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LC-MS/MS bioanalysis of peptides – How to manage non specific binding?

Anders Sonesson

Ferring Pharmaceuticals A/S
Bioanalysis Department
Copenhagen, Denmark

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Outline of this presentation

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- Background
  - Challenges in LC-MS/MS bioanalysis of peptides
  - Non-specific binding (NSB)
  - Consideration for peptides
- Strategies for minimising NSB in LC-MS/MS bioanalyis of peptides
- Two case studies where NSB have hampered bioanalytical method development
- Lessons learned
- Acknowledgement

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Challenges in LC-MS/MS bioanalysis of peptides

- Formation of multiple-charged ions
- Extensive fragmentation in MS/MS
- Adsorption problems → NSB issue
- Chromatographic issues → potential NSB issue

Non-specific binding (NSB)

- **Definition:** Non-specific binding – analyte losses that occur as a result of binding to any surface to which the analyte is exposed
- **Peptides:** Often demonstrate greater NSB issues than small molecules
- **Cause:** Physical/chemical properties e.g. Van der Waals interactions, ionic interactions
- **Problems:** Sensitivity and robustness. Most troublesome at low concentrations.

Non-specific binding

- NSB can occur to various material throughout the analytical procedure, e.g.
  - Vials
  - Plates

  } In pure solution and in evaporation/reconstitution

  - Tubing
  - Columns
  - Injection valves

  } In chromatography

Considerations for peptides

- Charged peptides
  - greater solubility in aqueous solution
  - less adsorption to plastics
- Uncharged peptides
  - can adsorb strongly to plastics
  - organic solvent may enhance solubility and prevent adsorption

- Need to be investigated during method development, generic approach will not work

Strategies for minimising NSB

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1. Use high concentration stock, spike into matrix and perform serial dilution in the matrix
2. Choice of material in tubes etc.
   - Evaluate glass or polypropylene vessel i.e. high quality/low binding polypropylene material or glass or glass coated material
3. Use additives
   - Organic solvent
   - Protein (e.g. HSA or BSA)
   - Surrogate peptide
   - Detergent (e.g. Triton-X, Tween or C_9-Glu)

Case 1- Sample plate material and use of additives

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20 AA charged peptide, Mw ~ 2400 Da

Start of non-clinical program

Evaluation of sample container material: glass or polypropylene (PP) 96-well plates

- Better precision with PP compared to glass

Dissolve peptide in ACN:5 mM Ammonium acetate (10:90) with and without additives (0.1% HSA + 0.01% Triton-X)

- With additives gave ~ 25% higher response in peak area

Case 1- Sample plate material and use of additives

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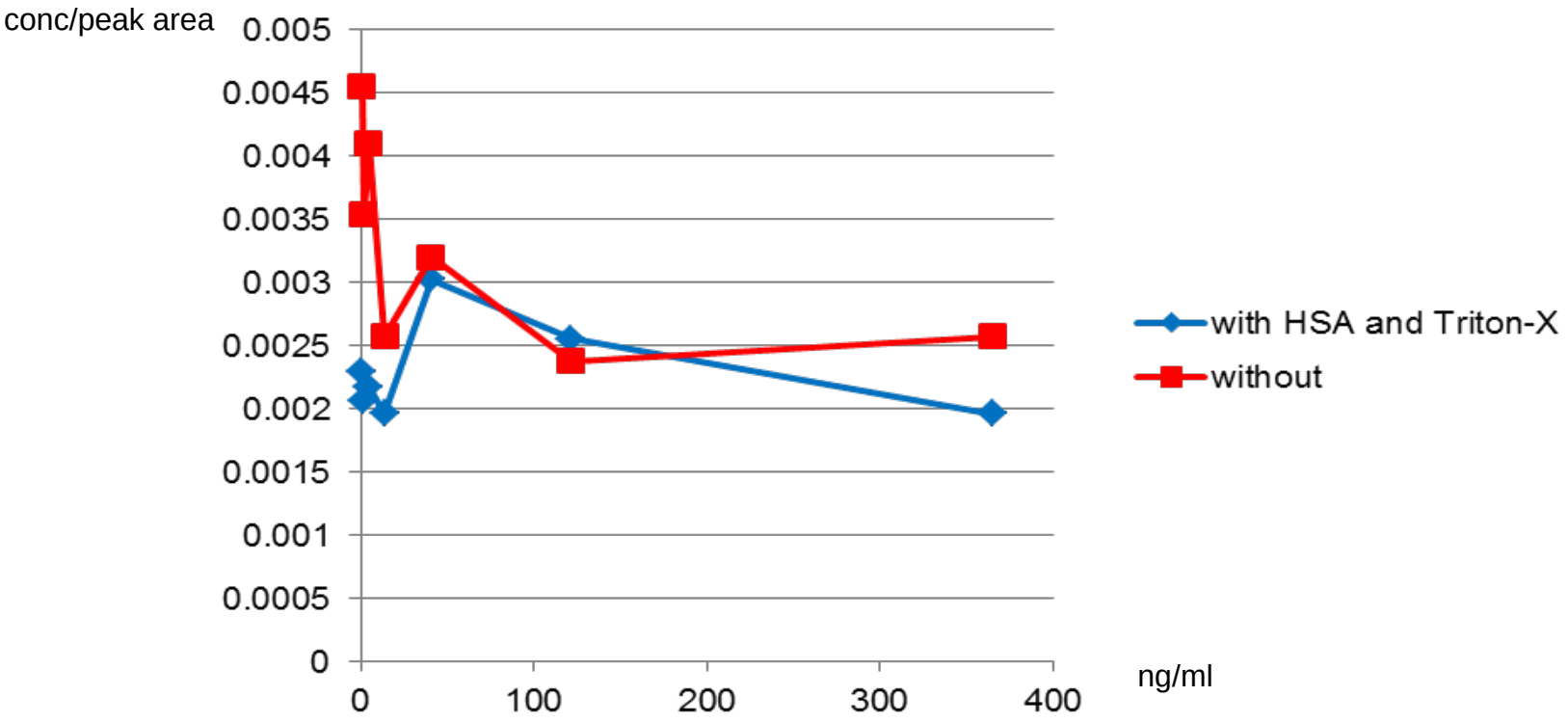
- Less absorption with additives (0.1% HSA + 0.01% Triton-X) as judged by looking at response factors



- Better precision and accuracy with additives (0.1% HSA + 0.01% Triton-X)

Case 1- Method for rat plasma validated

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Method for determination of peptide in rat plasma successfully validated

Occasion	**Nominal concentration (ng/mL)**	**0.50**	**1.50**	**7.91**	**106**
Validation run 1	Accuracy (% bias)	18	15	6	6
	Precision (%CV)	5	4	2	1
Validation run 2	Accuracy (% bias)	-6	0	-5	-3
	Precision (%CV)	20	6	6	3
Validation run 3	Accuracy (% bias)	17	16	8	7
	Precision (%CV)	6	7	4	1

Study samples analysed successfully.

Case 1 – start of validation for dog plasma

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The same method applied for dog plasma, same sample volume 50 µl

Occasion	Nominal concentration (ng/mL)	5.00	15.00	79.10	1060
QC test run	Accuracy (% bias)	-4	-5	3	-8
	Precision (%CV)	8	8	11	5
Validation run 1	Accuracy (% bias)	-8	-12	-13	-24
	Precision (%CV)	3	7	9	6
Validation run 2	Accuracy (% bias)	4	-14	-17	-20
	Precision (%CV)	19	10	8	9
Validation run 3	Accuracy (% bias)	-23	-10	-11	-16
	Precision (%CV)	4	11	5	4

→ Validation stopped – back to method development

Case 1 – start of validation for dog plasma

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According to EMA and FDA Guidelines on bioanalytical method validation,

The calibration curve should preferably be prepared using freshly spiked samples when appropriate stability data is not available.

→ can cause problems with bias especially for peptides if not handled carefully.

Case 1 - development for method in dog plasma

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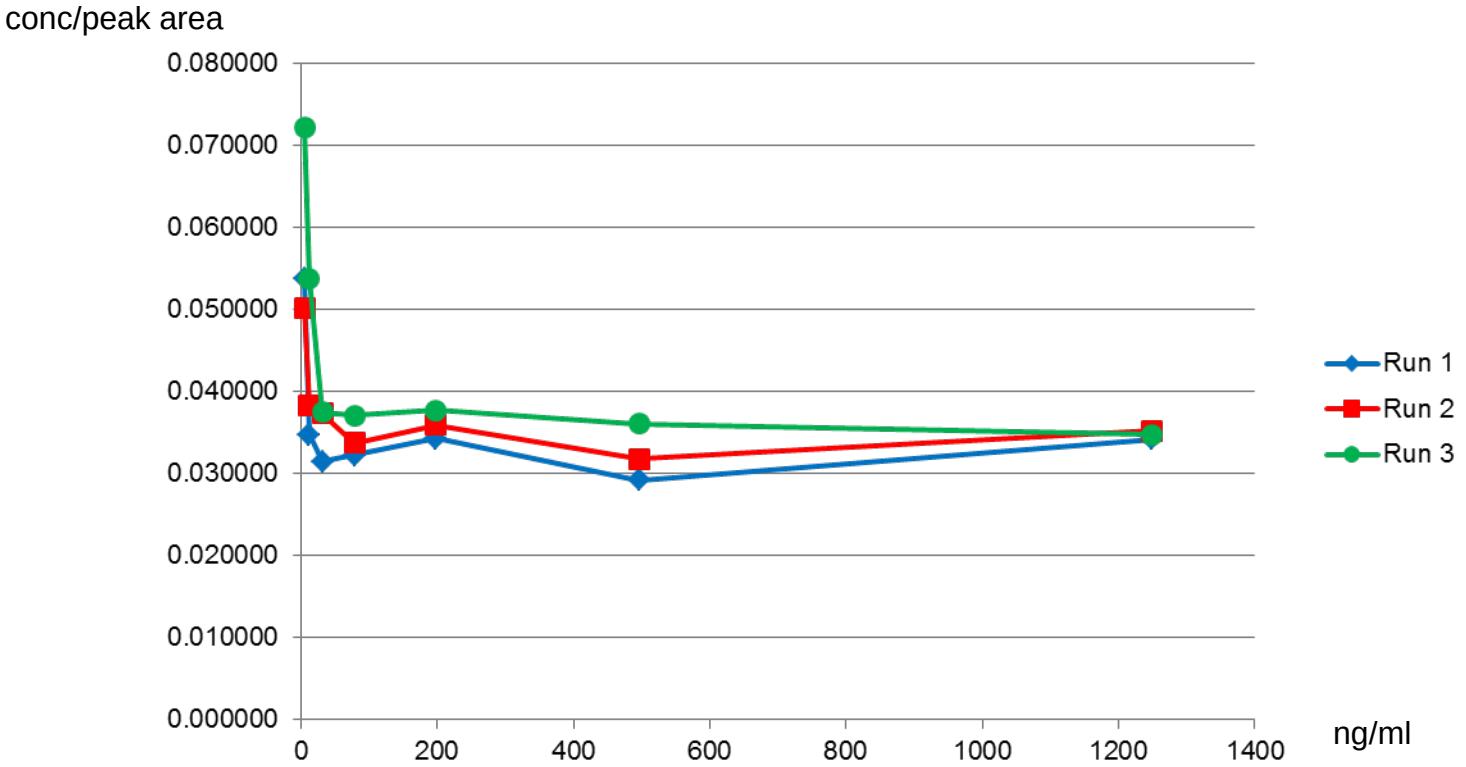
The CAL and QC samples were prepared by spiking from dilutions in ACN:5 mM Ammonium acetate (10:90) with additives (0.1% HSA + 0.01% Triton-X)

Response factors for CAL samples



Higher response factor at low concentrations indicted issue with NSB, affected slope on calibration curve with $1/x^2$ weighting factor.

Case 1 - development for method in dog plasma

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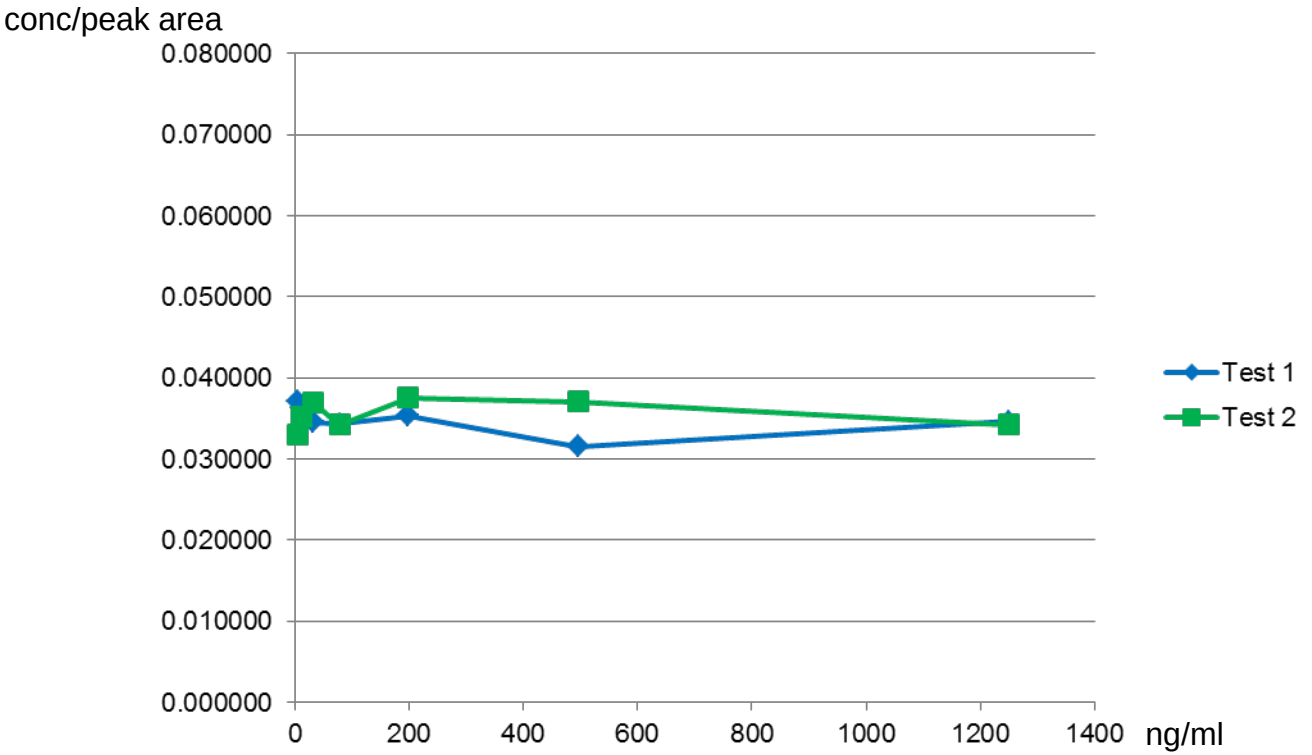
Preparation of CALs and QC:s from high concentration stock (5 times higher) with increased HSA concentration (from 0.1 % to 0.5 % HSA) and dilution in sample matrix (dog plasma).

Response factors for CAL samples



Case 1 - development for method in dog plasma

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Validation restarted

Occasion	Nominal concentration (ng/mL)	5.00	15.00	79.10	1060
Validation run 1	Accuracy (% bias)	8	3	-2	3
	Precision (%CV)	5	3	3	2
Validation run 2	Accuracy (% bias)	-7	-2	3	8
	Precision (%CV)	10	8	5	4
Validation run 3	Accuracy (% bias)	8	9	4	13
	Precision (%CV)	4	9	4	4

Validation successful for dog plasma

Case 2

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- 9 AA peptide, MW ~ 1000 Da
- Method successfully validated in plasma for three animal species + human (LLOQ - 5 pg/mL)
- However, problem occurred for fourth animal species + human LTS occasion

Case 2

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- Sheep plasma, 0.1 – 25 ng/mL
- 0.1% HSA and 0.01% Triton-X as additive in stock solution

Sample	Response per ng/mL
CAL 1	0.016
CAL 2	0.018
CAL 3	0.031
CAL 4	0.039
CAL 5	0.101
CAL 6	0.092
CAL 7	0.097

- Found to be related to PP plates used for preparation of calibration samples, from same manufacture as used for previous studies!

Case 2

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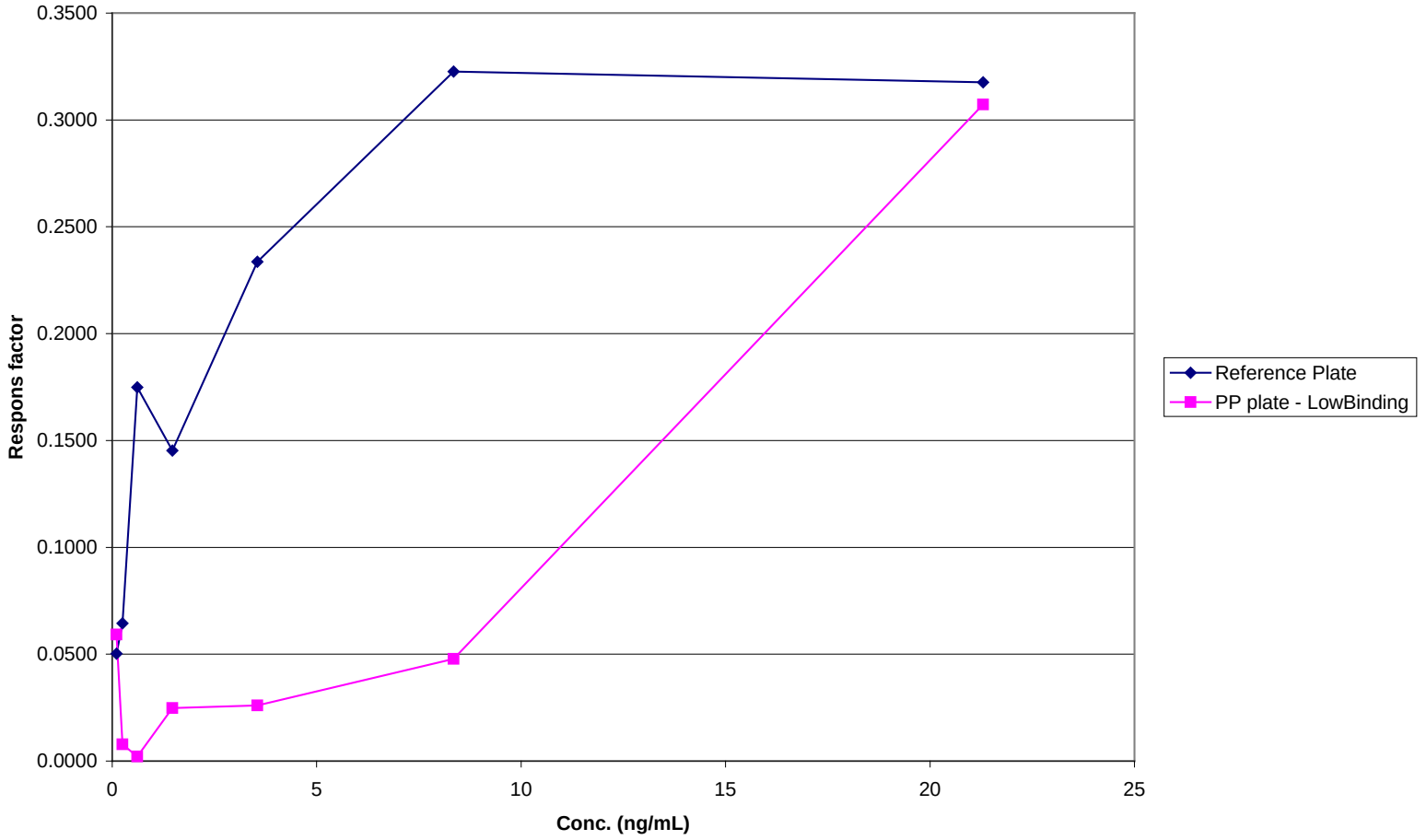

Severe NSB also found when using specific low binding PP plate, worse than in our reference PP plate!

Case 2

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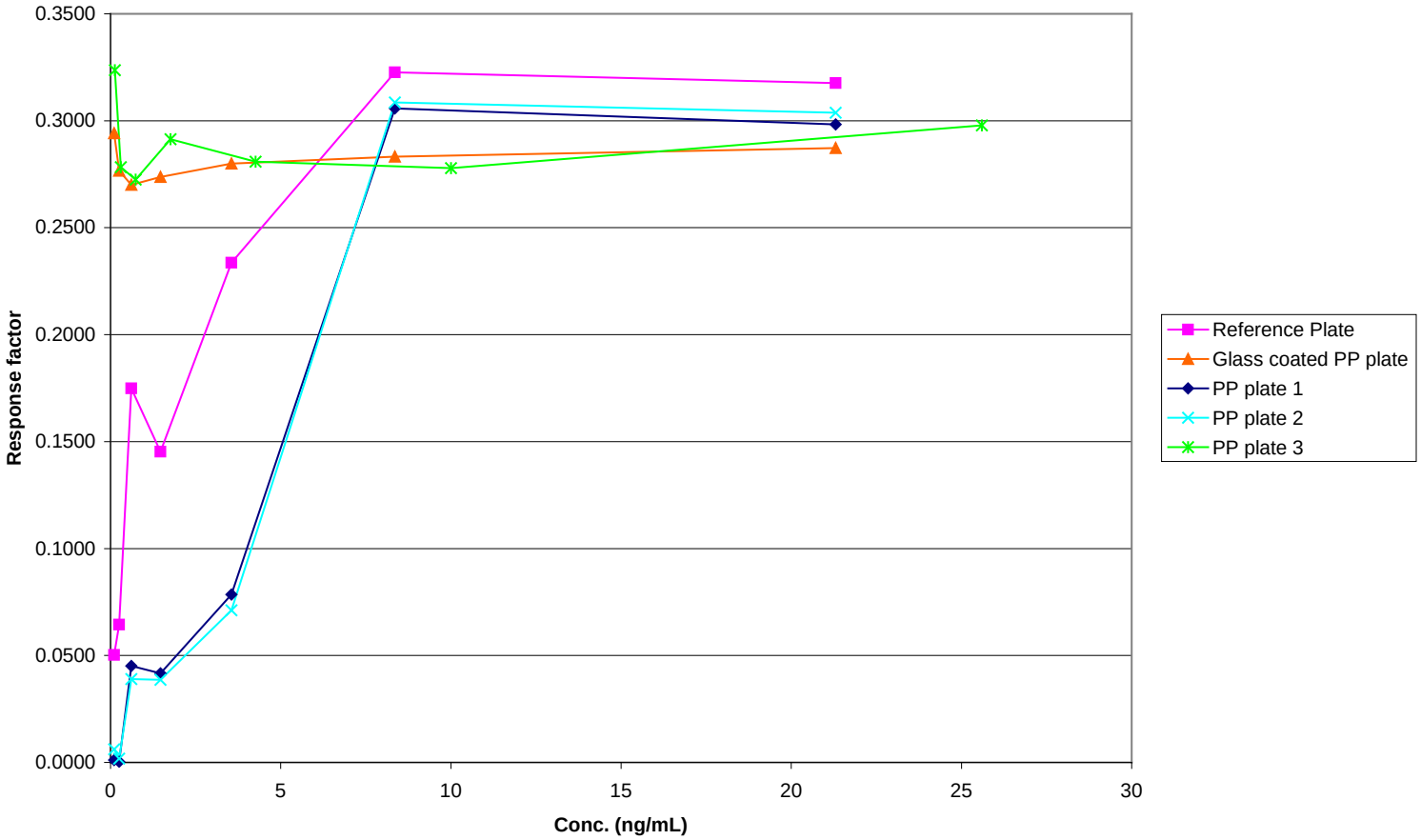

- Selecting another supplier of PP plate or using a glass coated PP plate solved the NSB issue.

- Method for sheep plasma was successfully validated with PP plate 3.

Lessons learned

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- Careful selection of material and additive might minimize problem
  - Test during method development
- Even for well established methods, NSB to material can all of sudden occur
  - As part of trouble shooting, good practice to start looking at the material involved (e.g. by looking at the response factors)

- Calibration standards and quality control samples of peptides should be prepared by spiking from a high concentration stock solution directly into plasma and subsequently serial-diluted in the same sample matrix.

Acknowledgements

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