Getting More with Less: Improving Sensitivity and Reducing Sample Consumption in LC/MS Assays for Endogenous and Injected Glucagon, 6 Insulins, and Teriparatide

Erin E. Chambers
Principal Scientist
Outline

- Background and Key Challenges
  - Practical Applications of Integrated Microscale LC
    - Routine Ultra-high Sensitivity Teriparatide Quantification: Adaption and Benefits from Analytical to Microscale LC
    - Endogenous and Therapeutic Glucagon Analysis
    - Increasing Sensitivity and Reducing Sample Volume Required for Quantification of Multiple Insulin Analogs
    - Reducing Sample Volumes for Small peptides
- Conclusions
Why LC-MS/MS?

Why an LC-MS/MS based assay?
- Challenges with ligand-binding assays
  - inability to distinguish closely related analogs
  - require separate assay for each peptide
  - limited linear dynamic range
  - Possible cross reactivity
  - Lack of standardization
  - Long development times

Benefits of LC-MS/MS
- Easy to multiplex
- Broad linear dynamic range
- Accurate, precise
- Universal
- Faster, cheaper method development
However:

- Ultra-high sensitivity required for most quantitative peptide assays
- Need to obtain the sensitivity of LBAs using LC/MS
  - Small sample volumes
- Need to obtain specificity that is comparable to LBAs using LC/MS
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Teriparatide (rhPTH)

- Recombinant form of 1 – 34 amino acids from human parathyroid hormone (PTH)

- Stimulates new bone formation leading to increased bone mineral density

- Use for people with osteoporosis at a high risk for fracture
Background

Why LC/MS for teriparatide?

1. Coming off patent 2018
   - Bioequivalence studies
   - Development of new versions

2. Replacement for original RIA method
   - Improved accuracy and precision through LC/MS
   - Avoid cross-reactivity and dilution issues
   - LC/MS can differentiate parent and metabolites and allows single assay for multiple compounds
Specific Challenges in Developing an LC-MS/MS Assay for Teriparatide

★ Obtain sensitivity and specificity similar to LBAs
  - Minimize sample volume
  - Specificity in matrix
  - High level of non-specific binding (NSB)

★ Low MS sensitivity
  - Poor fragmentation
  - Multiple precursors
  - Typical 20 µg clinical dose = plasma levels ~ 50 pg/mL

- Chromatographic peak shape
- Protein binding
Original **Analytical** Scale Method

- **Analytical Column:** ACQUITY UPLC 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 ACN
- **Gradient:** hold 15% B for 0.2 min, ramp to 50% B at 3.8 min, flush with 98% B, return to initial
- **Flow Rate:** 0.4 mL/min
- **Column Temp:** 60°C
- **Sample Temp:** 5°C
- **Injection Volume:** 30 μL

Chambers et al, *Journal of Chromatography B*, Volume 938, 1 November 2013, Pages 96-104
Extraction Conditions

PPT followed by Polymeric Reversed-Phase SPE in µElution 96-well plate

- **PPT**: 200 µL human plasma sample precipitated 1:1 with 5% NH4OH in ACN, vortex spin 15 min at 4000 rpm; dilute supernatant with 1 mL water

- **SPE**: Oasis® HLB µElution 96-well plate
  - Condition: 200 µL methanol
  - Equilibrate: 200 µL water
  - Load Sample: entire diluted supernatant in 2 steps
  - Wash: 200 µL 5% MeOH in water
  - Elute: 2X 25 µL 60/34/5/1 ACN/water/TFE/TFA
  - Dilute: 50 µL water
  - Inject 30 µL

PPT Improves Specificity by Eliminating High Abundance Proteins
Analytical Scale

75 pg/ml

35 pg/ml

20 pg/ml

Blank human plasma

200 µL sample, 30 µL injection
# Standard Curve Statistics

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<th>Mean Calculated Conc. (pg/mL)</th>
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Representative Standard Curve

Teriparatide
$R^2 = 0.998$
Quadratic fit, $1/x$ weighting

Teriparatide Extracted from Human Plasma: 15-500 pg/mL
## Representative QC Statistics

<table>
<thead>
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<th>Human Plasma Lot# (Biological Specialty Corp.)</th>
<th>Gender</th>
<th>QC Conc. (pg/mL)</th>
<th>Mean Calculated Conc. (pg/mL)</th>
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What’s Next?

- Desired Improvements
  - Reduce sample size
    • Preclinical tox
    • Pediatrics
  - Increase sensitivity
  - Simplify curve fit

- How do we get there?
Can I Successfully Adapt a Highly Optimized Analytical Scale Method??
Integrated Microflow LC: ionKey/MS Ion Source
Teriparatide ionKey/MS Analysis in Trap and Back-elute Mode

Analyte Band
Microscale Method: At-column-dilution and Trap and Back Elute

- **iKey**: 150 µm X 50 mm BEH PST C18, 1.7 µm
- **Trap column**: Symmetry C18 5 µm, 300 µm X 50mm
- **Mobile phase A**: 0.1% formic acid in water
- **Mobile phase B**: 0.1% formic acid in ACN
- **Loading time**: 2 minutes
- **iKey Temp**: 75°C
- **Injection Volume**: 10-15 µL
- **Gradient**:

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<th>Time (min)</th>
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Phase 2: Improve Method
Reduce Sample Size from 200 to 50 µL

50 µL human plasma extracted

50 pg/mL

25 pg/mL

15 pg/mL

Blank human plasma

MRM of 4 Channels ES+
687 > 787.1 (Teriparatide 6+)
6.13e4
Area

50 pg/ml qc
6.23;1950
Phase 2: Improve Method
Dynamic Range Increased, Linear Fit

Compound name: Teriparatide 687
Correlation coefficient: $r = 0.999254$, $r^2 = 0.998508$
Calibration curve: $0.010184 \times x + 0.0879951$
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Teriparatide Extracted from 50 µL Human Plasma: 10-1000 pg/mL
Linear fit and broader range than analytical
## Standard Curve Statistics: ionKey/MS

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<tr>
<th>Teriparatide Concentration (pg/mL)</th>
<th>Teriparatide/IS Ratio Response</th>
<th>Calculated Teriparatide Concentration (pg/mL)</th>
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## Representative QC Statistics

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Can I Successfully Adapt a Highly Optimized Analytical Scale Method?

**Yes!**
Absence of Carryover

High QC: 500 pg/mL teriparatide extracted from human plasma

Blank following high QC
Comparison of Original Analytical Scale and New ionKey/MS Method

20 pg/mL teriparatide extracted from human plasma

ionKey/MS method

50 µL plasma
15 µL injection
45:1 S:N

original 2.1 mm method

200 µL plasma
30 µL injection
11:1 S:N
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Glucagon
MW 3481

Glucagon secretion

- Sympathetic activity
- Secretin
- CCK
- Parasympathetic activity
- Amino Acids
- Insulin
- Glucose

↑ Fatty acids and ketones
↓ Glucose sparing
↓ Inhibition of anabolism
↑ Gluconeogenesis
↓ Glycogenolysis
↓ secretion of Insulin
Injection Volume Scalability on ionKey/MS

- **15µL injection**
  - MRM of 4 Channels ES+
  - 697.1 > 940.2 (Glucagon)
  - 3.87e4 Area

- **10µL injection**
  - MRM of 4 Channels ES+
  - 697.1 > 940.2 (Glucagon)
  - 3.87e4 Area

- **5µL injection**
  - MRM of 4 Channels ES+
  - 697.1 > 940.2 (Glucagon)
  - 3.87e4 Area

**Injection Volume Scalability**

- **Equivalent to ~3 mL**
  - Area: 7.19;1456
- **Equivalent to ~2 mL**
  - Area: 7.22;949
- **Equivalent to ~1 mL**
  - Area: 7.21;580
25 pg/mL Glucagon in Human Plasma: iKey vs. Analytical Scale

3X > S:N and 5X > intensity from 5 times less injected

5µL injection
15:1 S:N

ionKey/MS method
(150 µm ID)

25 µL injection
5:1 S:N

Original method
(2.1 mm ID)
Representative standard curve and statistics for glucagon extracted from human plasma 12.5-1,000 pg/mL

Compound name: Glucagon 1040
Correlation coefficient: \( r = 0.999889, r^2 = 0.997400 \)
Calibration curve: \( 42.6771 \times x - 120.577 \)
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

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Representative chromatograms from glucagon extracted from plasma at 12.5, 25, 50, 100, 250, 500 and 10000 pg/mL, compared to blank plasma.
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Critical Biotherapeutics Coming off Patent: Open to Biosimilar Competition

Modified slide from McKinsey and Company
Data Source: Evaluate Pharma
Insulin and Analogs

Human Insulin
MW 5808

Insulin A Chain
Insulin B Chain

Insulin glargine
(Lantus®)
Avg MW 6063

Insulin A Chain
Insulin B Chain

Insulin detemir
(Levemir®)
Avg MW 5917

Insulin A Chain
Insulin B Chain

Humalog
(insulin lispro)

Insulin aspart
(Novalog®)
Avg MW 5826

Insulin A Chain
Insulin B Chain

Insulin glulisine
(Apidra®)
Avg MW 5823

Insulin A Chain
Insulin B Chain

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### Xevo TQ-S Triple Quadrupole MS Conditions

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<td>Glulisine</td>
<td>1165-&gt;1370</td>
<td>14</td>
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<td>1165-&gt;346.2</td>
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<td>22</td>
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<tr>
<td>Bovine (IS)</td>
<td>956.6-&gt;1121.2</td>
<td>60</td>
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<td>Human insulin</td>
<td>1162-&gt;226</td>
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<td>40</td>
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<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
2.1 mm ID Scale: ACQUITY IClass with 2D Technology Valve Diagram

Pump 1 (Injector)
Pump 2
Pump 3

POSITION 1

Waste
Pump 3

TC

Pump 1: Loading pump
Pump 2: Dilution pump
Pump 3: Elution pump

TC= trapping column
AC= analytical column

POSITION 2

Waste
Pump 3

Pump 1

T

AC
MS

AC
MS
Analytical 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
Analytical Extraction Conditions

PPT followed by Mixed-mode Strong Anion Exchange SPE in 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% NH$_4$OH 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% NH$_4$OH 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

Analytical Scale LC, Plasma detection limit: 50 pg/mL
Analytical Scale Method: Lantus LOD and Low QC in Human Plasma

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

LOD 50 pg/mL (8.25 fmol/mL)

Blank human plasma

Lantus (insulin glargine)

- Top selling insulin analog ($6.6 billion)
- Off patent 2015
- Lots of requests for patent extensions
  - Pediatrics?
  - Different formulations?
- Requires more sensitivity and decreased sample volume
  - <50 pg/mL
  - ≤100 µL sample
- Analytical scale method uses 250 µL sample and reaches a LOD of 50 pg/mL
- Can integrated microscale LC/MS help??
IonKey/MS Lantus Results: 100 μL sample, 10 μL injection

Decrease sample volume, decrease injection volume, increase sensitivity!

200 pg/mL

100 pg/mL

25 pg/mL

Blank extracted plasma

~41 amol on column at LOD
Compound name: Lantus
Correlation coefficient: $r = 0.995564$, $r^2 = 0.991147$
Calibration curve: $0.000398185 \times x - 0.00299844$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Graphs showing concentration vs. response and residual vs. concentration.
# ionKey/MS: Representative Standard Curve Statistics 25 pg/mL to 10 ng/mL Lantus

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Std. Conc. pg/mL</th>
<th>Retention Time</th>
<th>Area</th>
<th>IS Area</th>
<th>Conc.</th>
<th>%Dev</th>
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<td>Blank Plasma</td>
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</tbody>
</table>
Insulin glargine (Lantus) from 50 µL human plasma sample, 10 µL injection

100 pg/mL Lantus

50 pg/mL Lantus

Blank extracted plasma

MRM of 9 Channels ES+
1011.3 > 1179.3 (Lantus)
2.49e4
Area

MRM of 9 Channels ES+
1011.3 > 1179.3 (Lantus)
2.80e4
Area

MRM of 9 Channels ES+
1011.3 > 1179.3 (Lantus)
2.27e4
Area

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Outline

- Background and Key Challenges
- Practical Applications of Integrated Microscale LC
  - Routine Ultra-high Sensitivity Teriparatide Quantification: Adaption and Benefits from Analytical to Microscale LC
  - Endogenous and Therapeutic Glucagon Analysis
  - Increasing Sensitivity and Reducing Sample Volume Required for Quantification of Multiple Insulin Analogs
  - Reducing Sample Volumes for Small peptides
- Conclusions
Enhanced sensitivity using ionKey/MS: Desmopressin in human plasma

2.5 pg/mL

Desmopressin MW 1069

200 µL extraction, 5 µL injection

100 µL extraction, 5 µL injection

25 µL extraction, 10 µL injection

~6.5 amol on column
Conclusions

- Integrated microscale LC facilitates increased sensitivity using small sample volumes
- **20-50X cumulative improvement** obtained over 2.1 mm ID scale through:
  - Decreasing sample volume
  - Decreasing injection volume
  - Increasing sensitivity
- Analytical scale quantification methods for teriparatide, glucagon, 6 insulins and small cyclic peptides were adapted to and significantly improved by ionKey/MS
  - Greater S:N, with less sample, and less injected
  - Single pM quantification limits from 25-100 µL of sample
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