Bioanalytical LC-MS/MS Method Development for Proteins without Sample Clean-up or Enrichment

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Purpose

A bioanalytical method development strategy is presented for quantitative protein analysis by LC-MS/MS when no capture antibody is available for bioanalytical sample clean-up or enrichment. Native human insulin was chosen as a model for generation of data at therapeutic concentrations.

Methods

For all experiments, a Perfinity Workstation was used for on-line sample preparation. MS data were acquired using a Thermo LTQ Velos ion trap mass spectrometer. Thermo Proteome Discoverer was used for peptide mapping and selecting surrogate peptides for SRM analysis. The 18 minute sample run time included a 4 minute tryptic digest at 50 C, desalting of the protein digest, and elution with a reverse-phase LC gradient of 10-70% mobile phase B in 5 minutes. The surrogate peptide selected for both insulin and internal standard contains the sequence GFFYTPK. MRM transitions were m/z 859.4 → 616.3+841.4 for insulin and m/z 869.5 → 626.4+851.5 for the isotope-labeled internal standard, Ac-LVCGERGF[F-^{15}N,$^{13}C_9$]YTPKT-OH.

Sample Preparation - Quantitative Screen

Reduced and alkylated insulin in a protein-based proxy matrix (10-10,000 ng/mL) demonstrated viable LC-MS/MS method performance when both an immunocapture step and internal standard were unavailable.

Sample Preparation - Quantitation in Biological Matrix

For preliminary work, native insulin spiked into K_2EDTA plasma (500-10,000 ng/mL) was diluted 10-fold in 2M Urea containing IS and centrifuged. 5 µL of the supernatant was injected.

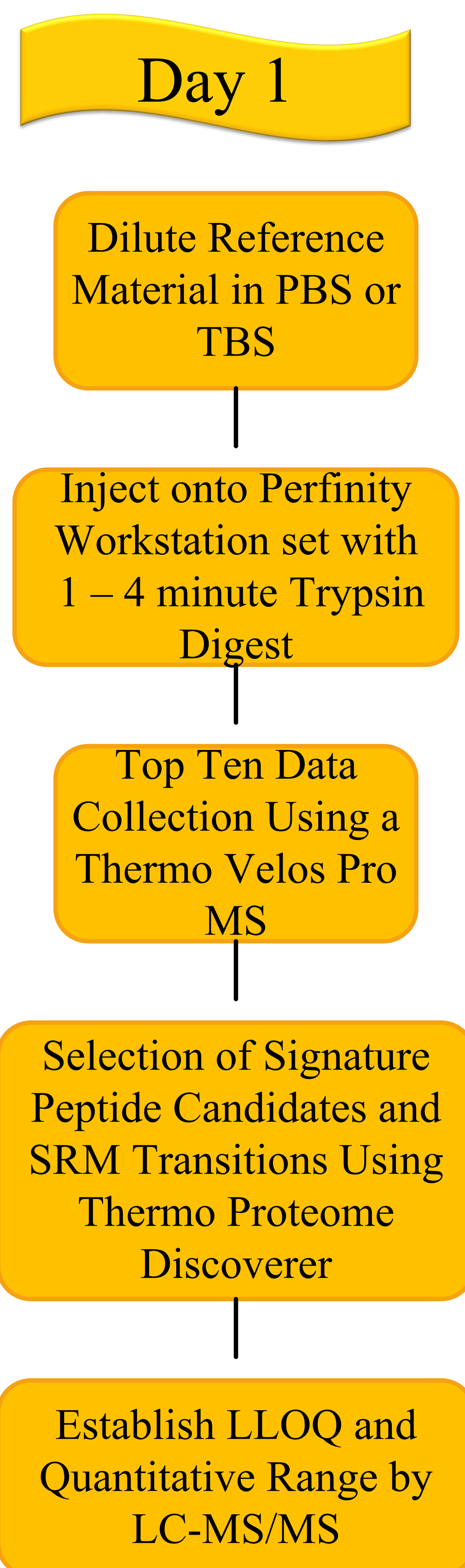
Sample Preparation and Instrumental Analysis in Serum

Method performance was evaluated using three analytical runs. Native insulin was spiked into human serum, filtered to 0.2 µm, and diluted 10-fold in 2M urea containing IS. Sample injection volume was 5 µL. Validation sample levels at the LLOQ, Low, Middle, and High portion of the quantitative range were used to evaluate both intra- and inter-assay accuracy and precision. Data sets were imported into Watson LIMS and regressed using a quadratic curve fit with $1/X^2$ weighting. Applied acceptance criteria included a %bias within 20.0% (LLOQ, 25.0%) and CV ≤ 20.0% (LLOQ, ≤ 25.0%).

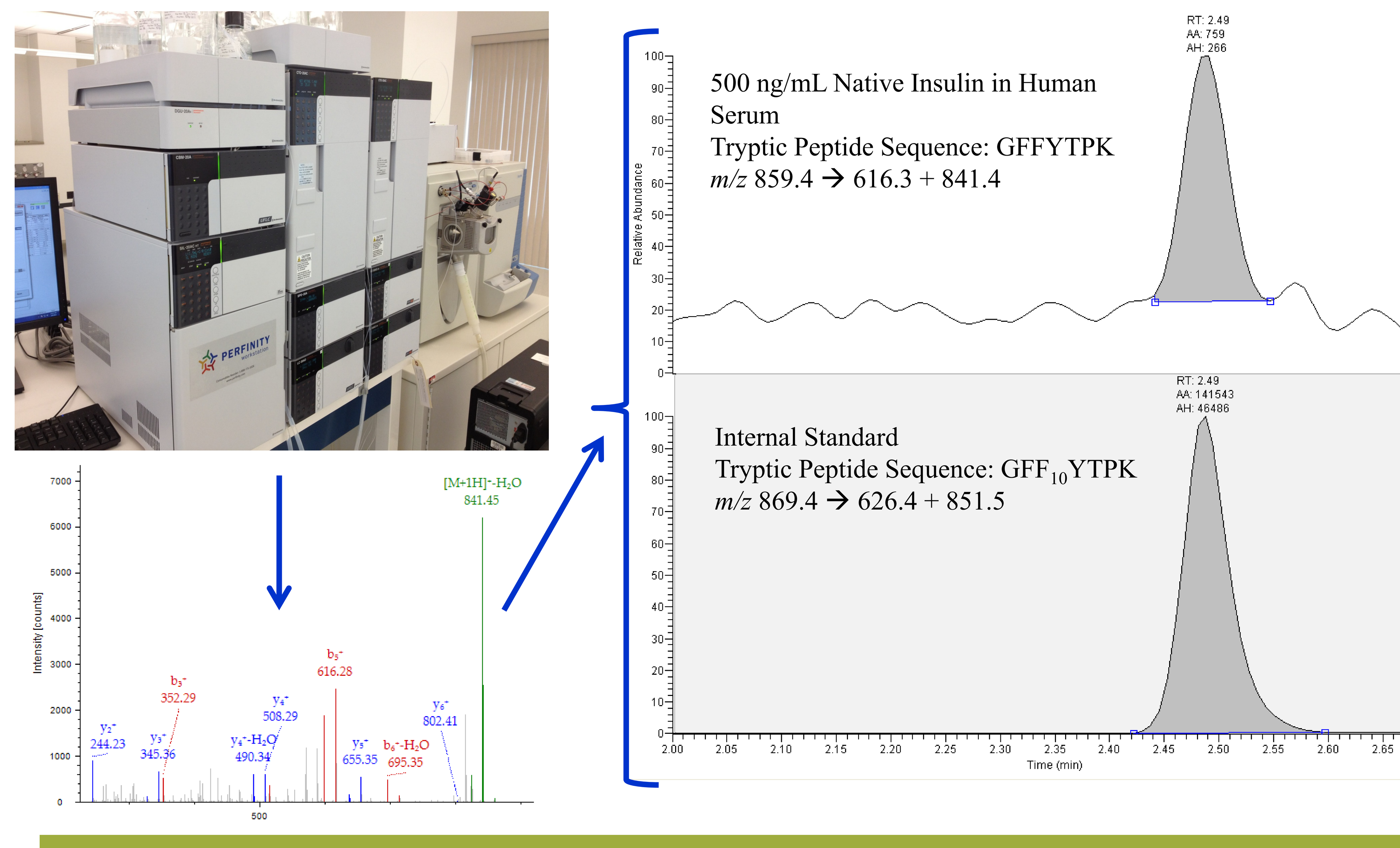
Instrumentation

Perfinity Workstation (Automated Protein Sample Preparation)
- Perfinity Digest Column (4.6 X 30 mm)
- Perfinity Desalting Column (1 X 20 mm)
- Halo Peptide ES-C18 RP Column (2.1 X 100 mm, 2.7 µm)
- Mobile Phase A – 2% Acetonitrile/98% H_2O/0.1% Formic Acid
- Mobile Phase B – 90% Acetonitrile/10% H_2O/0.1% Formic Acid

Thermo Scientific LTQ Velos Ion Trap Mass Spectrometer
- Positive Ion Electrospray

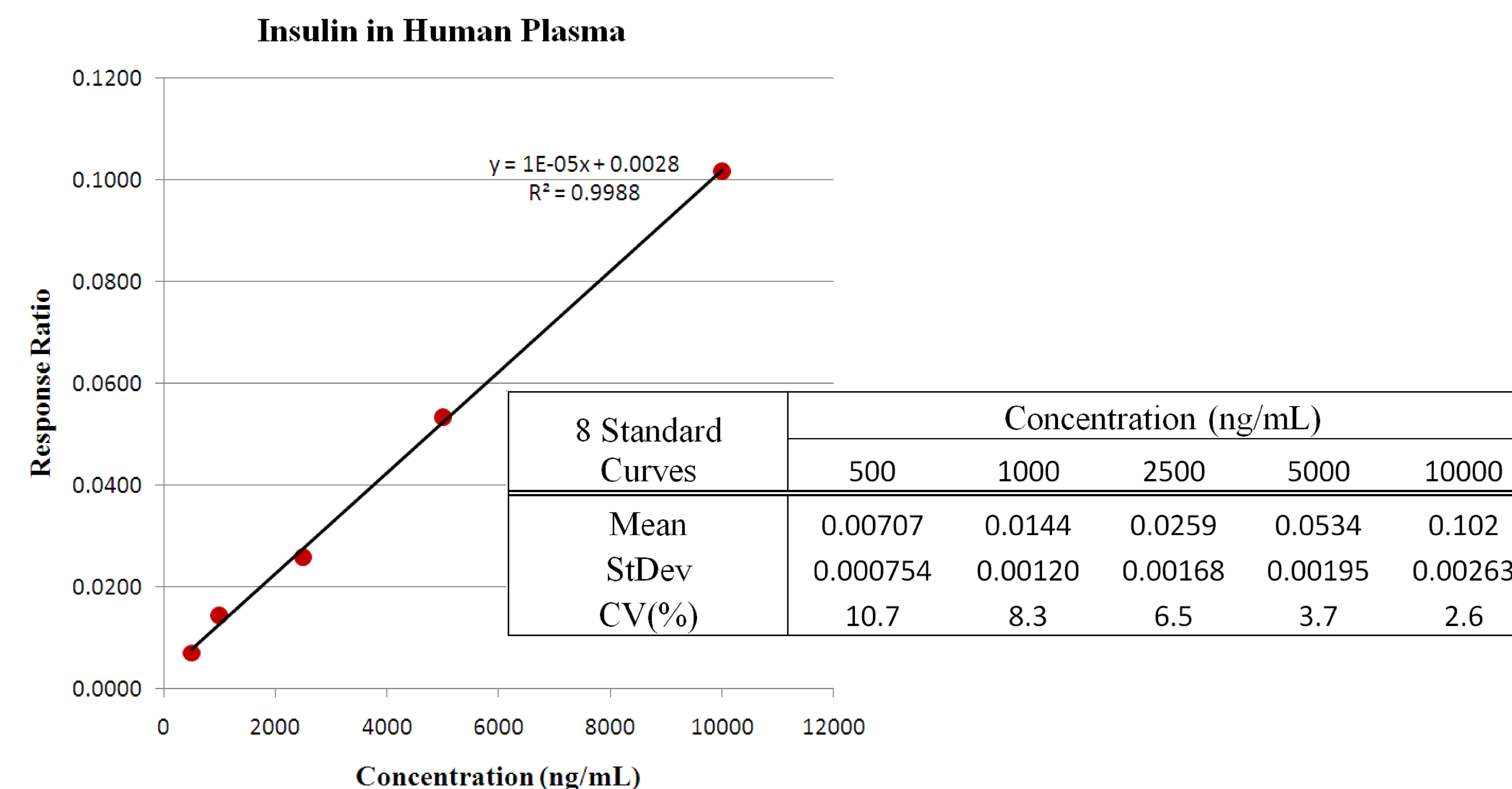


Methods (Cont.)



Results

Without internal standard and immunocapture, the CV values for reduced and alkylated insulin in proxy matrix ranged from 19.6% at the LLOQ to 1.7% at the ULOQ (data not shown). With internal standard but no immunocapture step, eight calibration curves of native insulin in plasma yielded CV values ranging from 2.6% to 10.7% with a linear fit (R^2 = 0.999).



8 Standard Curves	Concentration (ng/mL)				
	500	1000	2500	5000	10000
Mean	0.00707	0.0144	0.0259	0.0534	0.102
StDev	0.000754	0.00120	0.00168	0.00195	0.00263
CV(%)	10.7	8.3	6.5	3.7	2.6

Results (Cont.)

Method performance for quantitation of native insulin in human serum was evaluated using three analytical runs over three days in the presence of internal standard. The range observed for intra-assay %bias at each validation sample level was -15.4% to 12.2% (LLOQ), -4.0% to 5.6% (Low), -1.8% to 5.2% (Middle), and -3.7% to 3.6% (High). The intra-assay %CV was ≤ 14.9% for all VS levels. The inter-assay %bias for standard calibrators ranged from -5.1% to 5% while CV values ranged from 9.4% (LLOQ) to 3.9%. The inter-assay %bias observed for the validation samples ranged from 1.4% (LLOQ) to 0.8%, and CV values ranged from 16.6% (LLOQ) to 4.7%.

The % total error ranged from 18.0% (LLOQ) to 6.1% and indicates that this LC-MS/MS method would perform optimally when applying either traditional macromolecule or small molecule acceptance criteria.

Run Number	500 (ng/mL)	1000 (ng/mL)	1500 (ng/mL)	2000 (ng/mL)	3000 (ng/mL)	4000 (ng/mL)	6000 (ng/mL)	8000 (ng/mL)	10000 (ng/mL)
1	490	1140	1670	2260	3240	4060	6200	*14000	10900
	481	987	1340	1760	2800	3710	5600	6890	10400
2	484	1100	1480	2030	3100	4050	6060	7700	10700
	487	1040	1490	2080	2730	3590	6010	7970	9880
3	569	1000	1510	1980	3120	3840	6280	8460	10700
	425	1040	1450	1970	3220	3740	5980	6950	9730
Mean	489	1050	1490	2010	3040	3830	6020	7590	10400
S.D.	46	58.8	107	163	217	191	237	673	479
%CV	9.4	5.6	7.2	8.1	7.1	5	3.9	8.9	4.6
%Bias	-2.2	5	-0.7	0.5	1.3	-4.3	0.3	-5.1	4
N	6	6	6	6	6	6	6	5	6

** Abnormal Internal Standard Response*

Results from Three Analytical Runs	LLOQ (500 ng/mL)	Low (1250 ng/mL)	Mid (5000 ng/mL)	High (7500 ng/mL)
Mean Concentration	507	1260	5070	7580
Inter-run SD	84.3	82.7	238	406
Inter-run %CV	16.6	6.6	4.7	5.4
Inter-run %Bias	1.4	0.8	1.4	1.1
Inter-run %Total Error	18	7.4	6.1	6.4
n	18	18	18	18

Injecting larger sample volumes was attempted in order to lower the limit of quantitation. However, sample injection volume was limited by the quantity of proteinaceous material that could be loaded onto the desalting column during individual injections. It was determined that up to approximately 35-50 µg of protein on-column can be tolerated without causing a significant increase in back pressure or decreased column life. Therefore, an injection volume of 5-10 µL of plasma or serum diluted 10-fold with a non-protein based diluent was found to be optimal.

Conclusion

A robust quantitative LC-MS/MS method for protein therapeutics can be quickly developed and performed in less than a day without an immunocapture step for sample clean-up and/or analyte enrichment. Excellent accuracy and precision can be obtained without overnight digestion or multi-day sample preparation. This method development strategy is especially useful for early discovery screening of biological therapeutic candidates, drug safety studies or high-abundance biomarkers when no capture antibody is available.