Liquid Chromatography Mass Spectrometry Assay for Quantitation of Human Insulin

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Abstract: Human insulin is a peptide hormone which has a crucial role in regulating blood glucose. Monitoring insulin levels in patients can give diagnostic data regarding insulin resistance and pancreatic function. Insulin control is especially important in treating and diagnosing diabetes and medical conditions. Aptamers are short, single DNA or RNA strands that bind with high selectivity and specificity to molecular targets such as peptides and proteins. Isotope dilution is established as a gold standard for quantitation of biomarkers. Presented here is a high-performance liquid chromatography/mass spectrometry method enhanced by isotope dilution and aptamer enrichment for quantitation of human insulin. A QTRAP 6500+ (Sciex) coupled to a Shimadzu HPLC system was used. For selected ion monitoring the +5 and +6 ions were chosen. Calibration curves were generated from serial dilution of native and isotope labeled insulin. In summary, we have made progress in developing a novel method for quantitating human insulin using LC-MS.

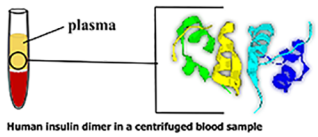
INTRODUCTION

Challenges in insulin measurement:
- Concentration of insulin in blood plasma is problematically low[1].
- Insulin enrichment methods must be specific for insulin and avoid extracting other proteins.
- Intact insulin does not have adequate MS sensitivity due to its high molecular weight[1].
- Insulin has a tendency to aggregate, posing difficulties for analysis[3].
- Insulin adsorbs to plastic and glass surfaces, making quantitation more difficult[3].

Features of an ideal method:
- Specific to insulin (as apposed to synthetic analogues)
- Is sensitive to low concentrations of insulin.
- Is applicable for both patient samples and samples with artificial matrix.

What is presented:
- Our current progress in developing a reference method for measuring insulin using HPLC-MS and aptamer technology

BACKGROUND



Human insulin dimer in a centrifuged blood sample

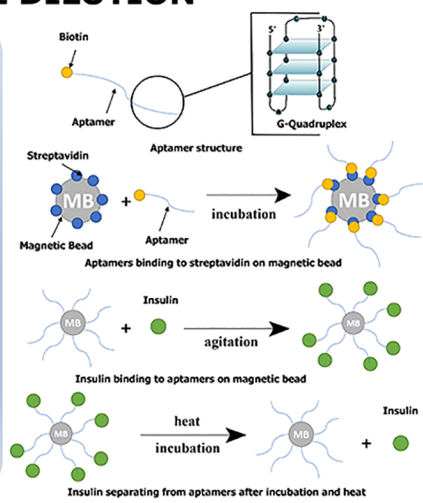
- Insulin is a peptide hormone consisting of two peptide chains: A and B. These chains are connected by disulfide bonds.
- Measuring insulin along with blood glucose creates a powerful metric that can help clinicians to diagnose diabetes.
- Diabetes is characterized by a dysregulation in blood glucose caused by either insulin deficiency or resistance to insulin[1].
- The fasting insulin level in blood for healthy individuals is in a narrow picomolar range. Ranges vary across assay to assay and require standardization.
- There are currently no standardized reference methods for measuring insulin in human samples.

SAMPLE PREPARATION

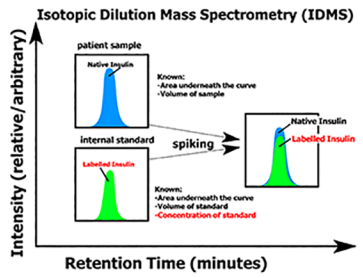
- Insulin and isotope labelled insulin solutions were prepared from recombinant human insulin and dissolved using 0.4% formic acid in H2O.
- Dithiothreitol was used to reduce the disulfide bonds between cysteines.
- Iodoacetamide was used to alkylate the thiol groups to prevent disulfide bond formation
- Insulin-binding aptamers bound to magnetic beads (Dynabeads) were diluted in a NaCl buffer solution to maintain aptamer stability
- Recombinant insulin and isotope labelled insulin was extracted using magnets after binding to aptamers (5′ biotin modification, IGA3 aptamer)

APTAMER ENRICHMENT & ISOTOPE DILUTION

- Aptamers are short, single DNA or RNA strands that bind with high specificity to molecular targets.
- The aptamer used for this study forms a non covalent bond with insulin via interaction with the G-quadruplex located on the aptamer.
- Magnetic beads can be modified with aptamers which allows for easy separation of target analytes that are bound to the aptamers·
- The magnetic beads used for this study were coated in streptavidin, which forms a strong non-covalent bond with the biotin label on the aptamer.
- Enrichment of the insulin is performed by separation of the magnetic beads with magnets from the solution after binding has occurred.

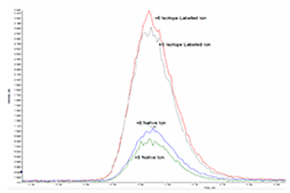


- Isotope labelled standards are chemically and conformationally equivalent to their native counterparts, but differ in mass.
- Using an isotope labelled internal standard allows us to more precisely determine the concentration of our target analyte in patient samples.
- By analyzing insulin as an absolute quantity, we increase method reproducibility and trueness.



MASS SPECTROMETRY ANALYSIS

Selected Ions	
+6 Native	968.0 Da
+6 Isotope Labelled	979.1 Da
+5 Native	1162.0 Da
+5 Isotope Labelled	1174.6 Da


Example extracted ion chromatogram with annotated ions


SCIEX QTRAP® 6500+ Mass Spectrometer

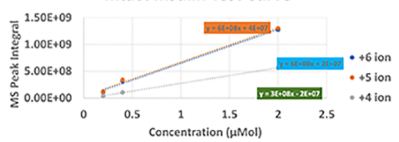
- Samples were analyzed on a SCIEX QTRAP® 6500+ mass spectrometer coupled to Shimadzu Prominence High Performance Liquid Chromatography unit.
- A full scan was performed on each solution (isotope labelled, native, etc.) beforehand to find ions for use in the quantitation step.
- Samples were quantitated using selected ion monitoring. Ions were selected according to the full scan.
- Each sample was submitted in triplicate or duplicate, with the average taken as the final measurement.
- Assigned insulin concentrations used for test curve were gravimetrically determined.
- A testing curve was constructed, relating peak integral values to concentration. This was used for ion selection purposes.
- Ions that were not adequately reproducible or whose peak integral values varied strongly with concentration were excluded from selected ion monitoring.

RESULTS

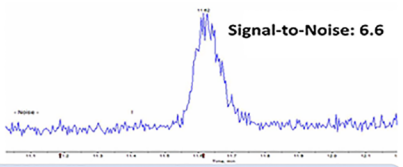
- A test curve for insulin was constructed and the ions with the best correlation with the peak integrals were the +5 and +6 ions. The +4 ion was not selected for quantitation.
- Ratios between the peak integrals for native and isotope labelled insulin were consistent between replicates and between sets of replicates.

MS Peak Integral Native:IS Ratios

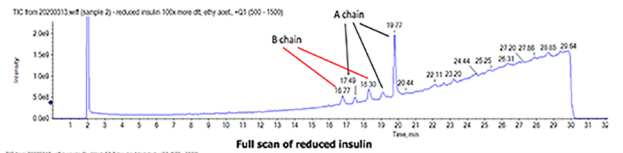
+6 ion	AVG	CV	+5 ion	AVG	CV
0.32	0.31	1.90	0.26	0.26	1.28
0.31			0.26		
0.31			0.26		



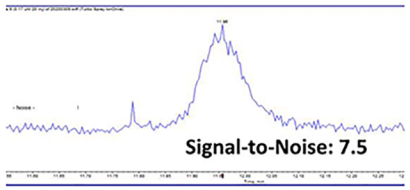
- Signal to noise ratios of all selected ion spectra were high, indicating adequate sensitivity at 28.3 pMol on the HPLC column.
- Despite good signal-to-noise at 28.3 pMol, the issue of low insulin concentrations in patient samples required us to get better sensitivity at even lower concentrations.



- To address the sensitivity issue, insulin solution was reduced by DTT. Reduction separated insulin into the A and B chains, which have better overall sensitivity on our system.
- Inefficiencies in the protocol or other potential factors resulted in heterogenous forms of reduced insulin.
- Possible interference from other proteins was detected in the reduced samples. Alkylation was performed to address the interference issues.
- Heterogeneity in both the reduced and alkylated runs can be explained by partial reduction/alkylation of insulin in different thiols. This can be mitigated with an improved protocol.



- Despite partial reduction/alkylation difficulties, alkylated and reduced insulin A and B showed higher sensitivity at the same concentrations.
- At 100 times more dilute concentrations than non-reduced samples, the same or better signal-to noise ratios were seen.



CONCLUSION

- In summary, we have made progress in developing a reference method for quantitating human insulin utilizing aptamer enrichment and isotope dilution.
- For future steps we will:
  - Improve the protocol and ensure total reduction and alkylation.
  - Consider alternative methods such as digestion by proteinase to address any other difficulties.

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