Investigating the Enhancement in Selectivity for the Analysis of Methyldienolone in Urine Samples by Differential Mobility Spectrometry

QTRAP® 5500 LC/MS/MS system equipped with SelexION™ differential mobility device

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Overview

Here we investigated the capabilities of a differential mobility spectrometry (DMS) device coupled with LC-MS/MS to provide a highly selective quantitation method for methyldienolone; separating interferences from the target analytes that cannot be achieved by tandem mass spectrometry alone. An investigation was performed to determine whether the use of the SelexION™ differential mobility separation technology device was able to resolve isobaric interferences in the analysis of methyldienolone in human urine samples. Selectivity, throughput and accuracy for detection of methyldienolone in doping control samples can therefore be potentially increased.

Introduction

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

Methyldienolone (Figure 1) is an anabolic androgenic steroid (AAS) prohibited by WADA in all types of sports 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 little or no sample preparation approach and short chromatographic runtimes can cause co-elution of these isobaric compounds and interfere with the analysis of methyldienolone. In order to maintain these high throughput approaches adopted by many labs, a solution was investigated that will separate these interfering compounds during the LC-MS/MS run.

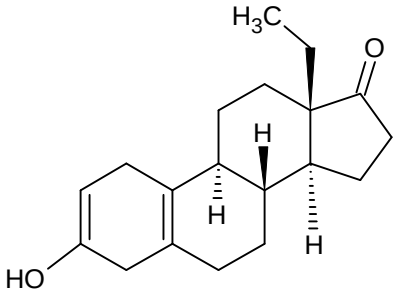

Figure 1: Structure of Methyldienolone ($C_{19}H_{26}CO_2$)

Differential mobility spectrometry is a method of separating ions based on difference between their ion mobility's in a high and low electric field in gases at or near atmospheric pressure. The SelexION™ technology is a Differential Mobility Spectrometer (DMS) placed in front of the inlet of the mass spectrometer (Figure 2). The ionized molecules travel into the orthogonal geometry shaped DMS cell. Advantages of the planar DMS geometry include:

- Short residence times
- Rapid voltage changes for MRM operation
  - MRM cycle times of 25 msec, (20 msec pause time)
  - Fast LC support
- Transparent Mode
  - Allows all ions to be transmitted by turning off voltages
- Minimal diffusion losses
- Uniform conditions for the addition of chemical modifiers

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]

Key Advantages of using SelexION™ Technology

- Separation of chemical noise from analyte of interest.
- Highly robust, reproducible and stable for use in analytical testing
  - Improved data quality with removal of unknown interferences
  - More consistent integration
- Hardware can be installed or removed in minutes with no need to break the vacuum, or use any tools
- Simplified sample preparation
  - Minimal or no sample preparation required; supports dilute and shoot method
- Reduced LC separation run times
- Improved LLOQ

Hence the aim of this study was to explore a more selective method for analysis of methyldienolone in urine samples using SelexION™ technology with rugged bio analytical performance and minimal sample preparation.

Experimental

Chemicals

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 & methanol were purchased from J.T. Baker, India and formic acid was obtained from Fluka, India.

Sample Preparation

1 mL of urine sample 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. For linearity, 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.

SelexION™ differential mobility device

Experiments were performed using a QTRAP® 5500 LC/MS/MS system equipped with SelexION™ technology, to provide the enhanced selectivity in the analysis. The ion mobility device was interfaced directly in the front of the mass spectrometer (Figure 2). The vacuum inside the instrument further draws the transport of gas through the ion mobility cell and hence into the interface region of the mass spectrometer.

Separation voltage (SV) was applied across the ion transport channel perpendicular to the transport gas flow in the DMS cell. During travel, ions migrated towards the walls of the DMS cell due to low and high field ion mobility coefficients. DC potential / compensation voltage (CV) was applied to correct the trajectory of ions of interest while interferences were deflected into the cell walls.

The compensation voltage was selected to a fixed value to pass only the ion species with a particular differential mobility. A flow of nitrogen gas, throttle gas was added to regulate the transport gas flow through the DMS cell. DMS mobility cell was optimized for selectivity by adjusting the throttle gas.

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.

Liquid Chromatography

A Shimadzu Prominence HPLC system equipped with a Waters BEH C18 column (2x100mm, 1.7µm) was used for this analysis. Compound elution was by gradient at a flow rate of 500 µL/min (Table 1). The column temperature was maintained to 60 °C and the injection volume was set to 10 µL.

Table 1: Chromatographic conditions

Time (min)	% Mobile Phase A (0.2 % formic acid in water)	% Mobile Phase B (acetonitrile)
0.1	95	*20*
5	*0*	*100*
5.1	*95*	*5*
7	*95*	*5*
8	*Controller stop*	

MS/MS Conditions

The SCIEX QTRAP® 5500 was operated in Multiple Reaction Monitoring (MRM) mode. The Turbo V™ source was used with an Electrospray Ionization (ESI) probe in positive polarity and parameters were optimized for optimum sensitivity (Table 2). Three selective MRM transitions (237.2: 135.1, 91.0, 77.0) were monitored for methyldienolone identification. Analyst

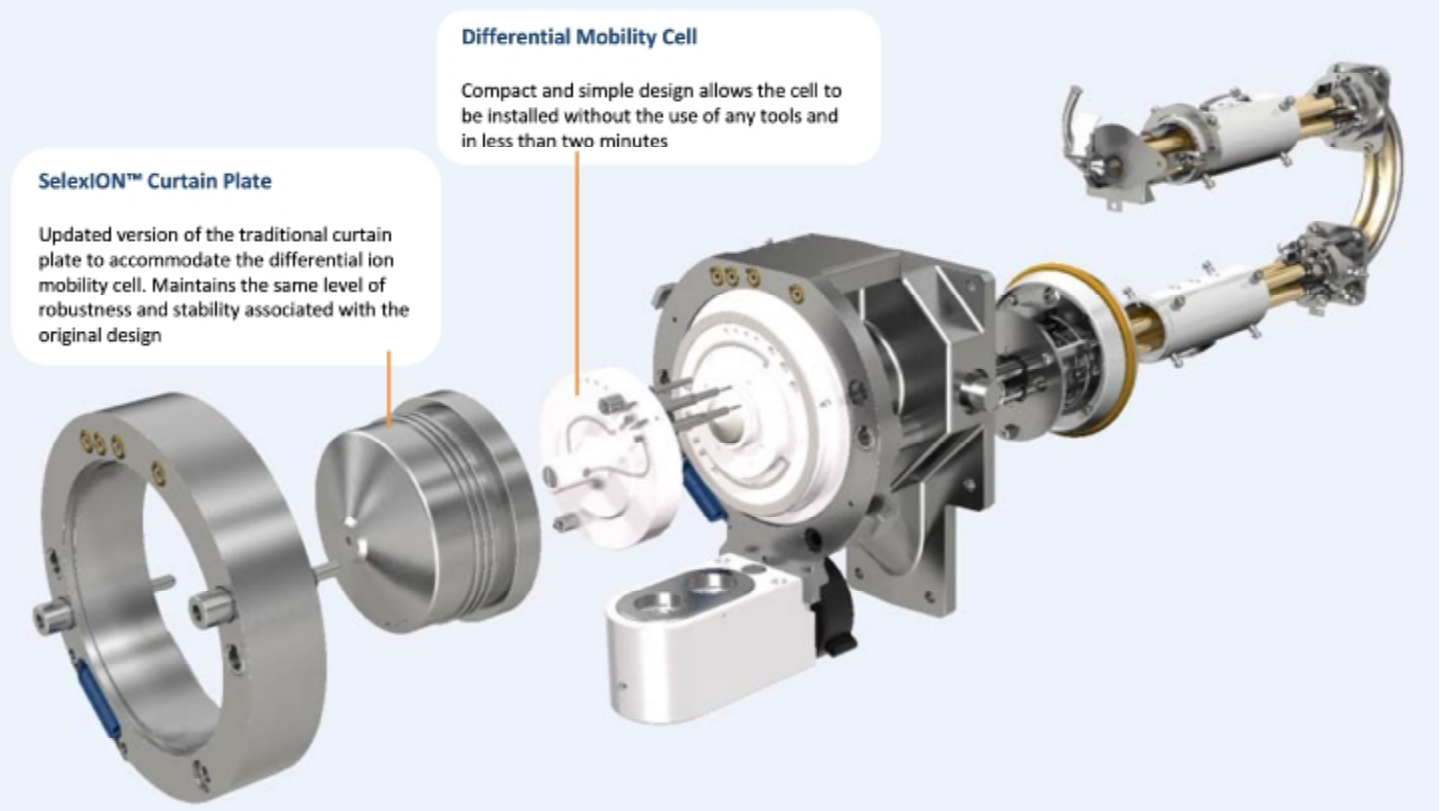

Figure 2. QTRAP® 5500 System Ion Path with SelexION™ Technology Components

1.5.2 software with patch for SelexION™ components was used for method development and data acquisition. LC-MS/MS data was processed using the MultiQuant™ software version 2.1.

Table 2: MRM parameters for Methyldienolone

MRM transition	DP	EP	CE	CXP
237.2/135.1	56	*10*	*61*	*8*
237.2/91.0	*66*	*10*	*37*	*8*
237.2/77.0	*46*	*10*	*89*	*14*

Results and Discussion

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 3) and in the spiked urine sample at 0.5 ng/mL (LOQ) (Figure 4).

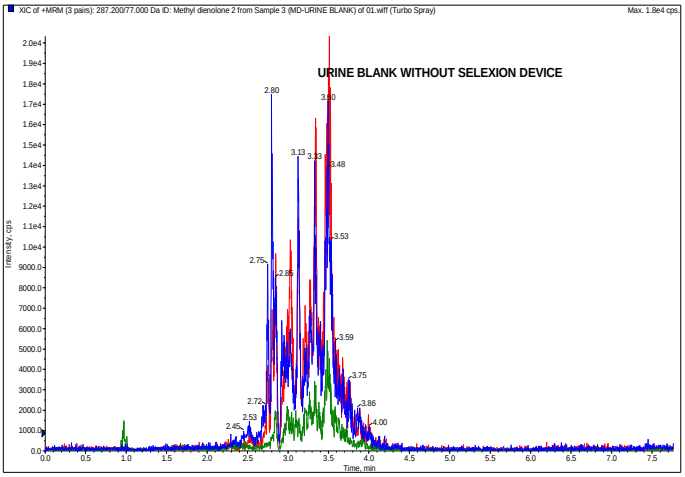

Figure 3: Chromatogram obtained for urine blank sample without SelexION™ technology

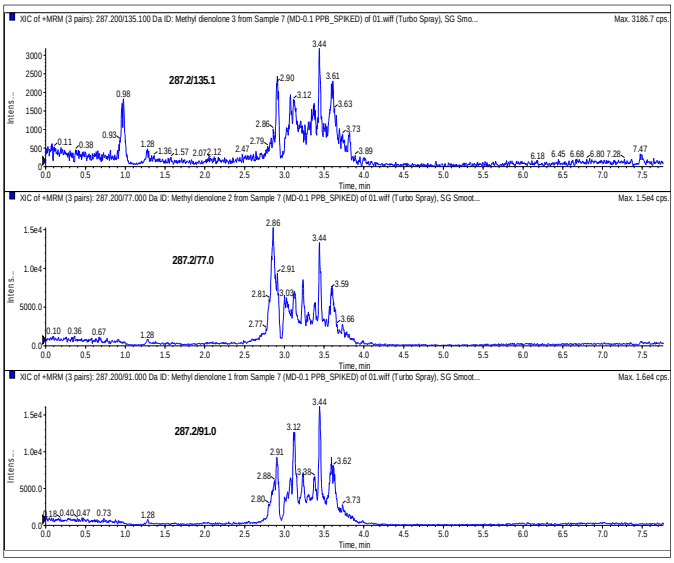

Figure 4: Chromatograms obtained for MRM transitions of methyldienolone at LOQ 0.5 ng/mL without SelexION™ technology

However, using the LC-DMS-MS/MS setup with the SelexION™ technology turned on and by selecting the appropriate value for compensation voltage all isobaric interferences and the high baseline were completely removed in the blank urine samples (Figure 5) as well as from the spiked urine sample at 0.5 ng/mL (LOQ) (Figure 6).

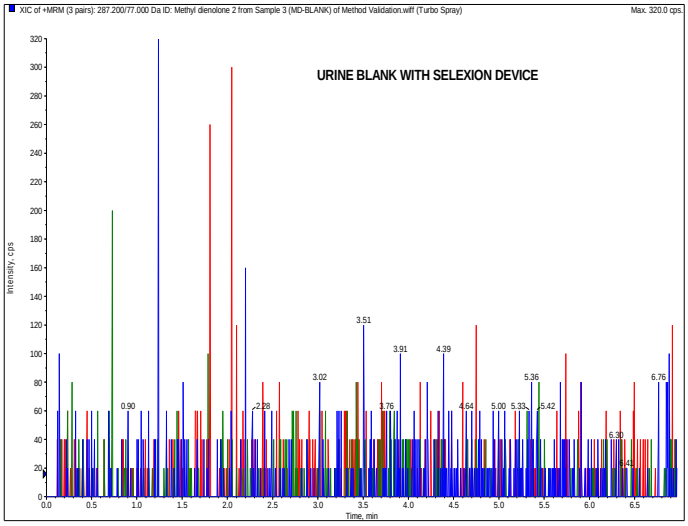

Figure 5: Chromatogram obtained for urine blank samples with SelexION™ technology

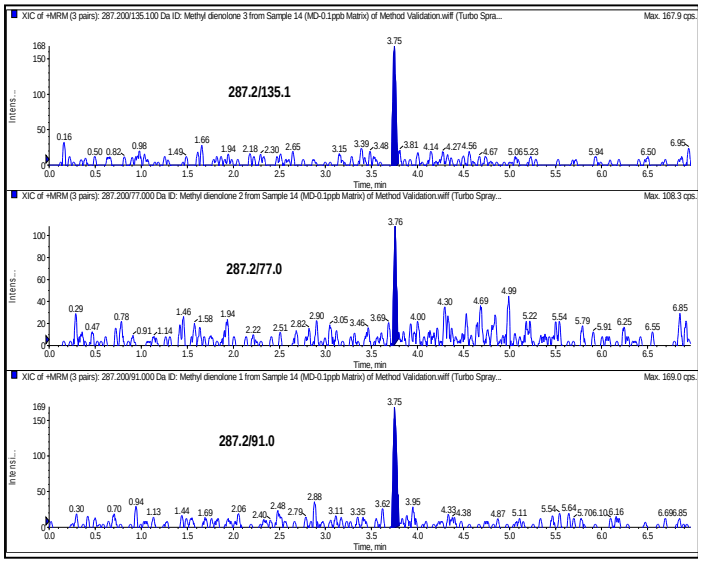

Figure 6: Chromatograms obtained for MRM transitions of methyldienolone at LOQ 0.5 ng/mL with SelexION™ technology

Aqueous linearity with a linear dynamic range of above 3.0 orders was made from a set of standard dilutions from 0.05 to 500 ng/mL (injected in triplicates). Calibration curve was found linear in the above range with regression co-efficient (r): 0.9964 using linear regression and weighing factor 1/X2. The limit of detection (LOD) obtained was 0.05 ng/mL with S/N> 30.

Table 3: Repeatability of Methyldienolone at LOD (0.05 ng/mL) level

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*

Aqueous solutions of 0.05 ng/mL were injected to check the repeatability of the results (Table 3). Intraday (n=8) and interday (n=16) precision of injections measured by % CV were found to be 3.18 % and 3.62 % respectively.

A calibration curve plotted using calibrators in urine matrix was generated from 0.1 to 500 ng/mL. Regression co-efficient obtained for the linear plot was (r): 0.9956 generated by applying linear regression with weighing factor 1/X2 (Table 4 & Figure 7).

Table 4: Accuracy obtained in calibration standards prepared in urine matrix

Actual Conc. (ng/mL)	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*

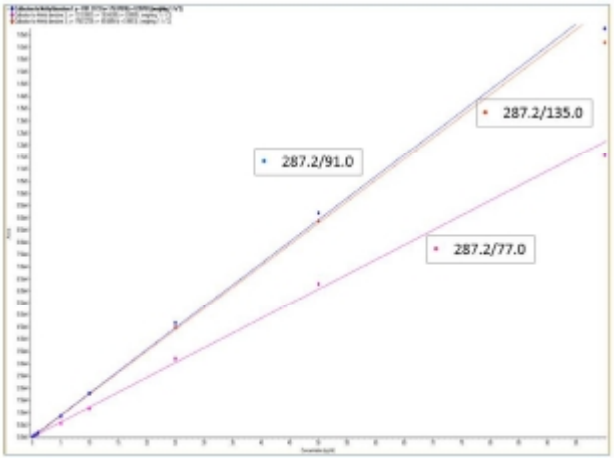

Figure 7: Calibration curve in urine matrix, r: 0.9956

Spiked concentration of 0.5 ng/mL (1 pg on column) extracted from matrix sample was selected as limit of quantitation with S/N> 70. Intraday precision (n=10) obtained as % CV was 2.05%. Interday precision of area at LOQ level was measured by repeated injections of spiked samples at 0.5 ng/mL (n=20) (Table 5). The obtained results at LOQ were reproducible with % CV of 3.26%. Average recovery at 0.5 ng/mL of urine was 101.6%.

Table 5: Repeatability of Methyldienolone Analysis at LOQ (0.5 ng/mL) level

Sample ID	Actual Conc. (ng/mL)	Area (counts)
MD-0.1ppb Matrix	0.5	*378.747*
MD-0.1ppb Matrix	0.5	*377.718*
MD-0.1ppb Matrix	0.5	*377.248*
MD-0.1ppb Matrix	0.5	*368.257*
MD-0.1ppb Matrix	0.5	*372.643*
MD-0.1ppb Matrix	0.5	*384.086*
MD-0.1ppb Matrix	0.5	*393.221*
MD-0.1ppb Matrix	0.5	*390.276*
MD-0.1ppb Matrix	0.5	*375.879*
MD-0.1ppb Matrix	0.5	*374.799*

Conclusion

The technique of DMS (SelexION™ differential mobility separation technology device) 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] From the results reported in this application note, the SelexION™ technology was demonstrated to be successful in the optimization of a dilute and shoot methodology for the analysis of methyldienelone; allowing the use of this simplified sample preparation approach by eliminating all interfering ions. This in turn reduces the background noise and increasing the S/N value and therefore allows an improvement in detection limits. The LOD of 0.05 ng/mL and LOQ of 0.5 ng/mL were achieved using SelexION™ technology which were otherwise 2 and 5 ng/mL respectively without SelexION™ technology.

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