Hybrid Immunoaffinity-LC-MS/MS Method for Quantifying Insulin Aspart in Human Plasma

SCIEX Hybrid Immunoaffinity Solution Featuring SCIEX 6500+ QTRAP® LC MS/MS System

Fan Zhang[1], Yihan Li[1], Sahana Mollah[1], Sean McCarthy[2], Lei Xiong[1]
[1]SCIEX Redwood City, California (USA); [2]SCIEX Framingham, Massachusetts (USA)

Introduction

Insulin aspart is a manufactured form of human insulin generated using recombinant DNA technology. It is a fast-acting insulin analog used to quickly control the rapid rise in blood sugar after meals and closely mimics the body's natural release of insulin at mealtime. Insulin aspart is often used by adults and children with type 1 diabetes and adults with type 2 diabetes.[1] Marketed as NovoLog, insulin aspart has been one of the top-selling pharmaceutical products in the past five years. As such, there is tremendous interest within the clinical research community in analyzing and studying insulin aspart in clinical samples.

Figure 1. SCIEX QTRAP 6500+ system coupled with ExionLC™ UHPLC system.

Key Challenges in Biologics Quantitation

- Achieving linearity across the expected PK sample range
- Lengthy and complicated procedures for measuring active or free circulating biotherapeutic drug are more challenging in complex matrices

Key Features of the SCIEX hybrid immunoaffinity-LC-MS/MS Solution

- Bioanalysis by immuno-affinity sample preparation to decrease sample complexity and to achieve desired assay linear dynamic range
- Simplified sample preparation protocol allows short sample preparation time
- A secondary sample cleanup before introduction to MS analysis is not required
- This workflow provides a high degree of reproducibility, with CV <10%
- IonDrive™ system technology on the QTRAP® 6500+ LC MS/MS system provides:
  - Increased ionization efficiency and ruggedness to provide superior quantitative performance
  - Enhanced dynamic range to cover the appropriate sample concentrations for biologics PK assays without sample dilution

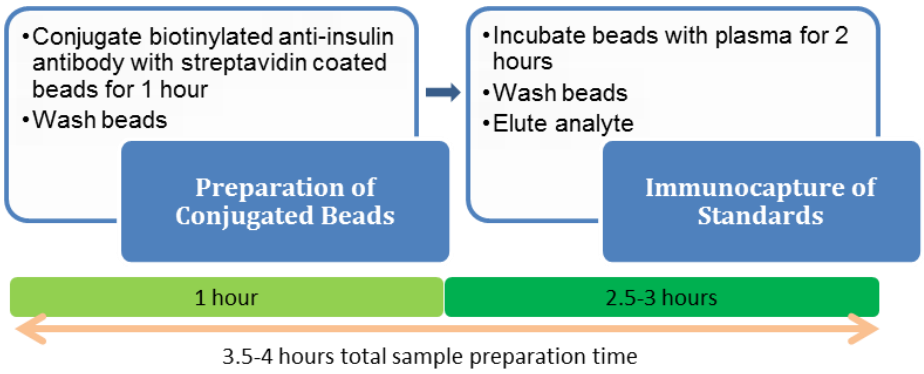

Figure 2. Sample Preparation Workflow

Experimental

Immunocapture of target analyte: A streptavidin coated immunoaffinity magnetic bead slurry was aliquoted and washed with PBS Buffer (1x) three times. Biotinylated anti-insulin and proinsulin antibody was added to the beads and incubated at room temperature for 1 hour. The conjugated beads were washed three times and re-suspended in PBS Buffer (1x). Bovine insulin was prepared as an internal standard. Serial dilution was performed from insulin aspart stock solution to prepare 250 µL of calibration standards by diluting in human plasma at final concentrations of 10,000, 5,000, 1,000, 500, 100 and 50 pg/mL. To each Insulin aspart calibration standard, 100 µL of PBS Buffer (1x) conjugated bead slurry and internal standard were added and the mixtures were incubated at room temperature for 2 hours. The beads were accumulated by magnetic stand and washed sequentially with PBS Buffer (1x) with 0.05% CHAPs. Elution buffer was added to the beads and vortexed for 10 min to elute both insulin aspart and bovine insulin. The eluents were collected and subjected to LC-MS/MS analysis.

LC Separation: Separation was accomplished using a Phenomenex® Kinetex®, 2.6 µm, C18 column, 3.0x50 mm at 40°C. Mobile phase A is 0.1% formic acid in water and mobile phase B is 0.1% formic acid in acetonitrile. For optimal performance, the column should be conditioned prior to use. Both internal and external rinses were used in this study to minimize carryover. The needle rinse solution was 33.3% water, 33.2% acetonitrile, 33.3% methanol with 0.2% acetic acid. The flow rate was set at 0.7 ml/min. Injection volume for each analysis was 30µL.The chromatographic gradient is shown in Table 1. Following the gradient, 4 wash steps were executed ramping from 10 to 95% mobile phase B. Overall run time was 9 min.

Table 1. LC conditions for signature peptide analysis.

Gradient profile	Time (min)	% B
	0.00	10
	0.20	10
	3.70	45
	4.00	95
	4.40	95
	4.50	10
	4.80	10

MS/MS Detection: Signature peptide MRM analysis was performed on a SCIEX QTRAP® 6500$^+$ LC MS/MS system equipped with an IonDrive^{TM} system technology in positive electrospray ionization (ESI) mode using an ion source temperature of 650ºC, IonSpray voltage of 5500, curtain gas pressure of 40 psi, Gas 1 of 65 psi and Gas 2 of 65 psi. The detailed MRM parameters are listed in Table 2. A divert valve was used to divert CHAPs and plasma to waste for long term performance.

Table 2. MRM transitions for insulin aspart and bovine insulin.

Name	Q1	Q3	DP	CE	CXP
Insulin Aspart 1[1]	971.6	1133.2	80	32	22
Insulin Aspart 2	971.6	1110.5	80	35	24
Insulin Aspart 3	971.6	661.1	80	35	12
Bovine Insulin 1[1]	956.6	1115.2	80	30	25
Bovine Insulin 2	956.6	637.0	80	32	17

[1]Most suitable transition for quantification

Results and Discussion

With significantly reduced matrix interference provided by immunoaffinity sample preparation and high sensitivity of QTRAP 6500$^+$ system, the assay we achieved an LLOQ 50 pg/mL (Figure 3). Lower levels may be possible but were not investigated in this work.

Each calibration standard was analyzed in triplicate. The accuracy and precision are summarized in Table 3. The calibration curve of insulin aspart in human plasma with bovine insulin as internal standard was linear between 50 and 10000 pg/mL (Fig.4) and displayed a regression coefficient (r^2) of 0.99509 using a weighting of $1/x^2$.

Importantly, insulin and insulin analogs are known to show significant carry-over in chromatographic separations due to non-specific binding to the auto-sampler needle, tubing, and the column stationary phase. Carry-over was significantly reduced by enabling both internal and external needle rinse in the auto-sampler and using the LC gradient with multiple washing steps (Table 1). 30 µL of blank sample was injected following the last injection of 10000 pg/mL calibration standard (ULOQ) after all the calibration standards were analyzed in triplicate fashion from low concentration to high concentration. The carry-over peak

area (Fig.3d) is <20% of LOQ (50 pg/mL calibration standard, Fig.3c).

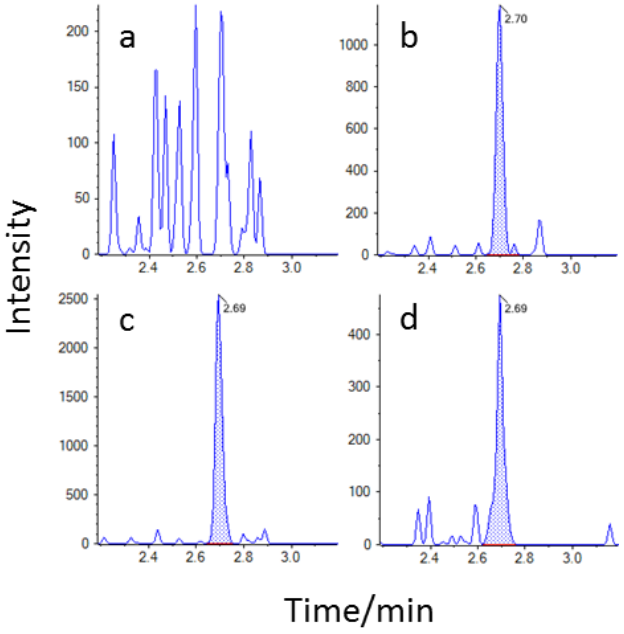

Figure 3. Extracted ion chromatograms of insulin aspart. a) blank; b) 25 pg/mL (LOD); c) 50 pg/mL (LOQ); d) carry-over in the 1st blank following the last injection of 10000 pg/mL calibration standard (ULOQ) after all the calibration standards were analyzed in triplicate fashion from low concentration to high concentration.

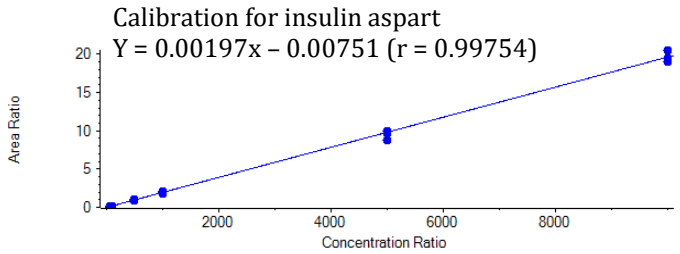

Figure 4. Calibration curve for quantitation of insulin aspart in human plasma (50 pg/mL to 10 000 pg/mL).

Table 3. Concentration-response linearity data of calibration curve for accuracy of insulin aspart in human plasma

Actual Concentration (pg/mL)	Mean Calculated Concentration (pg/mL)	Accuracy (%)	CV (%)
50.00	50.16	100.31	1.61
100.00	98.58	98.58	7
500.00	520.05	104.01	8.77
1000.00	1007.05	100.71	6.73
5000.00	4817.67	96.35	6.46
10000.00	10004.67	100.05	4.01

Conclusion

A hybrid immunoaffinity-LC-MS/MS method for quantifying insulin aspart in human plasma is reported. The QTRAP® 6500+ LC MS/MS system coupled with ExionLC™ system provides high sensitivity, robustness and broad dynamic range for MRM quantification of peptides. The immunoaffinity sample preparation significantly eliminates interference from matrix. Combined together, this method enables scientists to confidently quantify insulin aspart at 50 pg/mL in rat plasma.

References

1. www.novolog.com; the official site from Novo Nordisk.

Document number: RUO-MKT-02-7596-A

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

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices