Sensitive quantification of insulin lispro using accurate mass spectrometry

Increasing sensitivity for insulin quantification using the ZenoTOF 7600 system, powered by SCIEX OS software

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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).[1-3] Low-level quantification of insulin lispro was reached with exceptional accuracy and precision on an accurate mass spectrometry platform.

Key features of the insulin quantification workflow on the ZenoTOF 7600 system

- Achieve sensitive quantification of insulin lispro in rat plasma at 0.1 ng/mL using the ZenoTOF 7600 system with excellent reproducibility, accuracy and wide linearity up to 4.3 orders of magnitude
- Reach enhanced sensitivity for quantification with improvements in MS/MS sampling efficiency using the Zeno trap[1-3]
- Increase productivity with user-friendly interface and integrated platform for data acquisition, processing and management using the SCIEX OS software

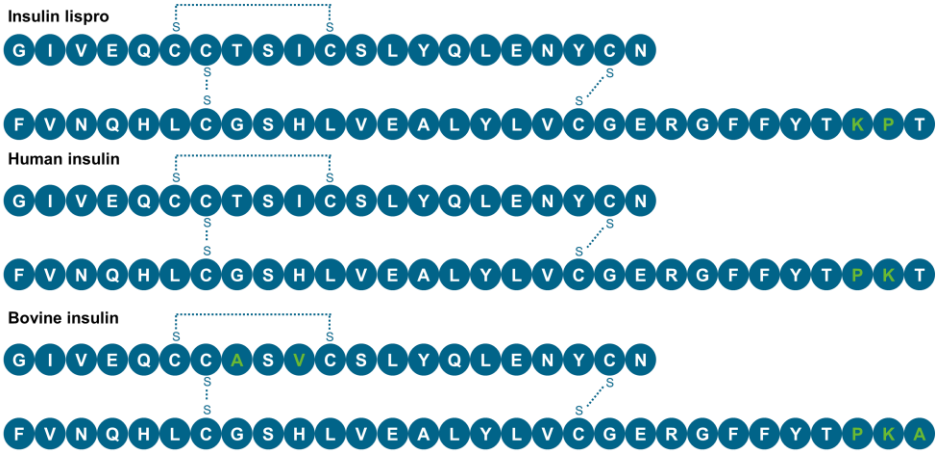

Figure 1. The amino acid sequences of insulin lispro, human insulin and bovine insulin. The difference in the sequences are labeled in green. All structures contain a total of 3 disulfide bridges. Bovine insulin was selected as the internal standard (IS).

Methods

Samples and reagents: Insulin lispro and bovine insulin were purchased from Sigma Aldrich, Inc.

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.

Chromatography: A SCIEX M5 MicroLC system was used for separation in trap-and-elute mode. A volume of 30 µL of the sample was loaded onto the trap column for analysis. The mobile phase A consisted of 0.1% formic acid in water and the mobile phase B was composed of 0.1% formic acid in acetonitrile.

Gradient conditions are summarized in Table 1. For analyte trapping, the operating flow rate was set to 50 µL/min using a Phenomenex Luna C18(2) column (20 x 0.3 mm, 5 µm, 100 Å). The column was operated at room temperature.

For analyte separation, the operating flow rate was 5 µL/min using a Phenomenex Kinetex XB-C18 column (50 x 0.3 mm, 2.6 µm, 100 Å). The column oven temperature was 50ºC.

Mass spectrometry: A ZenoTOF 7600 system with an OptiFlow Pro ion source with a microflow probe was used in positive Zeno MRM^{HR} mode. All source and MS parameters were optimized and are reported in Table 2.

Table 1. Microflow LC method operated in trap-and-elute mode.

Time (min)	Loading pump %B	Analytical pump %B	Trap valve position
0.0	*0*	*15*	*LOAD*
2.0	*0*	*15*	*INJECT*
3.0		*60*	
5.2	*90*	*90*	*LOAD*
6.8	*90*	*90*	
7.0		*15*	
10		*15*	

Table 2. Source and MS conditions.

Parameter	Value	Parameter	Value
Curtain gas	*30 psi*	*Source temperature*	*600˚C*
Ion source gas 1	*50 psi*	*Ion source gas 2*	*60 psi*
CAD gas	*7*	*Ion spray voltage*	*5500 V*
MS accumulation time	*80 ms*	*MS/MS accumulation time*	*60 ms*
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 cps*		

The Zeno MRM^{HR} parameters used for quantification are displayed in Table 3. All parameters were individually optimized for insulin lispro and bovine insulin.

Data processing: The Zeno MRM^{HR} data was processed using the Analytics module in the SCIEX OS software 2.0 using MQ4 integration algorithm. A weighting of $1/x^2$ was used for quantification.

Precursor and fragment masses of the peptides used for quantification are listed in Table 3. XIC peak width of 0.04 Da was applied for processing the Zeno MS/MS data.

Table 3. Zeno MRM^{HR} parameters used for quantification.

Compound ID	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	CE (V)
Insulin lispro	*1162*	*217.1194*	*80*	*55*
Bovine insulin (IS)	*955*	*1114.5069*	*80*	*40*

Insulin lispro quantification workflow

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 y_2 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.

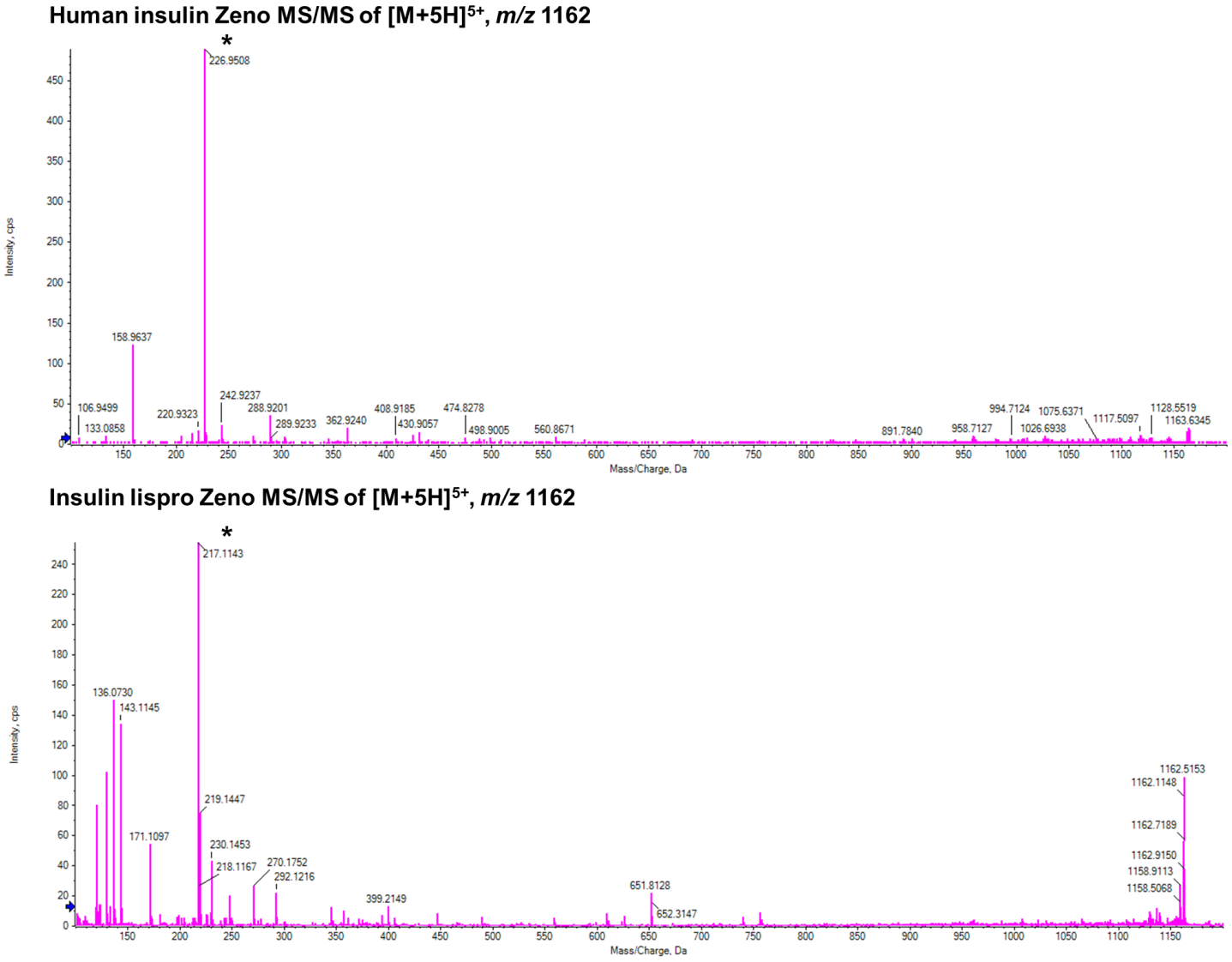

Figure 2. Zeno MS/MS spectra from CID of the [M+5H]5+ precursors of human insulin and insulin lispro. Specific y_2 fragment ions were selected for quantification to differentiate insulin lispro from human insulin at the *m/z* level (*).

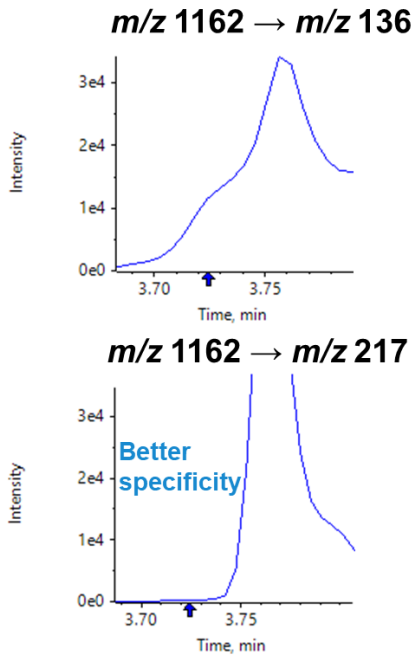

Figure 3. Extracted ion chromatograms (XICs) of 2 different MRM^{HR}. Tyrosine immonium ion (*m/z* 136) results in a lack of specificity (top) compared with the y_2 fragment ion from insulin lispro (bottom).

Quantitative performance on the ZenoTOF 7600 system

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.

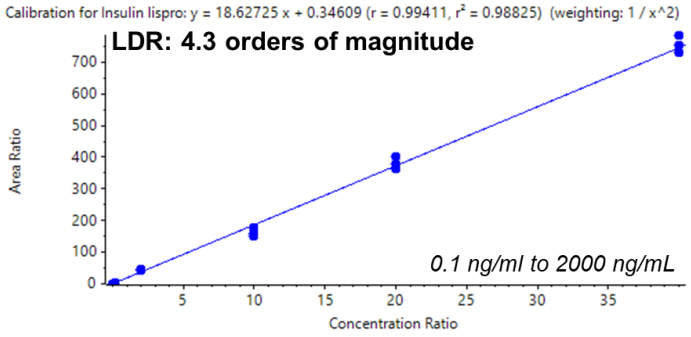

Figure 5. Calibration curve of insulin lispro in rat plasma. The concentration range was between 0.1 ng/mL to 2000 ng/mL resulting in 4.3 orders of magnitude in LDR.

The overall %CV was less than 9%, with accuracy within ±13% of the nominal concentration (Table 4). This demonstrates the overall assay sensitivity where low-level concentrations were detected and quantified with excellent accuracy and precision.

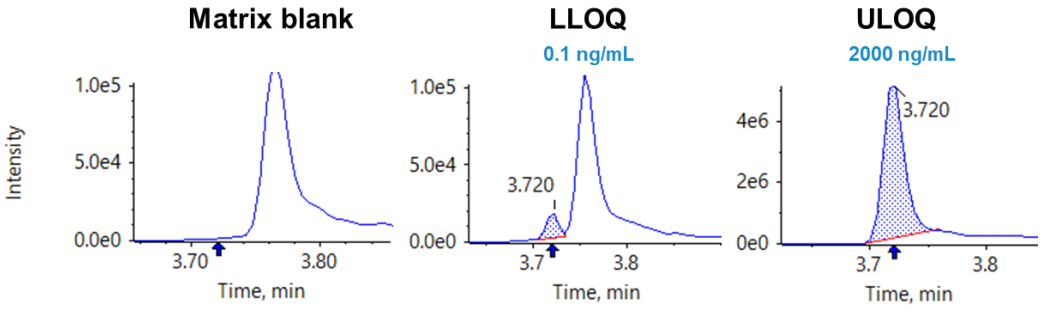

Figure 4. XIC of the matrix blank, LLOQ and ULOQ for insulin lispro quantification. No matrix interferences were observed at the retention time of the analyte. Low-level quantification of insulin lispro was achieved at 0.1 ng/mL with a ULOQ of 2000 ng/mL.

Table 4. Accuracy and precision results at each concentration level. Each concentration point was measured in triplicate.

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

References

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2. Enhanced sensitivity for peptide quantification in a complex matrix using high-resolution LC-MS/MS. SCIEX technical note, RUO-MKT-02-13324-A.
3. High-resolution LC-MS/MS solution for improved quantification of peptides in a complex matrix. SCIEX technical note, RUO-MKT-02-13323-A.

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