

# Utilizing High Resolution Accurate Mass for the Quantitation of Therapeutic Peptides in Human Plasma

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## ABSTRACT

**Purpose:** To benchmark assay performance utilizing high resolution accurate mass for the quantitation of Leuprolide.

**Methods:** Leuprolide was analyzed in the presence of human plasma sample matrix using Selected Ion Monitoring (SIM) and Parallel Reaction Monitoring (PRM) scanning functions. Data analysis were performed using Thermo Scientific™ TraceFinder™ software.

**Results:** The lower limit of quantitation (LOQ) for Leuprolide was determined to be 50 pg/mL for the SIM scan analysis and determined to be 10 pg/mL for the PRM scan analysis.

## INTRODUCTION

Therapeutic peptides represent a growing class of drug treatments that provide many benefits for a wide range of applications. Existing peptide drugs are known to be highly selective and effective at low levels. The increased efficacy of these therapeutic peptides requires both highly selective and highly sensitive quantitative assays to support the development of future treatments.

Here we investigate the advantages of a high resolution accurate mass LCMS assay for the quantitation of Leuprolide. A SIM scan experiment was performed to determine the lower limit of quantitation. Ultra high resolution settings of 70,000 for both SIM and PRM scan modes were investigated to assess and evaluate the increase of method selectivity on overall assay performance.

## MATERIALS AND METHODS

### Sample Preparation

Leuprolide and Nafarelin (Figure 1) standards were obtained from Sigma Aldrich, St. Louis, MO, and used to prepare calibration curves. The standard compounds were dissolved in Methanol at a concentration of 1 mg/mL. A series of standard working solutions was further prepared using Methanol and Water (50:50) at concentration range of 10 pg/mL to 250 ng/mL. Filtered human plasma was obtained from Bioreclamation/VT (Baltimore, MD) and used to generate subsequent calibration curves and QC samples.

300  $\mu$ L of plasma was spiked with the appropriate amount of the Leuprolide working solution and internal standard (Nafarelin) at each calibration curve concentration. Protein precipitation (ppt) was then performed on the plasma solution using a 200  $\mu$ L aliquot of methanol. The resulting sample solutions were vortexed for 30 seconds and then centrifuged at 4,000 rpm for 5 minutes. The supernatant from each sample was mixed with 300  $\mu$ L of water and transferred to a Thermo Scientific™ SOLA $\mu$ ™ HRP 2mg/1mL 96 well Solid Phase Extraction (SPE) plate for further sample clean up.

Prior to sample transfer, the SPE plate wells were pretreated with 2x 500  $\mu$ L portions of methanol followed by 2x 500  $\mu$ L of water. After plasma sample loading, the SPE wells were washed with 2x 500  $\mu$ L portions of water with 10% methanol. The analyte and internal standard were eluted with 2x 250  $\mu$ L of methanol with 0.1% formic acid. The eluate was then evaporated to dryness under a gentle stream of nitrogen. The resulting residue was reconstituted using 150  $\mu$ L of water with 20% methanol with 0.1% formic acid and agitated by plate mixer for 15 minutes. (Figure 2) The resulting solutions were then sampled directly in triplicate.

### Liquid Chromatography

Chromatographic separation was achieved using a Thermo Scientific™ Vanquish™ UHPLC system. Samples were injected at 20  $\mu$ L onto a 2.1 x 100mm, 1.9  $\mu$ m Thermo Scientific™ Hypersil GOLD™ Vanquish™ C18 UHPLC column. Gradient elution was accomplished using water + 0.1% Formic Acid (A) and acetonitrile + 0.1% formic acid (FA) (B), with a 34 minute gradient at a flow rate of 500 $\mu$ L/min (Table 1). Total run time including column equilibration was approximately 4.5 minutes.

### Mass Spectrometry

Compounds were analyzed utilizing a Thermo Scientific™ Q Exactive™ Focus MS with heated electrospray ionization (H-ESI II). Generic source conditions suitable for a 500 $\mu$ L/min LC flow rate were applied for all data collection (Table 1). Data were acquired at a resolution setting of 70,000 (FWHM) at m/z 200 utilizing both SIM and PRM modes with an external mass calibration.

### Data Analysis

All data were acquired and processed utilizing Thermo Scientific™ TraceFinder™ software. All chromatographic integration was accomplished using a 5 ppm mass extraction window and method defined processing settings. No manual integration or smoothing was applied to any chromatographic or spectral data.

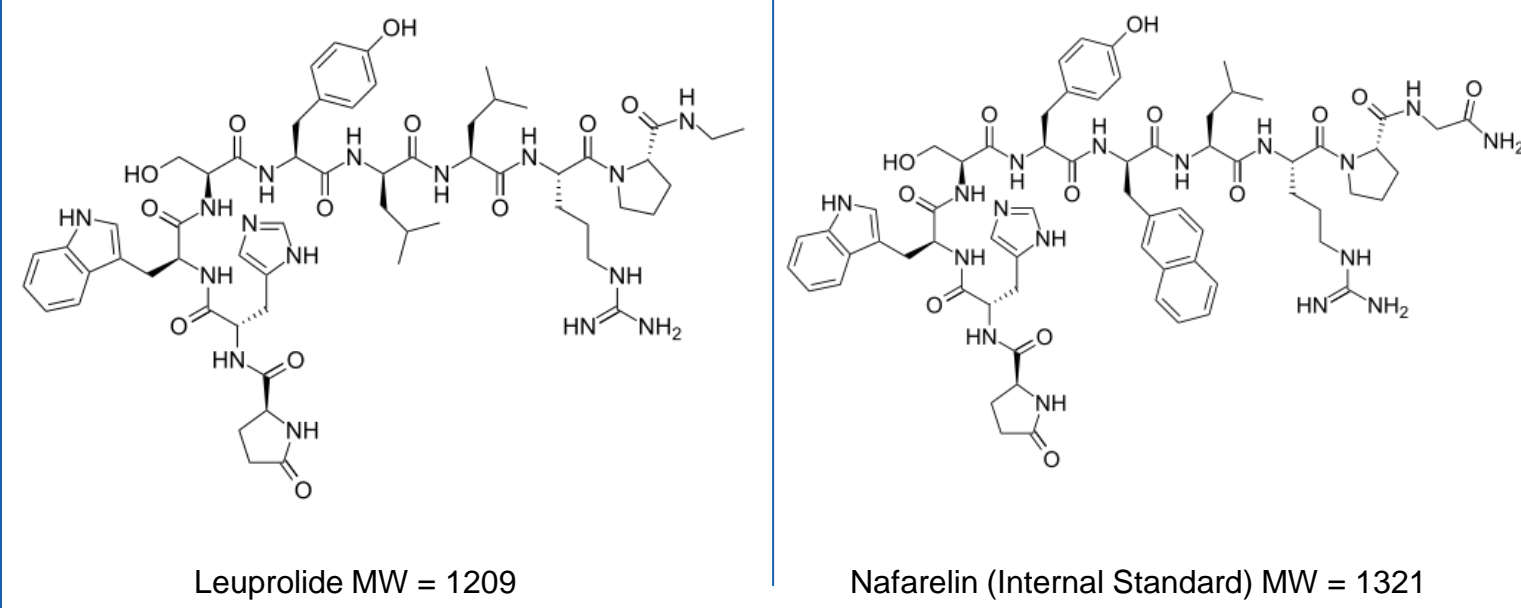


Figure 1. Compound structure for the nonapeptides Leuprolide (left) and Nafarelin (right)

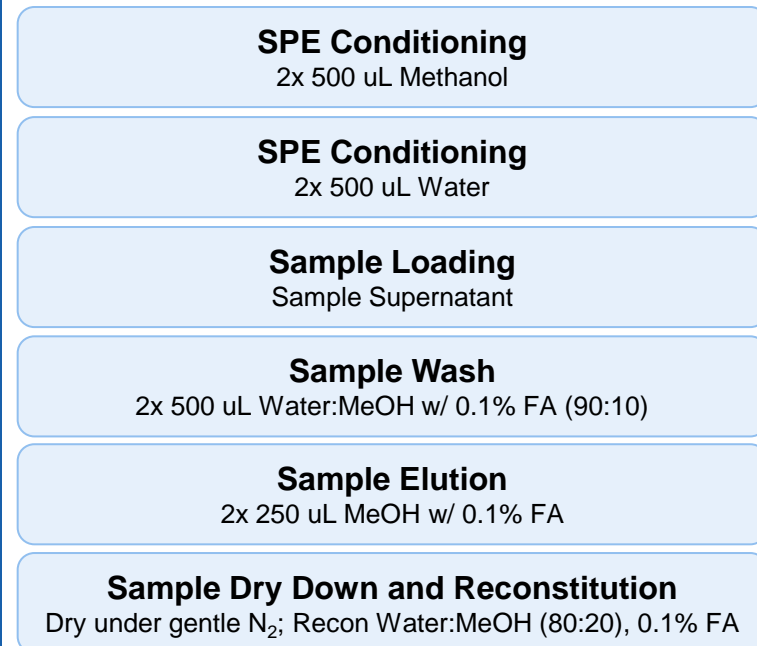


Figure 2. Solid Phase Extraction Workflow for Leuprolide (Analyte) and Nafarelin (Internal Standard).

Time (min)	Flow rate ( $\mu$ L/min)	%A	%B
0	500	95	5
0.5	500	95	5
1.0	500	70	30
2.5	500	65	35
2.7	500	5	95
3.5	500	5	95
3.6	500	95	5
4.0	500	95	5

HESI Source Settings	Value	Scan Settings	Value
Spray Voltage (V)	4000	Scan Type	SIM
Vaporizer temperature (°C)	450	Resolution	70,000
Capillary Temperature (°C)	350	AGC Target	2.00E+04
Sheath Gas Pressure (Arb)	45	IT Fill Time (ms)	240
Aux Gas Pressure (Arb)	15	Isolation Window	4
Ion Sweep Gas Pressure (Arb)	1	Scan Type	PRM
		Resolution	70,000
		AGC Target	1.00E+05
		IT Fill Time (ms)	240
		Isolation Window	4
		NCE Value	30

Table 1. LC gradient method (left) and MS conditions (right) utilized for sample analysis.

## RESULTS

### Target Ion Selection

Data acquisition utilizing an SIM scanning mode leverages high resolution settings to not only differentiate signal from the target analyte from unwanted interference without the need for MS/MS fragmentation but also enables the capability to fully resolve all isotopes in the isotopic envelope of a large molecule target. (Figure 3) This in turn allows each individual isotope to be monitored simultaneously and included in the post acquisition data processing. The ability to quantitate on and selectively sum multiple target ions while maintaining the flexibility to remove ion targets that introduce signal from unwanted interferences facilitates streamlined method development and assay robustness.

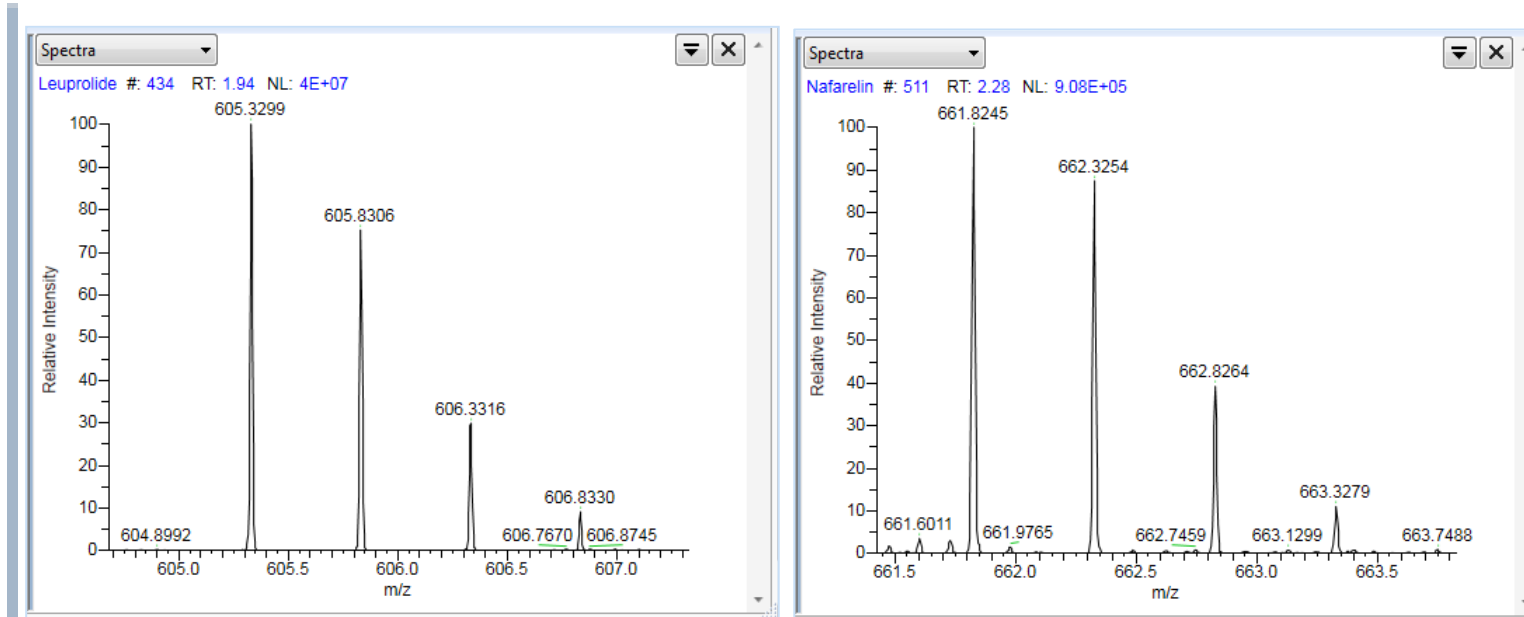


Figure 3. Isotopic envelop of the M+2 charge state for Leuprolide (left) and Nafarelin (right)

### SIM Analysis

The initial experimental analysis and assay performance was assessed using SIM mode. Assay performance and reproducibility were assessed across a calibration curve range of 10 pg/mL to 250 ng/mL. Each concentration level was analyzed in triplicate at an injection volume of 20  $\mu$ L. Linearity and reproducibility were calculated across the working range of the curve. The limit of quantitation (LOQ) was defined as the lowest concentration level that is both within <20% difference of the linear fit and <20% RSD for each group of replicate concentration points. Quantitative analysis and chromatographic peak generation was achieved through the quantitation of the two most intense isotopes of the M+2 charge state (A<sub>0</sub> and A<sub>1</sub>), which provide the most abundant signal and the least interference in the presence of the plasma matrix. The summed area of the two isotopes provide improved peak area reproducibility across the replicate injections as well as an improved % difference from the line fit, most significantly near and at the LOQ. (Figure 4) The overall assay LOQ using SIM mode for the plasma sample set was determined to be 50 pg/mL.

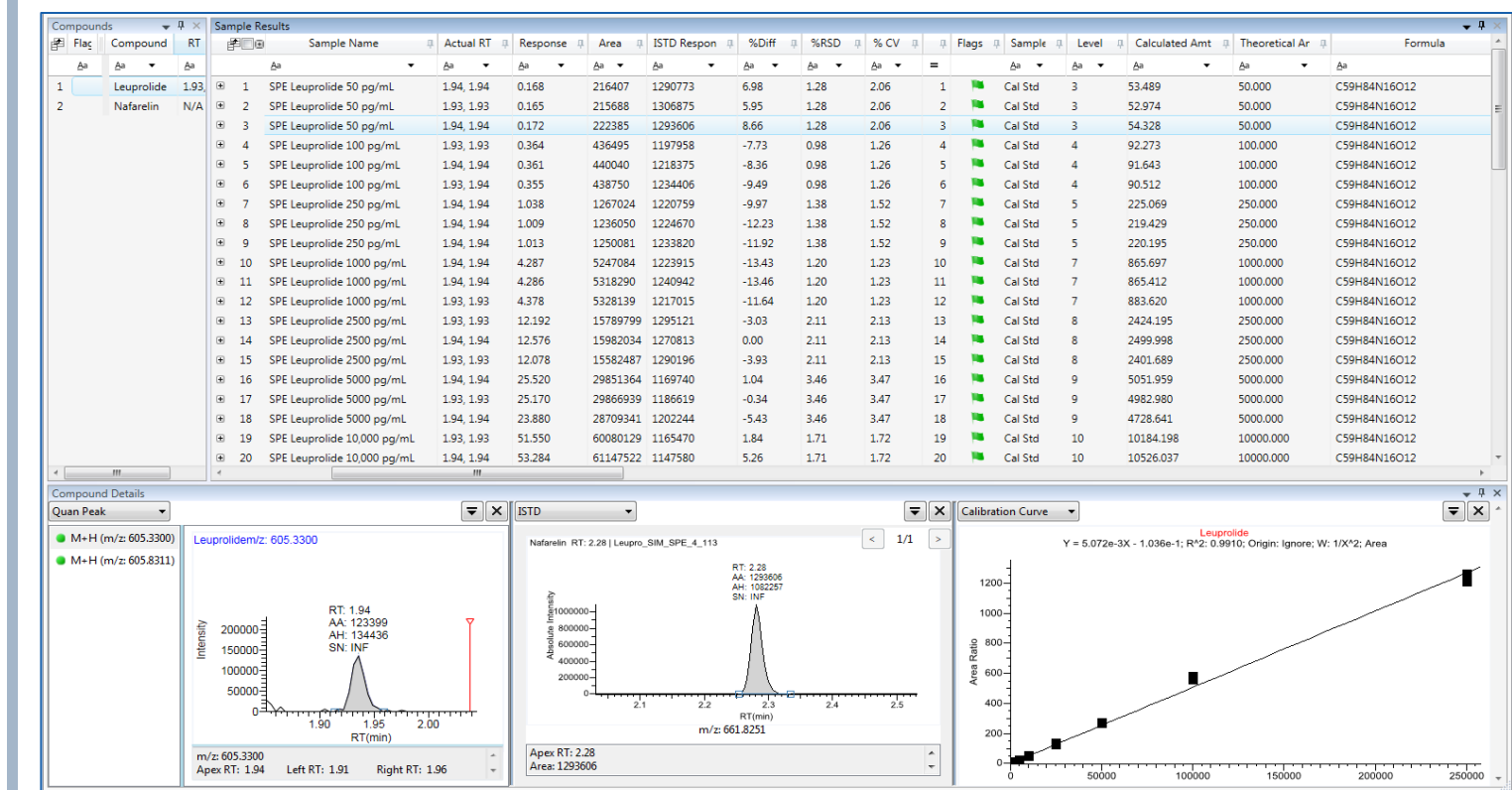
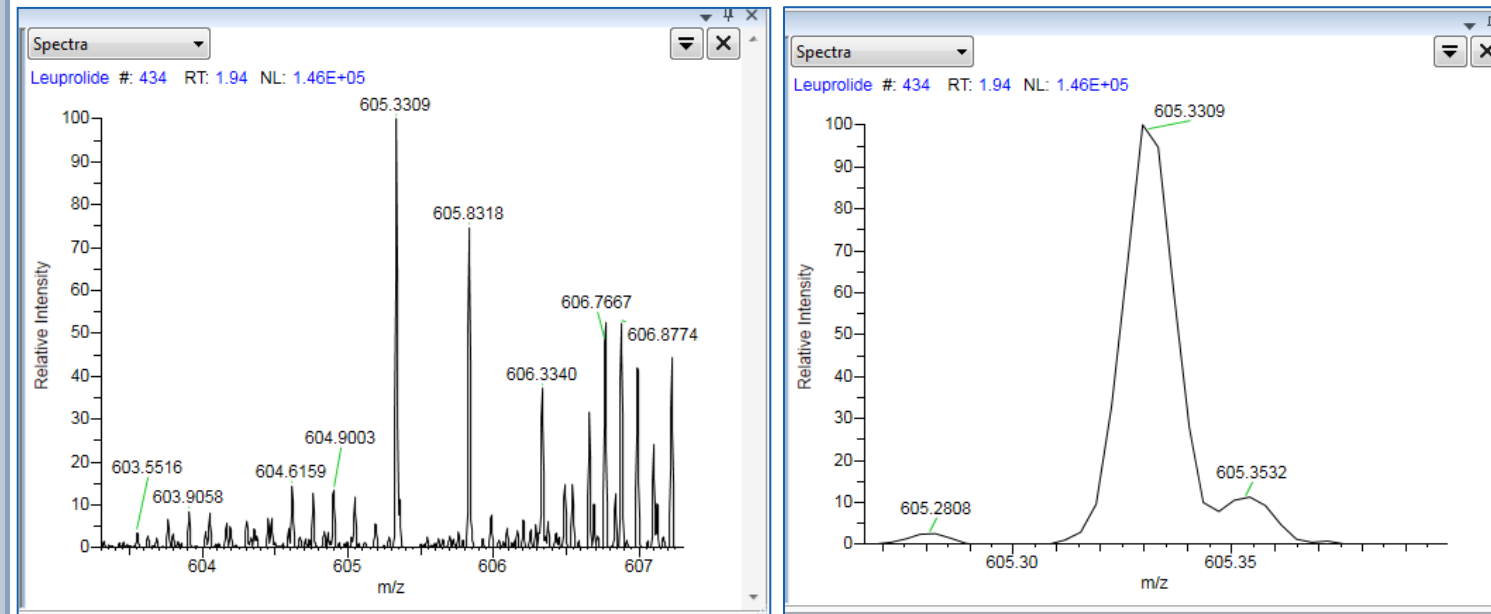


Figure 4. TraceFinder view of Leuprolide (left) and Nafarelin (right) for the SIM analysis at the 50 pg/mL LOQ.

Analysis of the SIM spectra of Leuprolide at the 50 pg/mL LOQ demonstrates an intense signal for the A0 and A1 isotopes with a mass accuracy of less than 2 ppm. Further spectral analysis at the A0 isotope reveals an interfering mass on the shoulder of the target mass. (Figure 5) The strong signal intensity at the LOQ indicates the need for improved selectivity in order to improve overall assay LOQ and performance. To obtain improved selectivity, samples were re-injected and analyzed utilizing the PRM mode.

Figure 5. Leuprolide spectra at the 50 pg/mL LOQ. The A0 isotope at 605.3300 and A1 isotope at 605.8316 (left) are present and intense. Further magnification of the A0 isotope displays an interference mass affecting assay performance at lower concentration levels.



Leuprolide SIM spectra at the 50 pg/mL LOQ ZOOMED - Leuprolide SIM spectra at the 50 pg/mL LOQ

### PRM Analysis

Results from the SIM analysis indicate the potential for improved assay performance arising from the increase of target selectivity and the further reduction of potential interferences. The experimental analysis and assay performance was reassessed using PRM mode. Potential fragmentation ions were predicted using Thermo Scientific™ Pinpoint™ software and the optimal product ion for quantitative performance was selected post acquisition. Fragmentation was achieved for both the Leuprolide and Nafarelin using the default Normalized Collision Energy (NCE) value of 30.

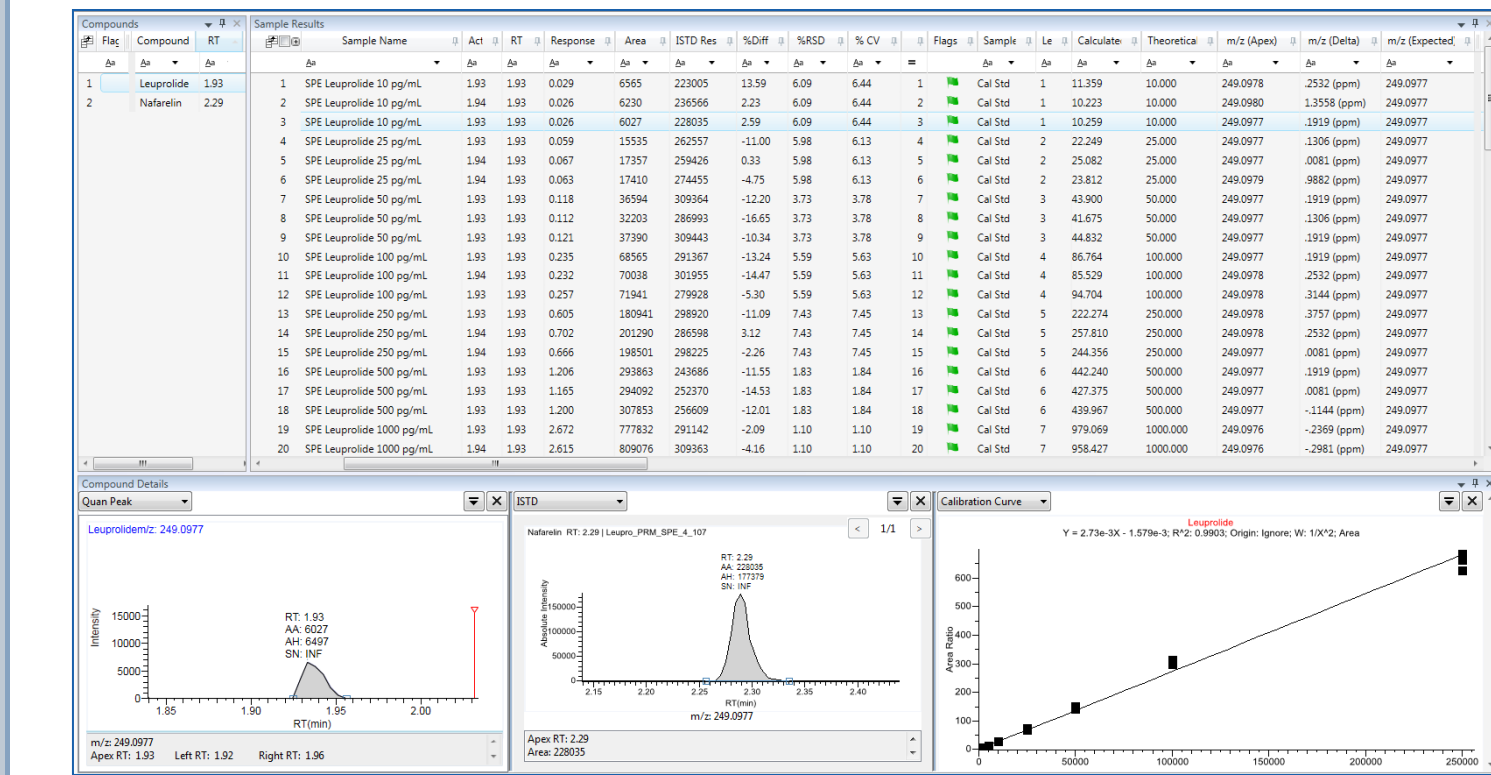
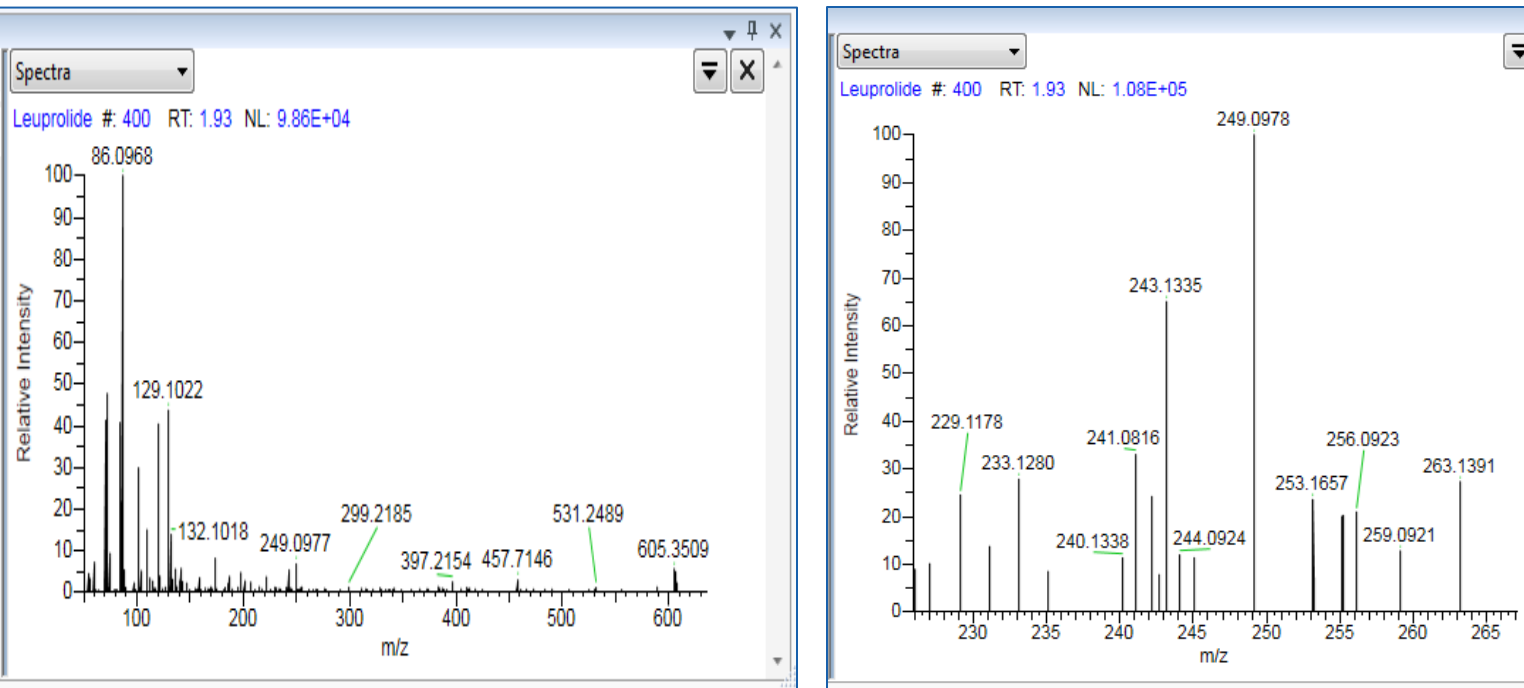


Figure 6. TraceFinder view of Leuprolide (left) and Nafarelin (right) for the PRM analysis at the 10 pg/mL LOQ.

Assay performance and reproducibility was again assessed across a calibration curve range of 10 pg/mL to 250 ng/mL. Each concentration level was analyzed in triplicate at an injection volume of 20  $\mu$ L. Linearity and reproducibility were calculated across the working range of the curve. The limit of quantitation (LOQ) was defined as the lowest concentration level that is both within <20% difference of the linear fit and <20% RSD for each group of replicate concentration points. Quantitative analysis and chromatographic peak generation was achieved through the quantitation of the b2 fragment ion, 249.0977 m/z, for both Leuprolide and Nafarelin, which provided the most abundant signal and the least interference in the presence of the plasma matrix. The improved selectivity of the PRM fragmentation provides excellent reproducibility across the replicate injections as well as a significant improvement in assay LOQ. (Figure 6) The overall assay LOQ using PRM mode for the plasma sample set was determined to be 10 pg/mL.

Analysis of the PRM spectra of Leuprolide at the 10 pg/mL LOQ demonstrates an intense signal for the b2 fragment ion with a mass accuracy of less than 2 ppm. Further spectral analysis of the b2 fragment ion isotope reveals the target m/z is fully resolved from all nearby noise and interference signals. (Figure 7) The strong signal intensity and the improved LOQ indicates that the improved selectivity provided by the PRM scan mode present an effective tool to further improve assay performance in the presence of complex biological matrices without the need for extensive re-optimization of the sample preparation procedure.

Figure 7. Leuprolide spectra at the 10 pg/mL LOQ. The b2 fragment ion 249.0977 (left) is present and intense at the analyte LOQ. Further magnification around the b2 fragment ion demonstrates resolution from interference ions facilitating assay quantitation at lower concentration levels.



Leuprolide PRM spectra at the 10 pg/mL LOQ ZOOMED - Leuprolide PRM spectra at the 10 pg/mL LOQ

## CONCLUSIONS

SIM scan analysis provided a sensitive and robust method that requires minimal method development. The LOQ for Leuprolide was determined to be 50 pg/mL in the human plasma matrix.

PRM scan analysis provided the highest assay selectivity while maintaining method simplicity and minimal method development. The LOQ was significantly improved with the increased selectivity and removal of interference and the LOQ for Leuprolide was determined to be 10 pg/mL.

The flexibility of the PRM scan mode presents an effective tool to further improve assay performance in the presence of complex biological matrices without the need for extensive re-optimization of the sample preparation procedure.

Detector sensitivity and scan method selectivity combined with an efficient SPE clean up procedure provide a robust and streamlined assay for the low level quantitative analysis of Leuprolide.

## REFERENCES (if necessary)

- D. Zhong, et al., Journal Chromatogr. B, 2009, 877, 3194–3200

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