# Bioanalytical Quantitation of Biotherapeutics Using Intact Protein vs. Proteolytic Peptides by LC-HR/AM on a Q Exactive MS

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# Overview

Quantitation of intact large molecules using high-resolution, accurate-mass spectrometry for biopharmaceutical applications is of increasing interest to bioanalytical scientists. These experiments use model molecules ranging from small proteins to a large therapeutic proteins in order to demonstrate the application of intact vs. peptide-based quantitation. The experiments include insulin quantitation at both the intact and peptide level and IgG1 light chain at the intact level. Calibration curves were prepared across three orders of dynamic range. For intact analysis, analyzed by full scan for *m/z* 800-6000 with resolutions 70,000 for insulin, and 140,000 for light chain. For peptide-based quantitation, peptide digests were analyzed by SIM at 70,000 resolution.

# Introduction

Quantitation of intact proteins is central for the protein-based biotherapeutic industry. Proteins may be present in multiple isoforms and fragments derived from variations in the genetic code, alternate splicing, processing events, post-translational modifications and catabolism. Because these can affect the protein's behavior, analytical methods capable of reliably and accurately identifying and measuring these variants is needed. High-Resolution, Accurate Mass (HR/AM) approaches to quantify intact proteins are of increasing interest. A high throughput method for the quantitation of biotherapeutics on intact proteins was developed on a Thermo Scientific™ Q Exactive™ mass spectrometer.

# Methods

**Sample Preparation**

For quantitation of intact insulin, the protein precipitation was performed by the addition of a 3:1 ratio of acetonitrile to rat plasma. The mixture was vortex mixed, flash frozen, thawed and centrifuged at 3000 rpm for 30 min. IgG1 light chain was produced by 10 mM DTT reduction of IgG1 at 37 °C for 30min. For peptide quantitation, insulins were reduced by 10 mM DTT and then digested by Trypsin at 37 °C overnight. Standard mAb was denatured by 8M Urea, followed by 10 mM DTT reduction, and then Trypsin digestion. Calibration solutions were prepared by serial dilution of stock solution.

**Liquid Chromatography**

Samples were analyzed on Thermo Scientific™ Dionex™ UltiMate™ XRS UHPLC pump and Thermo Scientific™ Accela™ Open autosampler interfaced with a Q Exactive Orbitrap™ MS with HESI source. Insulin was separated by 3-min gradient from 20% to 90% acetonitrile in water with 0.1% formic acid at flow rate of 500 µl/min on a PLRP-S (2.1x 50 mm) column heated to 30 ºC. Light chain of mAb was separated by 5-min gradient from 20 % to 90% acetonitrile in water with 0.1% formic acid at a flow rate of 500 µl/min on a PLRP-S (2.1x 50 mm) column heated to 70 ºC. For peptide analysis, insulin tryptic digests were separated by 5-min gradient from 10 % to 90% acetonitrile in water with 0.1% formic acid at flow rate of 50 µl/min on a Thermo Scientific™ Acclaim™ Pepmap™ 100 (1.0 x 50 mm) column; IgG1 tryptic digests were separated by 15-min gradient from 3 % to 90% acetonitrile.

**Mass Spectrometry**

Intact analysis of insulin and IgG1 light chain were performed in full scan mode from *m/z* 500 to 6000 at different resolutions for qualification. For quantitation, selected ion monitoring (SIM) was performed at resolution 70,000 and 140,000. Quantitation could be performed by summing several charge states and isotopes of analytes of interest.

**Data Analysis**

Data was acquired using Thermo Scientific™ Xcalibur™ 2.2 software. Quantitative and qualitative analysis of insulin was performed in Thermo Scientific™ LCQuan™ 2.7 and Thermo Scientific™ Protein Deconvolution™ 2.0 software, respectively.

# Results

**Qualitative Analysis of Insulin**

The isotopic-resolved full scan data (R=70,000) was deconvoluted in Protein Decovonlution 2.0 software using the Xtract algorithm. The monoisotopic mass of human insulin (*m/z* 5803.6397) are shown in Figure 1. The expanded table in Figure 2. demonstrated the result accuracy with Fit% >96.6% for their employed charge states.

**FIGURE 1. Charge Distribution of Human Insulin on Q Exactive MS.**

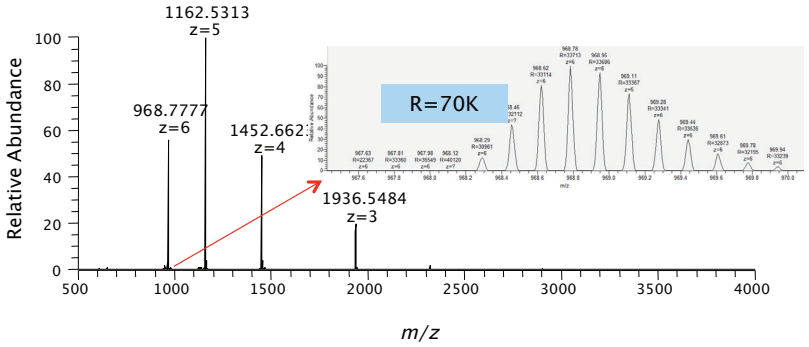
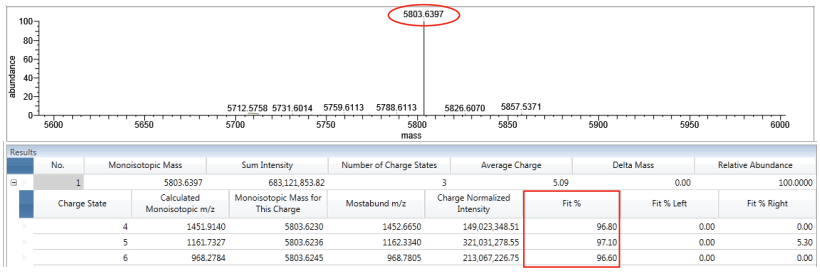



**FIGURE 2. Deconvoluted Spectra and Monoisotopic Mass of Human Insulin.**




| No. | Monoisotopic Mass | Sum Intensity | Number of Charge States | Average Charge | Delta Mass | Relative Abundance |
|---|---|---|---|---|---|---|
| 1 | 5803.6397 | 683,121,853.82 | 3 | 5.09 | 0.00 | 100.0000 |

| Charge State | Calculated Monoisotopic m/z | Monoisotopic Mass for This Charge | Mostabund m/z | Charge Normalized Intensity | Fit % | Fit % Left | Fit % Right |
|---|---|---|---|---|---|---|---|
| 4 | 1451.9140 | 5803.6230 | 1452.6650 | 149,023,348.51 | 96.80 | 0.00 | 0.00 |
| 5 | 1161.7327 | 5803.6236 | 1162.3340 | 321,031,278.55 | 97.10 | 0.00 | 5.30 |
| 6 | 968.2784 | 5803.6245 | 968.7805 | 213,067,226.75 | 96.60 | 0.00 | 0.00 |

**Quantitation of Insulin in Rat Plasma on Q Exactive MS.**

To reduce the insulin absorption on sample vials and the LC–MS system, depleted rat plasma was applied to give the least absorption. Insulin was quantified by summing isotopes of two most abundant charge states $[M+5H]^{5+}$ and $[M+6H]^{6+}$. Extracted ion chromatograms are shown in Figure 3.

The calibration curve and quantitation results are shown in Figure 4. Human insulin results indicate good linearity with a calibration range of 10 ng/mL to 200 ug/mL with an LOD of less than 1 pg on column. For insulin tryptic peptide, the LLOQ is 0.02 ng/ml with linearity over five orders of magnitude (Figure 5). The LOD for insulin tryptic peptide mixture is 0.125 pg on column.

**FIGURE 3. XICs of Human Insulin on Charge 5+ and 6+ in 5 ppm Mass Tolerance Window at Different Concentration.**

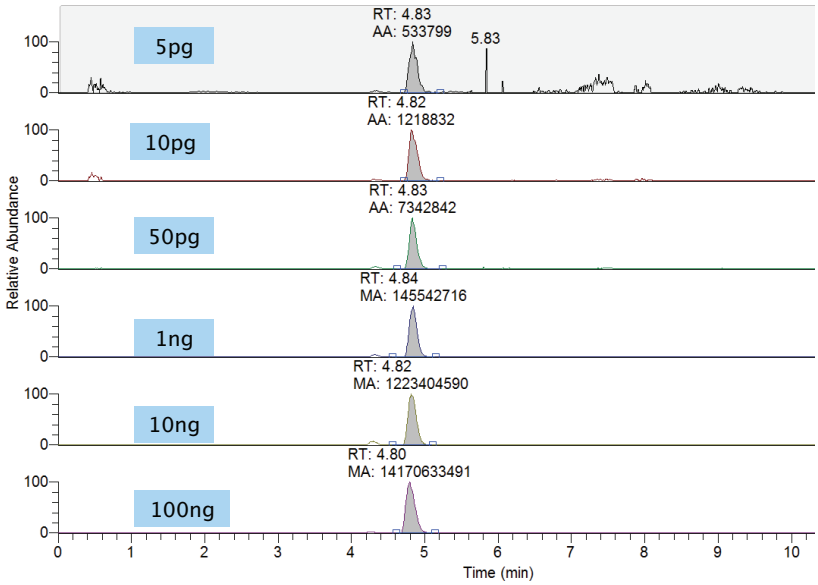


**FIGURE 4. Calibration Curve of Human Insulin in Rat Plasma by Full Scan Method.**

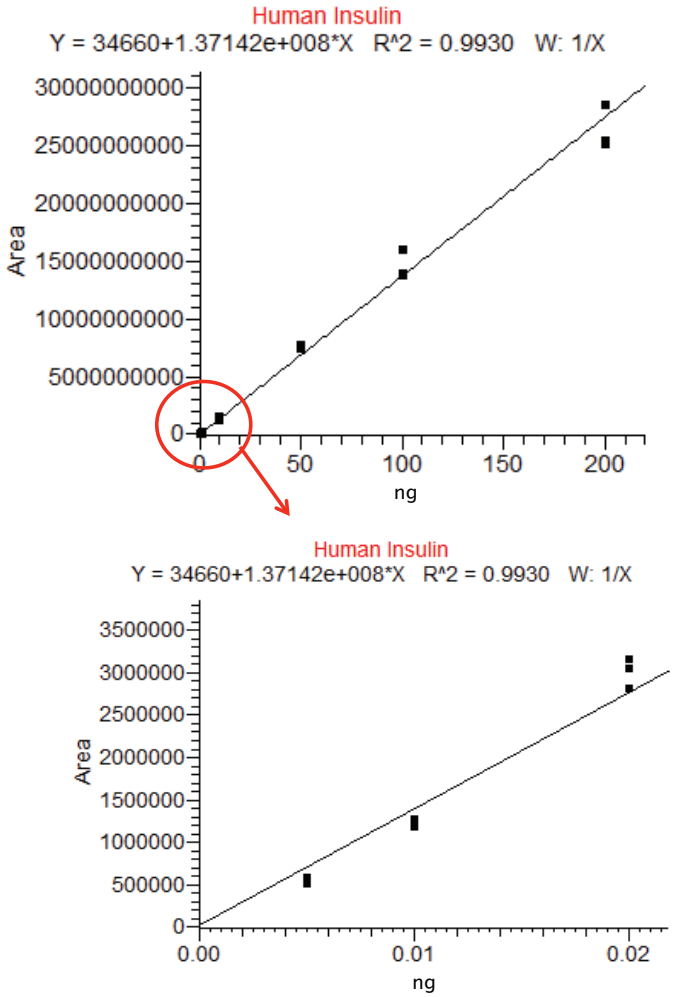
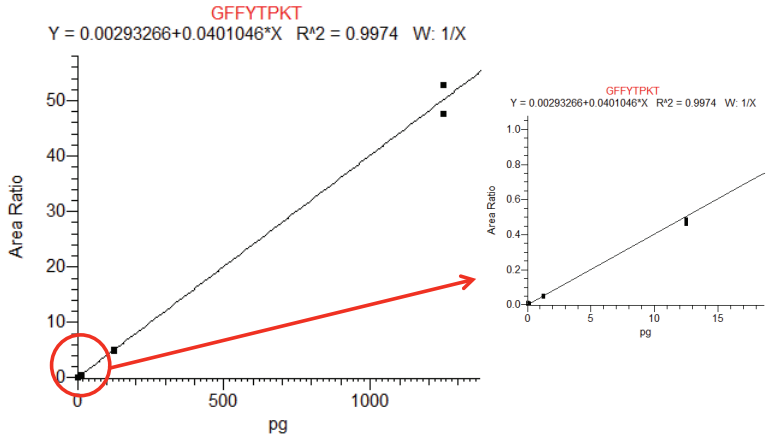


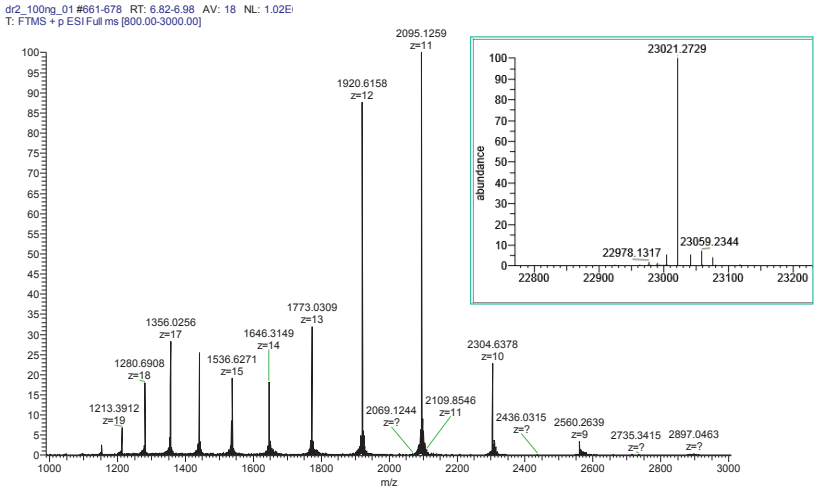

**FIGURE 5. Calibration Curve of Insulin Tryptic Peptide, 0.125–1250 pg*.**



### Qualitative Analysis of IgG Light Chain

The isotopically resolved full scan data (R=140,000) was deconvoluted in Protein Decovonlution 2.0 software using the Xtract algorithm. The monoisotopic mass of IgG light chain (*23021.2729*) is shown in Figure 6.

**FIGURE 6. Mass Spectrum of Ab Light Chain at 140,000 Resolution.**




### Quantitation of IgG Light Chain on Q Exactive MS

IgG light chain was quantified by summing isotopes of most abundant charge states $[M+10H]^{10+}$ and $[M+18H]^{18+}$. Extracted ion chromatograms are shown in Figure 7. The extraction mass tolerance window is 5 ppm.

The calibration curve and quantitation results are shown in Figure 8. IgG light chain results indicate good linearity with a calibration range of 5 ug/mL to 500 µg/mL with an LOD of less than 10 ng on column. For IgG light chain tryptic peptide, the LLOQ is 5 ng/ml , with quantitation linearity over three orders of magnitude (5 ng/mL to 5 µg/mL). LOD for insulin tryptic peptide mixture is 5 pg on column.

**FIGURE 7. XIC of Ab Light Chain($[M+10H]^{10+}$ to $[M+18H]^{18+}$) Within 5 ppm Mass Tolerance Window.**

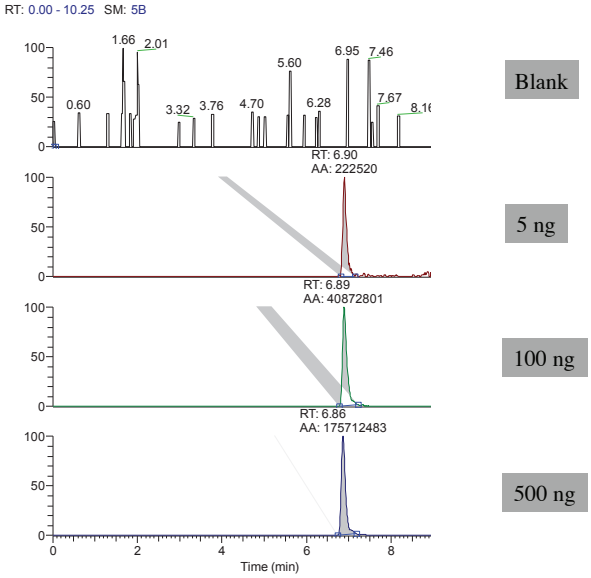


**FIGURE 8. Quantitation Curve of Light Chain 5–500 ng.**

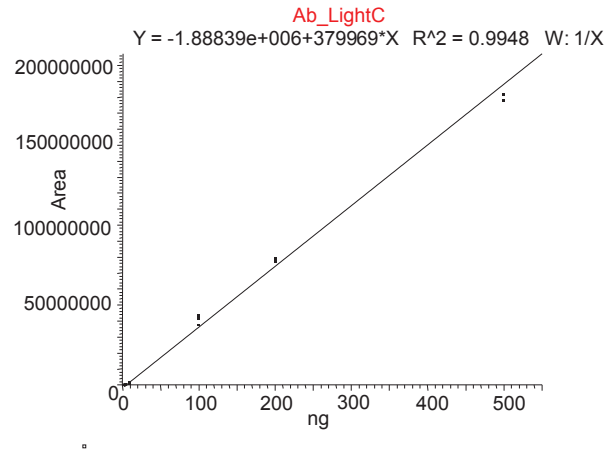
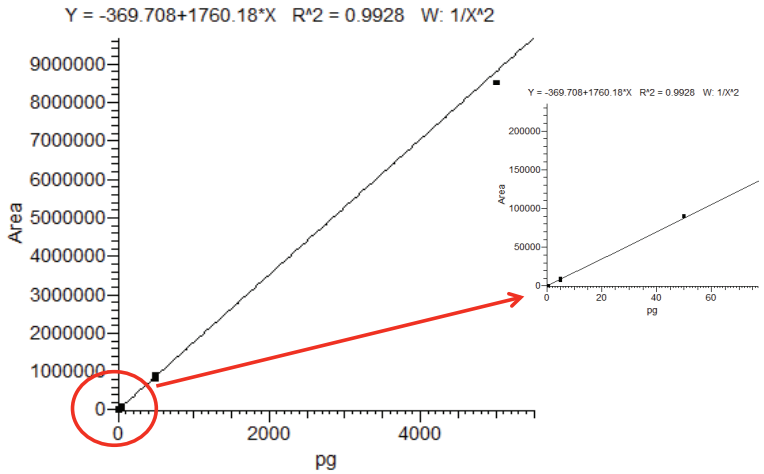



**FIGURE 9. Calibration Curve of IgG1 Light Chain Tryptic Peptide 5–5000 pg*.**




*: Loading amount here is the total digest amount.

# Conclusion

1. Human insulin and IgG1 light chain were identified by high resolution accurate mass data with charge distribution.
2. Human insulin and IgG1 light chain were quantified in full scan positive ion mode by LC–HR/AM without compound tuning and nearly zero method development.
3. The LOD for human insulin intact quantitation was below 1 pg on column. Quantitation results indicates good linearity with a calibration range of 10 ng/mL to 200 µg/mL. The accuracy within 25% and precision <15% at the LLOQ. The LOD for human insulin peptide-based quantitation was 0.125 pg on column, with good linearity from 25 pg/mL to 250 ng/mL. The accuracy is within 20% and precision <15% at the LLOQ.
4. IgG1 light chain (23 KDa) was quantified in full scan mode at resolution 140,000 with a 5-min gradient. The LOD was 5 ng on column with good linearity from 5 µg/mL to 500 µg/mL. The LOD for light chain peptide-based quantitation was 5 pg on column with good linearity from 5 ng/mL to 5 µg/mL. The accuracy for both methods is within 25% and precision <15% at the LLOQ.
5. For future work, different HPLC flow rates will be investigated to increase detection sensitivity of IgG1 light chain.

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