Bioanalysis of PEGylated Proteins by LC-MS

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Polyethylene Glycol (PEG)

- Water soluble (flexible and hydrated polymeric backbone)
- Soluble in several organic solvents
- Biocompatible
- FDA-approved for dermal, oral and intravenous applications
- Found in laxatives, toothpastes, skin creams, electronic cigarettes, paintball filling, etc.

But for our application of interest...
PEGylated Proteins

Improved Pharmacological Properties of Protein Therapeutics

Improved Pharmacokinetics

Human blood levels after subcutaneous injection of IFN-α2a and PEG-IFN-α2a.

- **Interferon-α2a**
- **PEG-Interferon-α2a.**

Better pharmacodynamics

TNF-related apoptosis-inducing ligand (TRAIL)

Kim et al. (2011) Bioconjug Chem.
PEGylated Protein Therapeutics

Great for patients...but what about analytical biochemists?
General Protein Quantification by LC-MS

Protein in Biological Matrix

Capture

Digestion

No Digestion

No Capture

Digestion

No Digestion

LC-MS
PEGylated Protein Quantification by LC-MS

Protein in Biological Matrix

Capture

Digestion

No Capture

LC-MS
Protein PEGylation-related LC-MS Analytical Challenges

• PEG size polydispersity – prevents intact protein quantification by LC-MS
Protein PEGylation-related Analytical Challenges

Intact PEGylated Protein by MS


Isotope distribution

Signal dilution over PEG size

Charge states
Protein PEGylation-related Analytical Challenges

• PEG size polydispersity – prevents intact protein quantification by LC-MS

• PEG attachment site - limits choice of signature peptide
PEGylated Proteins

Impact of PEGylation on Tryptic Digestion

Trypsin digestion impeded at PEGylated lysines resulting in miscuts, absent peptides and altered digestion profile

Protein PEGylation-related Analytical Challenges

• PEG size polydispersity – prevents intact protein quantification by LC-MS

• PEG attachment site - limits choice of signature peptide

• Internal Standard unavailability – risk of variability
Extraction Methods for PEGylated Proteins

Protein precipitation

Immunoaffinity purification

PEGylated protein solubility in organic solvents allows protein precipitation for extraction of PEGylated proteins and reduced sample complexity

Case Study: LC-MS Quantification of Pegvisomant

- PEGylated human growth hormone (hGH) structural analog
- hGH Receptor antagonist
- Used in treatment of acromegaly (excess pituitary hGH production)

LC-MS quantification of Pegvisomant: Tryptic digestion of a Lys-PEGylated protein

Surrogate peptide should be:
• Arginine-flanked
• Pegvisomant-specific
Direct Tryptic Digestion of Pegvisomant

Pegvisomant-spiked rat plasma

Dilution in NH$_4$HCO$_3$

Reduction (DTT) / Alkylation (IAA)

Tryptic digestion

LC-MS/MS
LC-MS Pegvisomant Quantification in Rat Plasma

Blank

LLOQ (50 ng/mL)

ULOQ (50 µg/mL)

Intensity (cps)

Time (minutes)
**LC-MS Pegvisomant Quantification in Rat Plasma**

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<tr>
<th>Calibrant</th>
<th>Nominal Conc. (ng/mL)</th>
<th>Calculated Conc. (ng/mL)</th>
<th>Nominal Conc. (%)</th>
<th>CV* (%)</th>
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*n=3

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**What if I need more sensitivity and selectivity?**
Immunoprecipitation (IP)

PEGylated Protein

anti-Protein Antibody

anti-PEG Antibody
Immunoprecipitation (IP) + LCMS

PEGylated Protein

Wash, Elute and/or Digest

LC-MS
Automated IP Sample Processing

KingFisher® Flex: Magnetic Particle Processor
Hybrid Extraction vs Direct Digestion

Rat Whole Blood Spiked with Pegvisomant at 5 µg/mL

Blood Plasma Separation

anti-PEG IP  anti-hGH IP  Dilution

Reduction (DTT) / Alkylation (IAA)

Tryptic digestion

LC-MS/MS
Hybrid Extraction vs Direct Digestion

anti-PEG IP

\[ R^2 = 0.997 \]

anti-hGH IP

\[ R^2 = 0.986 \]

Direct Digest

\[ R^2 = 0.993 \]
**Hybrid Extraction vs Direct Digestion**

- **anti-PEG IP**
- **anti-hGH IP**
- **Direct Digest**

**Blank**

**LLOQ (50ng/mL)**

**Intensity (cps)**

**Time (min)**

Bioanalysis of PEGylated Proteins by LC-MS
Case study: PEGylated Peptide

38 amino acids

Region of interest

20-kDa PEG
PEGylated Peptide Extraction: Protein Precipitation

1. Protein Precipitation
2. Keep Supernatant
3. Evaporate Dry
4. Resuspend in Digestion Buffer
5. Digest
6. LC-MS
PEGylated Peptide Extraction: Protein Precipitation

Blank                                      LLOQ (0.50 ng/mL)                       ULOQ (50.0 ng/mL)

S/N=5.1
### PEGylated Peptide Extraction: Protein Precipitation + Immunopurification

1. **Protein Precipitation**
2. **Keep Supernatant**
3. **Evaporate Dry**
4. **Resuspend in IP Buffer**
5. **IP with anti-peptide antibody**
6. **On-bead digestion**
7. **LC-MS**
PEGylated Peptide Extraction:
Protein Precipitation + Immunopurification

S/N=15.3

Bioanalysis of PEGylated Proteins by LC-MS
### Bioanalysis of PEGylated Proteins by LC-MS

**Precision and Accuracy: Protein Precipitation + Immunopurification**

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Summary

PEGylated Protein Bioanalysis by LC-MS

PEG:
Use it or Avoid it

PEG IP
Protein Precipitation

Use it

Avoid it

Protein IP
Direct Digestion
Peptide Selection

Implications for Assay Validation?
Acknowledgements

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