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Absolute Quantitation of Biotherapeutic Drug Product and Endogenous Protein in Human Serum Using a Hybrid Immunoassay-LC/MS Approach

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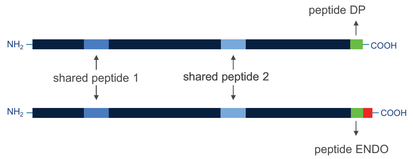
PURPOSE

- PK analysis of a biotherapeutic drug product (DP) designed to mimic an endogenous protein (ENDO). The DP and ENDO have identical sequences except the DP has a 3 AA truncation at the C-term.
- Immunoassay is unable to distinguish the DP from the ENDO due to insufficient antibody selectivity.
- A mass spectrometry-based assay was developed which could distinguish between the DP and ENDO proteins to provide independent measurements for each target. The assay used a single-antibody pulldown of both proteins and enzymatic digestion to peptides, followed by targeted LC-MS of multiple peptides (Figure 1) within the same analytical run.

Figure 1. Peptides targeted by the LC-MS assay are shown: Shared peptides 1 and 2 common to both proteins, and the unique C-term peptide of each protein.



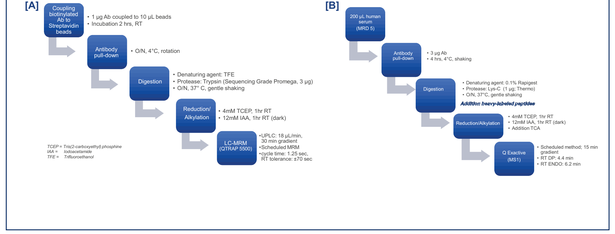
- Use of the DP and ENDO C-term peptides to obtain the required assay specificity presented various challenges to the LC/MS analysis:
  - [1] Digestion / Non-specific proteolysis causing conversion of the ENDO peptide to the DP peptide
  - [2] Sensitivity /Poor peak shape
  - [3] Linearity
- Extensive assay optimization and troubleshooting was performed to achieve the required level of assay performance in serum.
- Assay qualification was performed for the primary intended use: measurement of DP in dosed serum, and the secondary intended use for simultaneous measurement of the endogenous protein.

METHODS

ASSAY DEVELOPMENT AND OPTIMIZATION

Immunoaffinity pulldown conditions were developed using test samples generated by spiking ENDO and DP proteins in serum. Pulldown was performed using antibody-conjugated beads which recognized both target proteins. Captured proteins were enzymatically digested to generate peptides for mass spectrometry analysis. An LC-MRM/MS assay was developed to monitor the target peptides (two shared peptides and the unique C-term peptide from each target), followed by a preliminary assessment of the assay performance. Testing using the initial assay conditions (Figure 2A) raised several challenges due to the requisite use of the C-term peptides. Extensive optimization was performed (see RESULTS) to obtain the final assay method (Figure 2B) which provided the required performance for the intended uses.

Figure 2 [A] Initial assay conditions and [B] Final assay method after extensive optimization.



RESULTS

DIGESTION

Figure 3. Initial testing showed non-specific proteolysis and peptide conversion from ENDO to DP.

[A] Test samples generated by spiking pooled human serum with either protein ENDO or protein DP at a range of concentrations, were analyzed using the initial assay method. **[B]** In Sample 1 with spiked protein ENDO, the peptide ENDO showed a concentration-dependent response for all monitored transitions, while the peptide DP was unexpectedly detected in a concentration-dependent manner. *Data not shown*: Peptide DP was levels at ~30% of the spiked ENDO protein level across all ENDO concentrations and transitions. **[C]** For Sample 2 with spiked DP, the DP peptide showed a concentration-dependent response for all transitions, while the peptide ENDO was not detected, as expected. *Data not shown*: The pooled serum used as spiking matrix was selected based on having undetectable levels of endogenous ENDO protein.

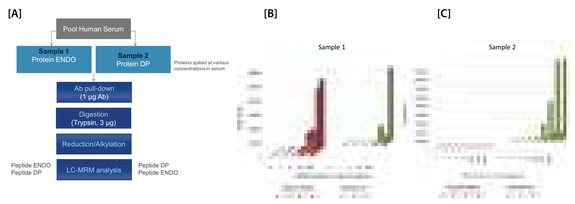


Figure 4. Testing of different trypsin enzyme amounts and impact on the peptide conversion activity.

Trypsin may exhibit autolysis and chymotrypsin-like activity under certain conditions. Chymotrypsin activity was hypothesized to cause the peptide conversion from ENDO to DP. **[A]** Trypsin was tested at lower concentrations, and the amount of antibody for pulldown was also increased to improve assay linearity on the higher end. **[B]** For Sample 1 spiked with protein ENDO, reducing trypsin resulted in a moderate, non-linear effect on signal intensity. *Data not shown*: A decrease in peptide DP levels was observed with reduced trypsin levels. **[C]** Sample 2 with spiked protein DP showed expected levels of peptide DP.

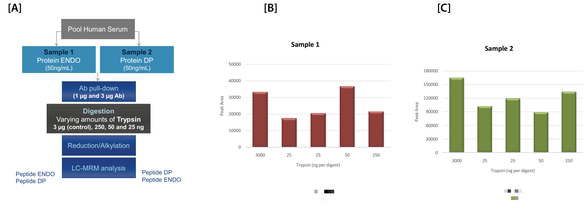
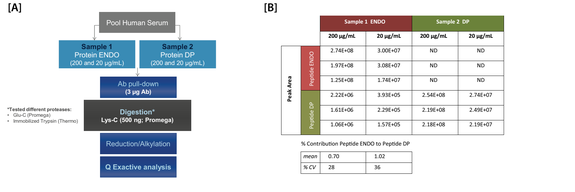


Figure 5. Testing of different proteases to minimize peptide conversion activity.

[A] To assess if the peptide conversion could be further minimized or eliminated through use of proteases other than trypsin, various enzymes were tested. **[B]** Use of 500ng Lys-C for digestion produced peptide ENDO and peptide DP for analysis, with a conversion rate of ~1%.



[B]

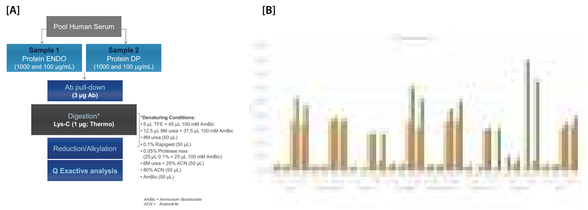
		Sample 1 ENDO		Sample 2 DP	
		200 µg/mL	20 µg/mL	200 µg/mL	20 µg/mL
Peak Area	Peptide ENDO	2.74E+08	3.00E+07	ND	ND
		1.97E+08	3.08E+07	ND	ND
		1.25E+08	1.74E+07	ND	ND
	Peptide DP	2.22E+06	3.93E+05	2.54E+08	2.74E+07
		1.61E+06	2.29E+05	2.19E+08	2.49E+07
		1.06E+06	1.57E+05	2.18E+08	2.19E+07

% Contribution Peptide ENDO to Peptide DP

mean	0.70	1.02
% CV	28	36

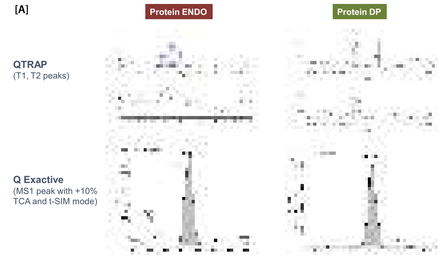
Figure 6. Optimization of Lys-C digestion protocol

[A] To determine the Lys-C digestion conditions with the best signal intensity and LOD for the ENDO and DP peptides, digestion was performed under various denaturing conditions. **[B]** Although Rapigest provided only slightly better signal intensity versus the TFE-based control protocol, the risk of evaporation and processing variability due to the small TFE volumes used, led to the selection of Rapigest for the final protocol, which also provided ease of use, good reproducibility and good signal intensity.



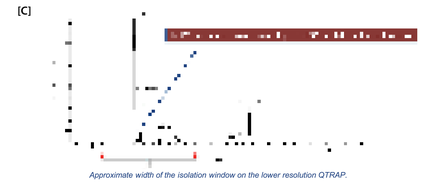
SENSITIVITY

Figure 7. Optimization of peak shape and LC-MS conditions to meet the target sensitivity of 1 ng/mL [A] Upper panel: Initial LC/MS analysis using MRM on a nanoAcquity UPLC (Waters) coupled to a QTRAP 6500 (AB Sciex) showed both poor peak shape for the ENDO peptide and low sensitivity. [B] To improve signal-to-noise and thus sensitivity, the assay was transferred to a high-resolution accurate mass (HRAM) Q-Exactive series mass spectrometer, which allows for analysis using either parallel reaction monitoring (PRM) or targeted Selected Ion Monitoring (t-SIM) mode. [C] Analysis of the MS1 peak using t-SIM mode provided better sensitivity than PRM or MRM, and the high resolving power of the instrument provided sufficient peak selectivity. [A] Lower panel: To improve the peak shape of the ENDO tryptic- and LysC- peptides, TCA was added to the samples as an ionic modifier, with a noted improvement in peak shape.



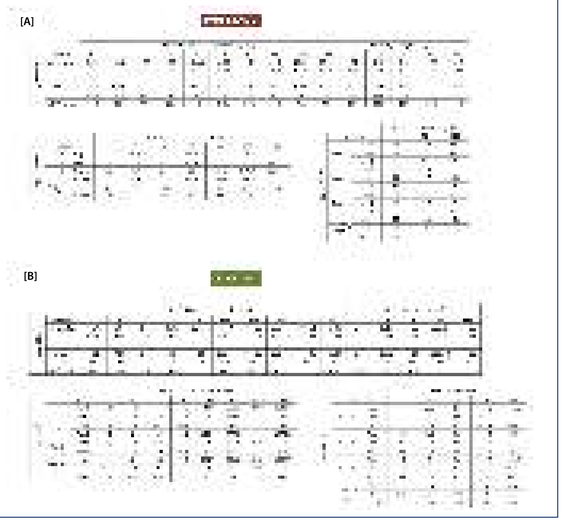
[B]

Instrument	Resolution	Scan Mode	Fragmentation
QTRAP 5500/6500	low	MRM	poor
Q Exactive	High (HRAM)	Parallel reaction monitoring (PRM)	poor
Q Exactive	High (HRAM)	Targeted Selected Ion Monitoring (t-SIM)	N/A



LINEARITY

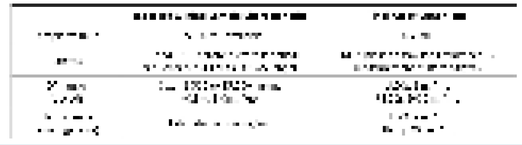
Table 1. Optimization of immunoaffinity pulldown. Initial conditions using 1 µg showed saturation at the upper range of the calibration curves and QC samples. Using different antibody amounts and incubation conditions. For **[A]** Protein ENDO and **[B]** Protein DP, the upper panels show the initial where use of sample results using 1 µg antibody. Lower panels show inter-assay precision and accuracy of calibration standards and QC samples using final assay conditions with 3 µg antibody improved the linearity of the upper ranges.



CONCLUSION

Whereas immunoassay provides insufficient antibody selectivity to distinguish between DP and ENDO forms, and provides a sensitivity of 1ng/mL for the combined measurement of DP and endogenous protein, this study shows the development and optimization of a hybrid immunoassay-LC/MS for independent measurement of DP and ENDO proteins within the same assay run, with similar assay sensitivity to immunoassay. The final assay characteristics versus the expected clinical sample characteristics are shown in Table 2.

Table 2. Expected clinical sample characteristics versus final assay characteristics.



Assay qualification was performed for the analysis of dosed clinical samples. The intended primary use was for DP measurement in serum at a high range of concentrations, while the secondary use was for ENDO measurement at the expected lower endogenous levels.