13-oxo-9Z,11E-octadecadienoic acid; 9,10-DiHOME, 9(10)-dihydroxy-12Z-octadecenoic acid; 12,13-DiHOME, 12,13-dihydroxy-9Z-octadecenoic acid; 9(10)-EpOME, 9(10)-epoxy-12Z-octadecenoic acid; 12(13)-EpOME, 12(13)epoxy-9Z-octadecenoic acid; 4-HDoHE, 4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid; 7-HDoHE, 7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-docosahexaenoic acid; 8-HDoHE, 8-hydroxy-4Z,6E,10Z,13Z,16Z,19Z-docosahexaenoic acid; 10-HDoHE, 10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-docosahexaenoic acid; 11-HDoHE, 11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid; 13-HDoHE, 13-hydroxy-4Z,7Z,10Z,14E,16Z,19Z-docosahexaenoic acid; 14-HDoHE, 14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid; 16-HDoHE, 16-hydroxy-4Z,7Z,10Z,13Z,17E,19Z-docosahexaenoic acid; 19,20-DiHDPA, 19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid; 10,17-DiHDoHE, 10,17-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid; RvD1, 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid; RvD2, 7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; C_5H_{10}, 2-pentene.

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and the kinetic flux through specific downstream oxidative pathways tailored to the function of each cell type to generate a highly specific array of oxidized polyunsaturated signaling metabolites. Prominent examples of oxidative enzymes leading to the generation of signaling fatty acids include the cyclooxygenases [11,12], lipoxygenases [13–15], and P450 monooxygenases [16,17]. Many hundreds to thousands of oxidized fatty acid metabolites are generated in mammalian cells that coordinate physiologic signaling processes and cellular adaptation in health but are subject to maladaptive alterations in disease processes promoting disease progression [18–20]. Accordingly, a major goal of lipid second messenger research is the identification of the diversity of oxidized fatty acids in biologic systems, quantitation of alterations in their amounts during physiologic and pathologic perturbations, and mechanistic identification of their roles in disease processes (see, e.g., Refs. [21–29]).

Recent approaches for the identification and quantitation of the diversity of oxidized fatty acids in biologic systems have largely relied on liquid chromatographic separation followed by electrospray ionization (LC–ESI) in the negative ion mode and selected reaction monitoring (SRM) [30–38]. However, ionization in the negative ion mode is inefficient in comparison with ionization in the positive ion mode. Although oxidized fatty acids in inflammatory cells, abscesses, exudates, or inflammatory fluids can be measured using negative mode ionization [39–51], the much lower abundance of oxidized fatty acid signaling metabolites that regulate cellular function in muscle, liver, brain, and other solid organs is typically 20- to 2000-fold lower than that present in inflammatory cells or fluids. Thus, the substantially lower ionization efficiency in the negative ion mode has placed severe limitations on the accurate identification and quantitation of oxidized fatty acids in non-inflammatory cells and tissues.

The inherent limitations in sensitivity in the analysis of oxidized fatty acids in the negative ion mode have been appreciated previously. Accordingly, multiple charge-switch chemical derivatization procedures to improve the ionization efficiency for detection of oxidized fatty acid signaling metabolites have been developed using positive ion mode ionization (see, e.g., Refs. [52–59]). Examples of charge-switch derivatization of the carboxylic acid group include derivatization with tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide [52], cholamine [53], 2-bromo-1-methylpyridinium iodide, and 3-carbinol-1-methylpyridinium iodide [54], *p*-dimethylaminophenacyl bromide [55], 2-hydrazinopyridine, and 2-picolylamine, among others [56–58]. However, these approaches are suboptimal for analysis of oxidized fatty acid moieties using ESI due to the undesirable fragmentation of the charge-switch tag in many cases. This weakness was recognized by Gelb and coworkers, who used molecular orbital calculations to develop a novel charge-switch reagent, *N*-(4-aminomethylphenyl)-pyridinium (AMPP) [59], which possessed the desired charge-switch mediated increase in signal/noise (S/N) and markedly improved the generation of informative fragment ions. Through using high mass accuracy product ion analysis in conjunction with AMPP charge-switch derivatization, we demonstrate the sensitivity and specificity of this method for the detection and quantitation of oxidized LA, AA, and DHA metabolites in the high attomole and low femtomole range. Furthermore, high mass accuracy analysis of diagnostic product ions collected during SRM dramatically decreases false positive interferences that compromise identification and quantitation of the targeted analytes that would otherwise go undetected or overestimated using traditional isobaric SRM analyses. The utility of this approach in biologic tissues was examined in hepatic tissue from wild-type (WT) and $iPLA_2\gamma^{-/-}$ mice that we generated and characterized previously [60–62]. Collectively, this approach provides a facile method to dramatically increase the sensitivity and accuracy of analysis of diminutive amounts of oxidized fatty acids in biologic samples while simultaneously decreasing false positive interferences through the use of high mass accuracy product ion analysis during monitored SRM transitions.

Materials andmethods

Materials

The following standards from Cayman Chemical (Ann Arbor, MI, USA) were used: thromboxane B_2 (TXB_2), TXB_2-d_4, 6-keto prostaglandin $F_{1\alpha}$ (6keto-$PGF_{1\alpha}$), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$), prostaglandin E_2 (PGE_2), PGE_2-d_4, prostaglandin E_1 (PGE_1), prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4), LTB_4-d_4, 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid (8-HETE), 11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HETE), 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE), 12-HETE-d_8, 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE), 20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (20-HETE), 5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-EET), 8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-EET), 11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET), 14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET), AA, 9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE), 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), 13-HODE-d_4, 9-oxo-10E,12Z-octadecadienoic acid (9-OxoODE), 13-oxo-9Z,11E-octadecadienoic acid (13-OxoODE), 9(10)-dihydroxy-12Z-octadecenoic acid (9,10-DiHOME), 9,10-DiHOME-d_4, 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME), 12,13-DiHOME-d_4, 9(10)-epoxy-12Z-octadecenoic acid [9(10)-EpOME], 12(13)epoxy-9Z-octadecenoic acid [12(13)-EpOME], LA, 4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid (4-HDoHE), 7-hydroxy-4Z,8E,10Z,13Z,16Z,19Z-docosahexaenoic acid (7-HDoHE), 8-hydroxy-4Z,6E,10Z,13Z,16Z,19Z-docosahexaenoic acid (8-HDoHE), 10-hydroxy-4Z,7Z,11E,13Z,16Z,19Z-docosahexaenoic acid (10-HDoHE), 11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid (11-HDoHE), 13-hydroxy-4Z,7Z,10Z,14E,16Z,19Z-docosahexaenoic acid (13-HDoHE), 14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid (14-HDoHE), 16-hydroxy-4Z,7Z,10Z,13Z,17E,19Z-docosahexaenoic acid (16-HDoHE), 19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid (19,20-DiHDPA), 10,17-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (10,17-DiHDoHE), 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (RvD1), 7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid (RvD2), and DHA. AMPP was obtained from Alchem Laboratories (Alachua, FL, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, chloroform, and water were purchased from Burdick & Jackson (Muskegon, MI, USA). Glacial acetic acid and *N,N*-dimethylformamide were obtained from Sigma–Aldrich (St. Louis, MO, USA), whereas *N*-hydroxybenzotriazole and (3-(dimethylamino)propyl)-ethyl carbodiimide hydrochloride were purchased from Advanced Chem Tech (Louisville, KY, USA) and TCI America (Portland, OR, USA), respectively. Ascentis Express C18 reverse phase HPLC columns (15 cm × 2.1 mm) were obtained from Supelco (Bellefonte, PA, USA), whereas solid phase extraction Strata-X columns were purchased from Phenomenex (Torrance, CA, USA).

Preparation of standard solutions and quantitation

Stock solutions of reference standards were prepared at a concentration of 10 to 100 pg/μl in ethanol and stored in a brown glass vial under nitrogen at −80 °C. The stock solutions were further diluted by ethanol to prepare standard working solutions. Appropriate amounts of internal standards of oxidized fatty acids in ethanol