TT26B: Experiences on LC-MS Analysis of Large Molecules

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Background and Aim

LC-MS Analysis of Large Molecules is:

- Hot topic in many BA labs
- Continued scientific challenge to perform
- Regulatory requirements

The survey

28 Questions divided into following chapters

- Experience
- Instrumentation
- Method validation
- Internal standard selection
- Acceptance criteria
- Assay comparability, MS vs. LBA

Team Members

- Dietmar Schmidt, Sanofi, TT lead
- Philip Timmerman, Janssen R&D, sponsor
- Alberto Guenzi, Hoffmann – La Roche
- Richard Kay, Quotient Bioresearch
- Michael Pilgaard Andersen, Novo Nordisk
- Christoph Siethoff, Swiss BioQuant
- Paul Lassahn, SwissBioanalytics
- Gabriella Rohde, Bayer
- Ronald Schmidt, Sanofi
- Simon Wood, Celerion
- Carsten Krantz, Novartis
- Magnus Knutsson, Ferring
- Michael Blackburn, Covance

Survey results

Introduction/general questions

Q1: Is your lab involved in regulated LC-MS/MS bioanalysis of large molecules?

Answer Options	Response
A) No, this is the end of the survey for you	7 (37%)
B) Yes, go to question 2	12 (63%)
Total responders	19

Q2: Which type(s) of large molecules do you analyse in your lab?

Answer Options	Response
A) Peptides (Definition of a Peptide = no pre-treatment required before analysis; intact analyte)	11
B) Proteins (Definition of a Protein = pre-treatment is required before analysis; digested analyte)	7
C) Monoclonal ABs	4
D) Oligonucleotides	2
E) other, please specify	0

BA method validation experiments

Q3: Do you perform additional validation experiments compared to a typical small molecule based method validation?

Answer Options	Response
A) No	8 (67%)
B) Yes, please specify: + Digest efficiency + Testing the cleavage from anti-drug antibody (positive control for ADA assay is used as "standard") + Additional stability experiments in whole blood (metabolites)	4 (33%)

Q4: Do you investigate matrix effects for protein quantitation?

Answer Options	Response
A) No	0 (0%)
B) Yes, please specify: + standard procedure as for SM + using labelled and unlabelled tryptic peptides, as you would do for small molecules + Digested matrix sample spiked with target peptide (stable labelled internal standards) vs pure target peptide in solution (stable labelled internal standards)	10 (100%)

Q5: Do you investigate recovery for protein quantitation?

Answer Options	Response
A) No	4 (36%)
B) Yes, please specify: + for peptides similar to small molecule methods + Digested matrix sample spiked with target peptide (stable labelled internal standards) vs pure target peptide in solution (stable labelled internal standards) + "We purchase a synthetic version of the 'signature' peptide to investigate recovery. We have only looked at recovery during the solid phase extraction step and haven't investigated peptide recovery throughout the procedure."	7 (64%)

Sample prep. - extraction methods

Q6: Do you see adsorptive losses of your analyte?

Answer Options	Response
A) No	1 (9%)
B) Yes , please specify: + Addition of BSA and/or surfactant to standard solutions + Use special plates + Low binding plates + High quality/purity PP plates + Use silanised glass ware + Block surfaces with similar peptides + Careful (or avoiding) evaporation	10 (91%)

Q7a: What kind of sample preparation do you use?

Answer Options	Response
A) Off-line SPE	10
B) On-line SPE	2
C) Plasma Protein Precipitation	9
D) Immuno Precipitation	6
E) Other techniques or combination of the above, please specify	0

Q7b: If you use SPE, what solid phase chemistries / mechanisms do you use

Answer Options	Response
A) Reverse Phase	8
B) Ion Exchange	4
C) Mixed phase	7
D) Other or combination of the above, please specify: + Varian / Agilent Bond-Elut Plexa plates – Size exclusion / RP	1

Internal Standardisation approaches

Q8a: Which kind of internal standard(s) do you use in your intact analyte assay:

Answer Options	Response
A) Stable labelled whole protein / peptide	9
B) Surrogate protein / peptide	3
C) Others - please specify + Halogenated Internal Standards	2

Q8b: Which kind of internal standard(s) do you use in your digested analyte assay:

Answer Options	Response
A) Stable labelled whole protein	3
B) Stable labelled tryptic peptide	7
C) Others - please specify	0

Q8c: If you use a stable isotope labelled whole protein/ peptide do you correct for isotope impurities?

Answer Options	Response
A) No	9 (90%)
B) Yes	1 (10%)

Q9: If you use a stable isotope labelled whole protein do you see differences in terms of digestion behaviour which can be related to process differences of the internal standard compared to the analyte protein (e.g. different cells, different purification etc.)?

Answer Options	Response
A) No, usually the stable labelled protein/ peptide mimics the behaviour of the analyte protein.	4 (80%)
B) Yes and therefore we try to follow the process of the analyte protein as closely as possible	1 (20%)

LC-MS Instrumentation

Q10a: Which kind of instrument(s) do you use for your large molecules quantitative assay(s):

Answer Options	Response
A) Triple-Quad Systems (tandem MS)	12 (80%)
B) High resolution mass spectrometry	2 (13%)
C) Other systems (please specify system)	1 (7%)

Q10b: What is the m/z range and resolution of the MS-instruments do you use in your large molecules assay(s):

Answer Options	Response
From 5 to 4000 m/z	12
Resolution , please specify: + Majority Unit Res. + Minority High Res.: 5000 to 40 000	9

Q11: What flow rate do you use in your LC-MS system (more than one can be selected):

Answer Options	Response
A) Normal flow rate: + Majority between 200 and 600µL/min + All between 100 µL and 1000 µL/min	10
B) Capillary flow rate + Down to 70 µL/min + 2-10 µL/min	3
C) Nano flow	0

Q12: Do you use 2D and 3D separation techniques?

Answer Options	Response
A) No	9 (75%)
B) Yes, please specify: + Home-build coupled column LC system + 2D/ 3D UPLC system from Waters. Both systems based on using multi-dimensional chromatography, with different separation mechanisms. + Column Switching + Offline	3 (25%)

Q13: Do you use other special equipment (e.g. FAIMS, Selexlon)?

Answer Options	Response
A) No	9 (75%)
B) Yes, please specify + Ion Mobility	3 (25%)

LC-MS/MS and SRM analysis quantitation

Q14a: If you perform tryptic digestion of a protein, how many peptides do you quantify:

Answer Options	Response
A) 1	3 (38%)
B) 2	3 (38%)
C) 3	1 (13%)
D) 4 or more – please specify reason (e.g. size of molecule): + start with more than 4, then narrow down to one or two, depending on the size and characteristics of the protein.	1 (13%)

Q14b: How many transitions per peptide do you monitor:

Answer Options	Response
A) 1	6 (38%)
B) 2	5 (31%)
C) 3	4 (25%)
D) 4 or more – please specify reason: + all transitions monitored during development	1 (6%)

Q14c: If you either monitor more than 1 peptide per protein, or more than one transition per peptide, do you apply any specific acceptance criteria to the data (e.g. difference in the concentration values assigned using two different peptides must be within 20%)

Answer Options	Response
A) No	6 (100%)
B) Yes	0 (0%)

Q14d: If you use additional qualifier peptides, which acceptance criteria do you apply

Answer Options	Response
A) No acceptance criteria defined	4 (100%)
B) Same as for the quantified peptides	0 (0%)
C) Different	0 (0%)

Acceptance Criteria

Q15: Do you have any experience from regulatory expectations on acceptance criteria?

Answer Options	Response
A) No	10 (83%)
B) Yes, please specify type of analyte(s), acceptance criteria and feedback from the regulators: + during FDA inspection of a peptide assay (6 kD), the acceptance criteria of 20 and 25% at LLOQ-Level were accepted (immunoaffinity extraction) by the inspectors without any furter questions + 1600 Da peptide, 4-6-15 acceptance criteria, no comments from FDA or EMA	2 (17%)

Q16a: Which acceptance criteria do you normally use when performing LC-MS/MS analysis of intact analyte.

Answer Options	Response
A) 15% (20% at LLOQ)	9 (69%)
B) 20% (25% at LLOQ) + Includes hybrid assay, immunoaffinity sample prep. + LC-MS/MS	3 (23%)
C) Other please specify + Depending on the complexity of the assay (e.g. sample-preparation, etc.)	1 (8%)

Q16b: Which acceptance criteria do you normally use when performing LC-MS/MS analysis of digested analyte

Answer Options	Response
A) 15% (20% at LLOQ)	3 (33%)
B) 20% (25% at LLOQ)	5 (56%)
C) Other, please specify + Depending on the complexity of the assay (e.g. sample-preparation, etc.)	1 (11%)

Q17a: According to your experience, what is the most important factor in setting the acceptance criteria for LC-MS/MS analysis of intact analyte?

Answer Options	Response
A) Please specify + Availability of internal standard + Behaviour in MS (fragmentation, charge-state) + Immunocapture + Performance and complexity of assay + Variability in extraction procedure	12

Q17b: According to your experience, what is the most important factor in setting the acceptance criteria for LC-MS/MS analysis of digested analyte?

Answer Options	Response
A) Please specify + Efficiency and reproducibility of digestion procedure + Immunocapture + Interferences + Performance and complexity of assay	7

Comparison of LC-MS/MS and ligand binding assays

Q18a: What is your experience when ligand binding assays are compared with LC-MS/MS data in clinical and preclinical studies?

Answer Options	Response
No experience	5
A) Good correlation, good agreement	5
B) Good correlation, poor agreement	2
C) Bad correlation, poor agreement	1
D) Please specify what you understand for + Good correlation: Correlation: R2> 0.8-0.9 Agreement: Slope 0.85-1.15 or RE: < ± 20-25% + Poor agreement: + Free vs. total + Catabolism, metabolism + IS working differently in spiked vs. incurred matrix	4

Q18b. If you answered B or C to the above question, did you initiate investigatory procedures as to the reason for the disagreement of the two quantitative techniques?

Answer Options	Response
A) No	2 (40%)
B) Yes - please specify + internal standard peptides being degraded differentially between standard/ QC/samples + not be folded correctly for the ELISA, + Total vs. free + Catabolims / metabolism	3 (60%)

Team Recommendation

- LC-MS analysis of peptides and proteins is scientifically challenging. The Experience in industry is growing, and we are still learning.
- LC-MS/MS acceptance criteria (4-6-15) in existing guidelines originates from SMOL, and should not be used by default for LC-MS analysis of LM.
- 4-6-15 will work for some LM, but when scientifically justified, a priori set wider acceptance criteria should be OK.
- A full concordance of concentration data generated by an LC-MS(/MS) and a LBA assay is not necessary

Future plans

Get further input from continued discussions in TT, EBF members and bioanalytical community prior to publication.

Continue discussion in topic team with focus on key scientific challenges:

- Scientific interface between LBA and LC-MS/MS technology in MVAL
- Assay comparability
- Acceptance criteria

Publication strategy:

- Publish the survey results as a separate paper
- Publish a recommendation on EBF's view