Low-pg/mL quantitation of long-acting insulin analog in rat plasma

A sensitive, quantitative analysis of insulin degludec in rat plasma using the SCIEX 7500 system

Ebru Selen, Rahul Baghla and Eshani Nandita
SCIEX, USA

This technical note demonstrates a sensitive method to quantify insulin degludec, an ultra-long-acting insulin analog, in rat plasma on a SCIEX 7500 system. A lower limit of quantitation (LLOQ) of 10 pg/mL was determined using a 6-minute LC-MS/MS method (Figure 1).

Insulin degludec regulates high blood sugar in adults and children with diabetes.[1] The structure of insulin degludec differs from human insulin by 2 modifications. Insulin degludec does not contain B30 threonine and the B29 lysine is conjugated to a 16-carbon fatty diacid chain (Figure 1B). The fatty acid chain in the latter modification binds to albumin and contributes to the ultra-long action profile of the analog.[1]

Due to their potential for diabetes management, sensitive and selective assays for high-confidence detection and quantitative performance in biological matrices are needed to ensure the safety and efficacy of promising therapeutic peptides. Quantitation of peptides and proteins has been commonly performed using immunoassays such as ELISA. However, immunoassays inherently face analytical challenges, such as poor selectivity to distinguish endogenous interferences, limited dynamic range and cross-reactivity. LC-MS/MS methods are increasingly applied for therapeutic quantitation because they offer superior selectivity. However, insulin analogs are often challenging to ionize and fragment, given their high molecular weight and structural complexity, conferred by disulfide bridging (Figure 1B). In addition, insulin analogs are challenging to detect and quantify owing to their ability to form complexes with circulating plasma proteins.

The presented method demonstrates a sensitive quantitation method for insulin degludec in rat plasma using a quick and efficient solid-phase extraction (SPE) technique. Sensitivity at the 10 pg/mL level was achieved in rat plasma using the SCIEX 7500 system. Front-end enhancements on the instrument facilitated improved overall ion generation, capture and transmission, providing optimal quantitative sensitivity.

Key features of the quantitation of insulin degludec using the SCIEX 7500 system

- **Low-pg/mL level quantitation of an ultra-long-acting insulin analog:** Achieve a 10 pg/mL LLOQ for insulin degludec in rat plasma on the SCIEX 7500 system
- **Ideal analytical performance:** Achieve accurate quantitative performance with %CV <7% at all concentration levels
- **Enhanced sensitivity unlocked:** Improved front-end technology with the D Jet ion guide, OptiFlow Pro ion source and E Lens probe enhanced the ion generation, capture and transmission, enabling users to reach desired quantitative sensitivity
- **Streamlined data management:** Data acquisition and processing are integrated into SCIEX OS software, a 21 CFR Part 11 compliance-ready platform

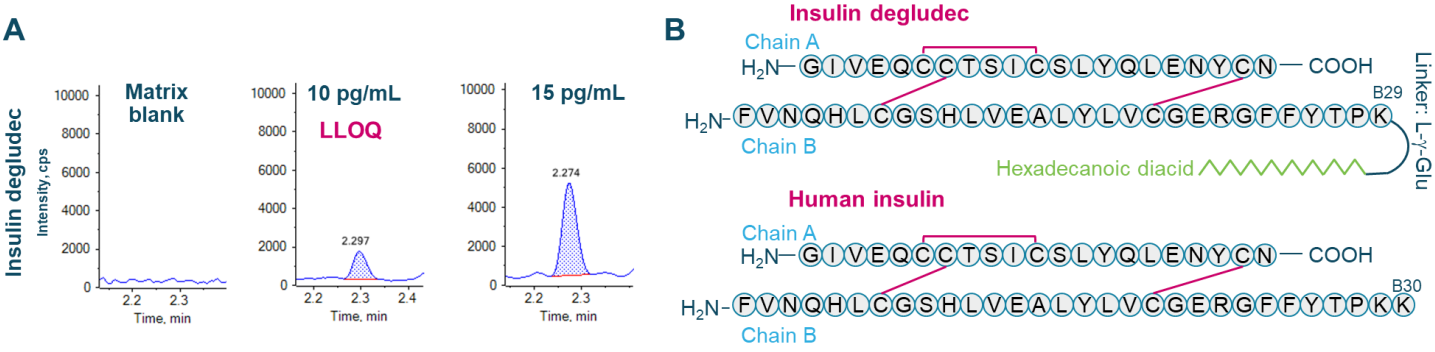

Figure 1. Representative extracted ion chromatograms (XICs) and structures of human insulin and insulin degludec. An LLOQ of 10 pg/mL (0.1 pg on column) was achieved for insulin degludec in rat plasma. No matrix interference was observed at the retention time of the analyte (A). Insulin degludec is a long-acting insulin analog. A glutamic spacer links a fatty acid chain to the B29 amino acid residue of insulin degludec. This modification is the hallmark of the long-acting profile of the analog. The structure of human insulin contains a B30 lysine and no linker on the B29 lysine (B).

Methods

Sample preparation: The commercially available insulin degludec (Figure 1B) was reconstituted in dimethylsulfoxide. Insulin degludec was diluted in dilution solvent containing 1% formic acid in a 75:25 (v/v), acetonitrile/water mixture. Individual concentrations were spiked into 100 µL of rat plasma at concentrations ranging from 10 pg/mL to 2500 pg/mL. Protein precipitation was performed with 100 µL of 4% phosphoric acid in methanol. Samples were vortexed for 30 seconds and centrifuged at 12000 rcf for 10 minutes at room temperature. The supernatant was transferred to a new Eppendorf tube containing 800 µL water and samples were briefly vortexed and centrifuged. Then, 500 µL of each sample was loaded twice on a Phenomenex Strata-X-A microelution plate operated under positive pressure. Samples were consecutively washed with 5% aqueous ammonia and 20% acetonitrile in water. Finally, elution was performed using 1% trifluoroacetic acid in a solution containing 70% acetonitrile, 20% water and 10% acetic acid by volume. The final elution volume was 100 µL.

Chromatography: Sample separation was performed using an ExionLC system at a 0.6 mL/min flow rate on a Phenomenex Kinetex XB C18 (2.1 x 50 mm, 1.7 µm, 100 Å) column. A 6-minute gradient was run using 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B (Table 1). The column temperature was maintained at 55°C. An injection volume of 10 µL was used for analysis. A mixture of equal parts by volume of acetonitrile, methanol and water was used as a needle wash solvent.

Table 1. Chromatographic gradient for insulin degludec.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	*90*	*10*
0.2	*90*	*10*
1	*70*	*30*
3	*60*	*40*
4	*55*	*45*
4.1	*2*	*98*
4.6	*2*	*98*
5	*60*	*40*
5.1	*90*	*10*
6	*90*	*10*

Mass spectrometry: The optimized source and gas parameters are listed in Table 2 and the optimized analyte-dependent MRM parameters are included in Table 3.

Table 2. Source and gas parameters.

Parameter	Value
Polarity	*Positive*
Ion source gas 1	*50 psi*
Ion source gas 2	*60 psi*
Curtain gas	*45 psi*
Source temperature	*500°C*
Ion spray voltage	*2500 V*
CAD gas	*9*

Table 3. MRM parameters used for quantitation.

ID	Precursor ion (*m/z*)	Fragment ion (*m/z*)	CE (V)	CXP (V)	Q0D (V)
Insulin degludec (+5)*	*1221.8*	*1366.9*	*40*	*15*	*-5*
Insulin degludec (+4)	*1526.9*	*1183.8*	*40*	*15*	*-5*

**Used for quantitation*

Data processing: Data collection and analysis were performed in SCIEX OS software, version 3.0. Peaks were automatically integrated using the MQ4 algorithm and a weighting of $1/x^2$ was used for quantitation.

Quantitative performance

This technical note demonstrates a low-pg/mL level quantitation assay of insulin degludec in rat plasma using the SCIEX 7500 system.

Compared to traditional antibody-based assays in protein and peptide bioanalysis, this LC-MS/MS platform overcomes the limitations regarding selectivity, cross-reactivity and sensitivity. The analog structures offer therapeutic benefits by forming complexes with plasma proteins but also pose a challenge for mass spectrometry-based detection. An optimized SPE procedure allows an effective protein precipitation and sample clean-up, contributing to the overall performance of the LC-MS/MS quantitation assay.

The calibration curve ranged from 10 pg/mL to 2500 pg/mL and was prepared as described in the sample preparation section. Individual concentrations were run in triplicate.

An LLOQ of 10 pg/mL was achieved for insulin degludec (Figure 1). No interferences were observed in the rat plasma matrix blank (Figure 1).

Linearity was achieved between 10 pg/mL and 2500 pg/mL with a coefficient of determination (r^2) of 0.991 (Figure 2).

Analytical performance was evaluated for accuracy and precision. The accuracy of the calculated mean was expected to be between 80% and 120% at the LLOQ and between 85% and 115% at higher concentrations. The %CV of the calculated mean for each concentration was expected to be <20% at the LLOQ and <15% at higher concentrations.

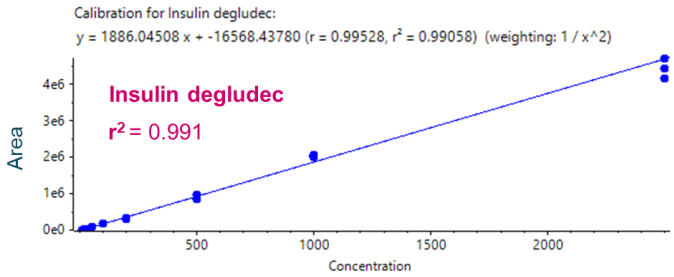

Figure 2. Calibration curve for insulin degludec. The peak area of insulin degludec was used to generate a calibration curve. Each concentration level was run in triplicate. Linearity was achieved between 10 pg/mL and 2500 pg/mL with an r^2 of 0.991.

Accuracy was within ±12% of the nominal concentration and the %CV was <7% for insulin degludec (Figure 3). Calculated accuracy and %CV values met the acceptance criteria at each concentration level.

Component Name	Actual Concentr...	Num. Values	Mean	Standard Deviation	Percent CV	Average Accura...
Insulin degludec	10.00	3 of 3	10.479	0.196	1.87	104.79
Insulin degludec	15.00	3 of 3	13.712	0.401	2.93	91.41
Insulin degludec	20.00	3 of 3	19.448	0.820	4.22	97.24
Insulin degludec	25.00	3 of 3	25.349	1.007	3.97	101.40
Insulin degludec	50.00	3 of 3	55.521	0.855	1.54	111.04
Insulin degludec	100.00	3 of 3	99.138	1.881	1.90	99.14
Insulin degludec	200.00	3 of 3	182.561	9.658	5.29	91.28
Insulin degludec	500.00	3 of 3	501.075	31.430	6.27	100.21
Insulin degludec	1000.00	3 of 3	1088.469	14.937	1.37	108.85
Insulin degludec	2500.00	3 of 3	2366.009	162.335	6.86	94.64

Figure 3. Quantitative performance for the analysis of insulin degludec. Reproducibility and accuracy results were determined from the calibration curve across 3 replicates at each concentration. Statistical results were summarized using the Analytics module in SCIEX OS software.

Compliance-ready SCIEX OS software

SCIEX OS software is a closed system and requires records and signatures to be stored electronically, meeting the regulations outlined by 21 CFR Part 11. SCIEX OS software can open raw data files from any visible storage location within a closed network by using designated processing workstations. Figure 4 illustrates the features of SCIEX OS software that are used for monitoring the audit trail, acquiring and processing data and configuring user access.

The audit trail feature enables users to audit critical user actions and locks in data integrity. The Central Administrator Console (CAC) feature allows users to centralize acquisition and processing using a single platform to maximize efficiency for multi-instrument laboratories, independent of compliance standards. The configuration module allows users to assign roles and access as the administrator, method developer, analyst and reviewer.

Figure 4. Features of the SCIEX OS software for monitoring user access and evaluating the audit trail. The audit trail view allows users to filter for high-risk events easily and enables data integrity features to meet compliance requirements. The software features a Central Administrator Console (CAC) to manage users and groups, role definitions, workstations and projects across all systems. The CAC feature supports both regulated and non-regulated compliance standards. The configuration module enables users to quickly set up roles and levels of access for the administrator, method developer, analyst and reviewer levels.

Conclusions

- An LLOQ of 10 pg/mL was reached for the quantitation of insulin degludec in rat plasma
- Linearity was achieved between 10 pg/mL and 2500 pg/mL, with an r^2 of 0.991
- The method demonstrated accurate and highly reproducible (%CV <7%) quantitative performance at all concentrations
- Sensitivity was achieved on the SCIEX 7500 system with an improved front-end technology for better ion generation, capture and transmission
- SCIEX OS software is compliance-ready to support 21 CFR Part 11 and integrates with an accurate mass spectrometer to support data acquisition, processing and management on a single platform

References

1. Stephen Atkin, Zeeshan Javed, Gregory Fulcher (2015). Insulin degludec and insulin aspart: novel insulins for the management of diabetes mellitus. Ther Adv Chronic Dis. 2015 Nov;6(6):375-88.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

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