Background

Ferring Pharmaceuticals

- Founded in 1950 and have since then worked with peptides
- Today, focus are on peptides as well as proteins
- Many of our peptides are agonists targeting the oxytocin or vasopressin receptor
  - Highly potent and selective peptides
  - Therapeutic doses at µg levels
  - Regulated Bioanalytical support at low pg/mL levels
  - Complex method needed to achieve required selectivity and sensitivity

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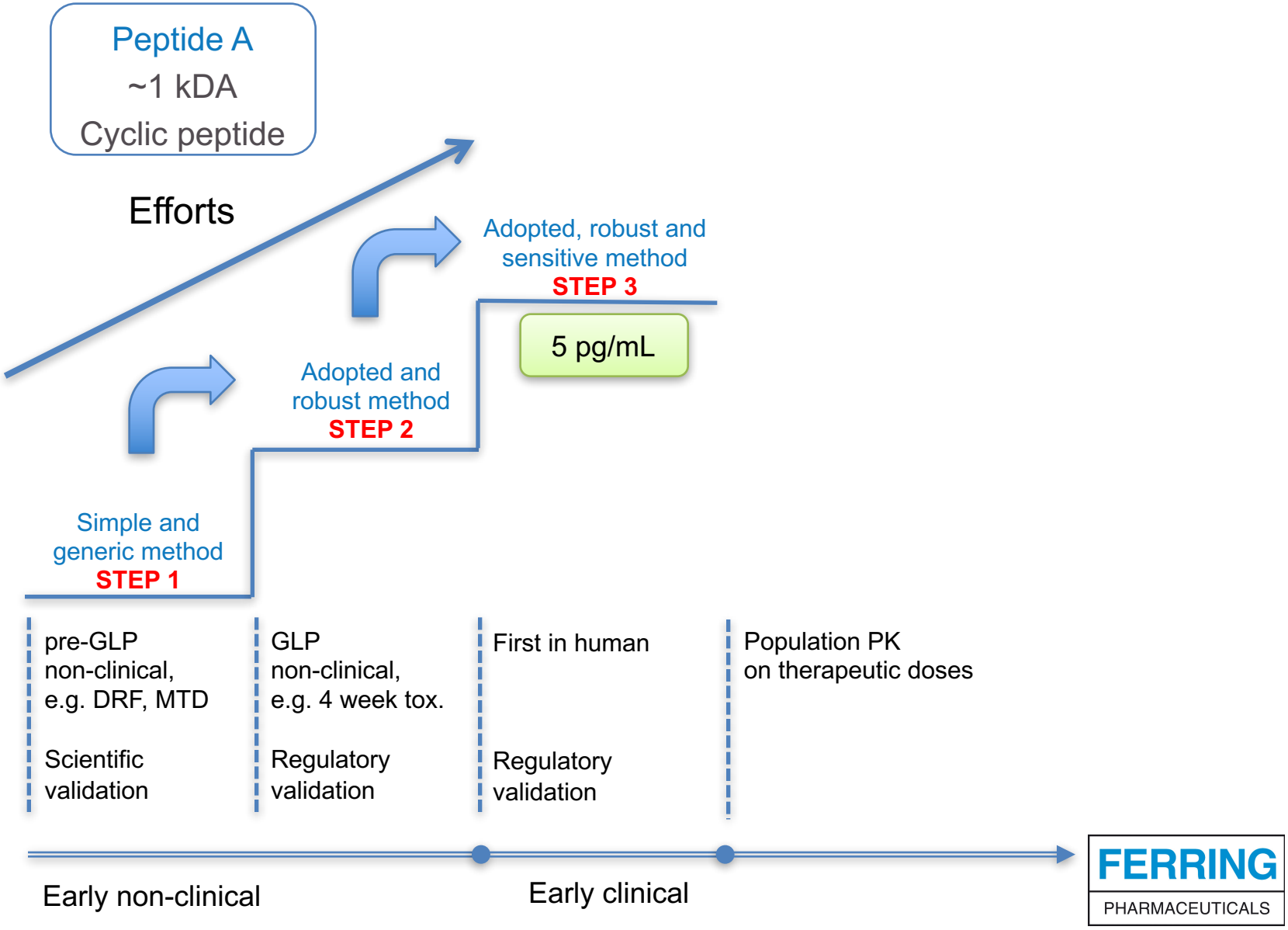
Bioanalytical support in early development



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Bioanalytical support in early development

Peptide A
~1 kDA
Cyclic peptide

Efforts

Simple and generic method
STEP 1

Adopted and robust method
STEP 2

Adopted, robust and sensitive method
STEP 3

5 pg/mL

Adopted, robust and even more sensitive method
STEP 4

Effective phase 2 doses:
50 – 500 ng

Target LLOQ:
0.5 - 1 pg/mL

pre-GLP	GLP	First in human	Population PK
non-clinical, e.g. DRF, MTD	non-clinical, e.g. 4 week tox.		on therapeutic doses
Scientific validation	Regulatory validation	Regulatory validation	

Early non-clinical

Early clinical

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Agenda

- The STEP 3 method (5 pg/mL)
- Investigated ways on how to reach Utopia
  - Superchargers
  - Immunoaffinity purification
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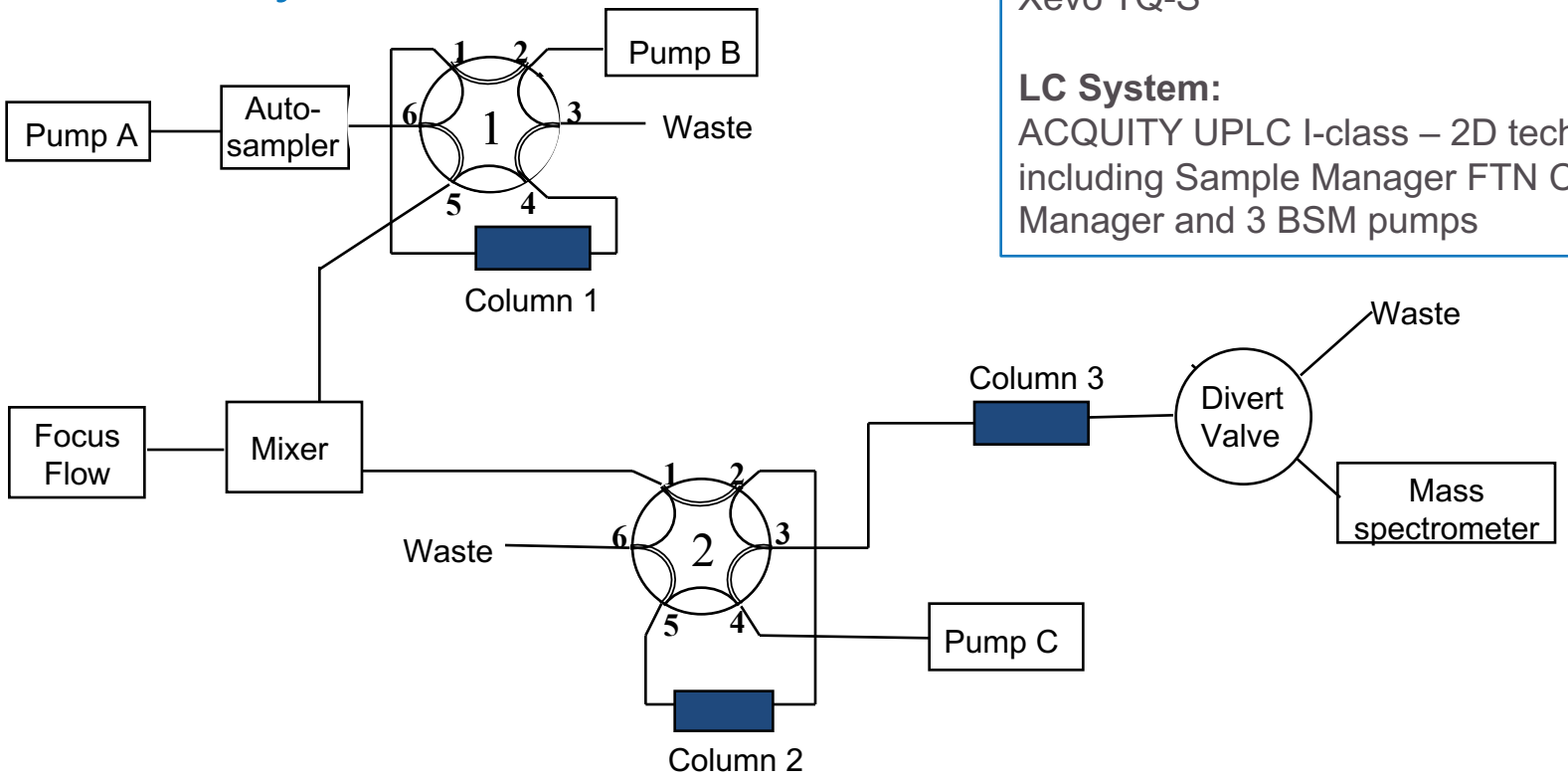
The STEP 3 method

2D-UPLC system overview

MS/MS System:
Xevo TQ-S

LC System:
ACQUITY UPLC I-class – 2D technology, including Sample Manager FTN Column Manager and 3 BSM pumps



Column 1: Beta Basic CN (50 × 2.1 mm, 5 µm)
Column 2: Waters Xbridge C8 (30 × 2.1 mm, 10µm)
Column 3: Waters ACQUITY UPLC HSS T3 (50× 2.1 mm, 1.8 µm)

Lövgren et al., Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 537-545

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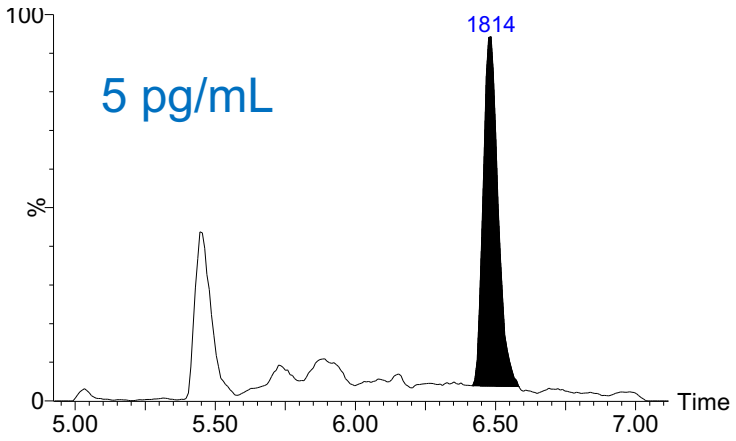
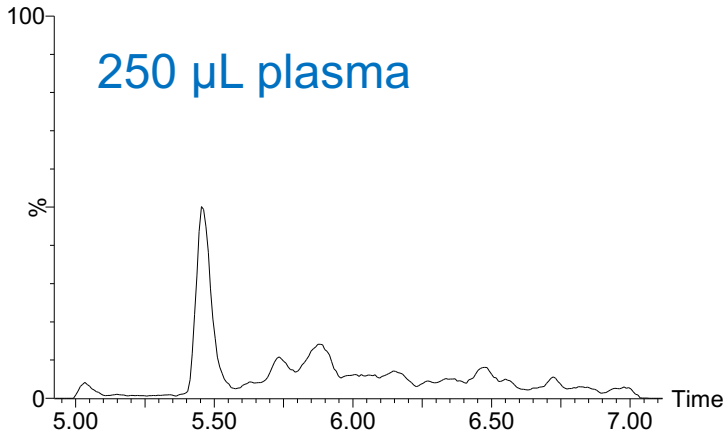
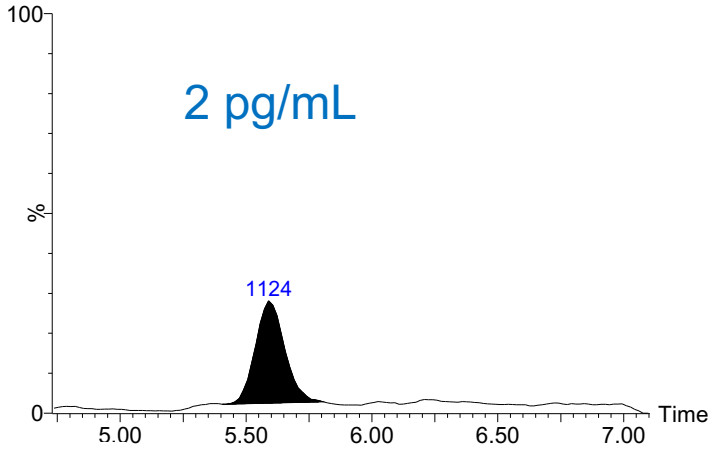
The STEP 3 method

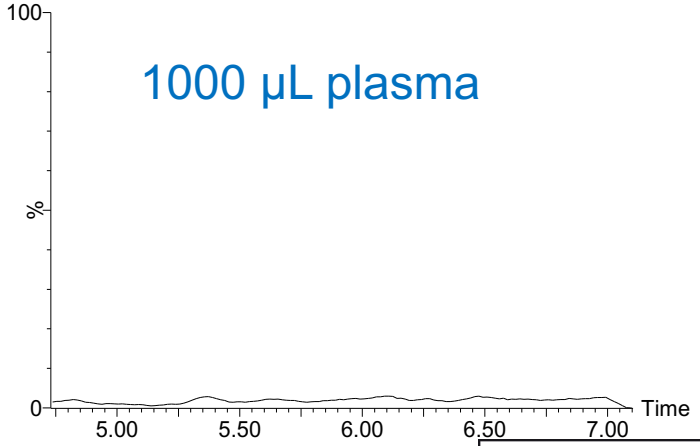
Peptide A in STEP 3 (FIH)





Peptide B (similar size)





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The STEP 3 method

Peptide A in STEP 3 (FIH)

- Very strong base, pI >12
- SPE (HLB)
  - Separation = hydrophobicity
- Mass spec behaviour
  - $Pep^{+} \rightarrow 211$

Peptide B (similar size)

- Weak/moderate base, pI=9
- SPE (WCX)
  - Separation = ion-exchange
- Mass spec behaviour
  - $Pep^{2+} \rightarrow 750 + Pep^{2+} \rightarrow 328$

1. Difficulties for Peptide A to elute from a WCX material – Strong interaction
2. Singly charged parent and a low mass fragment ion

►Non-specific MRM + HLB clean-up = High background in chromatogram

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Conventional SPE+LC-MS/MS + *Supercharger*

Supercharger theory

- A mobile phase additive = EASY TOOL
- E.g. m-NBA (*m*-Nitrobenzyl alcohol), Ethylene glycole, DMSO…
  - Boiling points higher than water and becomes enriched in the droplet as solvent evaporation occurs
  - Increase droplet surface tension → increased charge density of the droplet
- Influences the charge state envelope of a peptide
  - Theoretically, the maximum charge state of a peptide should correspond to the number of basic sites

For our small peptides:

- Charge state could theoretically be increased and/or concentrated to 2+/3+
- Dependent of the amino acid sequence and the ability to add charge i.e. (Basic sites, free N-terminus)
- Ionisation is affected, H^+ abdundance increase in favour over Na^+/K^+

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Conventional SPE+LC-MS/MS + *Supercharger*

Oxytocin (10 pg/mL)

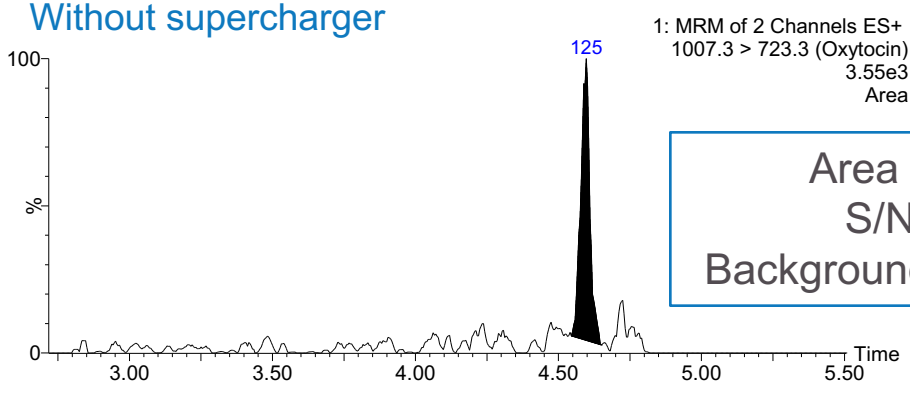
1 basic AA, free N-terminus

Singly charged

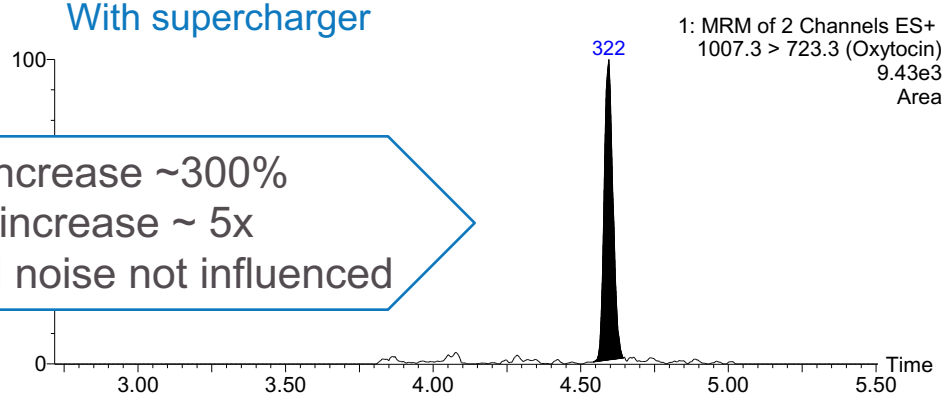
WCX extracted plasma (200 µL)

MRM transition, 1007→723

Without supercharger



With supercharger



Area increase ~300%
S/N increase ~ 5x
Background noise not influenced

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Conventional SPE+LC-MS/MS + *Supercharger*

Peptide A

1 Basic AA, no free N-terminus

Singly charged

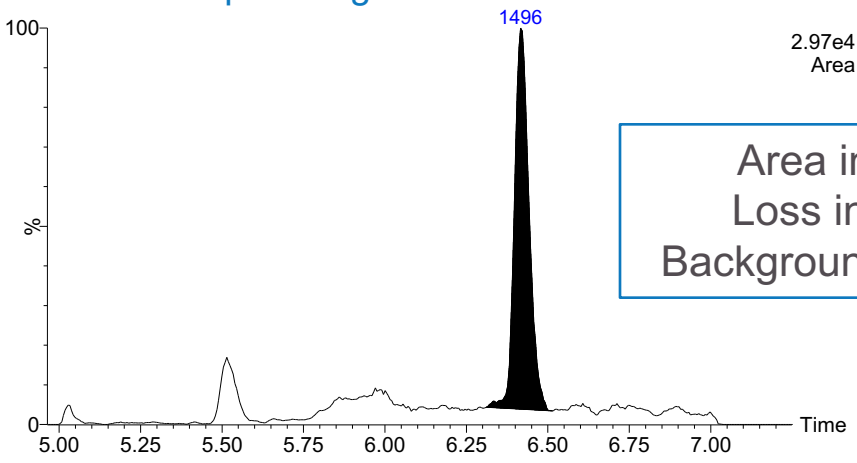
HLB extracted plasma 5 pg/mL (250 µL)

MRM transition ($Pep^{+} \rightarrow 211$)

Signal improvement:
m-NBA improved the robustness of the ionisations

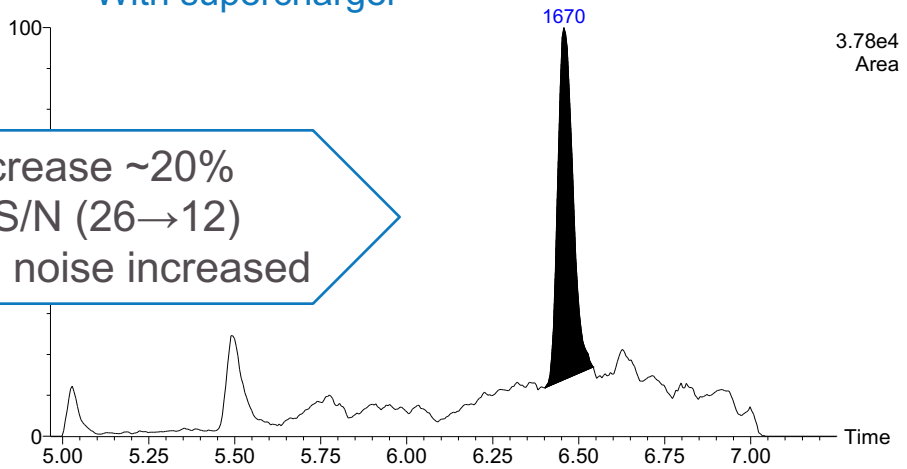
Effect on Bioanalysis:
Background ions due to remaining matrix in sample extract affect S/N

Without supercharger



Area increase ~20%
Loss in S/N (26→12)
Background noise increased

With supercharger



Kay, Howard and Stensson (2016) *Bioanalysis* 8(3) 157-161

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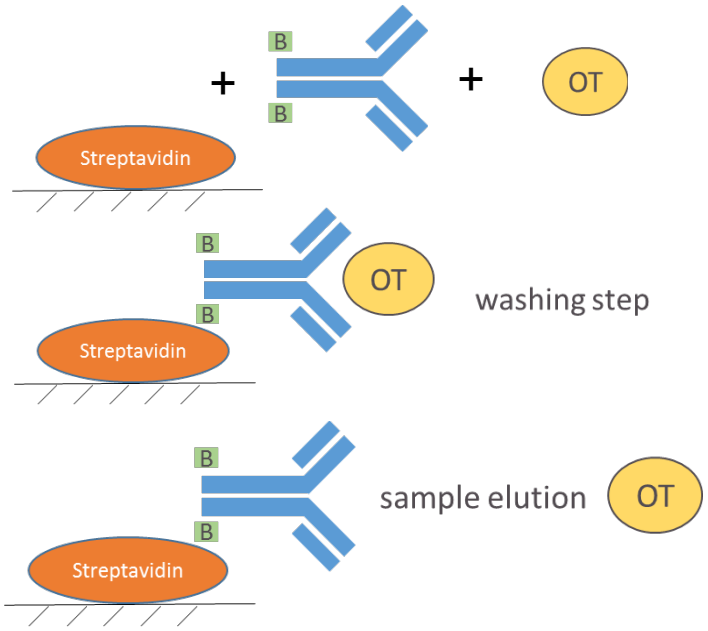
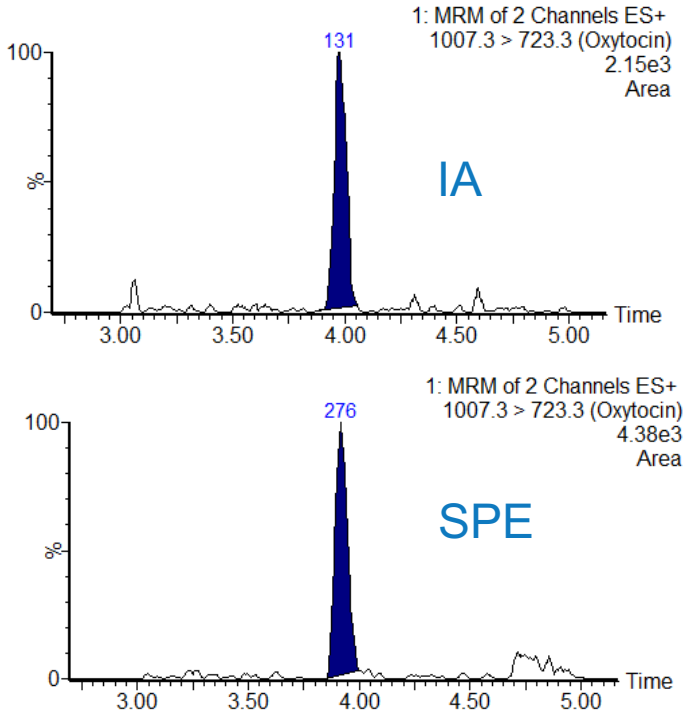
Immunoaffinity purification

Semi-homogen ligand binding assay



Oxytocin 10 pg/mL (200 µL)



Immunoaffinity purification is very specific and gives highly purified sample
Less signal compared to SPE but comparable S/N
Non-complex platform established to evaluate antibodies produced to Peptide A

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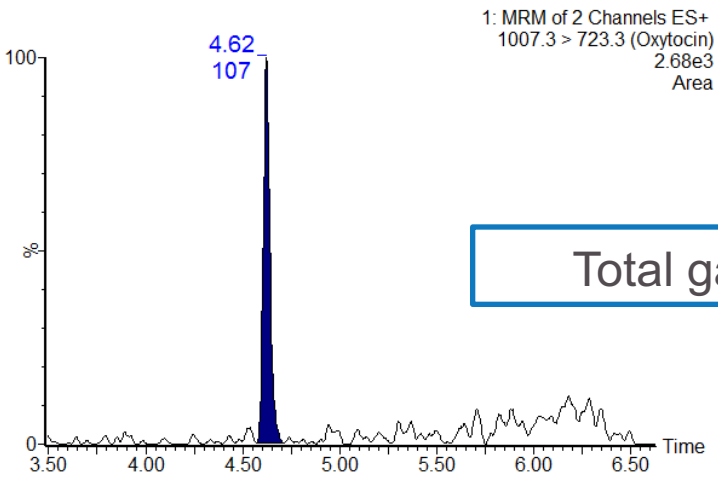
Microflow-LC-MS/MS

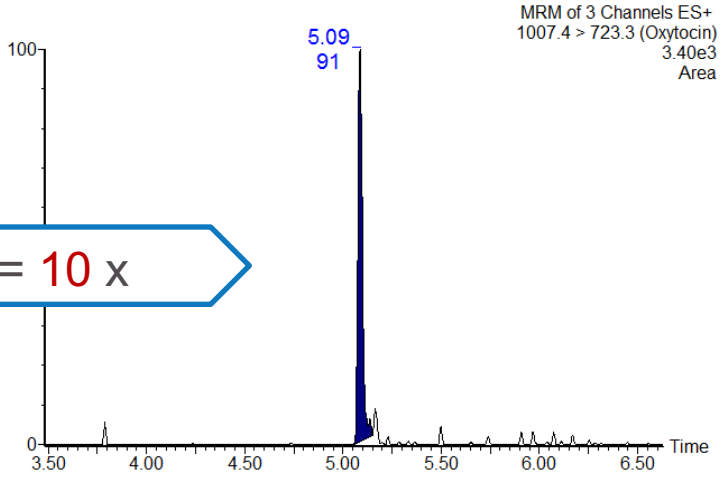
Oxytocin

Sample preparation: Immunoaffinity purification

10 pg oxytocin/mL plasma (200 µL)

Waters UPLC-TQS vs. IonKey-TQS





Total gain = 10 x

IA – 2D UPLC TQS

S/N = 25

Injection volume = 50 µL

IA – µUPLC TQS

S/N = 50

Injection volume = 10 µL

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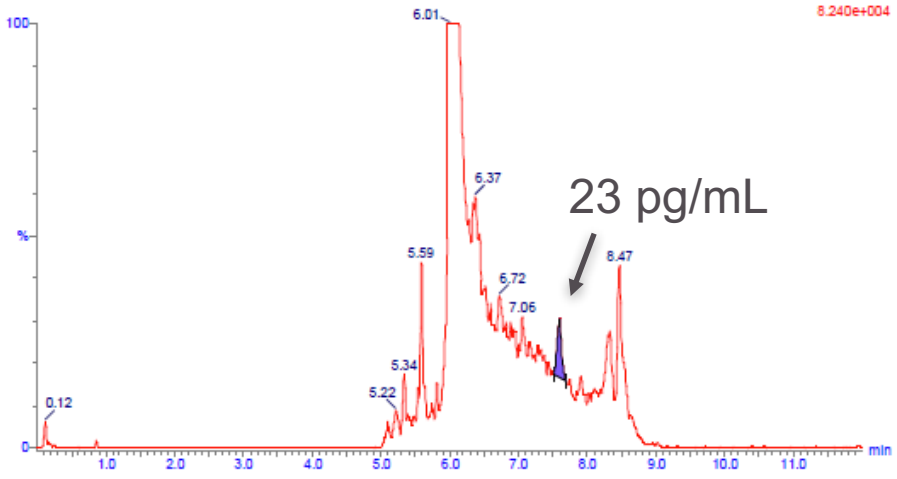
Microflow-LC-MS/MS

Peptide A

- Trap-elute IonKey TQS have shown 30 x improvement of S/N on neat solutions!
- We have not been able to transfer the signal improvement to extracted samples

  Neither with protein precipitated -, HLB extracted - nor WCX extracted samples
- Work is still ongoing to explore the possibilities of using different IKey stationary phases for this peptide.



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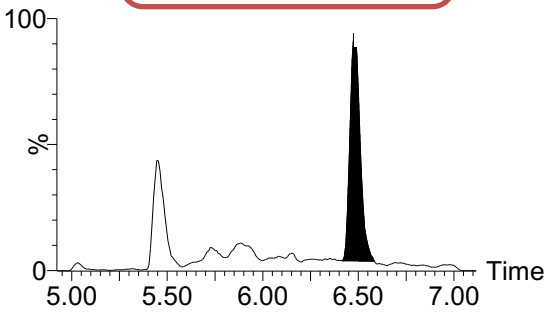
SPE improvement

Revisit the WCX material

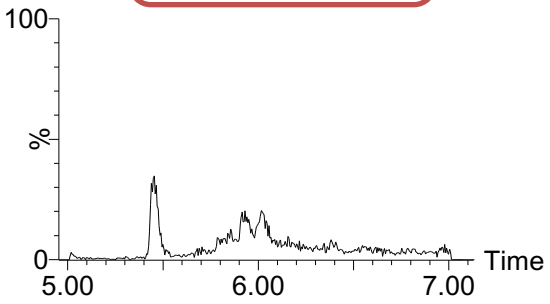
A different producer of WCX material (Brand 2) evaluated and optimised

Higher recovery achieved compared to previously tested material (Brand 1)

5 pg/mL
(HLB)

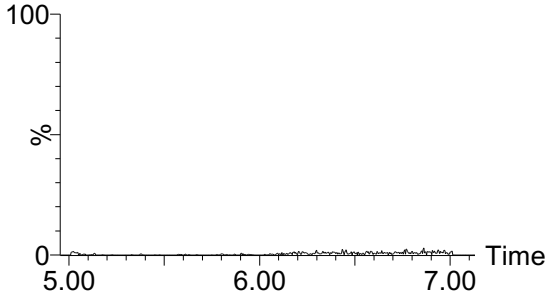


Wash 2
(WCX Brand 2)



Problematic background eliminated in Wash 2

Plasma Blank
(WCX Brand 2)



Clean background using WCX

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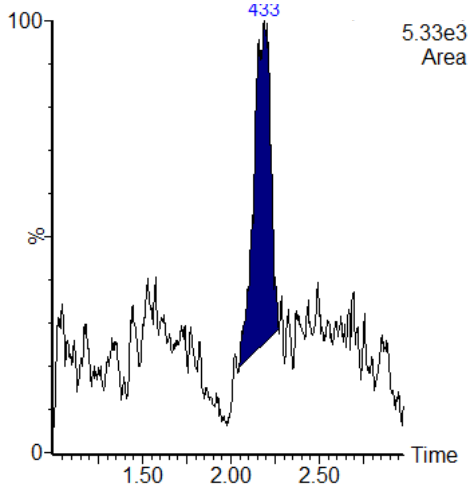
LC ortogonality

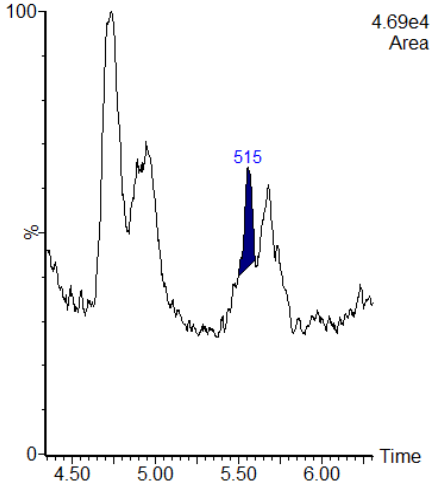
The WCX extract was used to evaluate HILIC phase (1D)

Good chromatography and short run time

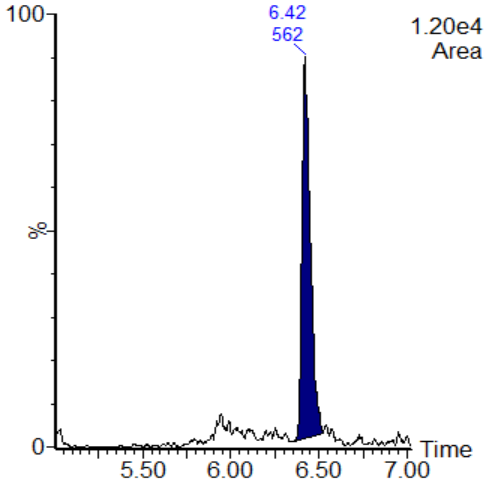
Robustness not satisfactory (new experience in lab)



1 pg/mL
WCX + 1D-HILIC



2 pg/mL
WCX + 1D-C18



1 pg/mL
WCX + 2D-CN/C18

1D HILIC better than 1D reversed phase!

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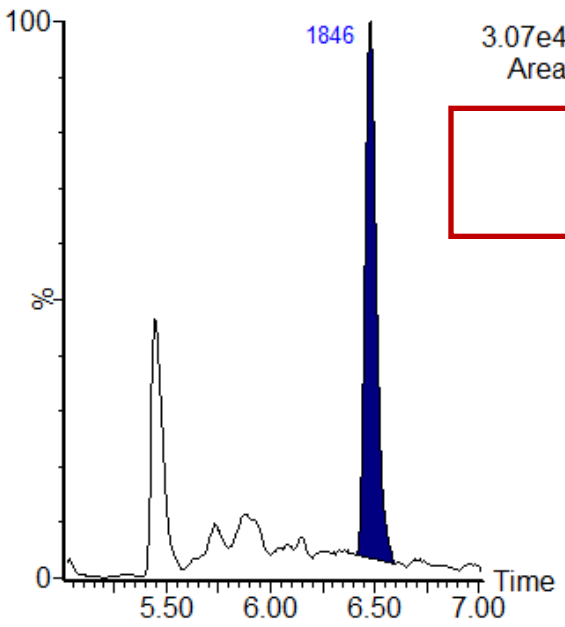
SPE-UPLC improvement

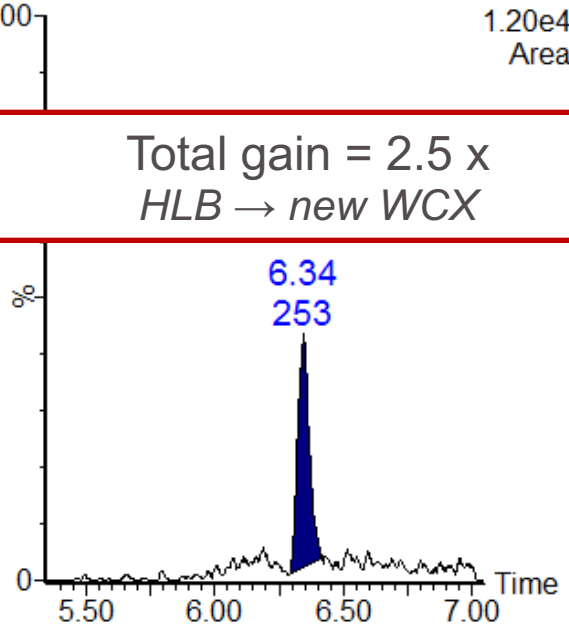
Total improvement from STEP 3 to STEP 4

5 pg/mL plasma
HLB (250 µL)



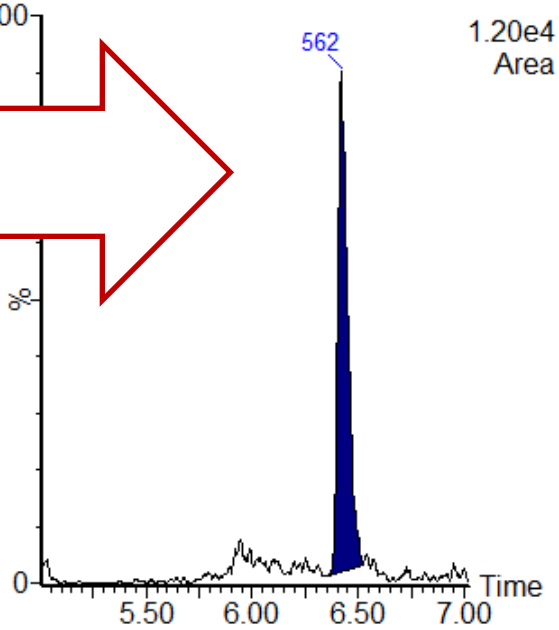
S/N =30

1 pg/mL plasma
WCX (250 µL)



S/N =15

1 pg/mL plasma
WCX (500 µL)



S/N =30

Total gain = 2.5 x
HLB → new WCX

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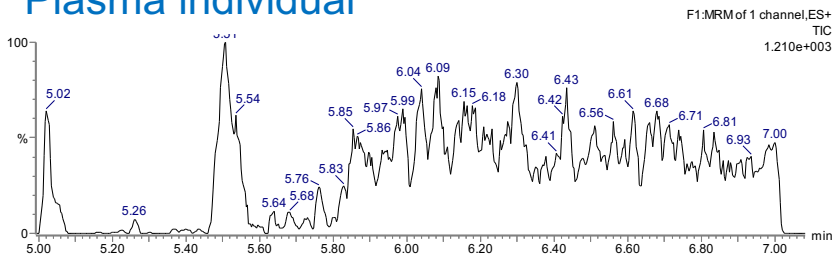
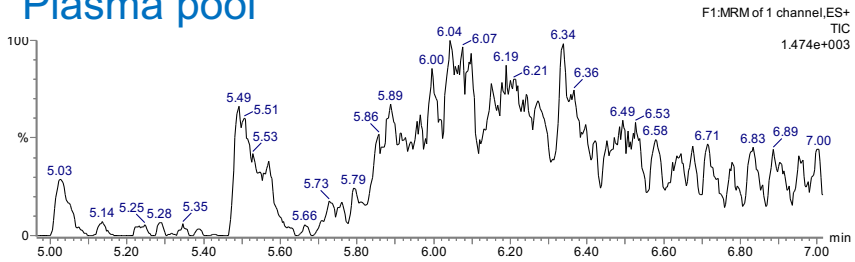
Validation data

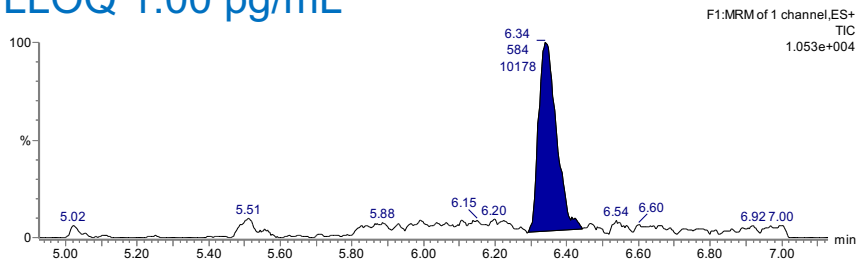
Plasma individual



Plasma pool



LLOQ 1.00 pg/mL



Sample preparation	
Plasma volume:	500 µL
WCX extraction and evaporation	
Reconstitution:	80 µL
Injection volume:	30 µL
Concentration factor:	~6

Between run precision and accuracy data				
	LLOQ 1.00 pg/mL	LOW 3.00 pg/mL	MID 10.0 pg/mL	HIGH 85.0 pg/mL
Mean:	0.984	2.97	9.90	79.4
SD:	0.134	0.329	0.947	6.559
CV (%):	14	11	10	8
Mean bias (%):	-2	-1	-1	-7

Final method: WCX + 2D(CN+C18)-UPLC-MS/MS

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Conclusions

SPE improvements → LLOQ =1 pg/mL
estimated to be sufficient to obtain full PK corresponding to the highest therapeutic dose to be tested in phase 2

Investigated tools

- Superchargers is an easy tool to boost sensitivity in detection of some small peptides
- Clean extract can be achieved with immunoaffinity purification
- Microflow LC-ES-MS improves the sensitivity

Utopia – how far is it?

Possible ways to reach LLOQ of ≤ 0.5 pg/mL

- Immunoaffinity purification
- Microflow using 2D

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Acknowledgements

- Collegues at Ferring
  - Magnus Knutsson
  - Anna Sterup
  - Anders Sonesson
- BioApp Solutions
  - Mohammed Abrar
- Waters (IonKey demos)

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