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Quantification of fatty acid oxidation products using online high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

Oxidized fatty acids formed via lipid peroxidation are implicated in pathological processes such as inflammation and atherosclerosis. A number of methods may be used to detect specific oxidized fatty acids containing a single or multiple combinations of epoxide, hydroxyl, ketone, and hydroperoxide moieties on varying carbon chain lengths from C8 up to C30. Some of these methods are nonspecific and their use in biological systems is fraught with difficulty. Measures of specific oxidized fatty acid derivatives help in identifying oxidation pathways in pathological processes. We used liquid chromatography coupled with electrospray ionization tandem mass spectrometry as an efficient, selective, and sensitive method for identifying and analyzing multiple specific fatty acid peroxidation products in human plasma and other biological matrices. We then distilled the essential components of a number of these analyses to provide an efficient protocol by which fatty acid oxidation products and their parent compounds can be determined. In this protocol, addition of a synthetic internal standard to the sample, followed by base hydrolysis at elevated temperature and liquid–liquid phase sample extraction with lighter-than-water solvents, facilitates isolation of the oxidized fatty acid species. These species can be identified and accurately quantified using stable-isotope dilution and multiple-reaction monitoring. Use of a coupled multiplexed gradient HPLC system on the front end enables high-throughput chromatography and more efficient use of mass spectrometer time.

Introduction

Assessment of lipid oxidation levels is essential to the study of biologically relevant reactive oxygen species [1]. Numerous methods exist to ascertain these levels through the systematic analysis of the oxidized fatty acids that originate from the oxidized lipids both in vivo and in vitro [2–25]. In addition to exploring the bulk oxidation, many of these oxidized lipids and their associated oxidized fatty acids have activities of significant clinical interest [3–6,10,14,18,20,22,26–31]. Numerous GC[1], LC [32], and hybrid methodologies such as GC–MS [3,5,14,18,20,22,31] and LC–MS [2–4,8–10,12,19,23,27,33] exist to quantify the levels of these compounds. These markers can be used clinically to ascertain the effectiveness of antioxidant therapies; however, there are no universal guidelines regarding the scale of the analyses required for the determination of lipid oxidation (and hence fatty acid oxidation) products in a large clinical study. We have performed and optimized these types of analyses for nearly 2 decades in our laboratory and are presenting our insights into what makes for a good high-throughput oxidized lipid assay.

Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepentaacetic acid; EET, epoxyeicosatrienoic acid; ESI, electrospray ionization; GC, gas chromatography; $PGF_{2\alpha}$, 9α,11α,15*S*-trihydroxy-5*Z*,13*E*-dien-1-oic acid; 15(*S*)-HETE-d_8, 15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; HODE, hydroxyoctadecadienoic acid; LC–MS/MS, liquid chromatography with electrospray ionization online tandem mass spectrometry; LA, linoleic acid; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; MRM, multiple-reaction monitoring; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; $PGF_{2\alpha}$-d_4, 9α,11α,15*S*-trihydroxy-5*Z*,13*E*-dien-1-oic-3,3,4,4-d_4 acid. See Materials for more specific abbreviations of oxidized fatty acid species.

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Principles

Liquid/liquid extraction of fatty acids and their oxidation products

Recovery of free fatty acids and their oxidation products using the classic Folch et al. [34] and Bligh and Dyer [35] extractions is not easily adapted to the small sample volumes required for high-throughput clinical analyses. Hara and Radin's [36] procedure using hexane and isopropanol can be modified and adapted to

these small sample volumes (see Protocol). With this method, in addition to avoiding the toxicity and regulatory issues associated with chlorinated solvents, the organic layer floats on top and is more easily removed. Hexane, being a lighter-than-water solvent, allows one to avoid pushing a pipette or needle through the aqueous layer and risking possible contamination that can occur with heavier-than-water chlorinated solvents. The extraction is efficient because the initial solution is homogeneous, giving a layer that fully permeates and extracts the entirety of the sample solution. In the next step more hexane is added to generate a heterogeneous mixture from which the top layer is easily removed after centrifugation. Even when this liquid–liquid extraction is done manually, its simplicity, low cost, and efficiency allow for rapid processing of multiple samples simultaneously in a short period of time.

Use of heavy isotope standards

Internal standards should be incorporated before the extraction step to control for any losses that may occur through poor extraction, inefficient removal of the organic phase, adherence to the container walls, etc. Additionally, one may incorporate a distinct stable-isotope-labeled internal standard of the parent fatty acid to monitor and control for any artificial oxidation products that form during lipid extraction [2,8,9,12,19,23,25,37]. Preferably these are universally ^{13}C-labeled unsaturated fatty acid derivatives; however, the deuterated analogs are generally less expensive and more readily available from commercial sources.

Advantages of column switching

For analyses of biological matrices composed of multiple distinct fatty acid species (both oxidized and unoxidized), a broad gradient elution is often useful to allow for chromatographic separation among the many different compounds present. Once the gradient has been run it is necessary to reequilibrate the column back to its initial state, which takes 10–15 min. This reequilibration period is followed by the injection of the next sample, which with an autosampler could take several minutes followed by 1–2 min (preferred—after the solvent peak) until any peak of interest elutes. During this period the mass spectrometer is sitting idle and not collecting any relevant data from analyte peaks. To make optimal use of the mass spectrometer, the most costly piece in the instrument string, a second column from another HPLC system that has already been injected with another sample immediately at the end of the first column's run can be brought online [28,33].

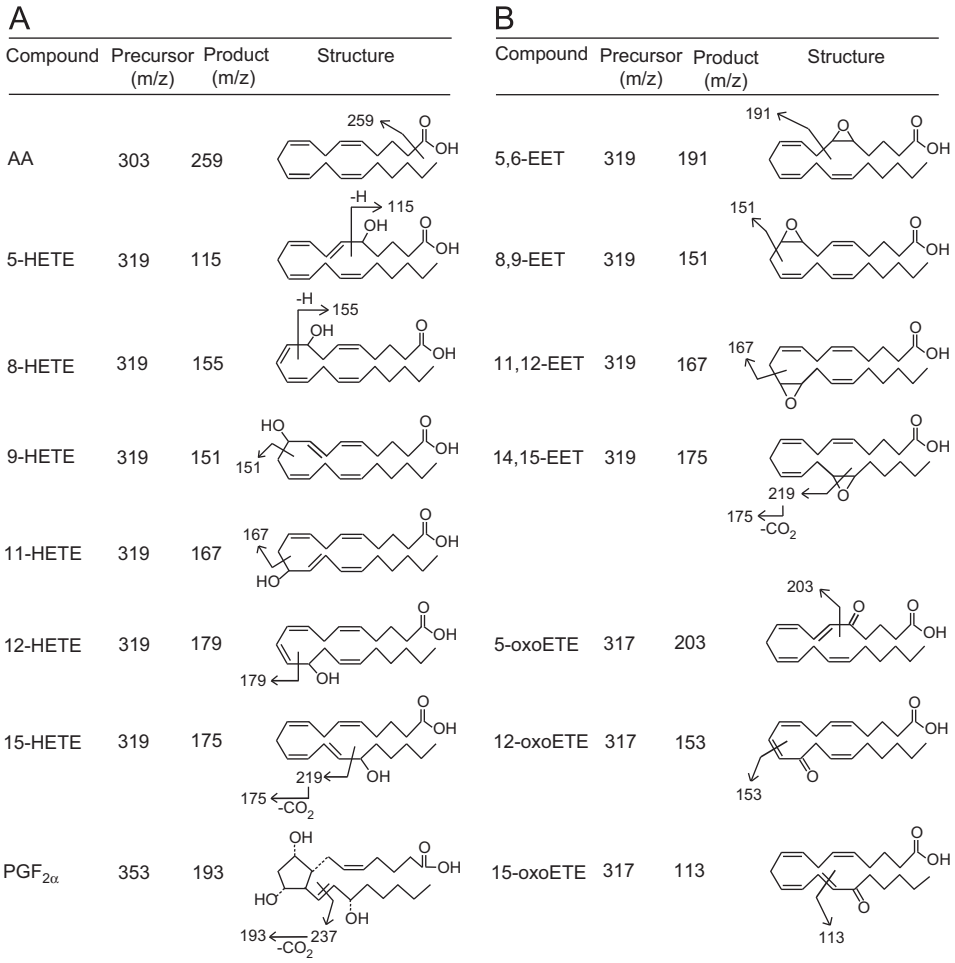

Fig. 1. Precursor ion→product ion transitions and suggested structure of the major product ion derived from each eicosanoid. ESI negative-ion full scan was used for identification of precursor ions for each of the analytes and ESI negative-ion product scan was used for identification of the specific product ions from each of the precursors. Suggested structures consistent with the product ions produced are depicted for each analyte.