Ultra-sensitive simultaneous LC-MS/MS quantification of human insulin, glargine, lispro, aspart, detemir and glulisine in human plasma using 2D-LC and a novel high efficiency column

Erin E. Chambers
Principal Applications Chemist
Outline

- Background and Goals
- Mass spectrometry development
- Chromatography development
- Sample Preparation Development
- Validation Data
- Conclusions
Insulin and Analogs

- **Human Insulin**
  - MW 5808

- **Insulin glargine** (Lantus®)
  - Avg MW 6063

- **Insulin aspart** (Novalog®)
  - Avg MW 5826

- **Insulin detemir** (Levemir®)
  - Avg MW 5917

- **Insulin glulisine** (Apidra®)
  - Avg MW 5823

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Background

Why bioanalysis for insulin analogs?

1. Many coming off patent between 2013 and 2015
   - Bioequivalence studies
   - Development of new versions
     • bioanalysis

2. Methods needed to identify/differentiate specific insulins
   • Need simultaneous quantification as combination therapies common
     - Forensic toxicology, cases of wrongful death
     - Anti-doping
     - Understanding/monitoring of patient dosing?

Current analytical methods

1. ELISA-based assays (lack of standardization)
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
Specific Challenges in Developing an LC-MS/MS Assay for Insulin Analogs

Key challenge: distinguish human insulin and Humalog (lispro) whilst obtaining adequate specificity for low level detection

Obtain sensitivity similar to LBAs

- Specificity in matrix
- High level of non-specific binding (NSB)
- Low MS sensitivity
  - Poor fragmentation
  - Multiple precursors
- Chromatographic peak shape
- Protein binding
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Lantus infused at 10 µL/min teed into LC effluent containing 40% ACN
MS Specificity: Avoiding Immonium Ion Fragments

867 -> 136 (tyrosine immonium ion)

Lack of Specificity

867 -> 984
MSMS spectra for insulin glulisine, aspart, detemir, and glargine

Insulin glulisine MSMS of 5+ 1165

Insulin aspart MSMS of 6+ 972

Insulin detemir MSMS of 5+ 1184

Insulin glargine MSMS of 5+ 1011
MSMS spectra from 5+ precursors of human insulin and insulin lispro

Human insulin MSMS of 5+ 1163

Insulin lispro MSMS of 5+ 1163
MSMS of different Humalog (lispro) Precursors

Lower m/z precursor yields higher intensity but lower signal to noise

MSMS of 5+ Precursor
1163 -> 217.3

S/N 633

S/N:RMS=633.04

MSMS of 6+ Precursor
969 -> 217.3

S/N 202

S/N:RMS=202.52

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Humalog Sample Analysis: Effect of Higher m/z Precursor

MSMS of 5+ Precursor
1163 -> 217.3

MSMS of 6+ Precursor
969 -> 217.3
### Xevo TQ-S Triple Quadrupole MS conditions

<table>
<thead>
<tr>
<th>Specific Insulin</th>
<th>MRM Transition</th>
<th>Cone Voltage (V)</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glargine</td>
<td>1011-&gt;1179</td>
<td>60</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>867-&gt;984</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>Lispro</td>
<td>1162-&gt;217</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>968.5-&gt;217</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Detemir</td>
<td>1184-&gt; 454.4</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1184-&gt; 1366.3</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Aspart</td>
<td>971.8 -&gt; 660.8</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>971.8 -&gt; 1139.4</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Glulisine</td>
<td>1165 -&gt; 1370</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1165 -&gt; 346.2</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>Bovine (IS)</td>
<td>956.6 -&gt; 1121.2</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>Human insulin</td>
<td>1162 -&gt; 226</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>968.5-&gt;217</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

Note: highlighting indicates the primary transitions used for quantification.
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Evolution of Insulin Method: Traditional C18 to Charged Surface Hybrid (CSH™) C18

Bovine Insulin MW 5734

Peak Width 11 sec

ACQUITY UPLC BEH C18
1.7 µm 2.1 X 50mm

Peak Width 3.6 sec

ACQUITY UPLC CSH C18
1.7 µm 2.1 X 50mm

1147.5 > 315.2
Improvement for insulin using solid core charged surface columns

Humalog

26% area increase

Apidra

52% area increase

Solid core

Fully porous

1162 > 217 (Humalog) 1.44e5

1165.2 > 1370 (Apidra) 3.87e5
LC Method: At-column-dilution and Trap and Back Elute

- Analytical Column: CORTECS C18+ 2.1 X 50mm, 1.7 µm
- Trap column: XBridge C18 IS, 3.5 µm, 2.1 X 20mm
- Mobile phase A = 0.1% formic acid in water
- Mobile phase B = 0.1% formic acid in ACN
- Loading time: 2 minutes
- At Column Dilution
- Elution
  - 15 to 40% B over 4 minutes
- Analytical Column Temp: 60°C
- Sample Temp: 15°C
- Injection Volume: 30 µL (can inject 45 µL without breakthrough)
- SNW: 50/25/24/1 ACN/IPA/H2O/FA
ACQUITY IClass with 2D Technology: Valve Diagram

**POSITION 1**

- Pump 1 (Injector)
- Pump 2
- Pump 3
- TC
- AC
- MS
- Waste

**POSITION 2**

- Pump 1
- Pump 2
- Pump 3
- TC
- AC
- MS
- Waste

**Legend**

- Pump 1: Loading pump
- Pump 2: Dilution pump
- Pump 3: Elution pump
- TC= trapping column
- AC= analytical column
UPLC-MS/MS Chromatograms of human insulin, insulin analogs, and bovine insulin (IS)

Insulin detemir
RT 5.52 min

Insulin glulisine
RT 4.29 min

Human insulin
RT 4.30 min

Insulin lispro
RT 4.28 min

Insulin glargine
RT 4.13 min

Insulin aspart
RT 4.27 min

Bovine insulin (IS)
RT 4.23 min
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Extraction Conditions

PPT followed by Oasis® MAX µElution 96-well plate

- **PPT**: 250 µL human plasma sample precipitated 1:1 with 50/50 ACN/MeOH + 1% FA, vortex spin 10 min at 13K rcf, dilute supernatant with 900 µL 5% NH₄OH in water
- **SPE**: Oasis® MAX µElution 96-well plate
  - Condition: 200 µL methanol
  - Equilibrate: 200 µL water
  - Load Sample: entire diluted supernatant in 2 steps of ~ 700 µL each
  - Wash: 200 µL 5% NH₄OH in water
  - Wash: 200 µL 5% methanol, 1% acetic acid in water
  - Elute: 2X 25 µL 60% methanol, 10% acetic acid in water
  - Dilute: 50 µL water
  - Inject 30 µL

Plasma detection limit: 50 pg/mL
Current LOD and LLOQ for Insulin Glulisine in **Human Plasma**

- **0.1 ng/mL Apidra**
  - 1165.2 > 1370 (Apidra)
  - 4.58e4 Area

- **0.05 ng/mL Apidra**
  - 1165.2 > 1370 (Apidra)
  - 4.58e4 Area

- **Blank human plasma**
  - 1165.2 > 1370 (Apidra)
  - 4.58e4 Area
Current Method: Insulin glargine (Lantus) at the LOD and the low QC in Human Plasma

Low QC 150 pg/mL (25 fmol/mL)

LOD 50 pg/mL (8.25 fmol/mL)

Blank human plasma

~248 amol on column at LLOQ
Insulin lispro (Humalog) at the LOD and the low QC

Low QC 150 pg/mL (26 fmol/mL)

LOD 50 pg/mL (8.6 fmol/mL)

Blank human plasma

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- Conclusions
## Standard Curve Statistics

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Std. Curve Range pg/mL</th>
<th>Std. Curve Range fmol/mL</th>
<th>$r^2$, linear fit, 1/x weighting</th>
<th>Mean % accuracy of all points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin lispro</td>
<td>50-10,000</td>
<td>8.6-1720</td>
<td>0.998</td>
<td>99.99</td>
</tr>
<tr>
<td>Insulin glargine</td>
<td>50-10,000</td>
<td>8.3-1650</td>
<td>0.996</td>
<td>99.98</td>
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<tr>
<td>Human insulin</td>
<td>50-10,000</td>
<td>8.6-1720</td>
<td>0.996</td>
<td>100</td>
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<tr>
<td>Insulin detemir</td>
<td>200-10,000</td>
<td>33.8-1690</td>
<td>0.998</td>
<td>96.4</td>
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<td>8.6-1720</td>
<td>0.995</td>
<td>100</td>
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<tr>
<td>Insulin Aspart</td>
<td>100-10,000</td>
<td>17.2-1716</td>
<td>0.995</td>
<td>100</td>
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</tbody>
</table>

For reference: 1µU/mL = 35 pg/mL = 6 fmol/mL

*Volund et al 1991*
### Human insulin QC Statistics

**Avg basal level was 1937 pg/mL**

<table>
<thead>
<tr>
<th>QC conc. (pg/mL)</th>
<th>Mean Calc. Conc.</th>
<th>Std Dev</th>
<th>% CV</th>
<th>Mean Accuracy</th>
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<tbody>
<tr>
<td>150</td>
<td>1915.1</td>
<td>125.4</td>
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<td>750</td>
<td>2542.5</td>
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<tr>
<td>7500</td>
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</table>

**Intra-day n=3**

**Basal level was 1872 pg/mL**

<table>
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<th>QC conc. (pg/mL)</th>
<th>Mean Calc. Conc.</th>
<th>Std Dev</th>
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<th>Mean Accuracy</th>
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<td>7500</td>
<td>10233.2</td>
<td>265.2</td>
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## Insulin lispro QC Statistics

### Insulin Lispro
#### Inter-day n=9

<table>
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<th>QC conc. (pg/mL)</th>
<th>Mean Calc. Conc.</th>
<th>Std Dev</th>
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<tr>
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<td>102.6</td>
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#### Intra-day n=3

<table>
<thead>
<tr>
<th>QC conc. (pg/mL)</th>
<th>Mean Calc. Conc.</th>
<th>Std Dev</th>
<th>% CV</th>
<th>Mean Accuracy</th>
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<tbody>
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<td>109.8</td>
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<tr>
<td>750</td>
<td>748.2</td>
<td>19.8</td>
<td>2.6</td>
<td>99.8</td>
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<tr>
<td>2500</td>
<td>2417.6</td>
<td>230.4</td>
<td>9.5</td>
<td>96.7</td>
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<tr>
<td>7500</td>
<td>8215.4</td>
<td>243.1</td>
<td>3.0</td>
<td>109.5</td>
</tr>
</tbody>
</table>
## Insulin glargine QC Statistics

### Insulin Glargine

#### Inter-day n=9

<table>
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<tr>
<th>QC conc. (pg/mL)</th>
<th>Mean Calc. Conc.</th>
<th>Std Dev</th>
<th>% CV</th>
<th>Mean Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>150.1</td>
<td>18.7</td>
<td>12.4</td>
<td>102.7</td>
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<tr>
<td>750</td>
<td>718.4</td>
<td>47.3</td>
<td>6.6</td>
<td>95.8</td>
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<td>2369.3</td>
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<td>94.8</td>
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<tr>
<td>7500</td>
<td>7648.5</td>
<td>511.3</td>
<td>6.7</td>
<td>102.0</td>
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#### Intra-day n=3

<table>
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<tr>
<th>QC conc. (pg/mL)</th>
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<th>Std Dev</th>
<th>% CV</th>
<th>Mean Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>167.4</td>
<td>16.6</td>
<td>9.9</td>
<td>111.6</td>
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<td>7500</td>
<td>7949.5</td>
<td>257.9</td>
<td>3.2</td>
<td>106.0</td>
</tr>
</tbody>
</table>
Further Method Assessment and Implementation

- Pilot Study with Patient Samples*
  - 22 type I and type II diabetic volunteers
    - Received one or several insulins
  - Dosage regime blind to analytical site
  - Results concur with multi-dosing therapies

- Human insulin over-spike
  - Samples spiked with human insulin at 200X the ULOQ
    - Represent possible high levels expected in diabetics
  - No interference with quantification of any analogs including lispro

*manuscript submitted
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Conclusions/Key Points

- Detection limits approx. 4X lower (than previous method) for most analogs
  - Only other LC/MS method that reaches these detection limits uses nano-flow and 3-step sample prep involving affinity purification followed by 2 SPE extractions
- The use of the CORTECS C$_{18}^+$ column provided significantly improved sensitivity and peak shape for insulin analogs versus charged-surface fully porous columns and traditional C18 columns
  - Excellent batch-to-batch reproducibility
- 2D LC enables higher loading and further cleanup
- Selective PPT/mixed-mode SPE cleanup significantly reduces endogenous interferences
- Test performed to verify absence of interference when human insulin present at >200X higher concentrations than other analogs
  - For example type II diabetics
  - No cross-talk or impact on quantification observed
- All FDA criteria for accuracy and precision met
  - Average accuracies for standard curve points and QC samples were >92%, with most being close to 99%
  - Inter- and intra-day precision for all QC samples better than 7.5%
  - CV’s of matrix factors, for all analogs, across 6 lots of human plasma were <15%
Acknowledgements

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