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From Clinical to Pre-Clinical Studies: LC-MS/MS Assay Challenges for Therapeutic Peptides

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OVERVIEW

PURPOSE

A case study for exenatide illustrates the challenges associated with the transfer of LC-MS/MS assays from human to animal matrices.

METHOD

Exenatide was extracted from plasma by Solid Phase Extraction using various protocols and analyzed by LC-MS/MS on a Sciex API 5000.

Exenatide stability in matrix was evaluated following storage in an ice-water bath at different time intervals. Information-dependent acquisition (IDA) was performed on a Sciex TripleTOF 5600^{TM} in order to identify exenatide degradation products in rat plasma.

RESULTS

Reducing sample volume 5-fold for the rat plasma assay decreased exenatide recovery by ~50% when compared to the human assay. Changing the SPE mechanism restored recovery to an acceptable level.

Exenatide stability in rat plasma failed to meet acceptance criteria under the conditions previously optimized for the human assay. Investigation performed by IDA on a Sciex TripleTOF 5600^{TM} revealed the presence of exenatide(3-39) degradation product.

INTRODUCTION

Over the course of a drug development program, methods are commonly transferred between pre-clinical and Phase I clinical studies, however the reverse is less frequent. While difficulties apply to both small and large molecule method transfers, the quantitation of biotherapeutic peptides and proteins present unique challenges. For example, peptide-based drugs are often predisposed to greater endogenous interference and enzymatic degradation as a function of species. Moreover, the limited sample volume associated with pre-clinical studies can translate to complications in achieving the targeted LLOQ. In the current investigation, we exemplify such bioanalytical hurdles for the quantitation of exenatide in an LC-MS/MS assay transferred from human plasma to rat plasma.

METHODS

SAMPLE PROCESSING

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

- Oasis WAX, 30 mg: Samples were diluted with 8M Guanidine HCl, loaded on the SPE, washed and eluted with methanolic ammonia
- Oasis MCX, 30 mg: Plasma samples were diluted with 10% H_3PO_4, loaded on the SPE, washed and eluted using methanolic ammonia

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:

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

Identification of exenatide degradation product:

- Information-dependent acquisition (IDA) was performed using AnalystTF version 1.6 on a Sciex TripleTOF 5600^{TM}
- 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

RESULTS

EXENATIDE EXTRACTION FROM RAT PLASMA

Sample extraction challenges were encountered during transfer of the exenatide LC-MS/MS method from human plasma to rat plasma. Reducing sample volume 5-fold decreased exenatide SPE recovery by ~50%. Lower SPE recovery was attributed to adsorptive losses during loading due to the insufficient presence of endogenous plasma components able to prevent non-specific binding to container surfaces.

Table 1. Relative Recovery of Exenatide by Weak-Anion Exchange SPE

Sample	Diluent	Loading Buffer	Relative Recovery
50 µL Rat Plasma	---	50 µL 8M Guanidine	0.459
50 µL Rat Plasma	200 µL H_2O	250 µL 8M Guanidine	0.465
50 µL Rat Plasma	200 µL Human Plasma	250 µL 8M Guanidine	1.002
250 µL Rat Plasma	---	250 µL 8M Guanidine	0.866
250 µL Human Plasma	---	250 µL 8M Guanidine	1.000

Changing the extraction mechanism from weak-anion exchange to strong-cation exchange allowed sample loading in diluted phosphoric acid, thereby circumventing adsorptive losses and restoring recovery to an acceptable level. The assay showed adequate sensitivity and selectivity at the LLOQ level.

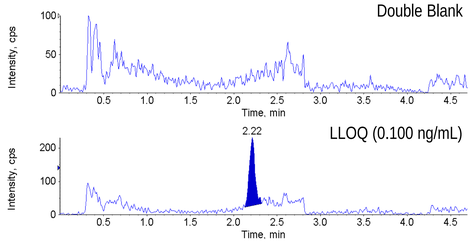

Figure 1. Representative Chromatograms of Exenatide Extracted from Rat Plasma (50 µL) by Strong-Cation Exchange SPE

EXENATIDE STABILITY IN MATRIX

Exenatide stability in rat plasma failed to meet acceptance criteria under the conditions optimized for the human assay. Stability was proven in human plasma for 22 hours on ice, however a (-) 25% deviation was observed in rat plasma after 18 hours under identical conditions.

Table 2. Exenatide Stability in Human and Rat Plasma

Matrix	Duration	Results – Accuracy (precision)
Human Plasma	22.7 hours	Low QC (30.0 pg/mL): 94.5% (6.0%) High QC (1500.0 pg/mL): 96.3% (2.1%)
Rat Plasma	18.5 hours	Low QC (0.300 ng/mL): 75.9% (5.0%) High QC (15.000 ng/mL): 76.9% (4.2%)

Investigations performed by high resolution mass spectrometry (TripleTOF 5600^{TM}) operated in IDA mode revealed the presence of a peptide with monoisotopic m/z 998.4873 (+4) in stability samples, but noticeably absent in freshly spiked extracts. Based upon parent mass assignment and MS/MS spectral information, this peptide was assigned to the exenatide degradation product 3-39 (N-terminal HG clipping).

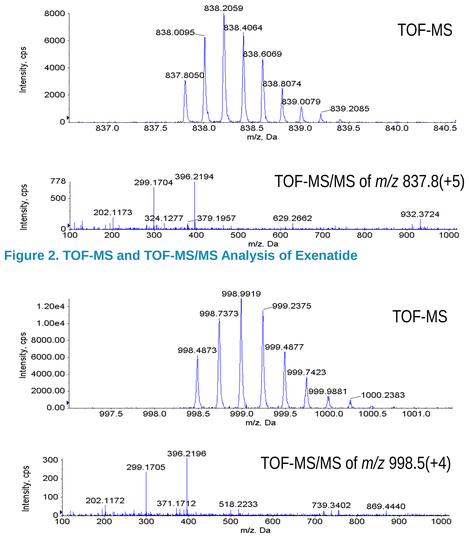

Figure 2. TOF-MS and TOF-MS/MS Analysis of Exenatide

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

Table 3. Accurate Mass Analysis of Exenatide Degradation Product

	Measured Accurate Mass (monoisotopic) m/z	Mass Accuracy ppm
Exenatide(3-39)	998.4873 (+4)	-6.7
y4	396.2196	-11.4
y3	299.1705	-3.0
y2	202.1172	-6.9

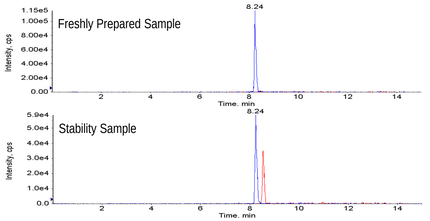

Figure 4. Extracted Ion Chromatogram from an IDA experiment of Exenatide (blue) and Exenatide(3-39, red)

METHOD VALIDATION

Table 4. Summary of Method Validation

Evaluation	Results
Precision and Accuracy (inter-day)	LLOQ QC: 108.6%, CV = 10.1% Low QC:103.8%, CV = 7.1% Mid QC(a) :104.4%, CV = 3.0% Mid QC(b) :102.2%, CV = 4.7% High QC:98.0%, CV = 3.1%
Percent Extraction Yield	47.2% to 50.0% through QC levels
Matrix Factor	Acceptable for 6 lots and one hemolyzed (5%)
Selectivity	Acceptable for 6 lots and one hemolyzed (5%)
Processed Samples Stability	163.9 hours at 4°C
Short-Term Stability (ice-water bath)	9.8 hours
Long-Term Stability (-80°C)	33 days
Freeze Thaw	4 cycles
Whole Blood Stability	2.1 hours

CONCLUSION

In overcoming the recovery challenge and elucidating the degradation pathway of exenatide in rat plasma, a newly developed LC-MS/MS assay was successfully validated and implemented for pre-clinical sample analysis.

ACKNOWLEDGMENTS

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