Altasciences CLINICAL RESEARCH ALGORITHME PHARMA

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Comparison of Bottom-Up and Top-Down Analytical Methodologies for the Quantitative Bioanalysis of Large Therapeutic Peptides in Biological Matrix

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OVERVIEW

PURPOSE

To highlight potential differences between bottom-up and top-down bioanalysis of biotherapeutic peptides due to *in vivo* protein catabolism.

METHOD

Exenatide was quantified by top-down (SPE-LC-MS/MS) or bottom-up (SPE-Digestion-LC-MS/MS) approaches on a Sciex API 5000 mass spectrometer.

Exenatide degradation products were identified by information-dependent acquisition (IDA) on a Sciex TripleTOF 5600™.

RESULTS

While both assays demonstrated acceptable precision and accuracy, discrepancies were noted for exenatide bench-top stability in matrix.

Exenatide was found to be stable in rat plasma when analyzed using the bottom-up approach. In contrast, using the top-down approach revealed a 60% difference in exenatide concentration.

Investigation performed by IDA on a Sciex TripleTOF 5600™ revealed the presence of exenatide(3-39) as a major degradation product.

INTRODUCTION

Quantitation of biotherapeutic proteins >10 kDa is generally performed using a bottom-up LC-MS approach. However, for large peptides <10 kDa, either top-down or bottom-up approaches may be implemented depending upon specific assay requirements, such as selectivity, sensitivity or throughput. An emerging concern in large molecule quantitation is whether a bottom-up approach adequately represents a biotherapeutic concentration. Indeed, protein catabolism could introduce a bias in the determination of the intact biotherapeutic concentration when using a bottom-up approach. Alternatively, a top-down approach only measures the intact peptide and therefore discriminates truncated forms that might still bear pharmacological activity. For the current investigation, top-down and bottom-up approaches are compared and contrasted using exenatide, a 4.2 kDa therapeutic peptide.

METHODS

SAMPLE PROCESSING

Exenatide-SIL ($^{13}C^{15}N$ phenylalanine) was used as internal standard.

- Plasma samples were diluted with 10% H_3PO_4, loaded on Oasis MCX SPE, washed and eluted using methanolic ammonia
- Following evaporation to dryness, eluates were either:
  - Reconstituted with mobile phase and analyzed, or
  - Reconstituted with trypsin, digested overnight and analyzed

CHROMATOGRAPHY

- Agilent Technologies Series 1100 pumps and autosampler
- XBridge Peptide BEH300 column (50 x 2.1mm, 3.5 µm)
- Gradient elution of 0.2% CH_3CO_2H and ACN

DETECTION

Exenatide quantitation (top-down):

- Sciex API 5000 operated in MRM mode. Exenatide and exenatide-SIL were detected as the $[M+5H]^{5+}$ ions with *m/z* 838.3 > 396.0 and *m/z* 840.3 > 396.0, respectively

Exenatide quantitation (bottom-up):

- Sciex API 5000 operated in MRM mode. Exenatide tryptic peptide LFIEWLK and exenatide-SIL tryptic peptide LF*IEWLK were detected with *m/z* 474.8 > 688.4 and *m/z* 479.8 > 688.4, respectively

Identification of exenatide degradation product:

- Information-dependent acquisition (IDA) was performed using AnalystTF version 1.6 on a Sciex TripleTOF 5600™
- MS/MS scans were triggered for the ten most abundant precursor ions detected per TOF-MS scan with intensity ≥ 100 cps and charge states from +1 to +5
- The Dynamic Background Subtraction algorithm was enabled

Figure 1. Exenatide Amino Acids Sequence
Trypsin cleavage sites are indicated by arrows. Exenatide tryptic peptide used for quantitation is underlined.

RESULTS

EXENATIDE STABILITY ASSESSMENTS

In the current study, two different analytical methodologies were compared to quantitate the therapeutic peptide exenatide in rat plasma. While both assays demonstrated acceptable precision and accuracy, discrepancies were noted for bench-top stability in matrix.

Exenatide was found to be stable in rat plasma for 24 hours when analyzed using the bottom-up approach. In contrast, the stability samples analyzed using the top-down approach revealed a 60% difference in exenatide concentration between freshly prepared and stability samples, thus suggesting a possible biotransformation of exenatide (Table 1, Figure 2).

Table 1. Exenatide Stability in Rat Plasma for 24 Hours at Room Temperature Analyzed Using Top-Down or Bottom-Up Approaches

Approach	Results – %Difference vs Fresh (%CV)
Top-Down	Low QC (0.600 ng/mL): -65.7% (4.3%) High QC (30.000 ng/mL): -61.9% (2.2%)
Bottom-Up	Low QC (0.600 ng/mL): -8.4% (4.9%) High QC (30.000 ng/mL): +1.4% (2.2%)

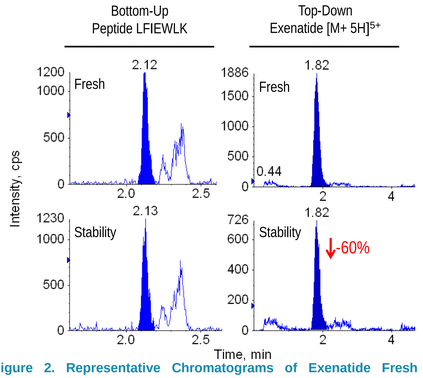

Figure 2. Representative Chromatograms of Exenatide Fresh and Stability Samples Analyzed Using Bottom-Up or Top-Down Approaches

EXENATIDE STABILITY INVESTIGATION

Top-down stability samples were reanalyzed using a TripleTOF 5600™ operated in Information-Dependent Acquisition (IDA) mode. Present only in stability samples, the analysis revealed a peptide with monoisotopic mass *m/z* 998.4884 (+4) whose product ion spectrum shared two diagnostic ions with exenatide: the y_3-ion (*m/z* 299.1680) and y_4-ion (*m/z* 396.2200, Figures 3 and 4).

This peptide was assigned to the N-terminal HG clipping biotransformation product exenatide(3-39). Notably, exenatide(3-39) once digested with trypsin would generate the surrogate peptide LFIEWLK and therefore be quantitated as exenatide, thus explaining why exenatide instability was noticed only with the top-down approach.

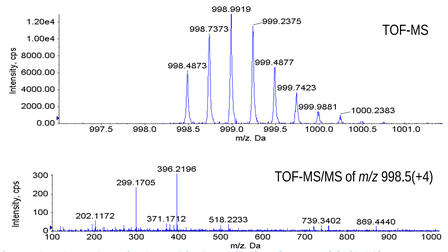

Figure 3. TOF-MS and TOF-MS/MS Analysis of Exenatide(3-39)

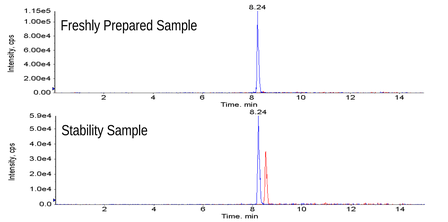

Figure 4. Extracted Ion Chromatogram from an IDA Experiment of Exenatide (blue) and Exenatide(3-39, red)

Further interrogation of the bottom-up stability samples using high resolution mass spectrometry confirm the presence of the N-terminal HG clipping biotransformation product exenatide(3-39). As shown in Figure 5, the exenatide(3-39) specific peptide EGTFTSDLSK is only detected in stability samples.

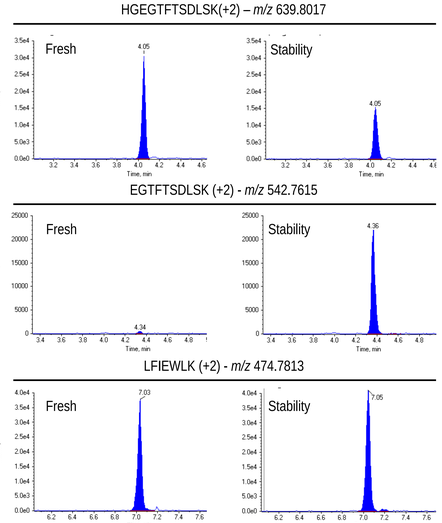

Figure 5. TOF-MS Analysis of Exenatide Bottom-Up Stability Samples

CONCLUSION

Due to the inherent differences in assay formats, results from bottom-up and top-down approaches may diverge. Although the observed discrepancies do not discredit either assay, their consideration is critical when interpreting data from different extraction approaches.

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