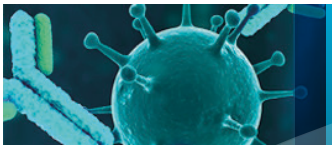

Quantitation of Insulin Glargine in Human Plasma with a Combination of Immunocapture-Based Target Enrichment and Trap-and-Elute Microflow LC-MS/MS

Using the SCIEX Biologics Bioanalysis (BioBA) assay and M3 MicroLC system to improve detection limits

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Overview

Insulin glargine, a recombinant protein, is a long-acting insulin analogue given daily to diabetes patients to help control blood sugar level. Marketed as Lantus, insulin glargine has been one of the best-selling pharmaceutical products for the past five years. As such, there is tremendous interest within the clinical research community in analyzing and studying insulin glargine in biological samples.

Who Should Read This: Senior Scientists, Lab Directors

Focus: Quantitation of insulin glargine through a combination of immunocapture-based target enrichment and trap-and-elute microflow LC-MS/MS.

Goal: Determine how much sensitivity can be gained by using trap-and-elute microflow liquid chromatography instead of traditional liquid chromatography in a method that combines immunocapture-based target enrichment with LC-MS/MS for quantitation of insulin glargine.

Problem: Traditional methods of quantifying insulin glargine, ELISA and LC-MS/MS, have significant drawbacks. ELISA provides poor specificity and cannot distinguish insulin glargine from other insulin analogs. Traditional LC-MS/MS provides specificity, but because of ion suppression and interference from the sample matrix can lack the sensitivity requiredfor contemporary clinical research.

Key Challenges:

- ELISA and LC-MS/MS, the traditional methods of quantifying insulin glargine, have significant drawbacks
- ELISA suffers from poor specificity; anti-insulin antibodies cannot distinguish insulin glargine from other insulin analogs
- Traditional LC at mL/min flow rates combined with MS/MS sometimes fails to provide the sensitivity needed when sample quantities are limited
- Insulin and insulin analogs are known to show significant carryover in HPLC due to non-specific binding to the stationary phase and this carryover negatively affects quantitative accuracy and method robustness

Key Features:

- Enrichment through immunocapture-based target enrichment
- High specificity from the use of unique MS/MS transitions
- 5x better sensitivity than traditional LC-MS/MS
- Increased robustness using trap-and-elute workflow

Experimental Design

Sample Preparation: Samples were prepared by spiking insulin glargine (Myoderm) and bovine insulin (internal standard, Sigma-Aldrich) into human plasma-K2 EDTA (BioreclamationIVT).

Streptavidin-coated magnetic beads were from SCIEX (BioBA High Capacity Enrichment Sample Preparation Kit, P/N 5041071). The antibody for immunocapture was biotinylated anti-insulin and proinsulin mouse monoclonal (P/N ab20756, Abcam). A 250 µL plasma sample was processed, resulting in 115 µL of enriched insulin glargine for injection into the LC-MS/MS system. See Reference 1 for details on the sample preparation method. Because leftover traces of the phosphate buffer solution can cause tailing of the insulin glargine peak, the washing buffer was replaced with 10 mM ammonium bicarbonate for the last wash step after immunocapture.

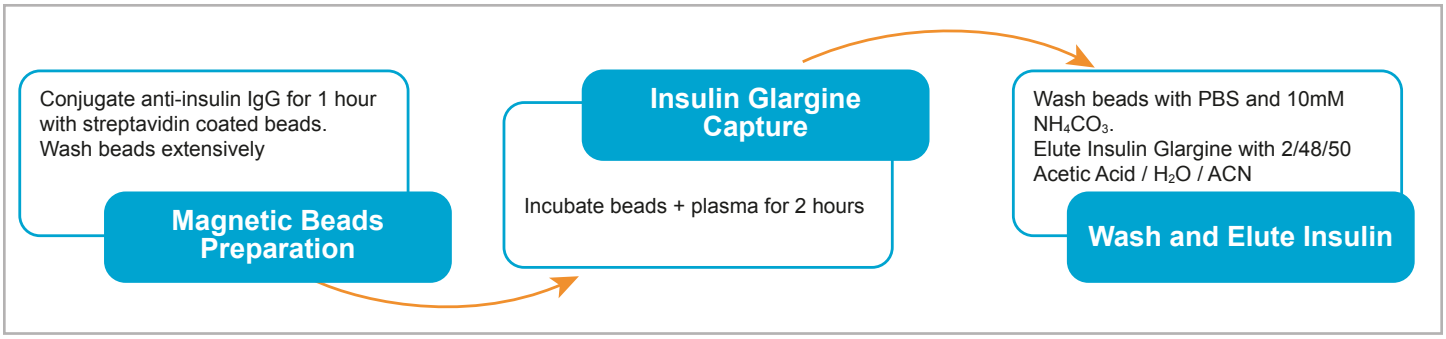

Figure 1. Immunocapture-Based Sample Preparation Workflow.

Figure 2. M3 MicroLC-TE System. The system includes two microflow LC gradient pumps and an integrated autosampler with a 6 plate capacity.

HPLC: For the traditional flow rate (mL/min) experiments, a SCIEX ExionLC™ AD HPLC system was used with the following column and conditions:

Injection Volume	30 µL
Autosampler Wash	20/20/60 methanol/acetonitrile/isopropyl alcohol
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Gradient	10-50% B over 5 min with a 4 min wash at 60% B
Flow Rate	500 µL/min
Column	100 x 2.1 mm Kinetex C18 2.6 µm 100 Å (Phenomenex)
Column Temperature	40° C

For microflow experiments, a SCIEX M3 MicroLC-TE system was used in trap-and-elute mode. The following columns and conditions were used:

Injection Volume	30 µL
Autosampler Wash	Wash 1: 66.5/30/3.5 acetonitrile/water/acetic acid Wash 2: water with 0.1% formic acid
Loading Solvent A	Water with 0.1% formic acid
Loading Solvent B	Acetonitrile with 0.25% trifluoroacetic acid
Trap Loading/Washing	Loading for 2 min at 50 µL/min with 0% B, washing for 10 min at 70 µL/min with 60% B
Trap Column	10 x 0.5 mm 5 µm 120 Å ChromXP™ C18 CL column (SCIEX)
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Gradient	10–50% B over 5 min with a 7 min wash at 60% B
Flow Rate	10 µL/min
Column	MonoCap Fast-Flow 100 x 0.2 mm (P/N 5020-10124, GL Sciences)
Column Temperature	40° C

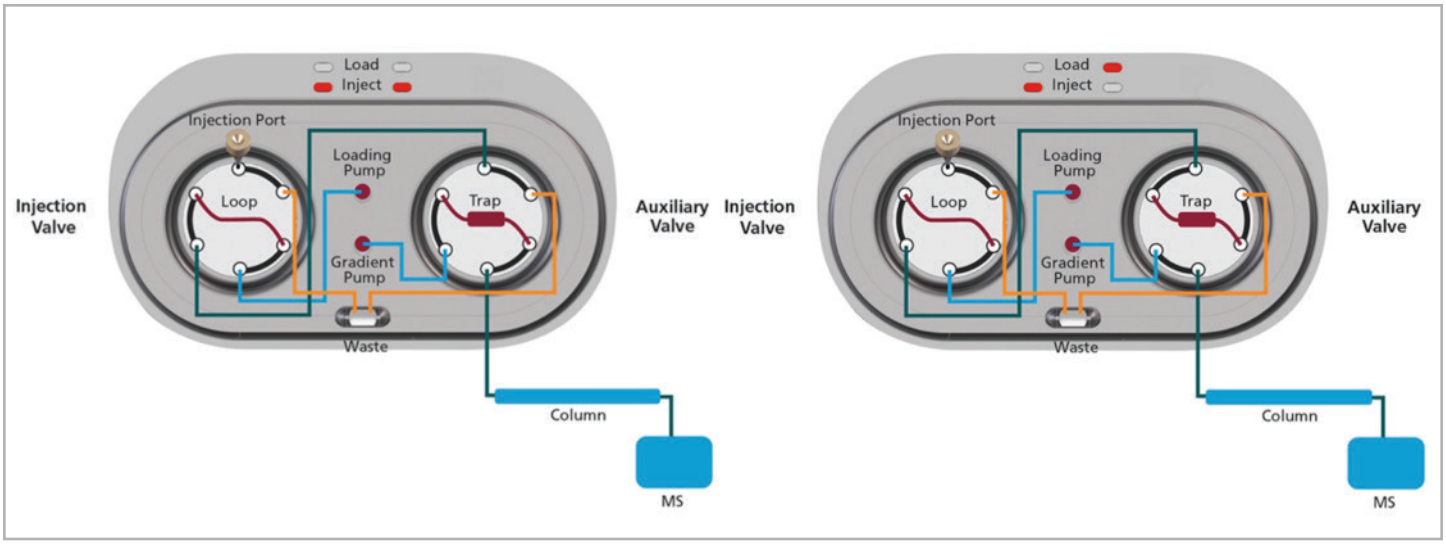

Figure 3. Valve Positions for the Trap-and-Elute Microflow LC Method. In the configuration on the left, sample is loaded onto the trap column at a high flow. Once sample loading and washing any non-retained matrix components to waste are completed, the auxiliary valve switches to Inject, and the LC-MS/MS analysis starts (configuration on the right). The trap-and-elute workflow provides faster loading of samples and removal of matrix components, thereby improving throughput and robustness.

Mass Spectrometry: A SCIEX QTRAP® 6500+ LC-MS/MS system with IonDrive™ was used in multiple-reaction monitoring (MRM) mode. For quantitation the transition *m/z* 867.0→984.0 was used, while two additional transitions (*m/z* 867.0→992.4 and *m/z* 1011.4→1179.5) of insulin glargine where monitored for confirmation. The *m/z* 956.6→1115.2 transition was used for the bovine insulin internal standard. For the microflow LC experiments, the standard electrode was replaced with a 25 µm ID electrode (SCIEX). Source conditions for both traditional LC and microflow LC are listed in Table 1. See Reference 1 for other MS parameters. SCIEX MultiQuant software version 3.0.2 was used for integration and data analysis. A 1 point Gaussian smoothing was applied, and a linear regression with weighting $1/x^2$ was used for all calibration curves.

	Traditional LC	Microflow LC
Electrode ID	100 µm	25 µm
Curtain Gas	20	20
IonSpray Voltage	5500	5000
Temperature (°C)	600	250
Ion Source Gas 1	50	20
Ion Source Gas 2	65	20

Table 1. Source Parameters.

Sensitivity Improvement

Microflow LC, using 0.3 mm ID columns at flow rates of 5–20 µL/min flow rates, can improve LC-MS sensitivity by a factor of up to 10 compared to traditional 2.1–3 mm ID columns at flow rates of 0.5–1 mL/min.[2] For insulin glargine, signal-to-noise was improved by a factor of approximately 3.5 times when using microflow LC (Figure 4). Calibration curves from 10 pg/mL to 10,000 pg/mL were acquired using both the traditional LC and trap-and-elute microflow LC methods. Each sample was injected in triplicate. Accuracy and precision using both traditional LC and microflow LC are summarized in Table 2.

LLOQ at traditional flow rates was 50 pg/mL, which was the same quantitation limit reported previously.[1,3] LLOQ using the microflow LC workflow was 10 pg/mL (Figure 5), which is an improvement of 5x. This is considerably lower than the required detection limit of 70 pg/mL for clinical use.[4]

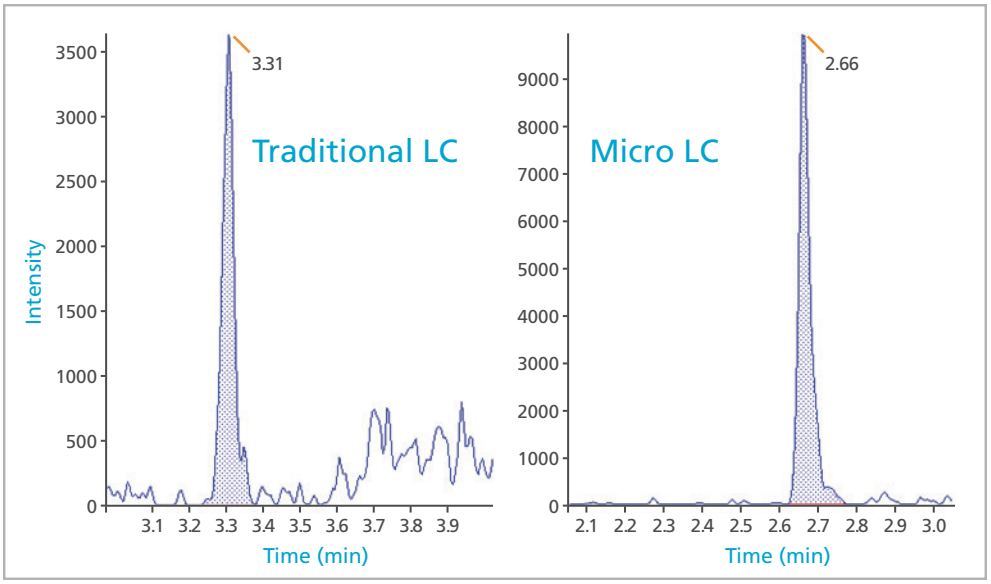

Figure 4. Sensitivity Comparison. Microflow LC–MS/MS analysis of insulin glargine in a plasma sample spiked at the 100 pg/mL level improved signal-to-noise by a factor of approximately 3.5 times compared to LC-MS/MS at traditional flow rates.

	Traditional LC-MS/MS			Microflow LC-MS/MS		
Actual Concentration (pg/mL)	Mean Calculated Concentration (pg/mL)	Accuracy (%)	CV (%)	Mean Calculated Concentration (pg/mL)	Accuracy (%)	CV (%)
10	N/A	N/A	N/A	10.2	102	10
25	N/A	N/A	N/A	23.0	92	14
50	49.2	98	13	50.9	102	3.4
100	95.7	96	9.6	104	104	5.8
500	482	96	1.4	486	97	10
1,000	990	99	0.61	1,056	106	3.4
5,000	5,276	105	1.3	4,888	98	1.3
10,000	10,300	103	1.3	9,922	99	0.50

Table 2. Standard Curve Data for the Traditional and Microflow LC-MS/MS Methods

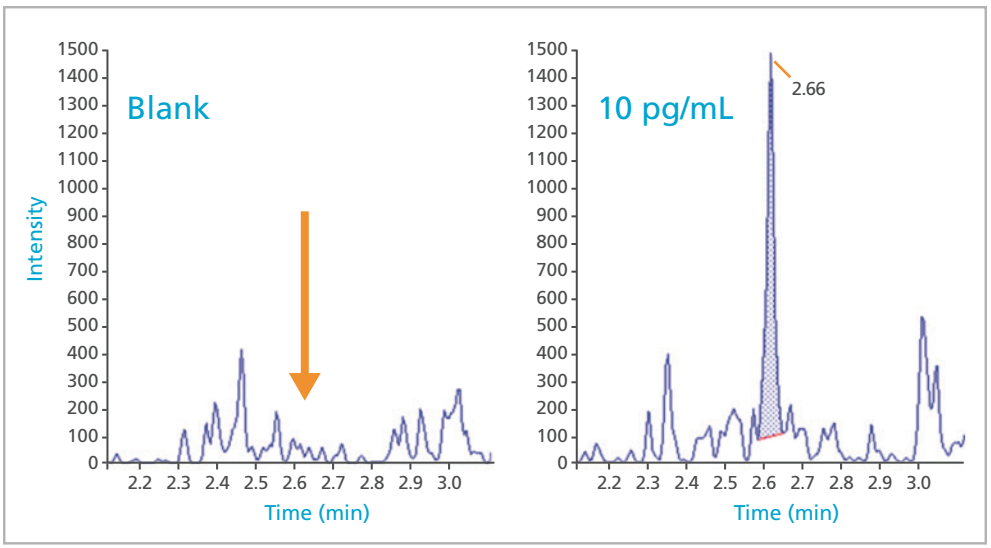

Figure 5. Extracted Ion Chromatograms for a Blank and Sample. At the 10 pg/mL LLOQ, the signal-to-noise ratio was approximately 10.

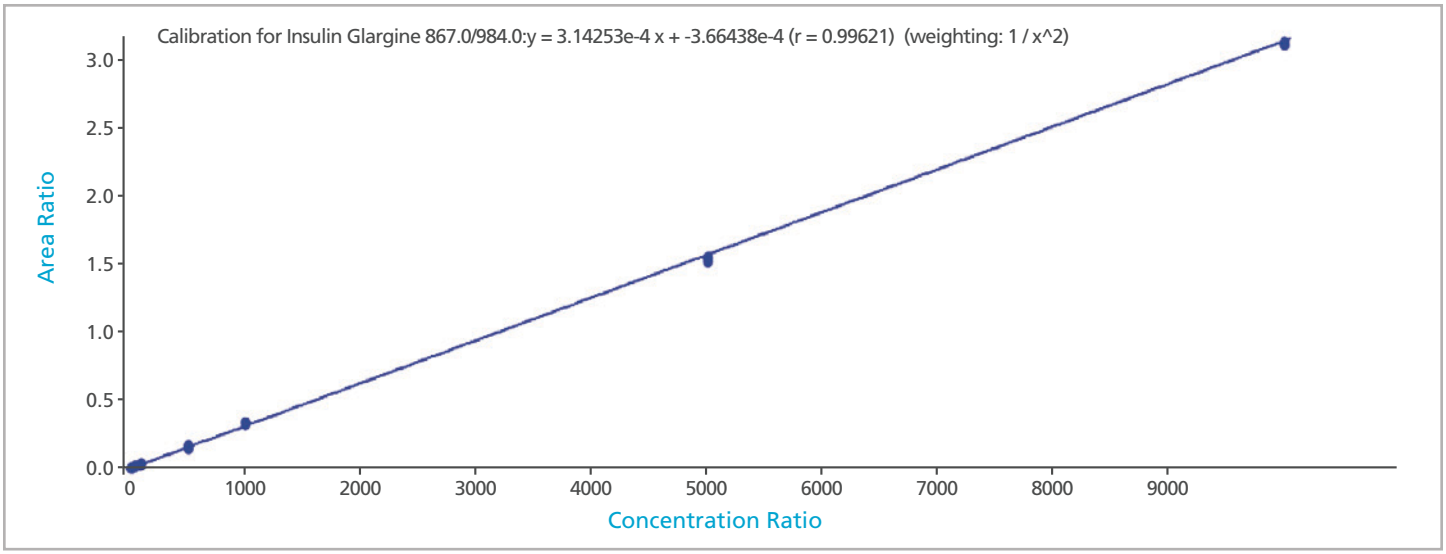

Figure 6. Calibration Curve for the Quantitation of Insulin Glargine using Microflow LC-MS/MS.

Carryover

Insulin and insulin analogs are known to show significant carryover in HPLC due to non-specific binding to the stationary phase. Carryover in the microflow LC method was reduced by using a silica-based monolithic column and by washing the trap off line from the analytical column for 10 minutes. Carryover was reduced to below the noise level within the calibration curve range measured (Figure 7).

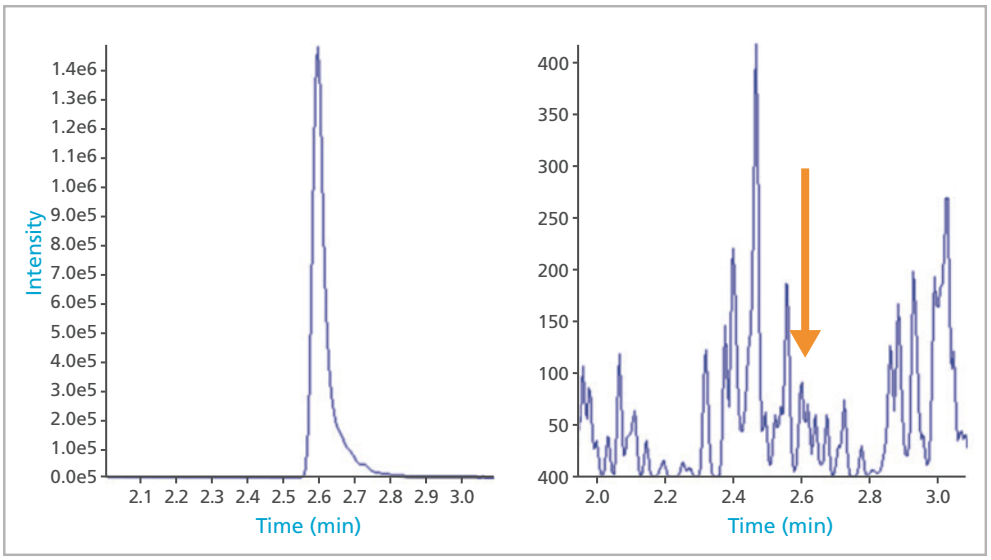

Figure 7. Carryover After Injecting the Extract of a Sample Spiked at the 10,000 pg/mL Level. Carryover based on peak intensity was less than 0.01%, and low enough to declare 10,000 pg/mL as the ULOQ.

Conclusion

The trap-and-elute microflow LC-MS/MS method, in combination with an immunocapture-based target enrichment workflow, achieved a LLOQ of 10 pg/mL insulin glargine in human plasma. This was 5x better than data generated by LC-MS/MS at traditional mL/min flow rates. By using a monolithic column and washing the trap column extensively off line from the analytical column, carryover of <0.01% was achieved, allowing quantitation up to 10,000 pg/mL. The trap-and-elute technique provided fast loading of large volume samples, while protecting the analytical column and mass spectrometer from contaminants, resulting in a more robust method.

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Publication number: RUO-MKT-02-5037-A

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