

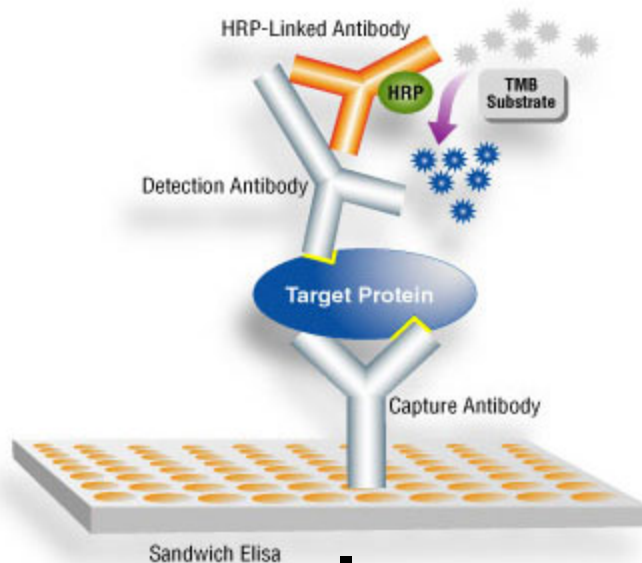
Getting More with Less: Improving Sensitivity and Reducing Sample Consumption in LC/MS Assays for Endogenous and Injected Glucagon, 6 Insulins, and Teriparatide

Erin E. Chambers
Principal Scientist

- ■ Background and Key Challenges
- Practical Applications of Integrated Microscale LC
 - Routine Ultra-high Sensitivity Teriparatide Quantification: Adaption and Benefits from Analytical to Microscale LC
 - Endogenous and Therapeutic Glucagon Analysis
 - Increasing Sensitivity and Reducing Sample Volume Required for Quantification of Multiple Insulin Analogs
 - Reducing Sample Volumes for Small peptides
- Conclusions

Why LC-MS/MS?

- Why an LC-MS/MS based assay?
 - Challenges with ligand-binding assays
 - inability to distinguish closely related analogs
 - require separate assay for each peptide
 - limited linear dynamic range
 - Possible cross reactivity
 - Lack of standardization
 - Long development times
- Benefits of LC-MS/MS
 - Easy to multiplex
 - Broad linear dynamic range
 - Accurate, precise
 - Universal
 - Faster, cheaper method development



Key Challenges in LC/MS Remain

However:

- Ultra-high sensitivity required for most quantitative peptide assays
- Need to obtain the sensitivity of LBAs using LC/MS
 - Small sample volumes
- Need to obtain specificity that is comparable to LBAs using LC/MS

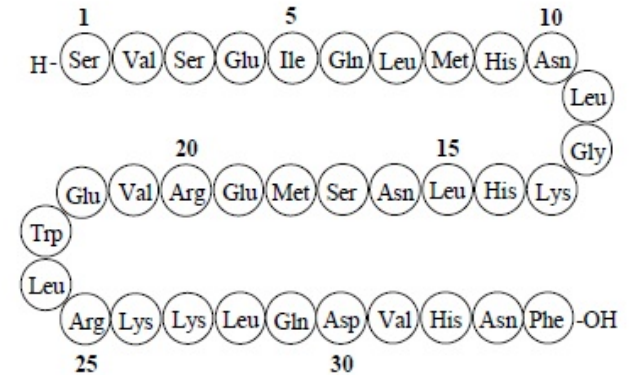
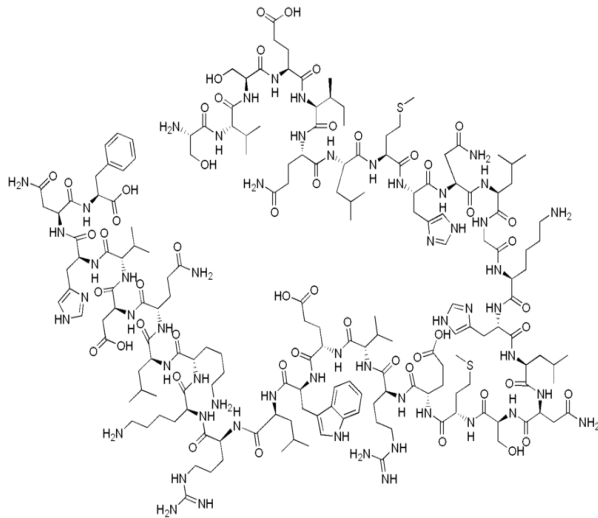


- Background and Key Challenges

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Teriparatide (rhPTH)

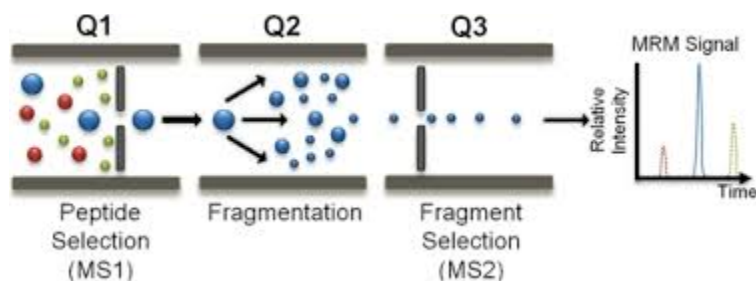
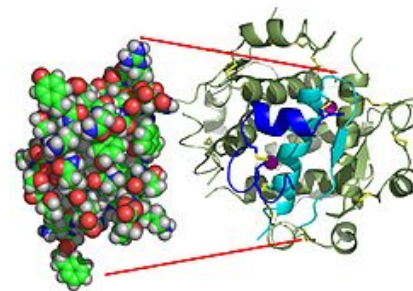
- Recombinant form of 1 – 34 amino acids from human parathyroid hormone (PTH)
- Stimulates new bone formation leading to increased bone mineral density
- Use for people with osteoporosis at a high risk for fracture



Teriparatide
(Forteo®)
Avg MWT 4117.7

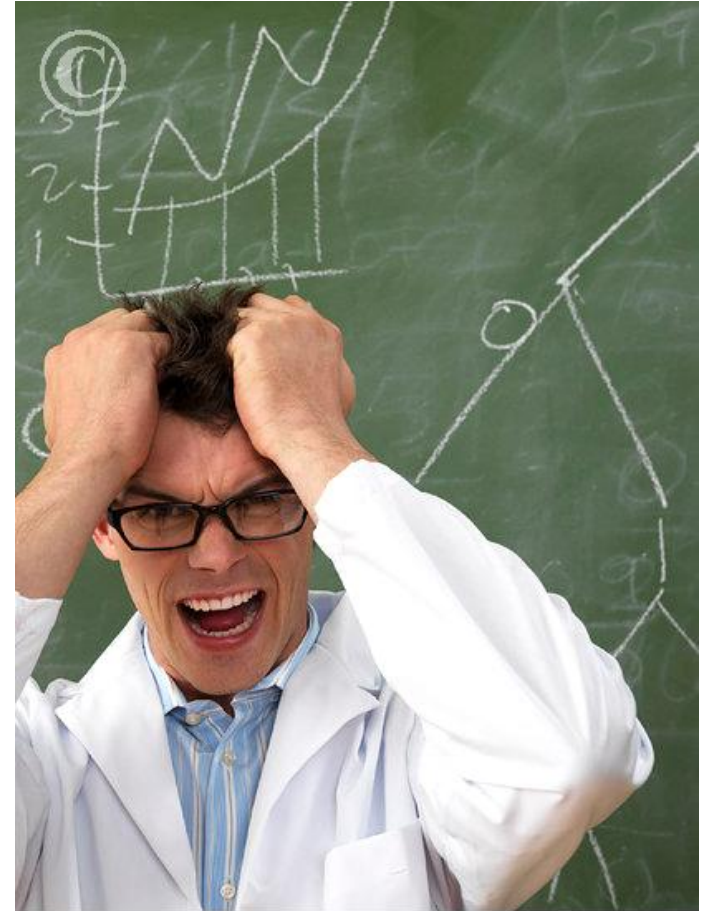
Why LC/MS for teriparatide?

1. Coming off patent 2018
 - Bioequivalence studies
 - Development of new versions
2. Replacement for original RIA method
 - Improved accuracy and precision through LC/MS
 - Avoid cross-reactivity and dilution issues
 - LC/MS can differentiate parent and metabolites and allows single assay for multiple compounds



Specific Challenges in Developing an LC-MS/MS Assay for Teriparatide

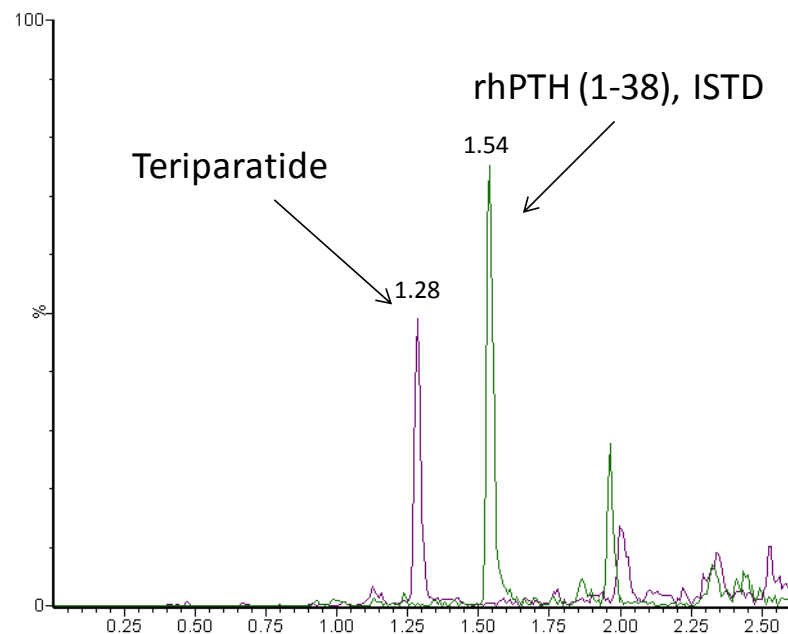
- ★ Obtain sensitivity and specificity similar to LBAs
 - Minimize sample volume
 - Specificity in matrix
 - High level of non-specific binding (NSB)
- ★ Low MS sensitivity
 - Poor fragmentation
 - Multiple precursors
 - Typical 20 μg clinical dose = plasma levels $\sim 50 \text{ pg/mL}$
 - Chromatographic peak shape
 - Protein binding



600-01695338 [RF] © www.visualphotos.com

Original **Analytical** Scale Method

- Analytical Column: **ACQUITY UPLC CSH C18** 2.1 X 50mm, 1.7 μm
- Mobile phase A= 0.1% formic acid in water
- Mobile phase B= 0.1% formic acid in ACN
- Gradient: hold 15% B for 0.2 min, ramp to 50% B at 3.8 min, flush with 98% B, return to initial
- Flow Rate: 0.4 mL/min
- Column Temp: 60°C
- Sample Temp: 5°C
- Injection Volume: **30 μL**



Chambers et al, *Journal of Chromatography B*, Volume 938, 1 November 2013, Pages 96-104

Extraction Conditions

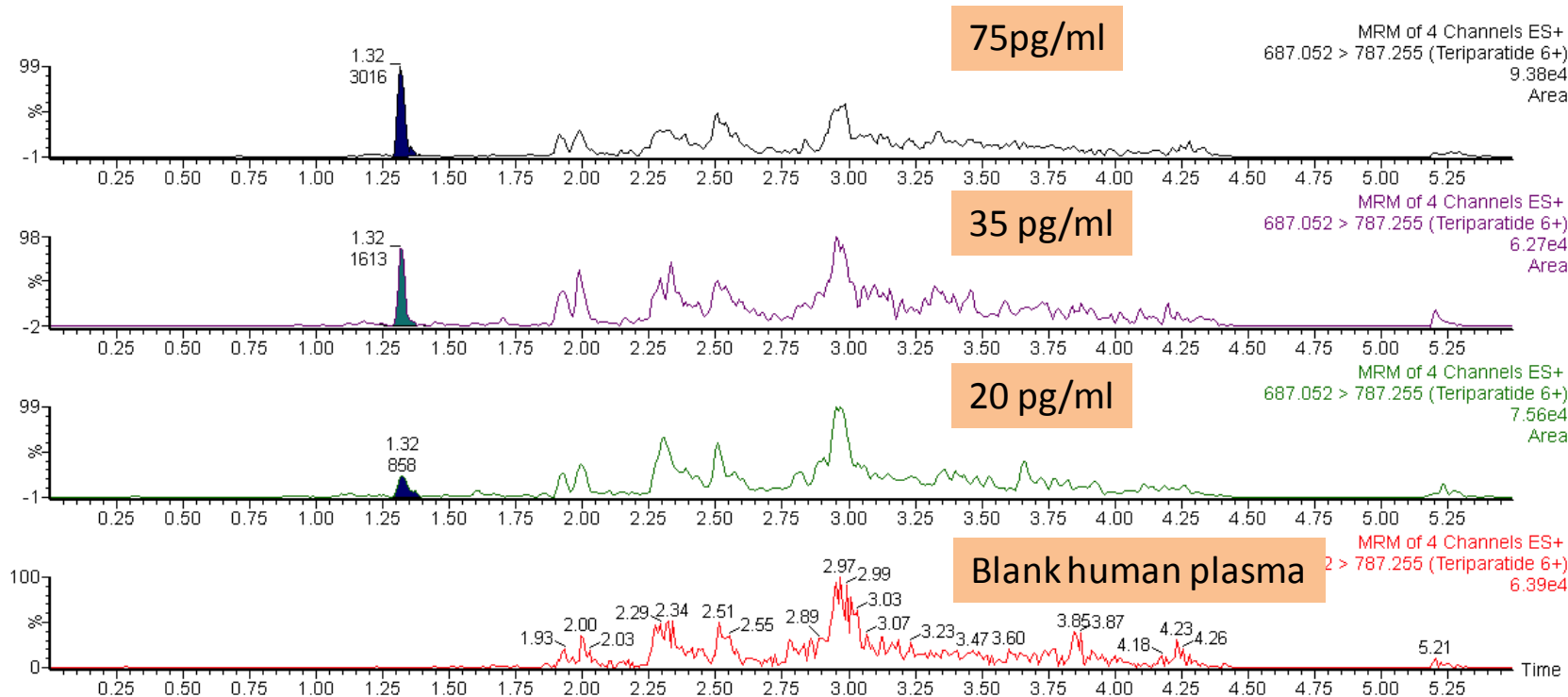
PPT followed by Polymeric Reversed-Phase SPE in μ Elution 96-well plate

- **PPT:** 200 μ L human plasma sample precipitated 1:1 with 5% NH_4OH in ACN, vortex spin 15 min at 4000 rpm; dilute supernatant with 1 mL water
- **SPE:** Oasis[®] HLB μ Elution 96-well plate
- Condition: 200 μ L methanol
- Equilibrate: 200 μ L water
- Load Sample: entire diluted supernatant in 2 steps
- Wash: 200 μ L 5% MeOH in water
- Elute: 2X 25 μ L 60/34/5/1 ACN/water/TFE/TFA
- Dilute: 50 μ L water
- Inject 30 μ L

PPT Improves Specificity by
Eliminating High Abundance Proteins



Analytical Scale



200 μ L sample, 30 μ L injection

Standard Curve Statistics

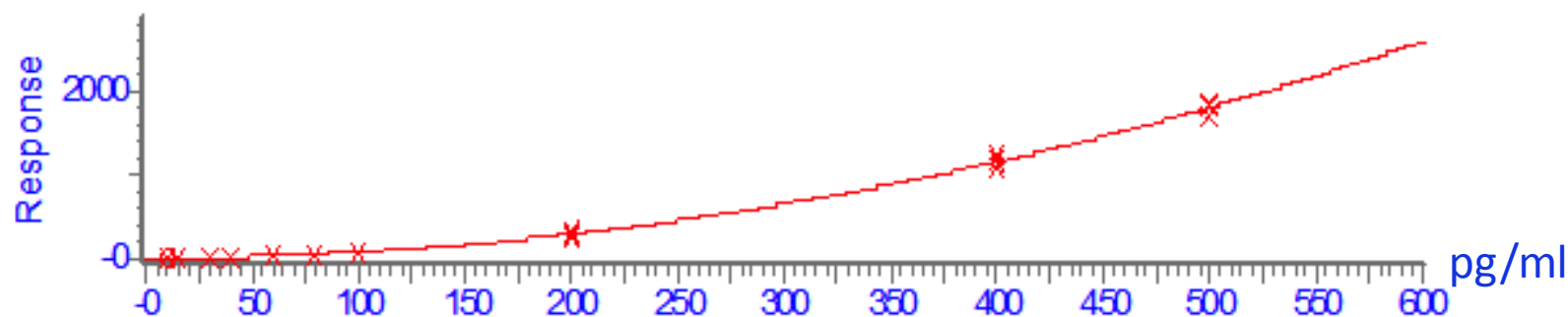
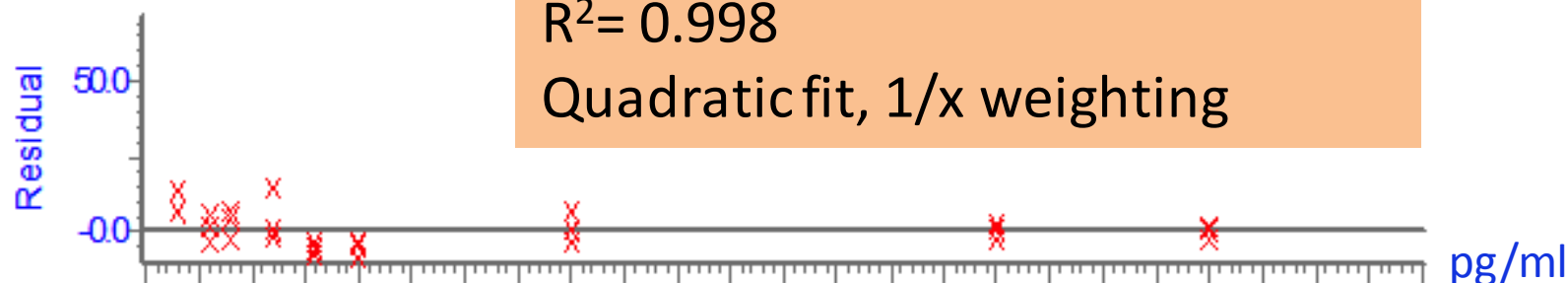
Human Plasma Lot# X1793C (Biological Specialty Corp.)	Std. Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
15pg/ml	15	16.65	0.64	3.9	3/3	111.0
30pg/ml	30	30.48	1.39	4.6	3/3	101.6
40pg/ml	40	41.09	1.95	4.8	3/3	102.7
60pg/ml	60	62.78	5.20	8.3	3/3	104.6
80pg/ml	80	75.38	1.56	2.1	3/3	94.2
100pg/ml	100	94.39	2.78	2.9	3/3	94.4
200pg/ml	200	202.60	10.63	5.2	3/3	101.3
400pg/ml	400	401.90	10.86	2.7	3/3	100.5
500pg/ml	500	498.47	10.36	2.1	3/3	99.7

Representative Standard Curve

Teriparatide

$R^2 = 0.998$

Quadratic fit, 1/x weighting



Teriparatide Extracted from Human Plasma: 15-500 pg/mL

Representative QC Statistics

Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	20	20.25	1.50	7.3	3/3	102.2
82111	Female	20	33.38	1.75	8.6	3/3	102.2
57298	Male	20	18.30	0.67	3.7	3/3	91.5
82740	Female	20	20.74	1.59	7.7	3/3	103.7
57901	Male	20	19.67	0.60	3.0	3/3	98.3
X1803C	Mixed	20	21.27	0.96	4.5	2/3	106.4
Human Plasma Lot# (Biological Specialty Corp.)	Gender	QC Conc. (pg/mL)	Mean Calculated Conc. (pg/mL)	Std. Deviation	%CV	Number of Replicates Passed	Mean Accuracy
X1793C	Mixed	35	33.35	1.99	6.0	2/3	95.3
82111	Female	35	34.48	1.84	5.3	3/3	98.5
57298	Male	35	31.64	1.81	5.7	3/3	90.4
82740	Female	35	33.29	1.73	5.2	3/3	95.1
57901	Male	35	34.71	0.30	0.9	3/3	99.2
X1803C	Mixed	35	34.24	2.66	7.8	3/3	97.8

What's Next?

■ Desired Improvements

- Reduce sample size
 - Preclinical tox
 - Pediatrics
- Increase sensitivity
- Simplify curve fit



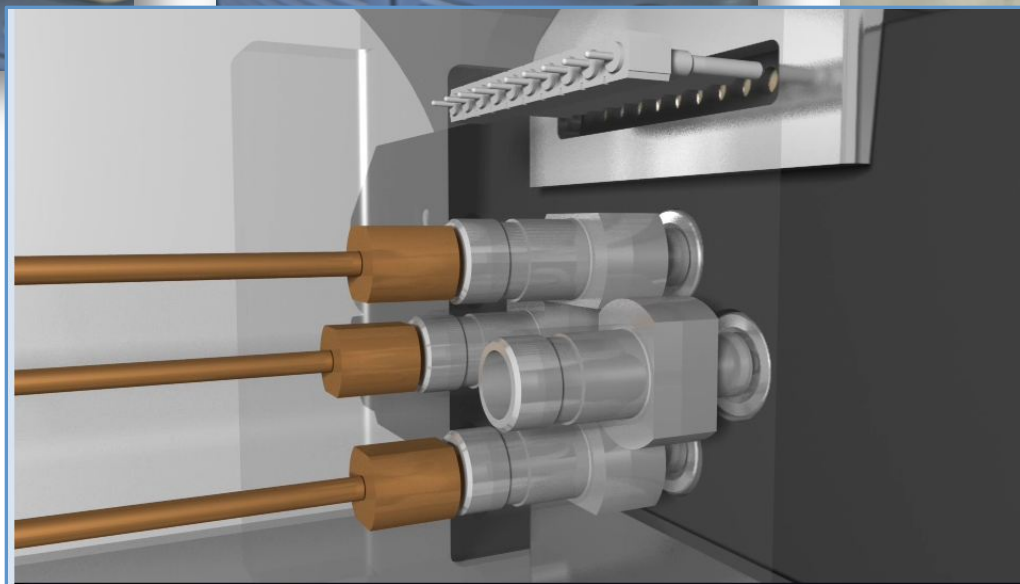
■ How do we get there?



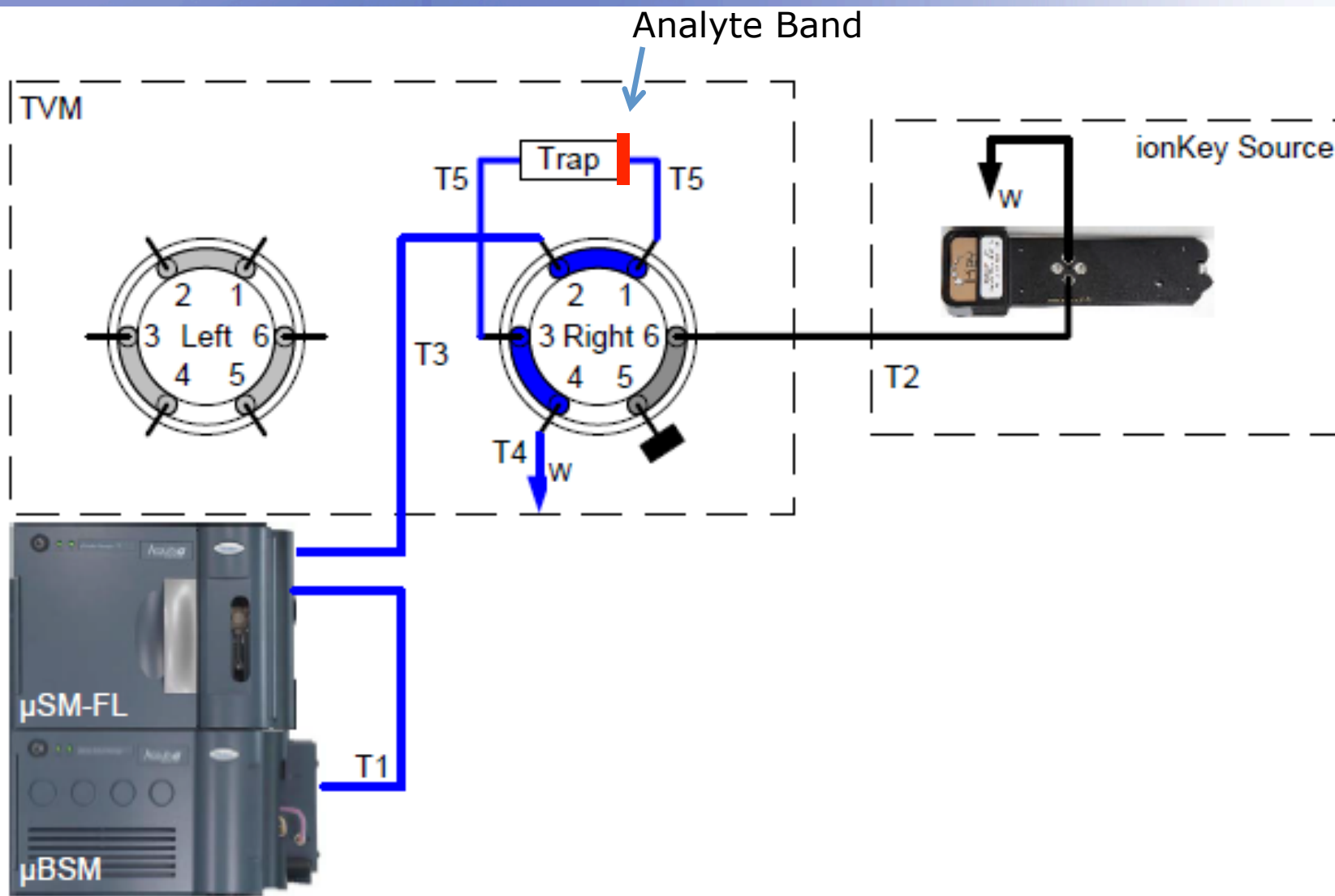
Can I Successfully Adapt a Highly Optimized Analytical Scale Method??

Integrated Microflow LC: ionKey/MS Ion Source

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®



Teriparatide ionKey/MS Analysis in Trap and Back-elute Mode



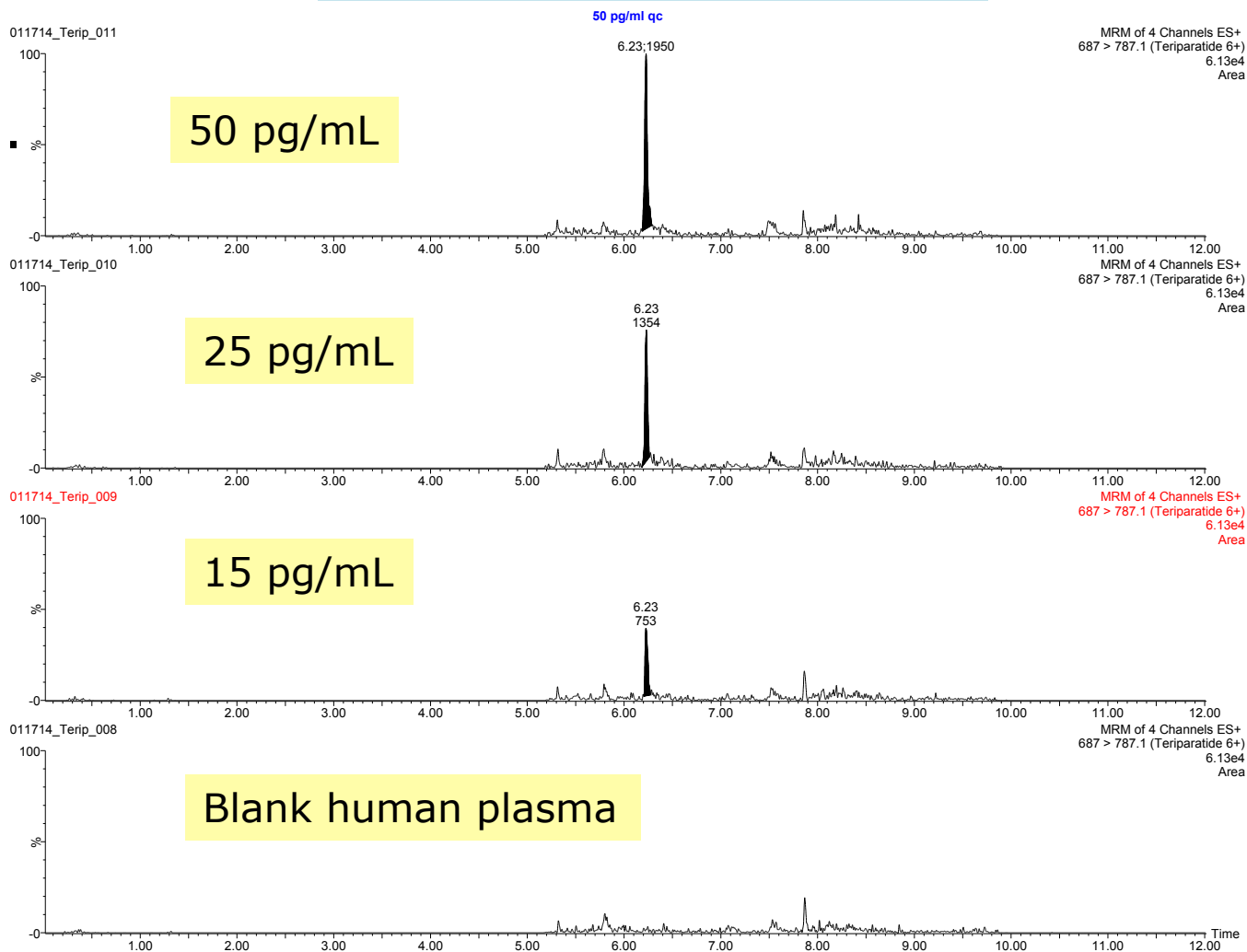
Microscale Method: At-column-dilution and Trap and Back Elute

- iKey: 150 μm X 50 mm BEH PST C18, 1.7 μm
- Trap column: Symmetry C18 5 μm , 300 μm X 50mm
- Mobile phase A= 0.1% formic acid in water
- Mobile phase B= 0.1% formic acid in ACN
- Loading time: 2 minutes
- iKey Temp: 75°C
- Injection Volume: 10-15 μL
- Gradient:

Time (min)	Flow Rate ($\mu\text{L}/\text{min}$)	Composition A (%)	Composition B (%)
0.00	2.0	85	15
5.00	2.0	55	45
6.00	2.0	5	95
8.00	2.0	5	95
9.00	2.0	85	15

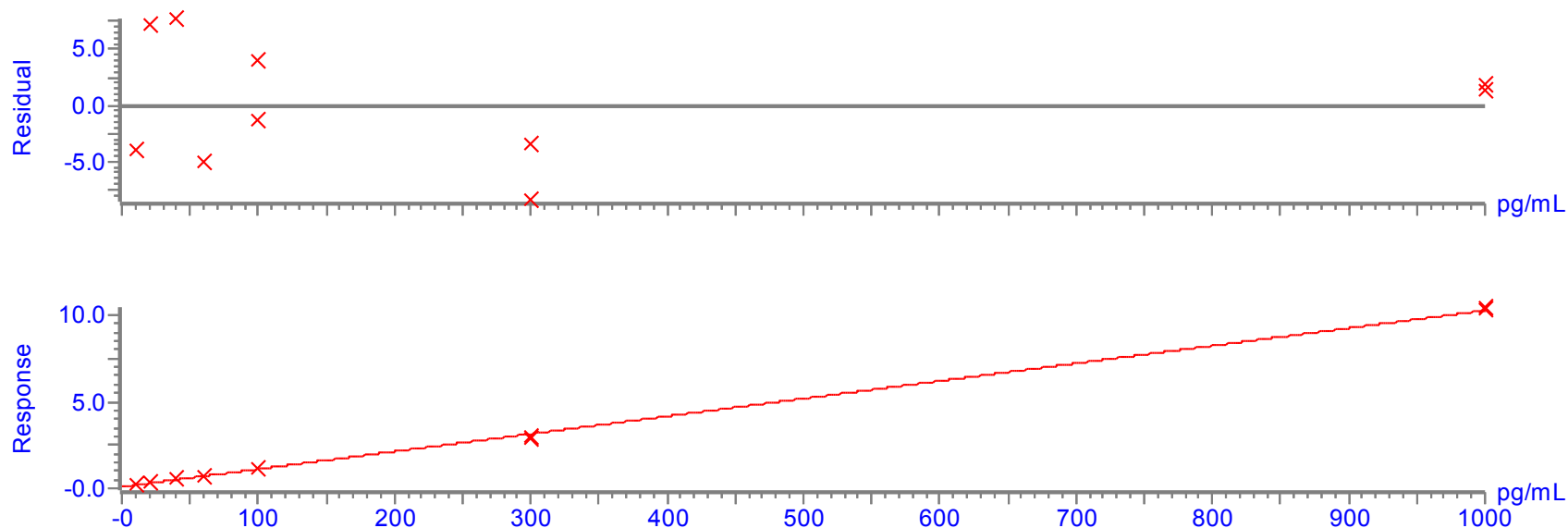
Phase 2: Improve Method Reduce Sample Size from 200 to 50 μL

50 μL human plasma extracted



Phase 2: Improve Method Dynamic Range Increased, Linear Fit

Compound name: Teriparatide 687
Correlation coefficient: $r = 0.999254$, $r^2 = 0.998508$
Calibration curve: $0.010184 * x + 0.0879951$
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Teriparatide Extracted from 50 μ L Human Plasma: 10-1000 pg/mL
Linear fit and broader range than analytical

Standard Curve Statistics: ionKey/ MS

Teriparatide Concentration (pg/mL)	Teriparatide/IS Ratio Response	Calculated Teriparatide Concentration (pg/mL)	Mean Accuracy
10.00	0.07	10.56	105.63
20.00	0.14	20.53	102.63
40.00	0.29	38.99	97.58
60.00	0.43	57.58	95.97
100.00	0.73	97.00	97.00
300.00	2.17	286.39	95.50
600.00	4.75	626.81	104.45
1000.00	8.05	1061.49	106.15
3000.00	22.31	2937.14	97.95

Representative QC Statistics

Teriparatide QC Concentration (pg/mL)	Mean (N=5) Calculated Concentration (pg/mL)	SD	%CV	Mean Accuracy
25	25.8887	1.32	5.09	103.6
50	51.4236	1.91	3.72	102.8
80	83.8803	2.15	2.56	104.9
200	202.3569	6.49	3.20	101.2
500	511.1018	15.23	2.98	102.2

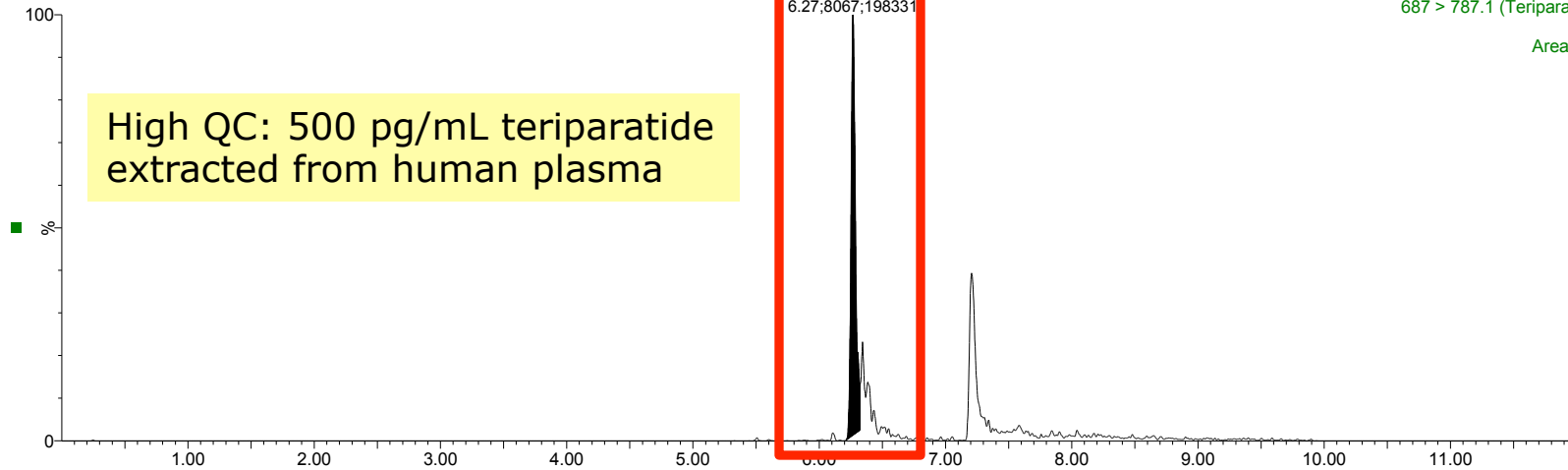
Can I Successfully Adapt a Highly Optimized Analytical Scale Method?

Yes!

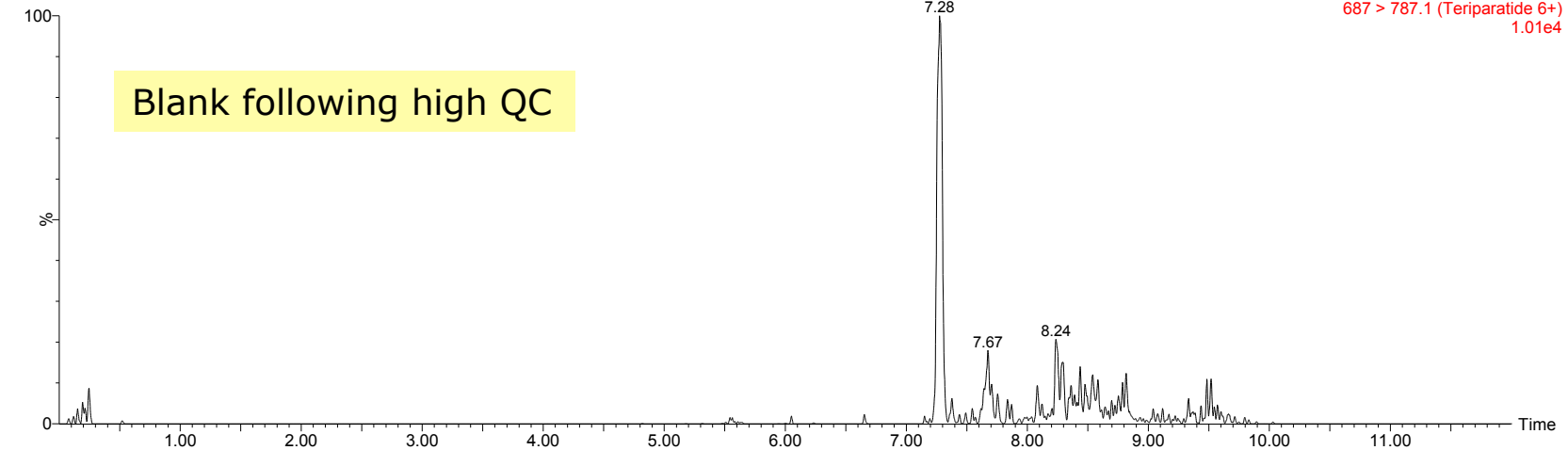
Absence of Carryover

500 pg/ml qc

012214_terip_011 Sm (Mn, 2x3)

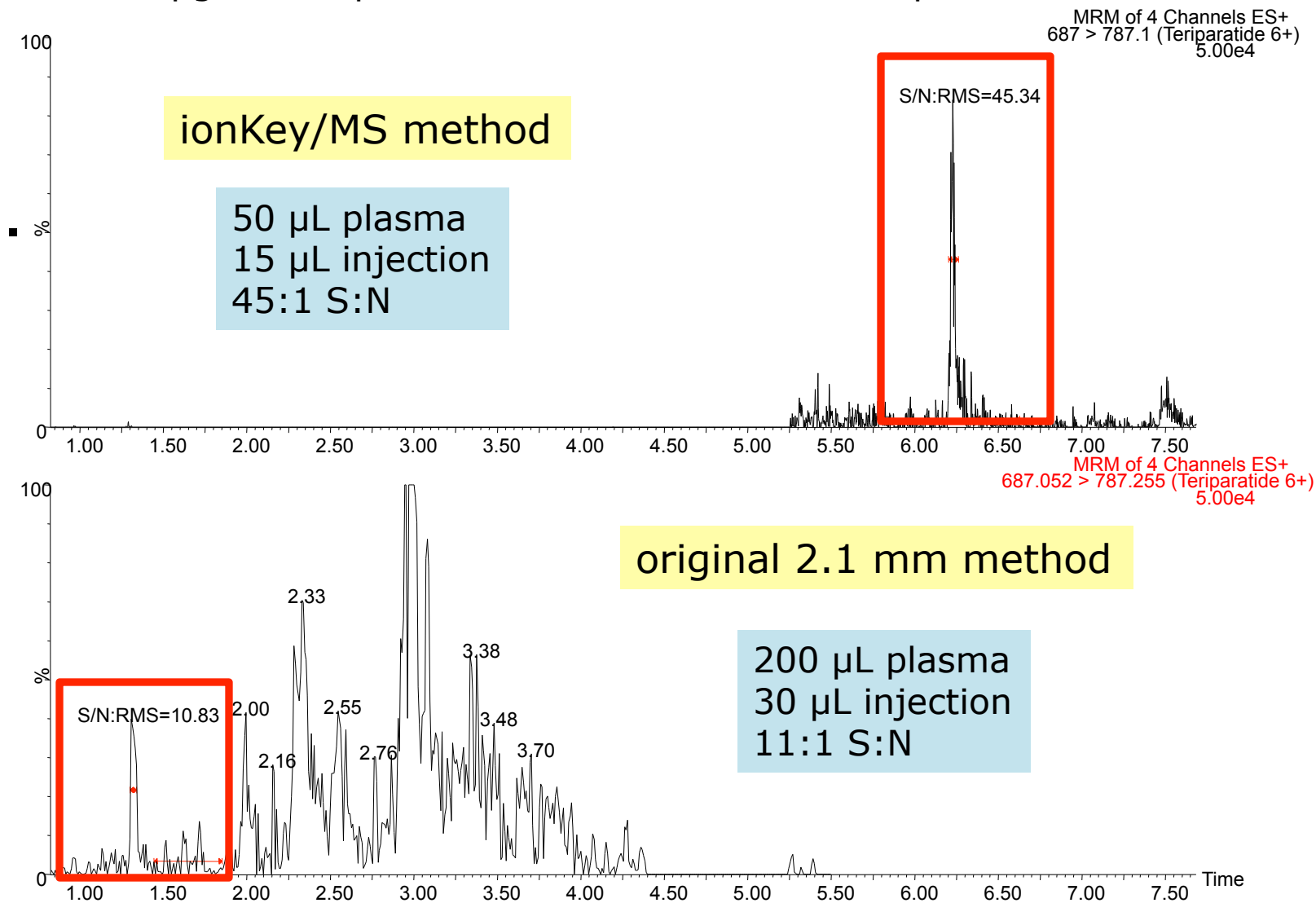


012214_terip_012 Sm (Mn, 2x3)



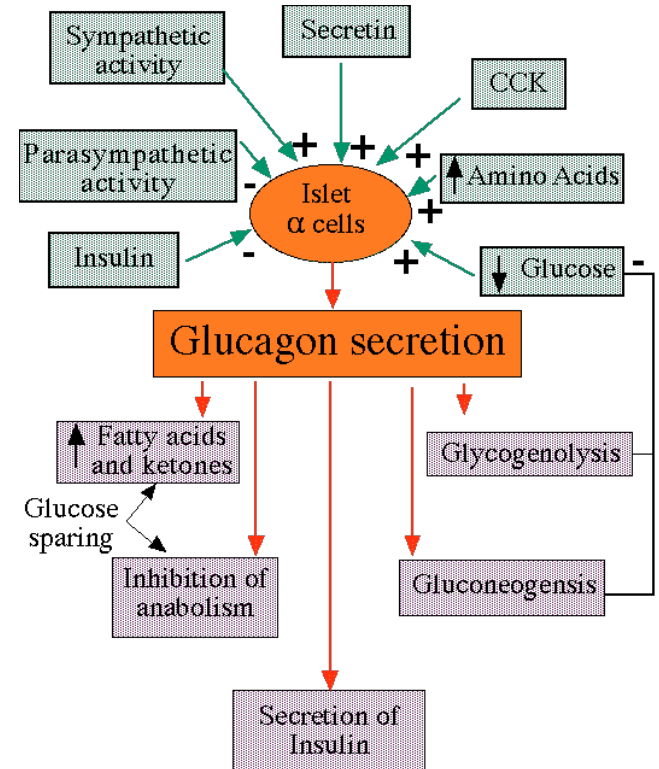
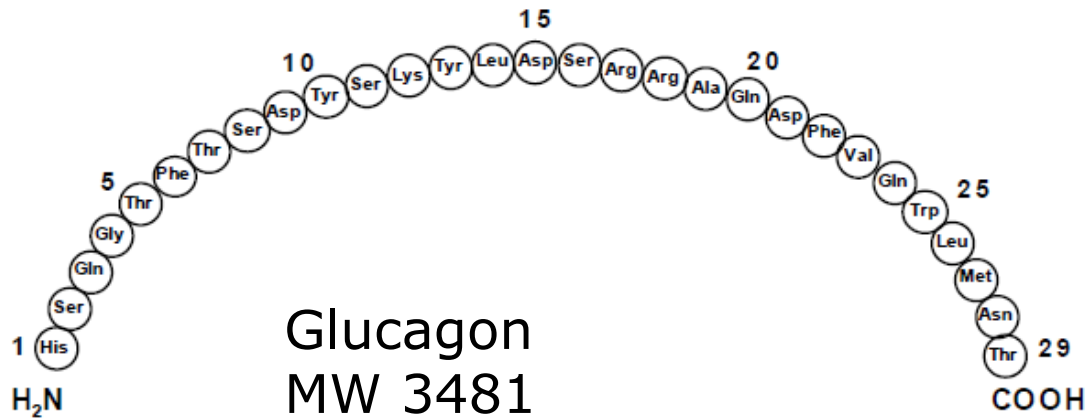
Comparison of Original Analytical Scale and New ionKey/MS Method

20 pg/mL teriparatide extracted from human plasma



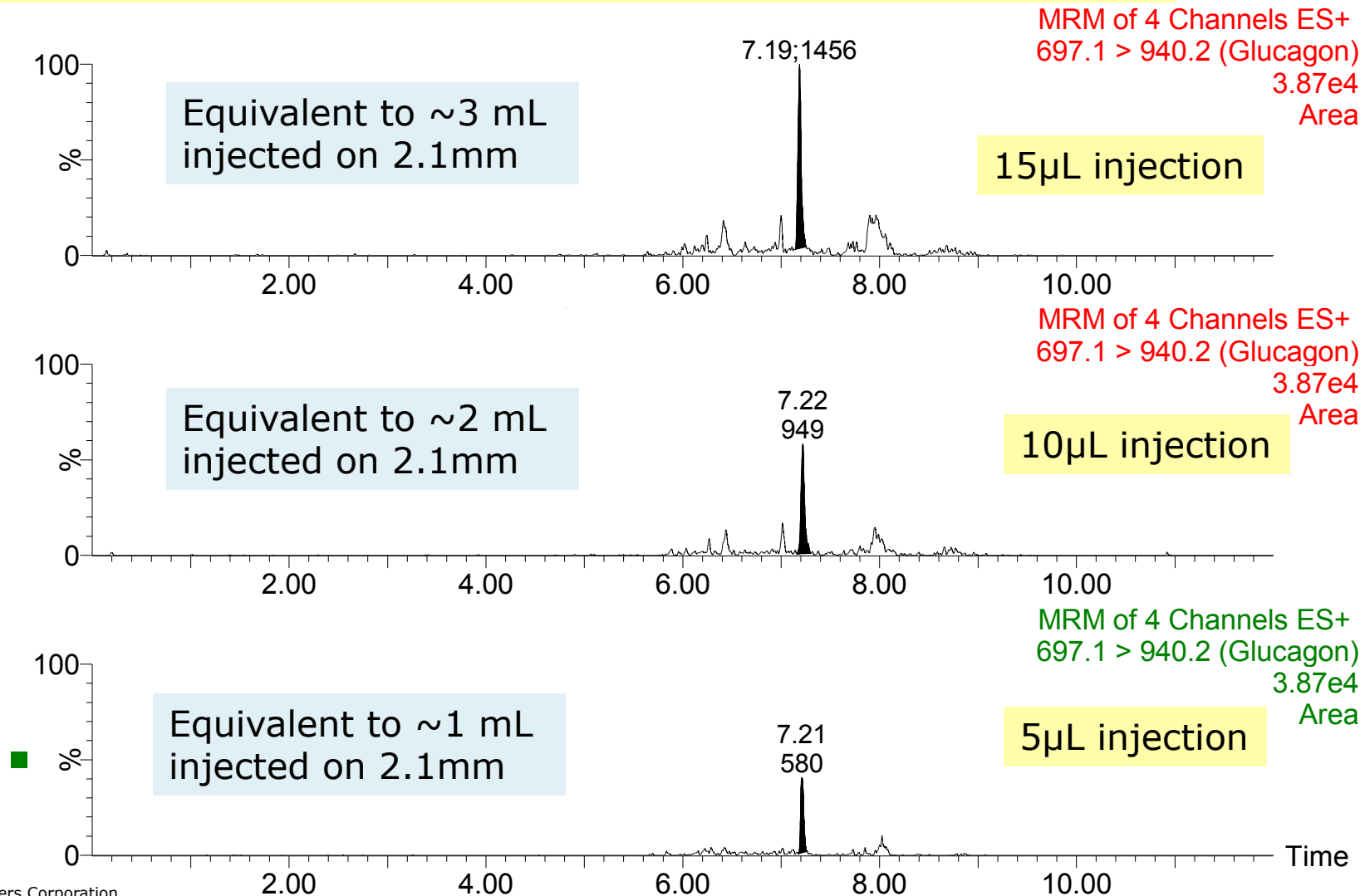
- Background and Key Challenges
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Glucagon



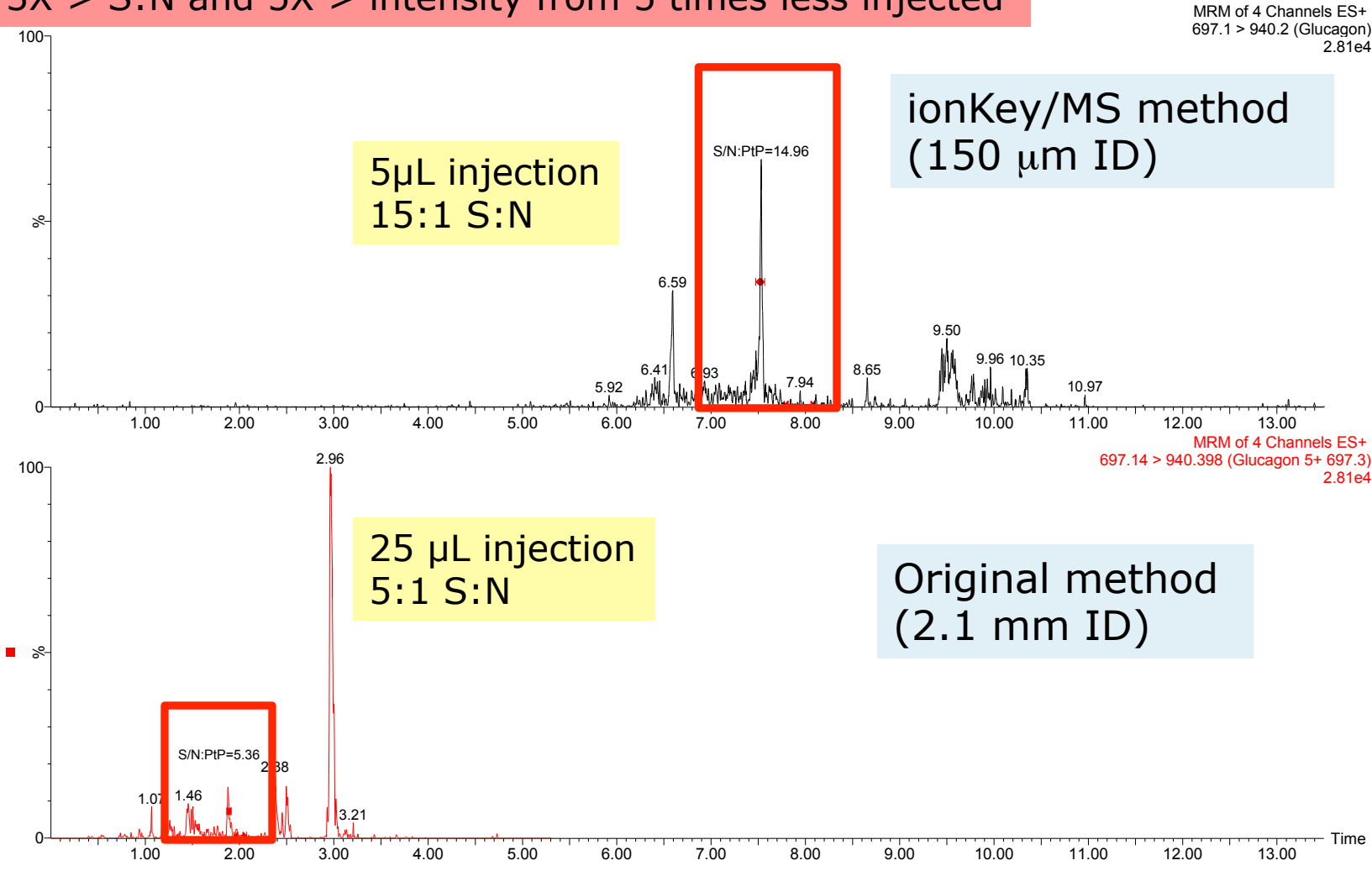
50 pg/mL Glucagon in Human Plasma, ionKey/MS

Injection Volume Scalability on ionKey/MS



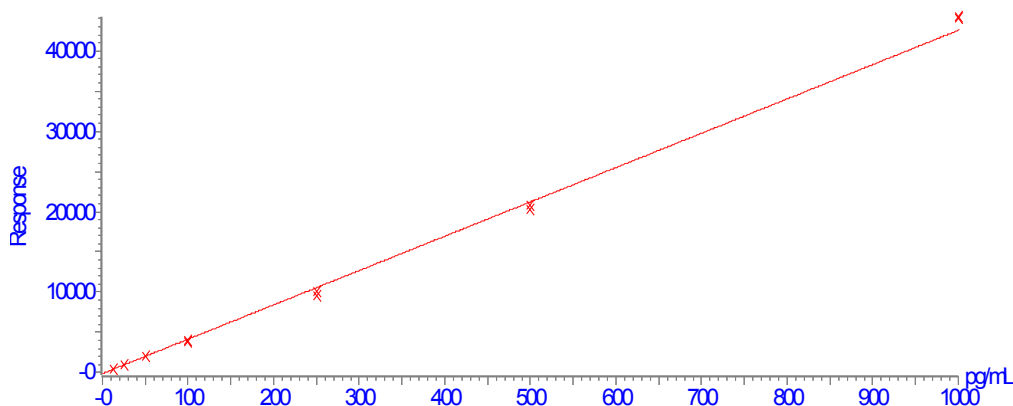
25 pg/mL Glucagon in Human Plasma: iKey vs. Analytical Scale

3X > S:N and 5X > intensity from 5 times less injected



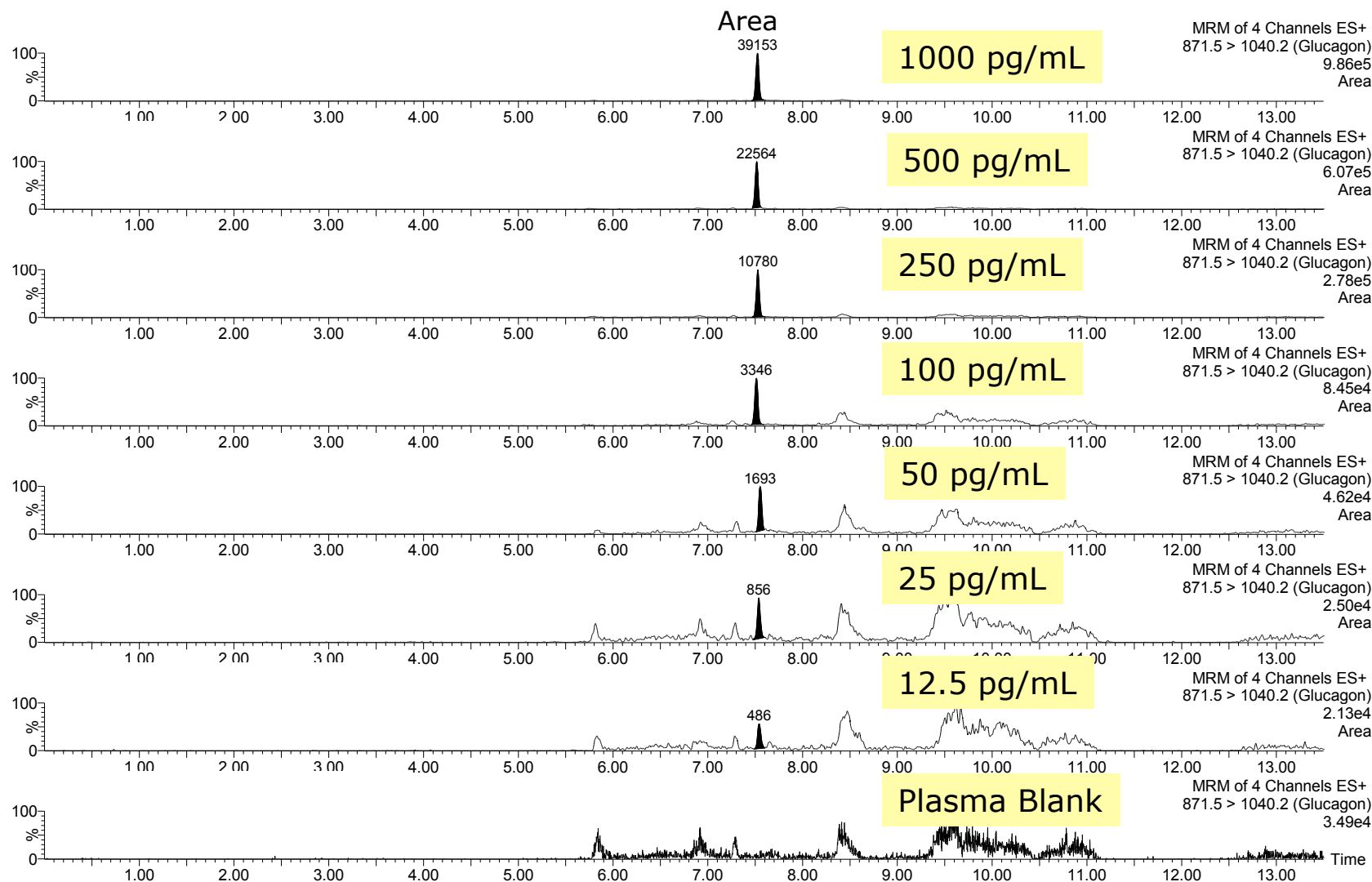
Representative standard curve and statistics for glucagon extracted from human plasma 12.5-1,000 pg/mL

Compound name: Glucagon 1040
 Correlation coefficient: $r=0.998699$, $r^2=0.997400$
 Calibration curve: $42.6771 * x + -120.577$
 Response type: External Std, Area
 Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



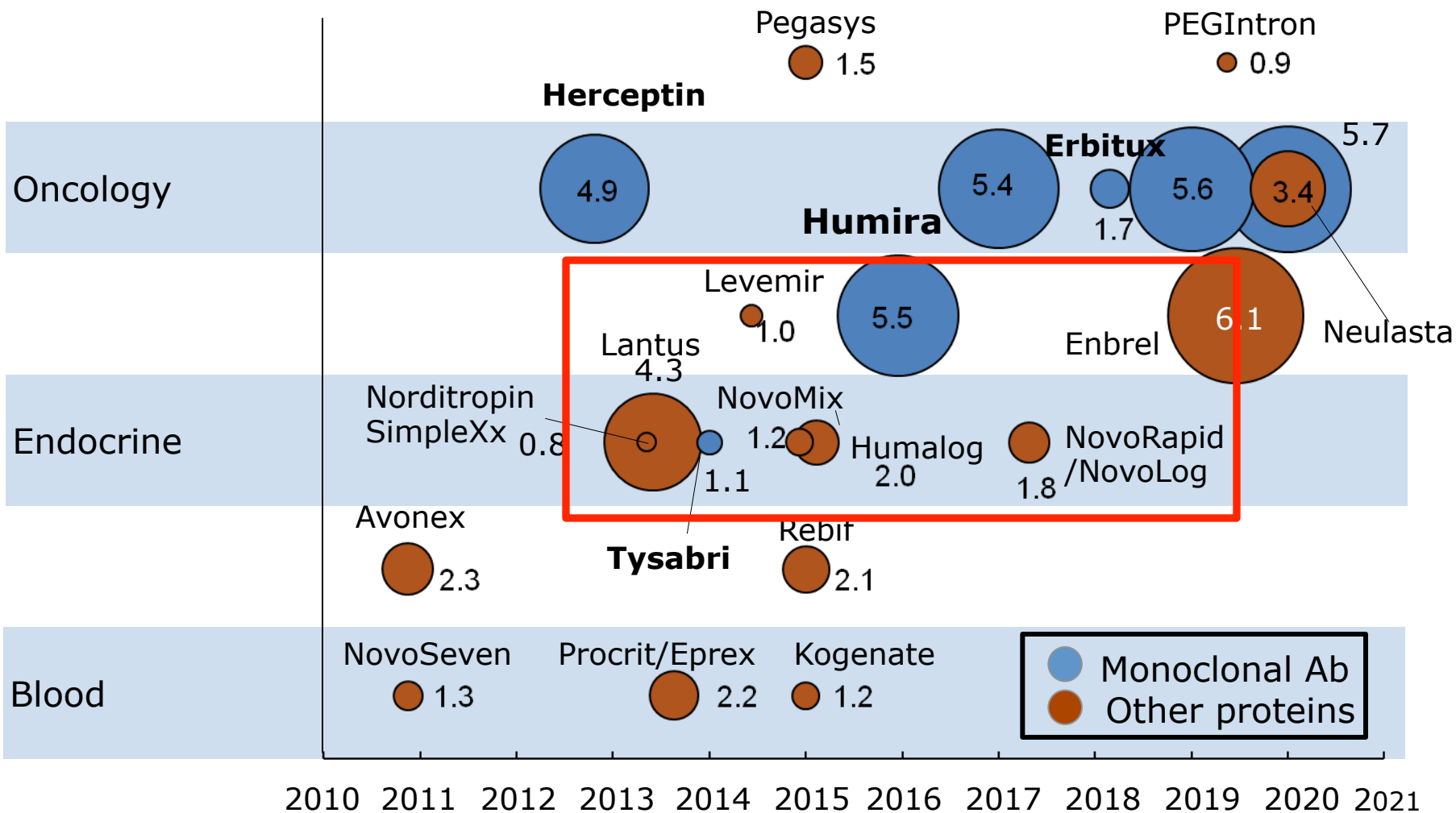
Std. Conc pg/mL	Area	Calc. Conc. pg/mL	%Dev	Accuracy
Blank	-	-	-	-
Blank	-	-	-	-
12.5	469	13.8	10.5	89.5
12.5	461	13.6	9	91
25	982	25.8	3.3	96.7
25	959	25.3	1.1	98.9
50	2005	49.8	-0.4	100.4
50	2080	53.5	6.9	93.1
100	3958	95.6	-4.4	104.4
100	3733	90.3	-9.7	109.7
250	10142	240.5	-3.8	103.8
250	9481	225	-10	110
500	20893	492.4	-1.5	101.5
500	20184	475.8	-4.8	104.8
1000	44244	1039.5	4	96
1000	44094	1036	3.6	96.4

Representative chromatograms from glucagon extracted from plasma at 12.5, 25, 50, 100, 250, 500 and 10000 pg/mL, compared to blank plasma



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Critical Biotherapeutics Coming off Patent: Open to Biosimilar Competition

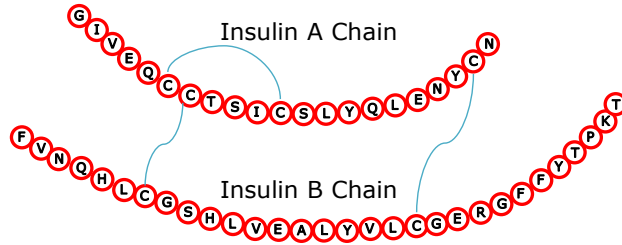


Modified slide from McKinsey and Company
Data Source: Evaluate Pharma

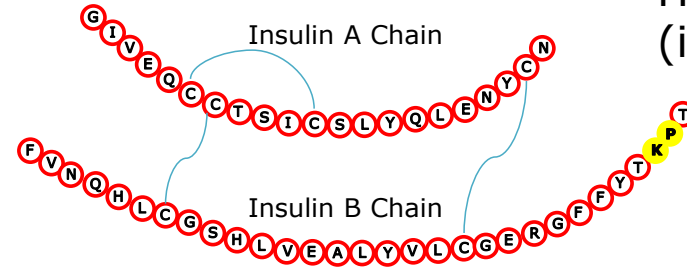
US Patent Expiration Date

Insulin and Analogs

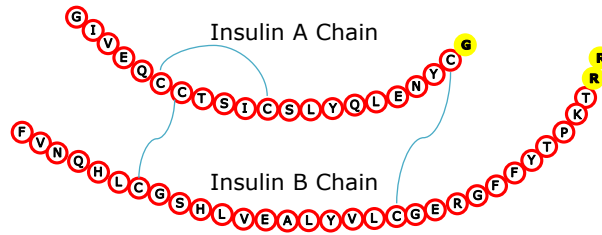
Human Insulin
MW 5808



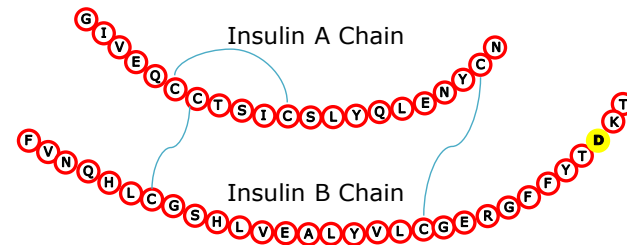
Humalog
(insulin lispro)



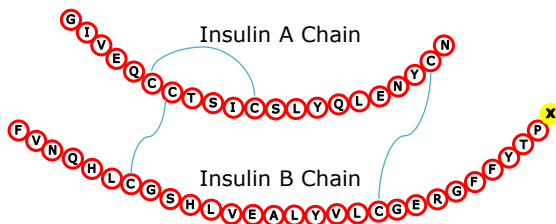
Insulin glargine
(Lantus®)
Avg MW 6063



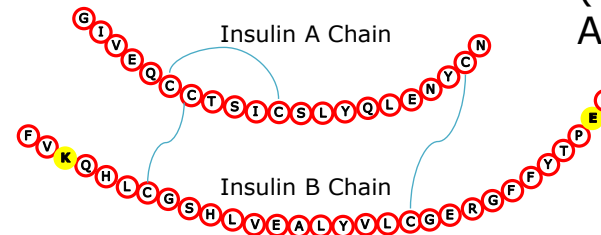
Insulin aspart
(Novolog®)
Avg MW 5826



Insulin detemir
(Levemir®)
Avg MW 5917



Insulin glulisine
(Apidra®)
Avg MW 5823



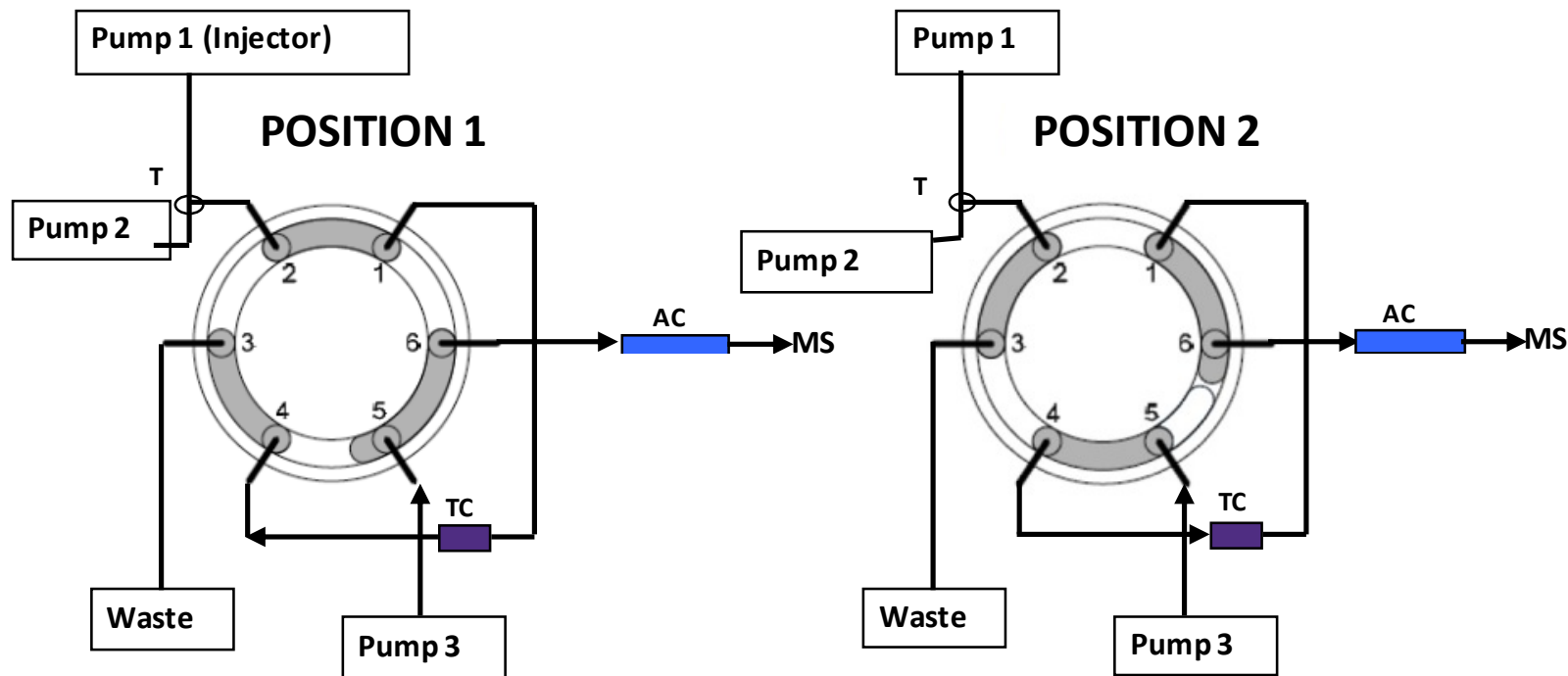
Xevo TQ-S Triple Quadrupole MS conditions



Specific Insulin	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Glargine	1011->1179	60	25
	867->984	60	18
Lispro	1162-> 217	50	40
	968.5->217	50	40
Detemir	1184-> 454.4	60	20
	1184-> 1366.3	60	20
Aspart	971.8 -> 660.8	60	18
	971.8 -> 1139.4	12	18
Glulisine	1165 -> 1370	14	22
	1165 -> 346.2	14	22
Bovine (IS)	956.6 -> 1121.2	60	18
Human insulin	1162 -> 226	50	40
	968.5->217	50	40

Note: highlighting indicates the primary transitions used for quantification

2.1 mm ID Scale: ACQUITY IClass with 2D Technology Valve Diagram

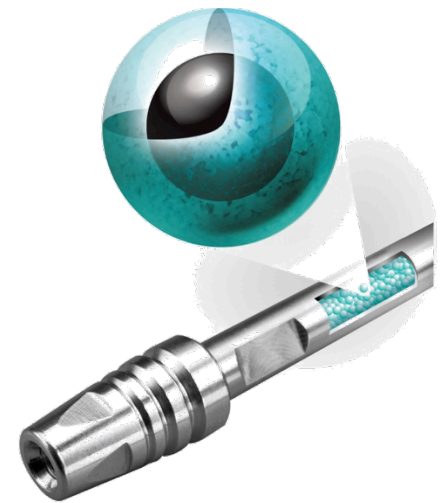


Pump 1: Loading pump
Pump 2: Dilution pump
Pump 3: Elution pump

TC= trapping column
AC= analytical column

Analytical LC Method: At-column-dilution and Trap and Back Elute

- Analytical Column: **CORTECS C18+** 2.1 X 50mm, 1.7 μm
- Trap column: XBridge C18 IS, 3.5 μm , 2.1 X 20mm
- Mobile phase A= 0.1% formic acid in water
- Mobile phase B= 0.1% formic acid in ACN
- Loading time: 2 minutes
- At Column Dilution
- Elution
 - 15 to 40% B over 4 minutes
- Analytical Column Temp: 60°C
- Sample Temp: 15°C
- Injection Volume: **30 μL** (can inject 45 μL without breakthrough)
- SNW: 50/25/24/1 ACN/IPA/H2O/FA

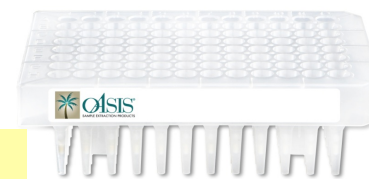


Analytical Extraction Conditions

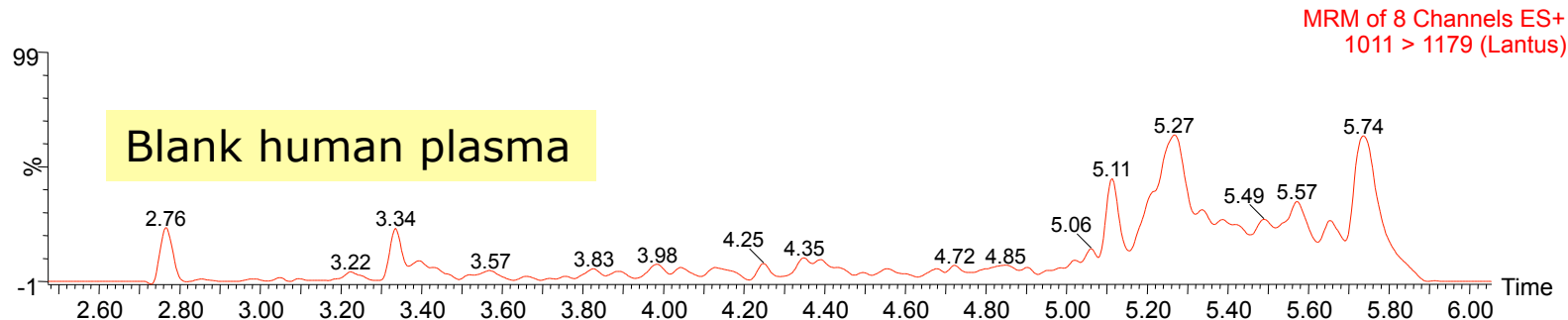
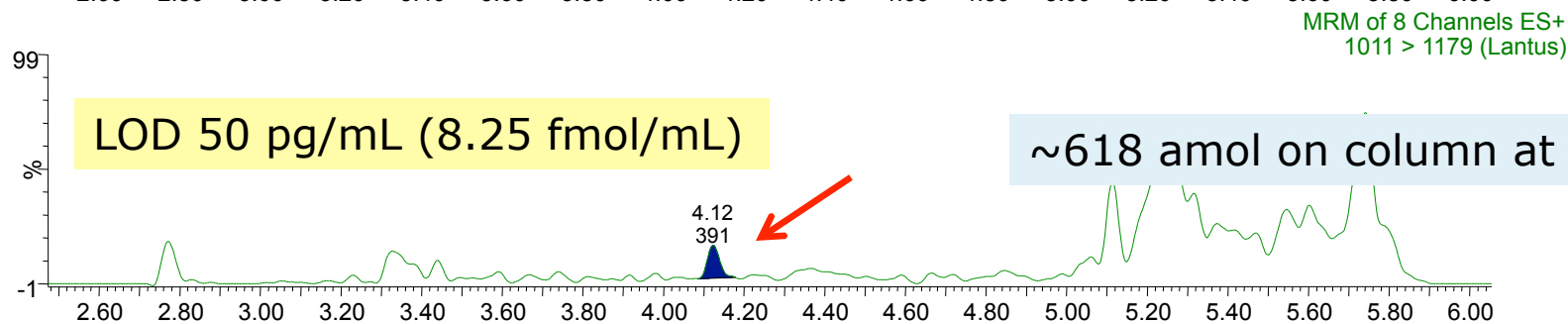
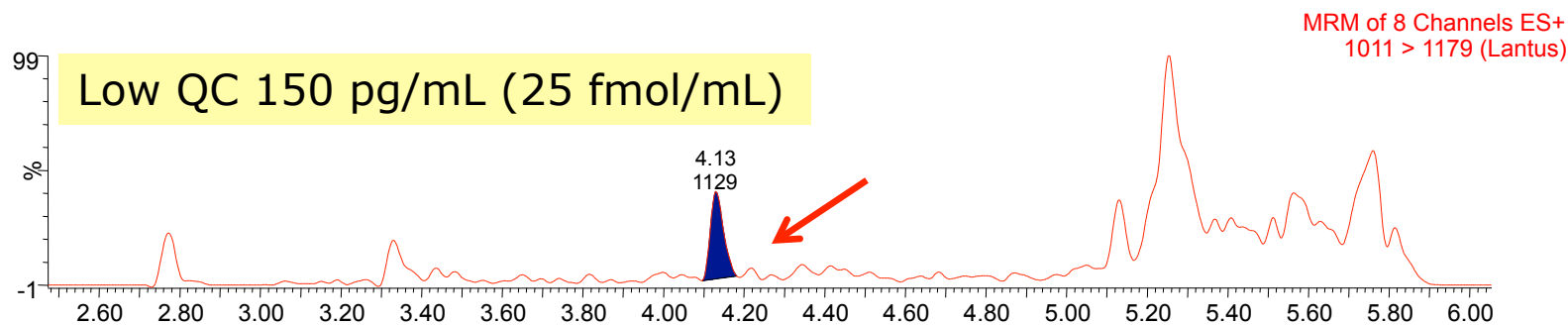
PPT followed by Mixed-mode Strong Anion Exchange
SPE in μ Elution 96-well plate

- **PPT**: 250 μ L human plasma sample precipitated 1:1 with 50/50 ACN/MeOH + 1% FA, vortex spin 10 min at 13K rcf, dilute supernatant with 900 μ L 5% NH_4OH in water
- **SPE**: Oasis® **MAX** μ Elution 96-well plate
- Condition: 200 μ L methanol
- Equilibrate: 200 μ L water
- Load Sample: entire diluted supernatant in 2 steps of \sim 700 μ L each
- Wash: 200 μ L 5% NH_4OH in water
- Wash: 200 μ L 5% methanol, 1% acetic acid in water
- Elute: 2X 25 μ L 60% methanol, 10% acetic acid in water
- Dilute: 50 μ L water
- Inject 30 μ L

Analytical Scale LC, Plasma detection limit: 50 pg/mL



Analytical Scale Method: Lantus LOD and Low QC in Human Plasma



Chambers et al, *Analytical Chemistry*, 2014, 86, 694-702.

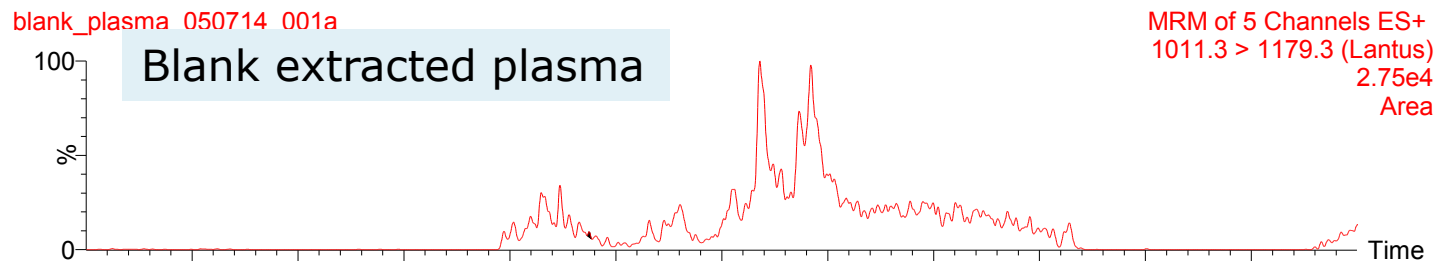
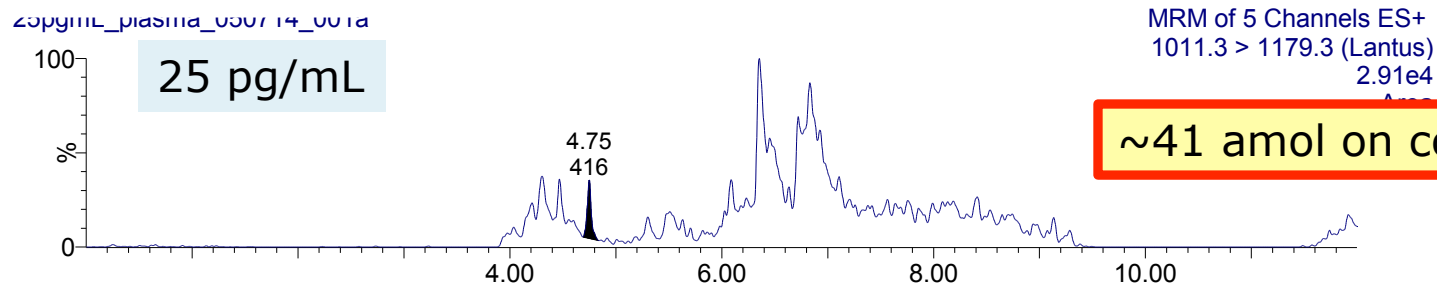
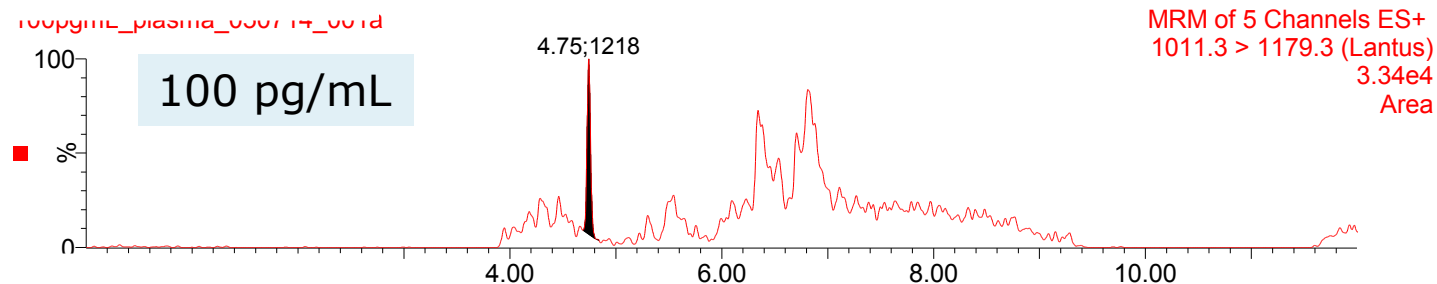
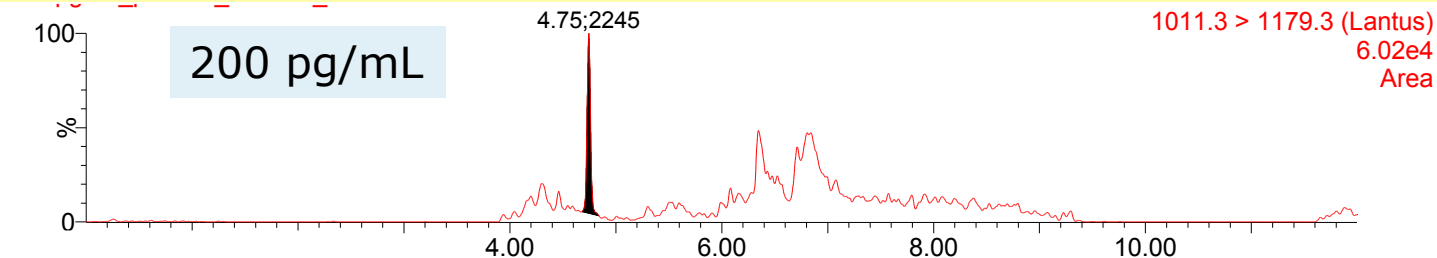
Lantus (insulin glargine)

- Top selling insulin analog (\$6.6 billion)
- Off patent 2015
- Lots of requests for patent extensions
 - Pediatrics?
 - Different formulations?
- Requires more sensitivity and decreased sample volume
 - <50 pg/mL
 - ≤100 µL sample
- Analytical scale method uses 250 µL sample and reaches a LOD of 50 pg/mL
- Can integrated microscale LC/MS help??



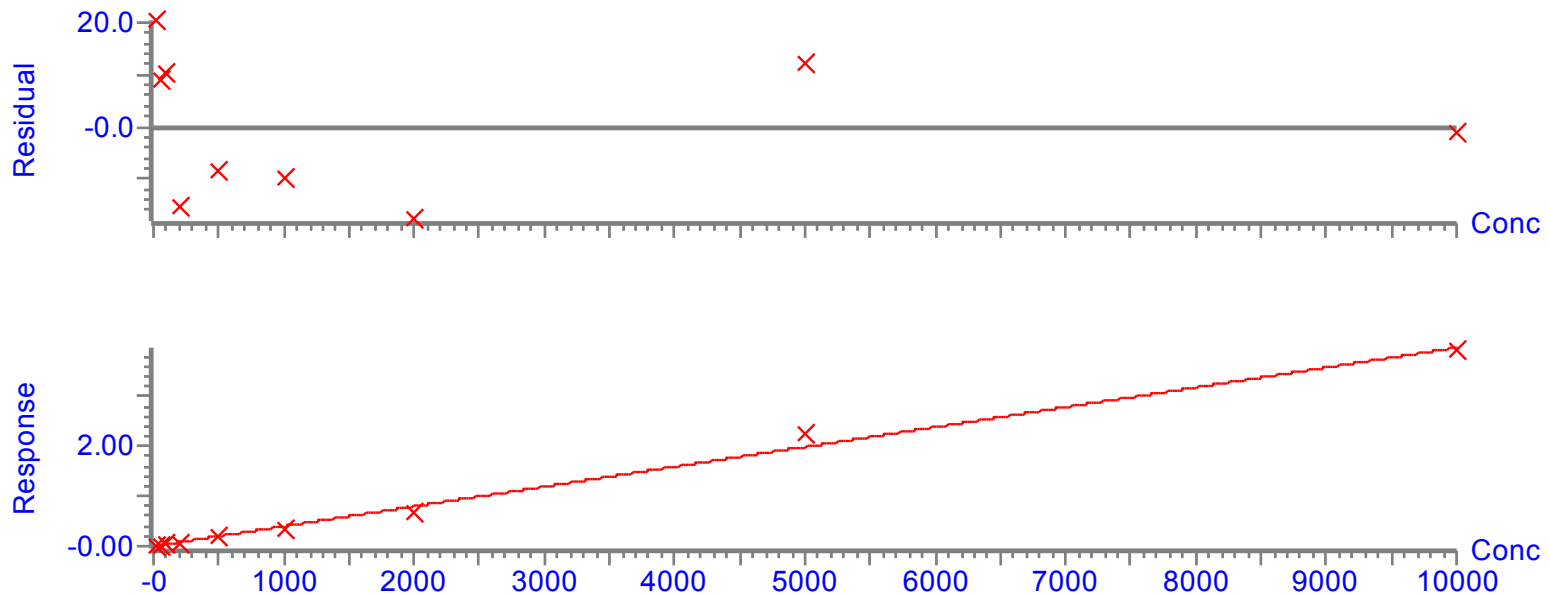
IonKey/MS Lantus Results: 100 μ L sample, 10 μ L injection

Decrease sample volume, decrease injection volume, increase sensitivity!



ionKey/MS: Representative Standard Curve 25 pg/mL to 10 ng/mL Lantus

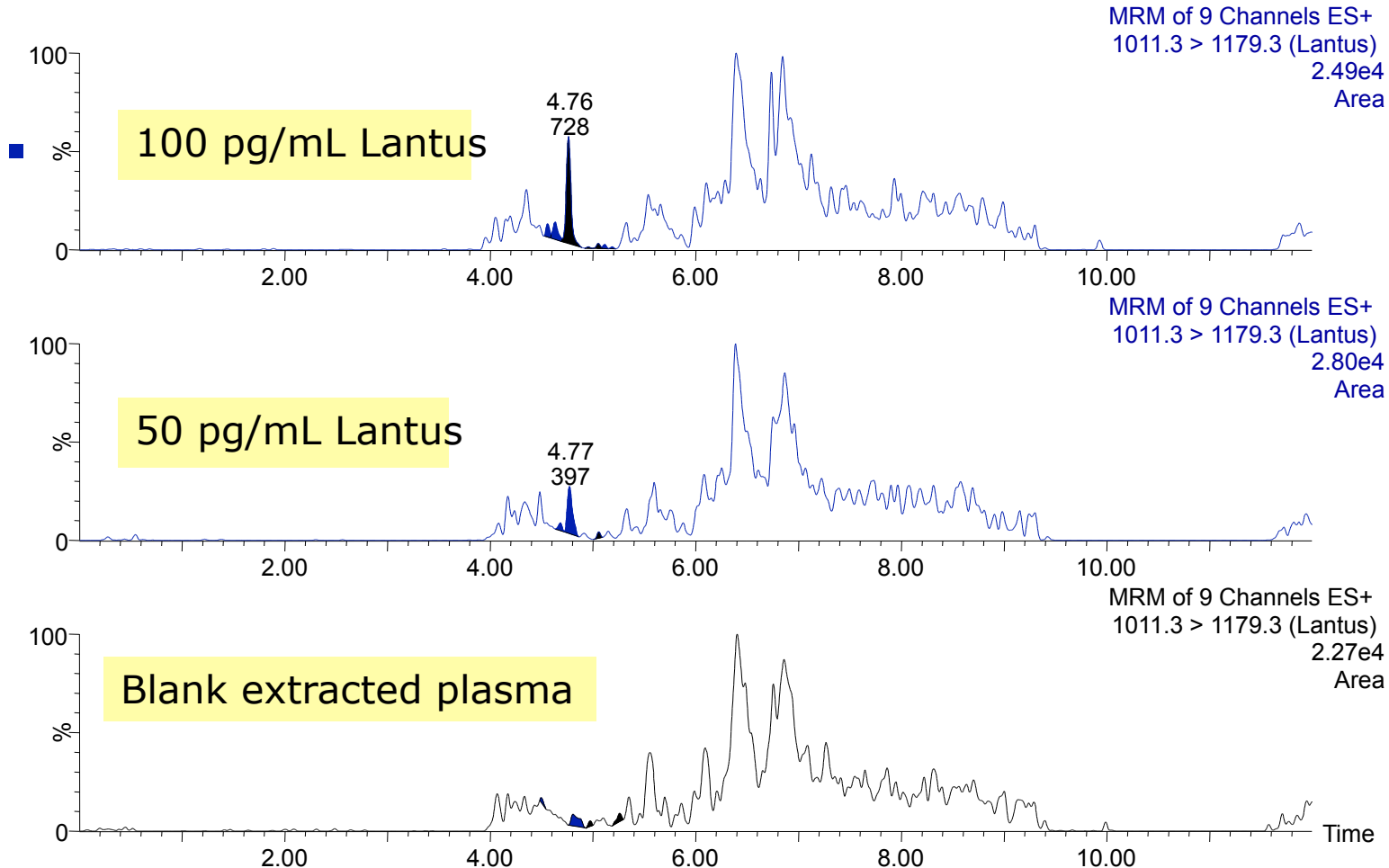
Compound name: Lantus
Correlation coefficient: $r = 0.995564$, $r^2 = 0.991147$
Calibration curve: $0.000398185 * x + -0.00299844$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



ionKey/MS: Representative Standard Curve Statistics 25 pg/mL to 10 ng/mL Lantus

Name	Type	Std. Conc. pg/mL	Retention Time	Area	IS Area	Conc.	%Dev
Blank Plasma			4.75	41			
25 pg/mL plasma	Standard	25	4.75	279	31017	30	20.4
50 pg/mL plasma	Standard	50	4.75	620	33096	55	9.2
100 pg/mL plasma	Standard	100	4.75	1313	32043	111	10.5
200 pg/mL plasma	Standard	200	4.75	2199	34167	169	-15.4
500 pg/mL plasma	Standard	500	4.75	5515	30733	458	-8.4
1 ng/mL plasma	Standard	1000	4.74	11575	32504	902	-9.8
2 ng/mL plasma	Standard	2000	4.75	20828	31912	1647	-17.7
5 ng/mL plasma	Standard	5000	4.76	59151	26498	5614	12.3
10 ng/mL plasma	Standard	10000	4.76	112246	28524	9890	-1.1
QC 1	QC	150	4.76	2013	33144	160	6.7
QC 2	QC	750	4.76	9477	33670	714	-4.7
QC 3	QC	2500	4.76	28692	31598	2288	-8.5
QC 4	QC	7500	4.75	90793	27901	8180	9.1

Insulin glargine (Lantus) from 50 μL human plasma sample, 10 μL injection



- Background and Key Challenges
- Practical Applications of Integrated Microscale LC
 - Routine Ultra-high Sensitivity Teriparatide Quantification: Adaption and Benefits from Analytical to Microscale LC
 - Endogenous and Therapeutic Glucagon Analysis
 - Increasing Sensitivity and Reducing Sample Volume Required for Quantification of Multiple Insulin Analogs
 - ○ Reducing Sample Volumes for Small peptides
- Conclusions

- Integrated microscale LC facilitates increased sensitivity using small sample volumes
- 20-50X cumulative improvement obtained over 2.1 mm ID scale through:
 - Decreasing sample volume
 - Decreasing injection volume
 - Increasing sensitivity
- Analytical scale quantification methods for teriparatide, glucagon, 6 insulins and small cyclic peptides were adapted to and significantly improved by ionKey/MS
 - Greater S:N, with less sample, and less injected
 - Single pM quantification limits from 25-100 μ L of sample

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