FERRING PHARMACEUTICALS

MEDICINE ON THE BODY'S OWN TERMS

LC-MS/MS Bioanalysis of Therapeutic Peptides in Plasma at Low pg/mL Levels

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Outline

- Background
- LC-MS/MS bioanalysis of peptides
    - Our experience and approaches in the last decade
- LLOQ at low pg/mL levels
    - SPE in combination with coupled column LC-MS/MS
    - Case studies, three peptides
- On-going and future work
- Conclusion

Background

- Ferring Pharmaceuticals was founded in 1950 and have since then worked with peptides
- Today, focus are on both peptides and proteins
- All approaches and data presented are performed in a Regulated Bioanalysis environment

Background
– Bioanalysis of peptides at Ferring

- Before the year 2000, all our peptide bioanalysis was performed using competitive immunoassay (RIA)

- Since the year 2000, LC-MS/MS bioanalysis is the preferred approach for all peptides (new entities as well as line extension activities)
  - MW < 5000 g/mol → LC-MS/MS
  - MW > 5000 g/mol → immunoassay

LC-MS/MS bioanalysis of peptides

- Challenges
  - Formation of multiple-charged ions
  - Extensive fragmentation in MS/MS
  - Chromatographic issues
  - Adsorption problems
    - More pronounced at low concentrations
    - Container material
    - Carry-over effects in LC system

LC-MS/MS bioanalysis of peptides - our current approaches

- Basic approach:
  Protein Precipitation with LC-MS/MS

- Sensitivity required:
  Solid Phase Extraction with LC-MS/MS

LC-MS/MS bioanalysis of peptides - the last 10 peptides that we assayed

peptide	number of AA	monoisotop mass	sample prep	Charge state precursor ion	LLOQ	
					animal	human
A	5	671	SPE	+1	1 ng/mL	N/A
B	7	830	PP/SPE	+1	5 ng/mL	500 pg/mL
C	9	988	PP	+1	N/A	250 pg/mL
D	9	994	PP	+1	N/A	5 ng/mL
E	9	1042	SPE	+1	20 pg/mL	5 pg/mL

LC-MS/MS bioanalysis of peptides - the last 10 peptides that we assayed

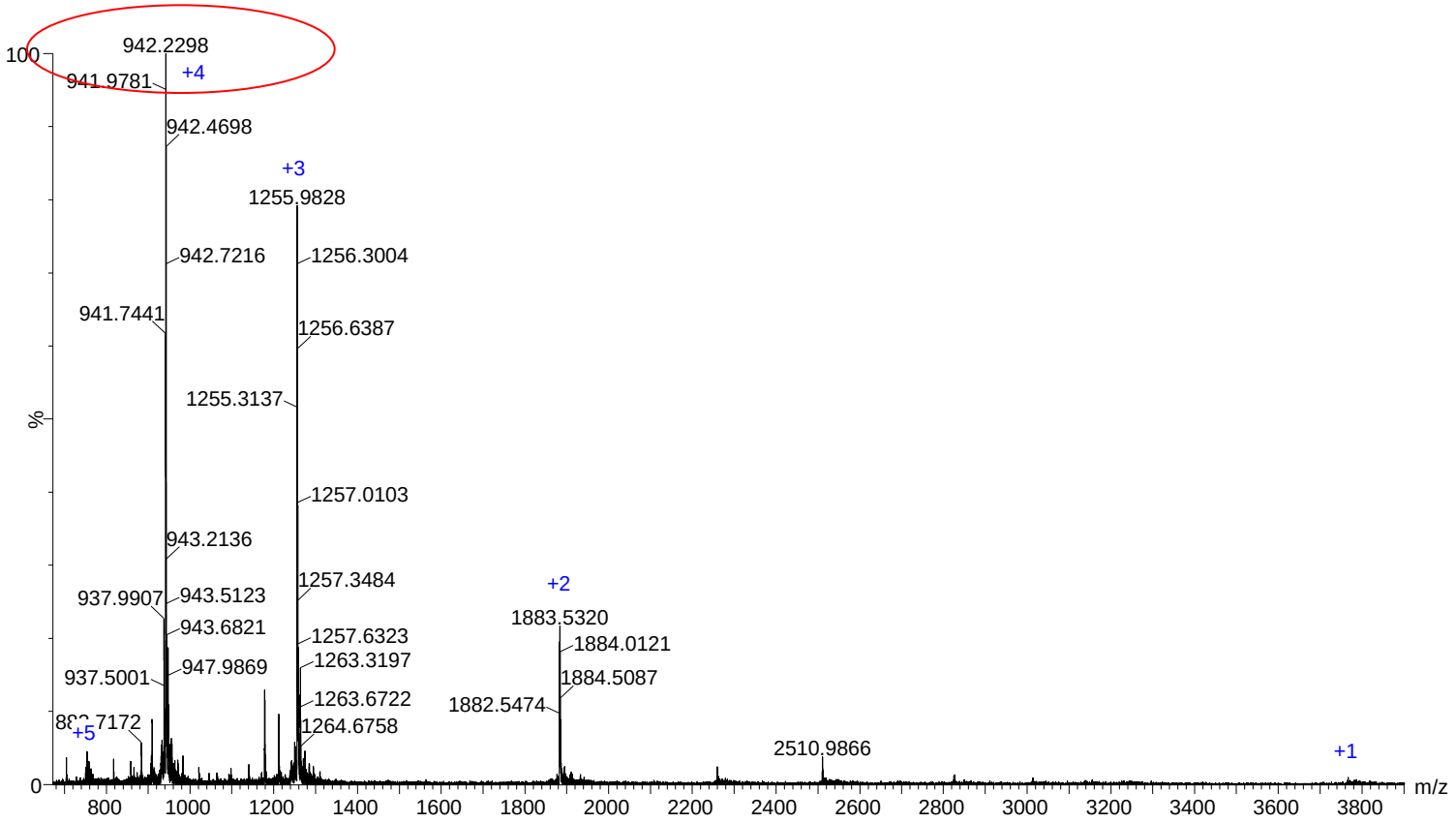
peptide	number of AA	monoisotop mass	sample prep	Charge state precursor ion	LLOQ	
					animal	human
F	9	1048	SPE	+2	20 pg/mL	5 pg/mL
G	9	1069	SPE	+2	4 pg/mL	2 pg/mL
H	9	1083	PP	+2	100 pg/mL	N/A
I	10	1631	PP/SPE	+2	1 ng/mL	100 pg/mL
J	33	3763	PP	+4	2 ng/mL	N/A

Acknowledgement to CRO's involved in some of the work:
Celerion, Covance and YBS

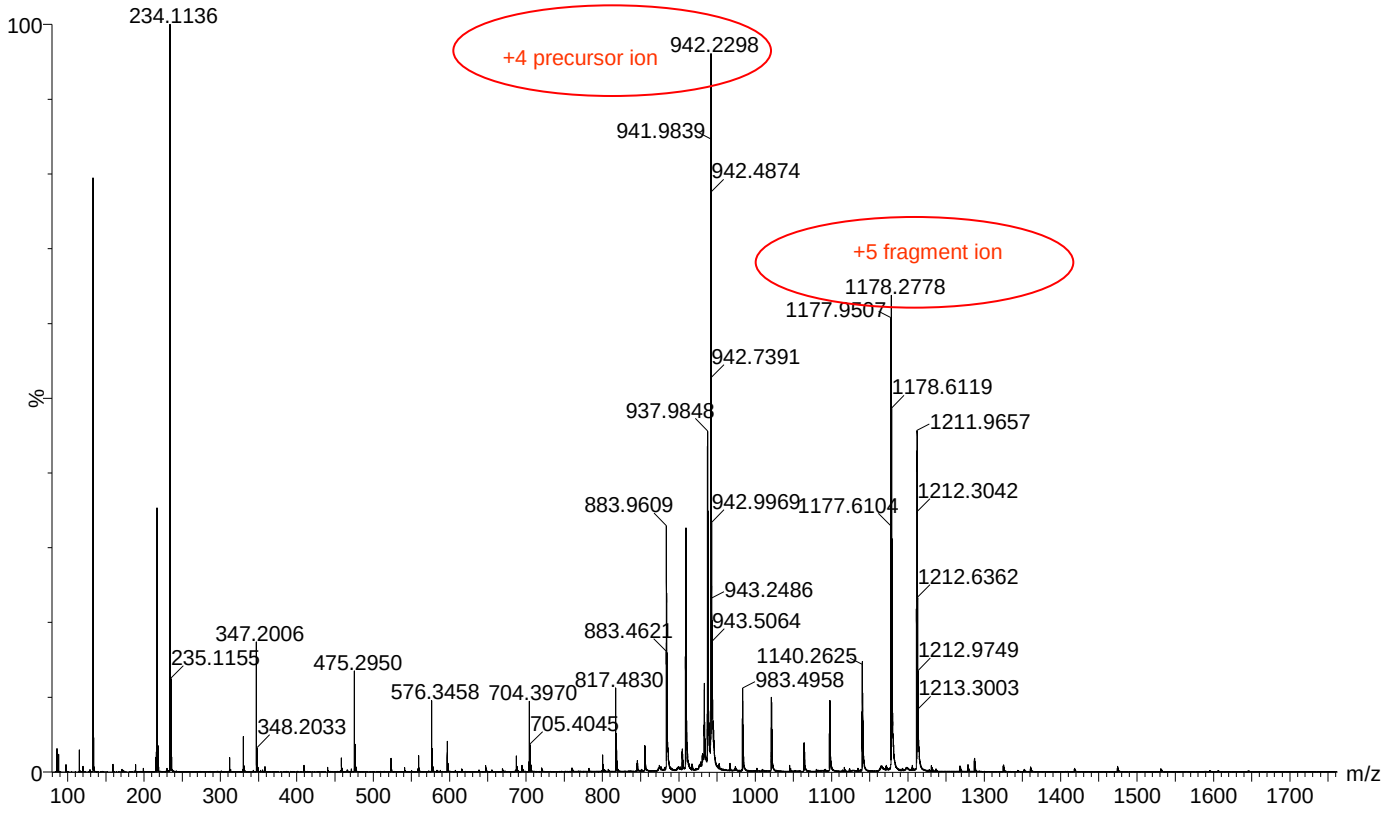
Protein Precipitation and LC-MS/MS

- Peptide J (monoisotopic mass = 3763), new entity
- Early stage → qualified method
- PP: 50 µL plasma + 150 µL EtOH:MeCN 70:30, supernatant diluted 1:4
- LC: Phenomenex Jupiter C18, 2.0×50 mm, 5 µm
- MS instrument: Sciex API4000

MS scan – Peptide J

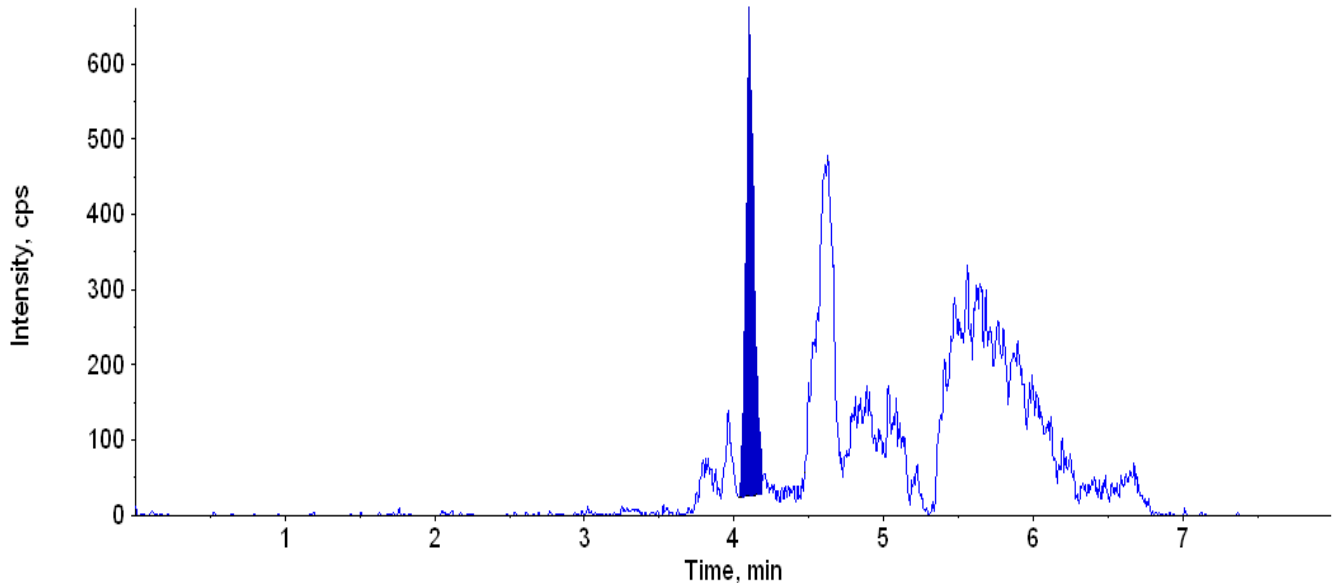


MS/MS scan – Peptide J



Chromatogram – Peptide J

2 ng/mL in rat plasma



Background – LLOQ at low pg/mL levels

- Many of our peptides target the oxytocin and vasopressin receptors
- These receptor agonists requires LLOQ at low pg/mL levels
- These peptides have molecular weights around 1000 g/mol

Our approach to improved sensitivity

- Use of larger plasma volume
  - Sample enrichment using SPE + solvent evaporation
- Improved MS sensitivity by use of miniaturized LC system
  - Concentrating effect in LC system
  - Decreased spray needle distance to orifice
- Improved chromatographic selectivity
  - Highly selective SPE (e.g. weak cation exchanger)
  - Coupled column LC (different types of stationary/mobile phases)

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

Solid Phase Extraction

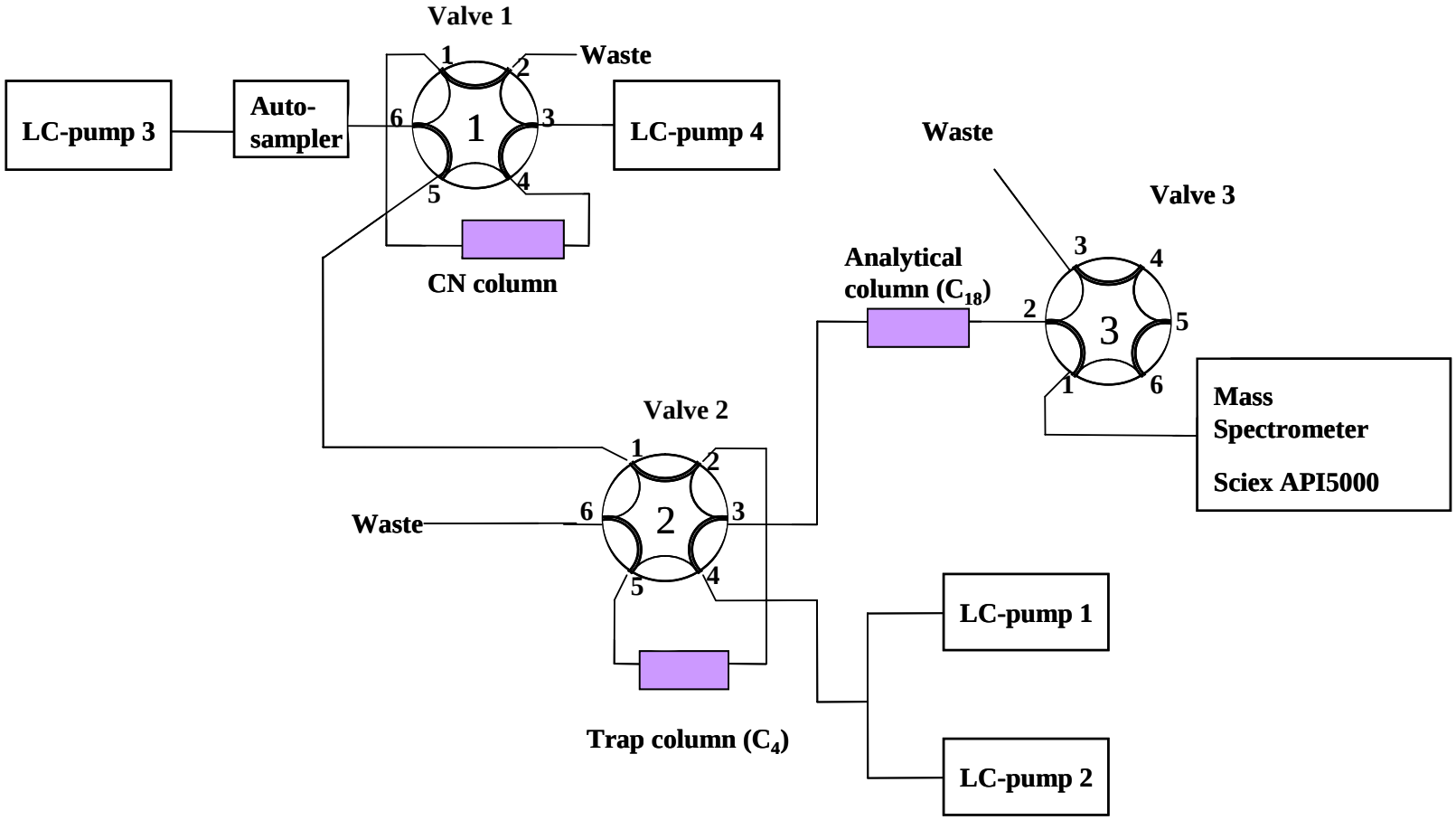
Oasis WCX, 30 mg, 1cc

1. Condition column under acidic pH
2. Load sample: Plasma : 2% phosphoric acid (1:1)
3. Wash step 1: 1 mL 10% MeOH in water
4. Wash step 2: 2 mL 2% ammonium acetate, pH 7
5. Wash step 3: 1 mL MeOH:MeCN (70:30)
6. Elution: MeOH:Water containing 2% formic acid (1:1)

Coupled Column LC

- High enrichment factor in coupled column system
  - Large injection volumes should be possible (up to 100µL)
  - Narrow peak at low flow rate from the analytical column preceding MS detection
    - 1 mm i.d. column as compromise between robustness and sensitivity
- High selectivity
  - LC separations based on different separation mechanisms

Coupled Column LC – the set-up

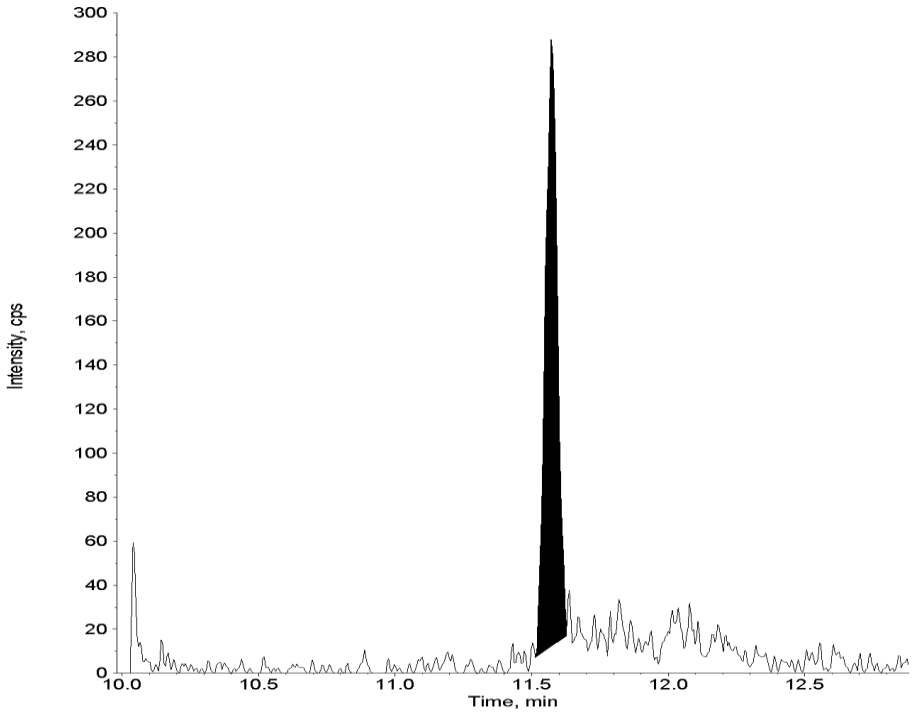


Solid Phase Extraction and LC-MS/MS – peptide F

- Peptide F (monoisotopic mass = 1048), new entity
- SPE: 0.5 mL plasma samples using Oasis WCX
- LC: Coupled column system
  - CN: Reprosil-Pur CN, 5 μm, 2.0 × 50 mm
  - C_4: Kromasil C4, 10 μm, 1.0 × 50 mm
  - C_{18}: Kromasil C18, 3.5 μm, 1.0 × 50 mm
- MS instrument: Sciex API5000

Chromatogram – peptide F

5 pg/mL in human plasma



Flow rate through analytical column: 70 μL/min

Total cycle time: 13 minutes

Gradient: 0 - 10 min: 90% A

10 - 13 min: 90%A →40% A

A: 10 mM NH_4Ac, pH 4.0

B: MeCN

Peptide F - Features of the system

- The Coupled Column LC system gave four-fold increase in S/N compared to a single column system
- Efficient removal of phospholipids (monitored m/z 496 →184, m/z 520 →184, m/z 525 →184, m/z 704 →184 and m/z 758 →184)
- Two of the five phospholipids are removed by SPE, the remaining three are removed by the Coupled Column LC system

Validation data – peptide F

	Peptide F in human plasma			
Nominal concentration, pg/mL	5.00	15.0	71.0	860
Mean measured concentration, pg/mL	4.80	14.5	72.7	885
Accuracy (% bias)	-4	-3	2	3
Inter-assay precision (%CV)	11	6	4	5
n	18	18	18	18

- ISR performed as part of FIH – 90% within acceptance criteria (20% from original)

Lessons learned from peptide F

- Despite that the approach were successfully used for validated methods, problems when validating the fourth animal specie and in LTS time point for human plasma method
  - Non specific binding to polypropylene (same material/manufacture as before) during preparation of calibration samples
  - Solution: change material
- Severe matrix effects in PoC study
  - Septic shock patients with a lot of co-medication
  - Solution: Less sensitivity required in study, effected samples diluted

Solid Phase Extraction and LC-MS/MS – peptide G

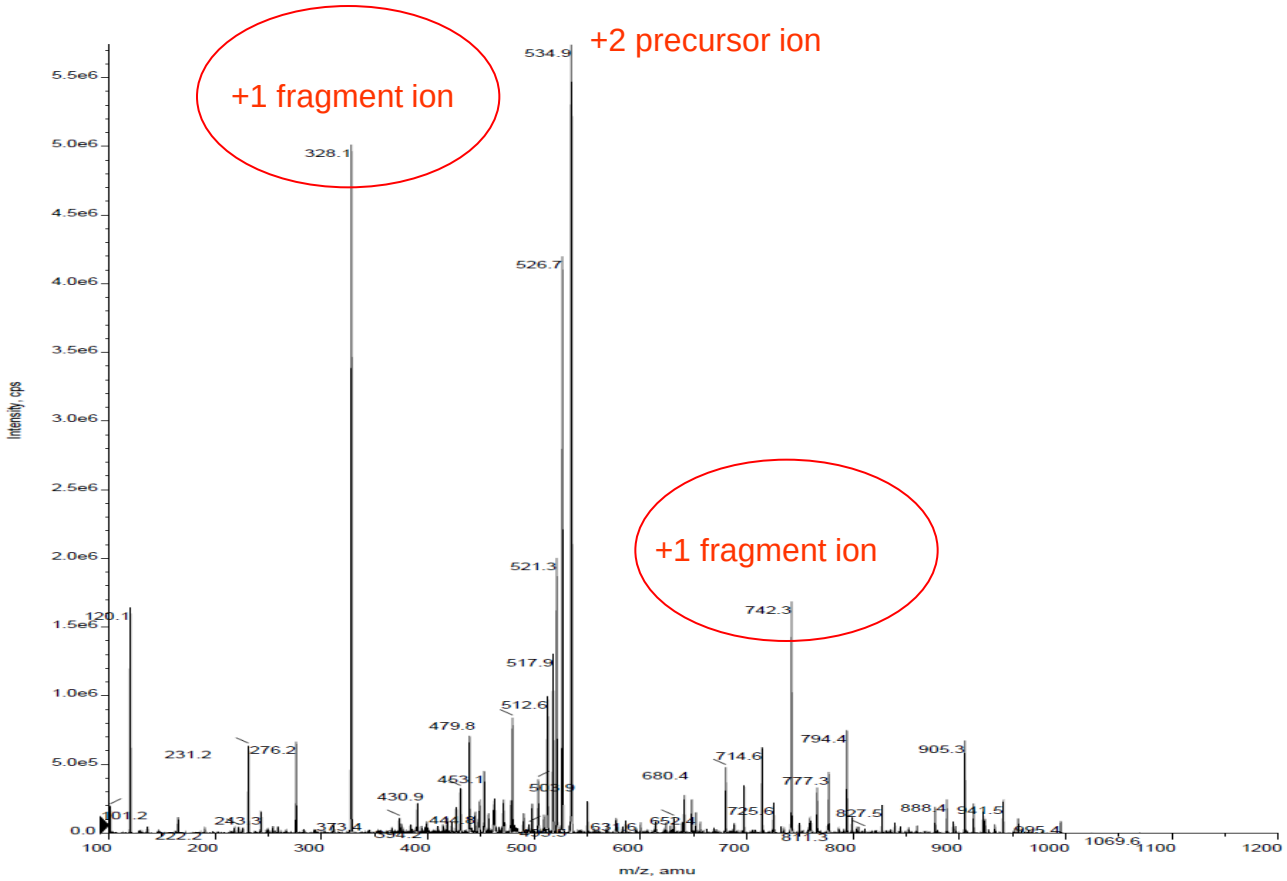
- Peptide G (monoisotopic mass = 1069), line extension
- "Old" immunoassay had LLOQ of 5 pg/mL, validated in the 1990's, precision and accuracy requirements < 20%
- Until recently, it has been hard to develop/validate LC-MS/MS method matching the LLOQ of the immunoassay

Solid Phase Extraction and LC-MS/MS – peptide G

- Approach as for previous peptide, minor modifications,
  - SPE of 1 mL plasma
  - C8 (2 x 50 mm) used as trap column in LC system
  - Mobile phase composition

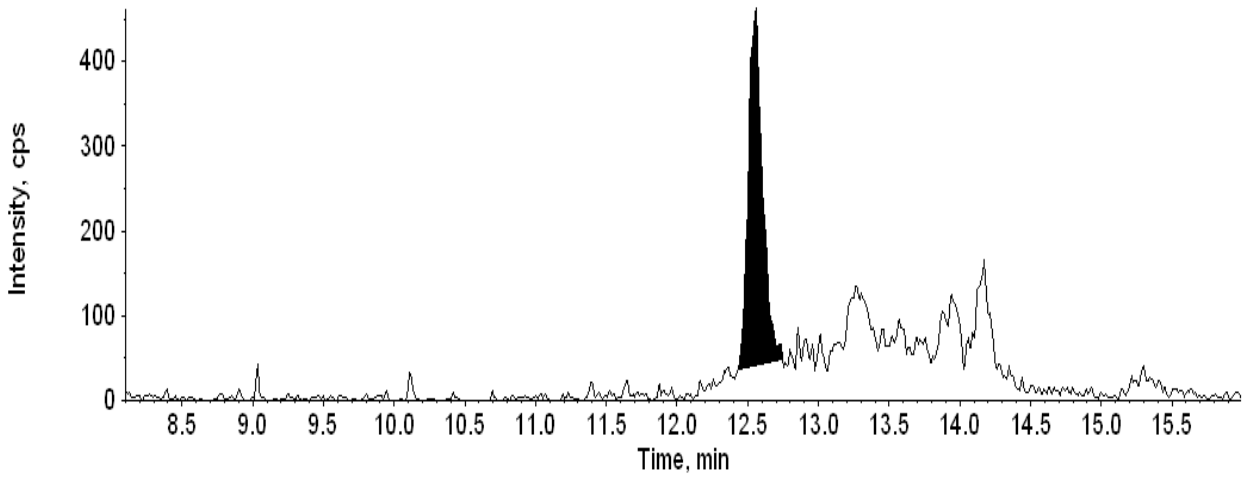
- Sum of two MRM transitions were monitored to achieve maximum sensitivity

MS/MS scan – peptide G



Chromatogram – peptide G

2 pg/mL in human plasma



Validation data – peptide G

	Peptide G in human plasma			
Nominal concentration, pg/mL	2.00	6.00	14.0	85.0
Mean measured concentration, pg/mL	2.04	6.10	13.9	88.3
Accuracy (% bias)	2	2	-1	4
Inter-assay precision (%CV)	10	6	3	3
n	18	18	18	18

- ISR performed as part of PK study – 90% within acceptance criteria (20% from original)

Solid Phase Extraction and LC-MS/MS – peptide E

- Peptide E (monoisotopic mass = 1042), new entity
- Uncharged peptide, cation SPE not suitable
  - other sample preparation approach needed
- Hydrophobic peptide, hard to get a good retention on the different columns thus the standard 3 column system not suitable
  - various chromatographic approaches tested
- Again, sum of two MRM transitions were monitored to achieve maximum sensitivity

Solid Phase Extraction and LC-MS/MS – peptide E

- 200 µL plasma on Waters Ostro
- UPLC using Waters Acquity UPLC BEH Phenyl column (2.1 x 50 mm, 1.7 µm)
- MS instrument: Sciex API5000

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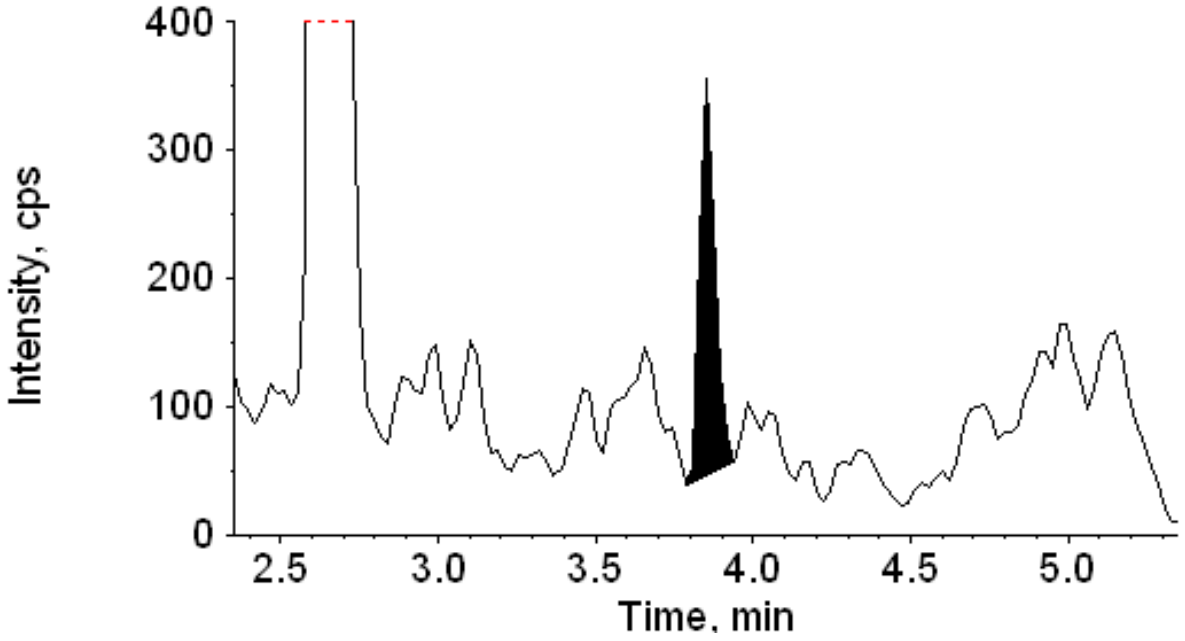
Chromatogram – peptide E

5 pg/mL in human plasma



On-going and future work

- Currently the bioanalytical ability to develop and validate methods with even lower LLOQ are crucial in two of our development projects

- With focus on sensitivity, we have lately tested different types of MS system,
  - for our peptides → QQQ instruments gives best sensitivity
  - on our tested peptides minor differences in sensitivity between the different vendors latest generation of QQQ instruments

Conclusion

- Presented different approaches for LC-MS/MS bioanalysis of peptides
- When focus on sensitivity, it is important to have selective sample preparation and chromatography
- SPE and coupled column system $\rightarrow$ LLOQ of 2 pg/mL

Acknowledgements

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