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Integrated identification/confirmatory and targeted analysis of epoxyeicosatrienosic acids in human serum by LC–TOF MS and automated on-line SPE–LC–QqQ MS/MS

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ABSTRACT

A combined strategy is here proposed for qualitative/quantitative targeted analysis of epoxyeicosatrienoic acids (EETs) in human serum. Identification of EET regioisomers was initially carried out by LC–TOF MS in high accuracy mode under optimum conditions for chromatographic separation of the four isomers with an isocratic method using 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid. Confirmatory analysis was supported on MS/MS experiments using the hybrid QqTOF mass analyzer by targeted fragmentation of the precursor ion fitting with the molecular formula $C_{20}H_{32}O_3$ (319.2279 m/z). Identification of selective fragment ions in high accuracy mode enabled the localization of the epoxy functional group and, therefore, the assignation of chromatographic peaks to each EET isomer. After qualitative analysis, an automated method was developed for analysis of EETs in human serum by direct analysis using an on-line platform based on SPE–LC–QqQ MS/MS in selected reaction monitoring. Recovery factors estimated with a dual-cartridge configuration were above 87% for all metabolites either using non-spiked and spiked serum at three different concentrations. Precision, calculated as within-laboratory repeatability and expressed as relative standard deviation, ranged from 2.5 to 9.9% with detection limits below 0.15 ng mL^{-1}. The optimization of the method was completed with a stability study under different conditions to assess the suited conditions for analysis of EET intermediate metabolites. Finally, concentration ranges of EETs were measured in nine healthy individuals.

1. Introduction

20-Carbon polyunsaturated fatty acids dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid (20:3$n-6$, 20:4$n-6$ and 20:5$n-3$, respectively) are the precursors of hormone-like eicosanoids, a wide variety of lipid mediators. Eicosanoids are formed through the action of a set of oxygenase enzymes such as cyclooxygenases, lipoxygenases and cytochrome P450 isozymes (CYP) [1,2]. The CYP class is divided into two groups: CYP epoxygenases, which catalyze formation of the epoxyeicosatrienoic acids (EETs), and the CYP *o*-oxidases, which catalyze hydroxylation of arachidonic acid at positions from ω16 to ω20 [3,4]. CYP epoxygenases produce four EET regioisomers from arachidonic acid: 5,6-, 8,9-, 11,12- and 14,15-EET. Each CYP epoxygenase catalyzes the formation of the four EET regioisomers indifferently, but one or two usually are the predominant products.

The primary function of EETs is to act as autocrine and paracrine mediators in the cardiovascular and renal systems. By modulating ion transport and gene expression, EETs trigger vasorelaxation and produce antiinflammatory and profibrinolytic effects [5]. EETs are potent vasodilators, especially in small arteries [6]. Also, they act on smooth muscle to open calcium-activated potassium channels, which results in membrane hyperpolarization and vasorelaxation [7–10]. Increased cerebral blood flow [11], protection of neurons [12] and astrocytes [13] from ischemic cell death by EETs have also been reported [14]. They also play a role in the regulation of angiogenesis and tumor growth [15].

Abbreviations: ACE, automatic cartridge exchanger; C18 EC, end-capped silica-based octadecyl phase; C18 HD, end-capped silica based phase with a high density of octadecyl chains; C2, silica-based ethyl phase; C8 EC, end-capped silica-based octyl phase; CN, silica-based cyanopropyl phase; CYP, cytochrome P450 isozymes; EETs, epoxyeicosatrienoic acids; FWHM, full width at half maximum; HPD, high-pressure syringe dispenser; JSESI, jet stream technology electrospray ion source; MM Anion, strong basic mixed-mode anion; Resin GP, polymeric polydivinylbenzene phase; Resin SH, strong-hydrophobic modified polystyrene-divinylbenzene phase; RSD, relative standard deviation

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Studies to determine the clinical importance of CYP arachidonic acid metabolites have created the need for sensitive, selective, and reproducible methods for measuring EET metabolites in human biological fluids. A number of methods have been developed for the detection and quantification of these metabolites: GC–MS, GC–MS/MS [16,17], capillary electrophoresis–UV [18], LC–fluorescence [19], radioimmunoassays [20], and enzyme immunoassays [21] have been used for quantitative analysis of these compounds in different tissues such as liver [22], human placenta [23], endothelial cells [24], and biofluids such as plasma [6] or urine [25]. GC–MS with chemical ionization has been the most commonly used approach for analysis of EETs by taking benefit of the selective information about the mass weight from each chromatographic signal. However, GC-based protocols seem not to be the most suited option for analysis of labile compounds such as EETs owing to tedious steps including TLC purification and derivatization required prior to analysis. Due to its combined sensitivity–selectivity, LC–fluorescence after derivatization seems to be a simple hyphenated approach [20], but the separation step is time-consuming, and the high background from the matrix largely interferes throughout the chromatogram, even after a solid-phase extraction clean-up step. Although immunoassay-based tests have been used for many different matrices, their limitations include high cost, low sensitivity, cross-reactivity, and long analysis time [26].

The most significant issue for quantification of these isomeric metabolites is selectivity when working with complex biological matrices. As a result, LC–MS and LC–MS/MS have been widely used as they are powerful analytical approaches that combine the resolving power of liquid chromatography with the detection selectivity of mass spectrometry. Several alternatives have so far been reported for the determination of EET such as LC–MS and LC–MS/MS. In general, these pose a number of disadvantages: either they do not exhibit the high selectivity necessary to work with complex matrices as serum, due to the fact that there exist a large number of compounds in these samples which have the same m/z as the interest compounds, which would make working in single ion monitoring (SIM) mode inadequate in terms of selectivity [27], or they require a prior derivatization step [28], or they are not fully automatic [29]. Similarly to other eicosanoids, the most important difficulty in analyzing EETs originates from rapid, extensive metabolism [30]. EETs degrade by several processes, including spontaneous hydration, conjugation to glutathione, and Ω or β oxidation [31].

The variability associated with sample storage or preliminary operations can be critical in targeted metabolomics approaches focused on a restricted set of metabolites that could be seriously affected [32]. It is known that unsuitable sample pretreatment protocols can lead to biased results owing to conversion or degradation of metabolites [33]. Increased interest exists in rapid handling of samples for metabolomics purposes, while turnover kinetics of some metabolites is known to be extremely fast. Accordingly, the time window between sampling and analysis has to be as short as possible; so, in this article we propose a fully-automated qualitative and quantitative methodology based on SPE–LC–MS/MS for the determination of EETs with previous confirmatory analysis by LC–QqTOF to ensure the separation–identification of the isomers under study.

2. Experimental

2.1. Chemicals

Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system was used to prepare all aqueous solutions. (±)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) and (±)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acetic acid, formic acid, methanol, ethanol and acetonitrile from Scharlab (Barcelona, Spain), and ammonium formate and acetate from Sigma (Sigma-Aldrich, St. Louis, MO, USA) were used for the development of the analytical method. All chemicals were LC-grade and used without further purification, except for the use of the respective LC–MS grade solvents in LC–QqTOF analysis.

2.2. Serum samples from human individuals

Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ USA) without additives (red top). The tube was not opened to ambient air and kept refrigerated at 4 °C until processing. Blood samples were processed within 1 h after collection and centrifuged at $4000 \times g$ for 10 min to separate serum, which was placed in plastic tubes and stored at −80 °C until analysis. All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki [34], which were supervised by the ethical review board of Reina Sofia Hospital (Córdoba, Spain) that approved the experiments. Individuals selected for this study were informed to obtain consent prior to this research.

2.3. Standard solutions and spiked serum samples

Stock standard solutions of all compounds were prepared in ethanol (250 μg mL^{-1}) and stored in amber vials at −80 °C under nitrogen atmosphere. Working solutions or spiked samples were prepared by dilution of the appropriate volume of stock solutions in ethanol or in serum pool aliquots, respectively, then used for optimization of the chromatographic steps by LC–MS, identification of EET isomers by LC–QqTOF MS/MS and sample preparation by coupling solid-phase extraction SPE and LC–QqQ MS/MS in an automated manner.

2.4. LC–QqTOF MS/MS analysis

Identification and confirmation of the presence of epoxyeicosatrienoic acids were supported on LC–QqTOF MS/MS in negative ionization mode. The analytes were separated using an LC (series 1200, Agilent Technologies, Palo Alto, CA) equipped with a reversed-phase C18 Mediterranea Sea analytical column (Teknokroma, Barcelona, Spain) with 150 mm × 4.6 mm dimensions packed with 3 μm particle diameter. Column temperature was maintained at 20 °C. Separation of analytes was carried out in isocratic mode with 40:40:20 (v/v/v) methanol–acetonitrile–water containing 0.02% acetic acid, mobile phase pumped at 1.1 mL min^{-1} and 20 μL of the sample extracts were injected. The injector needle was washed for 12 times with 80% acetonitrile to avoid cross-contamination. Furthermore, the needle seat back was flushed for 15 s at a flow rate of 4 mL min^{-1} with 80% acetonitrile to clean it. The LC system was connected to a hybrid mass spectrometer formed by a dual quadrupole connected to a time-of-flight analyser (Agilent 6540 UHD Accurate-Mass QqTOF) equipped with an electrospray interface under the following operating parameters: capillary 3500 V, nebulizer 40 psi, drying gas 10 L min^{-1}, gas temperature 350 °C, filtering voltage 175 V, skimmer 65 V, Oct rf Vpp 750 V. The mass axis was calibrated using the mixture provided by the manufacturer over the m/z 60–1700 range. The instrument gave typical resolution 15000 FWHM (full width at half maximum) at m/z 112.9856 and 30,000 FWHM at m/z 1033.9881. To assure the desired mass accuracy of