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Simultaneous lipidomic analysis of three families of bioactive lipid mediators leukotrienes, resolvins, protectins and related hydroxy-fatty acids by liquid chromatography/electrospray tandem mass spectrometry

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Abstract

Bioactive lipid mediators derived from polyunsaturated fatty acids (PUFA) and exhibit a range of tissue and cell-specific activities in many physiological and pathological processes. Electrospray tandem mass spectrometry coupled to liquid chromatography (LC/ESI-MS/MS) is a sensitive, versatile analytical methodology for the qualitative and quantitative analysis of lipid mediators. Here we present an LC/ESI-MS/MS assay for the simultaneous analysis of twenty mono- and poly-hydroxy fatty acid derivatives of linoleic, arachidonic, eicosapentaenoic and docosahexaenoic acids. The assay was linear over the concentration range 1-100 pg/μL, whilst the limits of detection and quantitation were 10-20 and 20-50 pg respectively. The recovery of the extraction methodology varied from 76-122% depending on the metabolite. This system is useful for profiling a range of biochemically-related potent mediators including the newly discovered resolvins and protectins, and their precursor hydroxy-eicosapentaenoic and hydroxy-docosahexaenoic acids, and, consequently, advance our understanding of the role of PUFA in health and disease.

Keywords

Hydroxy fatty acids; Eicosanoids; Arachidonic Acid; Eicosapentaenoic Acid; Resolvins; Protectin; Docosahexaenoic acid

Introduction

Polyunsaturated fatty acids (PUFA) exhibit a range of biological effects many of which are mediated through the production of lipid mediators. Such metabolites are formed through the action of cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P450

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monooxygenases (CYP450) or free radical oxidation mechanisms 1-3. Arachidonic acid (AA) (20:4n-6) is the precursor of many eicosanoids, a family of potent mediators of the inflammatory response. COX and LOX activities generate series-2 prostanoids, series-4 leukotrienes and hydroxy-eicosatetraenoic acids (HETE), whilst CYP450 and autoxidation reactions result in various hydroxy-, hydroperoxy- and epoxy-fatty acids, and F_2-isoprostanes. Eicosapentaenoic (EPA) (20:5n-3) and docosahexaenoic (DHA) (22:6n-3) acids are n-3PUFA major constituents of fish oil. EPA-derived mediators include series 3-prostanoids, series-5 leukotrienes, hydroxy-eicosapentaenoic acids (HEPE), and F_3-isoprostanes. Enzymatic oxidation or autoxidation of DHA produce hydroxy-docosahexaenoic acids (HDHA) and neuroprostanes. Recently, it has been shown that EPA and DHA give rise to novel di- and trihydroxy-containing mediators with anti-inflammatory and protective activities termed resolvins (RvE from EPA; RvD from DHA) and protectins (PD from DHA) 4-6. Finally, LOX metabolism of linoleic acid (LA) results in hydroxyoctadecadienoic acids (HODE) 7 (Figure 1 and scheme 1).

Those lipid mediators carry potent bioactivities functioning in a wide range of biological systems including the cardiovascular system, brain, eye, kidney, lungs and skin, and have been investigated for biomarker discovery and drug development. Specific examples include the role of CYP450 metabolites in the regulation of the vascular tone 8, 12-HETE and 13-HODE in cutaneous biology 7, 12-LOX products in hippocampal long-term potentiation and long-term depression 9, corneal injury 10 and angiogenesis 11, HETE, HODE and HDHA as markers of lipid peroxidation 12,13, and, finally, the anti-inflammatory and cellular protective activities of RvE1, RvD1 and PD14-6.

Methods currently used for the analysis of mono- and poly-hydroxy fatty acids include gas chromatography (GC), GC/mass spectrometry (GC/MS) and GC/MS/MS 14-17, HPLC/UV and HPLC/Fluorescence 17-19, liquid chromatography/MS (LC/MS) and LC-MS/MS 20-23, LC-atmospheric pressure chemical ionisation (APCI)-MS 24 and electron capture APCI-MS/MS 25,26, and enzyme immunoassays 27. HPLC-UV lacks sensitivity to detect low levels of metabolites, whilst immunoassays, although very popular, cannot be used for the analysis of more that one metabolite at a time. Furthermore, GC and GC/MS applications require lengthy sample preparation and derivatisation with the added danger of thermal decomposition for some hydroxy-fatty acids 28. LC-MS/MS combines the resolving power of liquid chromatography with the detection specificity of MS/MS and overcomes the limitations of the conventionally used approaches thus providing the means of a rapid, versatile and sensitive methodology.

Mediator lipidomic analysis concerns with the qualitative and quantitative analysis of bioactive lipid mediators in pathophysiological conditions, and when combined with information on metabolic, proteomic and genomic profiles can greatly assist in assessing the role of lipids at cellular, tissue and systems level 29,30. This approach has been greatly assisted by the development of LC-MS/MS-based analytical protocols that have great analytical power and allow the simultaneous detection and quantitation of a range of mediators in the same biological sample. Here we present an LC-MS/MS assay for the simultaneous analysis of twenty hydroxy-octadecadienoic acids, hydroxy-eicosatetraenoic acids, hydroxy-eicosapentaenoic acids, leukotrienes, resolvins and protectins. The method can be applied in a variety of tissues and body fluids and assist any lipidomic or systems biology applications targeted for biomarker discovery or other therapeutic and pharmacological approaches.

Experimental

Materials

Resolvin E1 (RvE1, 5S, 12R, 18R-trihydroxy-6Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid), resolvin D1 (RvD1, 5S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) and protectin D1 (PD1, 10R,17S-dihydroxy-docosa-4Z,7Z,11E,15Z,19Z-hexaenoic acid) were prepared by total organic synthesis 31-33. 9-Hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE), 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (5-HEPE), 18-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid (18-HEPE), 9-hydroxy-5Z,7*E*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (9-HEPE), 8-hydroxy-5*Z*,9*E*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (8-HEPE), 15-hydroxy −5*Z*,8*Z*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid (15-HEPE), 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaenoic acid (12-HEPE), 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), 8-hydroxy-5*Z*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid (8-HETE), 11-hydroxy-5*Z*,8*Z*,12*E*,14Z-eicosatetraenoic acid (11-HETE), 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE), 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE), 9-hydroxy-5*Z*,7*E*,11*Z*,14*Z*-eicosatetraenoic acid (9-HETE), leukotriene B_4 (LTB_4), 17-hydroxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (17*S*-HDHA) and 12S-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic-5,6,8,9,11,12,14,15-*d*8 acid (12-HETE-*d*8) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). HPLC grade solvents, glacial acetic acid and all other chemicals were from Sigma-Aldrich (Poole, UK). Solid phase extraction (SPE) cartridges (C18-E 500 mg, 6 mL) were purchased from Phenomenex (Macclesfield, UK).

Preparation of standards and calibration lines, linearity, detection and quantitation limits

Stock standard solutions of all compounds were prepared in ethanol (400 pg/μL) and stored in amber vials at −20°C under N_2. Composite standard solutions were prepared by mixing and diluting the appropriate stock solutions to the final concentrations of 100 pg/μL, 50 pg/μL, 20 pg/μL, 10 pg/μL, 4 pg/μL and 2 pg/μL. The internal standard (12-HETE-*d8*) was prepared in ethanol (2 ng/μL) and added to all composite standards at a final concentration of 800 pg /μL. The peak-area ratios of every compound to 12-HETE-*d8* were calculated and plotted against the concentration of the calibration standards. Calibration lines were calculated by the least squares linear regression method. To calculate the concentration of any given analyte the peak-area ratio to 12-HETE-*d8* was calculated and read off the corresponding calibration line. The limit of detection was calculated by using a signal to noise ratio of 3. The limit of quantitation was determined by using a signal to noise ratio of 10. Peak integrations and signal to noise calculations were performed using the MassLynx™ V4.0 software (Waters) using the manufacturers' instructions.

Sample preparation

Brain, liver and plasma samples were collected from male Wistar rats. Tissue samples (approximately 500 mg) were homogenised in water (35 up and down strokes) using a Dounce glass mini homogeniser (2 mL) with tight fitting pestle. During this process the homogeniser was kept on ice. The resulting solution was adjusted to 15% methanol (v/v) (final volume 3 mL). Plasma samples (500 μL) were diluted with water and adjusted to 15 % methanol (v/v), to a final volume of 3 mL. Internal standard 12-HETE-*d*8 (80 ng) was added to each sample. The samples were incubated on ice for 30 min and then centrifuged at 3000 rpm for 5 min to remove any precipitated proteins. The resulting clear supernatants were acidified with 0.025 M hydrochloric acid to pH 3.0 and immediately applied to SPE cartridges that had been preconditioned with 20 mL methanol followed with 20 mL water. The cartridges were then washed with 20 mL 15% (v/v) methanol, 20 mL water, and 10 mL hexane in succession. Finally, the hydroxy fatty acids were eluted with 15 mL methyl formate. The extraction procedure was performed using a vacuum manifold (Phenomenex);

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the vaccum was adjusted so that individual drops could be seen from each cartridge. The organic solvent was evaporated under a fine stream of nitrogen, the residue was dissolved in 100 μL ethanol, flushed with nitrogen and stored at −20°C awaiting LC-MS/MS analysis.

LC/ESI-MS/MS analysis

The LC-MS/MS analysis was performed on a Waters Alliance 2695 HPLC pump coupled to an electrospray (ESI) triple quadrupole Quattro Ultima mass spectrometer (Waters, Elstree, Hertsfordshire, UK). Instrument control and data acquisition were performed using the MassLynx™ V4.0 software. The instrument was operated in the negative ionisation mode. For optimisation of MS and MS/MS conditions, standards at a concentration of 10 ng/μL were individually introduced to the spectrometer by direct infusion through a syringe pump (flow rate of 10 μL/min) into the HPLC solvent flow (flow rate 0.2 mL/min). The capillary voltage was set at 3500 V, source temperature 120 °C, desolvation temperature 360 °C, and cone voltage 35 V. The collision energy was optimised for each compound to get optimum sensitivity using argon as collision gas. Dwell times were 0.2 s and the inter channel delay was 0.10 s giving a total cycle time of 4.920 s. The collision energy settings used for each one of the 21 MRM transitions are summarised in Table 1.

Chromatographic analysis was performed on a C18 column (Luna, 5 μ, 150 × 2 mm) (Phenomenex, Macclesfield, UK). Sample injections were performed with a Waters 2690 autosampler and the sample chamber temperature was set at 8 °C. The injection volume was 5 μL and the flow rate 0.2 mL/min. The column was maintained at ambient temperature. The analysis was performed using a methanol-based isocratic system obtained by mixing two solvents (A and B) at a ratio 95:5 (v/v). Solvent A was methanol:water:glacial acetic acid, 80:20:0.02 (v/v/v); Solvent B was acetonitrile:water:glacial acetic acid, 45:55:0.02 (v/v/v).

Recovery

Rat brain, liver and plasma samples were spiked with 20 ng of a mixed hydroxy fatty acid, leukotriene, resolvin and protectin standard. The metabolites were extracted as described above, and analysed in parallel with the extracts of un-spiked tissue samples to estimate the recovery. In detail: the extracts of un-spiked tissue samples allowed us to estimate the peak-area value corresponding to the amount of naturally occurring metabolites in those tissues. This figure was then subtracted from the corresponding peak-area value obtained from the spiked samples. The difference is equivalent to the amount of metabolite added to each tissue sample. This figure was then compared to the peak-area value that was obtained from the analysis of metabolites that did not undergo extraction thus representing 100% of the initial concentration. The resulting value shows the recovery of each metabolite expressed as percent of the initial concentration. Three separate sets of recovery experiments were performed per type of tissue or body fluid. Overall, there was no noticeable interference resulting from the biological matrices in any of the three types of material used in this study.

Results and Discussion

Profiles of PUFA-derived lipid mediators are tissue-dependent. Analytical methodologies that permit fast, sensitive and accurate, quantitative and qualitative analyses of the spatial and temporal profiles of those metabolites in pathological and physiological conditions are important. ESI has been widely applied in the analysis of all classes of eicosanoids, hydroxy- and epoxy-fatty acids, lipoxins, resolvins and protectins 6,34-37. Since these compounds have free carboxylic acid groups, ESI results in an abundant [M-H]-carboxylate ion that allows their detection at relatively low concentrations. Performing multiple reaction monitoring (MRM) assays leads to further improvement of the detection and quantitation

limits of ESI-LC/MS methods. To date, only a small number of HETE and leukotrienes have been studied together for the development of ESI-LC/MS-MS assays that can detect more than one lipid mediator 34,38-40, and there are no reports to date of such studies for the newly discovered resolvins and protectins. We have developed a sensitive LC/ESI-MS/MS assay applicable to the simultaneous detection and quantitation of twenty lipid mediators including LA-, AA-, EPA- and DHA-derived hydroxy fatty acids, EPA-and DHA-derived resolvins, and one DHA-derived protectin (Scheme 1).

In preparation for the development of the LC/MS/MS assay, product ion scanning experiments were conducted using argon as collision gas. The collision energy was optimised for each compound to generate the most abundant product ions. The product ion spectra were then used to select the precursor-product ion pairs that were included in the MRM assays (all ESI spectra are available as Supplementary Material). The ion pairs and corresponding optimal collision energies used in the LC-ESI-MS/MS assay are presented in Table 1. The choice of product ions were in accordance with the literature, when such information was available, i.e.: LTB_4 *m/z* 335>195, 5-HETE *m/z* 319>115, 8-HETE *m/z* 319>155, 9-HETE *m/z* 319>123, *m/z* 11-HETE 319>167, 12-HETE *m/z* 319>179 20,38-41. 15-HETE was detected using the transition *m/z* 319>175 instead of the reported *m/z* 319>219 40 because the fragment ion m/z 175 was found at higher abundance under the conditions used in our assay. The compounds were chromatographically resolved using a C18 column and an isocratic solvent system. Figure 2 shows representative chromatograms of this analysis. The run time of the assay was 35 min including a 10 min wash cycle programmed to run before the next injection.

The isobaric hydroxyeicosatetraenoic acids 8-HETE and 12-HETE did not resolve well chromatographically (retention times (r.t.) of 21.42 and 21.25 min respectively, Fig 2B). However, the choice of structure-specific fragment ions allowed their differentiation (8-HETE *m/z* 319>155, and 12-HETE *m/z* 319>179). Similarly, structure-specific fragment ions allowed the differentiation of the isobaric and coeluting 8- and 12-HEPE (r.t. 14.20 and 14.61 min, *m/z* 317>155 and *m/z* 317>179 respectively). Although the transition used for 9-HEPE (*m/z* 317>149) showed cross-reactivity with 5-, 8-, 12-, 15-and 18-HEPE, albeit at lower abundance, good chromatographic resolution (r.t. 18-HEPE=12.56 min, 8-HEPE=14.20 min; 9-HEPE=15.35 min; 5-HEPE=16.58 min) allowed their detection and quantitation without any problems. The choice of structure-specific fragment ions also allowed the differentiation of the two coeluting hydroxy-octadecadienoic acids 9- and 13-HODE (*m/z* 295>171 and 295>195 respectively). The fragment ion *m/z* 171 is formed at low abundance during CID of 13-HODE and therefore, it is possible that in the case of both isomers being present in a biological extract, the quantitation of 13-HODE may be overestimated due to the presence of 9-HODE. Finally, RvE1, RvD1 and PD1 were well separated chromatographically. The fragment ion selected for the MRM reaction of RvE1 (m/z 195) is in agreement with the recently reported low collision energy MS/MS spectra by Lu et al 37. However, the fragment ions used for the identification of RvD1 (m/z 141) and PD1 (m/z 206) were not the predominant species in the spectra shown by Hong et al 36 using ThermoFinnigan LCQ mass spectrometer. These differences can be attributed to the use of different mass spectrometers (ion-trap vs triple quadrupole instruments). Essentially identical spectra were obtained using ABI QSTAR (Hong and Serhan, data not shown).

Quantitation was performed using standard calibration lines, constructed for each analyte over the range 10 to 500 pg per injection. Calibration lines were calculated by the least-squares linear regression method, showing that the assay was linear over this range of concentrations (Table 2). The LoD was found to be in the range of 10-20 pg on the column and the LoQ was in the range of 20-50 pg on the column, depending on the compound. These results are shown in Table 2. The recovery of the extraction methodology was

calculated after spiking a range of biological matrices (rat plasma, brain and liver homogenates) with a cocktail of authentic standards (20 ng/compound). After extraction and LC-MS/MS analysis the peak area for each extracted analyte was compared with the one for the non-extracted standard from which recovery was calculated. Average recoveries ranged from 76 to 122 % depending on the compound and type of biological material; these results are shown in Table 3. Overall, the assay presented in this report was found to have good specificity, linearity, recovery and sensitivity.

Conclusion

The LC-/ESI-MS/MS assay reported here can be applied in a variety of biological materials for the qualitative and quantitative analysis of mono- and poly-hydroxy fatty acid derivatives of PUFA. This approach overcomes the lack of sensitivity of conventional methodologies and allows the simultaneous determination of a large number of biochemically-related PUFA-bioactive compounds including newly discovered potent mediators, without the need for derivatisation. The methodology is simple, sensitive, high-throughput and can be used in conjunction with the recently reported assay for prostanoids 42 for lipidomic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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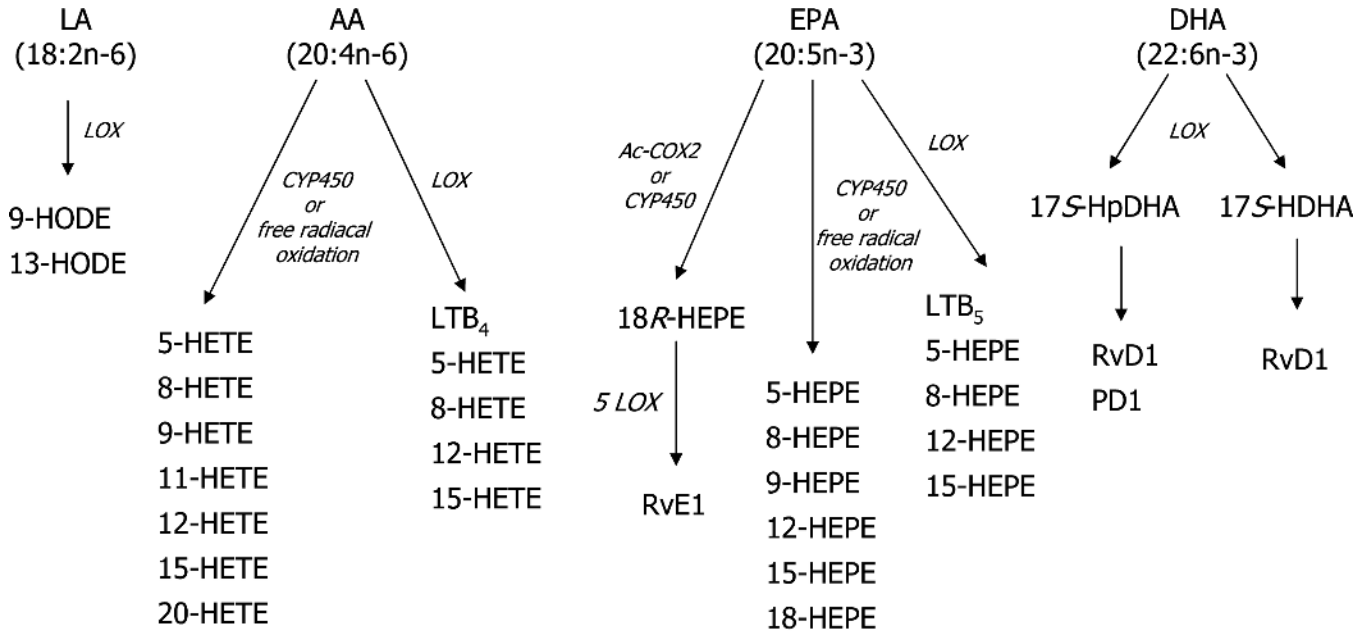
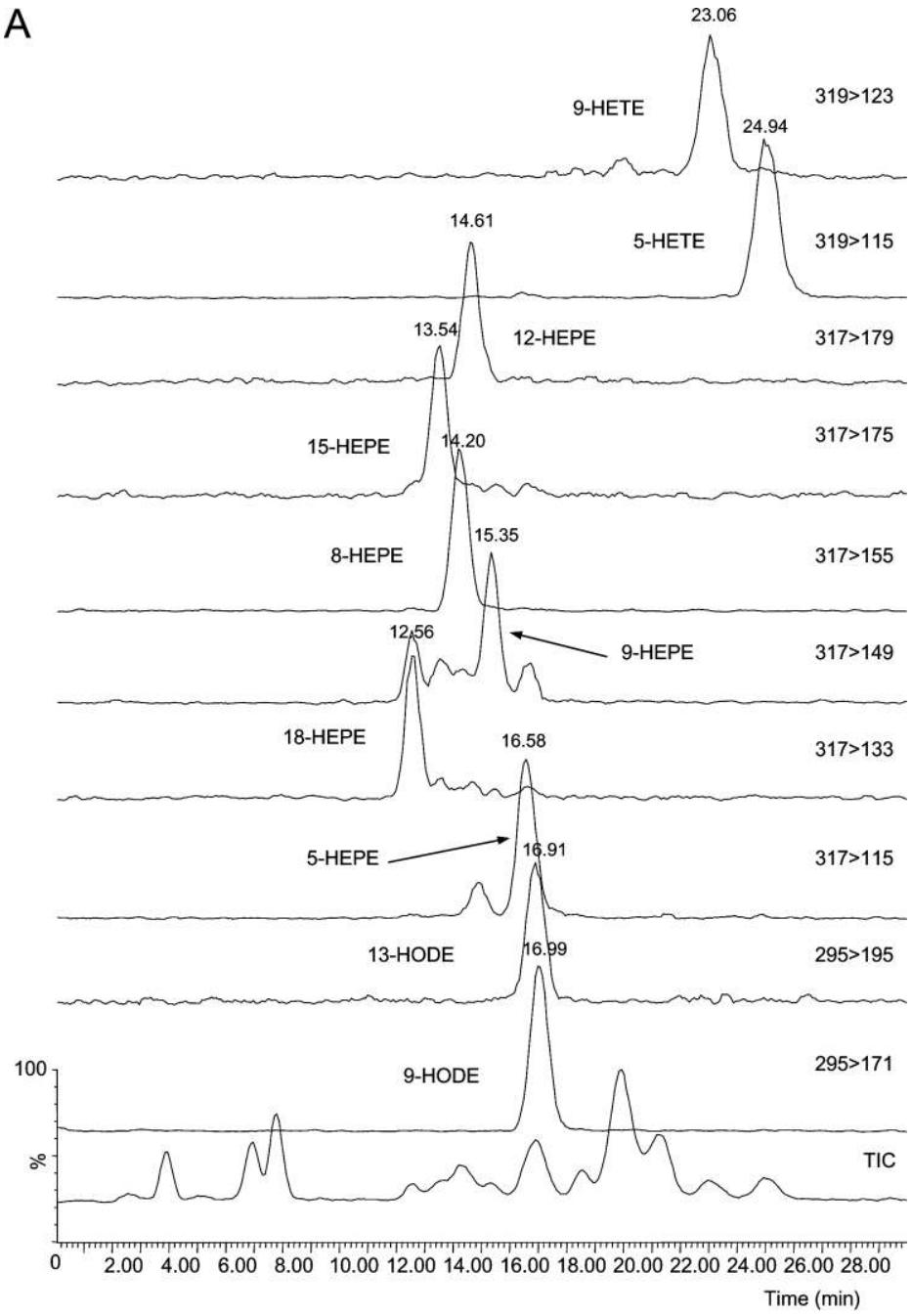


Figure 1.
Schematic outline of the hydroxy fatty acids, leukotrienes, resolvins RvE1 and RvD1, and protectin PD1 produced by linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via lipoxygenase (LOX), cytochrome P450 (CYP450), acetylated cyclooxygenase (Ac-COX2) or free radical catalysed pathways.



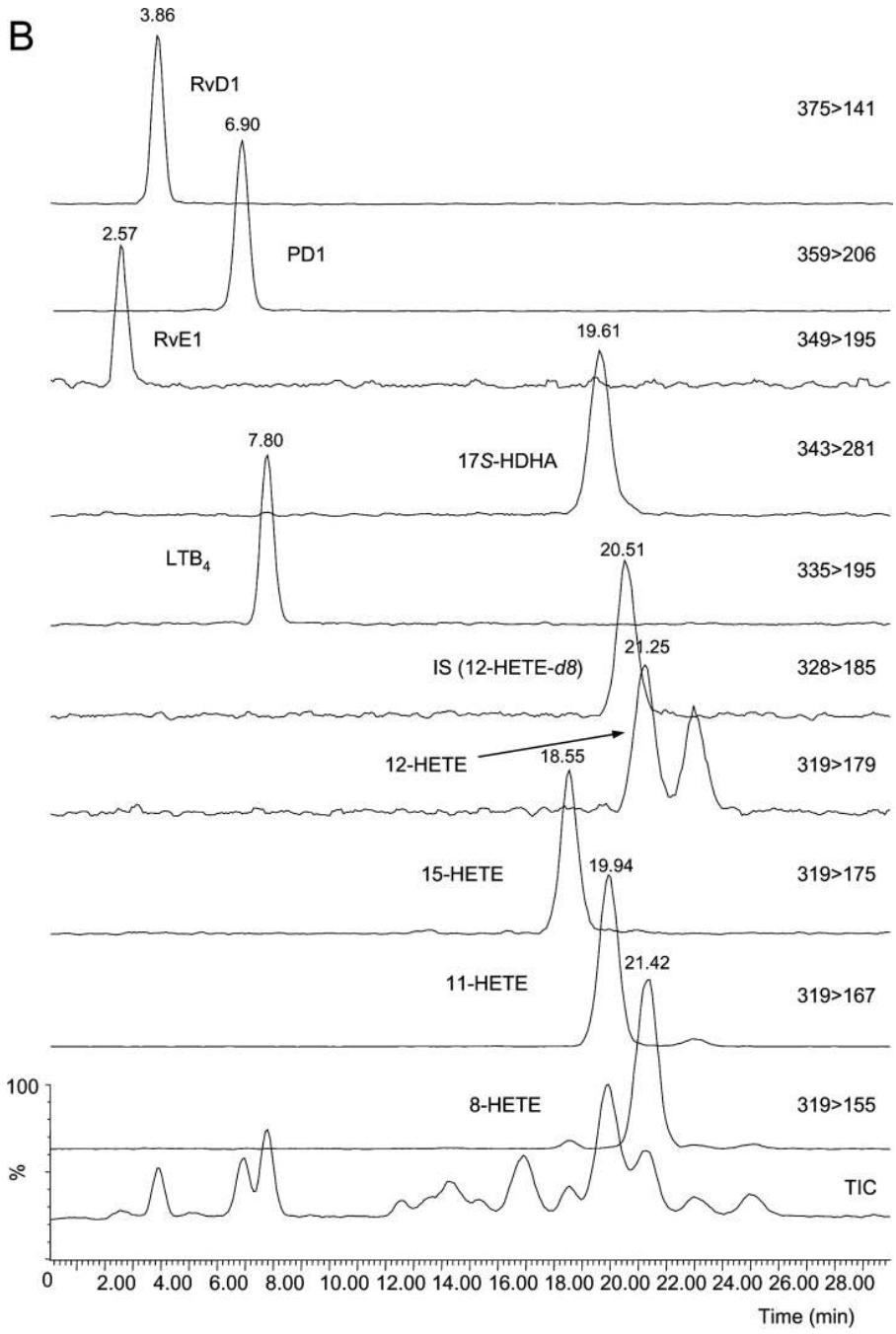


Figure 2.

Representative chromatograms showing the LC/ESI-MS/MS analysis of twenty hydroxy fatty acids, leukotrienes, resolvins and protectins (50 pg/mL per compound). IS: internal standard (12-HETE-*d*8).

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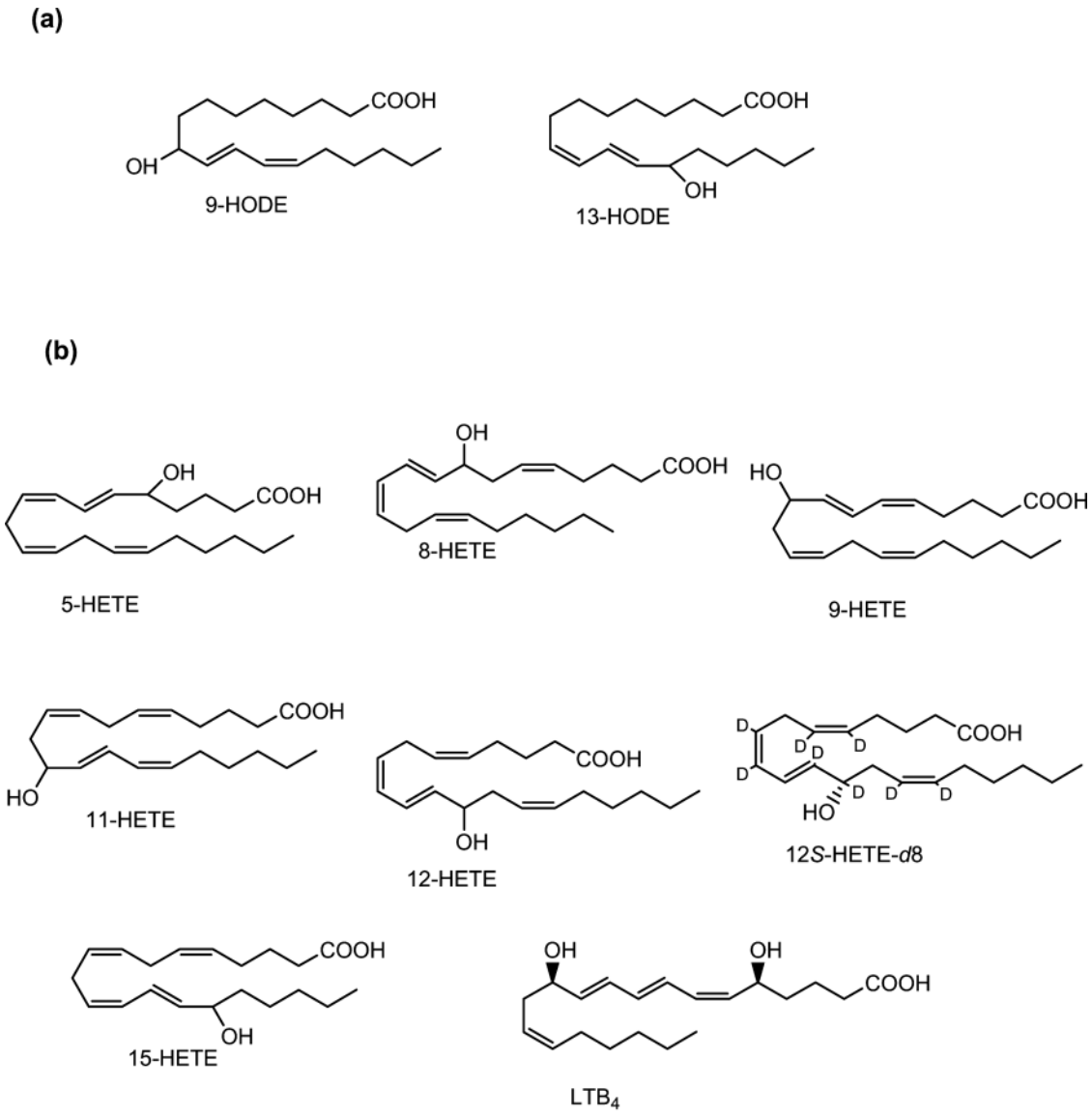

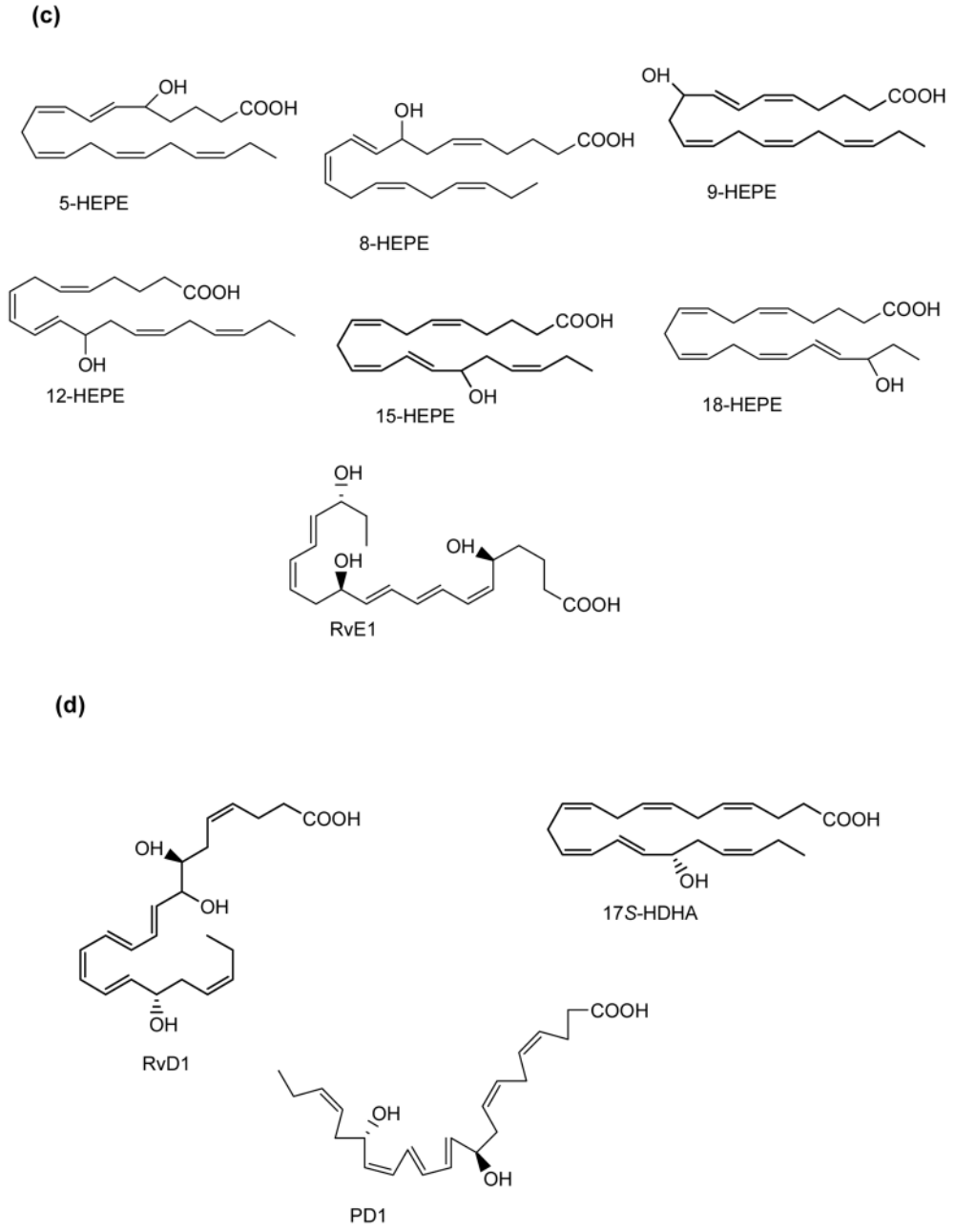

Scheme 1.

Structures of (a) linoleic acid, (b) arachidonic acid, (c) eicosapentaenoic acid and (d) docosahexaenoic acid-derived mono- and poly-hydroxy fatty acid mediators.

Table 1

Multiple reaction monitoring (MRM) transitions for the liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS) assay of hydroxy fatty acids, leukotrienes, resolvins and protectins.

Compound	MRM (*m/z*)	Collision energy (eV)
9-HODE	295→171	25
13-HODE	295→195	25
5-HEPE	317→115	20
18-HEPE	317→133	25
9-HEPE	317→149	20
8-HEPE	317→155	18
15-HEPE	317→175	18
12-HEPE	317→179	20
5-HETE	319→115	20
9-HETE	319→123	20
8-HETE	319→155	20
11-HETE	319→167	20
15-HETE	319→175	18
12-HETE	319→179	20
12-HETE-*d*8	328→185	17
LTB_4	335→195	17
17*S*-HDHA	343→281	15
RvE1	349→195	17
PD1	359→206	15
RvD1	375→141	15

Table 2

Linearity, limit of detection (LoD) and limit of quantitation (LoQ) of the ESI-LC-MS/MS assay for hydroxy fatty acids, leukotrienes, resolvins and protectins. (LoD and LoQ are expressed as pg on-column).

Compound	Equation	Correlation Coefficient	LoD (pg)	LoQ (pg)
9-HODE	y = 2.080x – 0.010	0.994	10	20
13-HODE	y = 0.483x – 0.028	0.987	20	50
5-HEPE	y = 0.511x + 0.021	0.998	20	50
18-HEPE	y = 0.279x + 0.010	0.994	20	50
9-HEPE	y = 0.321x – 0.016	0.996	20	50
8-HEPE	y = 0.877x + 0.037	0.972	20	50
15-HEPE	y = 0.459x + 0.016	0.999	20	50
12-HEPE	y = 0.484x – 0.030	0.999	20	50
5-HEPE	y = 0.564x + 0.050	0.986	20	50
9-HETE	y = 0.169x + 0.043	0.983	20	50
8-HETE	y = 2.688x + 0.073	0.997	10	20
11-HETE	y = 4.623x – 0.046	0.999	10	20
15-HETE	y = 1.083x – 0.013	0.998	10	20
12-HETE	y = 0.472x – 0.007	0.991	20	50
LTB_4	y = 2.127x + 0.031	0.991	10	20
17*S*-HDHA	y = 1.404x + 0.044	0.992	10	20
RvE1	y = 1.949x + 0.208	0.972	20	50
PD1	y = 0.606x + 0.125	0.984	10	20
RvD1	y = 2.979x + 0.008	0.996	20	50

Table 3

Recovery of 20 ng hydroxy fatty acids, leukotrienes, resolvins and protectins from spiked rat plasma, brain and liver. Data is shown as mean standard deviation (SD) (n=3 experiments).

	% Recovery		
Compound	**Plasma**	**Brain**	**Liver**
9-HODE	103±25	100±1	110±8
13-HODE	106±12	95±3	93±11
5-HEPE	84±4	77±7	111±6
18-HEPE	112±12	99±4	102±11
9-HEPE	106±3	101±5	123±9
8-HEPE	102±2	89±7	96±3
15-HEPE	106±7	97±8	83±6
12-HEPE	101±15	91±4	104±9
5-HETE	93±12	78±10	97±9
9-HETE	82±3	105±7	109±8
8-HETE	106±12	87±1	87±9
11-HETE	108±9	92±7	101±13
15-HETE	103±10	96±7	98±1
12-HETE	87±11	115±5	96±3
LTB_4	114±6	80±3	88±5
17*S*-HDHA	89±4	102±2	112±11
RvE1	117±13	102±9	117±5
PD1	109±6	79±4	104±1
RvD1	116±6	76±2	122±11