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SelexION™ Mobility Separation of Leukotriene Isomers

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ABSTRACT

Eicosanoids and docosanoids are bioactive lipids, featuring numerous stereoisomers with a highly specific structure-activity relationship. Moreover, the geometry of these compounds also reflects their biochemical origin. The unambiguous characterization of these isomers of the eicosanoid and docosanoid classes is thus important to the understanding of their origin and function. However, many eicosanoids are isoelemental and structurally closely related which make differentiation by high resolution ESI-MS or even ESI-MS/MS difficult or impossible.

SelexION™ technology can separate isomeric compounds based on their dipolar moment in an asymmetrically oscillating electrical field and is thus truly orthogonal to LC and MS. Here we developed a SelexION™ DMS method, that is capable of distinguishing at least five related leukotrienes which are (almost) unresolvable by using LC-MS/MS only.[1]

We applied this method to the separation of LTB_4 and its co-eluting isomer 5S,12S-diHETE in murine exudate cells. The results of this experiment show that LTB_4 is present only after zymosan A injection in murine peritoneal cells, while its isomer 5S,12S-diHETE is produced by resident murine peritoneal cells after control saline administration. Furthermore, we show that the SelexION™ technology can also separate two isomeric protectins PD1 and PDX (10S,17S-diHDHA).

INTRODUCTION

Eicosanoids and docosanoids are very low abundant (IC50 pM-nM) but highly bioactive lipid mediators (LM) with highly specific structural features like double bond geometry and stereo-centers which play a crucial role in defining their bioactivity and thus exhibit a strong structure-activity relationships.[2] Furthermore the exact stereochemical structures give insights into their biochemical origin.[3] Many eicosanoids are isoelemental and structurally closely related diastereomers and/or geometric E/Z-isomers, which makes characterization by high resolution ESI-MS or even ESI-MS/MS difficult or impossible.

In the present study, we developed a SelexION technology based differential ion mobility (DMS) method to separate isoelemental eicosanoids. SelexION™ technology is capable of separating ions based on their mobility in an oscillating asymmetric electrical field. Thus, it is "orthogonal" to chromatography or mass spectrometry. We show that microLC-DMS-MS/MS is a valuable tool for the unambiguous assignment of closely related eicosanoids and docosanoids. We investigated three sets of stereoisomers which are known to overlap in traditional LC analysis. First we studied the separation of the enzymatically generated chemotactic lipid mediator LTB_4[4], the non-enzymatic LTA_4 hydrolysis products 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and the isomer 12-epi-LTB_4. When compared to LTB_4, 6-trans-LTB_4 only differs in the double bond geometry at position 6; 12-epi-LTB_4 differs in the configuration of the stereocenter at position 12, while 6-trans-12-epi-LTB_4 is a combination of geometric double bond isomerism and enantiomeric stereocenter at position 12.

In addition, we investigated the separation of the platelet-neutrophil interaction product 5S,12S-diHETE[5] and LTB_4. While some of these isomers can be chromatographically resolved using reversed phase LC[3], LTB_4 and 5S,12S-diHETE as well as 12-epi-LTB_4 and 6-trans-12-epi-LTB_4 are almost completely co-eluting. Particularly interesting from a biochemical/biological perspective is the separation of the eicosanoid pair LTB_4 and 5S,12S-diHETE. It is known that these substances are difficult to separate using chromatographic techniques, but arise from different biochemical pathways and differ in their bioactivity.[4] Finally, we explored the separation of the closely eluting isomers PD1 and PDX, two components of recent interest due to the benefit of their biological effects[6], where confusion about the exact stereochemistry has however been apparent in recent literature

MATERIALS AND METHODS

Materials. The following synthetic standards were selected for analysis: Leukotriene B_4 (LTB_4), 6-trans-LTB_4, 6-trans-12-epi-LTB_4, 12-epi-LTB_4 and 5S,12S-diHETE. 5S,12S-diHETE and PD1 were prepared in-house. All other lipids were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Animal experiments. The zymosan A murine peritonitis model was produced by injecting 1 mg of zymosan A dissolved in PBS intraperitoneally (*i.p.*) as described elsewhere[7]. For comparison, mice were injected with PBS only. The study was approved by the Experimental Animal Committee, Ministry for the Environment in Iceland. At given time-points after induction of peritonitis (2 h for control mice and 24 h for zymosan A mice), peritoneal lavage was collected and cells obtained by centrifugation (10 min, 225 × g). The cells were extracted with 300 µL EtOH by shaking for 5 min and ultrasonification for 10 sec. Extracts (30 µL) from two mice were pooled, dried under a gentle stream of nitrogen and re-dissolved in 50 µL 40% MeOH.

microLC 200 Chromatography Chromatography was performed using an Eksigent MicroLC 200 system (SCIEX, USA) and a HALO C18 (2.7 µm; 0.5 × 50 mm) column kept at 50 ºC. The injection volume was 4 µL. Gradient elution was performed using 0.1 % formic acid in water (eluent A) and 0.1 % formic acid in acetonitrile (eluent B). The flowrate was 15 µL/min. The gradient was as follows: 0 – 0.5 min – 65% A; 1 min – 30% A; 3 – 4 min – 5% A; 4.02 – 5 min – 35% A.

QTRAP® 5500 mass spectrometry: For detection a QTRAP® 5500 system equipped with a SelexION™ DMS technology cell (SCIEX USA) was used. N_2 was used as resolution gas at 20 psi, modifiers were doped into the carrier gas when used. Further instrument parameters were: CUR: 20 psi; GS1: 30 psi; CAD: Medium; IS: -4500 V TEM: 0˚C; DP: -120 V; CE: -23 V; DT: 150 ˚C; DR: Low (N_2, 20 psi); SV: 4500 V; MRM: *m/z* 335.2 -> 195.2; Dwell time: 125 ms.

RESULTS

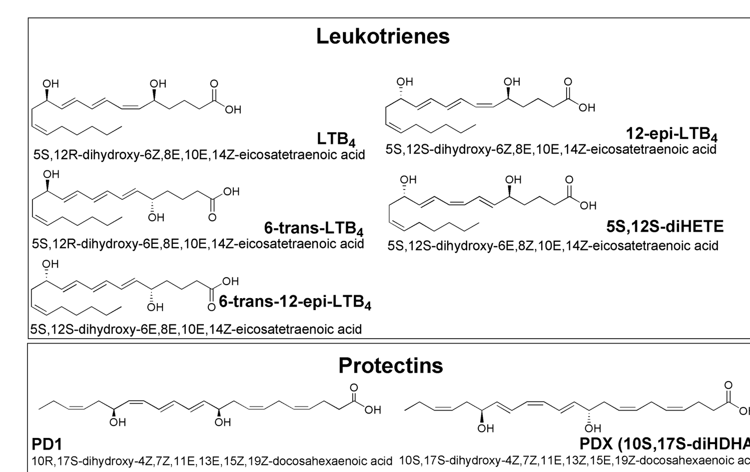

Figure 1 shows the structures and IUPAC names of the leukotrienes and protectins analyzed in this study. The molecular weight of the leukotrienes is 336.2 g/mol, and that of the protectins is 360.2 g/mol

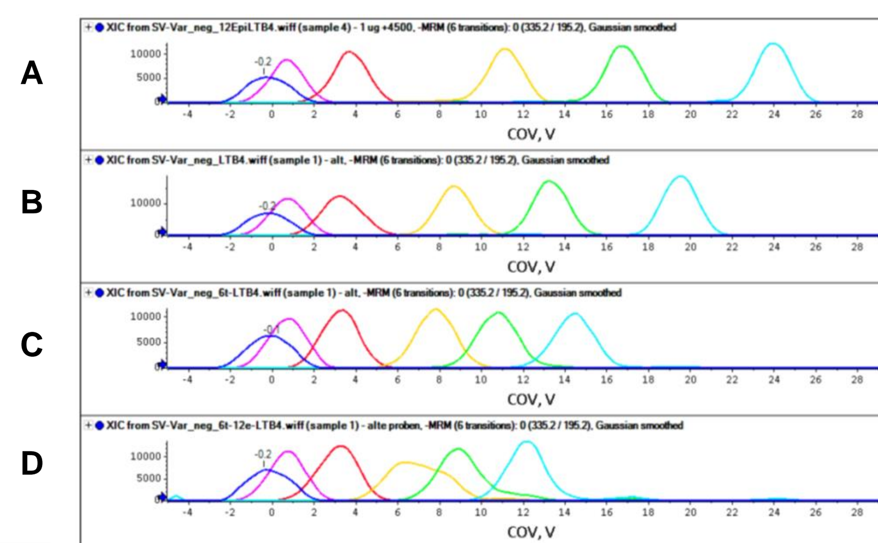

Figure 2 shows the ramping of the compensation voltages (COV) at six different separation voltages (SV) for separation, the four leukotrienes 12-epi-LTB_4 (A); LTB_4 (B); 6-trans-LTB_4 (C) and 6-trans,12-epi-LTB_4 (D) analyzed by direct infusion (100 ng/mL). The COV was ramped at six different Separation Voltages (0 V [blue], 1500 V [pink], 2500 V [red], 3500 V [yellow], 4000 V [green] and 4500 V [light blue]. The compound 6-trans-12-epi-LTB_4 (D) and 6-trans-LTB_4 (C) are not base line separated at SV 4500 V.

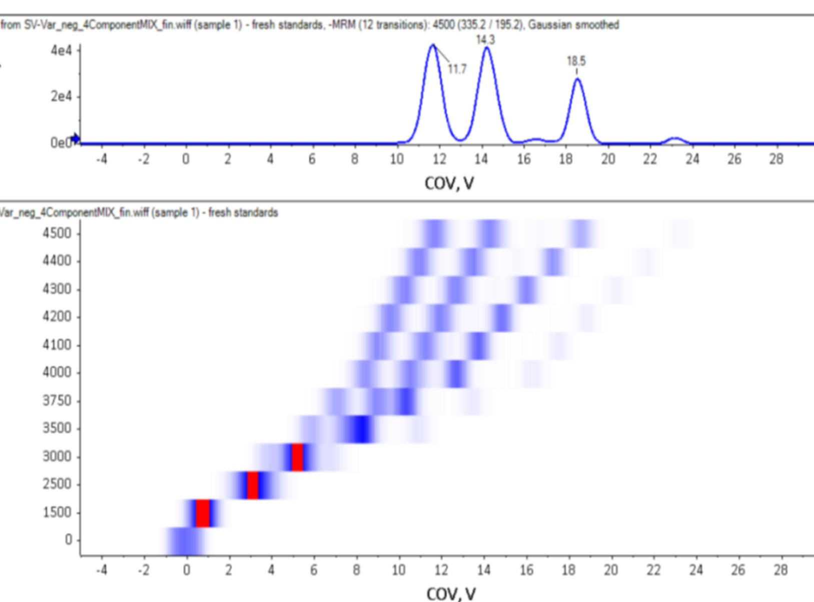

Figure 3 shows the SV mapping with direct infusion and the use of resolution gas of a mixture of four compounds 12-epi-LTB_4, LTB_4, 6-trans-LTB_4 and 6-trans-12epi-LTB_4 (all compounds 100 ng/mL, 12-epi-LTB_4 10 ng/mL). The COV was ramped at twelve SVs. Resolution gas was set to low (20 psi). The upper panel shows the separation of the four compounds at SV 4500 V. The lower panel shows the heat map of all SVs. Usage of the resolution gas now allows for base line separation of 6-trans-12epi-LTB_4 and 6-trans-LTB_4. Traces are: 12-epi-LTB_4, LTB_4, 6-trans-LTB_4 and 6-trans-12epi-LTB_4 - COV: 23.2; 18.5; 14.3; 11.7.

Analyte	COV [V], DI	COV [V], microLC	SV [V]	Rt [min]
LTB_4	18.5	18.3	4500	1.74
6-trans-LTB_4	14.3	14.9	4500	1.72
6-trans-12-epi LTB_4	11.7	12.3	4500	1.72
12-epi-LTB_4	23.1	n.d.	4500	n.d.
5S,12S-diHETE	n.d.	20.3	4500	1.77
PD1	13.4	n.d.	4500	n.d.
PDX	12.0	n.d.	4500	n.d.

Table 1 Comparison of the COV maxima between direct infusion (DI) and microLC. Thanks to the low flow rate of the microLC instrument of 15 µL/min, COV-values are similar between both infusion methods, COV: Compensation Voltage, SV: Separation Voltage; Rt: Retention time; n.d. not determined.

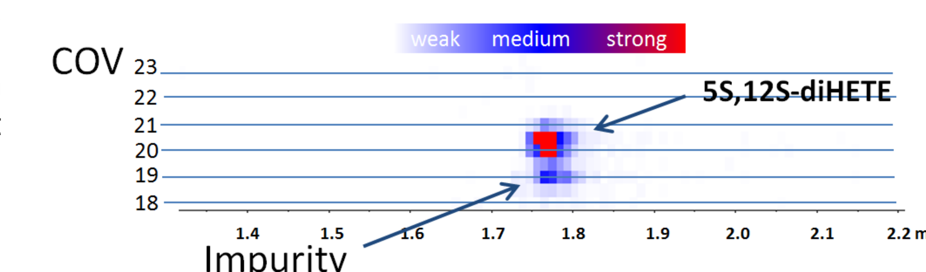

Figure 4: Heatmap of the on-column mapping of the 5S,12S-diHETE sample at SV 4500 V. The strongest signal for 5S,12S-diHETE can be seen at a CoV of approximately 20.3 V at an Rt of 1.77 min. A background impurity can be seen in the biologically synthesized 5S,12S-diHETE at COV 18.9 V.

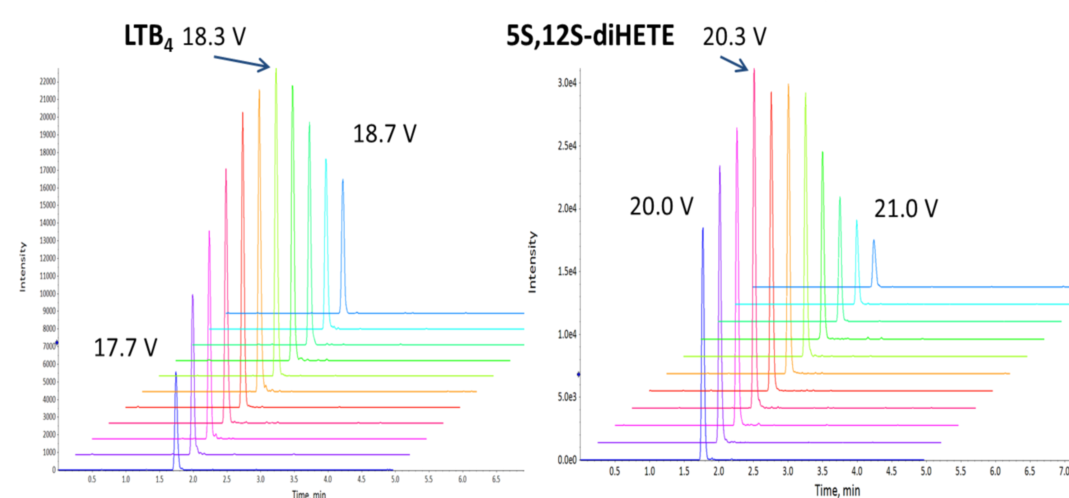

Figure 5: On-column COV mapping of LTB_4 (left) and 5S,12S-diHETE (right), SV 4500 V. As can be seen the maximum for LTB_4 is 18.3 V while for 5S,12S-diHETE is 20.3 V. A more detailed mapping of the COV values around the individual maxima.

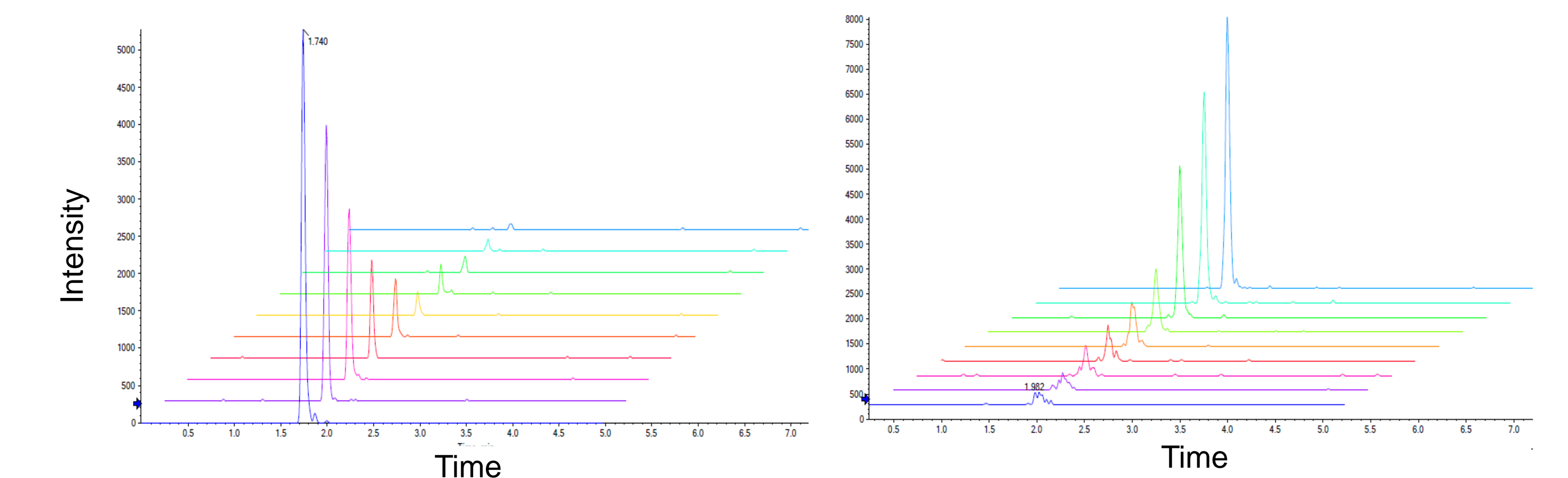

Figure 6: COV control experiment for the separation of 5S,12S-diHETE and LTB_4. Left panel: LTB_4 was injected and COV values were ramped from 19.2 V - 20.1 V with a step size of 0.1 V (COV range of 5S,12S-diHETE). Right panel: 5S,12S-diHETE was injected and COV values were ramped from 17.9 V - 18.7 V with a step size of 0.1 V (COV range of LTB_4). Starting from COV values of around 20 V selectivity for 5S,12S-diHETE is obtained as no LTB_4 signal is left in the trace *m/z* 335.2 -> 195.2 at an Rt of 1.75 min (left panel). *Vice versa* does the biologically synthesized 5S,12S-diHETE used in this experiment only show a minor background signal at the very specific COV value of 17.9 V and an Rt of 1.75 min (right panel). However a minor overlap/"cross-talk" with an unknown impurity was observed at 18.3 V. Hence, for the peritonitis sample we chose the more selective COV value of 17.9 V when monitoring LTB_4.Please also compare Figure 5.

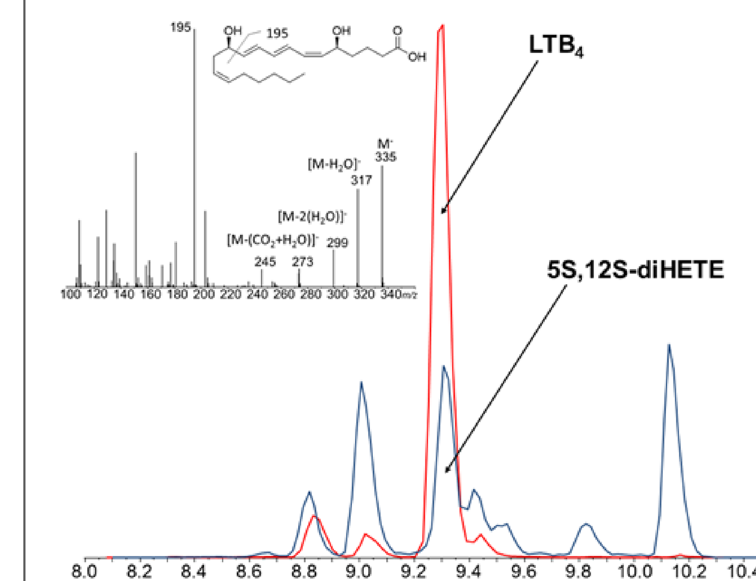

Figure 7: LC-MS/MS analysis of an extract from peritoneal cells measuring the transition *m/z* 335→195. Red trace: 24 h after zymosan A challenge; Blue trace: 2 h after PBS injection (control group). Upper left inset: Product ion spectrum of LTB_4 and its isomers, showing the typical fragment ion *m/z* 195.

Figure 8: Analysis of murine peritoneal cell extracts using microLC-DMS-MS/MS. Upper panel; control mice (PBS injection). Lower panel, zymosan A challenged mice. Red traces; SV 4500 V and COV 17.9 V, blue traces; SV 4500 V and COV 20.3 V. The biochemical pathways leading to LTB_4 (lower panel) and 5S,12S-diHETE (upper panel) are shown[8,9].

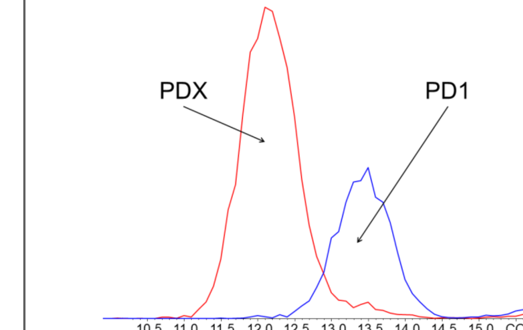

Figure 9: Direct infusion-DMS separation of PD1 (blue graph) and PDX (red graph). A solution of 50 ng/mL was used for the direct infusion experiments with a low resolution gas settings and a separation voltage (SV) of 4500V.

CONCLUSIONS

The bioactivity of eicosanoids and docosanoids is specific to their exact stereochemistry and LC-MS/MS has only limited suitability to resolve isomeric species. We showed that SelexION™ technology with its orthogonal selectivity to LC or MS/MS techniques allows for the unambiguous assignment of low abundant lipid species in biological samples. Our results show great promise for future applications of the SelexION™ technology in eicosanoid and docosanoid analysis, with a greatly improved assignment of isomeric components. Future applications may include COV voltages as additional physicochemical characteristics in libraries, high throughput DI based cellular screening assays, monitoring of biochemical pathway markers and rapid screening for drug development. Furthermore, the expansion to other substance classes, e.g. isomeric fatty acids would be interesting to explore. Finally, it will be important to further our capability to predict a compound's DMS behavior and therefore its COV values based on structural features.

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