Why do results for proteins differ?

A literature evaluation of different bioanalytical platforms

21 June 2017

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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

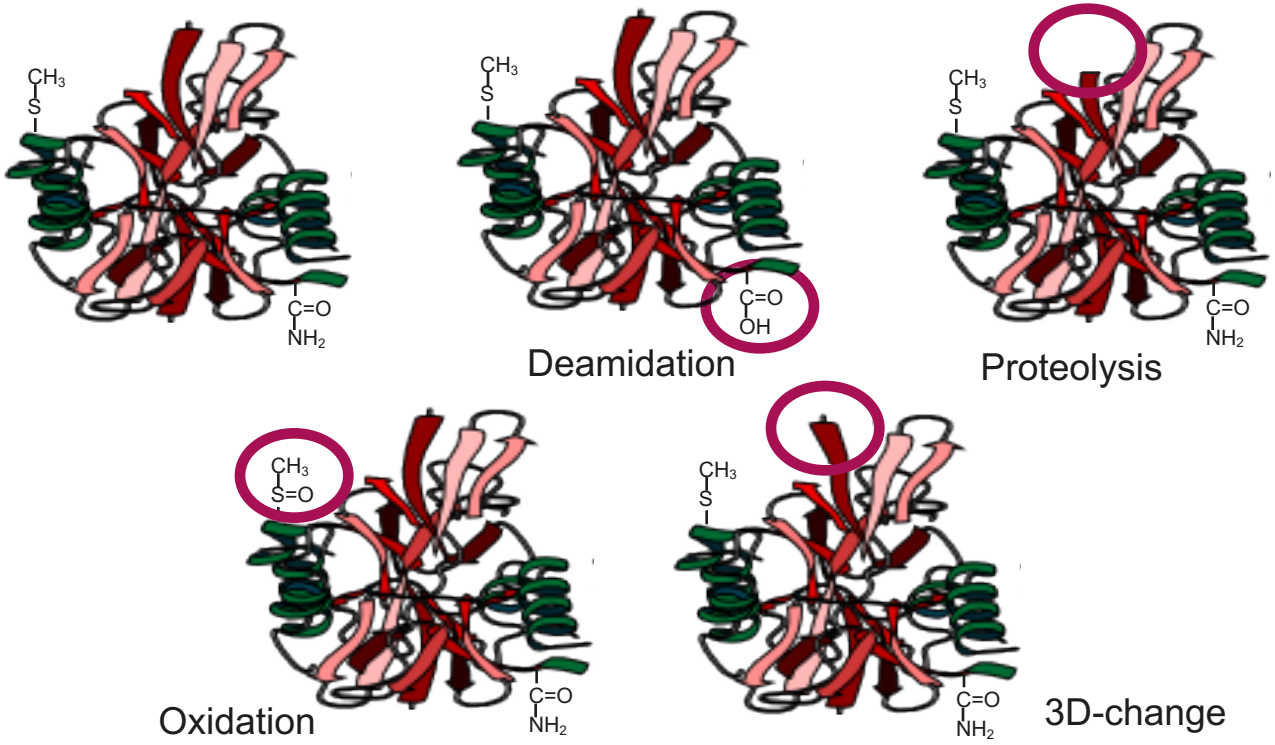
Differences usually are not convincingly explained

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

Proteins vs small molecules

Small molecules are well-defined, single species

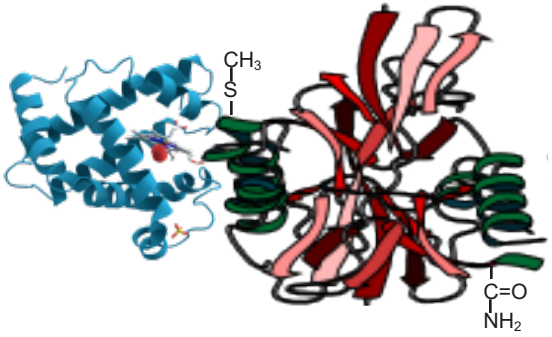
Proteins are often heterogeneous and may occur in several different (iso)forms



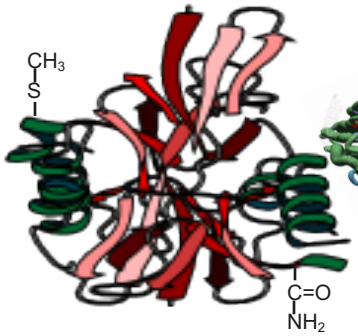
Proteins vs small molecules

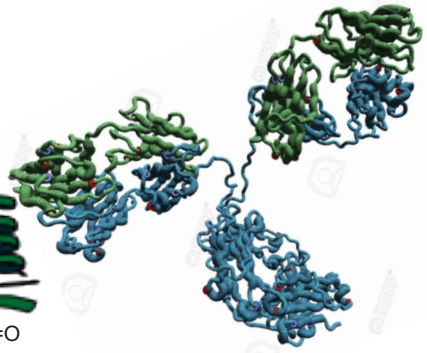
Small molecules are often loosely bound to plasma proteins and the binding is easily broken during analysis

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

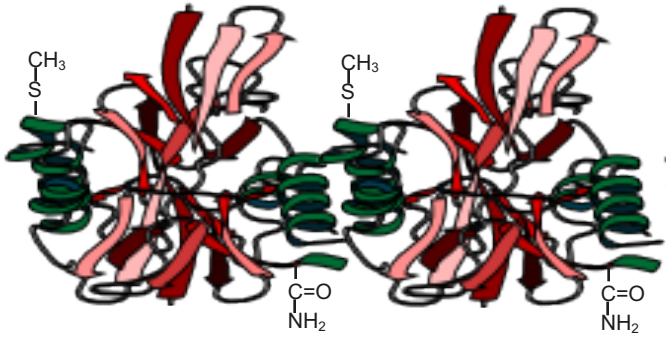


Target-bound





ADA-bound

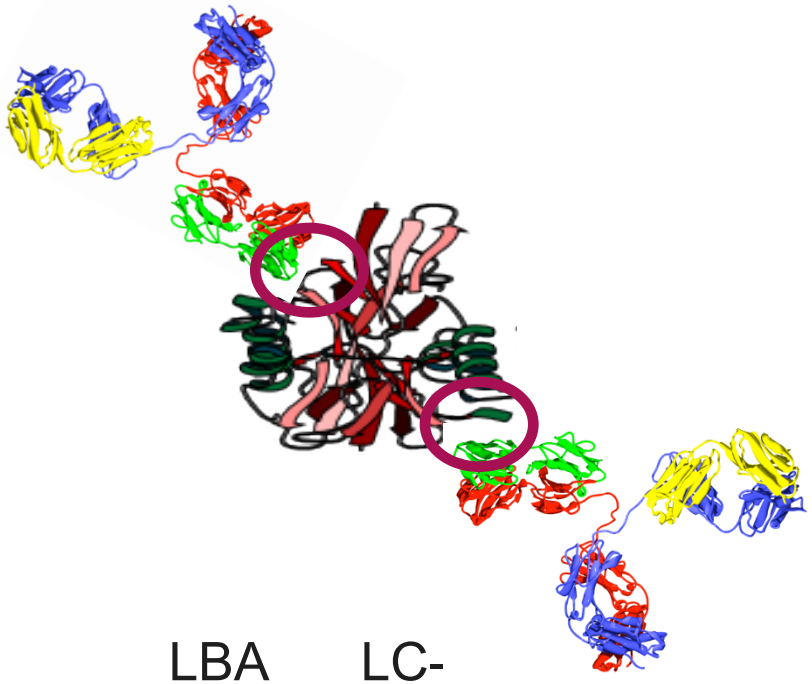


Aggregated

Protein quantification

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

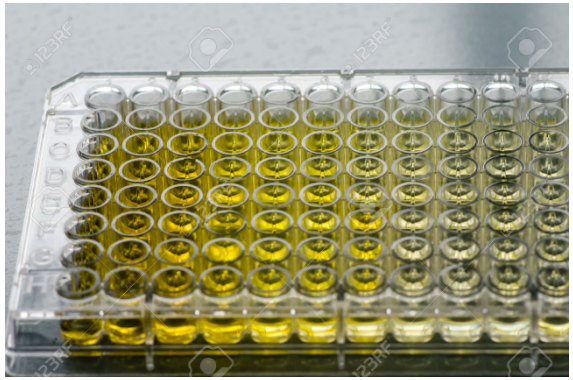
Depending on the analytical mechanism, modifications and/or binding events may or may not be picked up



LBA LC-

Some literature examples

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

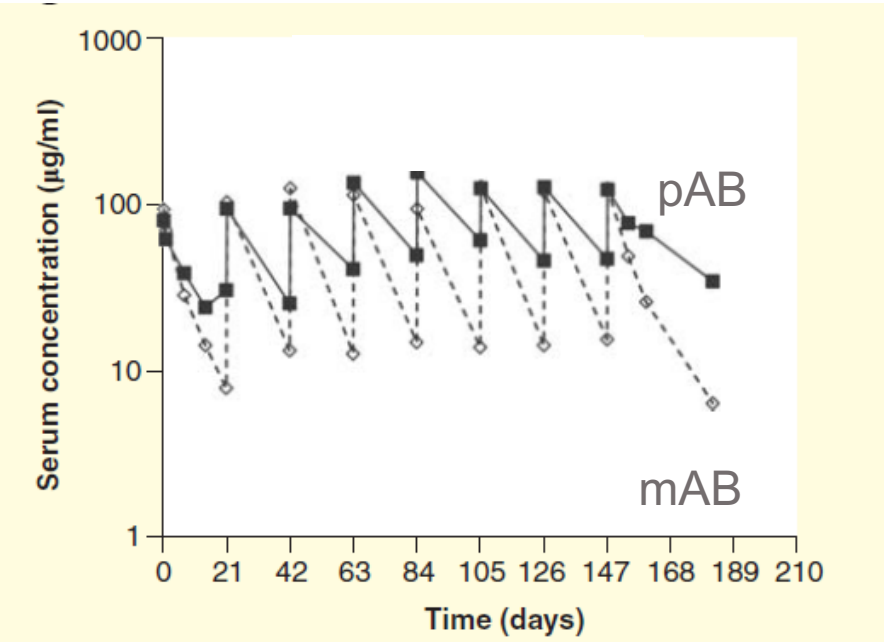


LBA vs LBA

Influence of capturing antibody: monoclonal vs polyclonal

Example: ocrelizumab

Concentrations in patient plasma ± 2–fold lower with mAB than with pAB



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

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

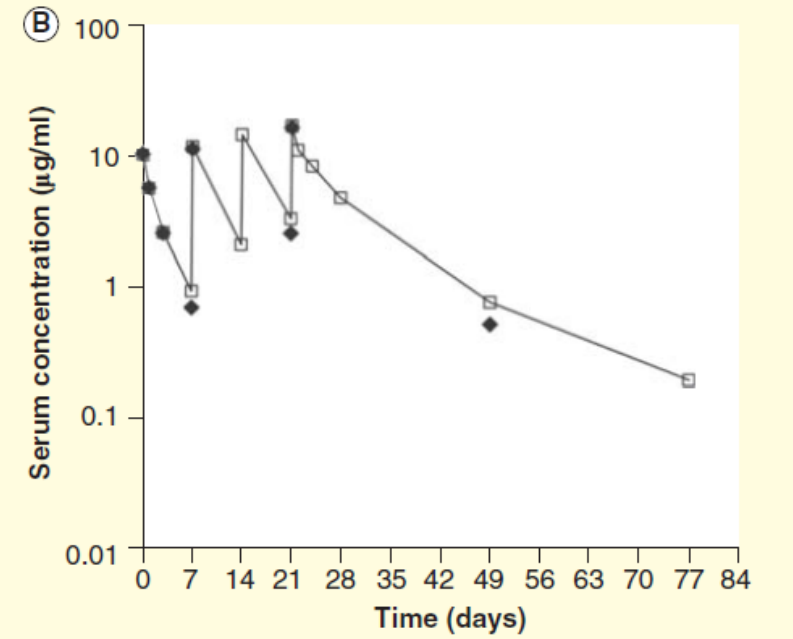
Polyclonal capturing antibody binds to multiple epitopes

- 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

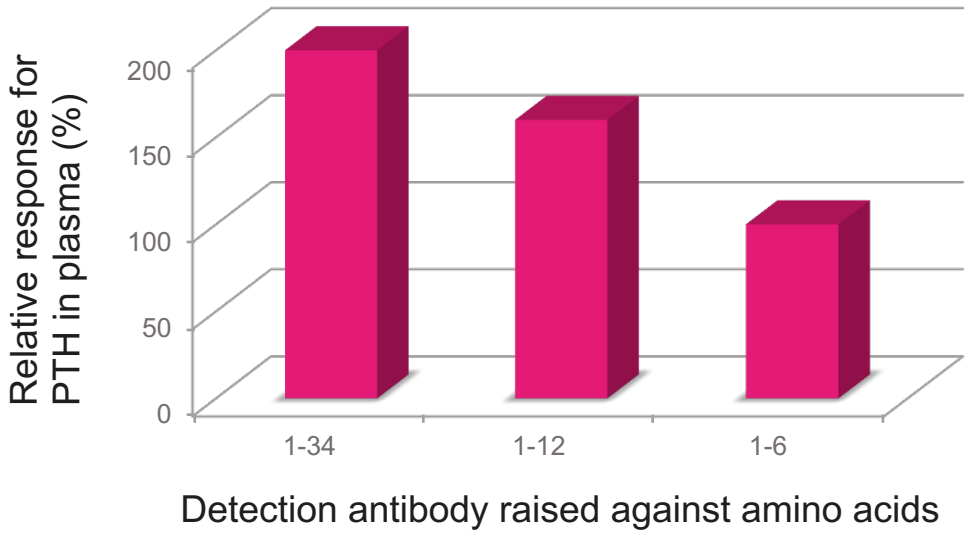


LBA vs LBA

Influence of detection antibody: different specificities of commercial ELISAs

Example: PTH

Concentrations depend on detection antibody specificity



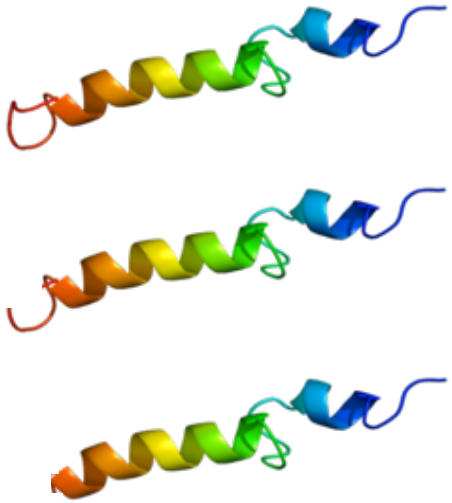
Sukovaty et al, J. Pharm. Biomed. Anal. 42 (2006) 261-271

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 detection reagent and the exact epitope to which it binds determine the concentration result



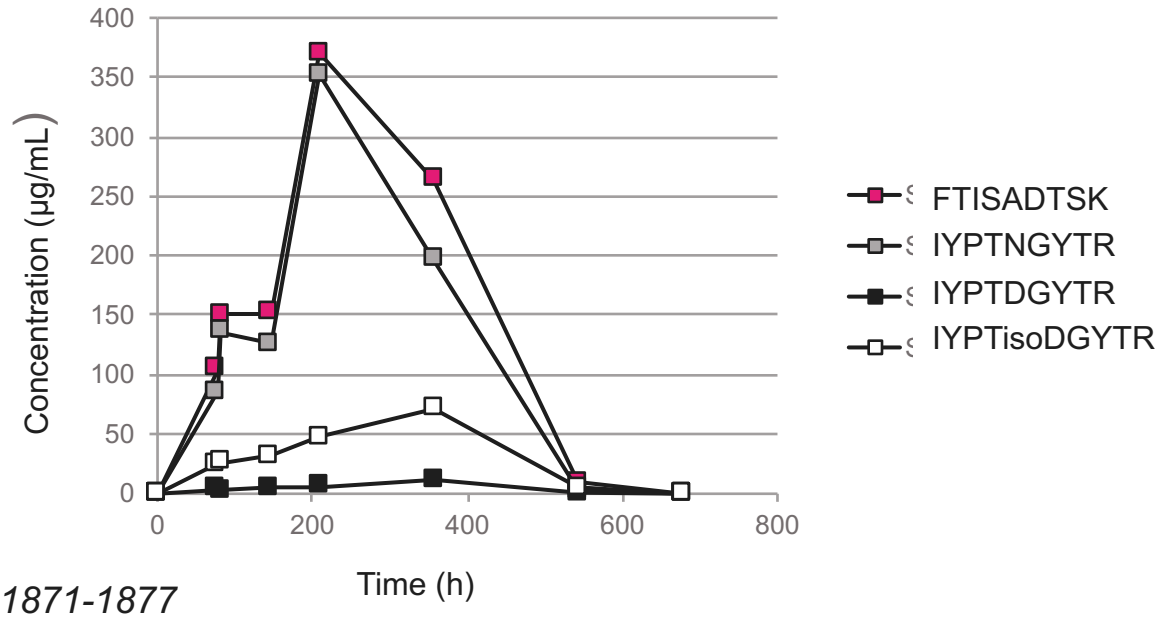
- LBA vs LBA
- LC-MS vs LC-MS
- LBA vs LC-MS

LC-MS vs LC-MS

Influence of signature peptide: stable vs unstable

Example: trastuzumab

Concentrations in patient plasma lower with unstable signature peptide

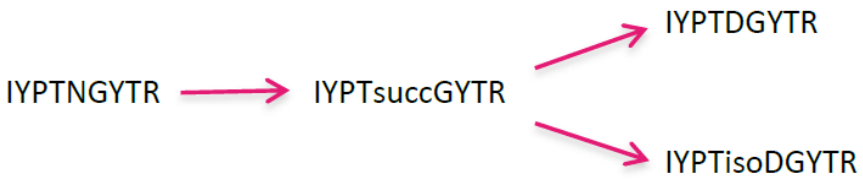


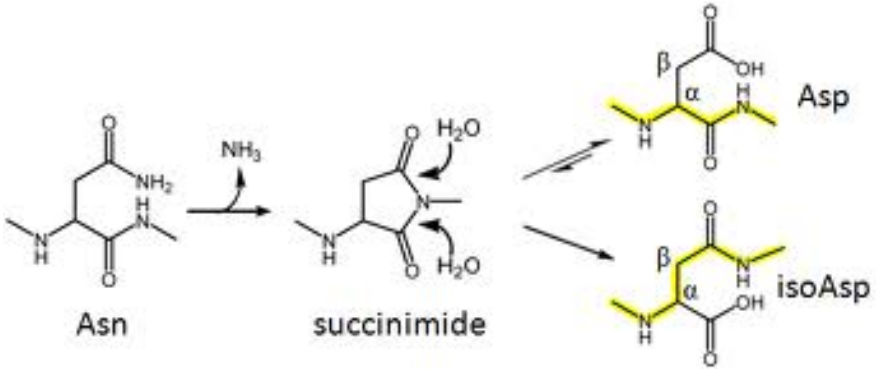
Bults et al, Anal.Chem. 88 (2016) 1871-1877

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 (active?) trastuzumab)



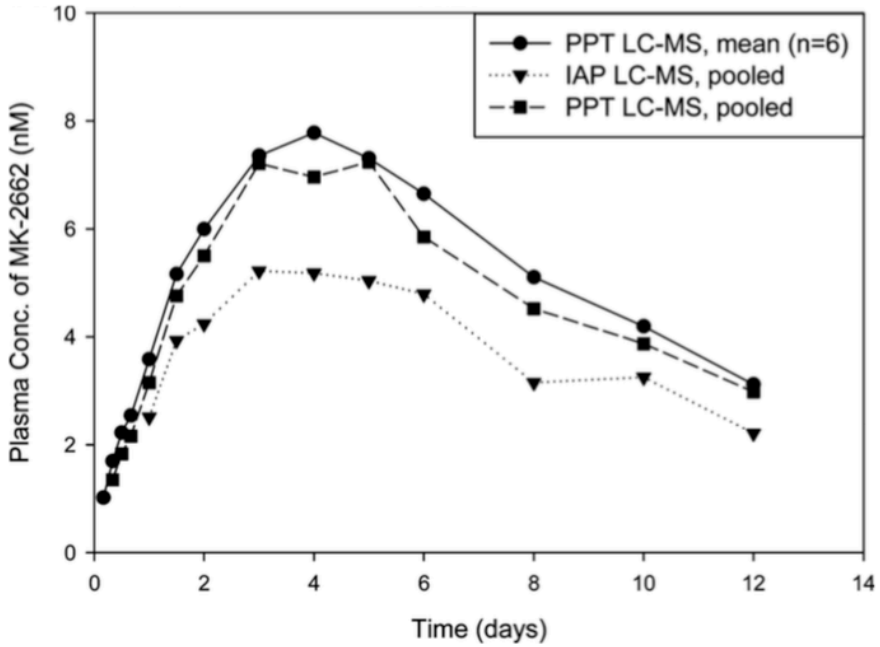


LC-MS vs LC-MS

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

LC-MS vs LC-MS

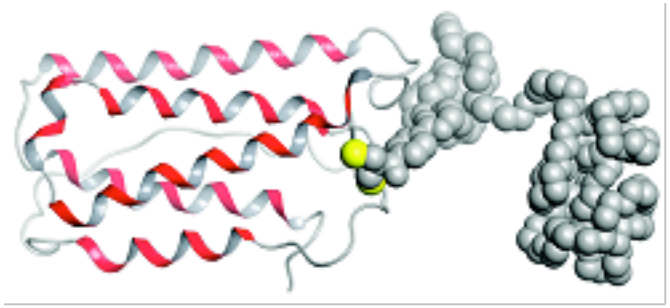
In vivo dePEGylation to a product that has the same signature peptide:

HAibDGTFTSDYSKYLDSRRAQDFVQWLMNTKRNNIAC-PEG →

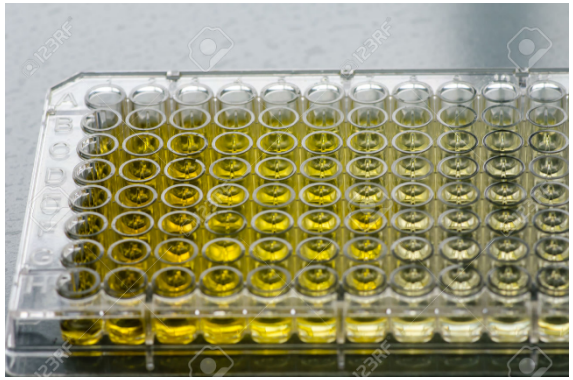
HAibDGTFTSDYSKYLDSRRAQDFVQWLMNTKRNNIAC

Direct digestion: concentration of PEGylated plus dePEGylated forms

Immunocapture: concentration of PEGylated form only



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

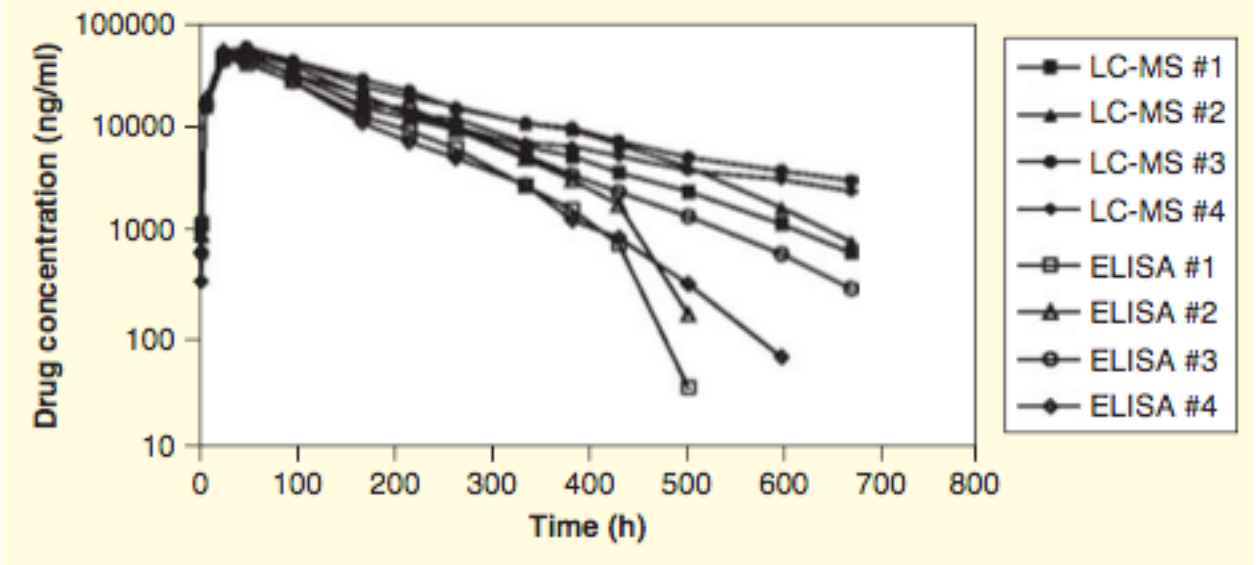


LBA vs LC-MS

Influence of anti-drug antibodies

Example: PEGylated protein:

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

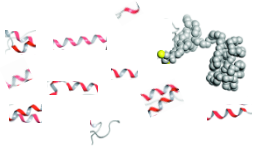


Wang et al, Anal. Bioanal. Chem., 402 (2012) 1229-1239.

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

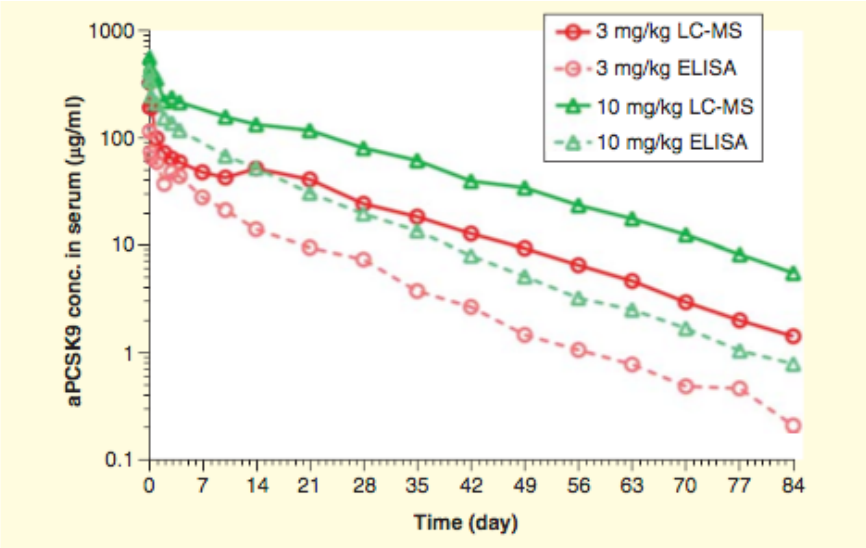
Note: discrepancy between ELISA and LC-MS/MS only at later time-points

LBA vs LC-MS

Influence of circulating target

Example: monoclonal antibody:

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



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

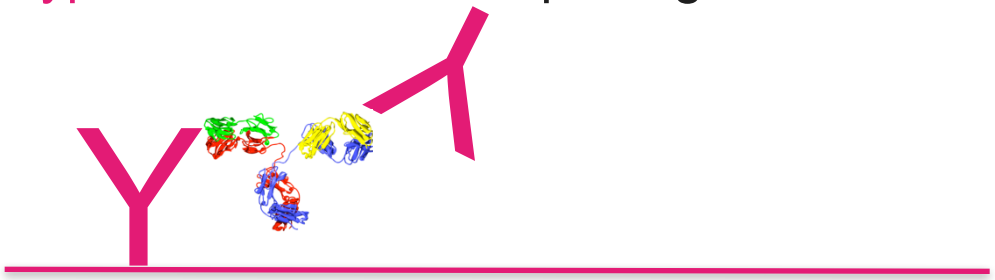
LBA vs LC-MS

LC-MS/MS:

- Immunocapture with anti-Fc antibody, digestion

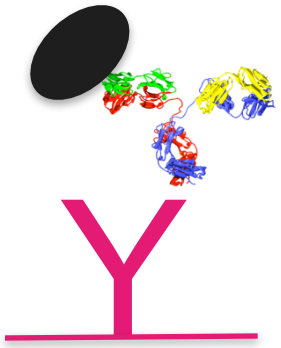
ELISA:

- 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



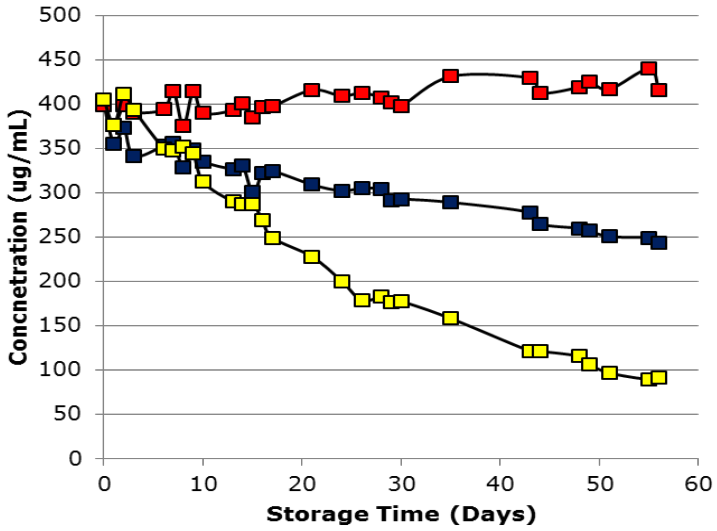
Presence of target interferes with the ELISA capturing and/or detection step and decreases the detectable concentration

LBA vs LC-MS

Influence of structural modification

Example: trastuzumab:

Lower concentrations with ELISA than with LC-MS/MS



LC-MS/MS: FTISADTSK

LC-MS/MS: IYPTNGYTR

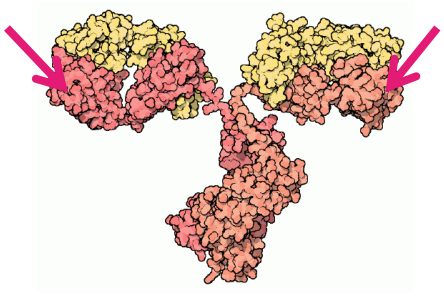
ELISA

Bults et al, Anal.Chem. 88 (2016) 1871-1877

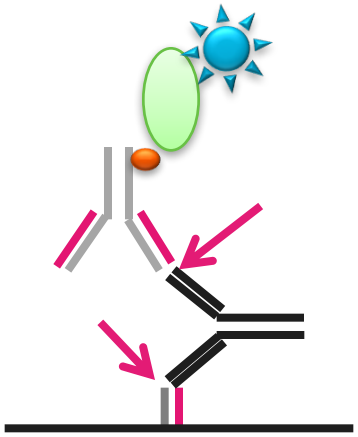
LBA vs LC-MS

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.