Highly Selective Analysis of Steroid Biomarkers using SelexION™ Ion Mobility Technology

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One of the most challenging aspects of LC-MS/MS analysis of steroids biomarkers is the presence of interferences that are typically caused by isobaric compounds or other matrix components. Interferences may present themselves in the form of unresolved chromatographic peaks, or as a high chromatographic baseline. In either case, if the analyte of interest cannot be adequately resolved from the interferences, this may compromise the analysis by adversely impacting the limit of quantitation (LOQ), dynamic range and compromise overall data quality.

The removal of interferences is typically addressed by adjusting HPLC conditions and sample preparation methodology. Usually this involves increasing the length of the chromatographic run, or pursuing a more complicated and labor-intensive sample clean-up. However both of these approaches are generally undesirable as they require more time and introduce considerable costs for sample analysis. Furthermore, even when these interferences can be chromatographically resolved, additional time is wasted for data review if the peak integrations must be manually adjusted on a sample-by-sample basis.

Significant advances have been made to increase MS/MS selectivity beyond the gold standard Multiple Reaction Monitoring (MRM) approach. For example, MRM^3 on the QTRAP® 5500 system adds additional selectivity by increasing the level of fragmentation. High resolution mass spectrometry systems also provide enhanced mass selectivity. However, in many cases even the use of accurate-mass / high-resolution systems cannot aid with differentiating between interfering species, since the exact mass of an interfering compound may be identical to that of the target analyte. This is a common problem in steroid biomarker analysis, as there are numerous endogenous interferences present in biological matrices. Ion mobility separation presents another attractive option by introducing selectivity during sample introduction, following atmospheric pressure ionization. Although ion mobility techniques have been used extensively for qualitative applications, they have traditionally lacked the ruggedness, selectivity, speed, and sensitivity required for routine high sample volume analysis.

The novel AB SCIEX SelexION™ ion mobility technology coupled to the QTRAP® 5500 system provides a means for differential ion mobility separation in complex matrices for steroids biomarker analysis. This has been enabled by several breakthrough innovations in ion mobility.

AB SCIEX QTRAP® 5500 LC/MS/MS System Equipped with SelexION™ Ion Mobility Technology

Key SelexION™ Technology Innovations

- Planar geometry results in minimal diffusion losses for maximum sensitivity.
- Rapid switching of voltages is compatible with multi-component MRM analysis and fast UHPLC;
- Highly robust, reproducible, and stable performance is suitable for use in routine analysis
- The option to introduce a chemical modifier to the transport gas further enhances the selectivity, and dramatically increases separation capacity.
- The easy to maintain ion mobility cell does not require breaking vacuum for installation or removal.
- In transparent mode, ions can pass through the device when it is not used, so it can remain in place during conventional non-ion mobility analysis.

Innovative Planar Design

SelexION™ technology brings differential mobility spectrometry (DMS) to the QTRAP® 5500 system in a compact device. It is integrated in the ion source directly in front of the orifice and behind the curtain plate (Figure 1).

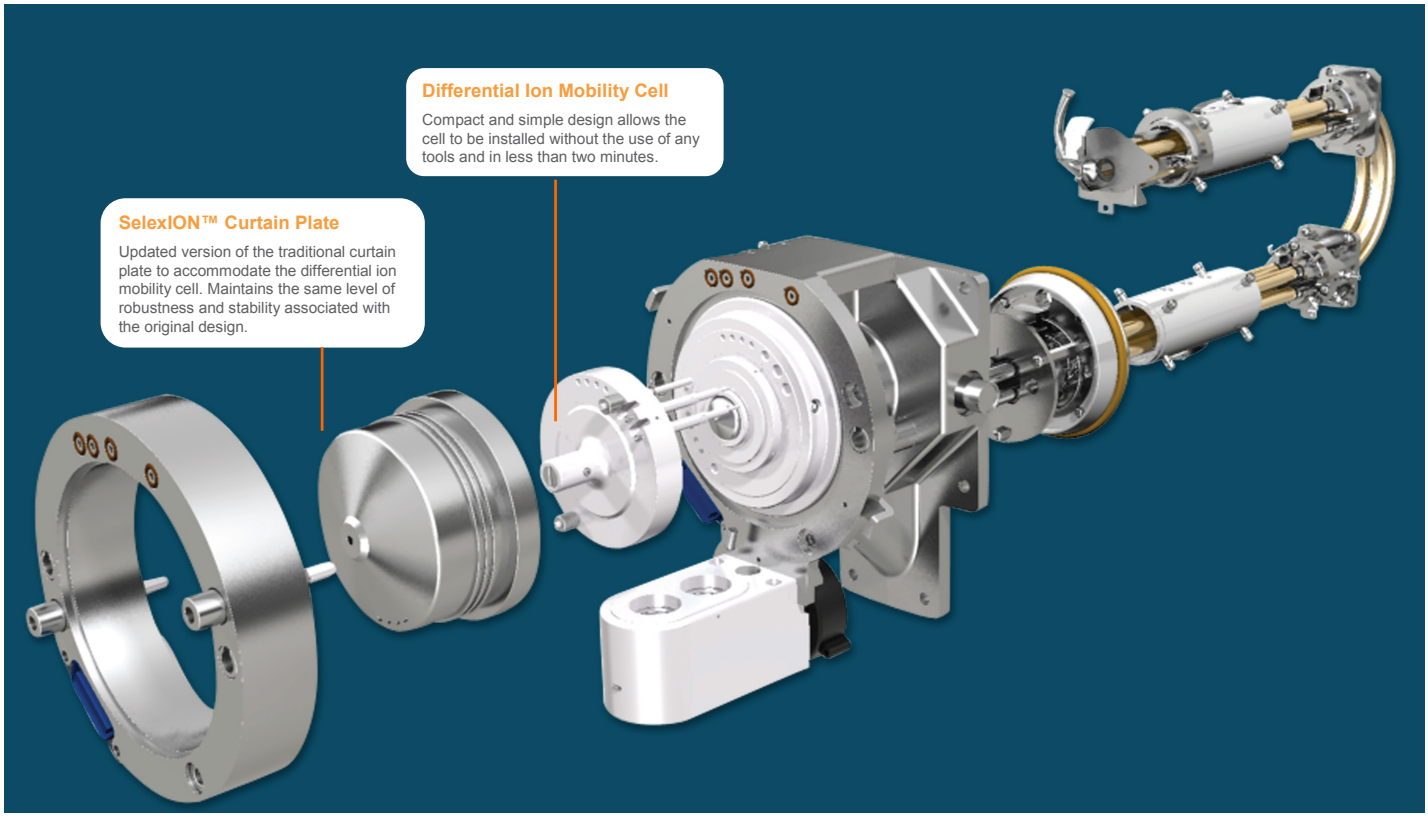
The DMS cell consists of two planar, parallel electrodes (10 x 30 mm, 1 mm gap) with an RF voltage (the Separation Voltage, SV) applied across the plates to generate an alternately high- and low-field. Unlike traditional ion mobility, ions are not separated in time as they traverse the cell. Rather, they are separated in trajectory based on differences in their mobility under the high-field and low-field conditions created by the applied RF waveform. The net motion of the ions resembles a saw-tooth pattern, and separation is achieved as the ions migrate at different rates towards one of the two electrodes. By applying a second DC voltage offset (the Compensation Voltage, COV), the trajectory of the desired ions can be corrected to remain along the centerline of the ion mobility cell, ensuring their transmission through the orifice. Since the COV is a compound-specific parameter, other species will migrate away from the centerline of the ion mobility cell, due differences in their mobility compared to the analyte of interest.

This planar design yields a stable, easy to tune system with high resolving power over a short distance. This gives high speeds and short residence times, resulting in minimal diffusion losses and enabling the use of short MRM dwell times. By simply turning off the separation voltage, the cell becomes transparent, with all the ions moving normally along the centerline. This means that it is possible to transmit ions through the mobility cell when not actively using the DMS device. Some signal loss is observed in transparent mode, so it is recommended that the device is dismounted for applications requiring maximum sensitivity when ion mobility separation is not required.

Chemical Modifiers

Volatile reagents may be introduced into the transport gas in the ion mobility cell, which chemically modifies how the analyte ions interact with the gas during the DMS separation. Different species will have different affinities to form clusters with this chemical modifier. As the applied electric field alternates between high- and low-field conditions, the ions will undergo clustering and de-clustering.

Figure 1. The SelexION™ differential mobility spectrometry (DMS) device is coupled directly to the orifice plate. A modified curtain plate accommodates the DMS cell, which can be easily removed without the use of any tools, and without venting the system.



Under the high-field conditions, ions will de-cluster due to the higher energy available. But under the low-field conditions, the clusters will form once again. This interaction dramatically increases the separation capacity of the DMS cell, taking advantage of chemical properties such as proton affinity to add another dimension in the differential mobility effect. Note that in the application presented here, no chemical modifier was needed in order to achieve the required level of selectivity.

Experimental

Study Design and Sample Preparation: 5 male and 5 female serum samples were obtained from a clinical research laboratory using a standard collection protocol and a gold top serum separating tube (SST). Calibrators were prepared from a pool of serum samples obtained from children under age 7 with the same collection conditions as above. Liquid-liquid extraction and protein precipitation sample preparation methods were both evaluated in these experiments. The liquid-liquid protocol utilized an aliquot of 200 µL of an unknown serum sample or calibrator, which was then spiked with labeled internal standard. The resulting solution was extracted with a mixture of 900 µL/100 µL hexane/ ethyl acetate. The organic supernatant was transferred and dried down. The protein precipitation protocol was carried out in parallel, using another aliquot of 200 µL of the same set of unknown serum samples or calibrators. These were spiked with labeled internal standard, and then precipitated with 600 µL of acetonitrile. The supernatant was transferred and dried down. All the samples processed from both methods were reconstituted in150 µL of 50/50 methanol/water.

Chromatography: A Shimadzu Prominence HPLC system equipped with a Kinetex 2.6u 50x2.1 mm C8 column was used for the analysis. Two chromatographic methods were evaluated consisting of a 7 minute long and a short 3 minute gradient. Water and methanol/acetonitrile containing 0.1% formic acid were used as mobile phases at a flow rate of 300 µL/min for both long and short gradient methods, which are shown in Table1 and 2. The analytical column was heated to 40 °C.

Differential Ion Mobility MS/MS: A QTRAP® 5500 system was equipped with and without the SelexION™ DMS interface for comparison purpose. The DMS cell was operated at a separation voltage (SV) of 3200V, with a Turbo V™ ion source operating in positive ESI mode. The compensation voltage (COV) was optimized at 4.8 V for testosterone, using an on-column injection method. The MRM transitions of m/z 289 → 109, and 289 →97 were monitored for testosterone.

Table 1. HPLC Conditions for 7-minute long gradient method.

Time (min)	%A	%B
0	90	10
0.5	90	10
1.5	35	65
2.5	30	70
4.3	30	70
5.5	5	95
5.7	5	95
5.9	90	10
7.0	90	10

Table 2. HPLC Conditions for 3-minute short gradient method.

Time (min)	%A	%B
0	85	15
0.25	85	15
0.5	35	65
2.15	20	80
2.25	5	95
2.4	5	95
2.41	85	15
3.0	85	15

Results

The optimization of compensation voltage using the SelexION™ DMS device for testosterone was evaluated by injecting sample from protein precipitated matrix on column, and the results are displayed using a heat map (Fig 2). The HPLC chromatogram for MRM transition 289/97 is shown in the top panel. The analyte of interest, testosterone, has an expected retention time of 3.4 minutes. It is quite apparent that it is co-eluting with the interferences from the SST tubes. As indicated by the heat map, all major interferences have higher compensation voltages (8-10V) in comparison to testosterone which optimized at COV 4.8V (Fig 2). Without the SelexION™ DMS device installed, testosterone was not well resolved from the interference peaks and the ion ratio is not in line with the expected MRM ratio value of 1.2, even when using the liquid-liquid sample preparation and a 7 minute long gradient (Fig 3, top panel). With the DMS on,

the interferences and testosterone were separated in the ion mobility cell. By applying a compensation voltage (COV) of 4.8V, which is specific for testosterone, the trajectory of the desired testosterone ions was directed to remain along the centerline of the ion mobility cell, ensuring efficient transmission through the orifice and detection by the mass spectrometer. Meanwhile, the isobaric interferences were directed away from the center line of the ion mobility cell, due to differences in their mobility compared to testosterone. This resulted in a clean Gaussian shaped LC peak for both MRM transitions used to monitor testosterone, and provided the correct MRM ion ratio for the matrix sample (Fig 3 bottom panel).

Figure 2. Heat map demonstrating the separation of Testosterone from potentially interfering components, based on differences in the optimized Compensation Voltage values (y-axis).

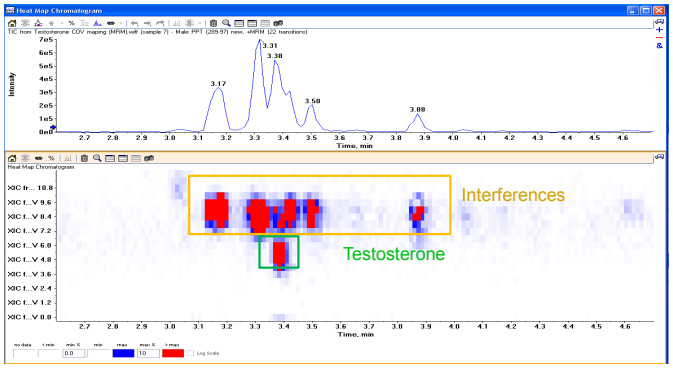


Figure 3. Analysis of testosterone in a female subject, using liquid-liquid extraction as the method of sample preparation.

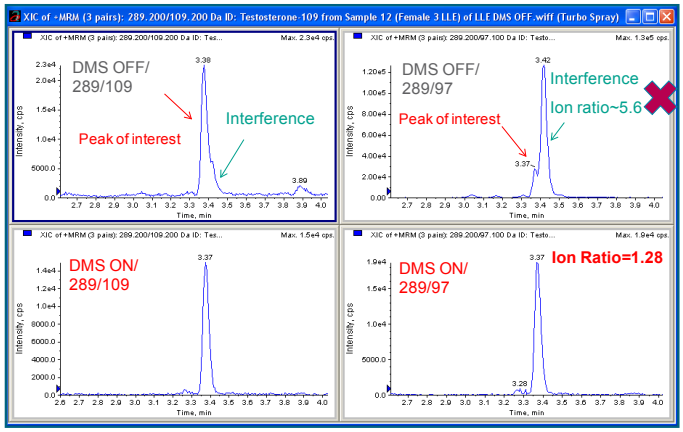


Figure 4. Analysis of testosterone in a pooled serum sample from children under age 7, using a protein precipitation method of sample preparation.

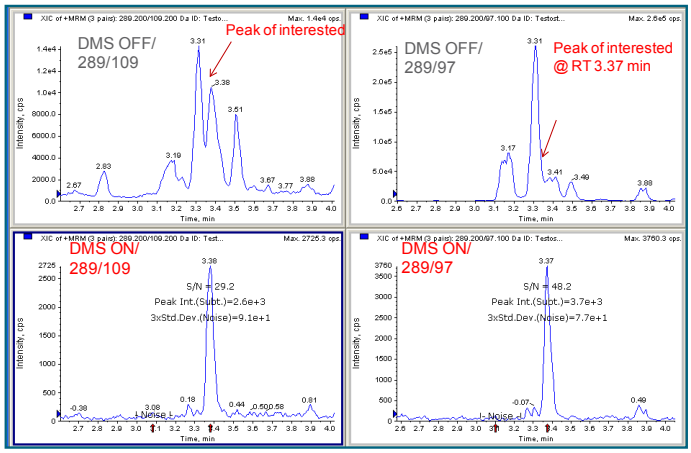
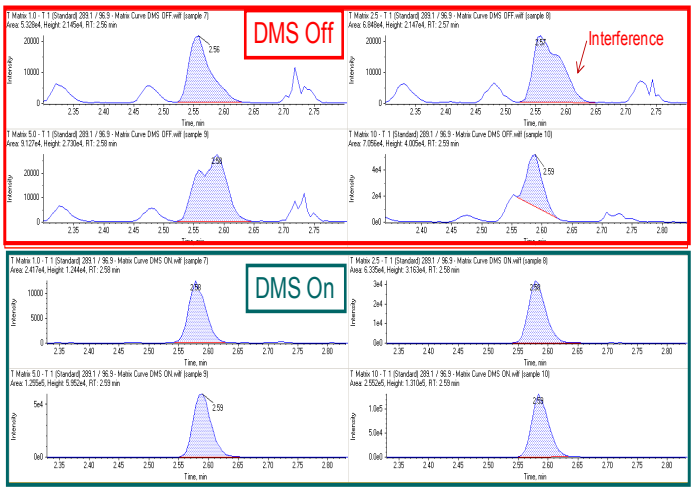


Figure 5. Chromatogram for unknown serum sample using a protein precipitation method of sample preparation, with a short 3-minute HPLC gradient. DMS "off" is shown in the top panel, and DMS "on" is shown in the bottom panel.



When the SelexION™ DMS device was not employed and the simplified protein precipitation procedure was used, no discernible testosterone peak was detected due to interferences and high background (Fig 4, top panel). Using the DMS device at a COV setting of 4.8V (optimized for testosterone transmission), the interferences were completely removed and testosterone was detectable at the pg/mL level in SST tube sample matrix. The peaks were easily integrated due to the absence of chromatographic interferences, and accurate concentrations were reported in all cases (Fig 4, bottom panel).

By taking advantage of the added selectivity of the SelexION™ device, we were able to improve the throughput of the sample analysis by shortening the length of the HPLC gradient from 7 minutes to 3 minutes, while maintaining an interference-free

chromatogram enabling more consistent integration. Example chromatograms for the analysis of a serum sample using the 3-minute gradient are shown in Figure 5, both with and without the DMS device installed.

A calibration curve was prepared and analyzed using the 3 minute gradient, with the protein precipitation sample preparation protocol, and employing the SelexION™ ion mobility device. Excellent linearity was observed for testosterone in serum sample matrix, with r>0.999 (Figure 6). The reproducibility and accuracy statistics over the dynamic range studied are shown in Table 3, where the %CV was less than 7% for all concentrations except for the lowest calibrator. The accuracy observed over all concentrations was within 3% of the expected value.

Figure 6. Calibration curve for testosterone in the SST serum matrix with DMS "on", using a short 3-minute HPLC gradient.

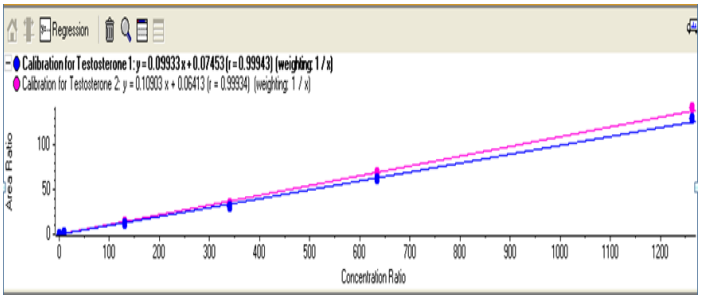


Table 3. Accuracy and precision statistics for the testosterone calibration curve, using a short 3-minute HPLC gradient.

Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy
1	T 1	0.05	3 of 3	0.05	0.01	13.26	98.55
2	T 1	0.10	3 of 3	0.10	0.01	6.12	103.22
3	T 1	0.25	3 of 3	0.25	0.00	1.73	99.46
4	T 1	0.50	3 of 3	0.51	0.00	0.05	102.20
5	T 1	1.00	3 of 3	0.97	0.03	3.01	97.44
6	T 1	2.50	3 of 3	2.46	0.02	0.87	98.56
7	T 1	5.00	3 of 3	5.01	0.13	2.63	100.15
8	T 1	10.00	3 of 3	10.04	0.04	0.39	100.42

Conclusion:

In this application we have demonstrated several key advantages of the SelexION™ differential mobility spectrometry (DMS) device:

- Optimization of the compound-specific Compensation Voltage (COV) parameter, in addition to the compound-specific Collision Energy (CE), enabled the development of an MRM assays with enhanced selectivity.
- Use of the SelexION™ ion mobility technology enabled the reduction of chemical background, and the removal of chromatographic interferences for the analysis of testosterone in serum samples, resulting in a net gain in signal-to-noise.
- The run-time for sample analysis was improved dramatically, from 7 minutes to 3 minutes, with no reduction in the selectivity of the method. The SelexION™ device removed interferences that would otherwise have required chromatographic separation.
- The sample preparation was simplified to a simple protein precipitation step, saving time and labor costs.
- Excellent linearity and reproducibility were observed across the entire calibration range, when the SelexION™ ion mobility device was employed.
- Using the SelexION™ device more reliable results were obtained, decreasing the potential for false positive results.

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