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Evaluating lipid mediator structural complexity using ion mobility spectrometry combined with mass spectrometry

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Aim: Lipid mediators (LMs) are broadly defined as a class of bioactive lipophilic molecules that regulate cell-to-cell communication events with many having a strong correlation with various human diseases and conditions. LMs are usually analyzed with LC–MS, but their numerous isomers greatly complicate the measurements with essentially identical fragmentation spectra and LC separations are not always sufficient for distinguishing the features. **Results/methodology:** In this work, we characterized LMs using ion mobility spectrometry (IMS) coupled with MS (IMS–MS). The collision cross-sections and m/z values from the IMS and MS analyses displayed distinct trend lines. Specifically, the structural trend lines for sodiated LMs originating from docosahexaenoic acid had the smallest collision cross-section values in relation to m/z , while those from linoleic acid had the largest. LC–IMS–MS analyses were also performed on LMs in flu infected mouse tissue samples. These multidimensional studies were able to assess known LMs while also detecting new species. **Conclusion:** Adding IMS separations to conventional LC–MS analyses show great utility for enabling better identification and characterization of LMs in complex biological samples.

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Keywords: collision cross-section • ion mobility spectrometry • lipid mediators

Over the last decade, there has been a growing interest in the study of lipid mediators (LMs) due to their connection with various diseases and conditions such as asthma, infertility, atherosclerosis, rheumatoid arthritis, ischemia, metabolic syndrome and cancer [1–3]. LMs include, but are not necessarily limited to, eicosanoids, endocannabinoids, sphingolipids, lysophospholipids, phospholipids, fatty acids, ether-linked lipids, platelet-activating factors (PAFs) and oxidized lipids. LMs comprise many different lipid categories, most are produced locally in response to extracellular stimuli and have pivotal roles in inflammation, host defense, immune response and maintenance of homeostasis [1,2]. Several LM categories are illustrated in Figure 1 to show their structural differences and similarities. LMs are initially derived from foods in our diet having omega-3 and omega-6 fatty acids. Their formation then occurs through oxidation of the polyunsaturated fatty acids (PUFA) such as linoleic acid (LA; 18:2), arachidonic acid (AA; 20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (DHA; 22:6). In LM synthesis, the PUFAs are cleaved from the phospholipid membranes by phospholipase (e.g., cPLA₂) and acted upon by stereospecific enzymatic reactions with monooxygenases such as cyclooxygenases, lipoxygenases and cytochrome P450, and/or nonenzymatic reactions with reactive oxygen species [1,3,4].

Despite only selective PUFAs being used to generate the LMs, many LM isomers are extremely difficult to distinguish. For example, the LipidMaps database downloaded in October 2017 had 158 isoprostane species where 118 either had the molecular formula C₂₀H₃₂O₅ or C₂₀H₃₄O₅. These two molecular formulas were also shared by 88 eicosanoid species (e.g., prostaglandins, lipoxins and levuglandins) with 65 corresponding to C₂₀H₃₂O₅ and 23 matching C₂₀H₃₄O₅, where the only differences in these isomers is the location and orientation of the hydroxyl groups, double bonds and side chains attached to the ring structures. Distinguishing these often slight structural and stereochemical differences, however, is crucial as many of the isomers have different and sometimes

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Figure 1. Lipid mediators are sourced from omega-3 and omega-6 fatty acids from foods in our diet such as corn, meat and nuts, and are also synthesized on demand upon extracellular stimuli such as stress, injury and bacterial/viral infections. Four example LM categories are shown with specific lipid species to illustrate both their diversity and structural similarity.

LM: Lipid mediator.

Table 1. HPLC gradient and column wash profiles used for the lipid mediator analyses.

Time (min)	% MPA	% MPB	Flow rate (ml/min)
0	60	40	0.25
2	50	50	0.25
3	40	60	0.25
12	30	70	0.25
15	25	75	0.25
17	22	78	0.25
19	15	85	0.25
20	1	99	0.25

MPA: Mobile phase A; MPB: Mobile phase B.

opposing functional roles. For example, Tam *et al.* [5] identified two LA-derived isomeric mediators 9-HODE and 13-HODE where the former is a pro-inflammatory mediator and the latter is an anti-inflammatory mediator. Serhan *et al.* [6] also compared six isomers of 10,17-dihydroxydocosatriene (protectin D1, DHA-derived mediators) and found that the chirality of the 10-carbon alcohol and geometry of the double bonds influenced the potency of the mediator toward anti-inflammatory activity. Thus, better methods to effectively obtain the exact LM assignment and biological interpretation are needed.

To date, the characterization of LMs has been extremely difficult as most have short half-lives ranging from seconds to minutes [1] and are only present at very low concentrations (~ 0.1 – 10 nM) [7]. These features, in addition to the large number of possible LM isomers present, make their detection and identification problematic. Methods that effectively distinguish, identify and quantify LMs and other signaling lipids are essential for gaining a mechanistic understanding of a host's response to stimulation, which in turn could aid in the development of novel therapeutic treatment targeting lipid metabolism. MS has played a key role in LM measurements to date. Eicosanoids and other fatty acid-derived LMs are currently measured using specific sample preparation methods, and then analyzed with LC–MS/MS measurements, which often use selected multiple reaction monitoring (MRM) in negative ionization [8–12]. Triple quadrupoles and QTRAPs are often utilized to obtain the sensitivity necessary

Figure 2. Ion mobility spectrometry collision cross-section versus m/z trend lines for the lipid mediators studied in both sodiated (left) and deprotonated (right) ionization modes. LMs were grouped based on their parent PUFA metabolites (docosahexaenoic acid, arachidonic acid or linoleic acid) or categories (LysoPCs or PAFs). Distinct patterns were observed for each in their sodiated forms, while the deprotonated forms all seemed to cluster together. CCS: Collision cross-section; IMS: Ion mobility spectrometry; LM: Lipid mediator; PAF: Platelet-activating factor; PUFA: Polyunsaturated fatty acid.

to characterize LMs [8–12]. In fact, Wang *et al.* recently published a method [10] for comprehensively monitoring 184 fatty acid LMs using a 5-min LC gradient and a QTRAP MS. In the study, the MS and MS/MS parameters, including collision energies and MRM transitions, were optimized for each of the 184 standards. This allowed LMs with slight structural variations to be separated, if not by retention time and/or MRM transitions. However, some isomers were still indistinguishable due to their extremely similar LC retention times and fragmentation profiles, thus requiring additional analyses [13]. One appealing technique for LM analyses is integrating ion mobility spectrometry (IMS) between LC and MS separations to obtain a third analytical dimension and add new structural information to the measurements. IMS–MS has been performed in previous lipid analyses to illustrate differences in lipid categories, subclasses and isomeric standards [14–26]. Additionally, LC–IMS–MS results have surpassed the sensitivity of LC–MS studies alone [27–29], indicating they are a promising approach for the detection of low concentration LMs. In this work, we analyzed the separation of different LM standards with IMS–MS, and then assessed the number of hydroxyeicosatetraenoic acid (HETE) isomers and other known LMs present in mouse lung tissue infected with influenza A virus [5].

Materials & methods

Chemicals, solvents, sample preparations & IMS–MS analyses

The LM standards were purchased from Cayman Chemicals and Avanti Polar Lipids, Inc. (Supplementary Table 1). These included 39 LMs derived from AA, LA and DHA, as well as 13 PAFs and lysophosphatidylcholine PAF isomers. Immediately prior to IMS–MS analysis, the LMs were diluted to 0.01 $\mu\text{g}/\mu\text{l}$ with 100% ethanol (Sigma-Aldrich, MO, USA) and stored in the dark. The remaining standards were brought up in 100% methanol (Sigma-Aldrich) to the same final concentration and directly injected into an Agilent 6560 IM quadrupole time-of-flight (QTOF) MS (Agilent Technologies, CA, USA) [30–32] using the nanoflow injection analysis system described in [33]. For the IMS–MS measurements, the Agilent 6560 was outfitted with the commercial gas kit (Alternate Gas Kit, Agilent) and a precision flow controller (640B, MKS Instruments) to allow for real-time pressure adjustment based on absolute readings of the drift tube pressure using a capacitance manometer (CDG 500, Agilent). In this platform, ions were passed through an inlet glass capillary, focused by a high-pressure ion funnel and accumulated in an ion funnel trap. Ions were then pulsed into the drift tube filled with ~ 3.95 torr of nitrogen gas, where they traveled under the influence of a weak electric field (10–20 V/cm). Ions exiting the drift tube were refocused by a rear ion funnel prior to QTOF MS detection and their arrival times were recorded. IMS–MS data were collected from 50 to 1700 m/z with a cycle time of 1 s/spectra to increase the signal of low abundance species. The detailed instrumental settings are given in the Supplementary Tables 2–4, and follow those previously published in an

Figure 3. Differences in LM separation based on ionization mode. IMS separations of **(A)** (±)9-HODE and (±)13-HODE, and **(B)** 9-Oxo-ODE and 13-OxoODE. The position of the double bonds and ketone or alcohol groups on the molecule seems to make a difference on their measured structural sizes for their sodiated (left) and deprotonated (right) forms.

HODE: Hydroxyoctadecadienoic acid; IMS: Ion mobility spectrometry; Oxo-ODE: Oxo-octadecadienoic acid.

interlaboratory study [34] and a drift-tube IMS collision cross-section (CCS) database study [35]. Each sample was measured in triplicate and the RSD values were measured for all analytes and noted in Supplementary Table 1. The Agilent IM-MS Browser software was utilized for all CCS calculations.

LC-MS & LC-IMS-MS analyses

To understand if the IMS dimension aided in better LM identifications in complex samples, mouse lung tissue infected with wild-type influenza A/H1N1, A/H5N1 and A/H7N9 were also studied. The lipids were extracted from the homogenized lung tissue using a modified Folch extraction [36] and reconstituted in 500 µl of methanol prior analyses. The LC-MS/MS studies in this manuscript were performed using a Waters NanoAquity UPLC system interfaced with a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, CA, USA) and the Agilent

Figure 4. Influence of sodium binding in LM separation. Ion mobility spectrometry separations of resolvin D1 and resolvin D2 in their sodiated (left) and deprotonated (right) forms. Again the position of the double bonds and alcohol groups seems to make a large difference on their structural sizes as their structural order swaps between positive and negative mode.

6560 Ion Mobility QTOF MS system [15,37]. For the LC analyses, 10 μ l of lipid extract was injected on to a reversed phase Waters CSH column (3.0 mm $\times$ 150 mm $\times$ 1.7 μ m particle size) and separated with the following solvents (mobile phase A: acetonitrile/water (40:60) containing 10 mM ammonium acetate; mobile phase B: acetonitrile/isopropyl alcohol (10:90) containing 10 mM ammonium acetate). All analyses were performed at a flow rate of 250 μ l.min $^{-1}$ and as previously published [38], except the gradient was stopped after 20 min to increase the LM analysis throughput. The gradient used for these studies is shown in Table 1.

Samples on the Velos were analyzed in both positive and negative ionization using higher energy collision dissociation (HCD) and collision-induced dissociation (CID) to obtain high lipidome coverage. Normalized collision energies of 30 and 35 were used for CID and HCD fragmentation. Both CID and HCD were set to a maximum charge state of 2, an isolation width of 2 m/z units, and an activation Q value of 0.18 for CID. LC–MS/MS raw data files from the pooled samples were imported into an in-house developed software Lipid Informed Quantitation and Identification for manual identification of lipid molecular species. Confident lipid identifications were determined by examining the tandem mass spectra for diagnostic ion fragments along with associated chain fragment information. In addition, the isotopic profile, extracted ion chromatogram and mass error of the measured precursor ions were examined for all lipid species.

The LC–IMS–MS analyses were performed in both positive and negative ion mode and collected from 100–3200 m/z at MS resolution of 40,000. The LC–IMS–MS data were analyzed using in-house Pacific Northwest National Laboratory (PNNL) software for deisotoping and feature finding the multidimensional LC, IMS and MS data [39]. Features were then compared with the accurate mass and time (AMT) tag database generated from LC–MS/MS for identification and quantitation.

Figure 5. Separation of R and S functional groups. Ion mobility spectrometry separations of 8(S)-HETE, 5(S)-HETE, 12(S)-HETE and 12(R)-HETE in their sodiated (left) and deprotonated (right) forms. The structural and R versus S functional differences in the HETEs illustrate how IMS is able to aid in their separations.

HETE: Hydroxyeicosatetraenoic acid; IMS: Ion mobility spectrometry.

Results & discussion

In this study, we utilized IMS–MS to characterize 52 LM standards consisting of isomeric pairs and groups to determine if their CCS separation was possible in either positive or negative ion mode. The study was then extended to the lipid extract of mouse lung tissue infected with wild-type influenza A/H1N1, A/H5N1 and A/H7N9 to determine how many additional LMs could be distinguished when the IMS dimension was added to LC–MS measurements. The results of these analyses are shown below and all utilized raw IMS–MS data files are available on the NIH Metabolomics Workbench website (DataTrack ID: 1316), if further evaluation is desired.

LM standard analyses in positive & negative polarity ionization modes

To understand the structural differences of LMs, 52 standards were characterized by IMS–MS (Supplementary Table 1). These LMs were from different PUFA sources such as DHA (22:6 PUFA), AA (20:4 PUFA) or LA (18:2 PUFA), where all double bonds have a *cis* orientation, and also belonged to different categories such as PAFs and monoacylglycerophosphocholines (lysoPCs), which are isomeric and have very similar fragmentation profiles making their confident identification and biological interpretations difficult. To date in most LM analyses, only negative ionization mode is utilized, however, positive ionization mode evaluations were also performed here to understand how ionization affects the IMS separation. Further since most LMs do not protonate, measured CCS and *m/z* values of either sodiated $[M+Na]^+$ or deprotonated $[M-H]^-$ ions are shown in Figure 2. As PAFs and lysoPCs do not deprotonate, they were not included in the negative ion analyses.

Upon analysis of all the standards purchased, the sodiated CCS versus *m/z* trend lines were found to have more width per PUFA group and specific trend lines, while all deprotonated ions mainly clustered together with the same trajectory. This indicated that sodium binding alters the structure of the LMs and allows better separation of the isomers and PUFA groups. Interestingly, the sodiated PUFA group sizes were ordered as DHA (22:6) < AA (20:4) < LA (18:2). Since there was no structural trend for the PUFA groups in negative mode, this showed that the number of double bonds had a huge effect on how sodium binds to the molecules. Finally, upon comparison of the sodiated PAFs and lysoPCs, the lysoPCs were found to have smaller sodium-bound structures than the PAFs. Both the PAFs and lysoPCs also had distinct trend lines, illustrating their structural differences. This CCS

Figure 6. Epoxyeicosatrienoic acid mediators are difficult to separate. Ion mobility spectrometry separations of 8(9)-EET, 14(15)-EET, and 11(12)-EET in their sodiated (**left**) and deprotonated (**right**) forms. The EETs lipids are difficult to separate, but some distinction was noted for 14(15)-EET in the deprotonated form. EET: Epoxyeicosatrienoic acid; IMS: Ion mobility spectrometry.

information provides the basis for future analyses of known and unknown LMs in complex environmental and biological samples.

LM isomer separations

To better understand the width of the CCS distributions for the PUFA groups observed in Figure 2, each isomer pair or group was analyzed individually. Out of the 52 LM standards studied, 42 were isomer pairs or groups. All 42 were found to be separated to some degree in either positive or negative ionization mode, however one isomer pair (5-iPF2 α -VI and PGF2 α ; Supplementary Table 1) was very similar in both modes. Interestingly, when all isomeric pairs were compared, there were times when both the positive and negative mode separations were comparable and other times when large difference occurred. For example, the hydroxyoctadecadienoic acid (HODE) isomers, ($\pm$)9-HODE and ($\pm$)13-HODE had similar IMS separations in both positive and negative mode (Figure 3a). However, the oxo-octadecadienoic acid (Oxo-ODE) isomers, which are metabolites of the HODEs, were quite different in the two modes. As shown in Figure 3b, 9-OxoODE and 13-OxoODE were baseline separated in positive mode with 13-OxoODE becoming much smaller than 9-OxoODE, whereas in negative mode the isomers were virtually inseparable.

Another interesting trend was when the LM isomers completely reversed IMS order upon sodium binding. This was the case for several of the isomeric pairs, but was most drastic for resolvins D1 and D2. As shown in Figure 4, these isomers swapped orders and additionally the change was so great that they were almost baseline resolved in both modes. Thus, sodium binding significantly influenced the structure of these molecules.

LM isomers only having R and S functional group variations are also very difficult to separate with current studies and not typically done [8–10]. In our previous studies with intact ceramides, we found that the R versus S orientations of hydroxyl groups could be separated in positive ionization upon sodium binding [22]. Here again, we show this outcome also applies to LMs. 7R- and 7S-maresin-1 were indistinguishable in negative mode, but could be separated in positive mode upon the addition of a sodium ion. 12R-HETE and 12S-HETE were also

Figure 7. The negative mode XIC for hydroxyeicosatetraenoic acid isomers in mouse tissue infected with H7N9. A similar MS/MS profile was seen for the whole XIC range indicating the peak matched to HETE species. IMS analyses illustrated five distinct CCS values in this XIC with three peaks corresponding to the 12S-, 8S- and 5S-HETE species and two additional HETE species being unassigned since we did not have corresponding CCS values in our database. CCS: Collision cross-section; HETE: Hydroxyeicosatetraenoic acid; IMS: Ion mobility spectrometry.

barely separated in negative mode, but their structural differences were greater in positive mode upon the sodium addition (Figure 5).

Finally, we note that the epoxyeicosatrienoic acid (EET) standards were difficult to separate in either positive or negative ion mode as shown in Figure 6. In fact, the EETs were almost better separated in their deprotonated forms and sodium binding seemed to make them all have a similar structure size. This occurs due to the structural similarity of the EETs and indicates that either additional separations (i.e. LC or GC) or higher resolution IMS techniques, such as those recently developed using structure for lossless ion manipulation devices [40–42], may be required for their separation.

LC–IMS–MS LM analyses of complex samples

To better understand the utility of IMS for LM analyses, LC–IMS–MS platform was used to study the total lipid extract of lung tissue from mice infected with different strains of influenza A virus. Influenza is one of the most common causes of human respiratory infections with 291,896 cases confirmed in 2014 alone [43]. Furthermore, it is thought that LMs may provide the novel, effective therapies needed for the treatment of severe influenza infection [5,44]. Tam *et al.* previously detected various LMs, such as PGE₂, PGF₂ α , 6-keto PGF₁ α , and the HODE and HETE isomers, as being upregulated in influenza samples [5]. Utilizing LC–IMS–MS and MS/MS in both positive and negative modes, we were able to detect and identify all of these LMs in our samples except for the HODEs, showing the sensitivity of the LC–IMS–MS approach [45,46]. Next, we utilized LC retention time and MS/MS information to identify HETE specific isomers. As shown in Figure 7, three main LC peaks were evident in the negative mode extracted ion chromatogram (XIC) for the HETE molecules in the H7N9 samples

($m/z = 319.23$), all having similar fragmentation spectra. Interestingly, when the IMS structural information for this XIC window was examined, five different CCS values were observed as shown by the arrows and assignments to the peaks. Upon matching to our LM CCS library, three of the IMS structures matched the 12S-HETE, 8S-HETE and 5S-HETE isomers in the LM CCS database, but two were not present and are therefore shown as question marks. Since all of these IMS peaks had HETE fragmentation profiles, these additional peaks are thought to just be two different HETE isomers, which we did not have standards for. Thus, this LC–IMS–MS study shows its potential for identifying known LMs and obtaining information about other LM species present in the samples for further investigation.

Conclusion & future perspective

In this work, we characterized the structural separation of 52 LMs using IMS–MS. The LMs were analyzed in both positive and negative ion mode as sodiated $[M+Na]^+$ and deprotonated $[M-H]^-$ ions, where more separation was observed for the sodiated species from different categories or those originating from distinct PUFAs than the deprotonated forms. Specifically, the structural trend lines for sodiated LMs originating from DHA had the smallest CCS values in relation to m/z , while those from LA had the largest. Interestingly, in negative mode all the LMs had a similar trend line and did not separate due to the PUFAs. This indicated that the number of double bonds had a great influence on the structure of the sodium-bound molecules, but did not affect the size of the negatively charged molecules. Analysis of the LM isomers illustrated that most could be distinguished by IMS with some almost baseline separated. These results showed great promise for IMS–MS separation of LMs in complex biological samples. Finally, LC–IMS–MS analyses were performed on flu infected mouse tissue. LMs in our database known to be overexpressed in these cases were detected in the samples and additional LM species were also observed. Since these species were not in our database, they could not be fully identified. However, the results possible from the LC–IMS–MS analyses show great promise for evaluating and detecting novel LM species in future studies.

Summary points

- There is a growing interest in the study of lipid mediators (LMs) due to their connection with various diseases and conditions.
- LMs are usually analyzed with LC–MS.
- The numerous LM isomers however greatly complicate LC–MS measurements.
- Adding an ion mobility spectrometry (IMS) separation into conventional LC–MS analyses provides a sensitive platform capable of better separating the diverse LM isomers.

Results & discussion

- LM standards and LMs in lung tissue were assessed with IMS–MS and LC–IMS–MS.
- In the standard analyses, 52 LM standards consisting of isomeric pairs and groups were analyzed in triplicate with IMS–MS.
- The IMS collision cross-section was determined for each standard in positive and negative ion mode.
- More structural separation was observed for the sodiated LM species than the deprotonated forms.
- The number of double bonds in each LM also had a great influence on the structure of the sodium-bound molecules, but did not affect the size of the negatively charged molecules.
- Mouse lung tissue infected with wild-type influenza A/H1N1, A/H5N1 and A/H7N9 was then assessed with LC–IMS–MS to determine how many additional LMs could be distinguished when the IMS dimension was added.
- These multidimensional studies were able to identify known LMs while also detecting new species.

Conclusion

- Collision cross-section values for 52 different LMs standards and isomer groups are presented in this manuscript.
- All the isomers could be distinguished in either positive or negative modes.
- Adding IMS separations to conventional LC–MS analyses shows great utility for enabling the better identification and characterization LMs in complex biological samples.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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