

The differences between digested and intact protein analysis: determination of somatropin in rat plasma

Bas Sleumer

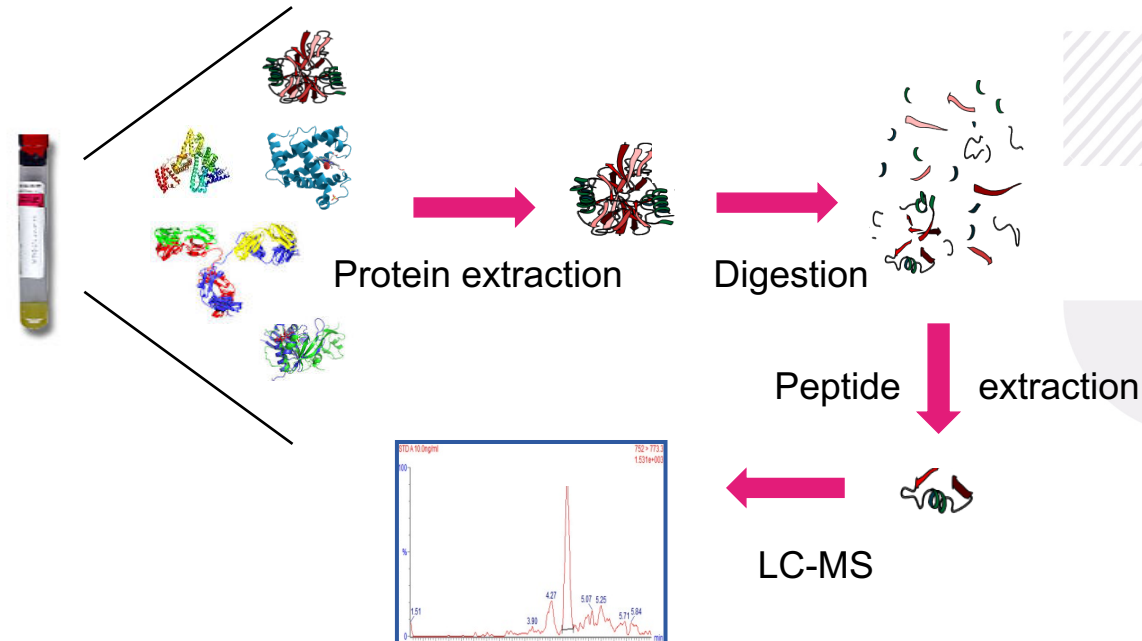
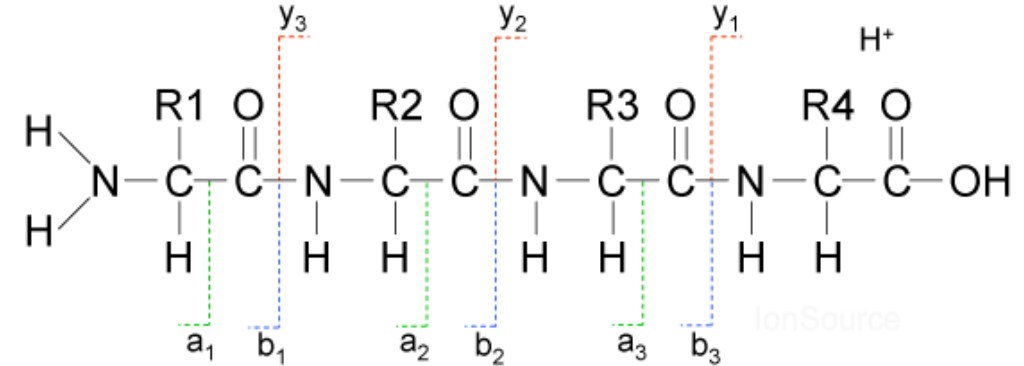
24-25 September 2020, YSS



Introduction

Digestion (bottom-up approach)

- Unique signature peptides after digestion
- Indirect measure of the intact protein
- Not representing the entire protein
- Triple quad mass spectrometer (low resolution)
- Fragmentation results in C-terminal y-ions and N-terminal b-ions

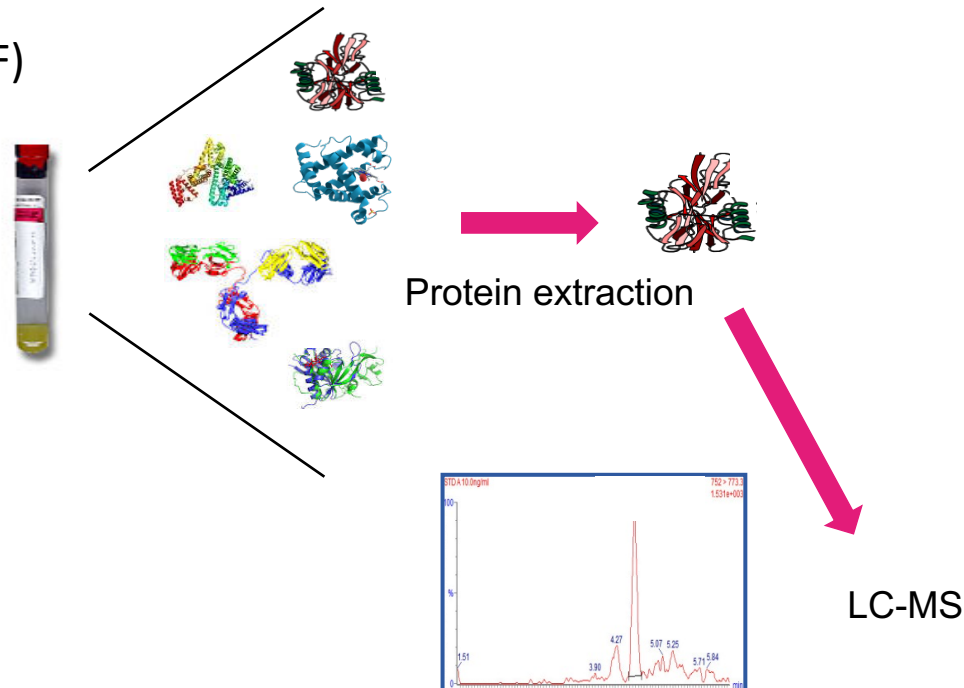




Introduction

Intact (top-down approach)

- Immunocapture
- Representing the entire protein
- No information lost
- High resolution mass spectrometer (LC-HRMS)
 - Quadrupole time of flight (TOF)
 - Orbitrap

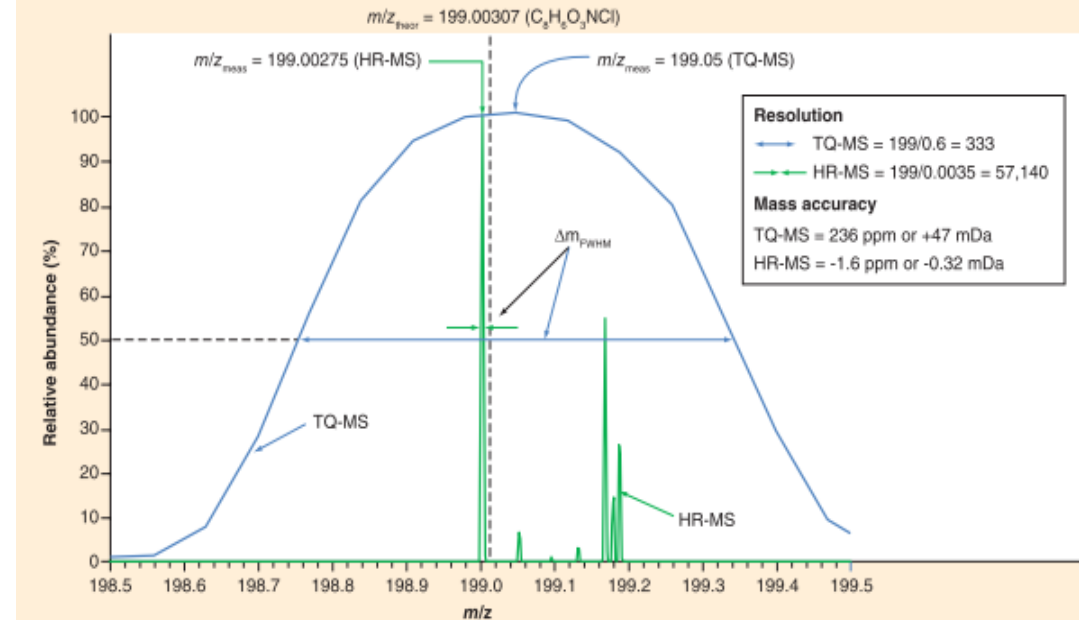
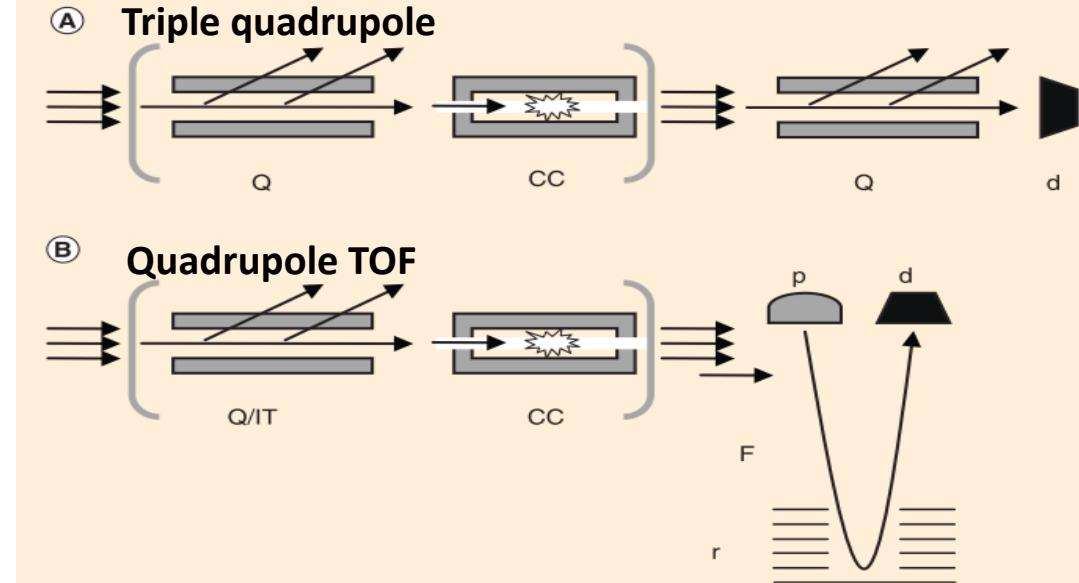




Introduction

Quadrupole time of flight (QTOF)

- Electrospray ionization
- Similar to triple quad but the third quad is a TOF
- Compared to triple quadrupole
 - Higher resolution
 - Higher mass range
 - Better mass accuracy
 - Adjustable mass extraction window

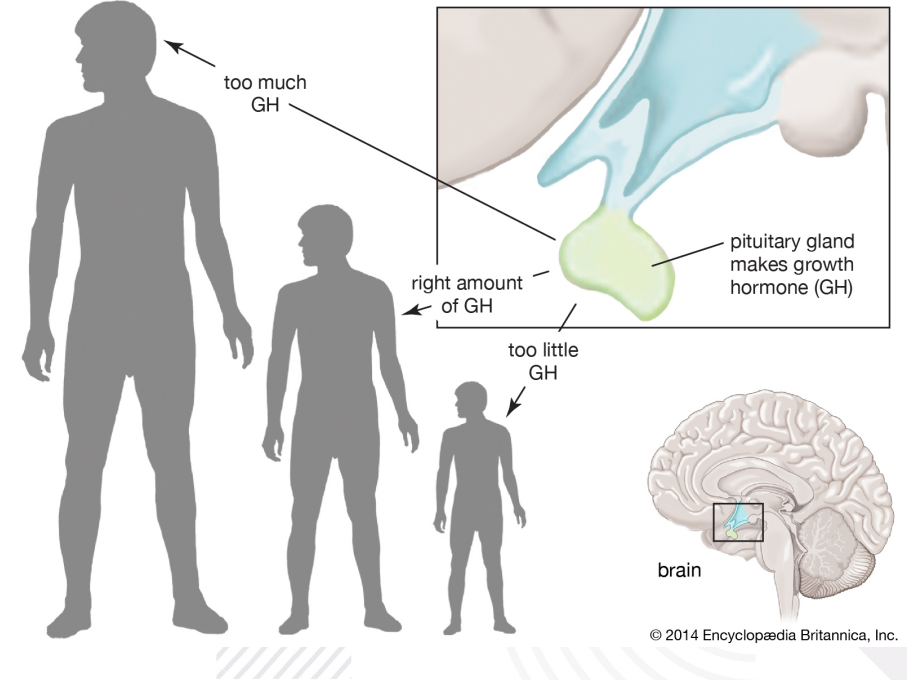




Somatropin

- Somatropin is the recombinant form of somatotropin (human growth hormone (hGH-1 22 kDa))
- Somatotropin is a heterogeneous and endogenous protein
- Main hGH isoform with 191 amino acids
- Expressed in the pituitary gland
- Biomarker in the diagnosis of growth disorders
- Drug to treat growth disorders (deficiency)

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FPTI PLSRLFDNAM LRAHRLHQLA FDTYQEFEEA YIPKEQKYSF LQNPQTSLCF
SESIPTPSNR EETQQKSNLE LLRISLLLIQ SWLEPVQFLR SVFANSLVYG ASDSNVYDLL
KDLEEGIQTL MGRLEDGSPR TGQIFKQTYS KFDTNSHND ALLKNYGLLY CFRKDMDKVE
TFLRIVQCRS VEGSGGF
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The determination of somatropin after digestion

Introduction

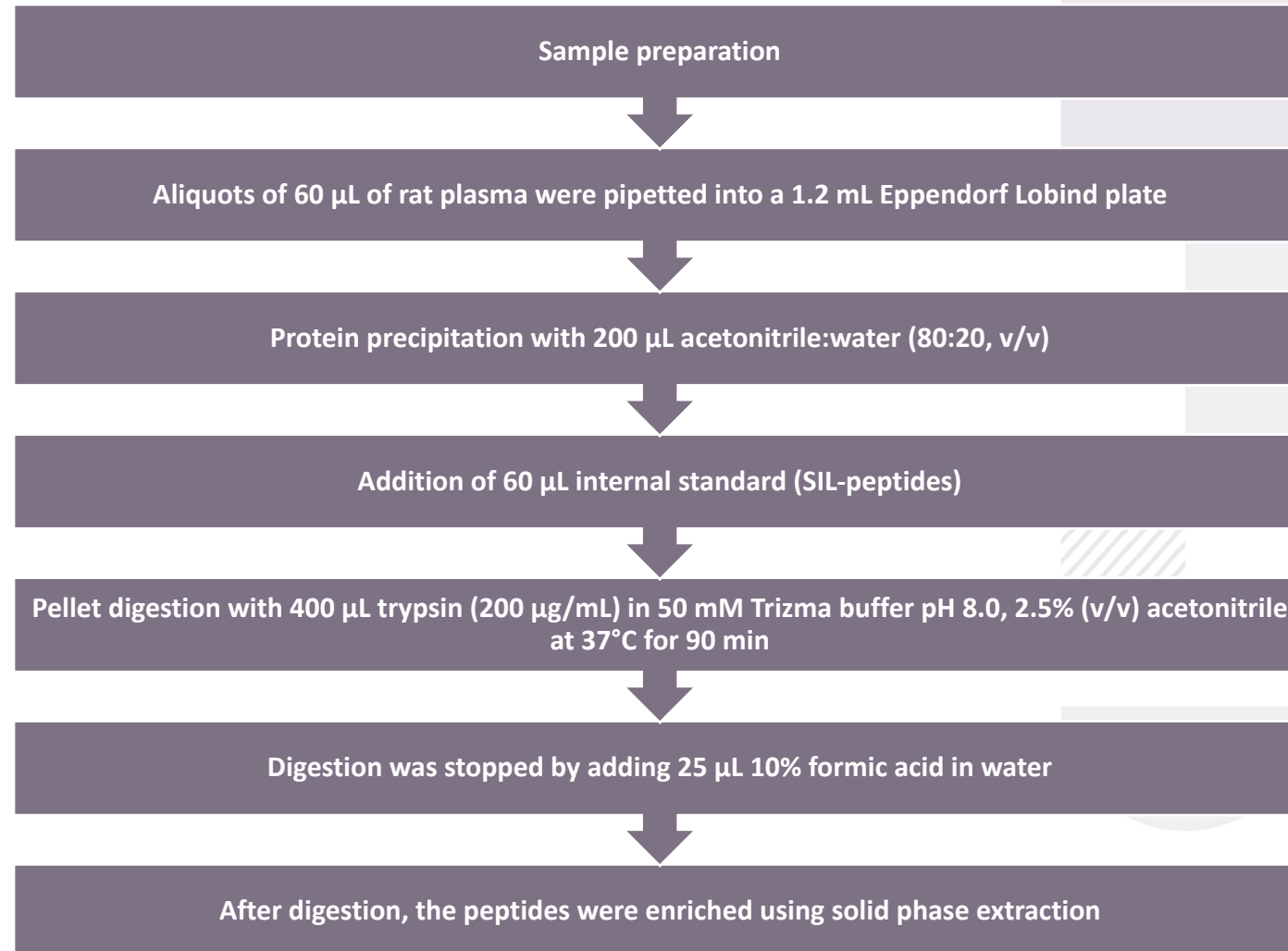
- To support pre-clinical research
 - Sprague Dawley rats were dosed with somatropin
- Quantification using 3 different signature peptides: LFDNAMLR, SNLELLR and FDTNSHNDDALLK
- Triple quadrupole compared to quadrupole TOF
- Effect of reduced mass extraction window (MEW)
- Range: 25.0-10000 ng/mL (somatropin)

FPTI PLSRLFDNAM LRAHRLHQLA FDTYQEFEEA YIPKEQKYSF LQNPQTS LCF
SESIPTPSNR EETQQKSNLE LLRISLLLIQ SWLEPVQFLR SVFANSLVYG ASDSNVYDLL
KDLEEGIQTL MGRLEDGSPR TGQIFKQTYS KFDTNSHNDD ALLKNYGLLY CFRKDMDKVE
TFLRIVQCRS VEGSGGF



The determination of somatropin after digestion

Material and methods





The determination of somatropin after digestion

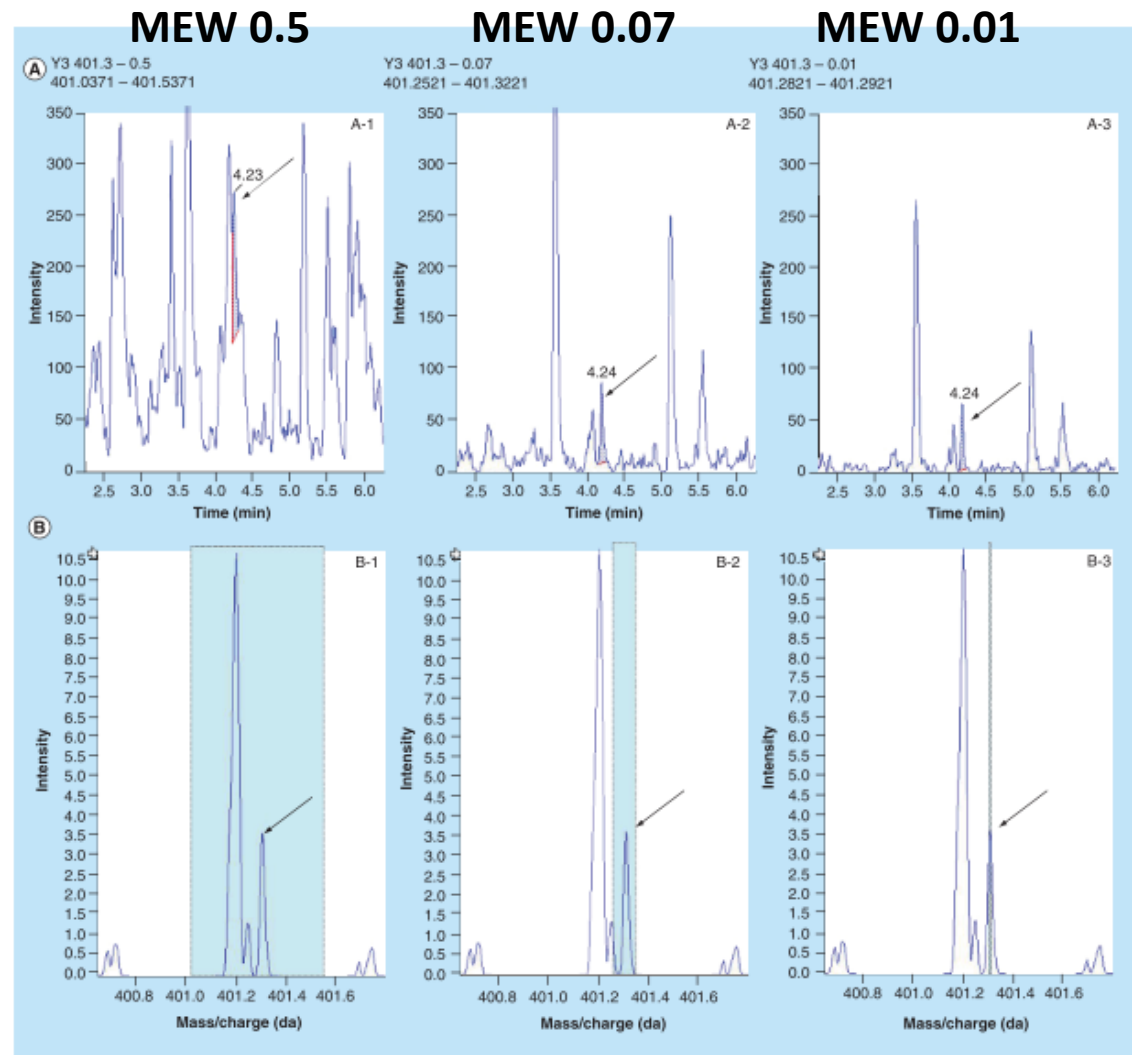
Material and methods

Chromatographic settings	
NexeraX2 (Shimadzu)	
Mobile phase A	0.1% formic acid in water:2-propanol (95:5, v/v)
Mobile phase B	0.1% formic acid in acetonitrile:2-propanol (95:5, v/v)
Column	100x2.1 mm (1.6 µm, 100 Å) Phenomenex Luna Omega C18
Flow rate (mL/min)	Gradient elution at 0.6 mL/min
Column temperature	60°C
Mass spectrometer settings	
Quadrupole TOF(Sciex TripleTOF 6600)/triple quadrupole (Sciex TripleQuad 6500)	
Quadrupole TOF	Operated in high sensitive mode in positive mode (ESI)
	MEW (Da): 0.07; FDTNSHNDDALLK, 0.01; SNLELLR and LFDNAMLRL
Triple quadrupole	Operated in unit-mass resolution in positive ion mode (ESI)
	MEW (Da): 0.7



The determination of somatropin after digestion

Results



A: Chromatograms of SNLELLR

B: Spectra of SNLELLR

Precision (CV) and accuracy for SNLELLR y3 under different MEW; somatropin concentration in plasma: 25 ng/mL, n=6

MEW (Da)	CV (%)	Accuracy (%)
0.5	61.4	222.0
0.07	6.0	104.2
0.01	19.6	94.4



The determination of somatropin after digestion

Results

Accuracy and precision results for both quadrupole TOF and triple quadrupole, n=6					
		Quadrupole TOF		Triple quadrupole	
Peptide	Concentration (ng/mL) LLOQ quadrupole TOF: 25.0 ng/mL LLOQ triple quadrupole: 100 ng/mL	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
FDTNSHNDDALLK	25.0	15.7	106.6	4.4	242.2
	50.0	10.6	103.7	4.5	143.6
	75.0	4.4	110.1	2.2	144.9
	100			2.1	94.7
SNLELLR	25.0	11.9	103.3	8.1	230.4
	50.0	10.4	98.7	7.3	135.8
	75.0	10.9	99.6	3.5	125.3
	100			5.3	87.5
LFDNAMLR	25.0	11.3	88.6	3.5	226.9
	50.0	16.3	80.8	4.5	131.6
	75.0	7.2	110.2	2.5	133.4
	100			0.9	93.0

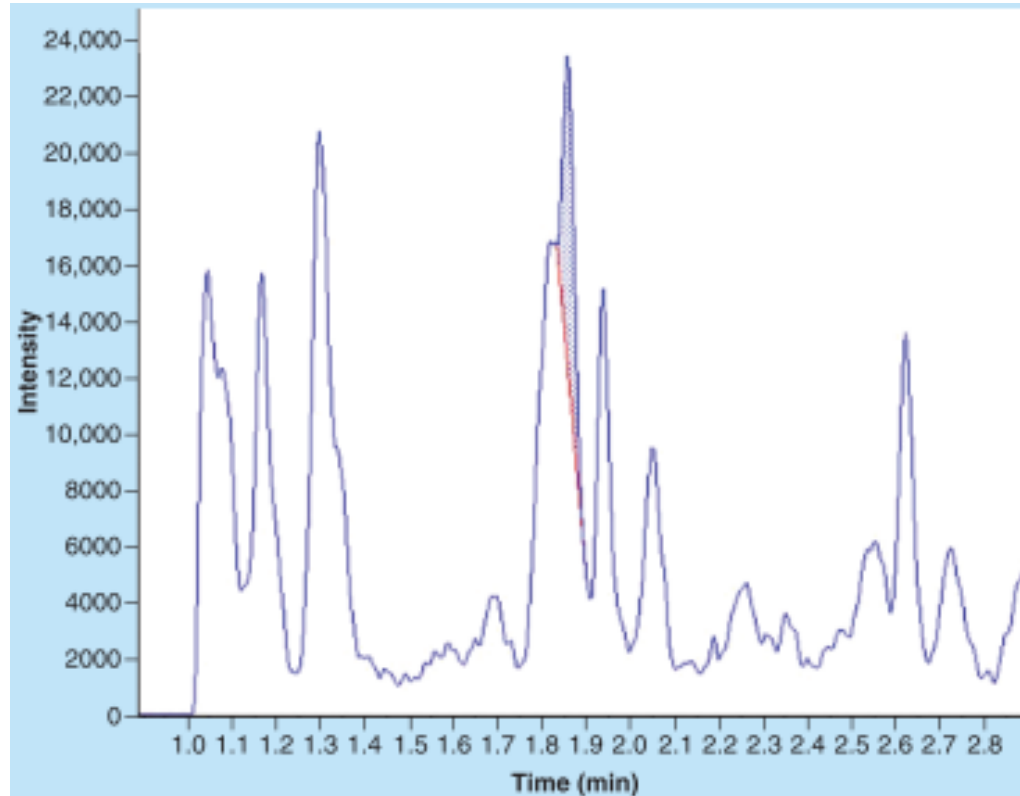


The determination of somatropin after digestion

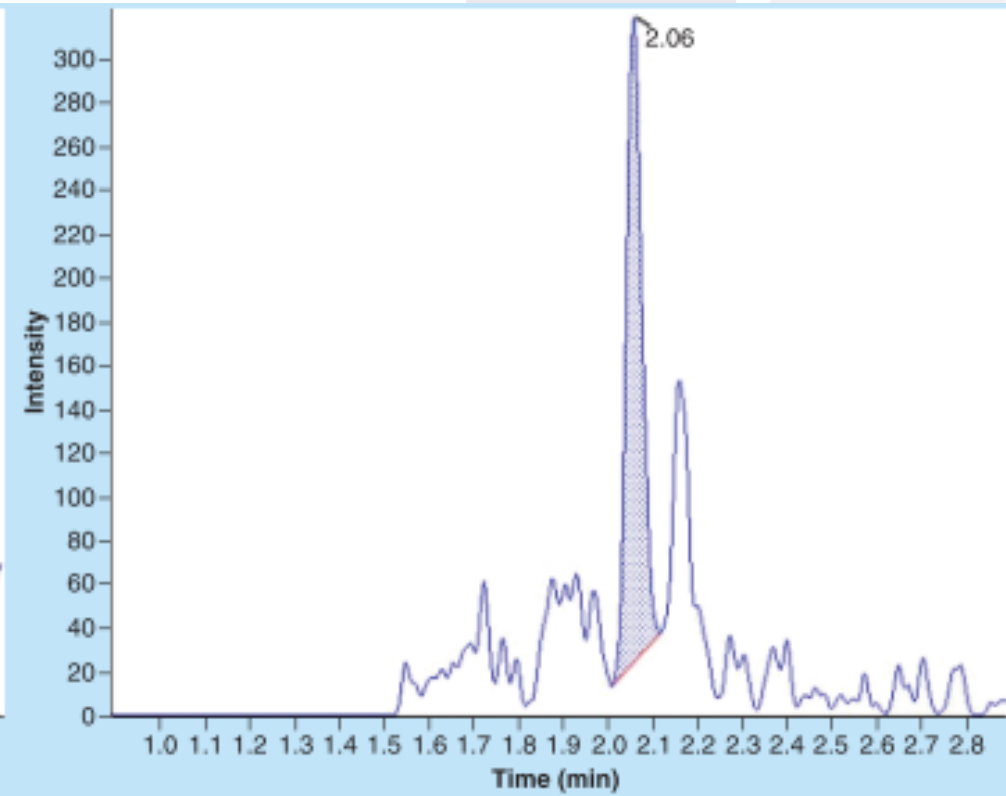
Results

Chromatograms of the SNLELLR peptide after digestion of rat plasma spiked with 25.0 ng/mL somatropin

Triple quadrupole



Quadrupole TOF

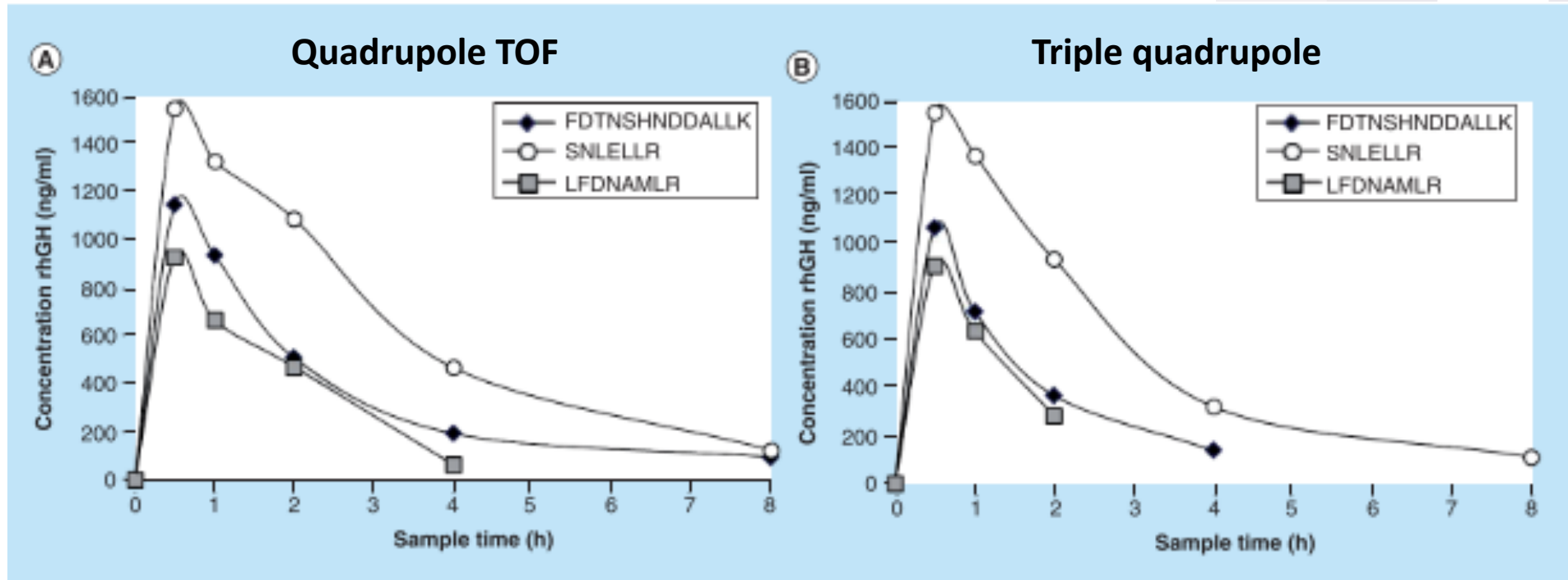




The determination of somatropin after digestion

Results

- Results comparable on triple quad and quadrupole TOF
- Later time points on triple quadrupole unquantifiable
- Results of different peptides dissimilar
- Possible in vivo effect

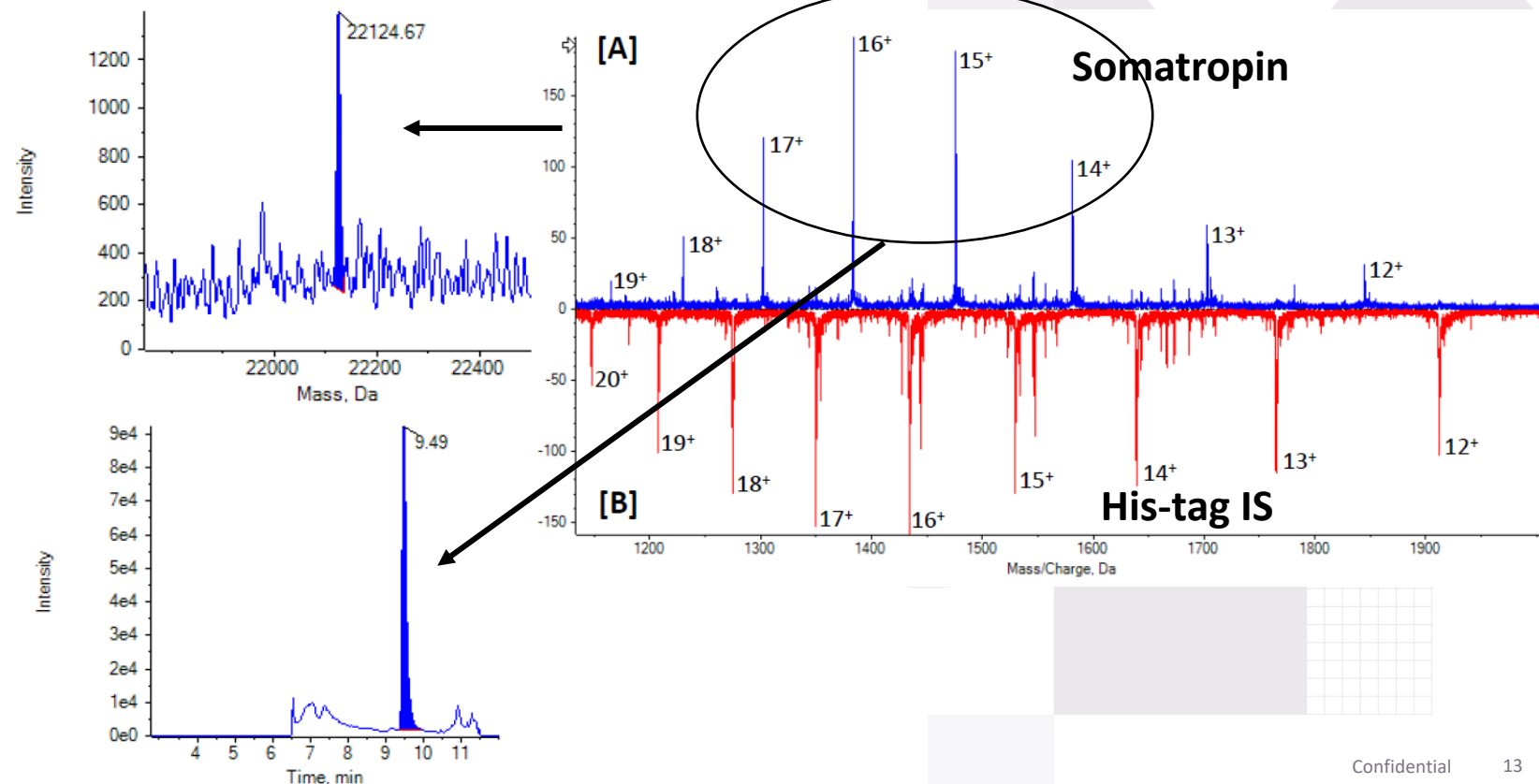




The determination of intact somatropin

Introduction

- To support pre-clinical research
 - Sprague Dawley rats were dosed with somatropin.
- Quantification of the intact protein
- Immunocapture
- His-tag internal standard
- Deconvolution compared to summation of charge states
- Range: 10-10000 ng/ml



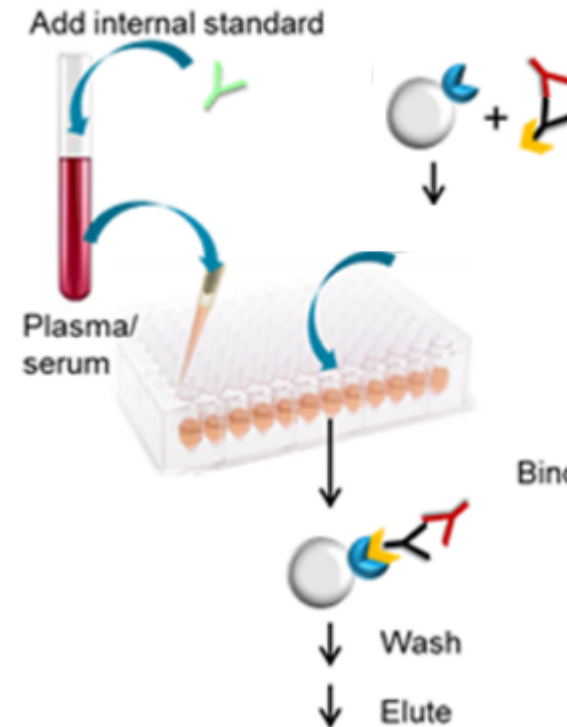


The determination of intact somatropin

Material and methods

200 μL in immunocapture buffer (75 mM NaCl and 8.4 mM aqueous Tris buffer at pH 7.2)

75.0 μL



50.0 μL capture antibody solution
(binding with somatropin)

15.0 μL streptavidin-coated beads

Binding Capturing of the antibody-somatropin
complex by the magnetic beads

Immunocapture buffer

60 μL elution buffer 0.1M citric acid in
water : acetonitrile (90 : 10, v/v))



The determination of intact somatropin

Material and methods

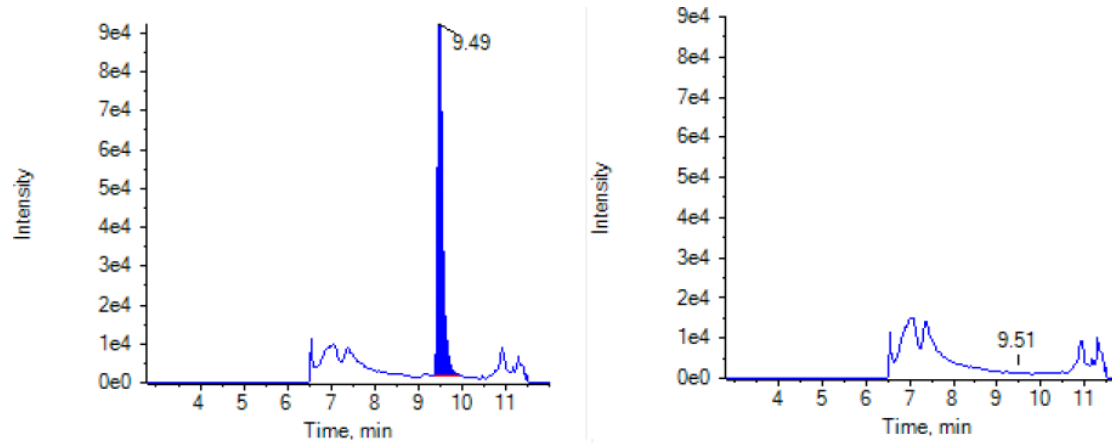
Chromatographic settings	
NexeraX2 (Shimadzu)	
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile
Column	100x2.1 mm (1.7 µm, 300 Å) ACQUITY UPLC Protein BEH C4
Flow rate (mL/min)	Gradient elution at 0.6 mL/min
Column temperature	80°C
Mass spectrometer settings	
Quadrupole TOF(Sciex TripleTOF 6600)	
Quadrupole TOF	Summation of the 14+, 15+, 16+ and 17+ charge states/deconvolution
MEW (Da)	1.0



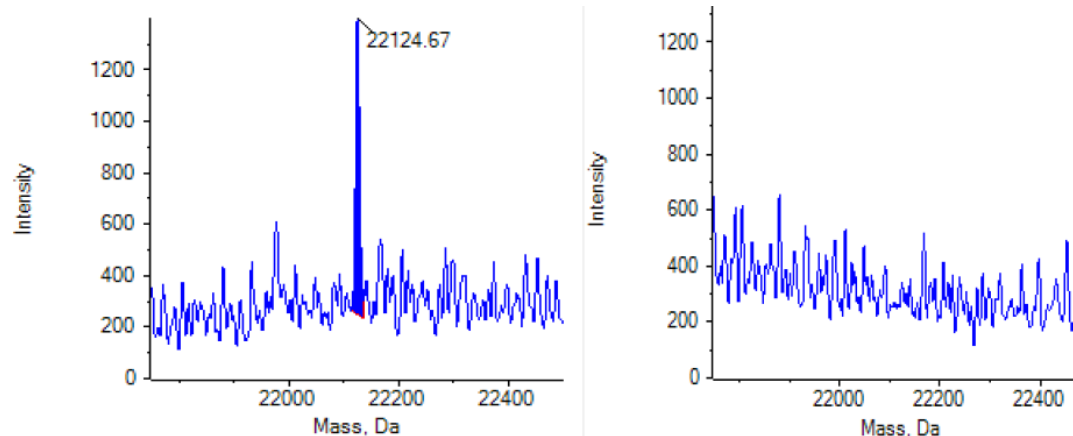
The determination of intact somatropin

Results

14+ to 17+ charge states summated (MEW 1.0 Da) **chromatograms** of a blank plasma and plasma spiked at 30.0 ng/mL



Deconvoluted **mass spectra** of a blank plasma and plasma spiked at 30.0 ng/mL





The determination of intact somatropin

Results

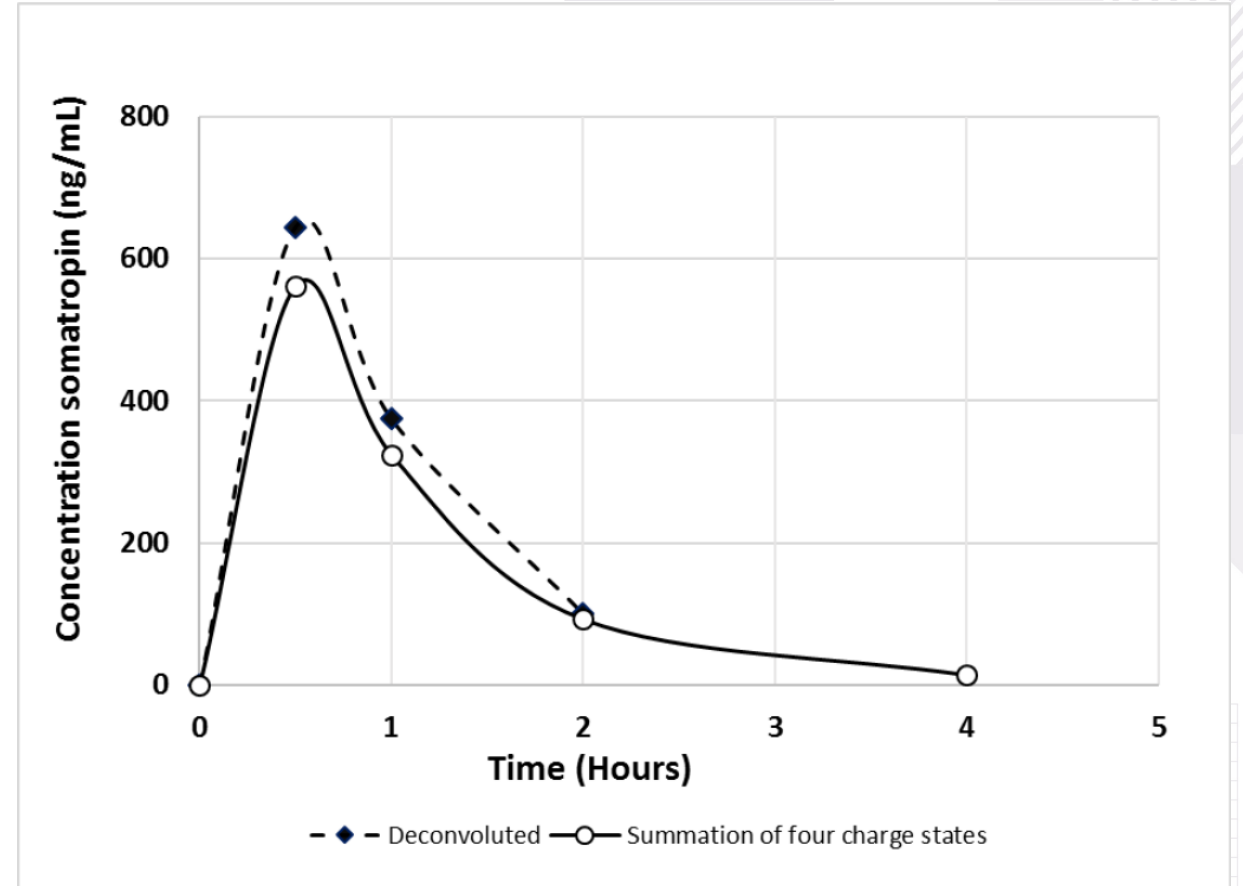
Summary of accuracy and precision results for two different detection settings, n=18				
Charge state	MEW (Da)	Nominal concentration (ng/mL)	CV (%)	Accuracy (%)
17-16-15-14	1.0	10.0	10.6	86.1
Deconvolution	NA	10.0	-	-
17-16-15-14	1.0	30.0	13.1	89.7
Deconvolution	NA	30.0	12.0	89.8
17-16-15-14	1.0	100	7.2	102.9
Deconvolution	NA	100	11.2	107
17-16-15-14	1.0	500	14.0	105.9
Deconvolution	NA	500	8.1	106.4



The determination of intact somatropin

Results

- Data of both quantification approaches agree quite well
- Latest time point after deconvolution is unquantifiable





Conclusion/discussion

- Digestion

- Reduction of MEW on quadrupole TOF improves selectivity and LLOQ
- LLOQ on quadrupole TOF (MEW 0.01/0.07 Da): 25.0 ng/mL, on triple quadrupole (MEW 0.7 Da): 100 ng/mL
- The three signature peptides gave dissimilar concentration results in the plasma samples.

- Intact

- LLOQ on quadrupole TOF is 10.0 ng/mL, using immunocapture and summation of charge states
- LLOQ after deconvolution: 30.0 ng/mL
- Concentration results in plasma samples after charge state summation and deconvolution agreed well
- Lower LLOQ compared to the digestion method



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