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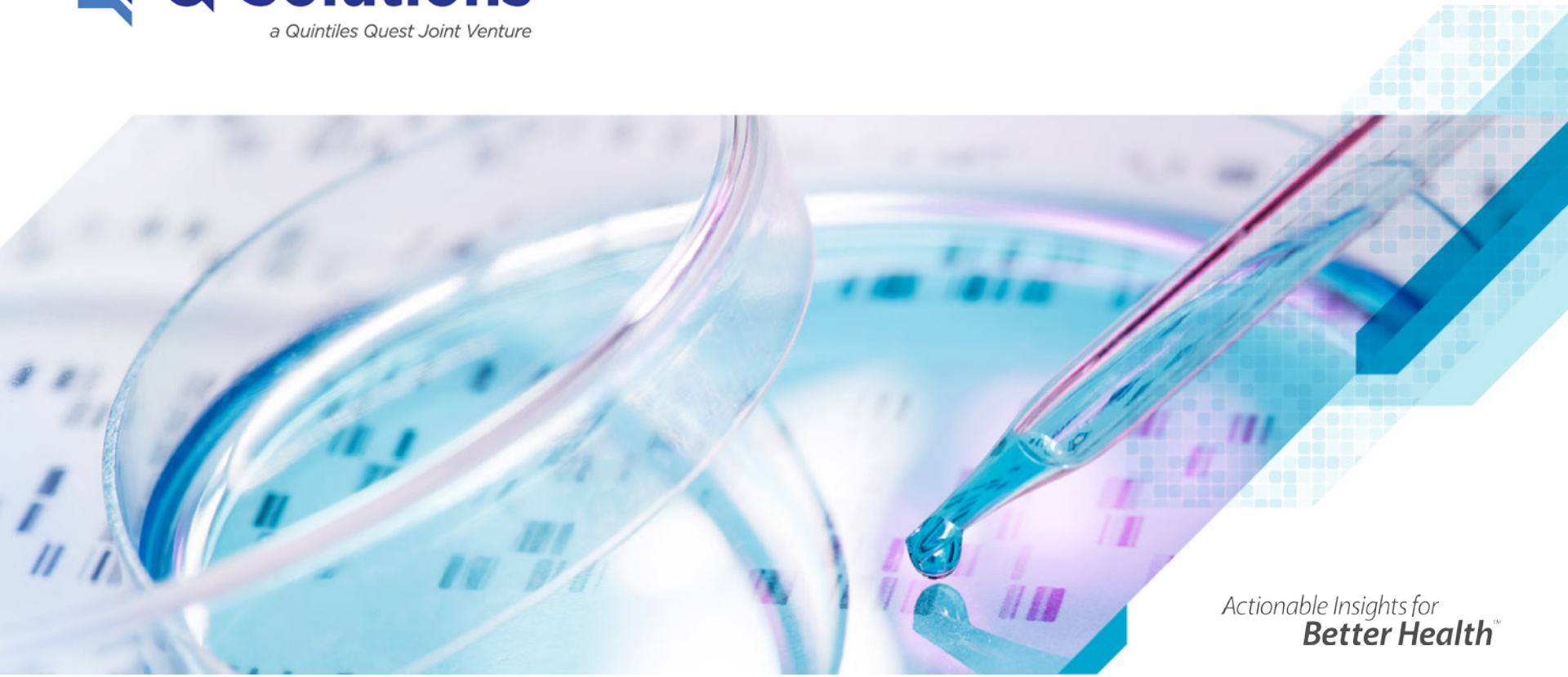

Revisiting the AAPS Recommendations Paper on Validating LC-MS Bioanalytical Methods for Protein Therapeutics: 3 Years of Progress

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Introduction

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White Paper

Recommendations for Validation of LC-MS/MS Bioanalytical Methods for Protein Biotherapeutics

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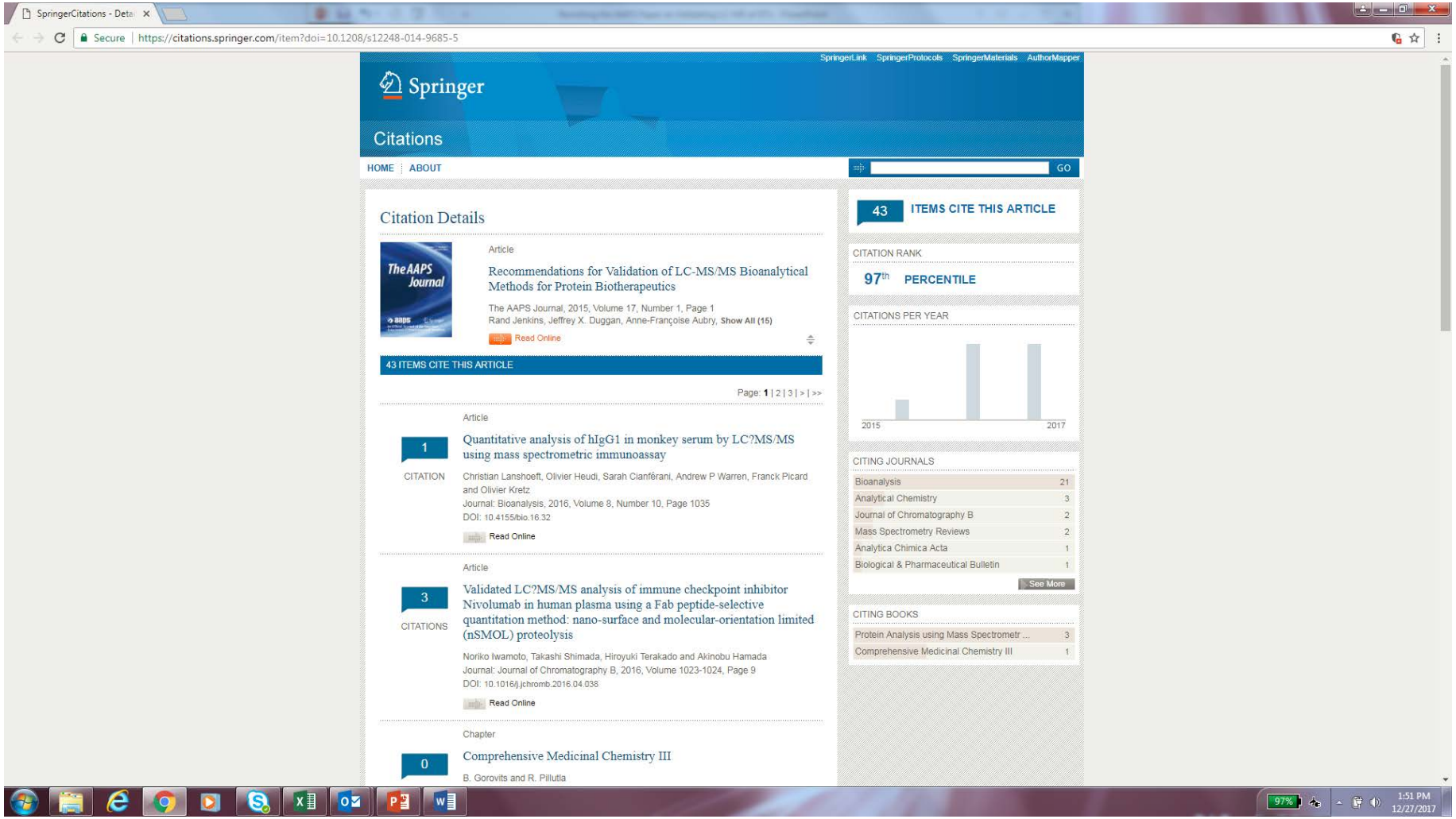
- Concept of the original paper:
  - Consensus view of status of applying LC-MS techniques to bioanalysis of protein therapeutics in support of regulated studies
  - Coordinated and authored by the Protein LC-MS Bioanalysis group - a subteam of the AAPS Bioanalytical Focus Group (BFG)
  - Input from others (EBF)

Introduction

- Key Points of Paper
  1. LC-MS can address some limitations of ligand binding assay (LBA) approaches
  2. Scope was limited to proteolytic digestion and measurement of resulting surrogate/signature peptide(s) a.k.a. PrD-LC-MS assays using triple quadrupole MS/MS instruments
  3. The principles of both small molecule drug (LC-MS) and large molecule (LBA) bioanalytical method validation apply to protein therapeutic LC-MS assays
  4. In general, ligand binding assay performance criteria may be more appropriate
- Caveats and Concerns
  - The development and validation of the assay needs to consider the intended application e.g. potential sample matrix interferences, correlation of the detected species to the intended analyte
  - Reference standard quality and COA
  - Recovery of analyte and digestion efficiency
  - Choice of internal standard(s)
  - Stability assessments
  - Regulatory authority input

Frequently Cited Paper



Key Follow Up Points Since January 2015 Publish Date

- Application of LC-MS across the biotherapeutic development spectrum as a complementary approach to LBA techniques
- Application to protein biomarker assays
- Developments in coupling immunoaffinity purification
  - Including multiple dimensions of immunocapture
- Tailoring validation strategy to specific aspects of the assay and the application intended
- Addressing potentially interfering ligands and matrix components
- The protein inference problem i.e. do peptide concentrations resulting from proteolytic digests accurately predict the parent protein quantitation?
- Intact analyte LC-MS quantitation is gaining attention
- Emerging interest of the regulatory authorities

Discovery to Clinical Trials

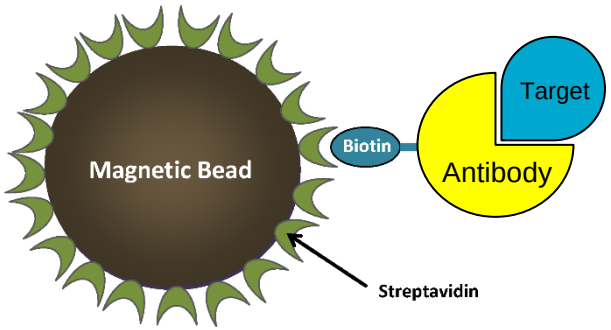
- Complementary approaches of LBA and LC-MS
  - Rapid method development of LC-MS assays without reliance on customized critical reagents for early (discovery stage) evaluations
    - Y. Zhang, T. Olah, J. Zeng, Bioanalysis 6(13), p1827-1841 (2014)
  - Generic/universal LC-MS methods for therapeutic mAb evaluation in non-clinical testing
    - J. Lee, Bioanalysis 8(1), p19-27 (2016)
  - Corroboration with LBA bioanalytical data
  - Building in LC-MS sensitivity with immunoaffinity purification = hybrid IA/LC-MS
    - J. Duggan, B. Ren, Y Mao, et al., Bioanalysis 8(18), p1951-1964 (2016)
  - Techniques have been adopted across the bioanalytical community - including CROs
    - J. Jones, G. Schultz, Bioanalysis 8(15), p1545-1549 (2016)
  - Has even reached the status of “plug-and-play” reputation with some laboratories
    - S. Kaur, L Liu, D Cortes, et al., Bioanalysis 8(15), p1565-1577 (2016)
- One notable trend:
  - The drive to understanding and improving assay selectivity/specificity

Predominance of Hybrid IA/LC-MS Assays

- Strengths
  - Selectivity
  - Sensitivity
    - Pre-concentration
    - Selectivity
  - Assay robustness

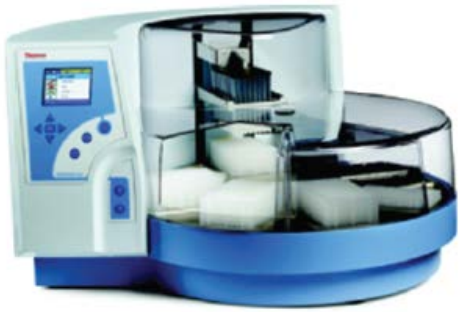
- Challenges
  - Critical reagent
    - Time
    - Cost
    - Reproducibility
    - Adds complexity to method validation
  - Combined expertise (LBA and LC-MS) is needed

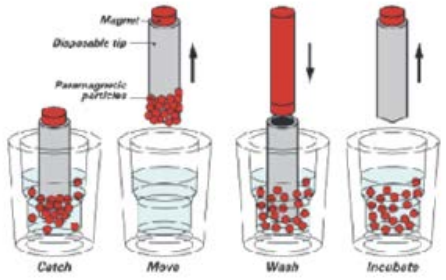
Coupling Immunoaffinity to LC-MS (pre-digestion)



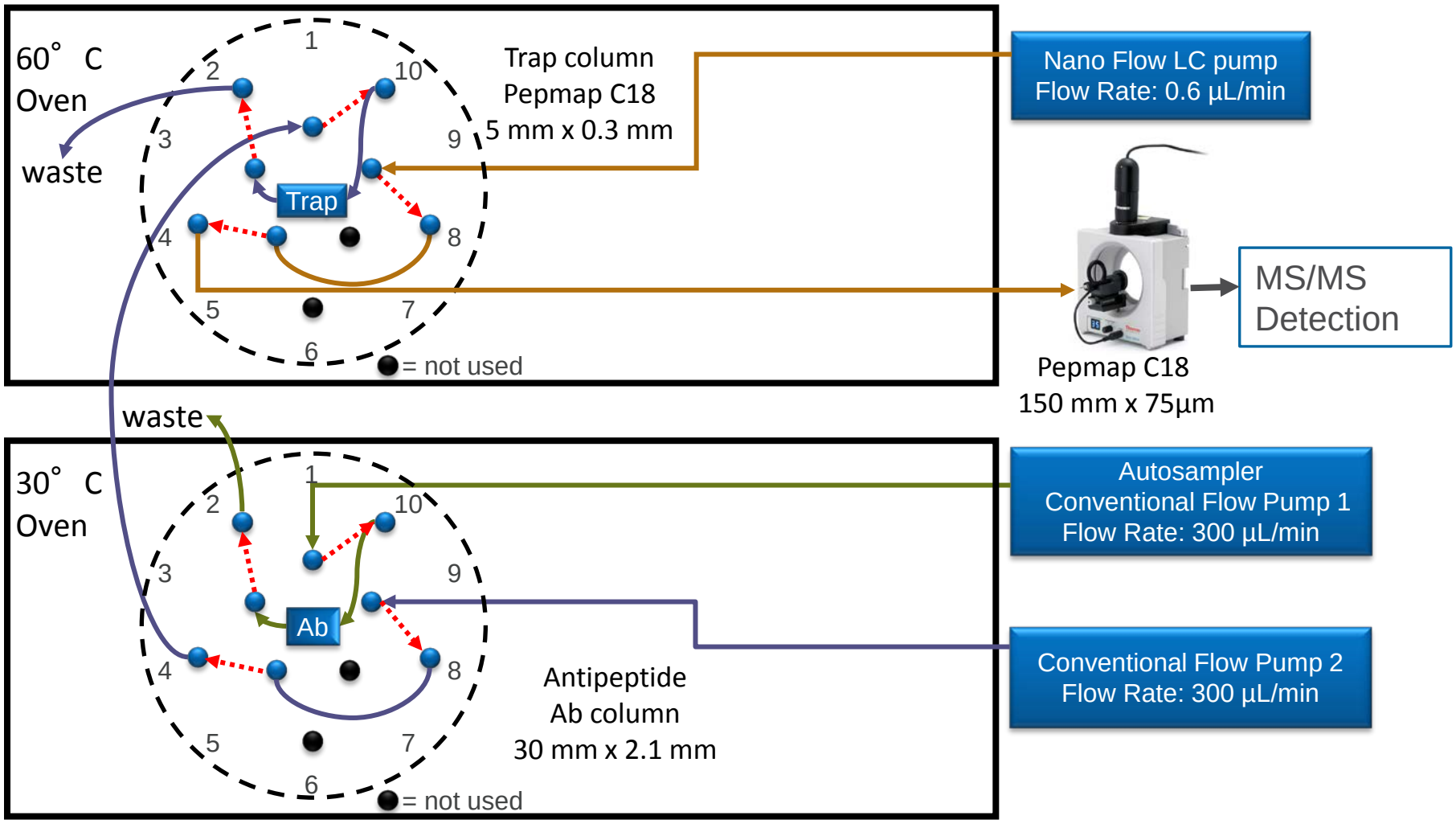
- Move the liquid
  - Capture
  - Wash
  - Elute analyte for LC-MS

- Move the bead
  - Capture
  - Wash
  - Elute analyte for LC-MS





Coupling Immunoaffinity to LC-MS (post-digestion)



Tailoring Validation Experiments to Specific Needs of Assay

Measuring Pre-digestion Immunocapture (IC) Efficiency

- Why?
  - Does the internal standard (IS) used accurately address inherent variables?
  - Circulating ligands
  - Added solubilizer or non-specific binding avoidance agent (e.g. BSA)
  - Anti-drug antibodies (ADAs)
- How?
  - Spike IS before and after IC step and compare surrogate peptide recoveries
  - Stress test further by spiking with suspected interferences
    - E.g. incorporate known ADA plasma into IC efficiency testing
  - Consider testing with disease state matrix
  - Conduct at low and high QC levels
  - Consult with LBA bioanalysts regarding specifics of the drug and the needed assay

Tailoring to Specific Needs of Assay

- Digestion step
  - Needs to generate a surrogate peptide with appropriate selectivity
    - This may not be always trypsin (also; surfactants, autolysis, commercial grades, age-stability)
    - Alternate endoproteases (e.g. Arg-C, Asp-N, Lys-C, Proteinase K)
  - Online *in-silico* evaluations are essential but confirm experimentally at the intended/needed sensitivity
  - Digestion efficiency should be reproducible across concentration range of protein analyte
    - Test empirically with QC performance Vs measure quantitatively by ratio comparison of post-digestion spike of equimolar amount of surrogate peptide ?
  - J. Duggan et. al., Bioanalysis 8(18), p1951-1964 (2016)
  - E. Fung, P. Bryan, A. Kozhich, Bioanalysis 8(8) p847-856 (2016)

Don’t overlook chemical digestion

- Large Molecule mAb Biotherapeutic, DX2930
  - Difficulty with human plasma LB Assay
- Hybrid IA-LC/MS method feasibility initiated.
  - Bottom-up digestion approach.
- No suitably selective tryptic signature peptides.
  - Required alternative digestion.
- *In silico* sequence homology searches utilizing alternative enzymatic digestion reagents did not provide unique peptides
- Putative unique peptides identified from *in-silico* formic acid chemical digestion (cleavage at aspartic acid residues)

Assay Details

Extraction *(Hamilton Microlab STAR)*

- Bead-based immunocapture of 100 µL plasma with Ab
- Elute at low pH, neutralize
- Add denaturant (Guanidine-HCl) and internal standard (winged SIL)
- Reduce (TCEP)
- Add formic acid (~2%)
- Incubate overnight at 95ºC

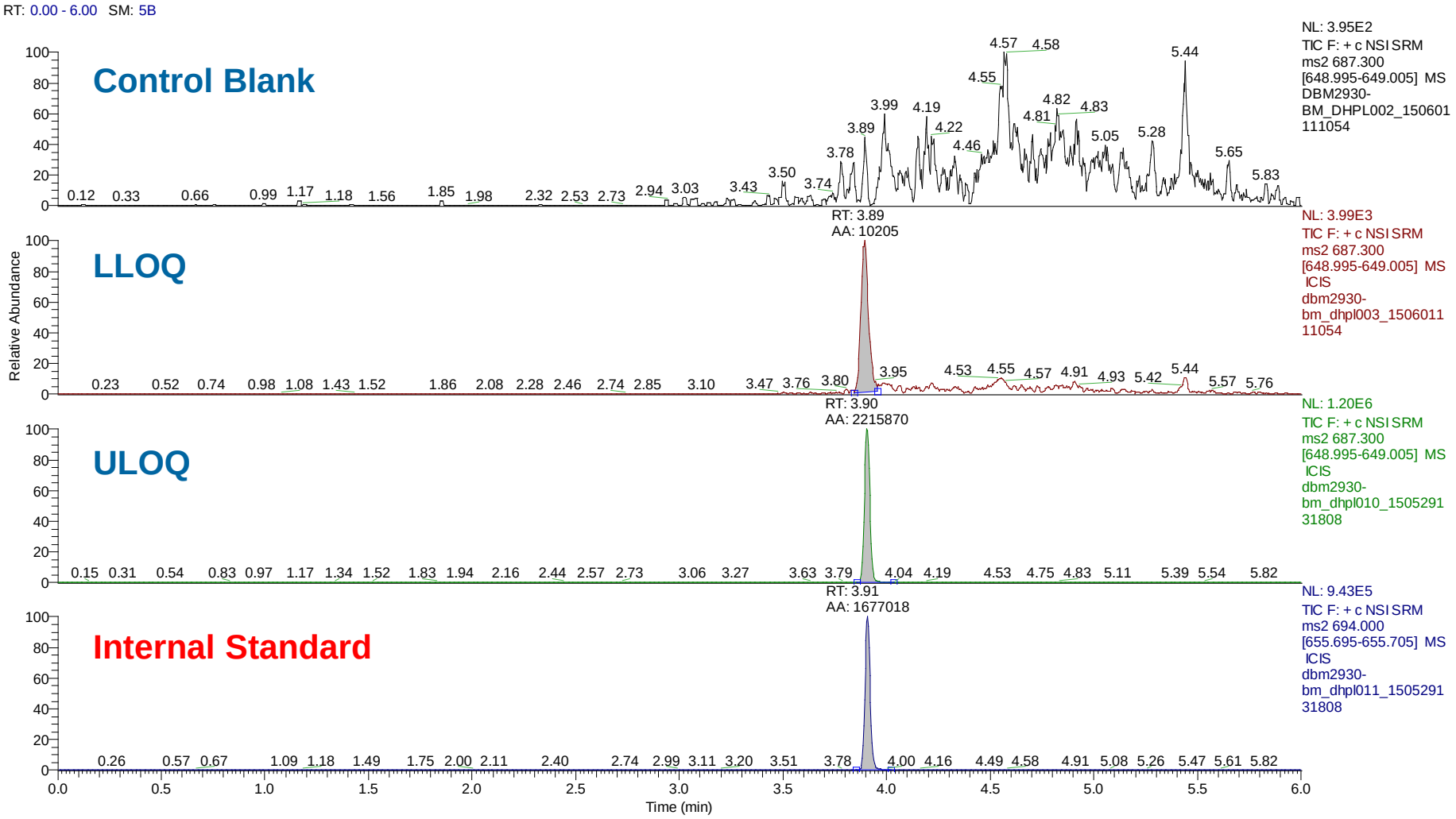
LC *(Dionex Ultimate 3000 multidimensional nano-LC system)*

- 40 µL injection to C18 trap µ-Precolumn™, 0.3 mm x 5 mm at 300 µL/min
- Elute to the HPLC column (PepMap C18, 75 µm x 15 cm) at 600 nL/min

MS *(Thermo TSQ Vantage QQQ MS)*

- Thermo EASY-Spray source

Representative XIC for Formic Acid Digestion Assay



Inter-Run Statistics

Two A&P test batches

		LLOQ	QC1	QC2	QC3	ULOQQC	100X DilQC
Theoretical Conc. (ng/mL)		**100**	**300**	**900**	**1200**	**1500**	**100000**
Run	3	111	302	1030	1170	1390	NA
		124	309	1030	1160	1450	NA
		117	310	1020	1150	1350	NA
		110	305	1030	1550	1560	NA
		120	305	1020	1160	1510	NA
		120	321	1090	1220	1440	NA
Run	4	108	332	951	1240	NA	93000
		107	357	971	1200	NA	94700
		111	337	944	1220	NA	92700
		111	346	1260	1240	NA	96000
		107	342	949	1240	NA	97600
		114	360	917	1230	NA	93100
Mean		**113.5**	**329.5**	**1016.5**	**1237**	**1462**	**94517**
S.D.		**5.66**	**21.20**	**91.12**	**105.99**	**76.68**	**1967**
%CV		**5.0**	**6.4**	**9.0**	**8.6**	**5.2**	**2.1**
%Theoretical		**113.5**	**109.8**	**112.9**	**103.1**	**97.5**	**94.5**
n		**12**	**12**	**11**	**11**	**6**	**6**

Acceptance Criteria

"Regarding accuracy and precision, in general, we recommend applying the LBA criteria as a starting point because of the inherent nature of these assays and the limited industry experience with the performance of the methods in regulated applications"

Jenkins R, Duggan J, Aubry A et al. AAPS J. 17(1), p1-16 (2015)

- Evidence to date is that this position has held
- Recent discussions around recommendations to ICH (M10) initiative did not move from this position other than to recommend that acceptance criteria should be supported by the validation of the assay and be appropriate for the intended use.

Protein Inference Challenge

- Does the chosen surrogate/signature peptide in a PrD-LC-MS assay accurately quantify the intact protein analyte?

- The original Jenkins et al. paper makes the case for simultaneously following “Monitoring Peptides” along with the quantitative surrogate peptide
  - May afford information on biotransformations, isoforms or degredation products
  - May help with troubleshooting

- Consensus seems to be that use of “monitoring peptides” should be considered in development of the assay but otherwise no specifics or standards have emerged.

- However the recognition of the value of intact protein LC-MS bioanalysis has been taken up by several groups

Intact Protein LC-MS Bioanalysis

- Also referred to as “Top-Down” approach
- Requires High Resolution Accurate Mass instrumentation (HRMS)
- Use immunocapture to pre-concentrate and selectively isolate protein of interest
- Quantify from response from one or more charge states

OR

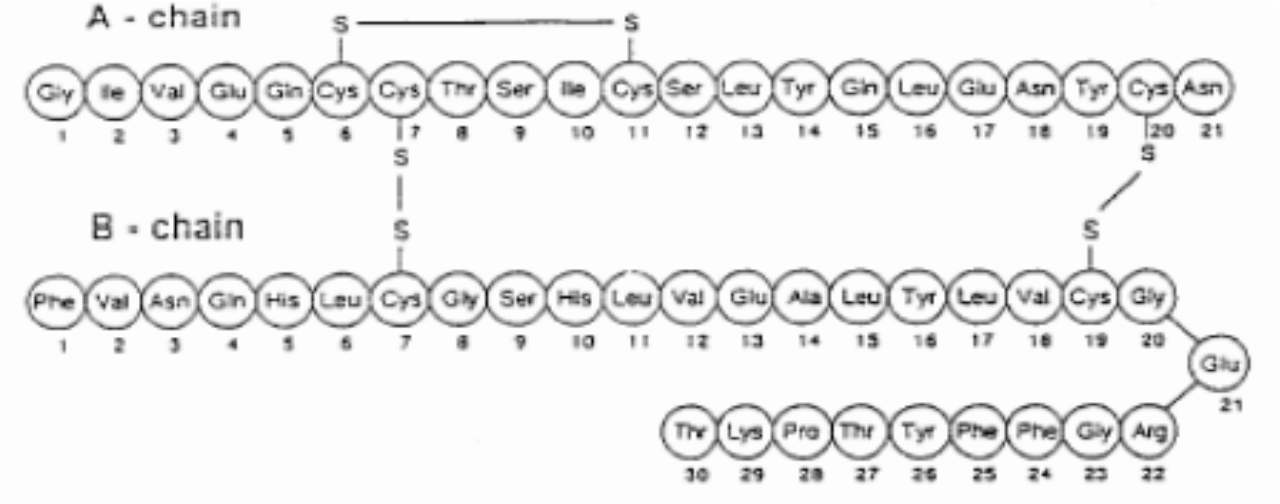
- Quantify from deconvoluted mass spectra

A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC-HRMS

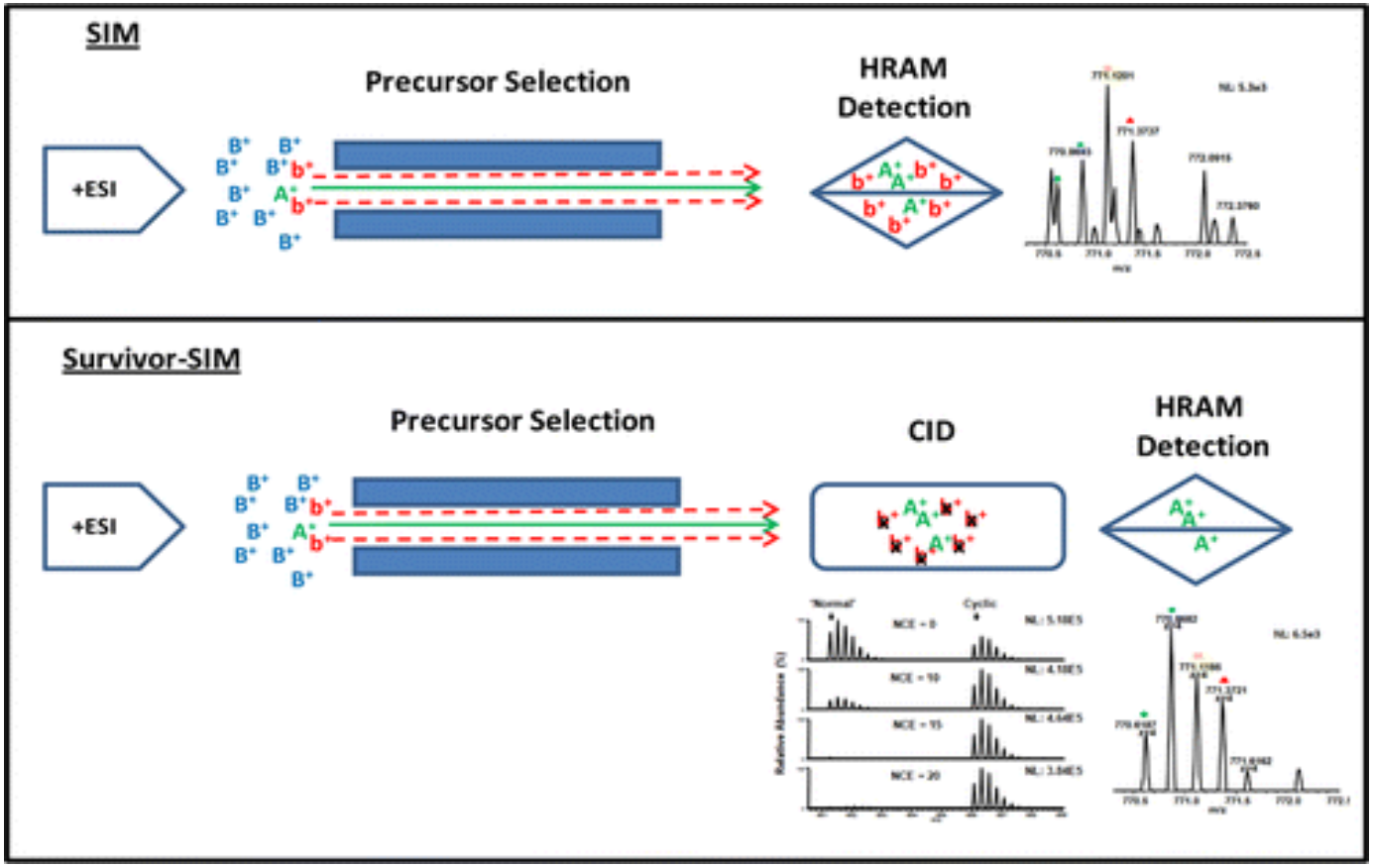
W. Jian, L. Kang, L. Burton, N. Weng, Bioanalysis 8(16) p1679-1691 (2016)

Bioanalytical Challenge

- **Need to measure human insulin in pig serum to 100 pg/mL levels**
- **LBA specificity challenges**
  - **Need to leverage LC-MS**
- **LC-MS sensitivity and selectivity challenges**
  - **Human insulin does not fragment well and high response fragments cannot be obtained for quantitation**

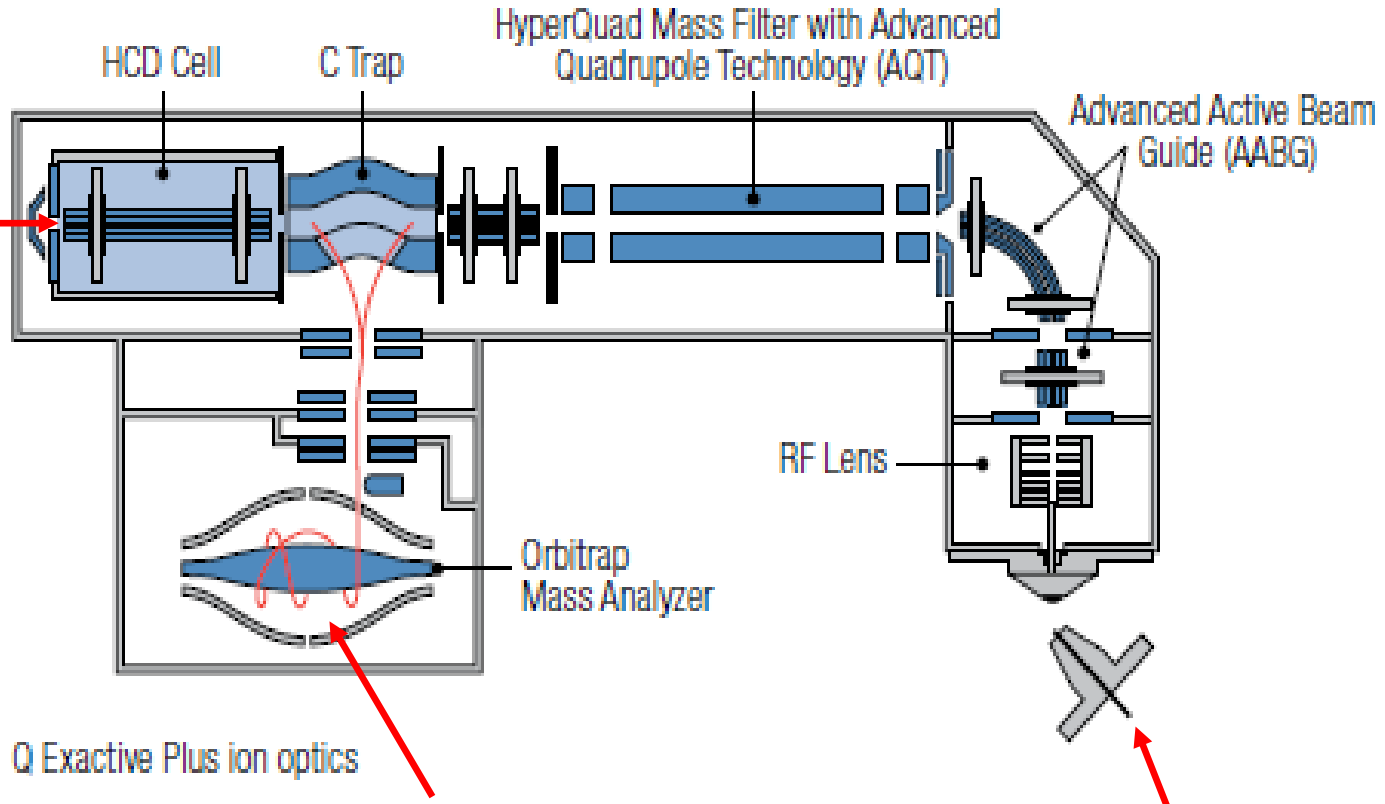


Survivor SIM of HRMS

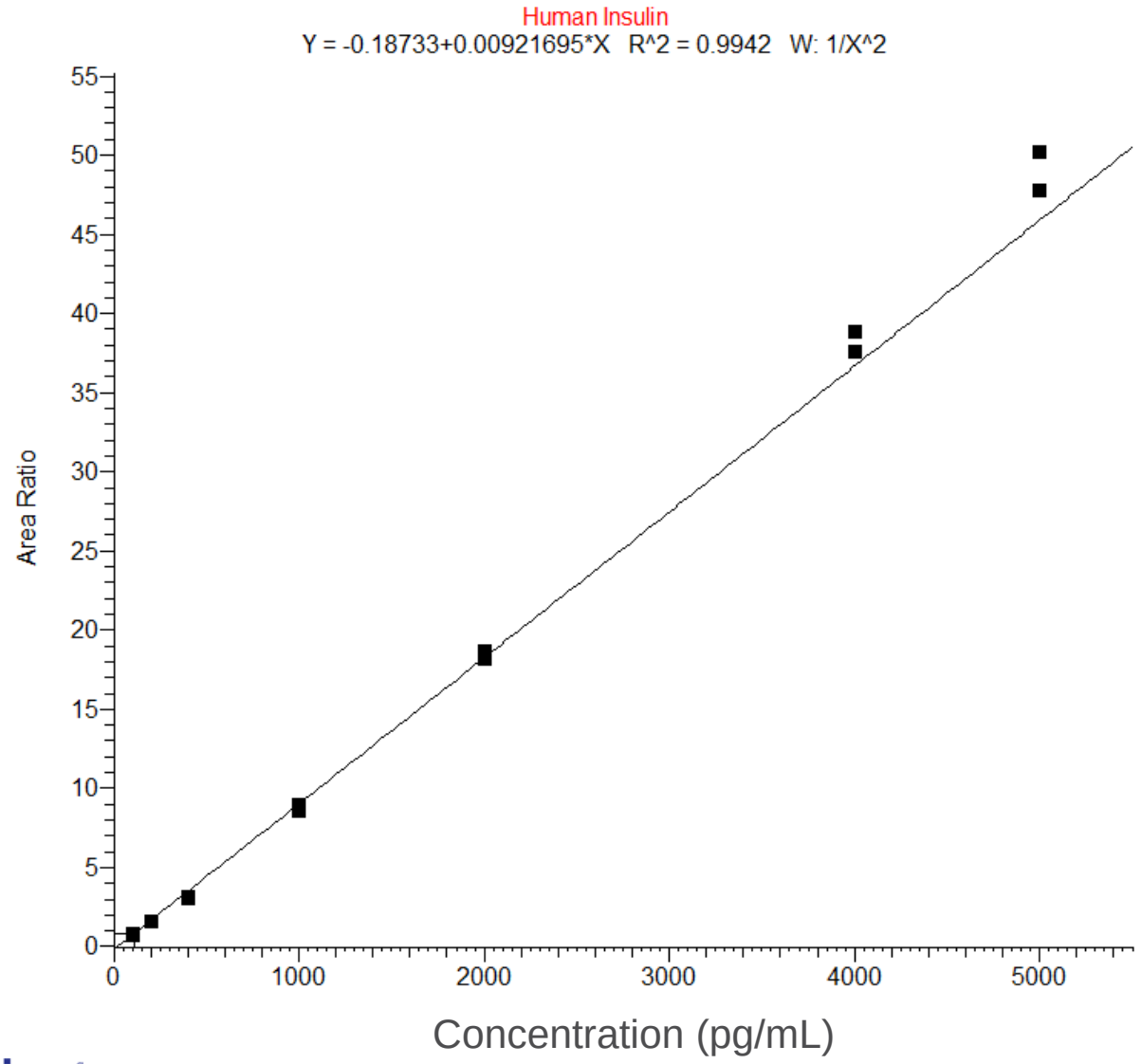


This figure is copied from Eugene Ciccimaro et al. Analytical Chemistry, 2014, *86* (23), pp 11523–11527

HRMS Methodology for Quantitation of Human Insulin: Survivor SIM, a PRM method



Calibration Curve



QC Statistics, Inter-days

	LLOQ	LQC	QC1	QC2	QC3
Theor. Conc. (pg/mL)	100	300	1000	2000	4000
#1	112.3	283.9	1001	1969	3923
#2	104.8	300.4	1063	2053	4290
#3	107.6	282.9	983.8	2188	4647
#4	103.5	252.1	976.9	1982	3962
#5	106.9	258.3	959	2140	3919
#6	103.8	274.1	938.6	1945	4149
#7	113.9	283.8	1085	2263	4315
#8	109.5	230.4	1037	2260	4307
#9	102.2	262.2	952.4	2287	3355
#10	107.7	282.7	889.1	2180	3831
#11	84.49	303.9	1211	2410	3998
#12	101.6	326.6	1307	2102	2845
Mean	105	278	1034	2148	3962
S.D.	7.47	25.6	120	144	476
%CV	14.6	10.6	11.5	5	12.3
%Theoretical	104.9	92.8	103.4	107.4	99.0
%Dev	4.86	-7.19	3.37	7.41	-0.96

Regulatory Authority Input

- There is still limited experience with Health Authority regulators around LC-MS of biotherapeutics
- Regulatory representatives have been present at many presentations on PrD-LC-MS and HRMS approaches to biotherapeutic bioanalysis
- There is not any specific guidance from any global regulatory body which addresses LC-MS bioanalysis of biotherapeutics
- Recommendations have been voiced from the FDA to engage in discussions of proposed bioanalytical strategies ahead of method implementation

Application of good science, documentation and method development/validation that is consistent with the well established pillars of BMV is expected

Future Predictions

- Multiplexed assays
  - Free Vs protein-bound
  - Bispecific therapeutics
  - Investigating biotransformations etc
- Biomarkers
  - Already a rapidly growing area of LC-MS bioanalysis and has paralleled the application to therapeutics
- Increasing selectivity/specificity
  - HRMS, micro-flow and nano-flow chromatography
- Intact analyte bioanalysis
- Native confirmation analyte determinations
  - Are these necessary?

Conclusions

- Protein therapeutic bioanalysis is now well established as a tool complementary to LBA approaches
- The most popular technique is a combination of immunocapture sample preparation with PrD-LC-MS (typically with a triple quadrupole mass spectrometer)
- Assay strategy needs to consider specifics of the bioanalytical need
- Assay performance criteria should be consistent with the intended application and the method validation.
  - Minimally, LBA BMV criteria are recommended
- LC-MS bioanalysis of proteins is expected to grow and evolve. There is much to be excited about as a bioanalyst when considering the potential of the technique.

Acknowledgments

- All authors of the original text:

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White Paper

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- Additional recognition in preparation of this presentation:
  - Barry Jones, Lian Shan