Separation of Diastereomeric Flubatine Metabolites using SelexION® Technology

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Positron emission tomography (PET) is a technique for in vivo imaging, that plays an important role for the diagnosis of brain diseases, for which selective radiotracers are required.[1] Use of PET is expected to increase as brain disease such as Alzheimer's Disease are on the rise.[2] Starting from the alkaloid epibatidine, which naturally occurs in the skin of an Equadorian poison dart frog (Epipedobates anthonyi), modifications of its structure led to the development of the PET tracers (+) and (-) [18F]Flubatine. Both enantiomers are suitable for the imaging of α4β2-nicotinic acetylcholine receptors[3] which are involved in learning and memory processes. Their levels are known to be reduced in certain brain areas of patients with early Alzheimer's disease.[4] Related clinical research studies have been conducted and are currently being performed.[5] In support of such trials, radiometabolites potentially formed in human are being investigated. Due to the low concentrations of a PET tracer administered, identification of radiometabolites is performed with the aid of non-radioactive reference compounds, using *in vitro* studies.

Here, isomeric Flubatine monohydroxy metabolites (Figure 3, M1 and M2) which have been produced after incubation with liver microsomes from mouse or human have been studied. Because analysis is performed from the complex matrix, use of differential mobility separation (Figure 1) was important for the reduction in isobaric interferences and the separation of stereoisomers.

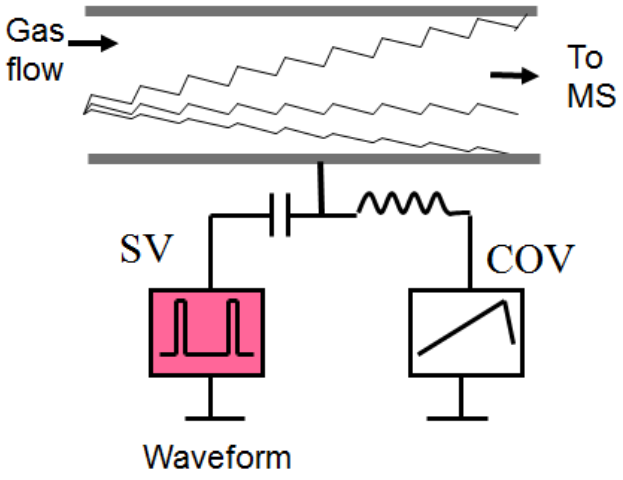

Figure 1: SelexION® Technology for Differential Mobility Separation (DMS). In the planar DMS cell, two potentials are applied to influence the trajectory of a traversing ion. RF separation voltages (SV) are applied across the ion transport channel. Due to differences between high and low field ion mobility coefficients, ions migrate toward the walls and leave the flight path. However, the trajectory of specific ions can be corrected by a counterbalancing compensation voltage (COV), a DC potential that steers select ions into the mass spectrometer.

Key Feature of SelexION® For Separation of Isomers

- SelexION® differential mobility separation technology (DMS, Figure 1) provides an additional dimension of selectivity and is easily combined with other common separation techniques e.g. chromatography or MS/MS.
- DMS allows distinguish of constitutional isomers and diastereomers to enable interference-free quantification
- A general background and interference elimination for MRM base quantification is possible.
- Mapping of compensation voltage, a compound specific DMS parameter, generates a unique fingerprint for future comparison of metabolite samples.
- DMS based isomer separation allows for shortened LC gradients, resulting in increased throughput.

Methods

Sample Preparation: Proteins from a mouse liver microsome incubation were precipitated via addition of cold acetonitrile (1:4 v/v). Afterwards, the mixture was centrifuged and the resulting supernatant was dried in vacuo. The sample was reconstituted using a mixture of 50% acetonitrile in water. Finally, this solution was diluted 1:10 in water yielding samples ready for LC-MS/MS injection.

Chromatography: Separation was performed using an Agilent 1290 HPLC system and a Kinetex PFP 50×2.10mm, 2.6 µm (Phenomenex). The flow rate was 0.5 mL/min and the column temperature was 60 ºC. Mobile phase A was water + 2.5 mM NH_4formate and Mobile phase B was acetonitrile + 2.5 mM NH_4formate and the gradient used is outlined in Table 1. Injection volume was 5 µL.

Table 1. Gradient for Rapid Separation.

Time	%A	%B
0.0	*95*	*5*
1.5	*95*	*5*
5.0	*5*	*95*
5.0	*5*	*95*
6.7	*95*	*5*
8.0	*95*	*5x*

Mass Spectrometry: Analysis was performed on a QTRAP® 5500 System equipped with a SelexION® Device and the Turbo V™ Source in positive ion mode. An MRM method consisting of two MRMs was used (223.1 → 110.0; 223.1 → 124.1) with a dwell time of either 150 or 200 msec and collision energy of 37 V. Source parameters were CUR of 15, IS of 5500V, Tem of 550 ºC, GS1 and GS2 of 60, and CAD at high.

Data processing: Data was reviewed in PeakView® Software.

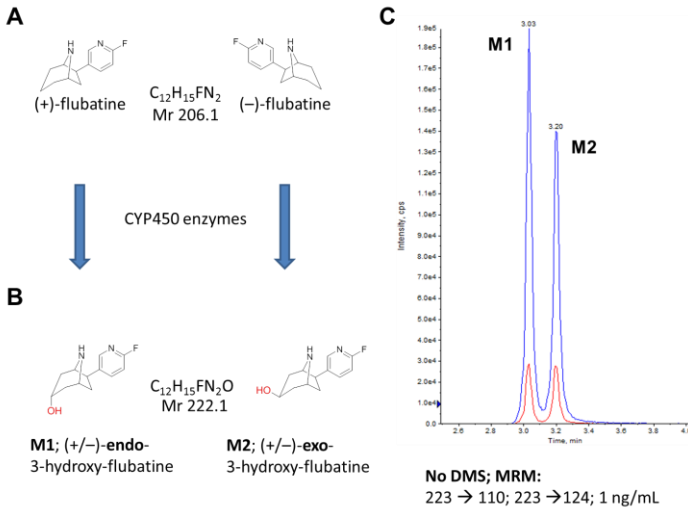

Figure 2. MRM Investigation of Flubatine. A) Flubatine enatiomers are enzymatically hydroxylated by mouse liver microsomes. B) M1 and M2 are possible mono-hydroxy products (shown for (+)-Flubatine), available as racemic reference compounds. C) MRM crosstalk of Flubatine metabolites M1 and M2.

Optimization of Isomer Separation

To first investigate the applicability of SelexION® Technology for the separation of Flubatine metabolite isomers, diastereomeric monohydroxylated products M1 and M2 were chosen for analysis (Figure 2, both available by chemical synthesis). After MS/MS parameter optimization, the DMS separation and compensation voltages were optimized. Differentiation of the two isomers was possible using SVs above 3600 V and resolution gas. This gas counterflow increases the residence time of ions traversing the DMS cell, to enhance the focusing effects. DMS peaks were sharpened, but a concomitant loss of sensitivity was observed (data not shown).

Next, the influence of gas phase modifiers on the DMS separation was investigated. Isopropanol (IPA), setting on “low”, featured a tenfold increase of the peak capacity in the COV space (Figure 3 A, B). A comparison of absolute signal intensity and necessary separation efficiency resulted in selection of an optimal SV of 3000 V with no additional use of resolution gas for the optimal separation of M1 and M2 (Figure 3 C). After adjustment, these results were verified on column. A 5 µL injection of a M1/2 mixed standard with a concentration of 1 ng/mL gave S/N ratios (3×Std.Dev.Noise; 3 points smooth) of 1040 and 464 for M1 and M2, respectively.

Analyzing from a Biological Matrix

Following the successful separation of diastereomeric mono-hydroxy Flubatine standards, a biological sample was investigated. Both, (+)- and (-)-Flubatine were incubated with mouse liver microsomes to result in products formed enzymatically. After precipitation of proteins, samples were analyzed using a standard LC-MS/MS MRM strategy. Retention time analysis of the chromatogram showed, that the sample contained M2 and two additional unidentified isobaric Flubatine metabolites (Figure 4A).

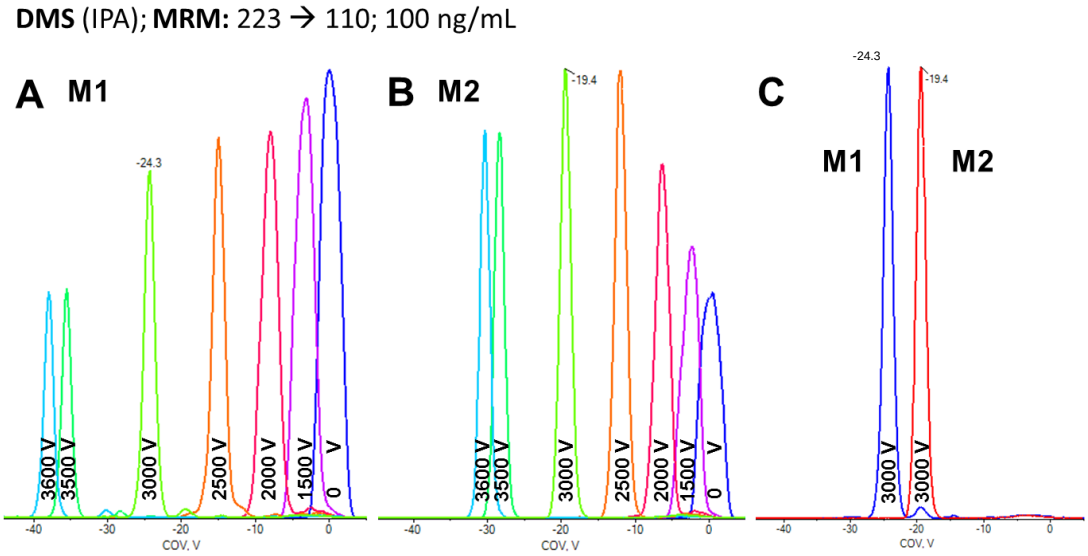

Figure 3. **Direct Infusion Experiments Showing the Correlation of DMS Separation and Compensation Voltages.** Each analyte was infused at a series of fixed separation voltages (SV) and the COV values were ramped to measure the optimal voltage and determine the transmission efficiency for M1 (A) and M2 (B). An evaluation of absolute signal intensities and necessary separation efficiency gives an optimal SV at 3000 V. C) An overlay of the traces for each at 3000V highlights the baseline separation obtained for M1 and M2.

For further characterization of the sample, an "on column" DMS COV mapping strategy was employed. A fixed SV was chosen and the same MRM (223.1 → 110.0) with stepwise varied COV values was added multiple times to an acquisition method. Hence, a COV profile for each individual LC peak could be recorded. This approach has several advantages:

1. DMS parameters are temperature sensitive. It allows an easy adjustment of direct infusion (DI) parameters to a high flow LC set-up,
2. It allows DMS parameter optimization of non-purified compounds, in biological matrix,
3. A significantly lower concentration/amount of sample is needed,
4. A LC-DMS fingerprint for sample characterization and comparison is generated. However, several injections with decreasing COV stepping size will be needed, consuming extra time compared to a sole DI workflow.

"On column" COV mapping of the sample from microsomal incubation using isopropanol as modifier showed a distinct overlap of M2 and the first unidentified Flubatine metabolite MX1 (see Figure 4B). Gas phase modifiers in a DMS cell can be compared to the solid phase in chromatography. Hence, selectivity of DMS separation can be influenced via usage of different gas phase modifiers. Classic candidates are isopropanol, acetonitrile, ethyl acetate, methanol, n-butanol and mixtures thereof.[6-8] Figure 4C shows the influence of methanol on the separation of the observed metabolites. Here, a parameter range in the COV space (1 V steps, SV 3000 V) exists that allows an interference free isolation of M2. An additional experiment with a very fine stepping of only 0.1 V provided an optimal COV of 13.6 V (see Figure 4D).

Reanalysis of the biological sample using only one MRM with the determined COV gave an inference free M2 signal with minimal background (5 µL injection, S/N 2400, Figure 5).

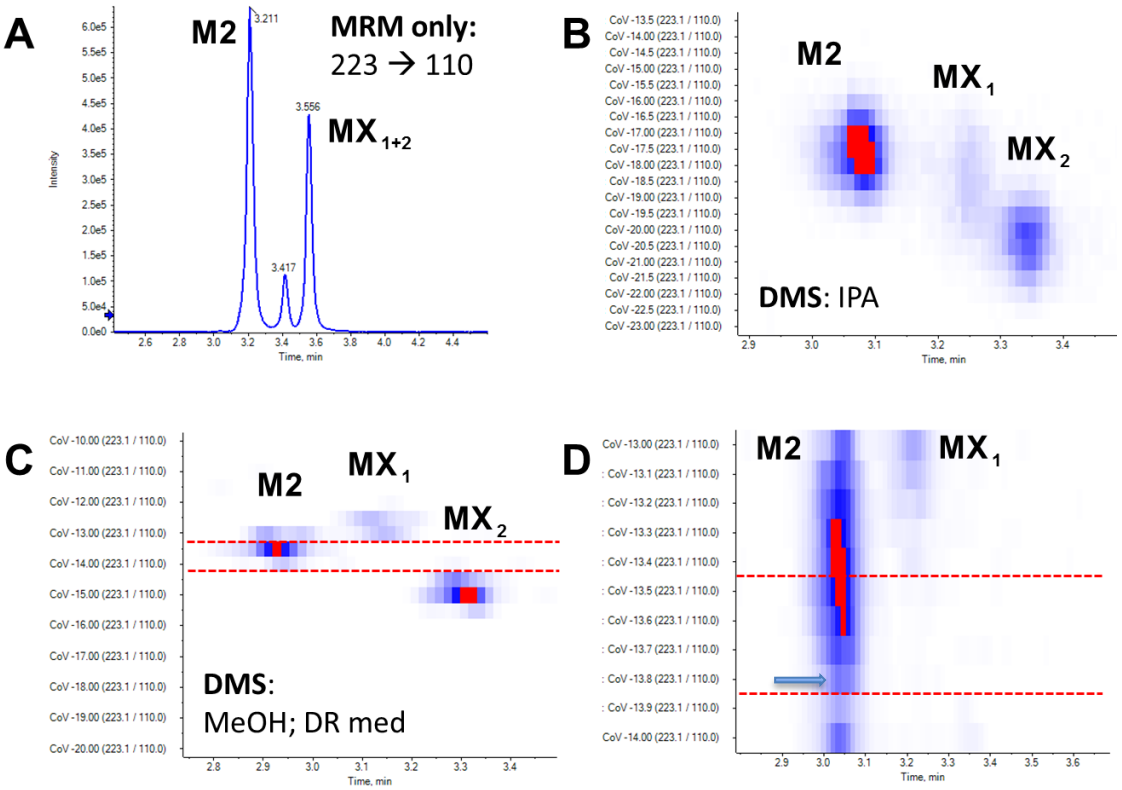

Figure 4. On-Column COV Mapping. Investigation of a metabolite mixture obtained from incubation of (-)-Flubatine with mouse microsomes. A) Conventional LC-MRM chromatogram of a Flubatine metabolite mixture derived from a mice microsome transformation, B) COV mapping of the same sample using SV 3000 V and isopropanol as a gas phase modifier; C) COV mapping using SV 3000V, a medium resolution gas and methanol as a gas phase modifier. The red lines highlight the COV range allowing for an interference free analysis of M2. D) A COV of -13.6 V is optimal, as determined by mapping with small 0.1V COV steps.

Conclusions

1. SelexION® DMS technology enables the separation of diastereomeric monohydroxylated Flubatine metabolites.
2. An "on column" DMS workflow that allows the optimization of COV values for unpurified metabolites and "fingerprinting" of the sample.

SelexION® DMS technology features a selectivity orthogonal to conventional chromatography and MS/MS. Additional selectivity reduces the risk of false positive assignments in complex samples and ultimately increases sample throughput as LC gradients can be significantly shortened.

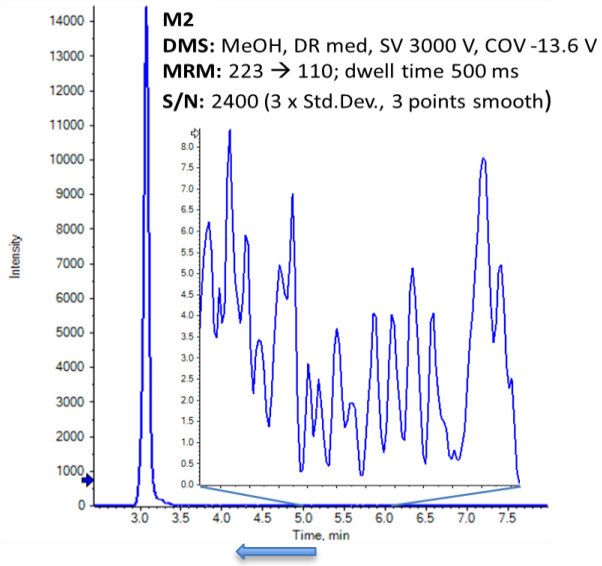

Figure 5. Final Assay for M2. Using the optimized COV value for the M2 provides full separation of M2 from isobaric interferences. Note the extremely low background signal (inset) and compare with Figure 5A.

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