The MSIA Insulin Workflow: An Accurate and Sensitive Method for the Qualitative and Quantitative Determination of Endogenous and Exogenous Insulins Using HRAM Detection

Application Note

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Introduction

Recombinant human insulin is a multibillion-dollar pharmaceutical used for the treatment of type 2 diabetes. Comprised of two covalently linked polypeptide chains (alpha and beta) created by enzymatic digestion of a proinsulin molecule *in vivo*, insulin regulates glucose uptake in eukaryotic cells.

Researchers must often quantify insulin levels in biological samples. Drug developers, for instance, may want to track the pharmacokinetics, pharmacodynamics, bioavailability, and biotransformation of different insulin variants following injection in human or animal subjects. Clinical researchers may need to quantify insulin levels over the course of a clinical trial. Forensic analysts must sometimes assess insulin levels in the case of a suspicious death. In the world of sports, anti-doping agencies increasingly are on the lookout for players using recombinant insulins to gain a competitive advantage.

Problem

Insulin levels traditionally are quantified in either of two ways. The antibody-based enzyme-linked immunosorbent assay (ELISA) is highly sensitive, but lacks the ability to distinguish different insulin variants in a single reaction, and measured concentrations can vary from vendor to vendor. Liquid chromatography-mass spectrometry-based approaches can easily differentiate between and quantify insulin variants, but generally lack the sensitivity of ELISA.

Solution

Recently, Thermo Fisher Scientific launched a workflow that combines the sensitivity of ELISA with the molecular specificity of LC-MS. The Thermo Scientific™ MSIA™ (Mass Spectrometric Immunoassay) Workflow combines a proprietary microfluidic column (contained within a functional pipette tip) that is derivatized with specific capture reagents and LC-MS detection designed to enable sensitive, reproducible, and confident quantification of specific analytes. The microcolumns of the MSIA Insulin D.A.R.T.'S (Disposable Automation Research Tips) are preloaded with a pan-insulin antibody that recognizes a conserved region within the insulin beta-chain to purify various insulin analogs, including endogenous human insulin, therapeutic analogs and non-human animal analogs, prior to mass spectrometric detection.

In this study, human plasma samples that contained varying amounts of four different insulins were purified using MSIA Insulin D.A.R.T.'S (Part No. 991001096) and analyzed on a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer. The high resolution accurate mass (HRAM) capabilities of the Q Exactive easily resolved and identified different insulin variants by a combination of mass-to-charge ratio (m/z) and isotope abundance. The recorded Limit of Quantification (LOQ) and Lower Limit of Detection (LLOD) values were clinically relevant at 15 pM. Inter- and intra-day repeatability was within 3%, and spike-in recoveries ranged from 96% to 100%.

Goal

The goal of this study was to assess the performance characteristics of the MSIA Insulin workflow and develop a robust method for insulin analysis.

Experimental

Plasma samples were spiked with known amounts of recombinant insulins plus a spike-in control for quantitation. The samples were then incubated with the MSIA Insulin D.A.R.T.'S to bind insulin to the anti-insulin antibodies located within their microfluidic channels. Because that process involves pipetting the solution up and down through the microcolumn several hundred times over the course of about 70 minutes, this step is easily automated using either an automated liquid handling robot, Thermo Scientific™ Versette™ automated liquid handler, or electronic pipettor, Thermo Scientific™ Finnpipette™ Novus i Multichannel Electronic Pipette. The microcolumn is then washed and the bound molecules eluted, dried and reconstituted for intact analysis by LC-MS on a Thermo Scientific™ Q Exactive™ mass spectrometer.

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Sample Preparation

For spike and recovery studies, both neat and donor plasma samples containing a mix of insulin and its analogs were prepared. Insulin was added to the donor plasma at three different amounts that spanned the dynamic range. Up to four analogs were prepared in a single sample. For limit-of-detection and limit-of-quantification studies, 1.5 pM to 960 pM insulin was added to bovine serum albumin in phosphate buffered saline. Additionally, 0.05 nM of either deuterated insulin (4[D10] Leu Insulin) or porcine insulin was added as an internal reference standard to each well of 500 µL of plasma.

Insulin analogs were purified with MSIA Insulin D.A.R.T.'S in accordance with the purification protocol in the MSIA Insulin D.A.R.T.'S Technical Manual. Affinity purification was automated with the Thermo Scientific Versette automated liquid handler which can process 96 samples simultaneously. (For individual samples, use a Thermo Scientific Finnpipette Novus i electronic pipette.) Following extraction, intact insulin analogs were eluted with 15 mg/mL adrenocorticotropic hormone fragment (ACTH) 1-24 in 75 µL of 70:30 water/MeCN with 0.2% formic acid in water. The final concentration was adjusted to 75:25 water/MeCN with 0.2% formic acid in water for LC-MS analysis.

Liquid Chromatography

A Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system controlled by Chromeleon™ was used for all experiments. Volumes of 84 µL for each sample were separated on a 500 um x 10 cm Thermo Scientific ProSwift RP-4H monolithic column (Part No. 164925) using a linear gradient 18-50% B (0.2% formic acid in MeCN) in 10 minutes at a flow rate of 150 µL/min. Mobile phase A was 0.2% formic acid in water. The column was heated to 60°C.

Mass Spectrometry

All data was acquired in positive-ion mode on a Thermo Scientific Q Exactive mass spectrometer operated in data-dependent mode with dynamic exclusion enabled. The HESI source parameters used were the following: sheath gas flow rate of 40, aux gas flow rate of 15, 4.50 kV spray voltage, 300°C capillary temperature, S-lens RF level of 100, and a heater temperature of 350°C.
Full MS scans were performed with the following settings: microscans = 1, resolution setting of 70 000 (@ m/z 200), AGC target of 3e6, maximum IT = 200 ms, and scan range of m/z 450 - 2000.

Data Analysis

LC-MS data were analyzed using Thermo Scientific™ Pinpoint™ software. HRAM measurements were used for qualitative and quantitative measurement of insulin and its analogs.

Results and Discussion

The MSIA Insulin workflow identifies and resolves multiple insulin variants

The Q Exactive has sufficiently high resolution to resolve multiple charge states and isotopic forms of intact insulin molecules. It is those features that the Thermo Scientific Pinpoint software uses to identify and quantify different insulin variants.

One key feature of the MSIA Insulin workflow is that it is designed to identify and quantify multiple insulin variants

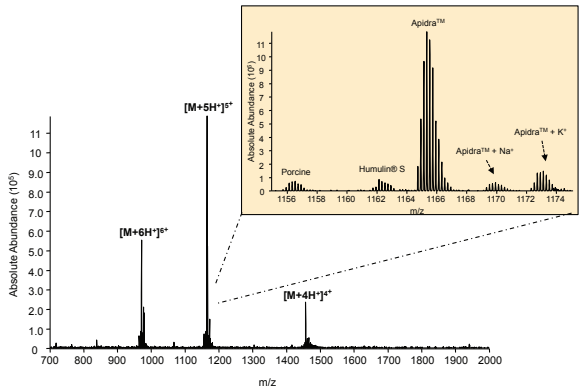

FIGURE 1. Simultaneous LC-MS detection of four different insulins. Lantus™ (0.48 nM) and Apidra™ (0.48 nM), Endogenous Human, and porcine as the internal standard were processed from the same sample and detected simultaneously. The full-scan spectrum was averaged across the three co-eluting variants. Lantus™ elutes 0.5 minutes prior to the three displayed insulin forms.

simultaneously in the same sample – for instance, to quantify molecular changes (biotransformations) occurring *in vivo*. Two recombinant insulin variants (Lantus™ and Apidra™) and porcine insulin were added to human plasma samples and processed via the MSIA Insulin workflow. As shown in **Figure 1**, endogenous human insulin, Apidra and porcine insulin all emerge from the LC column within the same timescale. Yet the Thermo Scientific Q Exactive mass spectrometer in this test resolved the three forms and their isotopic envelopes simultaneously in a single spectrum.

The MSIA Insulin workflow is linear over a wide dynamic range

The goal of the MSIA Insulin workflow is designed to enable reliable quantification of multiple insulin variants in a single sample.

Quantification curves for Lantus, Apidra (Glulisine) and endogenous human insulin are shown in **Figure 2**. Note that these curves are linear over three orders of magnitude from 1.5 pM to 960 pM, indicating the uncompromised performance of the MSIA Insulin D.A.R.T.'S to purify various concentrations of insulin variants over a wide dynamic range.

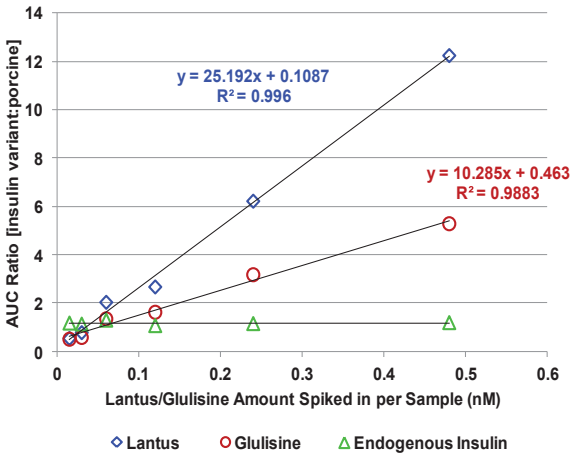

FIGURE 2. Quantification curves for Lantus and Apidra (Glulisine). Lantus and Apidra were spiked into donor plasma at different concentrations. The endogenous insulin from the donor plasma is also plotted. Since the same amount of donor plasma was used for each sample, the level of endogenous insulin remains static. All AUC values were normalized to the porcine AUC response.

Performance characteristics of the MSIA Insulin workflow

The performance characteristics detailed in Tables 1–5 demonstrate that the MSIA Insulin workflow in this example exhibits high sensitivity, reproducibility, and recovery efficiency.

The lower limit of quantification in plasma (LLOQ, defined as the lowest concentration which provides %CV that are below 20% with accuracy within ±20%). This was 15 pM (87 pg/mL) (**Table 1**). The Lower Limit of Detection (LLOD, the lowest concentration at which the mean total area was greater than four standard deviations away from the background signal) was < 15 pM (**Table 2**). (The LLOQ and LLOD values are identical as the dilution increments were too large to accurately discriminate these two points.)

Intra-day repeatability (a measure of how reproducible an observed value is over multiple experiments in a single day) was 3% (**Table 3**), and inter-day repeatability was 2% (**Table 4**). Those measurements were within 2.5% of the actual concentrations in those experiments and reliably report sample concentration.

Finally, spike-in recovery, a measure of workflow efficiency, ranged from 96.14% to 100.12% over three orders of magnitude in concentration, meaning little to no material was lost during sample processing (**Table 5**). Note that we recommend spike-in controls be added prior to MSIA Insulin D.A.R.T.'S processing, to ensure the most accurate quantification possible.

STD Conc (pM)	Mean (5-curves)	Stdev	%CV	Accuracy
0	7.42	1.02		
7.5	10.56	0.95	9.04%	40.80%
15	16378	1.42	8.46%	11.87%
30	28.96	1.12	3.85%	-3.46%
60	58.41	1.61	2.75%	-2.66%
120	115.93	1.96	1.69%	-3.39%
240	232.65	2.80	1.20%	-3.06%
480	473.25	14.41	3.04%	-1.41%
960	963.31	6.47	0.67%	0.34%

TABLE 1. Limit of Quantification

STD Conc (pM)	Mean Total File Area	4*STDev	Plus 4*STDev
0	2.37E+05	2.20E+05	4.57E+05
7.5	2.80E+05		
15	4.79E+05		
30	8.93E+05		

TABLE 2. Limit of Detection

STD Conc (pM)	Mean (3 controls x 5-curves)	Stdevp	%CV	Accuracy
50.00	51.21	1.33	3%	2.43%

TABLE 3. Intra-Day Repeatability

STD Conc (pM)	Mean (3 controls x 5-curves)	Stdevp	%CV	Accuracy
50.00	51.07	0.81	2%	2.15%

TABLE 4. Inter-Day Repeatability

Sample	Spike Conc. (pM)	Exp. Conc. (pM)	Average (pM)	Exp Recovery Conc. (pM)	% Yield
Neat 1	0.00	43.79	44.59		
Neat 2		45.59			
Neat 3		44.38			
Low 1	19.50	65.08	64.11	19.52	100.12%
Low 2		63.65			
Low 3		63.61			
Medium 1	199.50	241.19	237.56	192.97	96.73%
Medium 2		239.80			
Medium 3		231.70			
High 1	919.50	960.91	928.63	884.05	96.14%
High 2		905.35			
High 3		919.64			

TABLE 5. Spike and Recovery

Conclusions

Traditional insulin detection methodologies tend to exhibit either good sensitivity or molecular resolution, but not both. The MSIA Insulin workflow, however, is both discriminating and highly sensitive. Other LC-MS-based methods tend to rely on solid-phase extraction strategies, which yield impure products that limit the mass spectrometer's ability to detect low-abundance insulin variants. When the MSIA Insulin workflow is performed with the Thermo Scientific Q Exactive mass spectrometer, no sample fragmentation is required; the instrument is designed to detect sufficient charge and isotopic forms to confidently identify and quantify individual variants as intact molecules.

The MSIA Insulin Workflow yields a complete workflow that is simple, robust, and high-throughput. As demonstrated here, the method has a wide dynamic range, high sensitivity, high reproducibility, and efficient recovery, making it a reliable option for labs across a wide range of disciplines.

For more information on the MSIA workflow, **www.thermoscientific.com/msia**

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