Quantification of FE 202767, a small therapeutic peptide in milk samples from rat, rabbit and humans at low pg/mL levels using 2D- column switching liquid chromatography – tandem mass spectrometry

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INTRODUCTION

FE 202767 [1] is a therapeutic peptide for treatment within the field of reproductive health. For this therapeutic peptide, bioanalytical support was needed for non-clinical/clinical milk transfer studies. The neutral peptide is a selective oxytocin receptor agonist (~1040 amu) that is uncharged. This poster presents the approach to quantification of the peptide in milk from rat, rabbit and humans. Preclinical methods were developed at Unilab YBS followed by the development of the clinical method at Ferring Pharmaceuticals A/S.

The quantitative bioanalysis of peptide drugs is very challenging. Extremely low concentrations after administration are seen due to the high potency of this class of compounds. In this case, also the matrix is rare and demanding due to inhomogeneity with fat globules dispersed in water based liquid.

Peptide bioanalysis can be very difficult due to issues like non-specific binding , limited stock solution and sample stability and sometimes strong plasma-protein properties.

These difficulties were also seen for this peptide throughout the method development and the following additional steps were taken to ensure method robustness

- Calibration and QC samples were prepared by serial dilution in milk matrix
- QC:s were stored in appropriate sized vessels to ensure maximum volume/surface ratio (to minimize non-specific binding)
- QC:s were thawed on ice to inhibit changes in milk matrix during this process

We employed Waters Ostro protein/lipid depletion plates for sample pre-treatment to remove large proteins and lipids. The milk was first sonicated in acidic water/acetonitrile mixture to induce protein precipitation. The extracts were then put through the Ostro plate and the proteins were removed by filtration. The Ostro plate has chemistry to remove lipids with log p > than 6.

Key characteristics of the methods:

Sample volume:	Preclinical 50 µL, clinical 100 µL
Internal standard:	Analyte-d11
Analytical technique:	Online SPE-LC-MS/MS (preclinical)
	2D-UPLC-MS/MS (clinical)
Sample preparation:	Protein/lipid depletion plate
	and online extraction
Matrix:	Rat, rabbit and human milk
LLOQ (pg/mL):	Preclinical 100, clinical 25.0
ULOQ (pg/mL):	Preclinical 20000, clinical 1000
Total analysis time:	6.0 min (preclinical), 7.2 min (clinical)

EXPERIMENTAL

Offline extraction (preclinical/clinical method)

- Precipitation of proteins in the milk with acidic water/acetonitrile mixture
- Sonication
- Removal of large proteins and lipids with Waters Ostro protein/lipid depletion plate
- Evaporation
- Reconstitution

Online SPE (preclinical method)

Instrument:	Spark Holland Symbiosis
SPE :	10µM Hysphere-MM-anion cartridges
Sample load:	100 µL of 250 µL offline extract
Sample wash:	500 µL H_2O:ACN: Formic acid 80:20:0.2 v/v/v
Sample backward Wash:	1000 µL H_2O:ACN:Formic acid 90:10:0.2 v/v/v
Sample elution:	20 secs standard gradient

Chromatography (preclinical method)

Column:	Waters Xbridge BEH Shield RP18, 2.5 µm, 50 x 3 mm
Column Temperature:	60°C
Mobile phase A:	10 µM ammonium formate
Mobile Phase B:	Acetonitrile
Flow rate:	500 µL/min

Chromatography (clinical method)

Column 1:	Thermo BetaBasic CN 5 um, 50 x 2.1 mm
Mobile phase A:	Acetonitrile:H_2O:TFA (25:75:0.05 v/v/v)
Mobile Phase B:	Acetonitrile: H_2O:formic acid (80:15:5 v/v/v)
Backflush wash of column with Acetonitrile: H_2O: formic acid (35:63:2 v/v/v)	
Column 2:	Waters Acquity UPLC BEH Phenyl, 1.7 µm, 100 x 2.1 mm
Mobile phase A:	10 µM ammonium formate pH 6.0
Mobile Phase B:	Acetonitrile
Inj. Volume:	50 µL of 250 µL extract
Column Temp.:	20°C (CN column), 65°C (Phenyl column)
Flow rates:	300 µL/min

Heart cutting technique to concentrate and clean up sample.

Mass Spectrometry (preclinical & clinical)

Instrument: Waters Xevo TQ-S

Instrument Parameters

Interface:	Electrospray (+ve)	
	Preclinical	Clinical
Dwell time (each):	75 ms	80 ms
Cone voltage:	60 V	65 V
Collision energy:	26	25 & 28
Capillary voltage:	3.0 kV	3.5 kV
Temperature:	625°C	600°C

RESULTS

The methods have been validated in rat and human milk and qualified in rabbit milk. The validation comprised of three different runs to assess the precision and accuracy of the method and the qualification comprised of one run. Additional experiments were included to assess selectivity, matrix effects/modification of ionisation, freeze/thaw stability, short-term room temperature stability and long-term frozen storage stability. The peptide recovery from milk and the autosampler carry-over were also assessed.

Lower limit of quantitation (Preclinical)

The lower limit of quantitation (LLOQ), as defined by the lowest QC at which inter-batch accuracy and the inter-batch precision was ≤ 20%. The signal to noise ratio (S/N) at the LLOQ was approximately 200/1.
The LLOQ sample was 100 pg/mL (Figure 1).

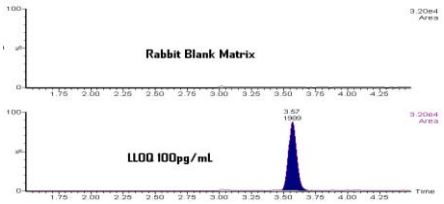

Figure 1: Chromatograms of rabbit blank matrix and LLOQ sample.

Linearity (preclinical)

Linearity was demonstrated from the LLOQ (100 pg/mL) to the highest evaluated concentration (ULOQ, 20000 pg/mL) using a linear regression of peak area ratio with a 1/x weighting.

Precision and Accuracy (Preclinical)

The overall intra-run and inter-run precision and accuracy data were within acceptable limits.

Table 1: Intra-run and Inter-run data for peptide in rat milk

Concentration (pg/mL)	Mean intra-run precision and accuracy		Inter-run precision and accuracy	
	Precision (CV%)	Bias (%)	Precision (CV%)	Bias (%)
100	12.0	-5.0	18.9	-4.2
300	5.4	-3.7	9.7	-3.7
4500	3.0	-4.8	6.3	-4.7
16000	1.8	-3.3	4.9	-3.1
40000[a]	2.3	2.0	5.2	2.0

[a] Diluted 5-fold with control rat milk prior to extraction

Figure 2: Preclinical method set up Waters Xevo TQ-S interfaced with Spark Holland Symbiosis

Lower limit of quantitation (Clinical)

The lower limit of quantitation (LLOQ), as defined by the lowest QC at which inter-batch accuracy and the inter-batch precision was ≤ 20%. The signal to noise ratio (S/N) at the LLOQ was approximately 80/1.

The LLOQ sample was 25.0 pg/mL (Figure 3).

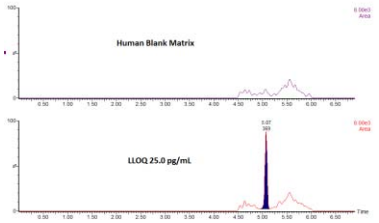

Figure 3: Chromatograms of human blank matrix and LLOQ sample

Linearity (Clinical)

Linearity was demonstrated from the LLOQ (25.0 pg/mL) to the highest evaluated concentration (ULOQ, 1000 pg/mL) using a linear regression of peak area ratio with a $1/x^2$ weighting.

Precision and Accuracy (clinical)

The overall intra-run and inter-run precision and accuracy data were within acceptable limits.

Table 2: Intra-run and Inter-run data for peptide in human milk

Concentration (pg/mL)	Mean intra-run precision and accuracy		Inter-run precision and accuracy	
	Precision (CV%)	Bias (%)	Precision (CV%)	Bias (%)
25.0	6.5	-18.1	5.6	-17.2
75.0	4.3	4.8	5.2	4.8
158	3.8	-6.2	4.1	-6.3
850	1.4	-0.6	2.0	-0.6

Figure 4: Clinical method set up Waters Xevo TQ-S interfaced with Waters 2D-UPLC.

Method summary

The optimum assay selectivity and sensitivity was achieved by interfacing the MS with a very selective online column switching employing a 'heart cutting' technique to concentrate and clean up the sample, followed by finely tuned ammonium formate/acetonitrile gradient chromatography on a hybrid HPLC column.

CONCLUSION

The method utilises offline protein/lipid depletion plates and online solid phase extraction and demonstrates the use of an orthogonal multidimensional approach. The preclinical method described for the determination of the peptide over the concentration range 100 to 20000 pg/mL has been successfully validated in rat milk and qualified in rabbit milk. The clinical method for the determination of the peptide in human milk has been validated over the concentration range of 25.0 to 1000 pg/mL. Both methods have been demonstrated to be sufficiently accurate and precise and to have sufficient selectivity, to reliably allow the determination of the peptide in rat, rabbit and human milk samples over the examined ranges. The pg/mL LLOQ has provided high quality PK data from early preclinical clinical studies, enabling the rapid progression of both programmes.

FERRING PHARMACEUTICALS

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Bioanalytical Solutions · York · Sandwich · Copenhagen

References

1.Wiśniewski K, et al. Synthesis and in vitro characterization of new, potent and selective oxytocin receptor agonists. Proceed 31st Eur Peptide Symp 2010 (Eds Lebl M, et al).