A Clinical Research Method for the Measurement of Low-Level Testosterone in Serum using Differential Ion Mobility Spectrometry-Tandem Mass Spectrometry (DMS-MS/MS)

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INTRODUCTION

It has been well documented that liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides excellent accuracy, precision and sensitivity for measurements of steroids in biological matrices compared to traditional techniques such as immunoassays, which may suffer from cross-reactivity.

Nevertheless, there are numerous uncharacterized, endogenous components in biological fluids which have the potential to interfere with the measurement of low-level steroids such as testosterone. In this work, we present a novel method employing differential ion mobility spectrometry (DMS) in conjunction with LC-MS/MS analysis to eliminate potential interferences, thereby simplifying sample pre-treatment and enabling reduced LC run-times.

MATERIALS AND METHODS

Figure 1. SCIEX Triple Quad™ 6500+ system, equipped with the SelexION®+ ion mobility device.

We have applied liquid chromatography (LC), differential ion mobility spectrometry (DMS), and tandem mass spectrometry (MS/MS) to enable the accurate quantation of low-level testosterone in human serum. The DMS cell filters out potential interferences prior to detection by MS/MS, ensuring that isobaric components do not obfuscate the analysis.
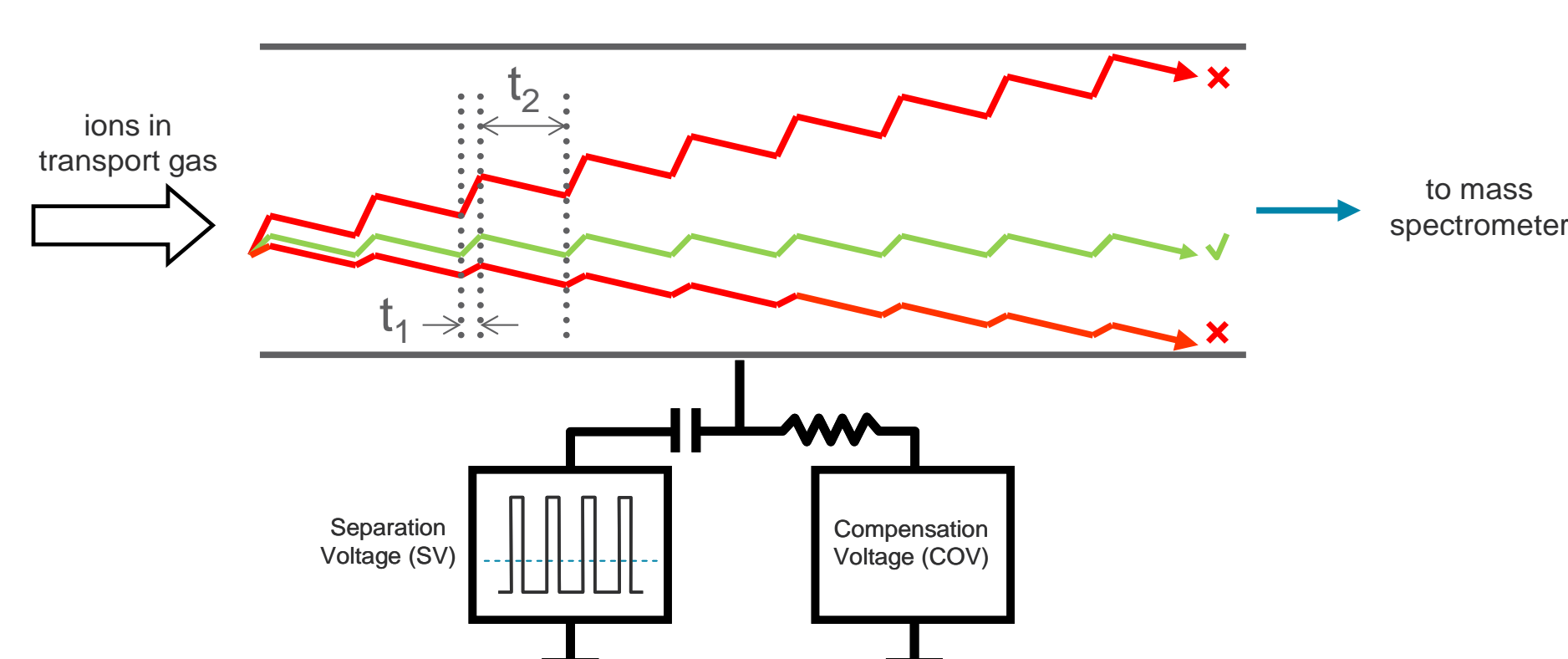

Figure 2. The SelexION+ differential ion mobility device consists of two planar electrodes. Voltages are applied, which serve to filter out isobaric interferences prior to detection by tandem mass spectrometry.

Sample Preparation:

Testosterone and Testosterone-d3 were obtained as 1mg/mL standards in methanol. Calibration curves were prepared by spiking known amounts of Testosterone into steroid-free serum obtained from Golden West Biologicals.

Sample preparation consisted of a one-step liquid-liquid extraction. 200uL of serum sample was combined with 50uL of internal standard solution and 1000uL of 90:10 hexane ethyl acetate in a micro-centrifuge tube. The sample was vortex mixed, centrifuged at 14,000 rpm for 15 minutes, and then 900 uL of the organic supernatant was removed and evaporated to dryness under a stream of nitrogen gas. The dried sample was reconstituted in 100uL of methanol, and then further diluted with 100 uL of deionized water.

HPLC Conditions:

Chromatographic separation was accomplished using the Shimadzu Prominence 20AD-XR HPLC system, with a Phenomenex Kinetex C18 column (100x2.1mm, 2.6μm), at a flow rate of 0.6 mL/min. Mobile phase A consisted of water containing NH_4F. Mobile phase B consisted of methanol. The method run-time was 7 minutes.

MS/MS Conditions:

MS/MS detection was performed using the SCIEX Triple Quad™ 6500+ system equipped with IonDrive™ Turbo V source and operated in electrospray ionization mode. Multiple Reaction Monitoring (MRM) mode was employed, with 2 MRM transitions monitored.

Optimization of SelexION+ parameters was performed using T-infusion of Testosterone at mobile phase flow rate of 0.6 mL/min. At a fixed separation voltage (SV) of 3700 V, the compensation voltage (COV) was ramped across a broad voltage range using a step-size of 0.5V. The optimum COV value, producing a maximum in signal intensity, was observed at a value of 8.9 V.
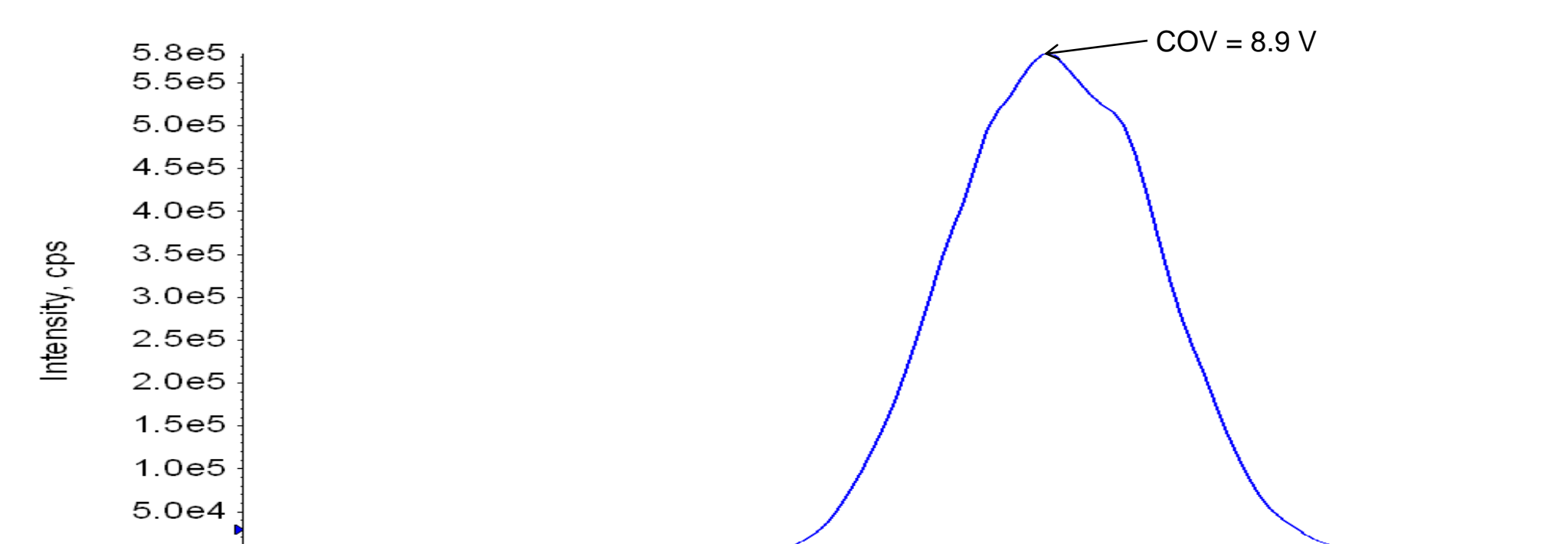

Figure 3. Optimization of the COV parameter, for Testosterone.

RESULTS

We have applied our LC-DMS-MS/MS method to the measurement of anonymized serum samples. As shown in Figure 4, the method displayed excellent sensitivity, and low chemical background due to the application of DMS. The limit of quantation (LOQ) was observed to be <1 pg/mL, and therefore this LC-DMS-MS/MS method is suitable for the measurement of total Testosterone, as well as the measurement of 'free' testosterone.

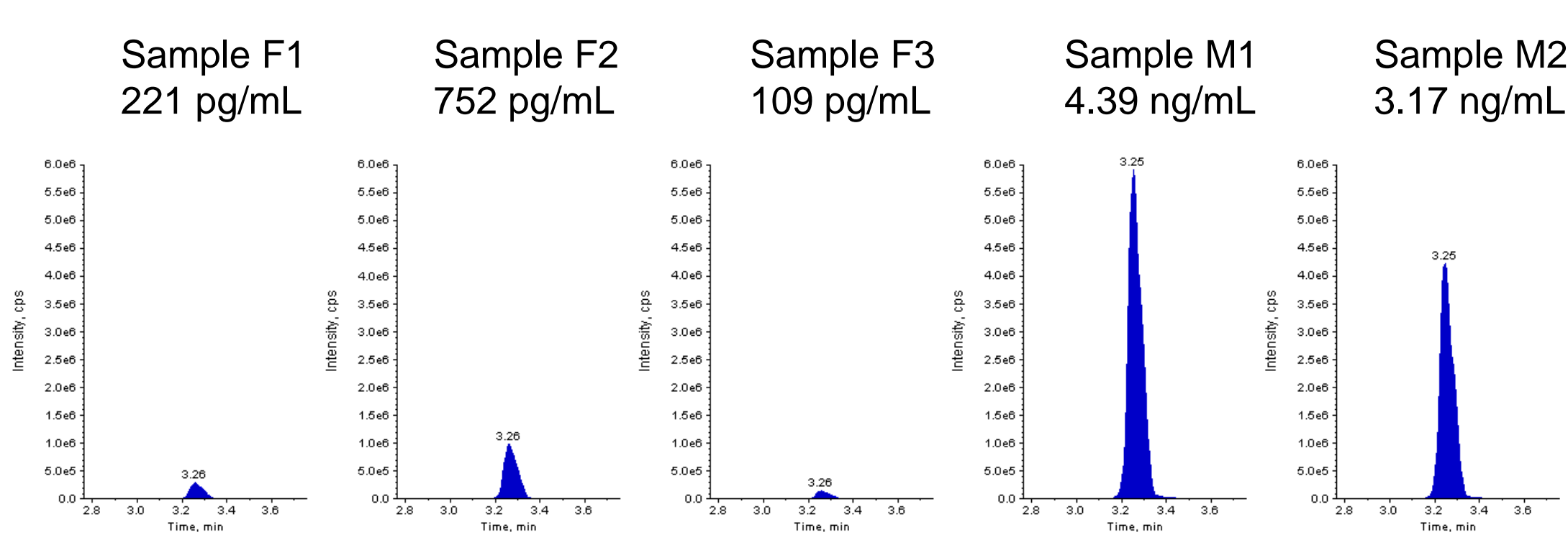| Sample F1 221 pg/mL | Sample F2 752 pg/mL | Sample F3 109 pg/mL | Sample M1 4.39 ng/mL | Sample M2 3.17 ng/mL |

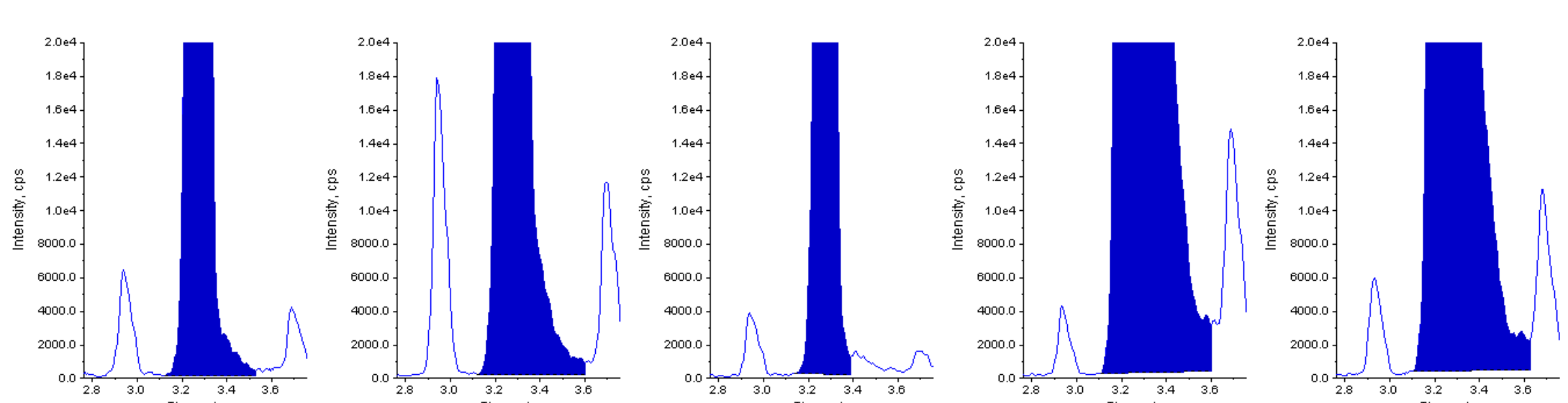Figure 4a. Example data for analysis of Testosterone in serum.

Figure 4b. Zoom on y-axis, to visualize separation of interferences, and low chemical background due to the use of the ion mobility filter.

DISCUSSION

The second generation SelexION+ ion mobility cell incorporates a jet injector lens and provides >2x improvement in ion transmission vs the original design.

To demonstrate the improved sensitivity when operating the ion mobility cell, a sensitivity comparison in neat solvent is presented in Figure 5. The total counts (cps) are lower when SelexION+ is employed, however the S/N and LOQ are equivalent, or better, when the ion mobility device is used.

Analysis on SCIEX 6500+ LC-MS/MS system (no ion mobility)
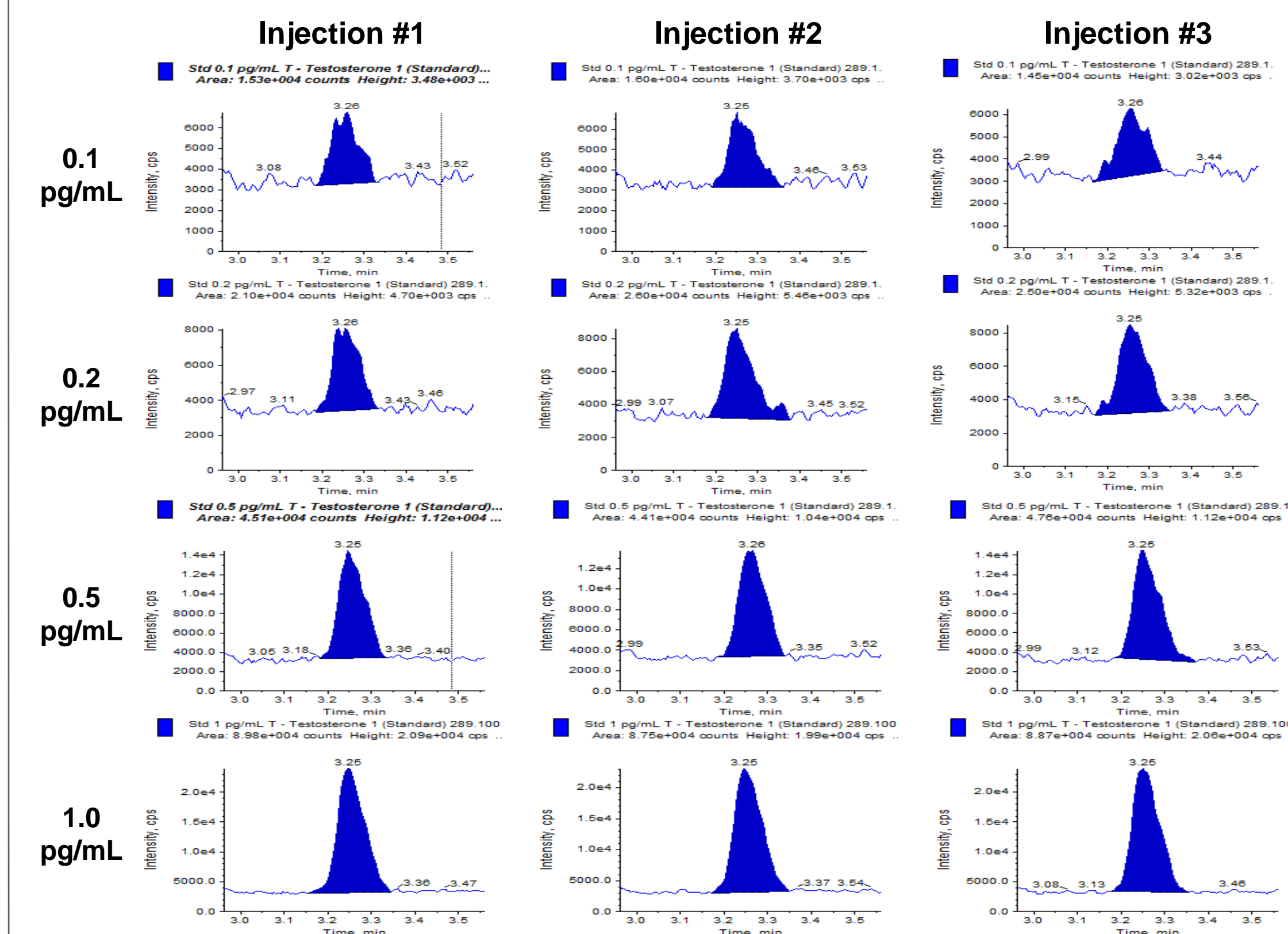
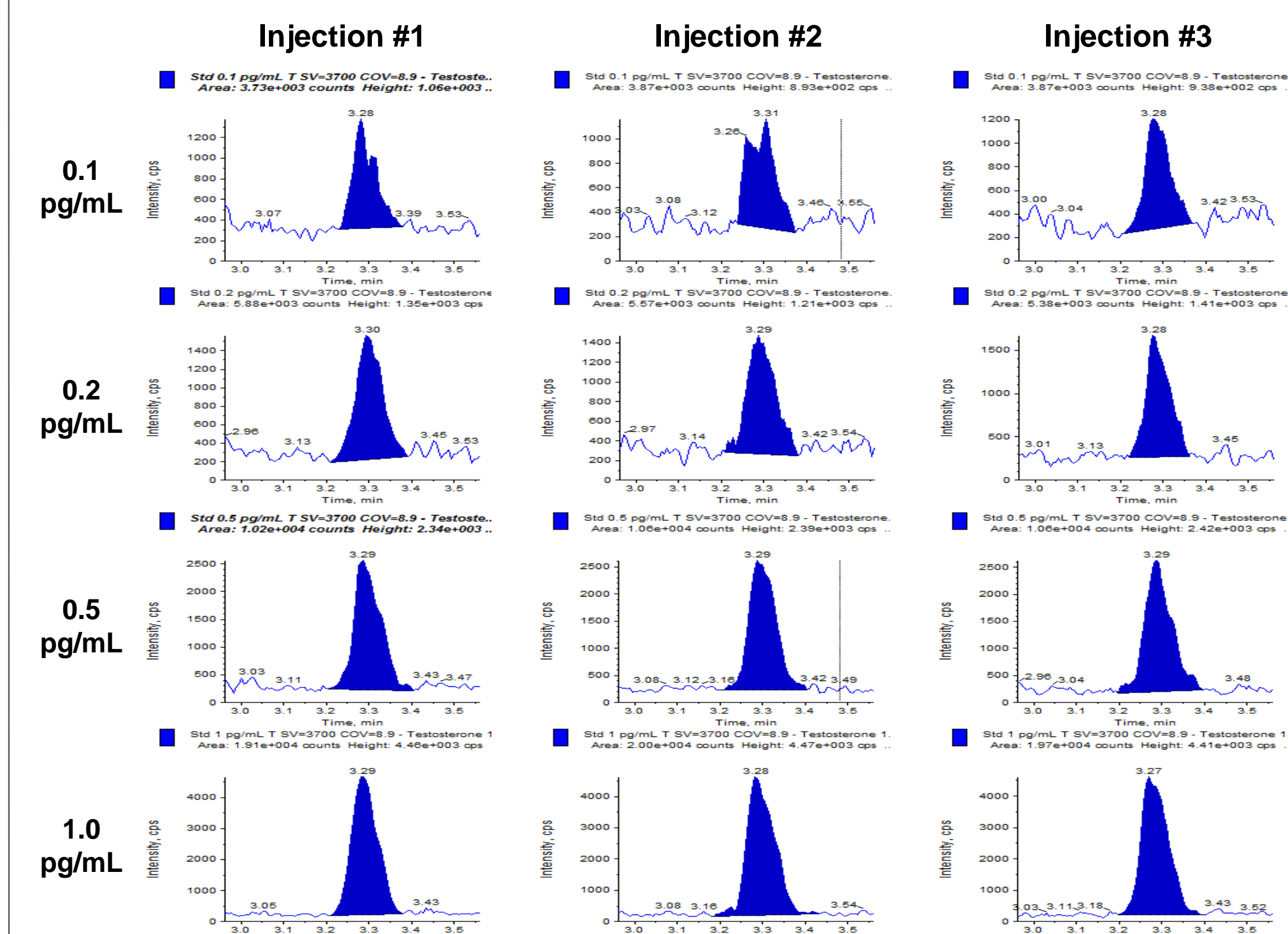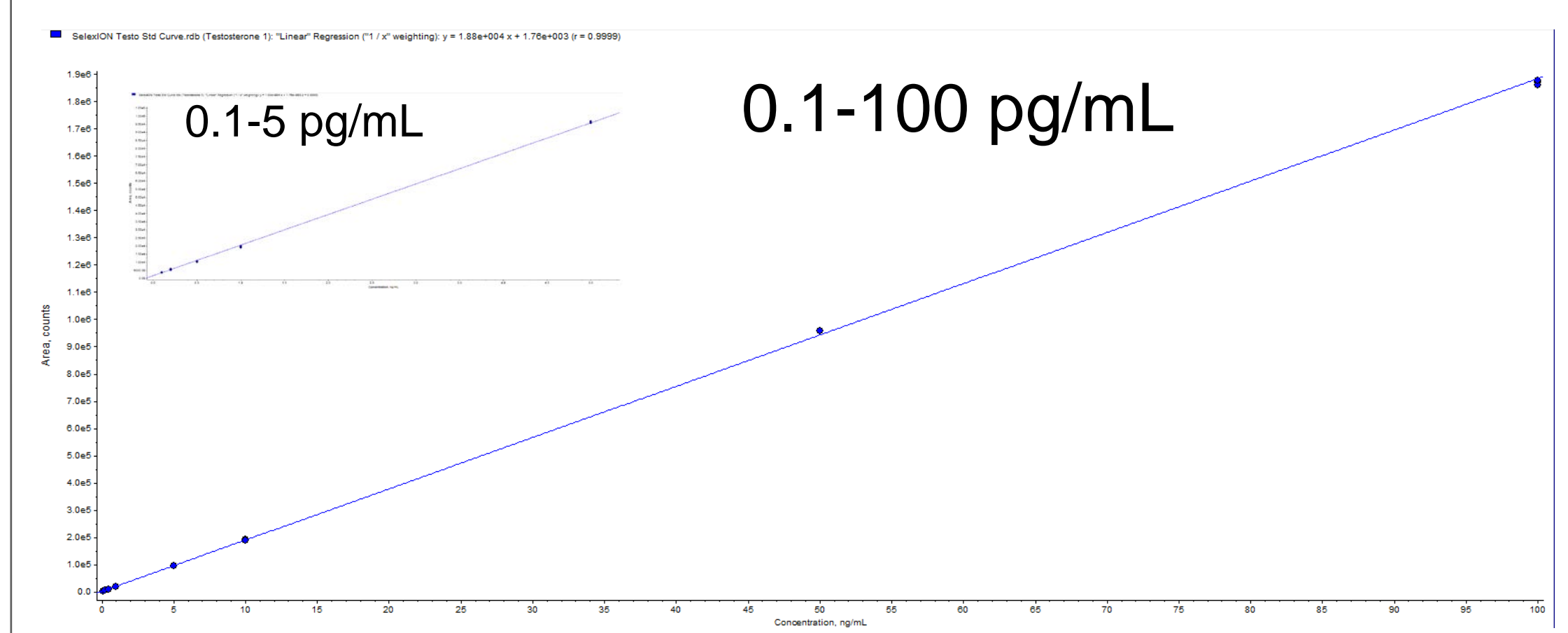### Analysis on SCIEX 6500+ LC-MS/MS system, equipped with SelexION+ ion mobility device

Figure 5. Comparison of LOQ and S/N, in neat solvent, with and without the SelexION+ ion mobility device.

Figure 6. Linear calibration curve (0.1 – 100 pg/mL) for Testosterone in neat solvent, using SelexION+ ion mobility device.

Expected Concentration	Sample Name	Number Of Values Used	Mean	Standard Deviation	%CV	Accuracy	Data Point #1	Data Point #2	Data Point #3
0.100000	Std 0.1 pg/mL, T, S	3 of 3	0.109250	0.004161	3.809138	109.250262	0.104445	0.111696	0.111609
0.200000	Std 0.2 pg/mL, T, S	3 of 3	0.204305	0.013413	6.564940	102.152492	0.218578	0.202374	0.191963
0.500000	Std 0.5 pg/mL, T, S	3 of 3	0.462214	0.014068	3.043539	92.442885	0.445996	0.471114	0.469533
1.000000	Std 1 pg/mL, T, S	3 of 3	0.948654	0.024199	2.550876	94.865365	0.922270	0.969815	0.953876
5.000000	Std 5 pg/mL, T, S	3 of 3	5.020899	0.026786	0.533493	100.417982	4.997296	5.049964	5.015227
10.000000	Std 10 pg/mL, T, SV	3 of 3	10.001103	0.043990	0.439853	100.011032	9.987918	10.050178	9.965214
50.000000	Std 50 pg/mL, T, SV	3 of 3	50.806408	0.071917	0.141552	101.612817	50.797449	50.739390	50.882386
100.000000	Std 100 pg/mL, T, S	3 of 3	99.247166	0.532913	0.536956	99.247166	99.351622	98.669759	99.720117

CONCLUSIONS

The LC-DMS-MS/MS method presented here enabled the quantation of Testosterone in human serum at <1pg/mL. No compromise in analytical sensitivity (LOQ) was observed when employing the ion mobility cell. This method boasts the added advantage of improved specificity, and therefore the possibility of simplified sample preparation.

TRADEMARKS/LICENSING