TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY

Insulin Analogs: From immunoassays to more specific LC-MS/MS methods

Vincent Trinh, M.Sc.

In collaboration with Merck&Co.

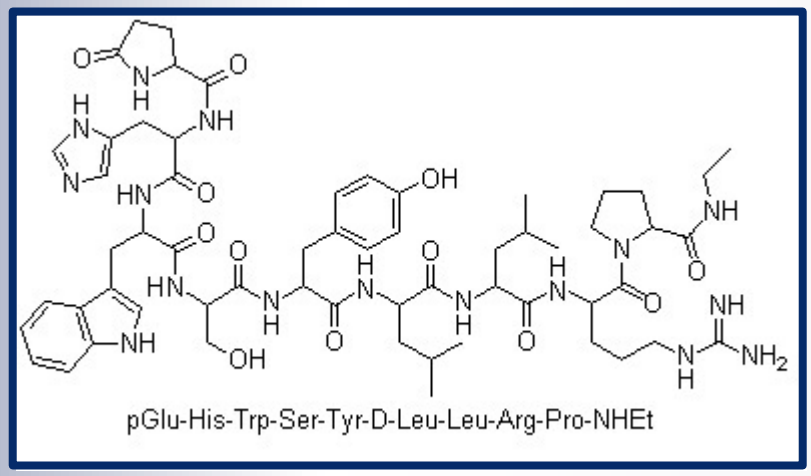
inVentiv Health clinical

Peptides and small proteins

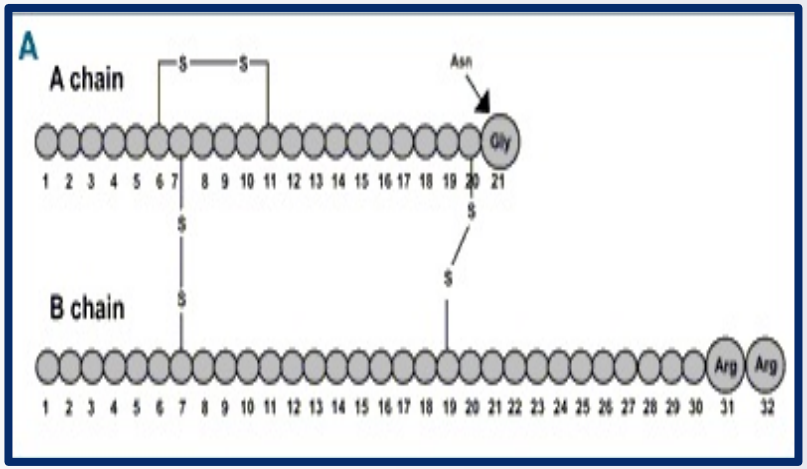
Peptides vary in size and sequence:

Examples:

Leuprolide: 9 residues, MW: 1269 g/mol



Insulin glargine: 53 residues, MW: 6063 g/mol



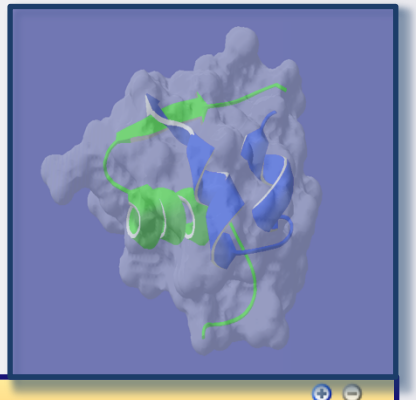
Chain A: GIVEQCCTSICSLYQLENYCG (21 res.)
Chain B: FVNQHLCGSHLVEALYLVCGERGFFYTPKTRR (32 res.)

Leuprolide: MW= 1209.4 g/mol

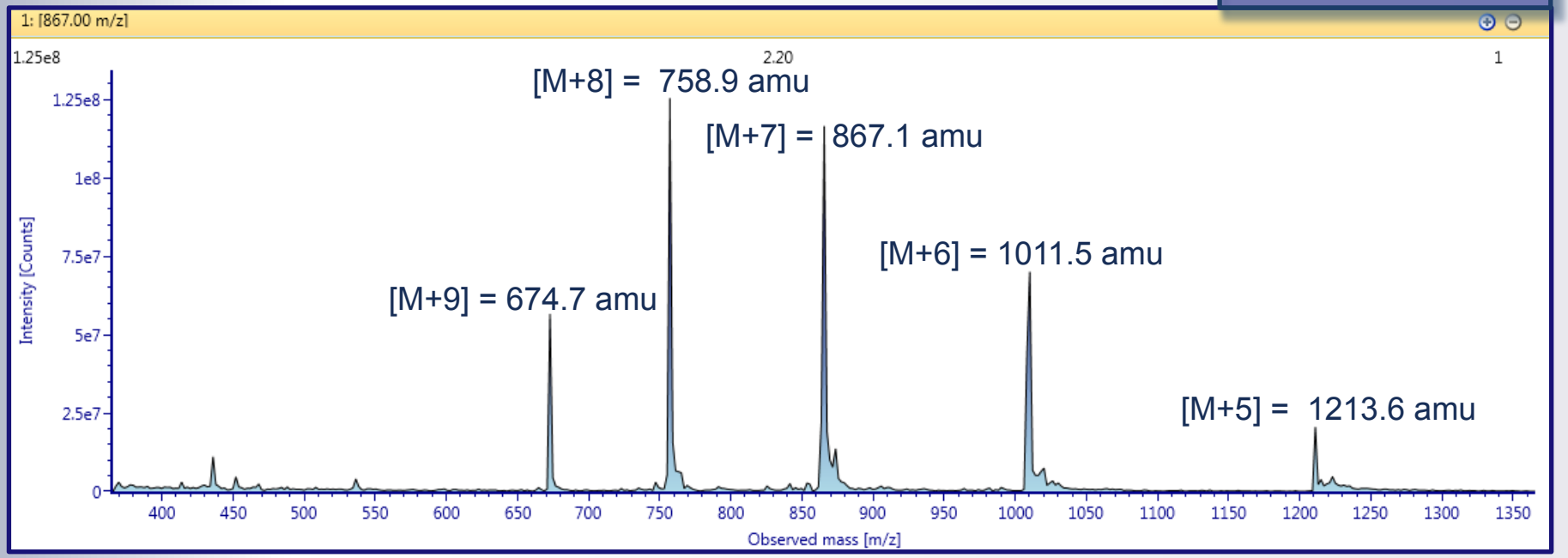
Insulin glargine: MW= 6063.1 g/mol

Peptide detection by mass spectrometry

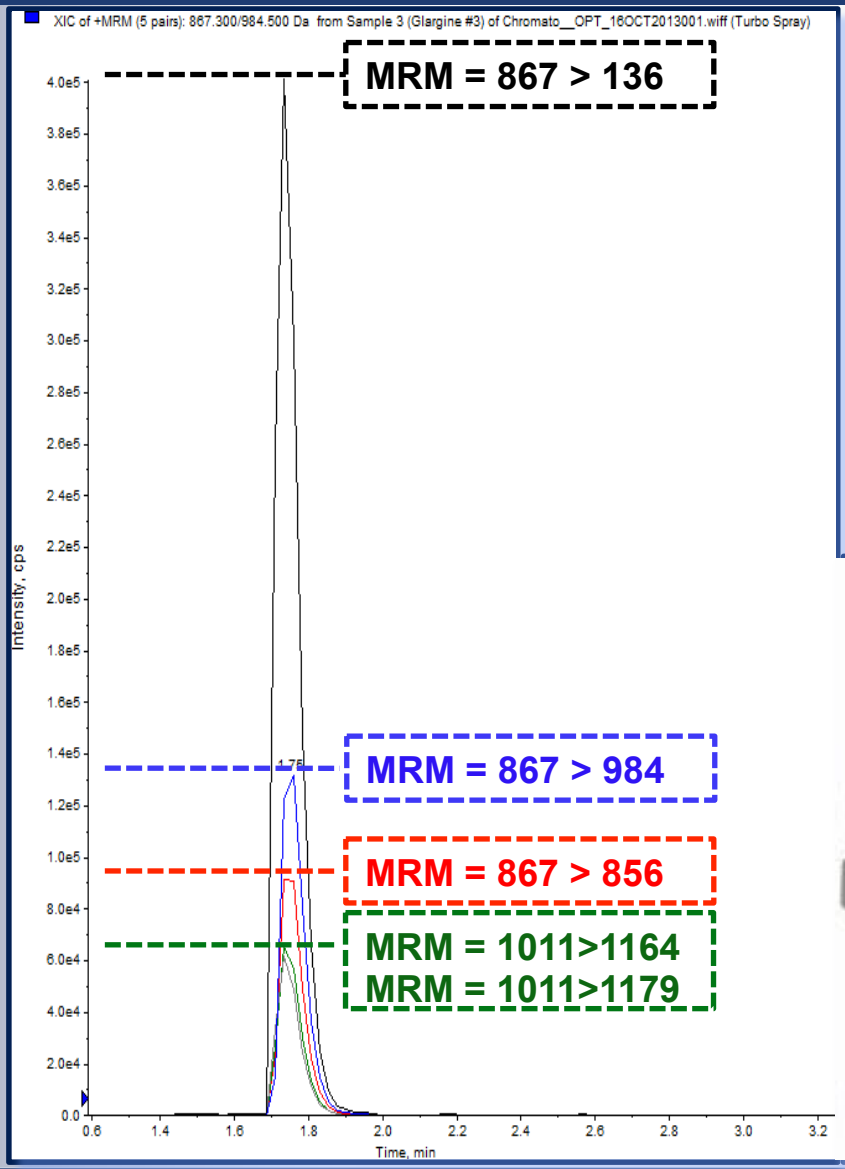
The analysis of peptides is possible on mass spectrometers because multiple charges are present, otherwise they would exceed the MS mass range.



For example:
Insulin glargine with a molecular weight of 6063 g/mol
→ 8 charges → [M+8] / 8 = 758.9 amu
→ 7 charges → [M+7] / 7 = 867.1 amu
→ 6 charges → [M+6] / 6 =1011.5 amu



Issues with Peptide Fragmentation



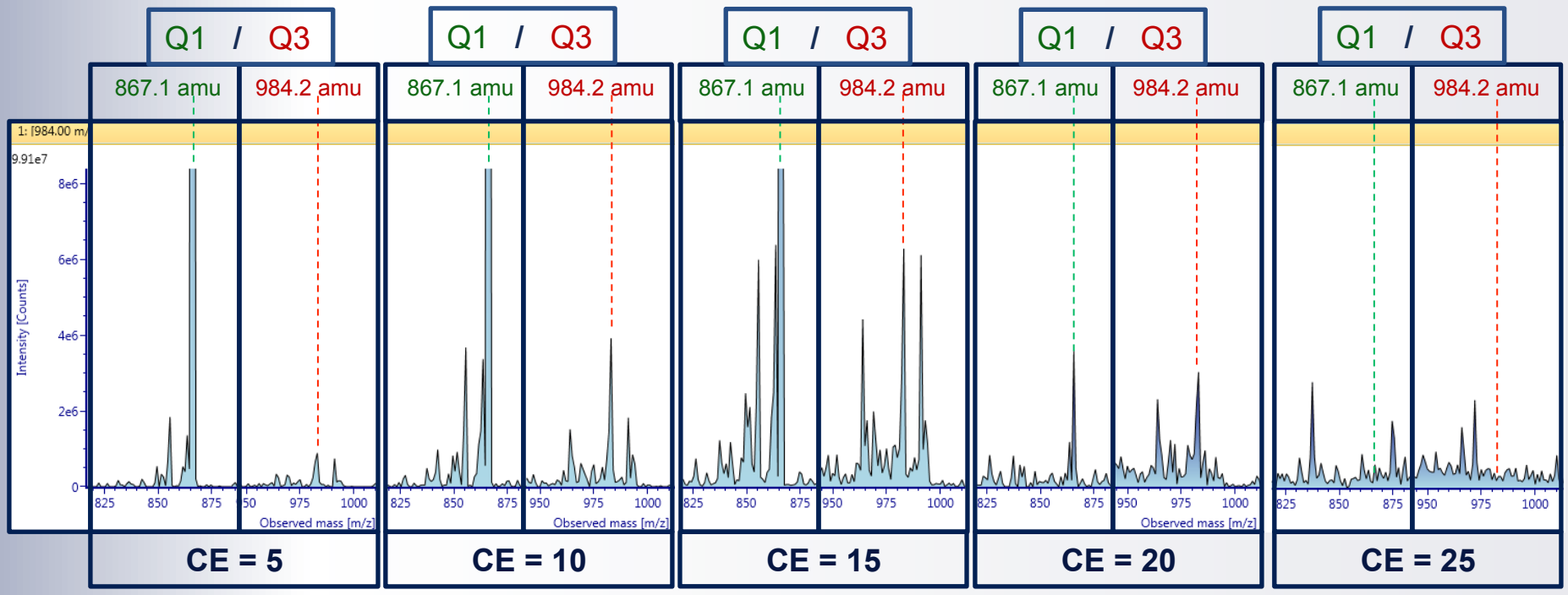
The most intense transitions usually offer poor specificity; this highlights the importance of optimizing as many transitions as possible.

It is also important to use instruments able to efficiently transmit these high masses through the ion path.

Peptide fragmentation

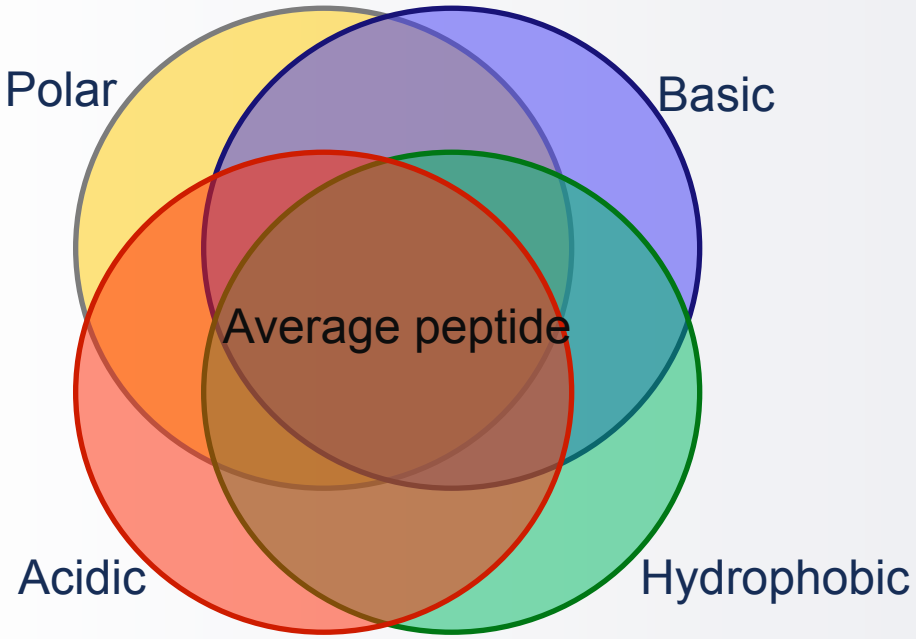
Fragmentation of peptides is not efficient as the most specific daughter ions can also fragment easily.

For example, at the optimal collision energy, the presence of the intact parent mass (867.1 amu) is still more intense than the generated daughter ion (984.2 amu).



Peptide extraction from plasma

Traditional extraction methods used in bioanalysis are designed to enrich the compound of interest. However for peptides, these methods often fail to specifically enrich the peptide of interest with respect to other peptides present in the plasma. This is likely due to the fact that its physico-chemical properties will be close to the average of the peptides present in the mixture.



Insulin glargine : First attempts

Determination of insulin glargine in human plasma
(100 pg/mL to 20 ng/mL)

Sample preparation = Protein precipitation followed by SPE.

Column: Kinetex XB-C18, 2.1X50mm, 5µm

Flow rate: 0.20 ml/min

MPA: 0.1% Formic Acid in H2O
MPB: 0.1% Formic Acid in ACN

Gradient from (85-15) to (75-25) ~ 3.0min.

Insulin glargine: Attempts on API5000

Determination of insulin glargine in human plasma
100 pg/mL to 20 ng/mL

Data from Intra-assay run acquired on API5000.(MRM = 867>136)

Sample preparation = Protein precipitation followed by SPE.

Major Issues

QC stats:

	LLQC (100 pg/mL)	QC1 (300 pg/mL)	ULOQ (20 ng/mL)
1	88.19	309.0	20557
2	110.2	272.9	20320
3	110.6	300.1	19386
4	88.30	290.2	19324
5	34.65	293.0	20011
6	107.6	254.9	24663
Mean	89.92	286.7	20710
STDEV	29.02	19.64	1998
C.V. (%)	32.3	6.9	9.7
R.E. (%)	-10.1	-4.4	3.6

Selectivity:

	Interference(%)
Blk-01	12.06
Blk-02	58.89
Blk-03	6.21
Blk-04	42.28
Blk-05	22.35
Blk-06	34.77
Blk-07	6.88
Blk-08	48.02
Blk-09	38.91
Blk-10	12.06

Insulin glargine: Second attempts

Signal-to-noise was insufficient on API5000 to expect a rugged method, it was decided to use XEVO-TQ-S

Sample preparation = Protein precipitation followed by SPE(mixed-mode).

Column: Kinetex XB-C18, 2.1X50mm, 5µm

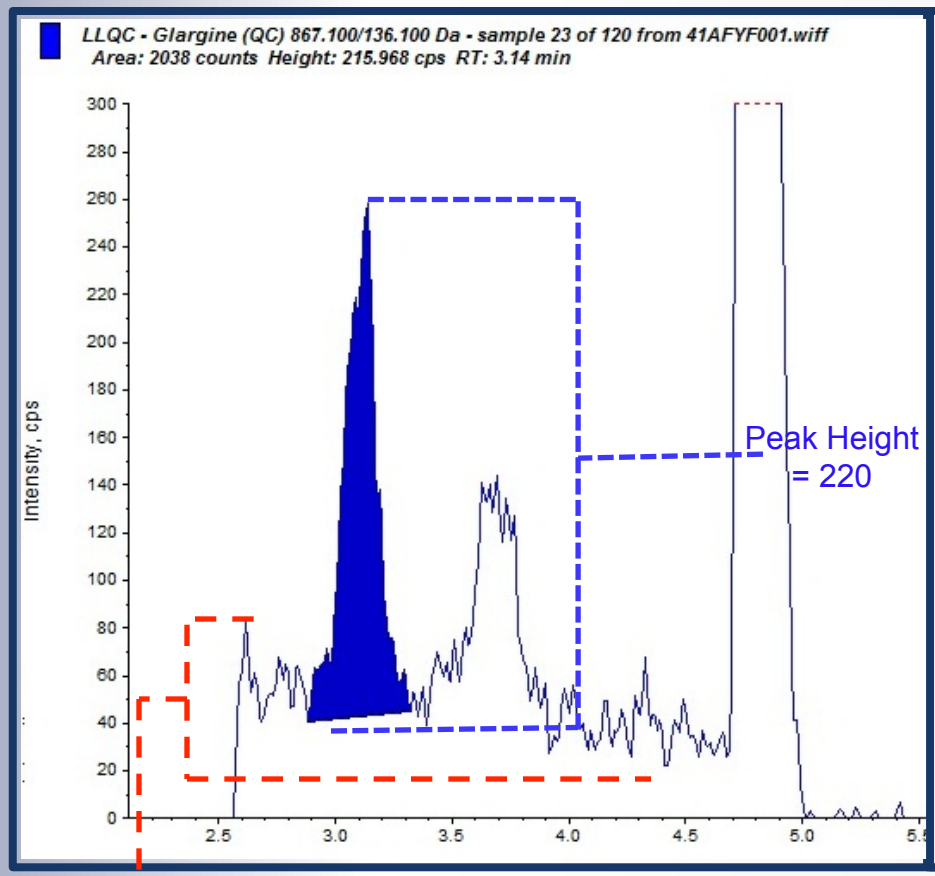
Flow rate: 0.20 ml/min

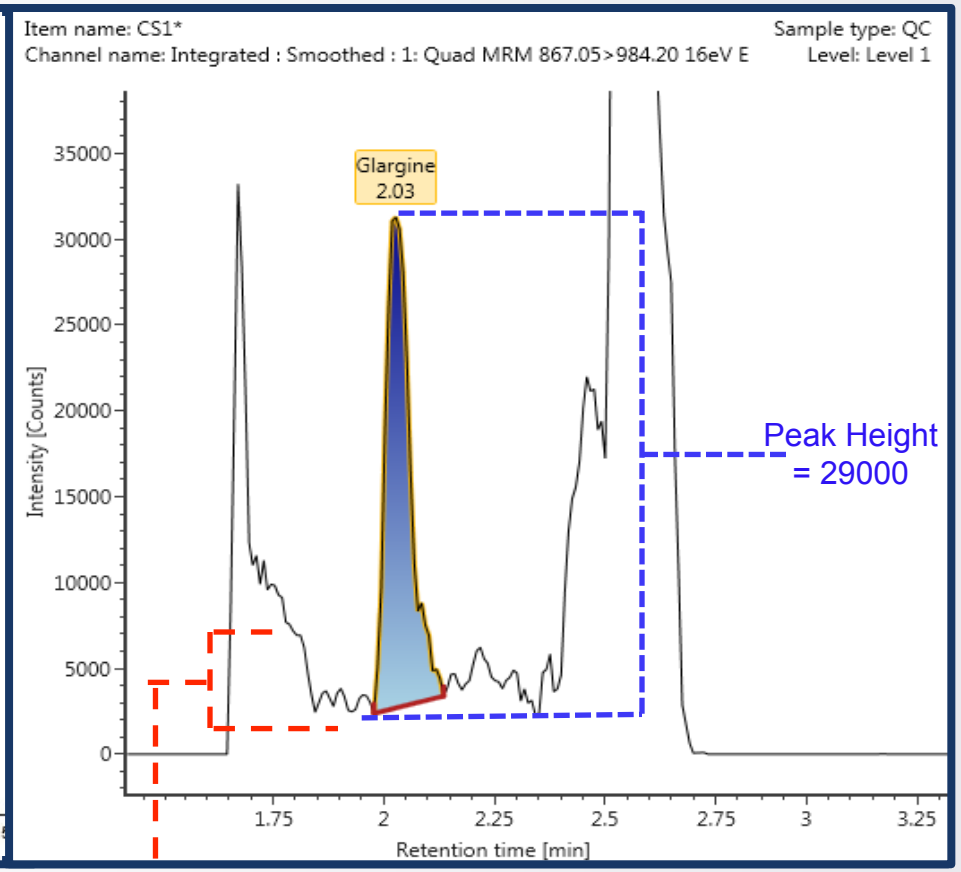
MPA: 0.1% Formic Acid in H2O
MPB: 0.1% Formic Acid in ACN

Gradient from (85-15) to (65-35) ~ 2.5min.

Insulin glargine

Comparison of the signal-to-noise obtained in LLOQC samples:





Noise = 65
Signal = 220
S/N = 3.4

API 5000

Noise = 5000
Signal = 29000
S/N = 5.8

XEVO-TQ-S

Insulin glargine

Attempts using traditional extraction analyzed on XEVO-TQ-S

Overall statistics are much better:

QC stats:

	LLQC (100 pg/mL)	QC1 (300 pg/mL)	ULOQ (20 ng/mL)
1	97.37	303.9	18065
2	97.38	291.4	20112
3	91.66	280.4	18753
4	83.03	285.2	18584
5	92.39	276.2	17902
6	89.82	301.1	18079
Mean	91.94	289.7	18583
STDEV	5.349	11.18	818.9
C.V. (%)	5.8	3.9	4.4
R.E. (%)	-8.1	-3.4	-7.1

Still some Issues:

Selectivity:

	Interference (%)
Blk-01	5.88
Blk-02	3.60
Blk-03	2.72
Blk-04	45.57
Blk-05	22.92
Blk-06	8.67
Blk-07	2.32
Blk-08	5.40
Blk-09	3.92
Blk-10	1.44

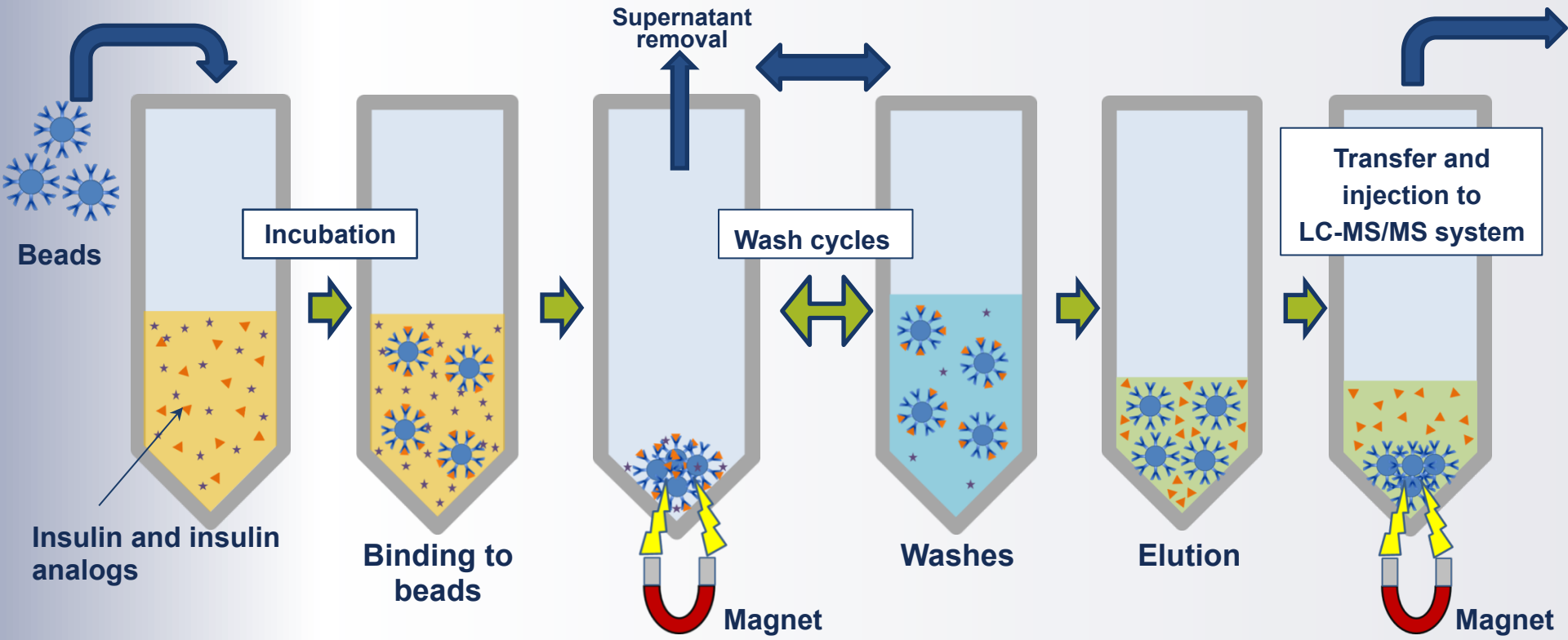
Glargine insulin

Alternatively, an immuno-capture method was developed by our client, and the assay was transferred:
Client: Merck
Group: Dr. Eric Woolf / Dr. Kevin Bateman / Dr. Yang Xu

To allow high-throughput, the method was tested and adapted for use with our automated liquid handling devices : Hamilton robotic systems.

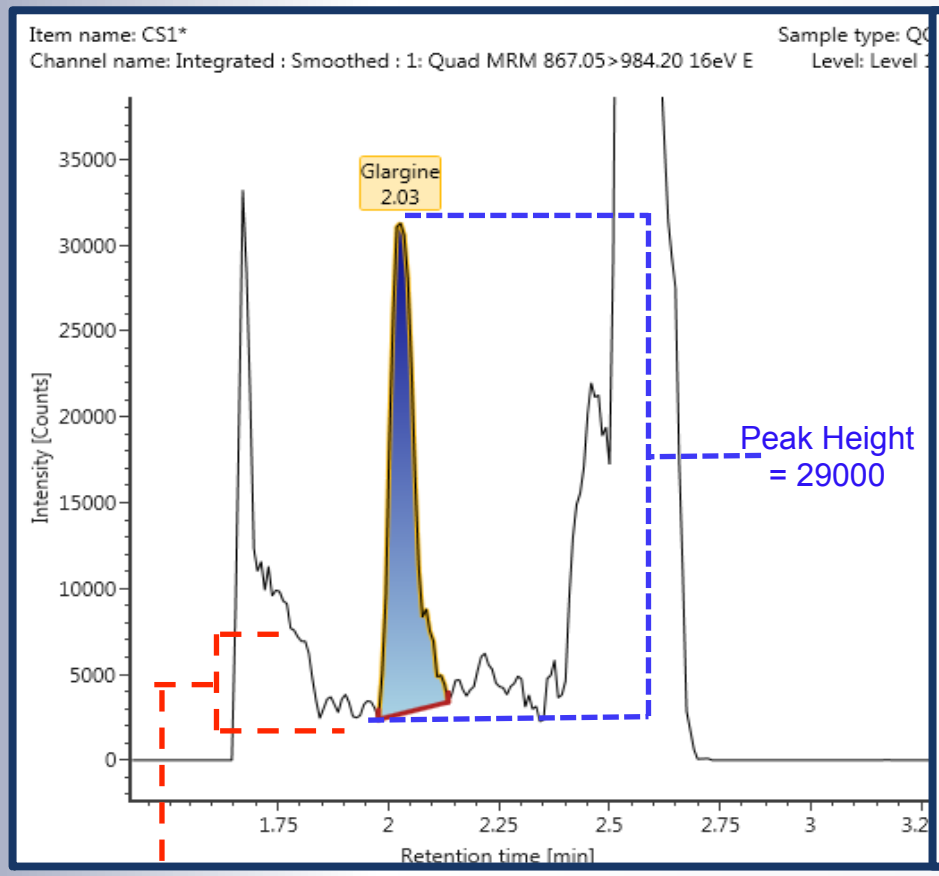
Insulin immuno-capture method

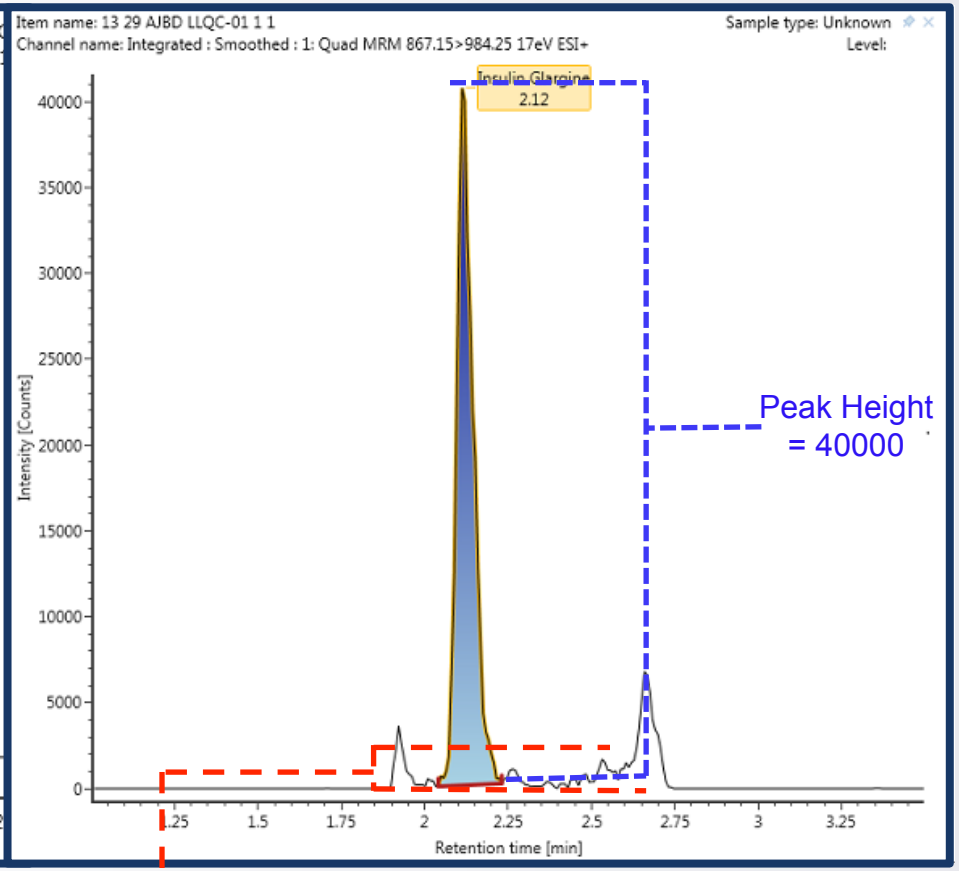
- **Anti-insulin antibodies are attached to magnetic beads.**
- **Samples are incubated with the beads, washed and eluted.**



Comparison of SPE-based method and immuno-capture

Comparison of extracts obtained using immuno-capture:





Noise = 5000
Signal = 29000
S/N = 5.8

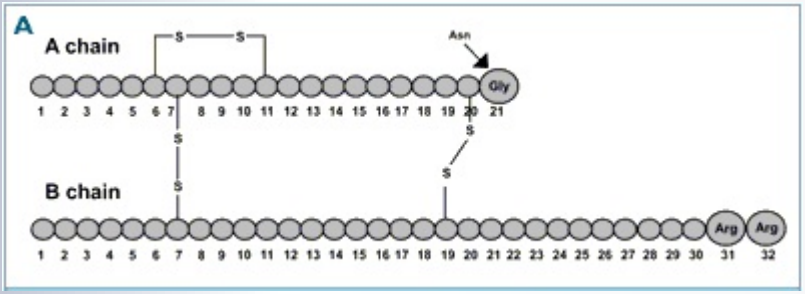
PPT + SPE
LLOQ extracts

Noise = 3000
Signal = 40000
S/N = 13.3

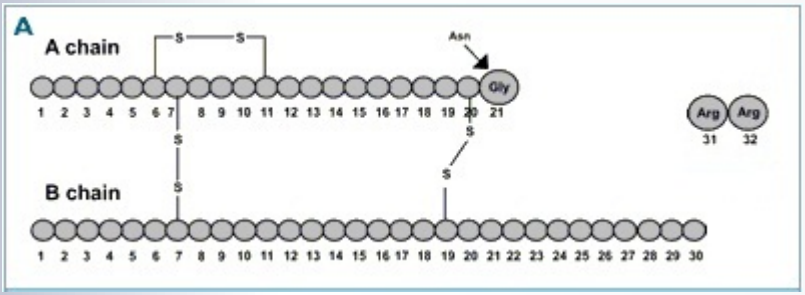
Immuno-capture
LLOQ extracts

Insulin glargine adding metabolites M1 & M2

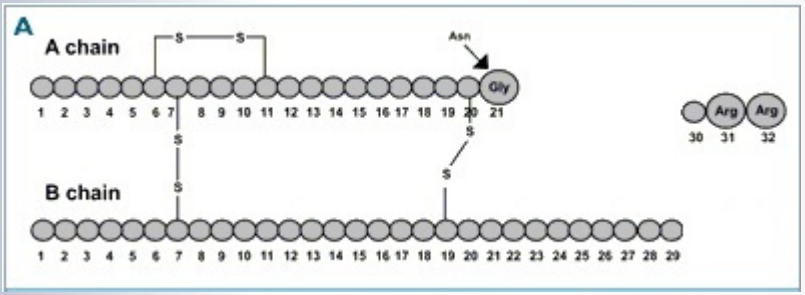
Two metabolites (M1 and M2) need to be added to the method, for the same calibration range (100 pg/mL to 10 ng/mL).



Insulin glargine



Insulin glargine-M1



Insulin glargine-M2

Insulin glargine : Final method

Determination of Insulin glargine, metabolites M1 and M2 in human EDTA K2 plasma (100 pg/mL to 10 ng/mL)

Sample preparation = Immuno-capture

Column: Acquity BEH C4, 2.1X50mm, 1.7µm

Flow rate: 0.20 ml/min

MPA: 0.1% Formic Acid in H2O
MPB: 0.1% Formic Acid in ACN

Gradient from (85-15) to (60-40) ~2.5min.

Insulin glargine + metabolites M1 & M2: Validation data

Summary of Between-Run Accuracy and Precision

Analyte		LLQC 100 pg/mL Measured Conc.	QC1 300 pg/mL Measured Conc.	QC2 5000 pg/mL Measured Conc.	QC3 7500 pg/mL Measured Conc.
	Mean	105.06	302.36	4976.5	7427.7
Insulin Glargine	CV(%)	6.39	4.13	4.34	3.35
	RE(%)	5.06	0.79	-0.47	-0.96
	SD(±)	6.71	12.5	216	249
	N	35	35	35	35
	Mean	98.942	290.03	4839.4	7241.2
Insulin Glargine-M1	CV(%)	9.55	4.18	3.95	3.48
	RE(%)	-1.06	-3.32	-3.21	-3.45
	SD(±)	9.45	12.1	191	252
	N	35	35	35	35
	Mean	102.16	297.35	4972.7	7433.2
Insulin Glargine-M2	CV(%)	6.61	4.65	4.37	3.96
	RE(%)	2.16	-0.882	-0.546	-0.890
	SD(±)	6.76	13.8	218	294
	N	35	35	35	35

Insulin glargine + metabolites M1 & M2: Validation data

Summary of Between-Run Accuracy and Precision

		LLQC	QC1	QC2	QC3
		100 pg/mL	300 pg/mL	5000 pg/mL	7500 pg/mL
Analyte		Measured Conc.	Measured Conc.	Measured Conc.	Measured Conc.
	Mean	105.06	302.36	4976.5	7427.7
Insulin	CV(%)	6.39	4.13	4.34	3.35
Glargine	RE(%)	5.06	0.79	-0.47	-0.96
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	N	35	35	35	35

Glargine insulin + metabolites M1 & M2: Validation data

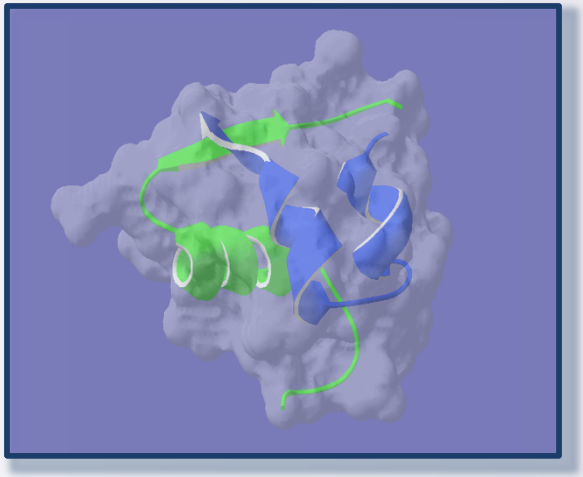
Selectivity

ID (Type)	Gender	Glargine % Interference	Glargine-IS % Interference	Glargine-M1 % Interference	Glargine-M2 % Interference	Glargine-M1-IS % Interference
BLK01	Female	1.23	0.0963	4.73	4.50	0.214
BLK02	Female	1.12	0.0000	1.91	2.19	0.095
BLK03	Female	1.04	0.0000	0.74	2.08	0.200
BLK04 (Diabetic)	Male	2.26	0.0000	1.82	1.33	0.166
BLK05 (Hyperlipemic)	Male	0.87	0.0000	0.64	1.36	0.000
BLK06 (3% Hemolyzed)	Male	0.00	0.0000	6.75	7.86	0.085

Immuno-capture combined to LC-MS/MS : Pros & Cons

PROS:
- Very clean extracts.
- Additional specificity achievable through modification of chromatography.
- Additional specificity achievable through optimization of the detector. (Use of highly specific transitions, summation, etc.)
- Easily adapted for high throughput.

CONS:
-More expensive with respect to conventional extraction methods.
-Requires available antibody



Interferences: Additional tests

Interferences with analogs, human insulin and anti-insulin antibodies

	Addition of Insulin:						Addition of anti-Insulin Antibody:			
	N/AP	Human	Lispro	Aspart	Detemir	Glulisine	N/AP	Anti-Human	Anti-Glargine	Anti-Glargine
	Conc.	500 µU/mL	500 µU/mL	500 µU/mL	500 µU/mL	500 µU/mL	Conc.	497.60 ng/mL	9.94 ng/mL	49.70 ng/mL
Insulin Glargine										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	3.68	3.41	2.76	3.26	4.54	3.63	3.73	2.06	4.35	5.95
% Change	N/AP	-0.85	-0.82	-1.14	0.43	-1.82	N/AP	1.16	1.08	-23.87
Insulin Glargine-M1										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	4.02	5.82	1.22	3.42	3.30	5.56	2.19	2.42	4.14	2.25
% Change	N/AP	0.48	2.78	3.78	4.56	3.10	N/AP	-5.85	-1.84	-32.96
Insulin Glargine-M2										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	5.66	3.89	2.76	2.03	2.48	4.24	3.97	4.14	4.54	2.32
% Change	N/AP	2.99	4.70	4.21	1.71	3.53	N/AP	-3.44	-3.68	-31.94

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	Addition of Insulin:						Addition of anti-Insulin Antibody:			
	N/AP	Human	Lispro	Aspart	Detemir	Glulisine	N/AP	Anti-Human	Anti-Glargine	Anti-Glargine
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	Addition of Insulin:						Addition of anti-Insulin Antibody:			
	N/AP	Human	Lispro	Aspart	Detemir	Glulisine	N/AP	Anti-Human	Anti-Glargine	Anti-Glargine
	Conc.	500 µU/mL	500 µU/mL	500 µU/mL	500 µU/mL	500 µU/mL	Conc.	497.60 ng/mL	9.94 ng/mL	49.70 ng/mL
Insulin Glargine										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	3.68	3.41	2.76	3.26	4.54	3.63	3.73	2.06	4.35	5.95
% Change	N/AP	-0.85	-0.82	-1.14	0.43	-1.82	N/AP	1.16	1.08	-23.87
Insulin Glargine-M1										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	4.02	5.82	1.22	3.42	3.30	5.56	2.19	2.42	4.14	2.25
% Change	N/AP	0.48	2.78	3.78	4.56	3.10	N/AP	-5.85	-1.84	-32.96
Insulin Glargine-M2										
Nominal concentration (pg/mL)	298	298	298	298	298	298	298.01	298.01	298.01	298.01
%CV (n=6)	5.66	3.89	2.76	2.03	2.48	4.24	3.97	4.14	4.54	2.32
% Change	N/AP	2.99	4.70	4.21	1.71	3.53	N/AP	-3.44	-3.68	-31.94

Immunocapture method: Additional tests

Stability of antibody-coated beads

	Insulin Glargine				Insuline Glargine-M1				Insulin Glargine-M2			
	QC1 (300pg/mL)		QC3 (7500 pg/ml)		QC1 (300pg/mL)		QC3 (7500 pg/ml)		QC1 (300pg/mL)		QC3 (7500 pg/ml)	
Days	T=0	T = 19	T=0	T = 19	T=0	T = 19	T=0	T = 19	T=0	T = 19	T=0	T = 19
Mean	297.01	302.17	7388.4	7433.0	289.81	281.94	7179.0	7115.4	286.33	283.40	7304.2	7234.2
SD(±)	14.0	8.6	213.8	209.6	13.5	11.3	183.6	176.6	9.4	23.2	287.7	246.0
CV(%)	4.70	2.85	2.89	2.82	4.67	4.00	2.56	2.48	3.28	8.20	3.94	3.40
RE(%)	-1.00	0.72	-1.49	-0.89	-3.40	-6.02	-4.28	-5.13	-4.56	-5.53	-2.61	-3.54
N	5	5	5	5	5	5	5	5	5	5	5	5

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From inVentiv Health Clinical

- Philippe Bélanger, Senior Research Scientist
- Marie-Josée Marcoux, Bioanalytical Project Coordinator
- Patricia Lavallée, Analyst
- Nadine Boudreau, Bioanalytical Associate Director
- Ann Lévesque, Senior Bioanalytical Director

Thank you for listening !

For additional information, please contact us.

>> TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY

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