



Why do LC-MS and LBA results differ? A literature evaluation

18 November 2015

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An overview

In most published BA methods for large molecules, **no comparison of platforms** (LBA vs LBA, LBA vs LCMS or LCMS vs LCMS) is undertaken

If there is a comparison, in just **a few cases** there is a difference of <20%, this is mainly for

- smaller proteins
- spiked samples
- incurred samples after short exposure

Differences usually are **not convincingly explained**

Bults et al, Expert Rev. Proteomics, 12 (2015) 355-374



Proteins vs small molecules

Small molecules are well-defined, **single species**

Proteins are often heterogeneous and may occur in several different **(iso)forms**

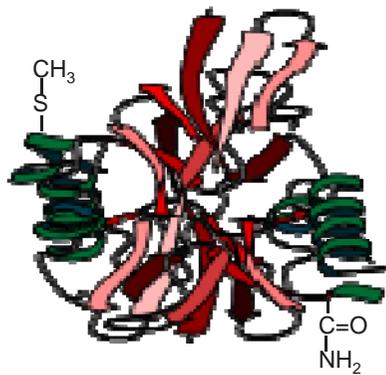
Small molecules are often **loosely bound** to plasma proteins and the binding is easily broken during analysis

Proteins may form **very stable complexes** with other plasma proteins and the binding can be difficult to disrupt during analysis

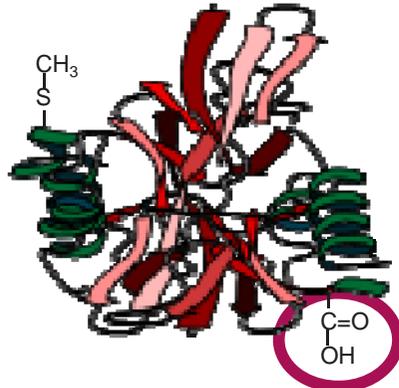


Protein forms

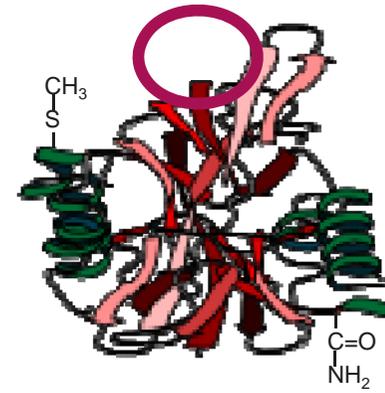
Modifications:



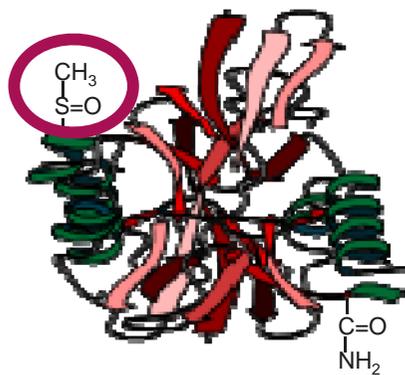
native



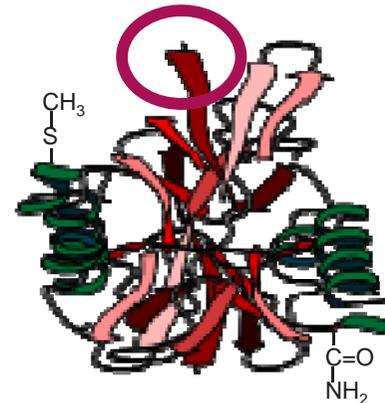
deamidation



proteolysis



oxidation

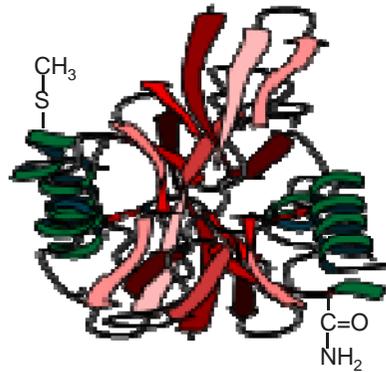


3D change

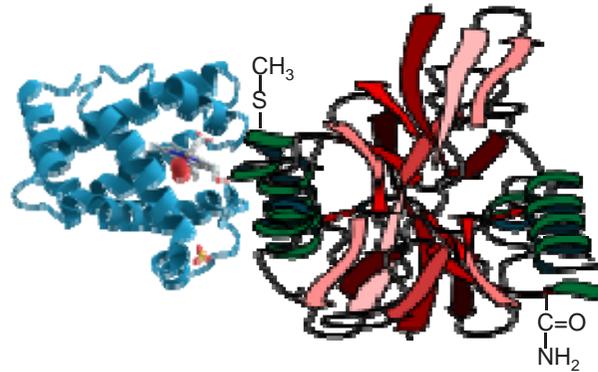


Protein forms

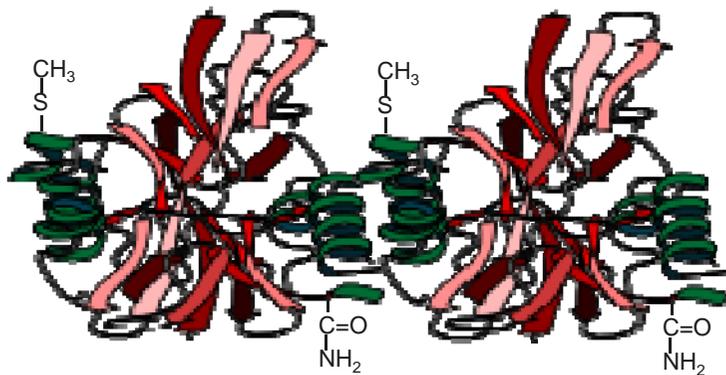
Binding:



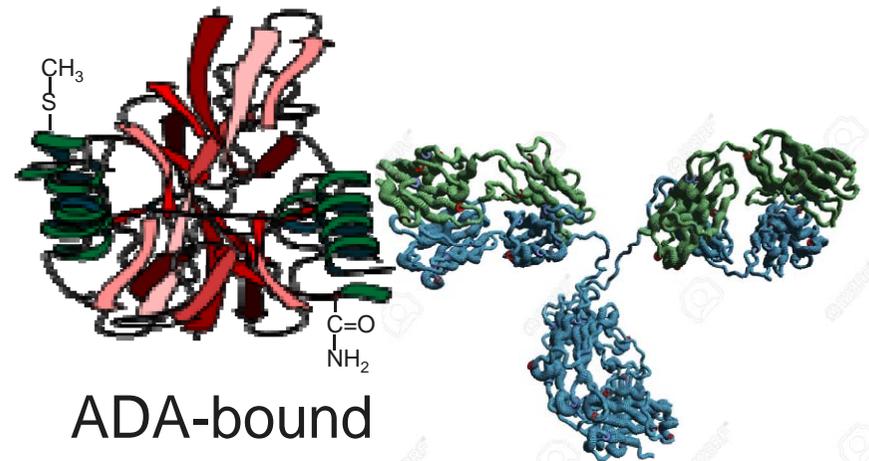
unbound



target-bound



aggregated



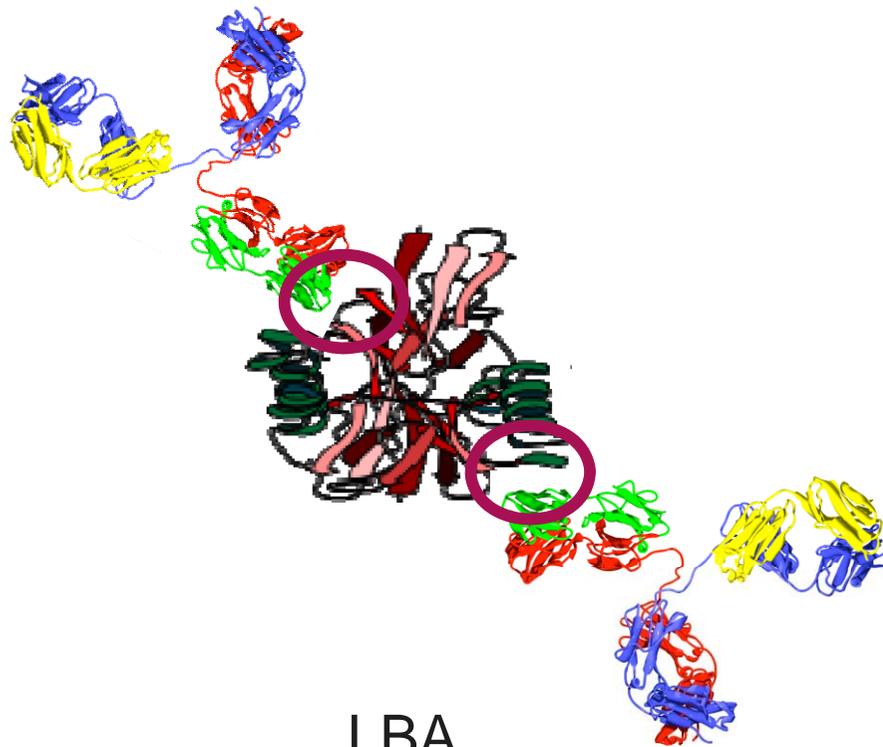
ADA-bound



Protein quantification

Both LBA and LC-MS/MS respond to only a (small) **part of the protein**

Depending on the analytical mechanism, modifications and/or binding events may or may not be picked up



LBA



LC-MS/MS



Some literature examples

- LBA vs LBA
- LC-MS vs LC-MS
- LBA vs LC-MS

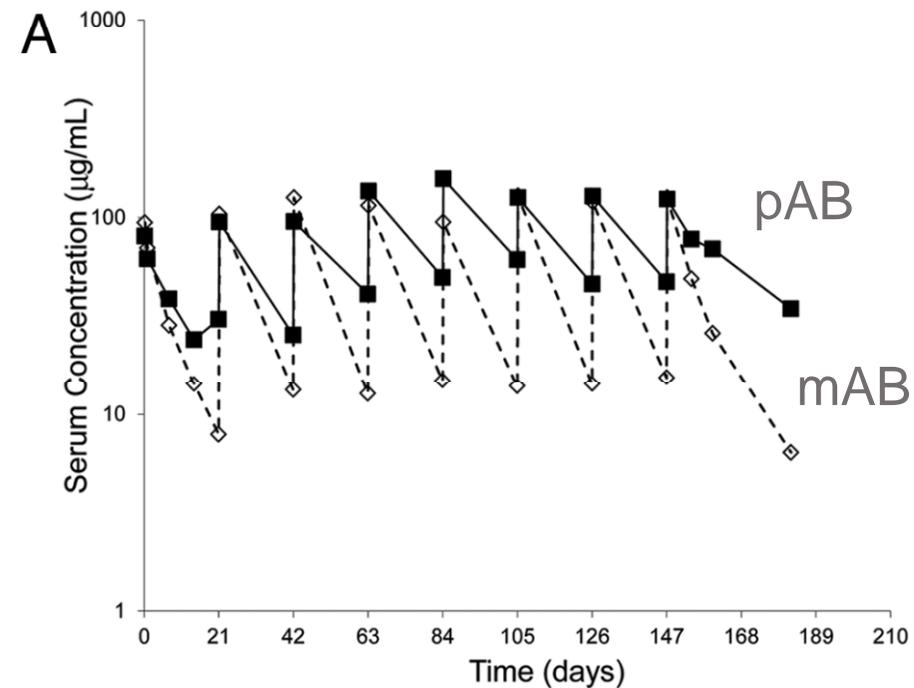


LBA vs LBA

Influence of **capturing antibody**: monoclonal vs polyclonal

Example: ocrelizumab

Concentrations in patient plasma \pm 2-fold lower with mAB than with pAB



Fischer et al, mAbs, 4 (2012) 623-631

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LBA vs LBA

Pharmacological target CD20 and monoclonal capturing antibody bind to the exact same epitope of ocrelizumab

- no (or reduced) analyte recognition by ELISA when target-bound

Polyclonal capturing antibody binds to multiple epitopes

- also (partial) analyte recognition by ELISA when target-bound

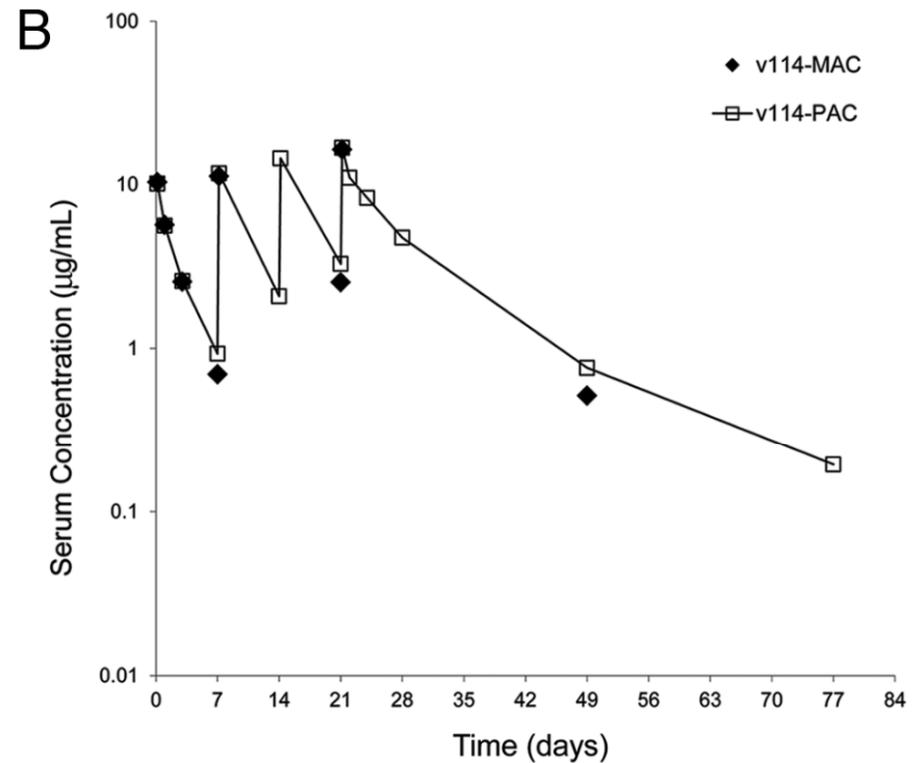




LBA vs LBA

No such effect for another CD20-directed therapeutic antibody

The nature of the capturing reagent and the exact epitope to which it binds determine the concentration result



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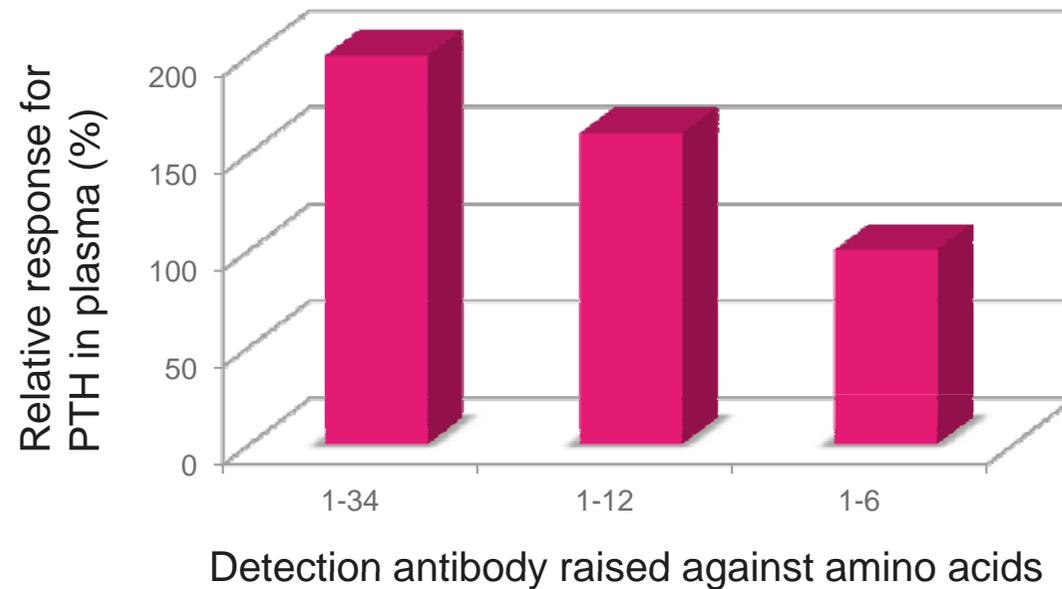


LBA vs LBA

Influence of **detection antibody**: different specificities of commercial ELISAs

Example: PTH

Concentrations depend on detection antibody specificity



Sukovaty et al, J. Pharm. Biomed. Anal. 42 (2006) 261-271

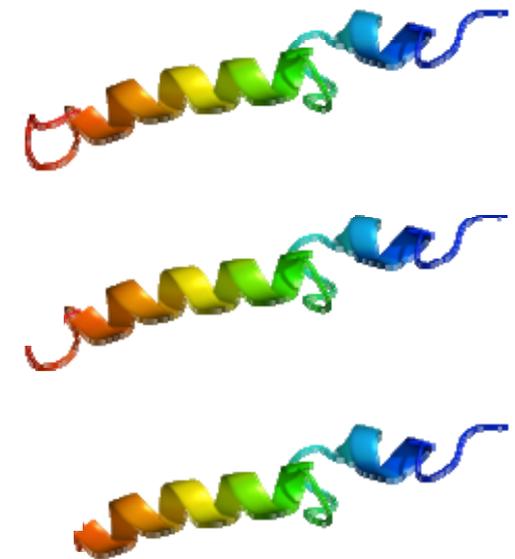


LBA vs LBA

The truncated forms of PTH, [3-84] and [7-84], are present in plasma at similar concentrations as full length PTH [1-84]

- They cross-react with the detection antibodies directed to amino acids 1-34 and 1-12, which leads to overestimation of PTH concentrations
- They don't cross-react with the detection antibody directed to amino acids 1-6

The nature of the capturing reagent and the exact epitope to which it binds determine the concentration result



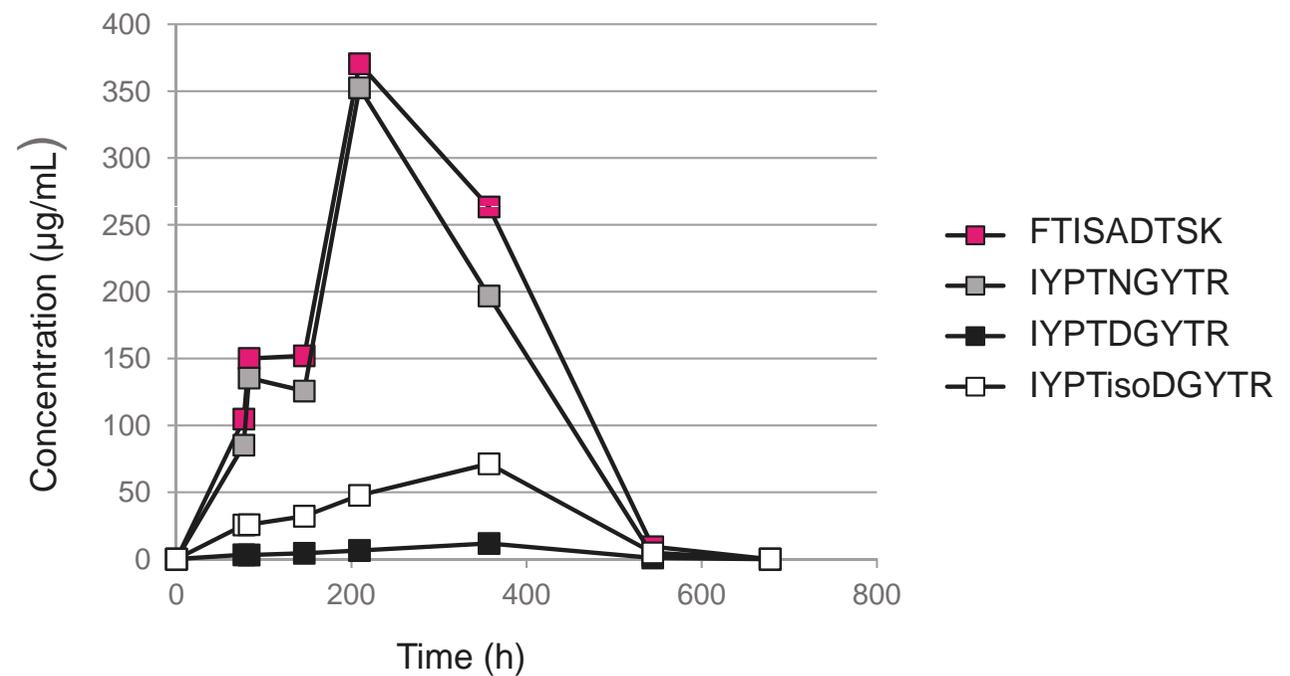


LC-MS vs LC-MS

Influence of **signature peptide**: stable vs unstable

Example: trastuzumab

Concentrations in patient plasma lower with unstable signature peptide



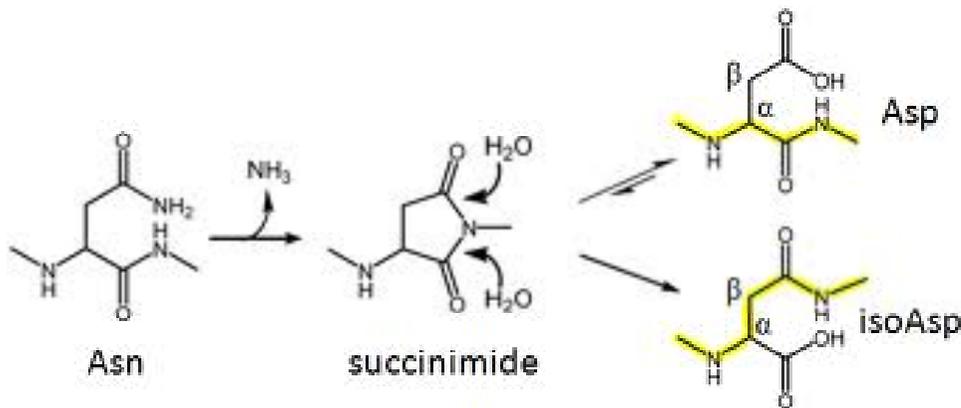
Bults et al, submitted for publication



LC-MS vs LC-MS

Stable part of the protein is not metabolized (**total** trastuzumab),

Unstable part is *in vivo* deamidated and decreases in concentration (**non-deamidated** trastuzumab)



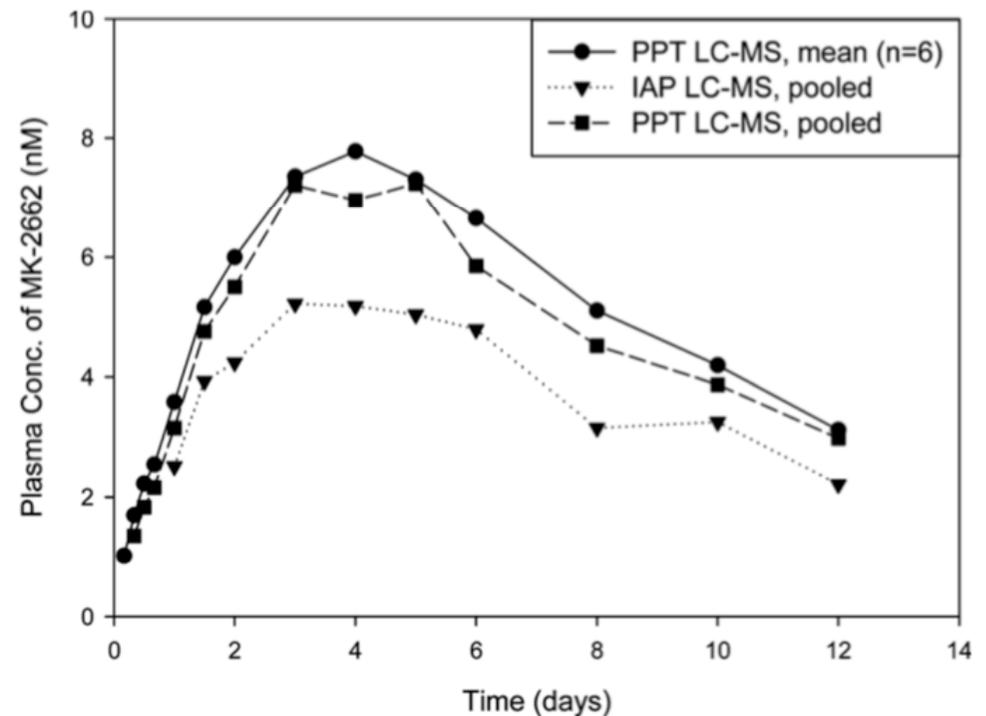


LC-MS vs LC-MS

Influence of **extraction approach**

Example PEGylated protein:

Digestion without further treatment gives up to 35% higher concentrations than immunocapture (PEG-directed) plus digestion



Xu et al, *Anal. Chem.* 82 (2010) 6877-6886

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LC-MS vs LC-MS

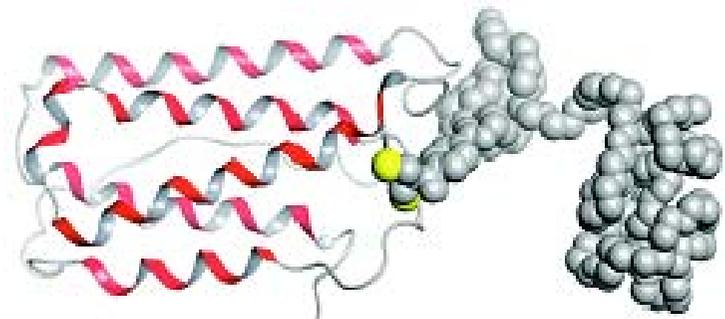
In vivo dePEGylation to a product that has the same signature peptide:

HAibDGTFTSDYSKYLDSRRAQDFVQWLMNTKRNNIAC-PEG →

HAibDGTFTSDYSKYLDSRRAQDFVQWLMNTKRNNIAC

Direct digestion: concentration of PEGylated plus dePEGylated forms

Immunocapture: concentration of PEGylated form only



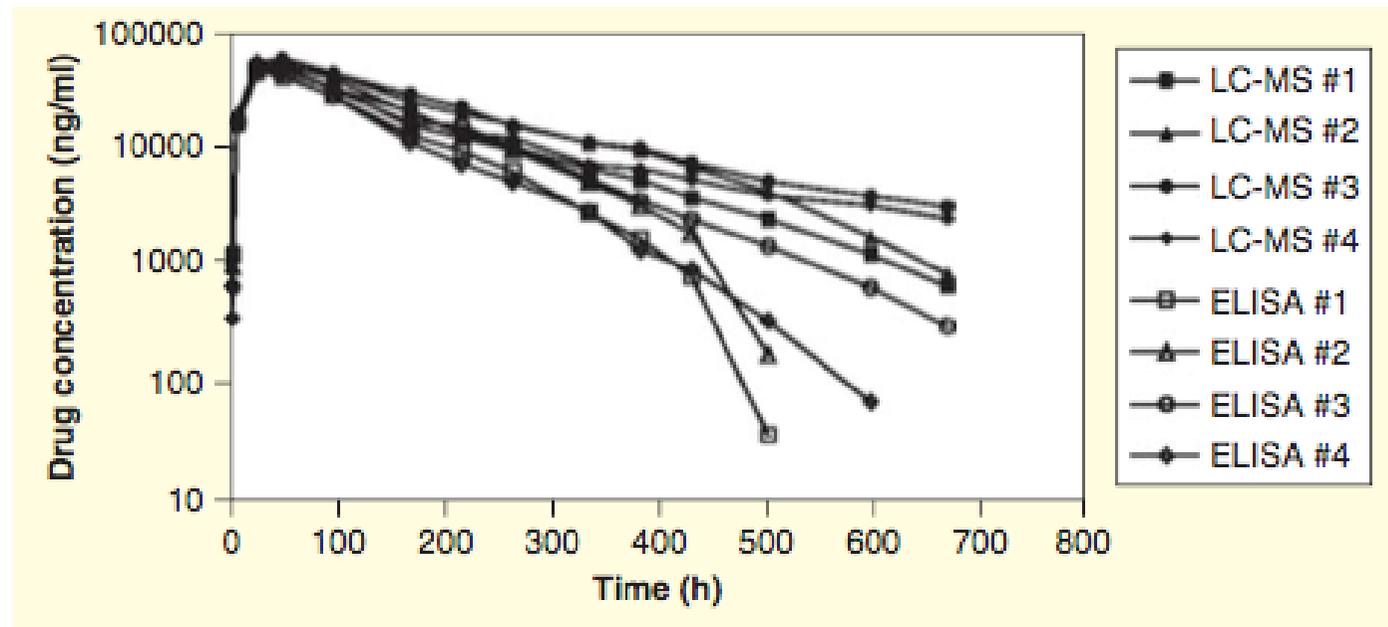


LBA vs LC-MS

Influence of **anti-drug antibodies**

Example: PEGylated protein:

Lower concentrations (up to 8-fold) with ELISA than with LC-MS/MS



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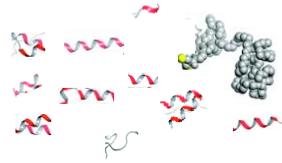
Wang et al, *Anal. Bioanal. Chem.*, 402 (2012) 1229-1239.



LBA vs LC-MS

LC-MS/MS:

- Extraction into isopropanol, digestion



ELISA:

- Capturing with pharmacological target, detection with anti-PEG antibody



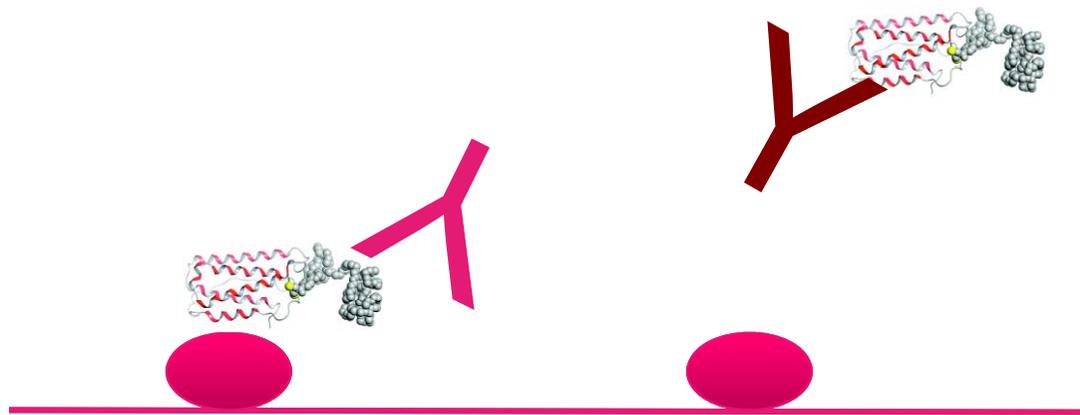


LBA vs LC-MS

Presence of ADAs specific to target binding site was demonstrated

These interfere with the ELISA capturing step and **decrease the detectable concentration**, but do not interfere with extraction and digestion in the LC-MS/MS assay

Note: discrepancy between ELISA and LC-MS/MS only at later time-points



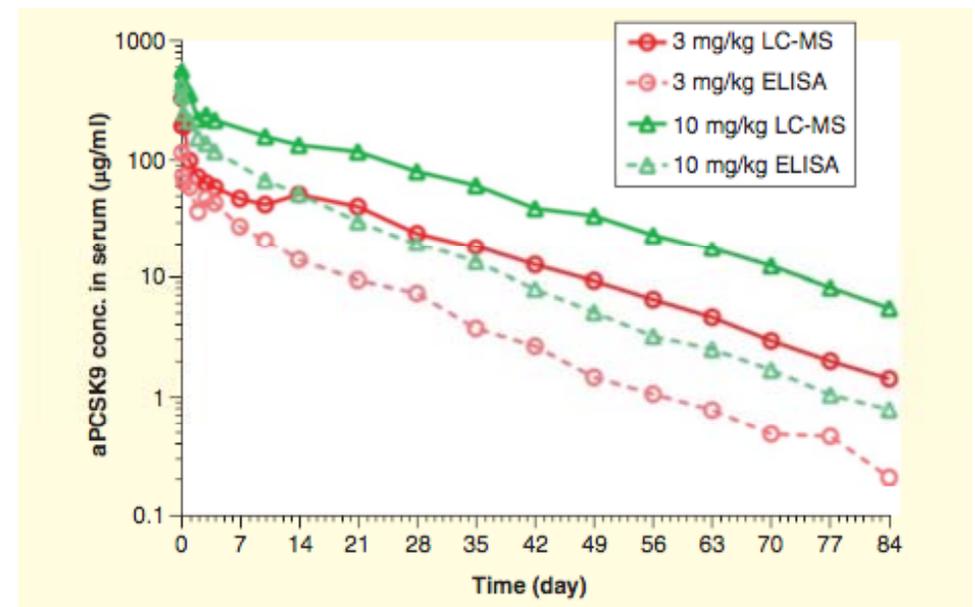


LBA vs LC-MS

Influence of **circulating target**

Example: monoclonal antibody:

Lower concentrations (up to 3-fold) with ELISA than with LC-MS/MS



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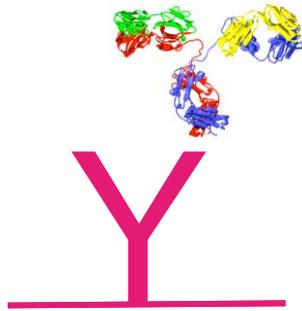
Zhang et al, *Anal. Chem.*, 86 (2014) 8776-8784.



LBA vs LC-MS

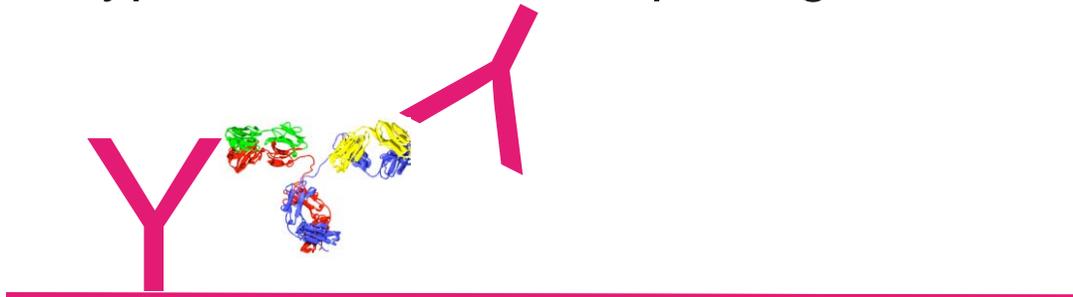
LC-MS/MS:

- Immunocapture with anti-Fc antibody, digestion



ELISA:

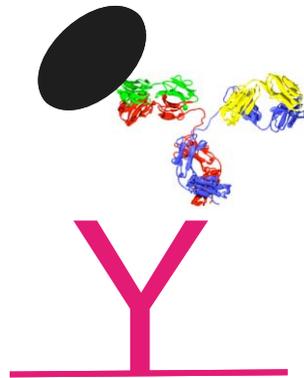
- Anti-idiotypic antibodies for capturing and detection



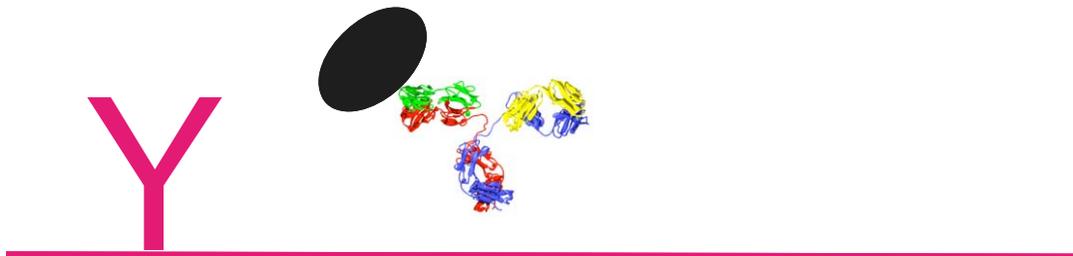


LBA vs LC-MS

Presence of target does not interfere with the LC-MS capturing step and digestion



Presence of target interferes with the ELISA capturing and/or detection step and **decreases the detectable concentration**



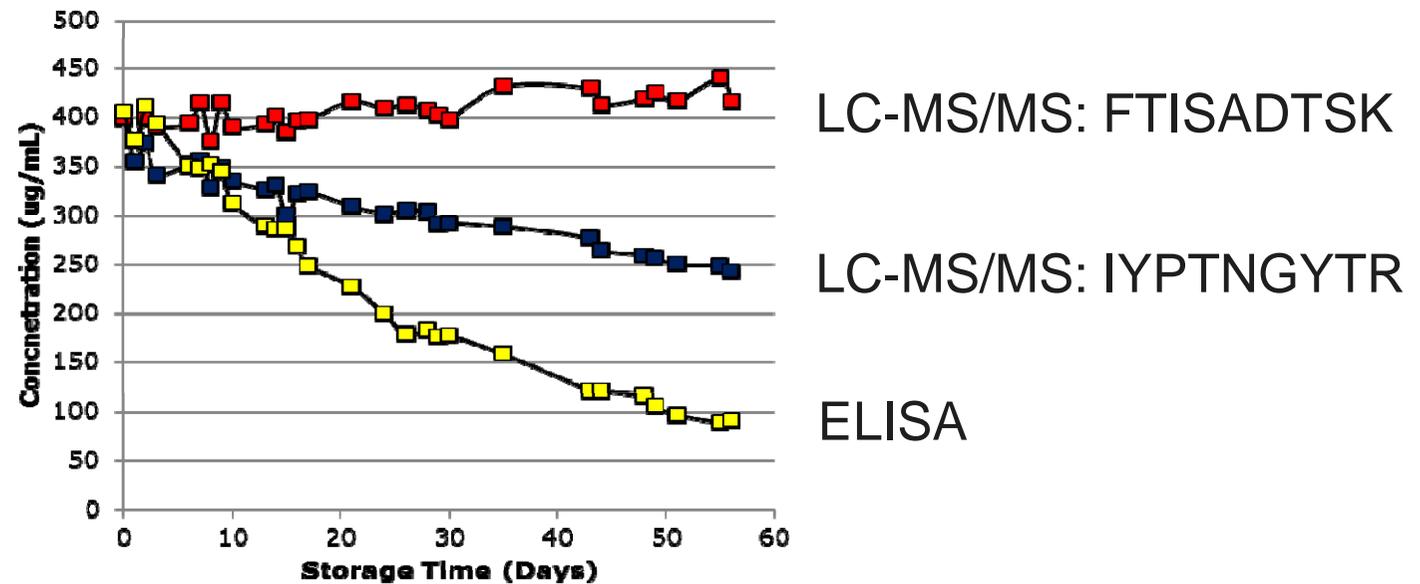


LBA vs LC-MS

Influence of structural modification

Example: trastuzumab:

Lower concentrations with ELISA than with LC-MS/MS



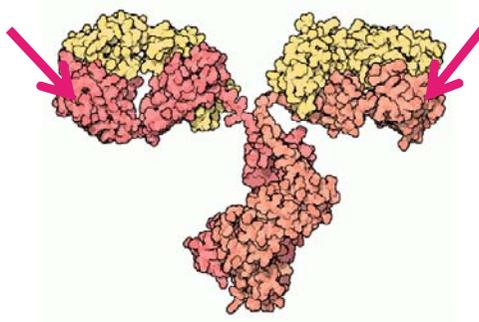
Bults et al, submitted for publication



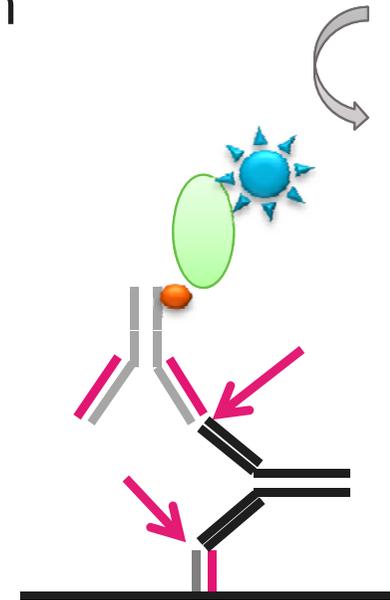
LBA vs LC-MS

Epitope / signature peptide is deamidated

This leads to a decrease in concentration for LC-MS/MS and a two-fold faster decrease for ELISA due to lost recognition



LC-MS/MS (YPTNGYTR):
Non-deamidated: full response
Singly deamidated: **half response**
Doubly deamidated: no response



ELISA:
Non-deamidated: full response
Singly deamidated: **no response**
Doubly deamidated: no response



Conclusion

- Proteins are large, complex molecules which often occur **in multiple structural forms** and may be **bound to other proteins** in a sample
- Quantification by LC-MS and LBA is based on only **a smart part** of a protein structure and disregards a major part of the analyte molecule
- The **design of an analytical method** determines whether or not a structural modification or binding event is picked up
- “The” protein concentration is not a meaningful result, unless it is defined which molecular property the method responds to.



Acknowledgment

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