LC-MS for large molecules vs. Ligand Binding Assays: orthogonal readout or contradictory methods?

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EBF Open Symposium, 18-11-2015

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Introduction

- Bioanalysis of Biologics long dominated by Ligand Binding assays
- LC-MS/MS techniques gain more and more momentum for protein therapeutics
- Change of paradigm in BA analyte and complexity of analyte directs technology to be used for drug development
- Combination of LBA and LC-MS/MS assays can help to better characterize the drug candidate

In the next 5-10 years....an increasing number of assays might be required to justify the bioactivity of biotherapeutics for regulatory applications (Dudal S, Bioanalyis 2014 6(10))

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Ligand binding assays vs large molecule LC-MS/MS

LBA	LC-MS/MS
Requires specific antibodies which can be time-consuming and costly	Short development times if whole matrix digest is used w/o immunocapture; generic assays for mAbs
Very sensitive to pg/ml	Without enrichment tools limited sensitivity
Limited selectivity	High selectivity due to different mass transitions, HRMS, ISTD
Can be used to measure free bound and total fraction	Free, and bound only detectable with Hybrid LBA LCMS
Fast due to parallel assay	Laborious workup and sequential assay in autosampler
Limited multiplexing	Multiplexing of different peptides from same or different proteins
High Throughput	Complicated workup, low throughput

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Ligand binding assays vs LC-MS/MS: It depends what is measured

Can be in good accordance
(Law et al, Bioanalysis 2014)

LBA = LCMS ?

	LBA	LC-MS/MS
Assay interference by ADA	ADA block binding for capture or detection Ab leading to over/underestimation (Wang et al , Anal Bio Chem 2012)	No interference if direct approach without immunocapture
Formation of active metabolites	Metabolite can distort LBA signal (Wang et al, Bioanalysis 2012)	Multiplexing of parent and metabolite at the same time
Detection of free vs. total	Capture Ab may detect only free or a combination of free and total (Heudi et al , Anal Chem 2008)	Direct methods detect total only

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Biologics have special demands on BA strategy

- Approximately 40% of biologics are non-antibody protein entities which require distinct bioanalytical considerations
- Therapeutic proteins demand different BA strategies
- Low MW (<50kDa) often associated with short terminal half-life due to renal filtration and proteolytic degradation
- To prevent high dosing frequencies half-life extension strategies are developed:
  - Fc/Albumin fusions using the FcRn recycling
  - Increase of hydrodynamic radius (e.g. PEGylation, PASylation)...

→ BA strategy needed to understand stability and eliminations mechanisms in vivo

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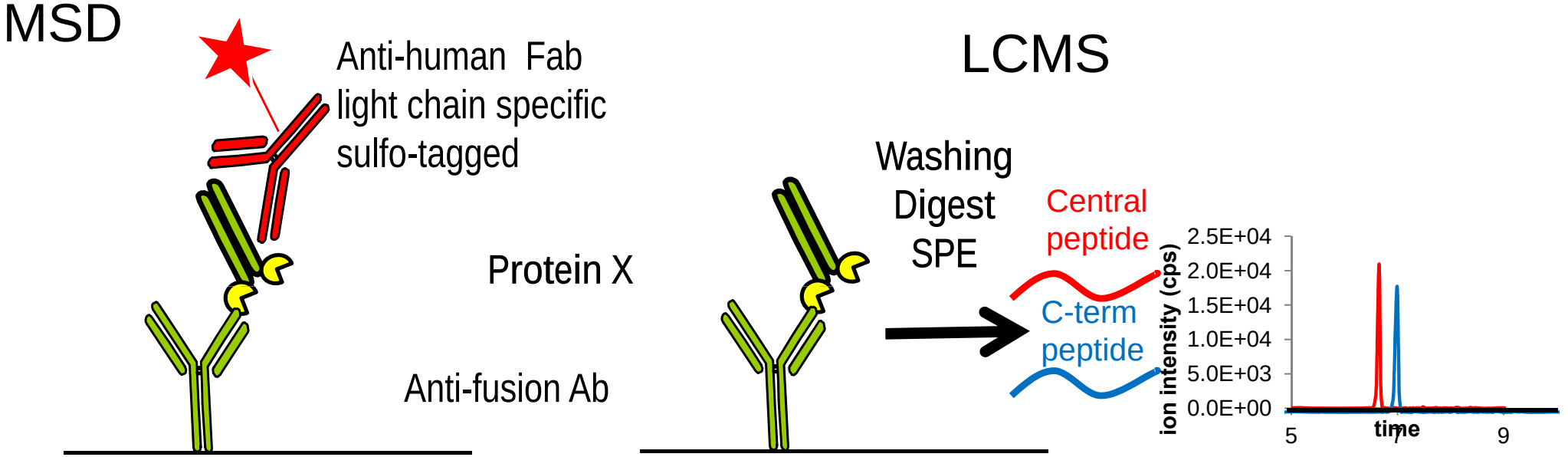
Case Study I: Stability of fusion protein

- Protein X is Fab that is half-life extended by protein fusion
- Integrity of the fusion part is essential to preserve half-life extension capacities
- Using both LBA and LCMS to characterize in vivo integrity of the molecule



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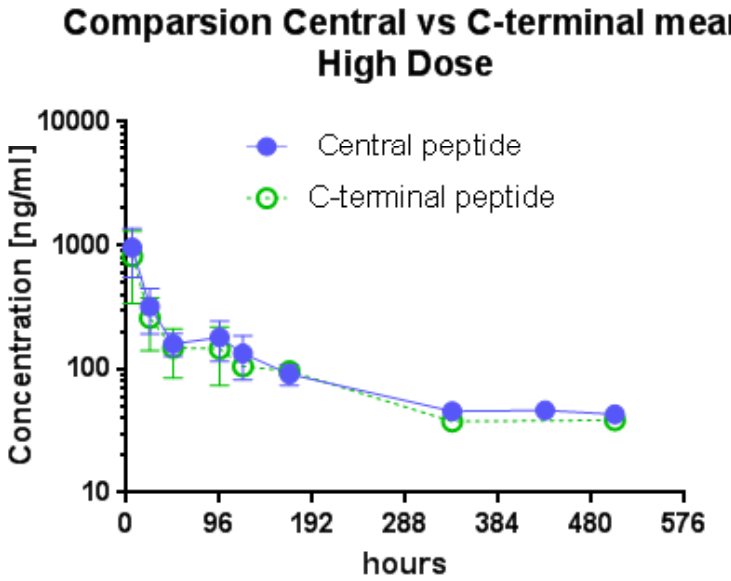
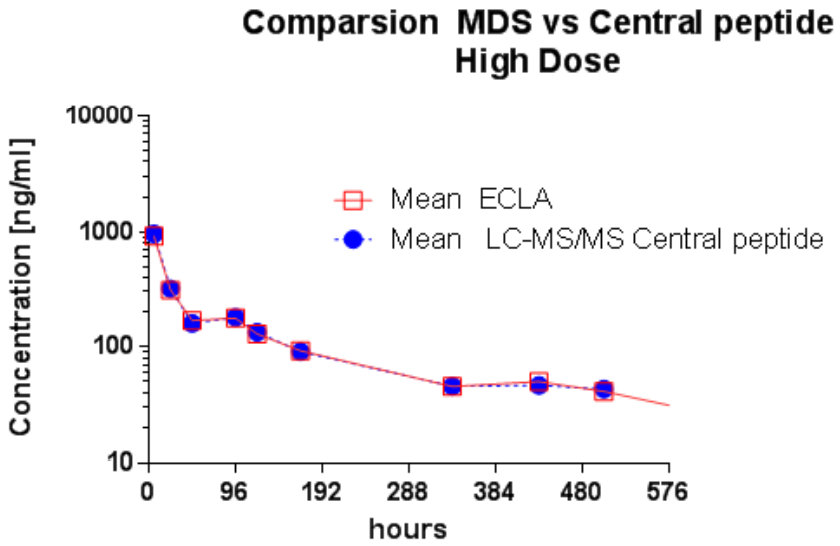
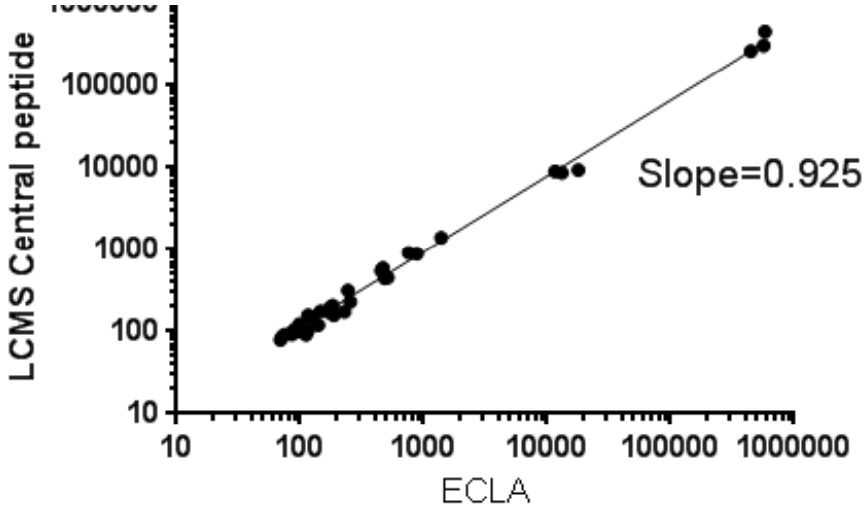
Case study I: PK results of Fab fusion protein







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Summary Case Study I

- LC-MS and LBA showed a good correlation
- No cleavage was detected by multiplexing LC-MS
- Project team decided that information about drug integrity is sufficient and project can be continued with one assay only
- MSD was selected to proceed because of higher sensitivity and throughput

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Case Study II: PEGylated Protein Y

- Protein Y is a soluble protein that was PEGylated using 40kDa branched PEG maleimide to increase half-life
- It was known that the protein is proteolytically unstable at the C-terminus whereas the core domain was shown to have a long systemic persistence presumably due to the PEG moiety
- An integrated BA strategy was selected encompassing a LBA assay using an anti-Protein Y pAb for capture and a anti-PEG detection antibody
- Results were compared to a multiplexing LC-MS/MS assay where several peptides N-terminal (Pep 1,2) and C-terminal (Pep 3) to the PEG moiety were monitored

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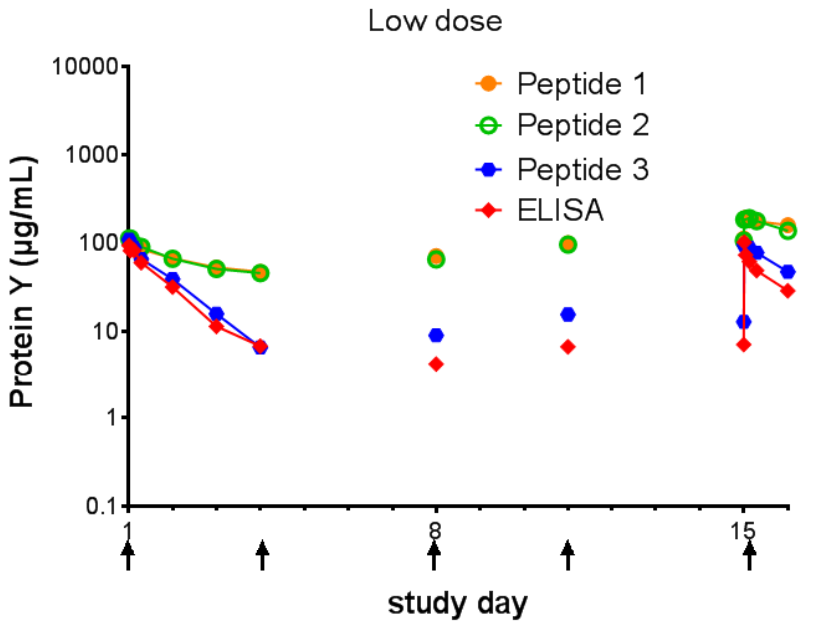
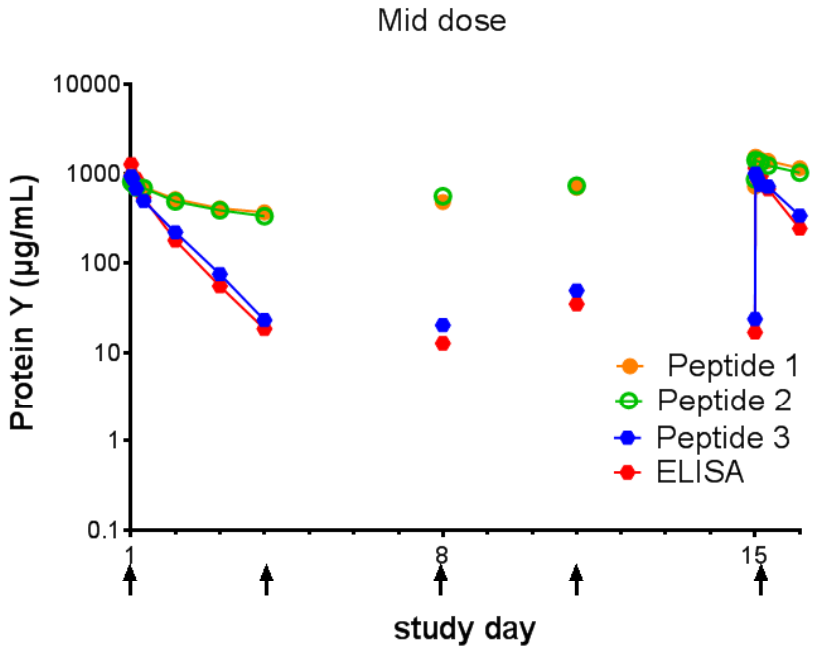
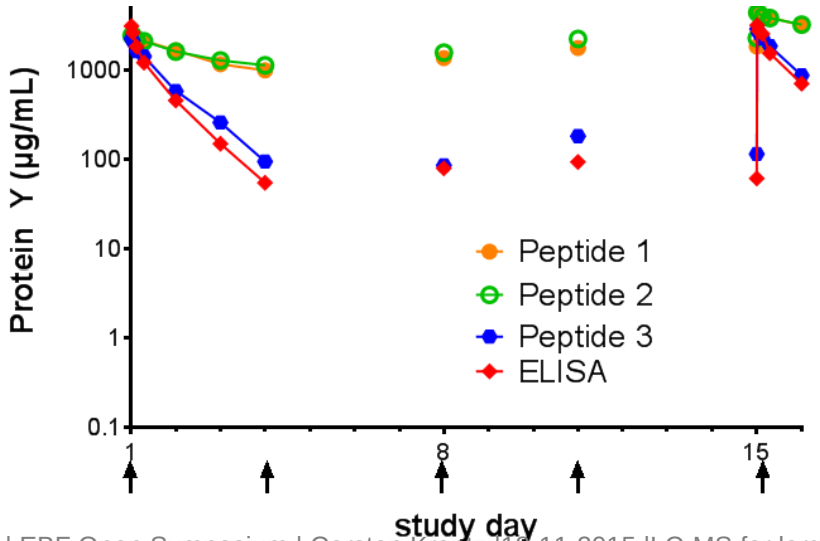
Case study II: Results pre-clinical cyno study







Analyte	Mean T1/2 (h)	CV (%)	Min T1/2 (h)	Max T1/2 (h)
Peptide 1	68.4	26	49.5	99.7
Peptide 2	78.6	23	60.3	105.4
Peptide 3	16.1	40	7.4	25.6
ELISA	14.6	36	7.9	21.1

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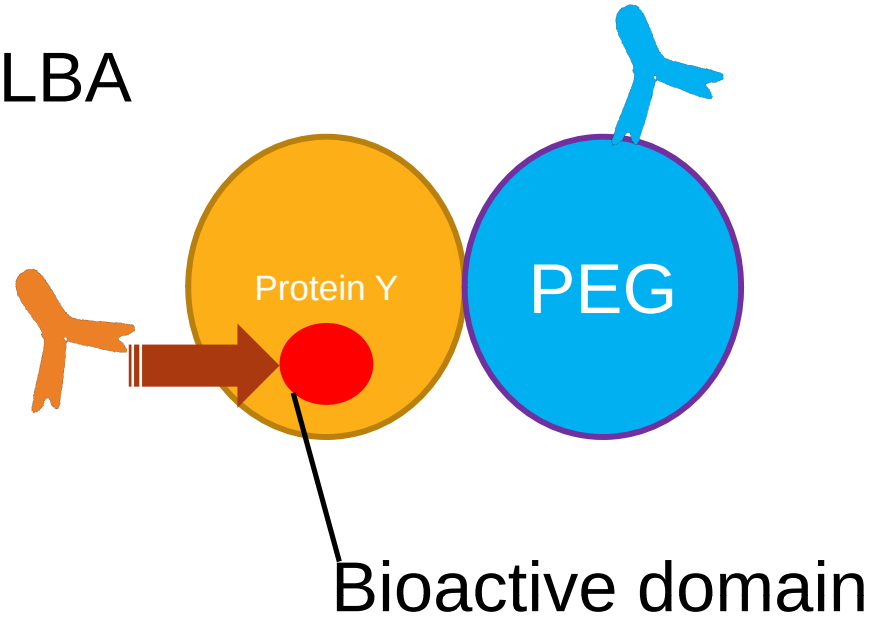
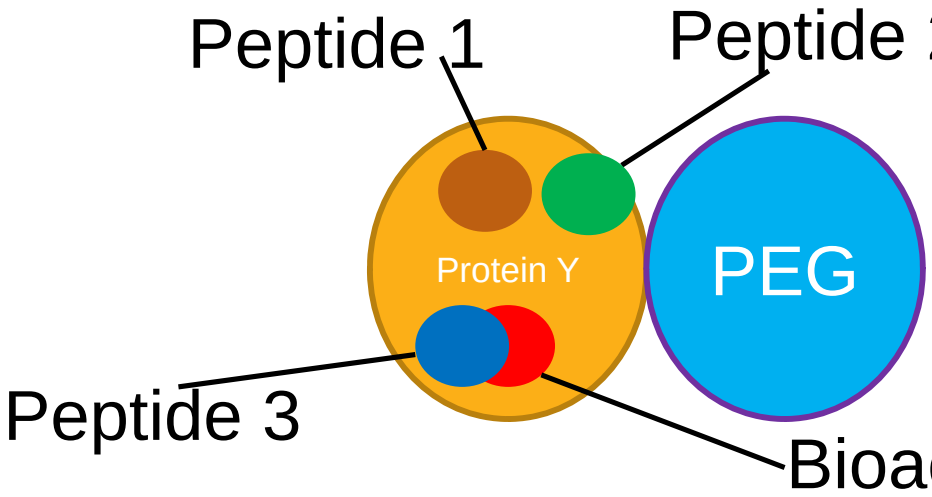
Case study II: What do we measure?

- LBA



Intact
Bioactive but truncated

- LC-MS/MS



Intact (Pep 1-3)
Bioactive but truncated (Pep 3)
Truncated but inactive (Pep 1, 2)

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Case study II: In-vivo instability of Protein Y

- It was confirmed that ELISA-based results and Peptide 3 for LC-MS represent the bioactive fraction of the drug
- However, the **bioactive fraction was cleaved relatively rapidly *in-vivo*** and the predominant circulating moiety in serum **are truncated inactive PEGylated forms of Protein Y**
- LBA assay not able to detect metabolite seen in LC-MS/MS
- Variability of half-life for full length protein attributed to different proteolytic activity

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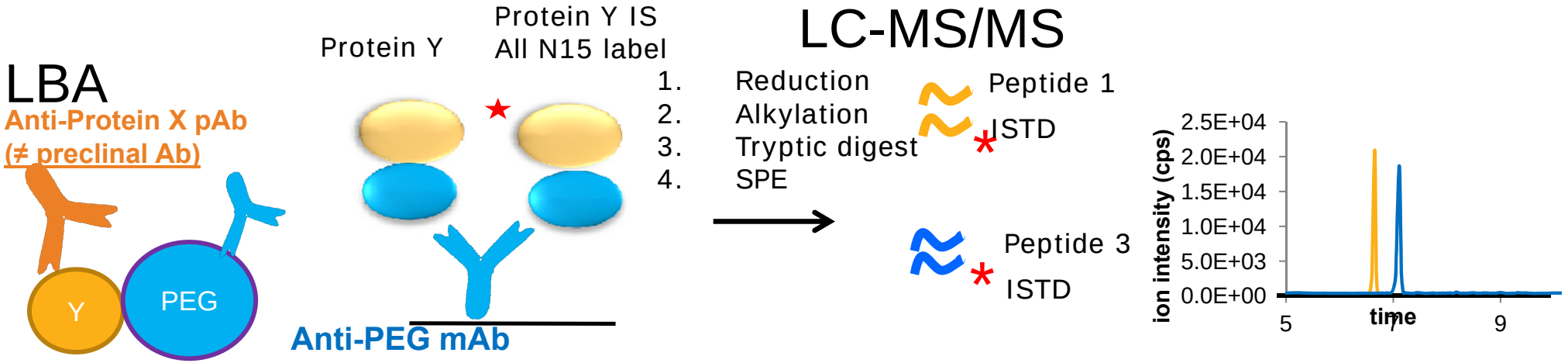
Case study II: Regulatory Input

- HA requested that truncated and intact Protein Y to be measured

...Provide pharmacokinetic, pharmacodynamic and safety data from single intravenous data of Protein Y in humans. Please determine serum pharmacokinetics of **both active and total** Protein Y ...



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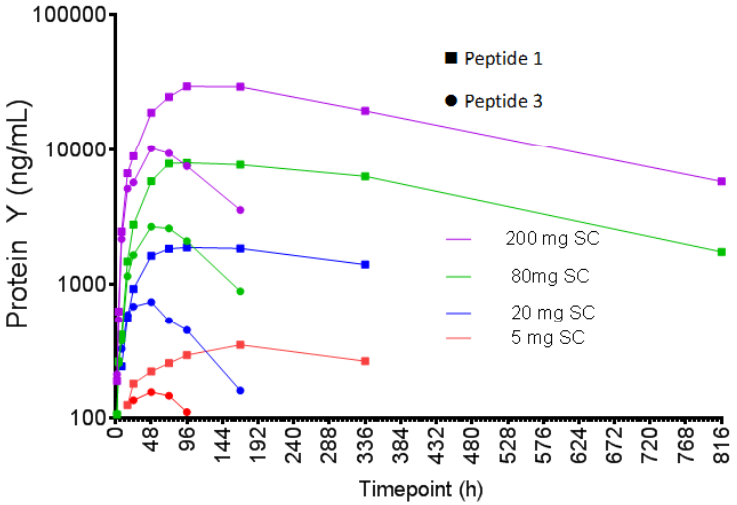
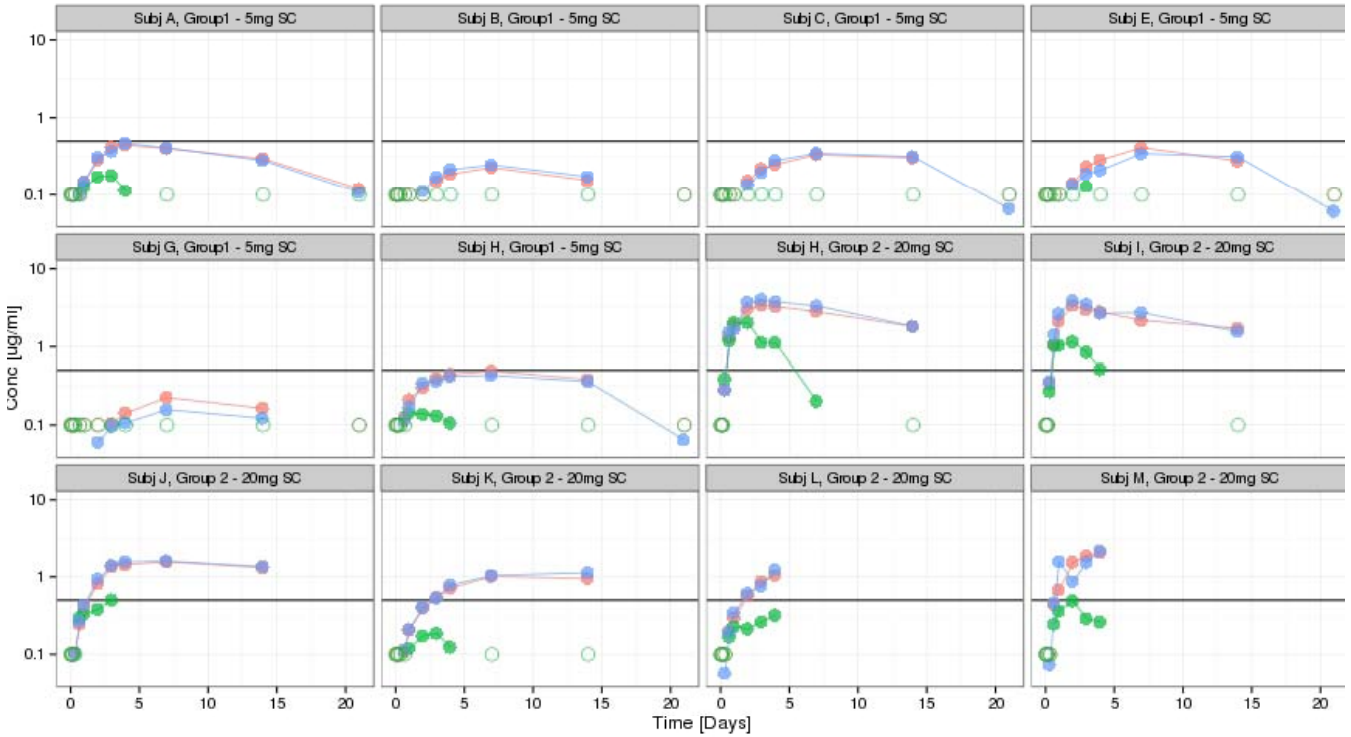
Case study II: Results clinical study

- Peptide 1 (intact + truncated)
- Peptide 3 (bioactive)
- **ELISA (intact + truncated)**
- o – BLQ





Conversely to pre-clinics ELISA does not track with bioactive part (Peptide 3) but with the intact/truncated surrogate (Peptide 1)

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Summary Case Study II

- Protein Y was shown to undergo proteolytic cleavage in pre-clinics and clinics
- Using a careful selection of peptides a bioactive PK could be measured
- LBA and LC-MS/MS gave a complementary picture of the characterized drug and helped to elucidate PK behavior of active and truncated PEGylated Protein Y
- LC-MS/MS characterized LBA tools and helped to understand what the LBA measured

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Conclusions

- LBA and LC-MS can be used in an orthogonal manner and both can provide complementary read-outs which can help decision making
- Differences between LCMS and LBA need to be investigated and characterized
- When LCMS and LBA are used in combination it is important to know what is measured

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Acknowledgements

- Bx-MS

Andrew Warren

Olivier Heudi

Arlette Garnier

Gwénola Tréton

Christian Miess

- DMPK PK/PD

Igor Vostiar

Mark Milton

- CVM DA

John Diener

- DMPK-Bx

Matthias Hofmann

Birgit Jaitner

- PCS

Ron Newton

- OPH DA

Joy Ghosh

- Others

Pete Lloyd

Rick Schiebinger

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