Development of a Bioanalytical Method for the Simultaneous Quantification of Synthetic Insulin Analogs in Human Plasma: Challenges of Working with Large Peptides

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Waters Corporation
Acknowledgement
Erin Chambers
Outline

- Background
- Mass Spectrometry
- Liquid Chromatography
- Solid Phase Extraction and Plasma Data
Background

- Why bioanalysis for insulin analogs?
  1. Many coming off patent between 2013 and 2015
     - Bioequivalence studies
  2. Methods needed to identify/differentiate *specific* insulins
     - Forensic toxicology, cases of wrongful death
     - Anti-doping
     - Understanding/monitoring of patient dosing?

- Current analytical methods
  1. ELISA-based assays
  2. Nano-flow or low flow LC-MS/MS assays
  3. SPE-immuno affinity LC-MS/MS assays
  4. Assays where insulin has been digested or disulfide bonds reduced
Why LC-MS/MS?

- Why an LC-MS/MS based assay?
  - ELISA assays cannot differentiate between human insulin and analogs
  - Challenges with ELISA assays
    - Time consuming, expensive to develop
    - Require separate assay for each peptide
    - Limited linear dynamic range
    - Possible cross reactivity

- Benefits of LC-MS/MS
  - LCMSMS provides single assay for multiple insulin analogs
  - Broad linear dynamic range
  - Accurate, precise
  - Universal
  - Faster, cheaper method development
Insulin and Analogs

Human Insulin
MW 5808

Insulin A Chain

Insulin B Chain

Insulin glargine
(Lantus®)
Avg MW 6063

Insulin aspart
(Novalog®)
Avg MW 5826

Insulin detemir
(Levemir®)
Avg MW 5917

Insulin glulisine
(Apidra®)
Avg MW 5823

Slide courtesy of Martha Stapels.
Specific Challenges in Developing an LC-MS/MS Assay for Insulin Analogs

- Specificity in matrix
- High level of non-specific binding (NSB)
- Aggregation
- Low MS sensitivity
  - Poor fragmentation
  - Multiple precursors
- Chromatographic peak shape
- Protein binding
Outline

- Background
- Mass Spectrometry
- Liquid Chromatography
- Solid Phase Extraction and Plasma Data
Precursor Formation During Tuning

Human Insulin

200 µL/min

10 µL/min

2.1 x 50 mm, 1.7 µm
Lantus infused at 10 µL/min teed into LC effluent containing 40% ACN
MSMS of m/z 867
7+ Insulin Glargine Precursor

Collision Energy = 18 eV

Collision Energy = 35 eV
Immonium Ions: high intensity, however...

### Insulin B-Chain

**Average Mass = 3740.3317, Monoisotopic Mass = 3737.8711**

**Residues: 1-32**

**N-Terminus = H, C-Terminus = OH**

**Fragment ions: Monoisotopic/Average (1750) m/z ratios with 1 positive charge(s).**

<table>
<thead>
<tr>
<th></th>
<th>a'</th>
<th>b'</th>
<th>c''</th>
<th>i</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<td>72.1</td>
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</table>

**Residues:**
- **Gly:** 25
- **Ser:** 24
- **His:** 23
- **Leu:** 22
- **Val:** 21
- **Glu:** 20
- **Ala:** 19
- **Leu:** 18
- **Tyr:** 17
- **Val:** 16
- **Leu:** 15

**x**
- 2927.4
- 2870.3
- 2783.2
- 2646.1
- 2532.9
- 2433.8
- 2304.7
- 2233.6
- 2120.4
- 1957.3
- 1858.1

**y''**
- 2901.4
- 2844.3
- 2757.2
- 2620.1
- 2506.9
- 2407.8
- 2278.7
- 2207.6
- 2094.4
- 1931.3
- 1832.1

**z**
- 2884.3
- 2827.3
- 2740.2
- 2603.1
- 2489.9
- 2390.8
- 2261.7
- 2190.6
- 2077.4
- 1914.2
- 1815.1
Avoiding Immonium Ion Fragments

867 -> 136 (tyrosine immonium ion)

Lack of Specificity

867 -> 984
Fragmentation Patterns for Insulin Analogs

Insulin Glulisine
MSMS of 1165 \(^{5+}\)

Insulin Aspart
MSMS of 972 \(^{6+}\)

Insulin Glargine
MSMS of 867 \(^{7+}\)

Insulin Detemir
MSMS of 1184 \(^{5+}\)
MS conditions for Insulin Analogs

- MS system: Xevo TQ-S triple quadrupole
  - Capillary: 3.00 kV
  - Desolvation temperature: 600 °C
  - Desolvation flow: 1000 L/hr
  - Dwell time 0.025 sec per transition

<table>
<thead>
<tr>
<th>Specific Insulin</th>
<th>MRM Transition</th>
<th>Cone Voltage (V)</th>
<th>Collision Energy (eV)</th>
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<tr>
<td>Glargine</td>
<td>867-&gt;984</td>
<td>60</td>
<td>18</td>
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<tr>
<td></td>
<td>1011-&gt;1179</td>
<td>60</td>
<td>25</td>
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<td>Detemir</td>
<td>1184-&gt;454.4</td>
<td>60</td>
<td>20</td>
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<tr>
<td></td>
<td>1184-&gt;1366.3</td>
<td>60</td>
<td>20</td>
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<td>Aspart</td>
<td>971.8-&gt;660.8</td>
<td>60</td>
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<td>971.8-&gt;1139.4</td>
<td>12</td>
<td>18</td>
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<td>Glulisine</td>
<td>1165-&gt;346.2</td>
<td>14</td>
<td>22</td>
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<tr>
<td></td>
<td>1165-&gt;1370</td>
<td>14</td>
<td>22</td>
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<tr>
<td>Bovine (IS)</td>
<td>1147.5-&gt;315</td>
<td>50</td>
<td>35</td>
</tr>
</tbody>
</table>
Predicted Van Deemter Using Flow Rate: Influence of Analyte Size on Optimal Flow Rate Range

Plate Height vs. flow Rate

3.5 µm particle

2.1 x 50 mm
Predicted Van Deemter Using Flow Rate: Influence of Chromatographic Particle Size

Plate Height vs. Flow Rate

- 3.5 um
- 1.7 um

2500 MW Peptide

2.1 x 50 mm

Chromatographic Peak Shape Using Formic Acid Mobile Phase

Bovine Insulin MW 5734

ACQUITY UPLC BEH300 C18
1.7 µm 2.1 X 50mm

Peak Width 11 sec

1147.5 > 315.2
Alternative Column for Peptide Analysis: Charged Surface Hybrid (CSH™) C18

ACQUITY UPLC BEH300 C18
1.7 µm 2.1 X 50mm
Peak Width 11 sec

ACQUITY UPLC CSH C18
1.7 µm 2.1 X 50mm
Peak Width 3.6 sec

Bovine Insulin, IS
MW 5734

1147.5 > 315.2
Lesson Learned: Column Conditioning

After 9 injections of precipitated plasma

2nd injection after solvent blanks

New column: 1st injection after solvent blanks
UPLC conditions for Synthetic Insulin Analogs

- Instrument: ACQUITY UPLC I-Class with FTN or ACQUITY
- Column: ACQUITY CSH™ C₁₈ 2.1 X 50mm, 1.7 µm
- Mobile Phase A: 0.1% formic acid in water
- Mobile Phase B: 0.1% formic acid in acetonitrile
- Weak wash: mobile phase A
- Strong wash: 50/25/24/1 ACN/IPA/water/formic acid
- Flow rate: 0.25 mL/min
- Column temperature: 60° C
- Sample Manager temperature: 15° C
- Gradient: 20% B to 65% B in 2 min, to 98% B at 2.1 min, hold for 0.5 min, return to initial at 2.7 min
- Total cycle time: 3.5 min
- Injection volume: 15 µL
Separation of 4 Synthetic Insulins and the Internal Standard, Bovine Insulin
Detection Limit for Insulin Glargine in Solvent Standards

100 pg/mL

50 pg/mL

Solvent Blank
Linearity for Insulin Glargine in solvent Standards: 50 pg/mL to 50 ng/mL

<table>
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<th>% Dev</th>
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<tr>
<td>3.4</td>
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<td>-7.2</td>
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<td>1.5</td>
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<td>-4.4</td>
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<td>3.4</td>
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<tr>
<td>-0.1</td>
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<tr>
<td>-13.9</td>
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</table>

The table shows the % Dev for Insulin Glargine standards ranging from 50 pg/mL to 50 ng/mL.
insulin glargine solution: <30 min on benchtop

30% MeOH, 10% acetic acid, 0.05% rat plasma

30% MeOH, 10% acetic acid
Outline

- Background
- Mass Spectrometry
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- Solid Phase Extraction and Plasma Data
Extraction Conditions for Insulin Analogs from Human Plasma

Oasis® HLB µElution 96-well plate

- **Condition**: 200 µL methanol
- **Equilibrate**: 200 µL water
- **Load Sample**: 300 µL human plasma diluted with 300 µL 10mM TRIS Base
- **Wash**: 200 µL 5% methanol, 1% acetic acid in water
- **Elute**: 2X 25 µL 60% methanol, 10% acetic acid in water
- **Inject 15 µL**
Extraction from Human Plasma: Impact of Pretreatment Prior to SPE

Final Eluate

TRIS Base dilution

TFA dilution

Human Serum Albumin
LOD and LLOQ for Insulin Glargine in Human Plasma

0.5 ng/mL Lantus

0.2 ng/mL Lantus

Blank human plasma
LOD and LLOQ for Insulin Detemir in Human Plasma

20 µL injected
LOD and LLOQ for Insulin Glulisine in Human Plasma

MRM of 3 Channels ES+
1165.032 > 1369.904 (Apidra)
2.58e5
Area

0.5 ng/mL Apidra

MRM of 3 Channels ES+
1165.032 > 1369.904 (Apidra)
9.83e4
Area

0.2 ng/mL Apidra

MRM of 3 Channels ES+
1165.032 > 1369.904 (Apidra)
9.66e4
Area

Blank human plasma

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LOD and LLOQ for Insulin Aspart in Human Plasma

1 ng/mL Novolog

0.5 ng/mL Novolog

Blank human plasma

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Linearity for Insulin Detemir and Glulisine in Human Plasma

Insulin Detemir
$R^2 = 0.997$
Linear fit, $1/x$ weighting

Insulin Glulisine
$R^2 = 0.998$
Linear fit, $1/x$ weighting
## Standard Curve and QC Samples: Insulin Detemir in Human Plasma

<table>
<thead>
<tr>
<th>Name</th>
<th>Conc ng/mL</th>
<th>Area</th>
<th>Response</th>
<th>IS Area</th>
<th>%Dev</th>
<th>Calc Conc ng/mL</th>
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</thead>
<tbody>
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<td>Blank plasma</td>
<td></td>
<td>31.523</td>
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<tr>
<td>200 pg/mL plasma</td>
<td>0.2</td>
<td>153.33</td>
<td>0.012</td>
<td>13239.484</td>
<td>16.6</td>
<td>0.233</td>
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<tr>
<td>500 pg/mL plasma</td>
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<td>375.641</td>
<td>0.03</td>
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<tr>
<td>1 ng/mL plasma</td>
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<td>750.185</td>
<td>0.06</td>
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<td>25007.11</td>
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<td>241.626</td>
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<td>733.632</td>
<td>0.058</td>
<td>12560.556</td>
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<td>0.35</td>
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<td>20.091</td>
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</table>
Conclusions

- Importance of: column chemistry, sample pretreatment, addressing NSB, concentration without evaporation, proper fragment choice
- One extraction method was developed for 4 insulin analogs from human plasma
- A single simple, analytical scale LC method was developed for separation of 4 synthetic insulin analogs
- Limit of detection for all 4 insulins was 50 pg/mL in solvent standards
- Detection or quantitation limits of 0.2 to 0.5 ng/mL were achieved for all 4 insulin analogs extracted from 250-300 µL human plasma
- Shows promise for direct quantification of intact insulins in plasma
Acknowledgements

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