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Sensitive Quantification of Insulin Lispro Using Accurate Mass Spectrometry

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Introduction

In this technical note, a sensitive workflow was developed for the quantification of Insulin Lispro, an altered form of natural Insulin implemented for glycemic control. Excellent sensitivity was achieved for the quantification of Insulin Lispro at a lower limit of quantification (LLOQ) of 0.1 ng/mL in rat plasma with outstanding reproducibility, accuracy, and wide linearity up to 4.3 orders of magnitude. Short-acting Insulin analogs such as Insulin Lispro, Aspart, and Glulisine are more readily absorbed and exhibit faster biological actions in comparison to human Insulin. As a result of their key role in Insulin therapeutics, it is important to study the pharmacokinetic and pharmacodynamic profiles of the Insulin analogs. While LC-MS based methods remain the most sensitive and selective platforms for the analysis of Insulin therapeutics, key analytical challenges still prevail.

Sensitivity is one of the most critical analytical challenges because of the low ionization and low CID efficiency given the high molecular weight of Insulin analogs. In addition, Insulin analogs are difficult to quantify at low concentrations in biological matrices given the presence of endogenous interference from natural Insulin. Recently, accurate mass spectrometers such as time-of-flights (TOFs) have been implemented for quantitative analysis because of greater selectivity. However, the low duty cycle (<30 %) directly impacts the overall quantitative sensitivity. Herein, a highly selective and sensitive microLC-MRM^{HR} workflow was developed for the quantification of Insulin Lispro in rat plasma. Greater sensitivity for quantification was achieved using a combination of enhanced in-source gas phase ion production from the micro-LC and the Zeno™ trap with improved MS/MS sampling efficiency (~ 90 % duty cycle). Low-level quantification of Insulin Lispro was reached with exceptional accuracy and precision on an accurate mass spectrometry platform.

Sample Preparation

The rat plasma was protein precipitated and the supernatant was diluted 1:1 (v/v) by water and served as the processed biological matrix. Insulin Lispro and Bovine Insulin (internal standard) were prepared in 1:1 (v/v) processed rat plasma. Serial dilution was performed to prepare calibration curve standard samples. The samples were processed by mixed-mode SPE. The eluents from the SPE plate were diluted in 1:1 (v/v) water and injected directly into the LC-MS/MS for analysis.

LC Conditions – Trap Column

Column: Luna™ 5 µm C18(2)
Dimensions: 20 x 0.3 mm
Part No.: 05M-4252-AC
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient: Time (min)	%B
0	0
2.	0
5.2	90
6.8	90

Flow Rate: 50 µL/min
Injection Volume: 30 µL
Temperature: Ambient
LC System: SCIEX® M5 MicroLC

LC Conditions – Analytical Column

Column: Kinetex™ 2.6 µm XB-C18
Dimensions: 50 x 0.3 mm
Part No.: 00B-4496-AC
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

Gradient: Time (min)	%B
0	15
2	15
3	60
5.2	90
6.8	90
7	15
10	15

Flow Rate: 5 µL/min
Injection Volume: 20 µL
Temperature: 50 °C
LC System: SCIEX M5 MicroLC
Detection: MRM
Detector: SCIEX Zeno MRM^{HR} on a ZenoTOF® 7600

MRM Conditions

Polarity: Positive
Source Temperature: 600 °C
GS1: 50
GS2: 60
CUR: 30
CAD: 7
IS: 5500 V
MS Accumulation Time (ms): 80
MS/MS Accumulation Time (ms): 60
TOF MS Start Mass (m/z): 600
TOF MS Stop Mass (m/z): 1200
TOF MS/MS Start Mass (m/z): 100/600
TOF MS/MS Stop Mass (m/z): 600/1200
Zeno Threshold: 20,000

MRM Parameters

Compound	Precursor Ion (m/z)	Product Ion (m/z)	DP	CE
Insulin Lispro	1162	217.1194	80	55
Bovine Insulin (IS)	955	1114.5069	80	40

Results and Discussion

Insulin Lispro was selected as a model analyte to evaluate the quantification of complex Insulin therapeutic structures on the ZenoTOF® 7600 system. The calibration curve included concentrations ranging from 0.1 ng/mL to 2000 ng/mL. The concentration of the IS (Bovine Insulin) was 50 ng/mL. Each calibration point was measured in 3 replicates.

Since human Insulin is often encountered as an endogenous interference, CID was employed to differentiate Insulin Lispro from human Insulin. As shown in **Figure 2**, human Insulin and Insulin Lispro are differentiated using m/z 226 and m/z 217, respectively. Both y2 fragments arise from the last 2 amino acids in the B chain (**Figure 1**). For human Insulin, fragment m/z 226 is composed of Proline and Tyrosine while for Insulin Lispro, fragment m/z 217 is composed of Lysine and Tyrosine.

For quantification of Insulin Lispro, fragment ions m/z 136 and m/z 217 were considered. Fragment ion m/z 136 corresponds to a Tyrosine Immonium ion. As shown in **Figure 3**, the XIC using m/z 136 generates significant background interference at the retention time of the analyte (3.72 min). However, XIC generated using m/z 217, shows no interference at the retention time of the analyte and therefore, was selected for quantification of Insulin Lispro.

Significant analytical challenges such as low gas-phase ionization and low CID efficiency impede low-level quantification of Insulin analogs. For this analysis, an OptiFlow® Pro ion source was employed under classical microflow conditions to deliver sensitive and robust ionization. In addition, greater MS/MS sampling efficiency of the Zeno™ trap improved the overall sensitivity for quantification of Insulin Lispro.

The quantitative criteria for %CV were less than 20 % and accuracy was within ±20 % of the nominal concentration at the level of the LLOQ. For the remaining concentrations, the %CV was required to be less than 15 %, while the accuracy was required to be within ±15 % of the nominal concentration.

As shown in **Figure 4**, the LLOQ for Insulin Lispro was determined to be 0.1 ng/mL. No matrix interferences were observed in the blank. The upper limit of quantification (ULOQ) was determined to be 2000 ng/mL.

Strong linearity was observed across the concentration range between 0.1 ng/mL to 2000 ng/mL with a LDR of 4.3 orders of magnitude (**Figure 5**). Solvent blank injected after the ULOQ indicated 10 % carryover relative to the LLOQ. This could be further reduced by adding longer columns and trap washes if the full linear range is needed.

The overall %CV was less than 9 %, with accuracy within ±13 % of the nominal concentration (**Table 1**). This demonstrates the overall assay sensitivity where low-level concentrations were detected and quantified with excellent accuracy and precision.

Figure 1. Amino Acid Sequence of Insulin Lispro, Human Insulin, and Bovine Insulin.

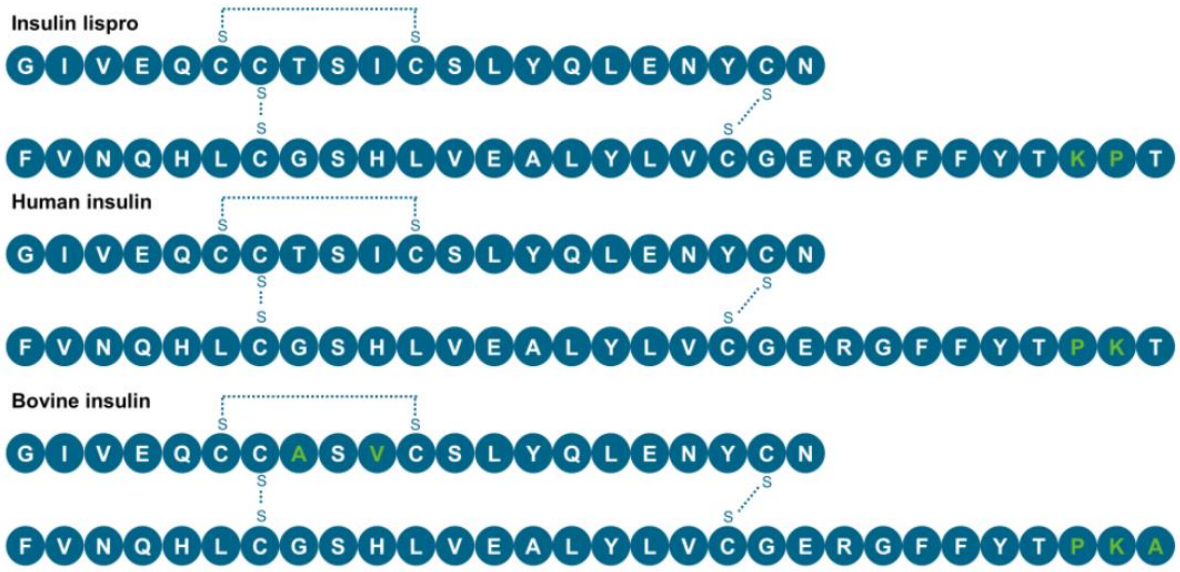

Figure 2. Zeno™ MS/MS Spectra from CID of the $[M+5H]^{5+}$ Precursors of Human Insulin and Insulin Lispro.

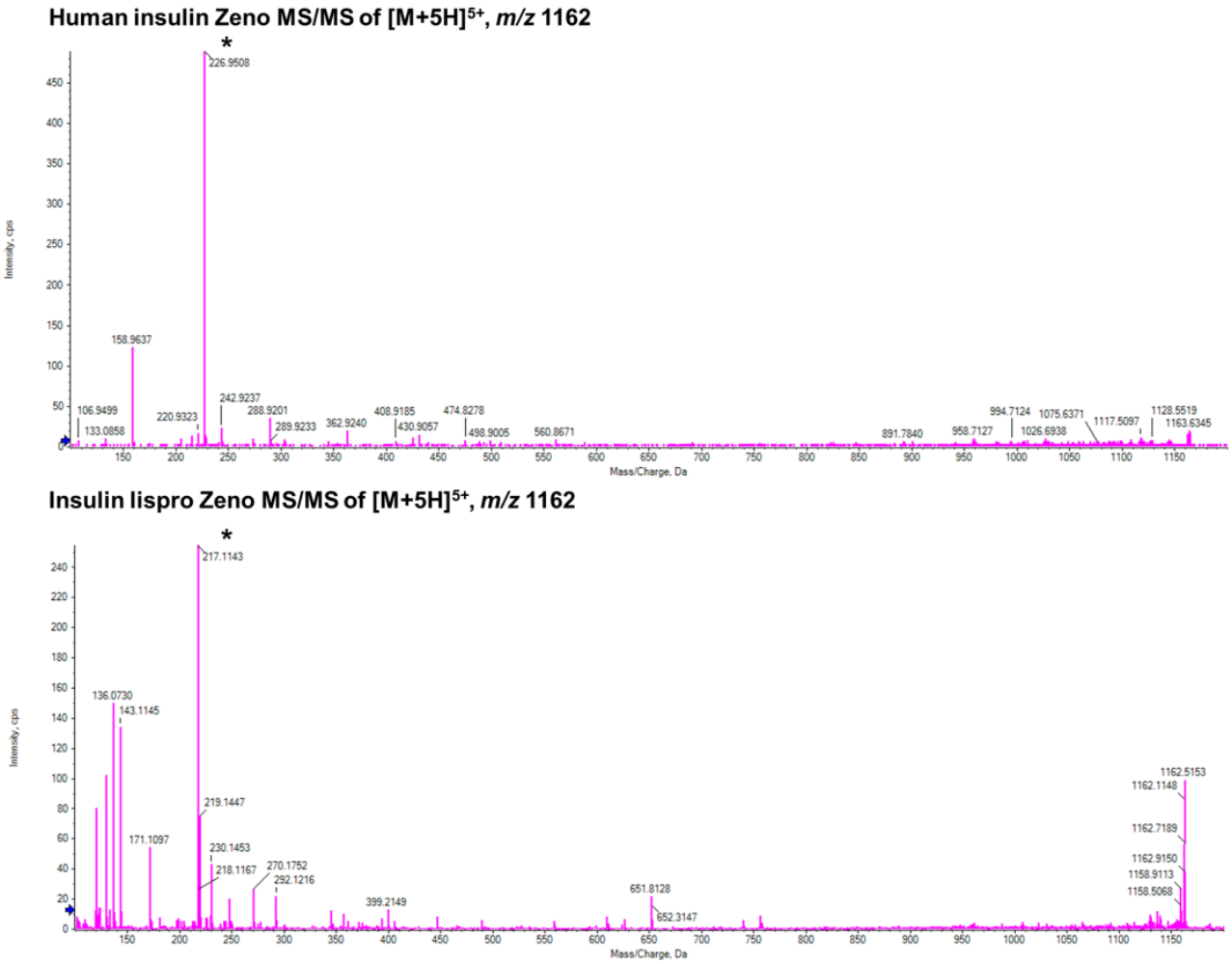

Figure 3. Extracted Ion Chromatograms (XICs) of Two Different MRM^{HR} for Tyrosine Immonium Ion.

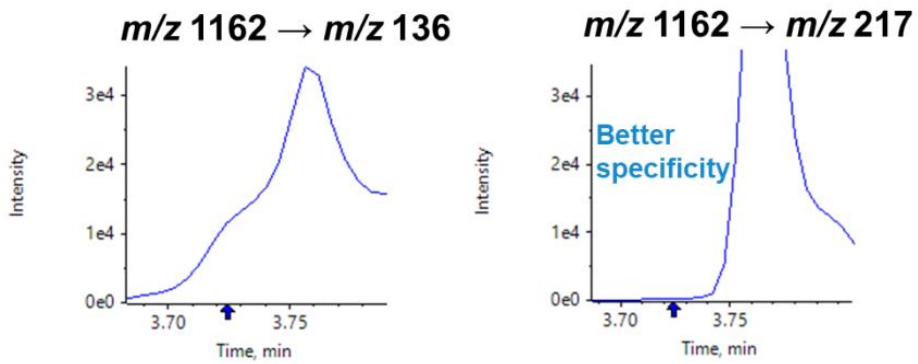

Figure 4. XIC of the Matrix Blank, LLOQ, and ULOQ for Insulin Lispro Quantification.

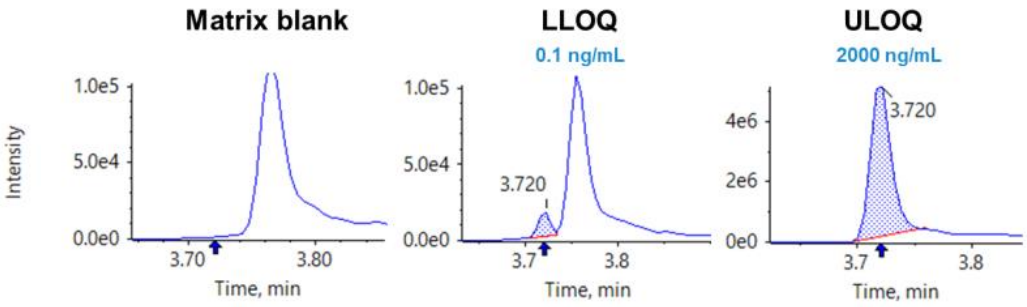

Figure 5. Calibration Curve of Insulin Lispro in Rat Plasma.

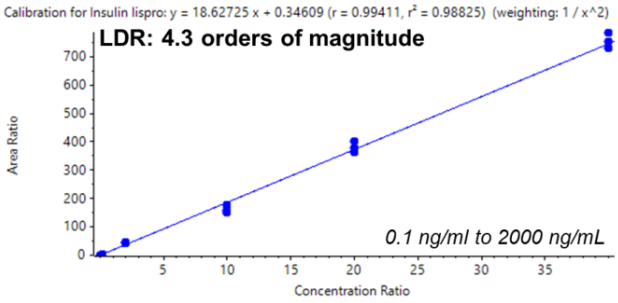

Table 1. Accuracy and Precision Results at Each Concentration Level.

Concentration (ng/mL)	Accuracy (%)	CV (%)
2000	101	3.68
1000	102	4.99
500	87.2	7.92
100	114	4.89
10	105	6.30
1	88.8	2.86
0.1	101	8.76

Conclusions

Low-level LLOQ for quantification of Insulin Lispro was achieved with GLP accuracy, precision and wide linearity up to 4.3 orders of magnitude. Sensitive quantification of insulin analogs was achievable with improved MS/MS sampling efficiency provided by the Zeno™ trap on the ZenoTOF® 7600 system seamlessly interfaced with a micro-LC front end. A streamlined data reduction platform was demonstrated on SCIEX® OS software for data acquisition, analysis and management.

Luna™ Ordering Information

3 µm and 5 µm Micro LC Columns (mm)								Trap Column	Trap Column
Phases	**50 x 0.30**	**100 x 0.30**	**150 x 0.30**	**50 x 0.50**	**100 x 0.50**	**150 x 0.50**	**250 x 0.50**	**20 x 0.30**	**20 x 0.50**
3 µm C8(2)	00B-4248-AC	—	—	00B-4248-AF	—	—	—	—	—
3 µm C18(2)	00B-4251-AC	00D-4251-AC	00F-4251-AC	00B-4251-AF	00D-4251-AF	00F-4251-AF	—	—	—
3 µm Phenyl-Hexyl	—	00D-4256-AC	—	—	00D-4256-AF	—	—	—	—
3 µm NH2	—	—	00F-4377-AC	—	—	—	—	—	—
3 µm HILIC	—	—	—	00B-4449-AF	—	—	—	—	—
5 µm C8(2)	—	—	00F-4249-AC	—	—	—	—	05M-4249-AC	05M-4249-AF
5 µm C18(2)	—	—	00F-4252-AC	—	—	00F-4252-AF	00G-4252-AF	05M-4252-AC	05M-4252-AF
5 µm Phenyl-Hexyl	00B-4257-AC	—	—	00B-4257-AF	—	—	—	—	—

Kinetex™ Ordering Information

2.6 µm Micro LC Columns (mm)						
Phases	**30 x 0.3**	**50 x 0.3**	**100 x 0.3**	**150 x 0.3**	**50 x 0.5**	**150 x 0.5**
XB-C18	00A-4496-AC	00B-4496-AC	00D-4496-AC	00F-4496-AC	00B-4496-AF	00F-4496-AF
Biphenyl	—	00B-4622-AC	—	00F-4622-AC	00B-4622-AF	—
C18	00A-4462-AC	00B-4462-AC	—	00F-4462-AC	00B-4462-AF	—
EVO C18	—	00B-4725-AC	—	00F-4725-AC	00B-4725-AF	—
F5	—	00B-4723-AC	00D-4723-AC	00F-4723-AC	00B-4723-AF	—

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