Investigating the enhancement in selectivity for the analysis of methyldienolone in urine samples by differential mobility spectrometry

Using SelexION®+ Differential Mobility Separation Technology on the SCIEX QTRAP® 5500 LC-MS/MS System

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The use of liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) is currently considered the method of choice for analysis of compounds in biological matrices. This has led to the development of high throughput methods with little or no sample preparation and short chromatographic run times.

Methyldienolone is an anabolic androgenic steroid (AAS) prohibited by the World Anti-Doping Agency (WADA) in all types of sports, and for which the minimum required performance limit (MRPL) is set at 5 ng/mL.[1, 2] The detection of methyldienolone in human urine is made challenging because biological matrices often contain various substances similar in structure and mass to the target analyte. This means that an approach with little or no sample preparation and short chromatographic runtimes can cause co-elution of these isobaric compounds that interfere with the analysis of methyldienolone. In order to maintain this type of high throughput approach that is adopted by many labs, a solution was investigated to separate the interfering compounds during the LC-MS/MS run.

In this technical note, an innovative and selective method for the analysis of methyldienolone in urine samples is presented using a SCIEX QTRAP 5500 System equipped with the SelexION+ Differential Mobility Separation Device. The presented method enables accurate quantification of methyldienolone with rugged bioanalytical performance and minimal sample preparation, enabling high-throughput and sensitive detection of this substance without the need for derivatization or additional sample preparation steps.

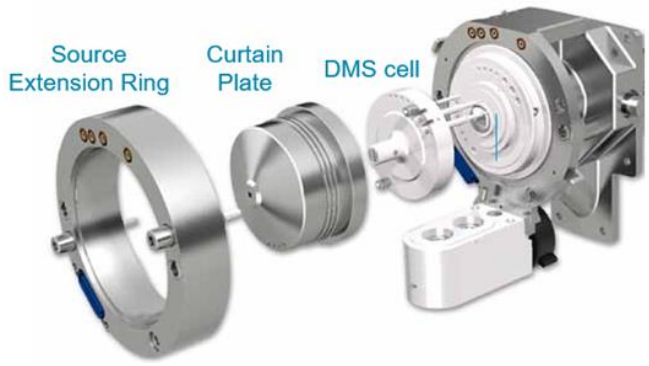

Figure 1: Assembly of the SelexION+ Device. The DMS cell is installed between the orifice plate and a modified curtain plate. The source extension ring enables use of the standard SCIEX sources. The DMS cell is easily installed or removed in minutes, with no need to break the vacuum or use any tools.[8]

Key advantages of SelexION+ Technology for methyldienolone analysis in urine samples

- The SelexION+ Differential Mobility Separation Device is shown to resolve isobaric interferences that cannot be achieved by tandem mass spectrometry alone
- The combination of SelexION+ Technology with MRM analysis on the SCIEX QTRAP 5500 System enabled sensitive and accurate quantitative analysis of methyldienolone using a dilute and shoot methodology in doping control samples
- The loss in background noise improved the S/N value, resulting in improvement of detection limits
- The use of SelexION+ Technology resulted in LOD of 0.05 ng/mL and LOQ of 0.5 ng/mL for methyldienolone in matrix

Experimental details

Reagents: Methyldienolone was purchased from National Measurement Institute (NMI), Australia. Stock solution was prepared by weighing 10 mg of standard in 100 mL methanol. Mass spectrometric grade acetonitrile and methanol were purchased from J.T. Baker, India and formic acid was obtained from Fluka, India.

Sample preparation: A dilute and shoot sample preparation procedure was used to analyze the urine samples. In short, 1 mL of blank human urine was centrifuged and spiked with working standard of methyldienolone at desired concentration. The sample was further diluted 5 times with water to perform dilute and shoot analysis. To investigate the linearity of the assay, aqueous spiked dilutions were prepared in the range of 0.05 - 500 ng/mL. A calibration curve was prepared by diluting spiked urine at 0.1-500 ng/mL.

Liquid chromatography: HPLC separation was performed on a Shimadzu Prominence HPLC system equipped with a Waters BEH C18 column (2 mm x 100 mm, 1.7 µm) held at 60°C. Mobile phases consisted of water, acetonitrile and modifiers. The LC flow rate was 0.5 mL/min and the total run time was 8 min. The injection volume was 10 µL.

Mass spectrometry: MS data were collected on the SCIEX QTRAP 5500 System equipped with SelexION+ Technology using Analyst® Software. The Turbo V™ Ion Source was used with an electrospray ionization (ESI) probe operating in positive mode. The SelexION+ Technology was operated at low temperature and low resolution setting without the use of any modifier. Separation voltage and compensation voltage were set at 3000 V and 3.0 V respectively while the offset voltage was kept at -3.0 V. Comparative experiments were performed with the SelexION+ Device “on”, and in “transparent” mode.

Data analysis: Data were processed in MultiQuant™ Software where calibration curves and concentration calculations were automatically generated.

SelexION+ Technology reduces background interferences in urine samples

The SelexION+ Technology coupled with LC-MS/MS has proven to resolve ions that are indistinguishable by mass spectrometry alone.[3, 4] Ions are separated on mobility scale due to differences in molecular size and shape, thereby providing highly selective analysis with minimal background interferences.[4,5,8] The SelexION+ Technology with the SCIEX QTRAP 5500 System brings the power of differential ion mobility separation to quantitative analysis in complex matrices, enabled by multiple innovations in ion mobility.[6] This approach is particularly suited for the analysis of analytes that are challenging to resolve from the matrix background. The use of the SelexION+ Technology coupled with LC-MS/MS was investigated for the analysis of methyldienolone in urine samples.

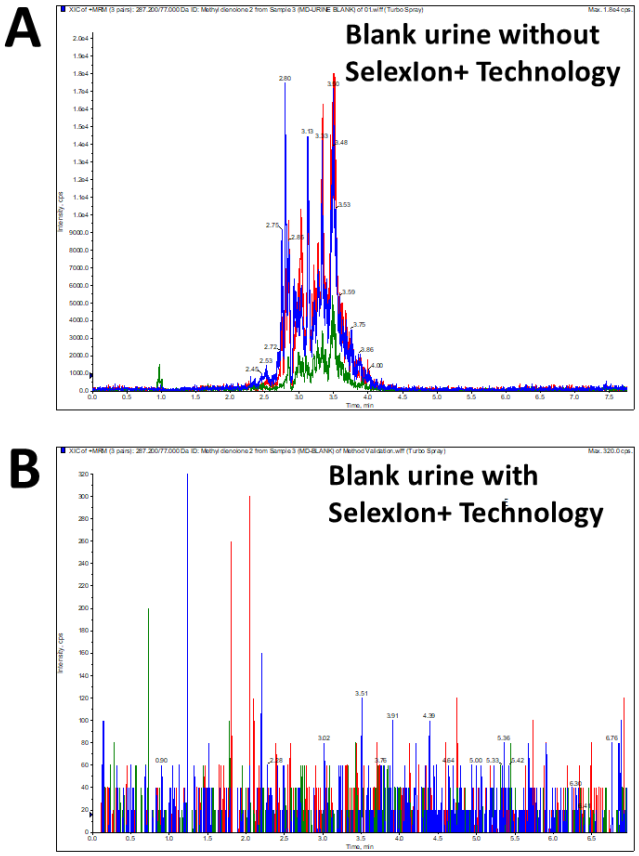

Figure 2: Extracted ion chromatogram (XIC) traces of blank urine samples. Chromatogram were obtained A) without and B) with the SelexION+ Technology. The use of the SelexION+ Technology removed all isobaric interferences and high basline noise associated with injecting diluted urine sampes directly into the LC-MS/MS system.

Figure 2A shows the extracted ion chromatogram of a blank urine sample analyzed without SelexION+ Technology. With a dilute and shoot LC-MS/MS method based on MRM (without SelexION+ Technology), isobaric interferences and high baseline were observed at the retention time of methyldienolone in the blank urine sample. Figure 2B shows the resulting extracted ion chromatogram of the same blank urine sample analyzed using the LC-DMS-MS/MS setup with the SelexION+ Technology turned on, and after selecting the appropriate value for compensation voltage. The chromatogram shows that the use of the SelexION+ Technology removed all isobaric interferences and the high baseline from the blank urine sample.

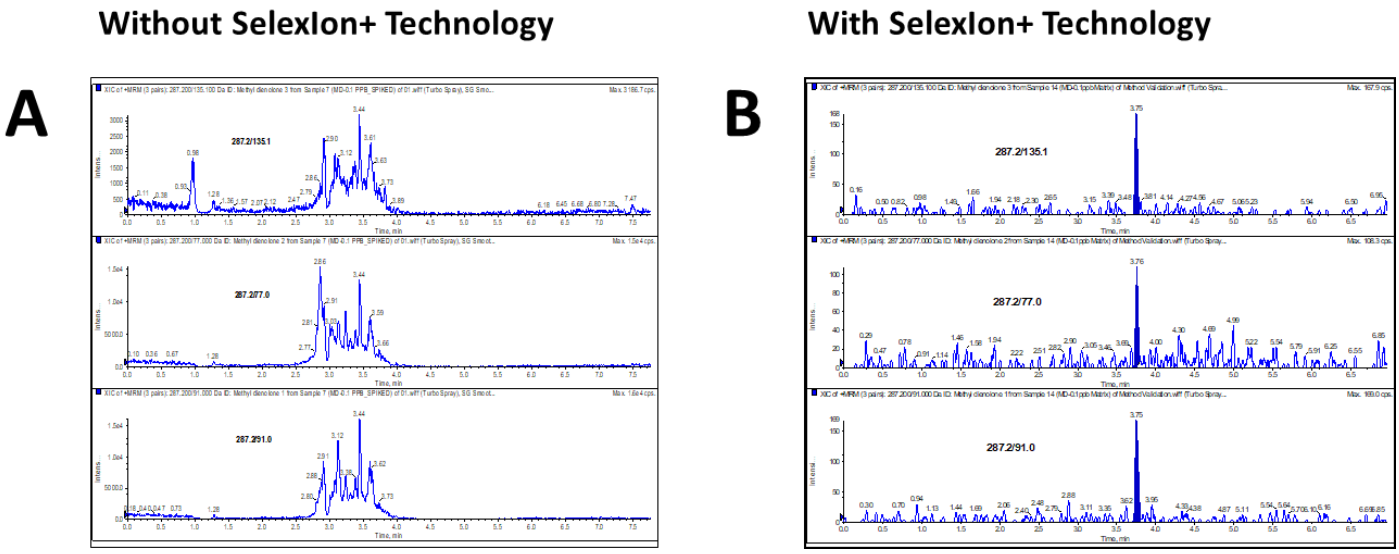

Figure 3. Extracted ion chromatogram (XIC) traces for each of the three MRM transitions monitored for methyldienolone spiked in urine at 0.5 ng/mL (LOQ). A) Resulting chromatograms without the use of SelexION+ Technology show isobaric interferences and high baseline at the retention time of methyldienolone. B) Resulting chromatograms with the use of SelexION+ Technology show a drastic decrease in background and an increase in signal-to-noise ratio (S/N).

Reduction in background interferences leads to sensitive and accurate quantification of methyldinolone in urine samples

The ability of the SelexION+ Technology to reduce background interferences and improve detection of analytes that would be otherwise indistinguishable by mass spectrometry alone was further investigated. Control urine samples spiked with methyldienolone were prepared at concentration levels ranging from 0.05 to 500 ng/mL and injected in triplicate. Figure 3 shows the extracted ion chromatogram for each of the three MRM transitions monitored for methyldienolone spiked in urine at 0.5 ng/mL (LOQ). Figure 3A shows the chromatograms for the three methyldienolone MRM transitions monitored in this study without using SelexION+ Technology. The chromatograms show isobaric interferences and high baseline at the retention time of methyldienolone. The same sample was re-injected with the SelexION+ Technology on. Figure 3B shows a drastic decrease in background and an increase in signal-to-noise ratio (S/N) for each of the MRM transitions monitored for methyldienolone. The limit of detection (LOD) obtained was 0.05 ng/mL with S/N>30 for all three MRM transitions monitored. Intra-day precision (n=10) obtained as %CV was 2.05% for methyldienolone at the LOD (0.05 ng/mL). The results indicate that the use of the SelexION+ Technology greatly reduces background interference, hence improving detection of methyldienolone in urine samples.

Calibration curves were generated to investigate the performance of the SCIEX QTRAP 5500 System with SelexION+ Technology for accurate quantification of methyldienolone detection in urine samples. Figure 4 shows the calibration curves for each of the three MRM transitions monitored for methyldienolone at concentrations ranging from 0.05 to 500 ng/mL. Excellent linearity was observed across the concentration ranges analyzed with R^2 values greater than 0.99 for all of the three MRM transitions monitored.

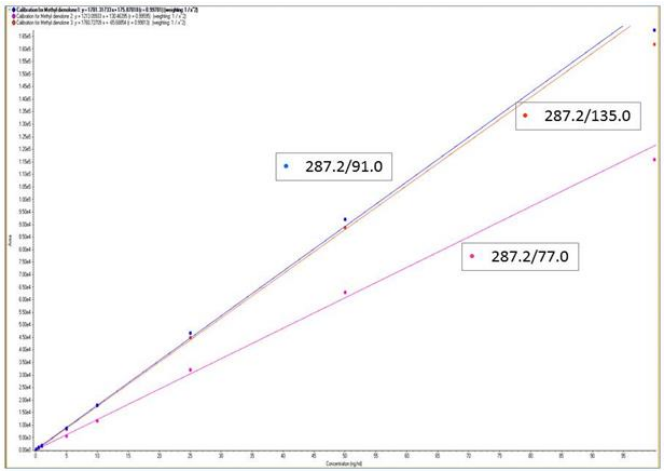

Figure 4. Excellent linearity for each of the three MRM transitions monitored for methyldienolone. Linear regression curves resulting from the calibration series of methyldienolone from 0.05 to 500 ng/mL. R^2 values greater than 0.99 were observed for all of the three MRM transitions monitored.

The quantitative performance of the SCIEX QTRAP 5500 System with SelexION+ Technology was investigated by monitoring the intra-day (N=8) and inter-day (N=16) precision of injections for aqueous solutions of methyldienolone. Table 1 shows the repeatability for 8 consecutive injections of aqueous methyldienolone at the LOD (0.05 ng/mL). Intraday (n=8) and interday (n=16) precision of injections measured by %CV were found to be 3.18% and 3.62% respectively.

Table 1: Repeatability of measurements for 8 consecutive injections of aqueous solutions of methyldienolone at the LOD (0.05 ng/mL).

Sample ID	Actual Concentration (ng/mL)	XIC Area (counts)
MD- 0.05 ppb Aq._1	0.05	*232.376*
MD- 0.05 ppb Aq._2	0.05	*230.995*
MD- 0.05 ppb Aq._3	0.05	*250.123*
MD- 0.05 ppb Aq._4	0.05	*267.827*
MD- 0.05 ppb Aq._5	0.05	*225.036*
MD- 0.05 ppb Aq._6	0.05	*209.26*
MD- 0.05 ppb Aq._7	0.05	*200.817*
MD- 0.05 ppb Aq._8	0.05	*246.47*
MD- 0.05 ppb Aq._1	0.05	*232.376*

The quantitative performance of the SCIEX QTRAP 5500 System with SelexION+ Technology at the low end of the calibration range was further investigated by monitoring the accuracy of measurements for a series of 9 calibrators prepared in urine matrix ranging from 0.1 to 250 ng/mL. Table 2 shows that the accuracy of measurements ranged between 82.64 and 110.19% in urine matrix.

Table 2: Accuracy obtained in calibration standards prepared in urine matrix

Actual Conc (ppb)	XIC Area (counts)	Calc. Conc (ng/mL)	Accuracy (%)
0.1	307	*0.1*	*100.07*
0.5	*987*	*0.51*	*101.14*
1	*1743*	*0.96*	*95.64*
5	*8646*	*5.07*	*101.42*
10	*17961*	*10.62*	*106.24*
25	*46426*	*27.59*	*110.37*
50	*92813*	*55.24*	*110.49*
100	*154450*	*91.99*	*91.99*
250	*346698*	*82.64*	*82.64*

The reproducibility of injections for extracted urine samples was also investigated at the LOQ. Table 3 shows the repeatability for 8 consecutive injections of matrix extracted urine samples at 0.5 ng/mL (1 pg on column). This concentration was selected as limit of quantification with S/N>70. The obtained results at the LOQ were reproducible with %CV of 3.26%. Average recovery at 0.5 ng/mL of urine was 101.6% (data not shown).

Table 3: Repeatability of methyldienolone injections at the LOQ (0.5 ng/mL – 1 pg on column).

Sample ID	Actual Concentration (ng/mL)	XIC Area (counts)
MD-0.1ppb Matrix Aq._1	0.5	*378.747*
MD-0.1ppb Matrix Aq._2	0.5	*377.718*
MD-0.1ppb Matrix Aq._3	0.5	*377.248*
MD-0.1ppb Matrix Aq._4	0.5	*368.257*
MD-0.1ppb Matrix Aq._5	0.5	*372.643*
MD-0.1ppb Matrix Aq._6	0.5	*384.086*
MD-0.1ppb Matrix Aq._7	0.5	*393.221*
MD-0.1ppb Matrix Aq._8	0.5	*390.276*
MD-0.1ppb Matrix Aq._1	0.5	*375.879*

Conclusions

The technique of differential mobility spectrometry (DMS) was investigated to determine its use as an ion-pre-filter for mass spectrometry, to provide reduced chemical noise, and improved quantitative accuracy.[6,7,8] The SelexION+ Technology coupled with LC-MS/MS has proven to resolve ions that are indistinguishable by mass spectrometry alone.[3, 4] Ions are separated on the mobility scale due to differences in molecular size and shape, thereby providing highly selective analysis with minimal background interferences.[4,5,8]

From the results presented here, the SelexION+ Technology was demonstrated to be successful in the optimization of a dilute-and-shoot methodology for the analysis of methyldienelone. This allows the use of a simplified sample preparation approach by eliminating all interfering ions. It reduces the background noise and increases the S/N value, improving detection limits. The LOD of 0.05 ng/mL and LOQ of 0.5 ng/mL were achieved using SelexION+ Technology. They were 2 and 5 ng/mL, respectively, without SelexION+ Technology. The innovative SelexION+ Technology, with the SCIEX QTRAP 5500 System, brings the power of differential ion mobility separation to quantitative analysis in complex matrices.[6]

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