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Original Research Article

Comparison of derivatization/ionization techniques for liquid chromatography tandem mass spectrometry analysis of oxylipins

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ABSTRACT

The performance of two derivatization and ionization techniques for the quantitative reversed phase liquid chromatography (LC)- mass spectrometry (MS) analysis of hydroxy fatty acids (OH-PUFA) in plasma was evaluated: One used AMPP (*N*-(4-aminomethylphenyl)pyridinium chloride) leading to a positive charged amid-derivate which can be detected by electrospray ionization (ESI)-MS. Second yielded penta fluorobenzyl bromide (PFB) ester derivates allowing detection in electron capture atmospheric pressure chemical ionization (ecAPCI)-MS. The sensitivity of detection of a comprehensive set of hydroxy fatty acids of n6- and n3- poly unsaturated fatty acids was investigated. On the SCIEX3200 MS the applied PFB derivatization led to poor limits of detection (LOD) of 10–100 nM (0.1–1 pmol/0.03–0.3 ng on column). By contrast, AMPP derivatization led to a similar sensitivity compared to the standard ESI(-) of non derivatized analytes (LOD about 1 nM (10 fmol/3 pg on column)). For several analytes, including 9-HETE, 11-HETE and 17-HDHA the AMPP derivatization improved sensitivity enabling their detection in human plasma. However, precision was reduced by AMPP derivatization and variation in IS recovery indicated a strong matrix influence on the MS-signal. In sum, with the instrumentation used, neither of these derivatization methods improves in our hands the LC–MS based quantification of oxylipins.

1. Introduction

The analysis of oxidation products – oxylipins – of polyunsaturated fatty acids (PUFA) formed within the arachidonic acid cascade is essential for the understanding of biology of inflammation, pain and related diseases. Furthermore, a reliable, robust and sensitive analysis of products of the arachidonic acid cascade is crucial to monitor effects of diet and pharmaceuticals on these lipid mediators [1,2].

Eicosanoids and other oxylipins are derived by three different pathways. One is catalyzed by constitutively expressed cyclooxygenase 1 (COX-1) and inducible COX-2 leading to prostaglandins or thromboxanes. Products in the second pathway are formed by lipoxygenases (LOX), in humans namely 5-LOX, 12-LOX, 12R-LOX, 15-LOX and 15-LOX-2 (8-LOX) [3,4]. The formed hydroperoxy-fatty acids can be reduced leading to mono- and di-hydroxylated PUFA such as hydroxyeicosatetraenoic acids (HETEs), hydroxyeicosapentaenoic acids (HEPEs), hydroxydocosahexaenoic acids (HDHAs) or be converted to leukotrienes. The last pathway is catalyzed by cytochrome P450 monooxygenases (CYP), leading to several hydroxy-FA as well as epoxy-FA. The conversion of the initial formed products by further enzymes and formation by non-enzymatic (aut)oxidation (e.g. hydroperoxy-FA, hydroxy-FA, epoxy-FA, isoprostanes, neuroprostanes) lead to a large variety of oxylipins [3,4].

To date, targeted analysis of oxylipins in biological samples is commonly carried out by means of reversed phased LC–MS/MS [3]. These methods need to be sensitive and selective to allow individual quantification of oxylipins in low nM concentrations. Additionally, they need to cope with a large concentration range of different oxylipins in one sample (up to 10^4 fold). Modern LC–MS/MS methods use the high separation power of LC columns filled with sub 2 μm particles with reversed phase (RP) material. Detection is commonly carried out by highly sensitive triple quadrupole mass spectrometers using negative electrospray ionization (ESI(-)) [5–9]. This combination allows sensitive and selective detection of isomers which cannot be separated by

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reversed phase liquid chromatography (e.g. 9-HETE and 12-HETE) or show similar fragmentation behavior (e.g. PGE_2 and PGD_2). Typically, these methods have limits of detection between 0.1 and 2 nM (0.5–10 fmol on column) and allow the selective parallel detection and quantification of about 100 different oxylipins [6,7,9].

However, due to the ESI ionization these methods often suffer a loss of sensitivity and precision because of matrix effects particularly by suppression [10–12]. Additionally, for reasons not well understood, the positively charged ions are formed more efficiently in commercial ESI sources than negatively charged ones [13]. Moreover, the RP-chromatographic separation of oxylipins – bearing a carboxyl group – requires acidic conditions and volatile acids (e.g. acetic acid or formic acid) are generally used as additive to the solvents. However, the low pH further reduces the formation of $[M\text{-}H^+]^-$, i.e. carboxylate anions, of oxylipins in the ESI source and thus diminishes sensitivity.

In order to circumvent the problems with ESI(-), different derivatization techniques have been developed. One is using a positively charged agent (AMPP (*N*-(4-aminomethylphenyl)pyridinium chloride)) forming an amide with the oxylipins which can be analyzed using ESI(+) [13]. A second derivatization utilizes penta fluorobenzyl bromide (PFB) to form an ester with the oxylipins allowing detection in electron capture atmospheric pressure chemical ionization (ecAPCI(-)) [14,15]. Though both methods are used by several groups [13–16], their performance on quantitative analysis of oxylipins has not been systematically evaluated. Therefore, we compared in the presented study the performance of LC–MS methods using the two derivatization techniques with direct ESI in the negative mode. For this purpose, the sensitivity of detection of a comprehensive set of hydroxy-FA derived from all relevant n6- and n3-PUFA was evaluated and the methods were applied on the analysis for the quantification of oxylipins in human plasma.

2. Materials and methods

2.1. Chemicals and biological materials

LC–MS grade acetonitrile (ACN), methanol (MeOH), *n*-hexane, and methyl formate were purchased from Fisher Scientific (Schwerte, Germany). Acetic acid (HAc) LC–MS grade, pyridine anhydrous (99.8%), ethanol p.a., 1-chloro-2,4-dinitrobenzene, 4-[(*N*-Boc) aminomethyl]aniline, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxybenzotriazole, *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid, 2,3,4,5,6-pentafluorobenzyl bromide, *N*,*N*-diisopropylethylamine, and all other chemicals were from Sigma Aldrich (Steinheim, Germany). Oxylipin standards (Table 1) and internal standards $^2H_8$-5-HETE, $^2H_8$-12-HETE, $^2H_8$-15-HETE were purchased from Cayman Chemicals (local distributor: Biomol, Hamburg, Germany). Desalted and purified water (>18 M Ohm) was generated by a Milli Q Gradient A10 – System (Millipore, Schwalbach, Germany). AMPP was synthesized as described [13] and details are summarized in the SI. Pooled human plasma as well as serum was obtained from healthy male volunteers.

2.2. Sample preparation and derivatization

Oxylipine extraction from plasma and serum samples was carried out utilizing C18 solid phase extraction columns as described [9]. Briefly, 500 μL of freshly thawed human plasma or serum was mixed with 1450 μL MeOH/water (20/80; v/v) and 50 μL of internal standards in MeOH ($^2H_8$-5-HETE, $^2H_8$-12-HETE, $^2H_8$-15-HETE each 100 nM) and centrifuged (10 min, 4 °C, 20,000 × *g*). After acidification with glacial HAc to a pH of 3.0 (approx. 80 μL) samples were loaded onto the preconditioned C18-SPE column (6 mL, 500 mg, 37–55 μm particles from Macherey-Nagel, Dueren, Germany). Samples were washed with 10 mL water and 6 mL *n*-hexane and dried before elution with 8 mL of methyl formate. The organic phases were collected into sample tubes and spiked with 6 μL of 30% glycerol in MeOH. Samples were evaporated in a stream of nitrogen at 30 °C and resuspended in 50 μL MeOH. Extracts were directly analyzed by LC/MS or directly derivatized. Recovery of internal standards was evaluated using by an external calibration of $^2H_8$-5-HETE, $^2H_8$-12-HETE, $^2H_8$-15-HETE 30–100 nM as described [9].

For AMPP derivatization of oxylipins 20 μL MeOH of a standard solution or of the obtained SPE extract was dried under a stream of nitrogen. The residue was resuspended in 5 μL cold ACN/DMF (4/1; v/v) and 5 μL of cold EDC (640 nM in water). The sample was vortexed for 30 s and 10 μL of AMPP (30 mM in ACN) was added. Finally, the mixture was incubated at 60 °C for 60 min and analyzed by LC-ESI(+)-MS.

For PFB derivatization of oxylipins 20 μL of a standard solution or of the obtained SPE extract was dried under a stream of nitrogen. The sample was then derivatized in 20 μL of diisopropylethylamine in dichloromethane (1/19; v/v) and 20 μL of pentafluorobenzyl bromide in dichloromethane (1/9; v/v). After vortexing for 30 s the samples were incubated at 50 °C for 60 min and dried under a stream of nitrogen. The residue was resuspended in 20 μL MeOH analyzed by LC-ecAPCI(-)-MS.

2.3. LC–MS/MS analysis

All LC–MS analysis were carried out using an Agilent 1260 LC system (Agilent, Waldbronn, Germany) on a Nucleoshell Gravity C18 reversed phased column (2.1 mm × 100 mm, 1.9 μm particle) equipped with a guard column (2 mm × 4 mm); Macherey-Nagel, Dueren, Germany kept at 50 °C. For all derivatives optimized separation conditions were used utilizing a binary gradient at a flow rate of 400 μL/min, with 95% water, 5% B and 0.1% HAc as solvent A and ACN/MeOH/HAc (800/150/1; v/v/v) as solvent B. The gradient for the separation of non-derivatized oxylipins was: 0-0.25 min isocratic 55% B, 0.25-1.00 min linear from 55% to 60% B, 1.00-8.00 min linear from 60% to 73% B, 8.00-8.01 min linear from 73% to 100% B, 8.01-8.50 min isocratic 100% B, 8.50-8.51 min linear from 100% to 55% B followed by reconditioning for 5 min. The gradient for the separation of AMPP derivates was 0-0.25 min isocratic 40% B, 1.00-8.00 min linear from 40% to 53% B, 8.00-8.01 min linear from 53% to 100% B, 8.01-8.50 min isocratic 100% B, 8.50-8.51 min linear from 100% to 55% B followed by reconditioning for 5 min. The gradient for the separation of PFB derivatized oxylipins was 0-0.25 min isocratic 80% B, 1.00-8.00 min linear from 80% to 85.5% B, 8.00-8.10 min linear from 85.5% to 100% B, 8.50-8.51 min linear from 100% to 55% B followed by reconditioning for 5 min. Samples were injected (10 μL) by a CTC-Pal autosampler (CTC Analytics, Zwingen, Switzerland).

Mass spectrometric detection was carried out using a API 3200 triple quadrupole mass spectrometer (Sciex, Darmstadt Germany) following negative electrospray ionization for non-derivatized oxylipins, positive electrospray ionization for AMPP derivatized oxylipins and negative electron capture atmospheric pressure chemical ionization (ecAPCI(-)) for PFB derivatized oxylipins. Detection was carried out in scheduled selected reaction monitoring within a window of 40 s and a cycle time of 0.9 s. Taking the average peak width (e.g. for ESI(-) approx.17 s; Table 1) into account, these parameters allow the acquisition of at least 15 data points per peak. Optimized source settings as well as detection parameters for each method are reported in the supporting material (Table S1–S4).