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Increasing Throughput for the Analysis of Human Insulin and Related Biotherapeutic Analogs using the ionKey/MS System

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The ionKey/MS System enables the flexibility to modulate between ultra-sensitivity and higher throughput with ease.

GOAL

To improve throughput for the microflow LC-MS analysis of human insulin and related biotherapeutic analogs, while maintaining pg/mL sensitivity.

BACKGROUND

Recombinant human insulin and its analogs are well known as the standard of care for insulin dependent diabetes. With the increasing incidence of type 2 diabetes and limited alternative treatments, numerous insulin biosimilars are being developed. LC-MS has emerged as a key platform for quantification across the drug discovery and development continuum, showing advantages over ligand binding assays (LBAs) which are challenged with specificity issues and multiplexing capabilities. Previous work has highlighted the ability of microflow LC-MS using the ionKey/MS® System as an effective approach to obtaining ultimate sensitivity for insulin and its analogs with low sample volume.[1]

Microflow LC-MS as an alternative to analytical scale is well known to address high sensitivity requirements, yet often with the compromise of longer cycle times.

Figure 1. The ionKey/MS System with the ACQUITY® UPLC® M-Class System and the Xevo® TQ-XS Mass Spectrometer.

The system configurations and limited throughput of traditional microflow LC has been a barrier to entry into routine bioanalysis labs. Recent work has shown that the ionKey/MS System can achieve 3 minute cycle times with up to a 6-fold increase in sensitivity when moving from analytical scale.[2] The ionKey/MS System's integrated turn-key design improves usability and reproducibility because it reduces system dispersion and eliminates the expertise needed to make multiple capillary connections.

This work described herein, demonstrates that sensitive, accurate, robust and high througput bioanalytical analysis can be achieved with microflow regimes using the ionKey/MS System and 300 μm I.D. iKey™ HT Separation Device for insulin and five of its analogs extracted from plasma.

METHOD CONDITIONS

LC conditions

LC system:	ACQUITY UPLC M-Class, configured with trap and back flush elution
Analytical column:	Peptide BEH C_{18} 130 Å, 1.7 µm, 300 µm x 50 mm iKey HT
Trap column:	Symmetry® C_{18}, 5 µm, 300 µm x 50 mm
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Trap loading:	85:15 mobile phase A:B, 25 µL/min for 2 minutes
Analytical gradient:	15–55% B over 2 minutes, re-equilibrate to 5 minutes
Flow rate:	6 µL/min
Column temp.:	75 °C
Sample temp.:	12 °C
Injection volume:	15 µL
Total run time:	7 minutes
Sample volume extracted:	250 µL

Table 1. LC conditions utilizing single pump trap and elute.

EXPERIMENTAL

Sample preparation

Plasma samples were pretreated using protein precipitation (PPT) and extracted using an Oasis® MAX µElution SPE protocol previously outlined by Chambers, et. al.[3]

Step 1: Protein PPT:
250 µL plasma + 200 µL 1:1 ACN:MeOH containing 1% acetic acid
Supernatant transferred and diluted with 900 µL of 5% NH_4OH in H_2O

Step 2: SPE using Oasis MAX µElution 96-well Plate

SPE Extraction Protocol

Condition:
200 µL MeOH
Equilibrate:
200 µL H_2O
Load:
Entire diluted PPT supernatant (2x 700 µL)
Wash 1:
200 µL 5% NH_4OH in H_2O
Wash 2:
200 µL 5% MeOH + 1% acetic acid in H_2O
Elute:
2 x 25 µL of 1% TFA in 60:30:10 MeOH:H_2O:acetic acid
Dilute:
50 µL H_2O

Figure 2. Insulin and insulin analogs sample extraction protocol using Oasis MAX µElution 96-well plate.

Specific insulin	MRM transition	Cone voltage (V)	Collision energy (eV)
Human insulin	1162 -> 226	50	40
Lispro	1162.2 -> 217	80	45
Glargine	1011 -> 1179	60	25
Detemir	1184 -> 454.4	60	20
Aspart	971.8 -> 660.8	60	18
Glulisine	1165 -> 1370	60	22
Bovine (IS)	956.6 -> 1121.2	60	18

Table 2. MS conditions for human insulin, insulin analogs, and the internal standard bovine insulin.

RESULTS

LC-MS analysis of insulin and five analogs, Levemir (insulin detemir), Apidra (insulin glulisine), Humalog (insulin lispro), Lantus (insulin glargine) and Novolog (insulin aspart), was performed on an ionKey/MS System comprised of an ACQUITY UPLC M-Class System coupled with a Xevo TQ-XS Mass Spectrometer (Figure 1). The LC conditions are summarized in Table 1, while the MRM transitions and MS settings for insulin, analogs, and the internal standard bovine insulin are summarized in Table 2. Chromatographic separation was achieved using a 2D trap-and-elute configuration. Preliminary trapping was achieved using a Symmetry C_{18}, 5 μm, 300 μm x 50 mm trap column (p/n 186007498) in combination with a 20 μL loop, while the analytical separation was performed on an iKey BEH C_{18}, 1.7 μm, 300 μm x 50 mm (p/n 186008725) and a linear gradient from 15–55 %B over 2 minutes. Total analysis time was 7 minutes. Use of this trap-and-elute strategy provided additional sample cleanup, increased sample loading of the high organic SPE eluate, and improved peak focusing.

LINEARITY AND SENSITIVITY

Human plasma was fortified with human insulin and the 5 analogs at concentrations ranging from 25–10,000 pg/mL. Bovine insulin was used as the internal standard (IS). SPE of the fortified plasma sample was performed as previously described[3] and is highlighted in Figure 2. Calibration curves were linear over 3 orders of magnitude with R^2 values >0.99 (1/x weighted regression). A representative standard curve for glulisine is shown in Figure 3. Lower limits of quantification ranges from 25–100 pg/mL with mean accuracy values >93% were achieved achieved for insulin and its analogs. Demonstration of chromatographic performance, using the lowest 3 standards of Apidra (glulisine) compared to the human plasma blank, is illustrated in Figure 4.

While microflow LC-MS has been shown to offer significant sensitivity gains over traditional 2.1 mm x 50 mm UPLC approaches,[4,5] its use often translates into longer run times, making it less than ideal for some bioanalytical assays. Use of
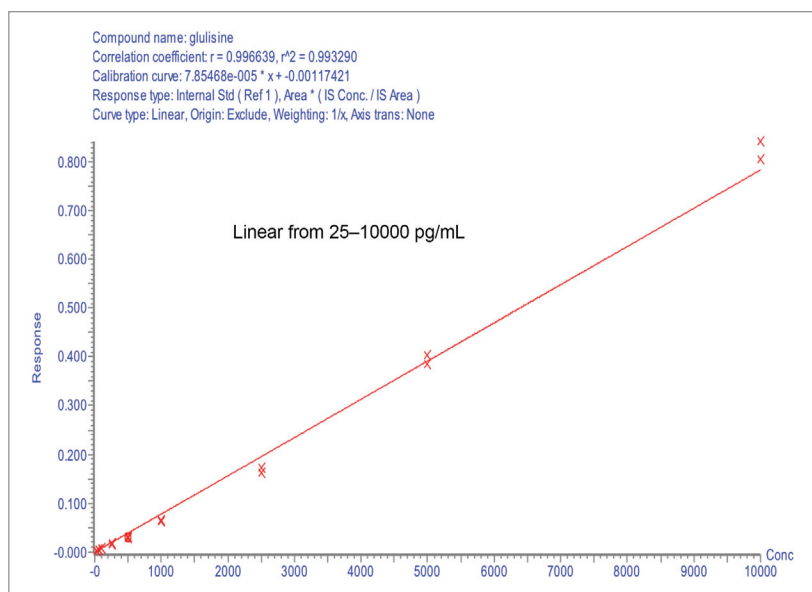

Figure 3. Representative calibration curve performance (25–10,000 pg/mL) for the insulin analogue Apidra (glulisine) extracted from plasma.
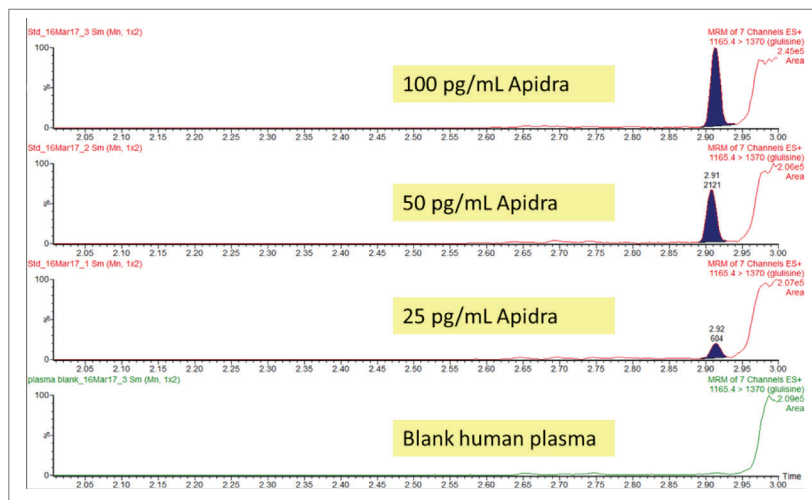

Figure 4. Representative chromatographic performance of Apidra (glulisine) extracted from plasma using the iKey HT.

the 300 μm I.D. iKey HT Separation Device not only enhances sensitivity compared to 2.1 mm analytical separations, but with its larger microfluidic channel, enables operation at higher pressures and facilitates use of higher flow rates, resulting in shorter run times compared to microflow analysis using the 150 μm I.D. iKey. In a previous method,[1] ultra-high sensitivity (25 pg/mL) for insulin and its analogs was demonstrated using an integrated microflow LC-MS system and chromatographic separation with a 150 μm I.D. iKey and analysis time of 13.5 minutes. Overall comparison of sensitivity and run times for conventional UPLC and microflow LC (150 μm iKey and 300 μm iKey HT) is highlighted in Figure 5. Among all three methods, the iKey HT yielded the shortest run time (7 minutes) with minimal compromise on

sensitivity compared to 150 µm iKey. It should be noted that there are some differences among the methods; the UPLC method extracted 250 µL and injected 30 µL. The ionKey HT method extracted the same volume, but only injected 15 µL. The ionKey 150 µm method extracted only 100 µL of sample and injected 10 µL.

SUMMARY

This study highlights the novel and highly efficient ionKey/MS System, combined with the robust and high throughput 300 µm I.D. iKey HT separation device for the highly sensitive and accurate quantification of therapeutic insulin and five analogs using tandem quadrupole LC-MS. For ultra sensitivity, the iKey 150 µm I.D. columns achieved LLOQ of a 25 pg/mL for most analogs while using the least amount of sample, but with 1.7X run time compared to the 2.1 mm analytical scale method. The iKey HT (300 µm x 50 mm) delivered a minimal compromise on sensitivity with a LLOQ of 50 pg/mL and a 2-fold improvement in run time compared to the 150 µm microflow method, which is more appropriate for routine bioanalysis labs. The fully integrated ionKey/MS System enables bioanalysts the flexibility to modulate a microflow LC-MS system between ultra sensitivity analysis and higher throughput by simply switching between the novel iKey columns.

	Standard curve range (pg/mL)		
Analyte	2.1 x 50 mm	300 µm x 50 mm	150 µm x 100 mm
Lispro	50–10,000	100–10,000	25–10,000
Glargine	50–10,000	50–10,000	25–10,000
Detemir	200–10,000	100–5,000	50–10,000
Glulisine	50–10,000	25–10,000	25–10,000
Aspart	100–10,000	50–10,000	25–10,000
Sample volume	250 µL	250 µL	100 µL
Injection volume	30 µL	15 µL	10 µL
Run time	8 minutes	7 minutes	13.5 minutes

UPLC 2.1 mm method

iKey 300 µm HT method
Fastest run time: Same sample volume, 1/2 the injection volume, and 2X more sensitive than the 2.1 mm method

iKey 150 µm method
Greatest sensitivity (2–4X): Using 2.5X less sample, 1/3 injection volume, 2X longer column, and 1.7X longer run time.

Figure 5. Insulin performance comparison using conventioanl UPLC (2.1 x 50 mm), microflow LC iKey HT (300 µm x 50 mm) and microflow LC with standard iKey (150 µm x 100 mm).

References

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