Development of a hybrid method for simultaneous quantification of two near identical proteins in plasma

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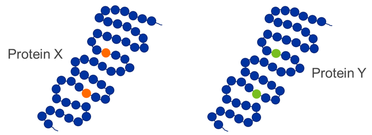
1- ABSTRACT

The number of biotherapeutic drugs is increasing yearly. Traditionally these molecules have been detected using ligand-binding assays. However, hybrid LC-MS/MS methods, which rely on immune-capture of the molecule of interest prior to LC-MS/MS analysis, are becoming increasingly popular because of their power to enrich samples for the analyte, increasing both selectivity and sensitivity.
Here we present data on the development of a method for simultaneous quantification of two near identical proteins in plasma samples. A ligand binding or functional assay would not be suitable, due to the near identical nature of the analtyes. Hence, hybrid LC-MS/MS was chosen as the way forward.

2- INTRODUCTION

Protein X is a 63 kDa endogenous protein

Protein Y is a modified form of Protein X – differs by two amino acids



Goal: Develop assay for simultaneous quantification of both proteins in plasma

3- CHALLENGES

Sensitivity

LLOQ as low as possible – low ng/mL (below 100 pM) required

Sample volume below 100 µL

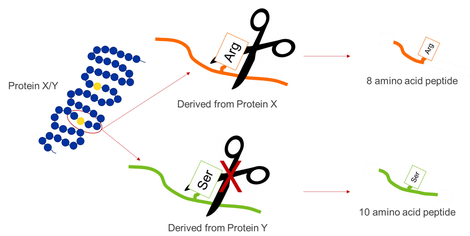
Selectivity

High background likely from plasma

Protein X and Protein Y display identical structures and functions

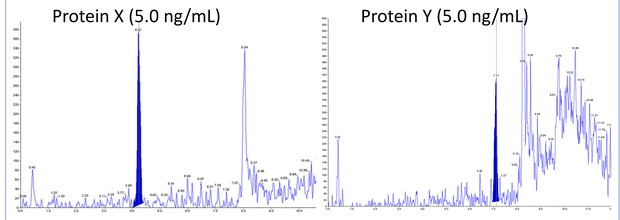
4- IN SILICO DIGESTION

Arg-Ser mutation results in different tryptic peptide



5- LLOQ IN BUFFER

LLOQ of 5.0 ng/mL (79 pM) possible for both proteins

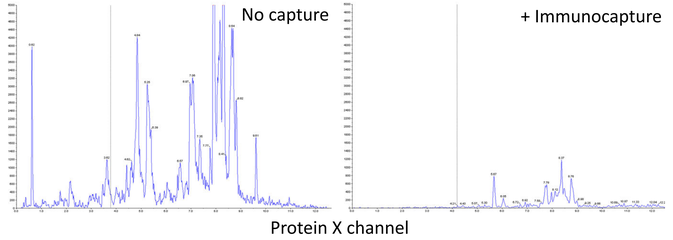


6- SAMPLE PREPARATION

- Sample volume 50 µL
- Commercially available biotinylated antibody combined with Streptavidin magnetic beads
  → 5.0 µg antibody per capture
- Samples treated with TCEP and Iodoacetamide
- Trypsin digestion
- Injection volume 20 µL

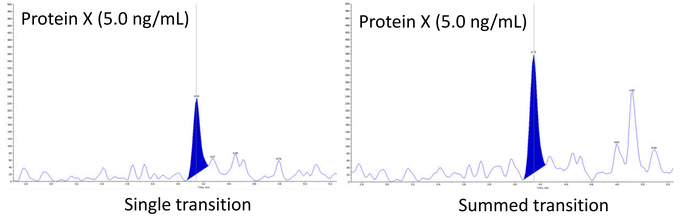
7- GAIN FROM HYBRID ASSAY

More than 10 fold reduction in background for both surrogate peptides



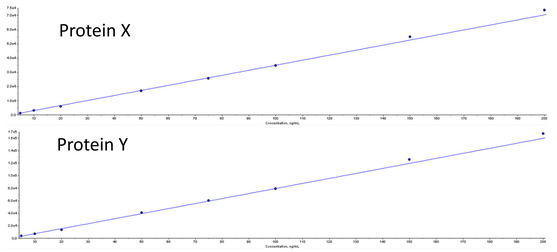
8- SUMMING OF TRANSITIONS

Summing of transitions results in ~60% increase in response



9- LINEARITY

Response for both proteins linear between 5.0-200 ng/mL



10- PRECISION AND ACCURACY

Protein X (ng/mL)			
Sample	LQC	MQC	HQC
	15.0	75.0	175
1	17.3	82.7	206
2	15.4	71.6	194
3	15.1	85.9	192
4	14.4	79.9	199
Mean	15.55	80.0	198
SD	1.24	6.13	6.24
%Bias	3.67	6.70	13.0
%CV	7.97	7.66	3.15
n	4	4	4

Protein Y (ng/mL)			
Sample	LQC	MQC	HQC
	15.0	75.0	175
1	16.2	82.6	200
2	14.6	68.3	196
3	16.4	76.6	192
4	14.4	75.4	193
Mean	15.4	75.7	195
SD	1.05	5.87	3.59
%Bias	2.67	0.967	11.6
%CV	6.79	7.75	1.84
n	4	4	4

11- INTERNAL STANDARD

Data generated so far without internal standard

Stable labelled version of protein not commercially available

Stable labelled peptide chosen as internal standard

Based on surrogate peptide from Protein Y

Added after affinity purification step

12- CONCLUSIONS

Here, we have successfully developed a 2in1 hybrid LC-MS assay for the quantification of two proteins that differ by only two amino acids in plasma samples. The time spent on optimizing the experimental setup (antibody incubation step, reducing agent etc) was crucial for success while summing of the transitions significantly helped to improve sensitivity. The resulting method is sufficiently sensitive and accurate to support pre-clinical trials.

Hybrid LC-MS assays are a valuable addition to ligand-binding assays for the analysis of biotherapeutics.

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