‘A new hybrid immuno-affinity mass spectrometric method for dosed or endogenous human insulin in clinical samples’

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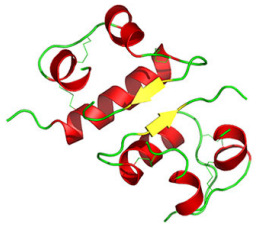
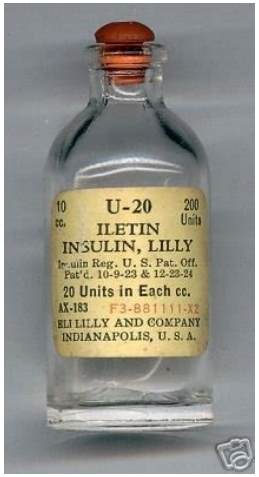
Contents



- Why use a hybrid assay - expectations?
- What form of the protein are we measuring?
- Human vs. animal insulins and their mass spectrometry
- Endogenous concentrations and required analytical range
- Example: an assay for human insulin
- Fed versus fasted insulin levels in human volunteers
- Assay performance and cross-reactivity experiments
- Immunogenicity effects (ADA)
- Conclusions and references



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Immunoassays vs hybrid IA-mass spectrometry

Example from 2015 publication:

Bioequivalence trial for an insulin analogue in healthy subjects:

1. Dose with analogue
2. Measure total insulin activity with RIA
3. Correct for endogenous insulin by using a C peptide assay
4. Perform PK for dosed drug with corrected value

2 approximations or extrapolations here:

C peptide is a by-product of the formation of human insulin:

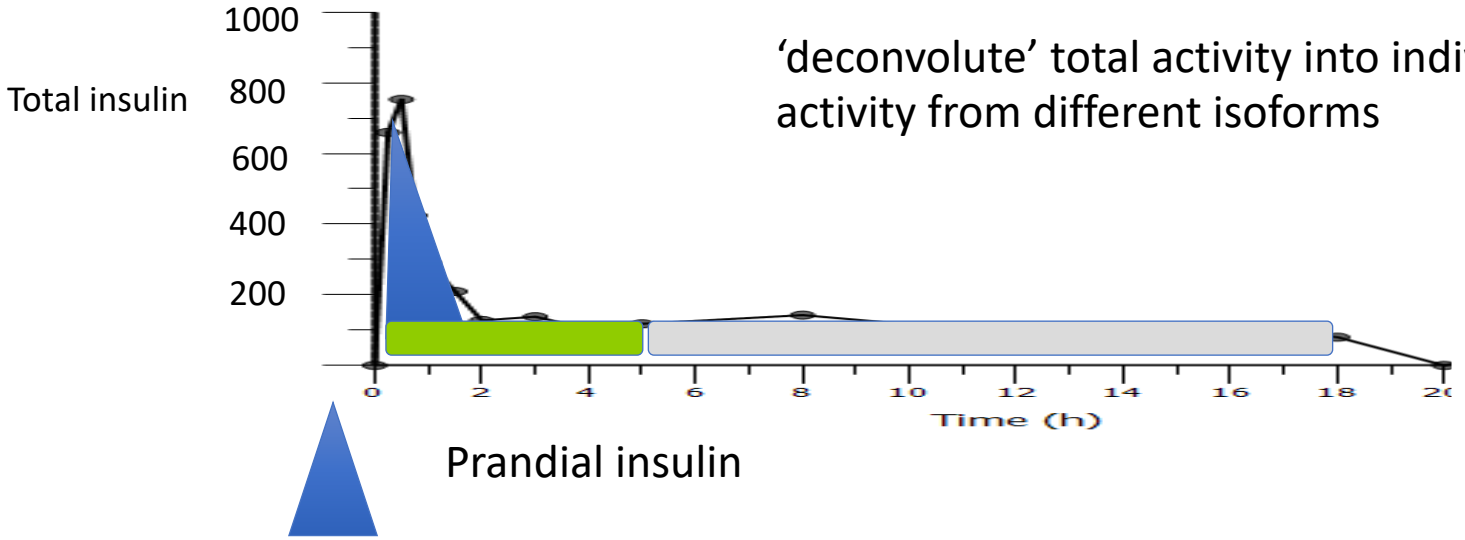
for every molecule of human insulin produced, so is one of C peptide

C peptide is used as a marker of insulin secretion

However different assay approaches (for example RIA, ELISA) can give different measures for C peptide, and the total assay is not a direct measurement of free circulating drug rather of activity.

Should use specific assays for PK, to **attribute** the measured activity

Why mass spectrometry



'deconvolute' total activity into individual activity from different isoforms

Prandial insulin

Basal insulin or endogenous if healthy patients

Basal insulin metabolite or endogenous if healthy patients

'When healthy volunteers are enrolled in the clamp studies, their endogenous insulin production may interfere with PK and/or PD measurements. For some insulin analogues, specific assays, capable of distinguishing between exogenous and endogenous insulin, exist. If available, the use of such assays should be considered.'

'Where necessary, samples should be referred to specialist centres for insulin analysis and ideally by a validated and fully quantitative mass spectrometry based method' (in the case of hypoglycaemia where standard kit tests fail to identify exogenous insulins)

EUROPEAN MEDICINES AGENCY
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National Institute for Health Research

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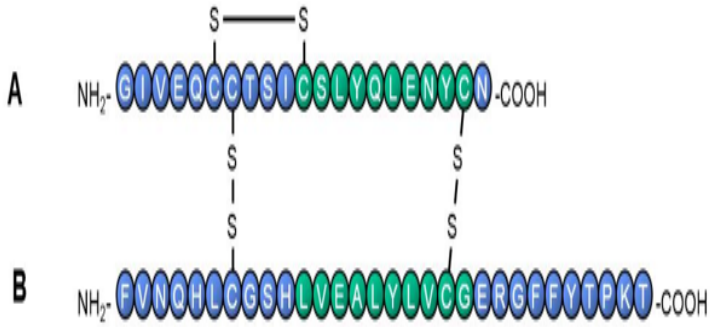
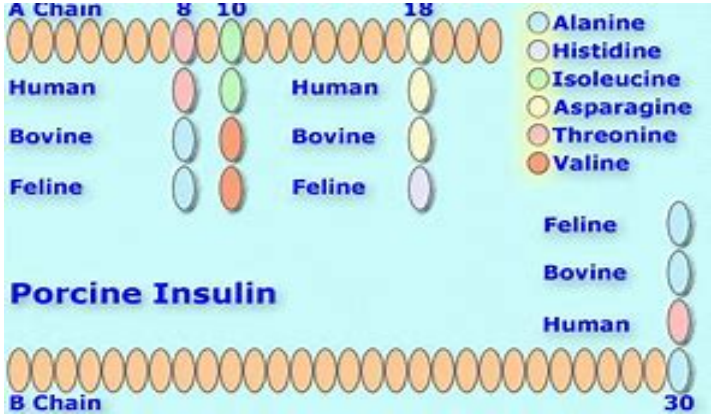
Insulins structures and sequences





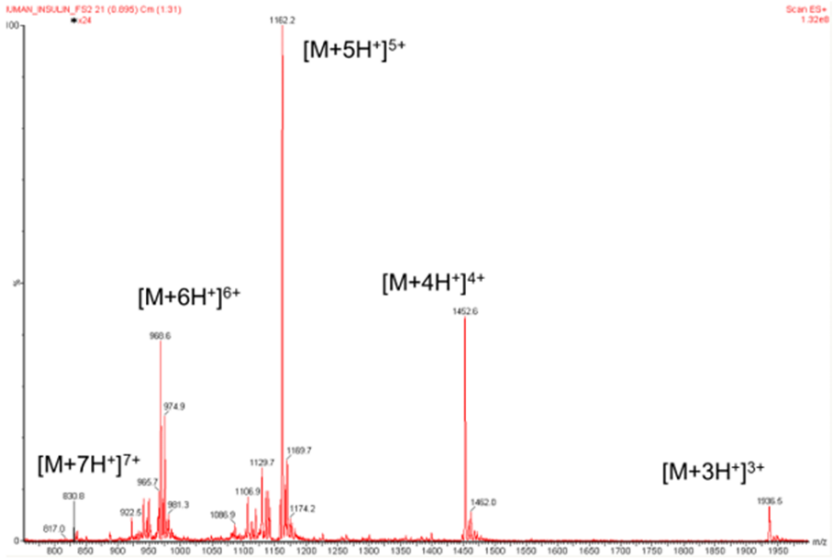
Human: 51 amino acids, molecular weight approx. 5.8 KDa

Porcine insulin has one different residue on the B chain C terminus

Bovine insulin typically used as an internal standard

Mass spectrometry of human insulin

Q1MS



‘top down’ of intact clusters.

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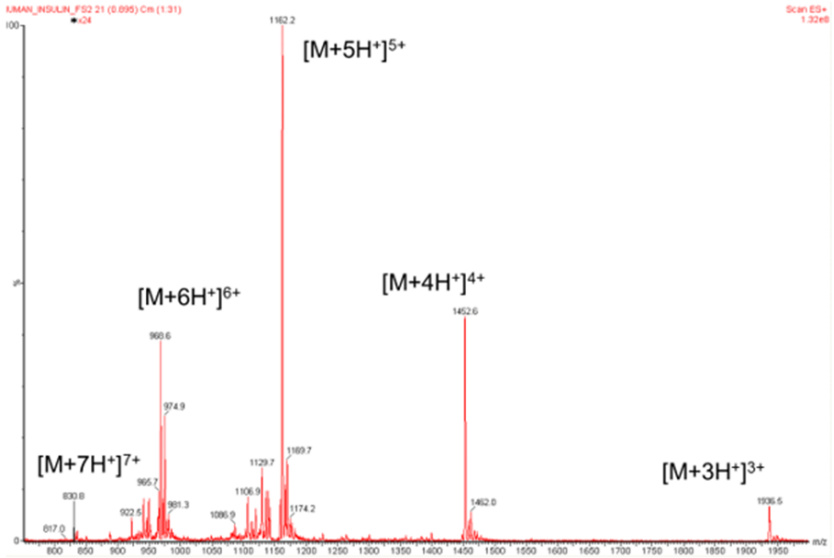
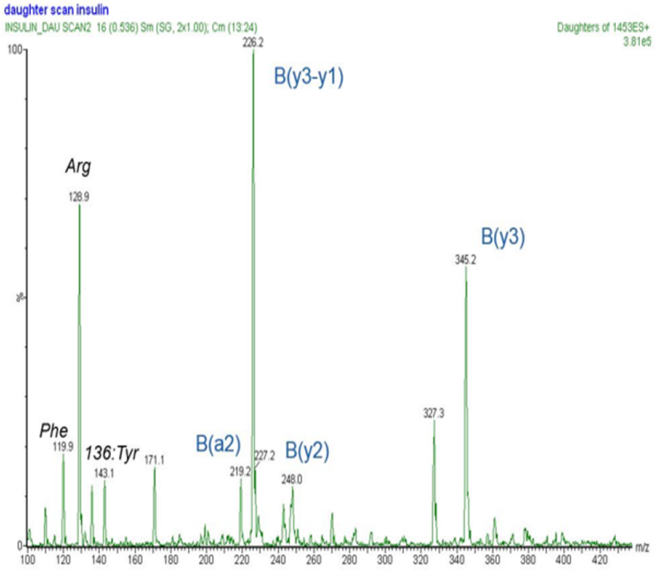
Mass spectrometry of human insulin

Q1MS

MS - MS





'top down' of intact clusters. Select specific product ion

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What are we measuring with this assay?

"The protein concentration is not a meaningful result, unless it is defined which molecular property the method responds to."
Van der Merbel, European Bioanalytical Forum 2017

FREE vs. ACTIVE (target binding) vs. TOTAL

MSIA^{TM} extracts target binding insulins, with a capturing epitope on the mid-point of B chain.
To measure TOTAL, crash plasma first & dilute
To prevent non specific binding in the well plate, use a carrier peptide e.g ACTH fragment, leucine enkephalin

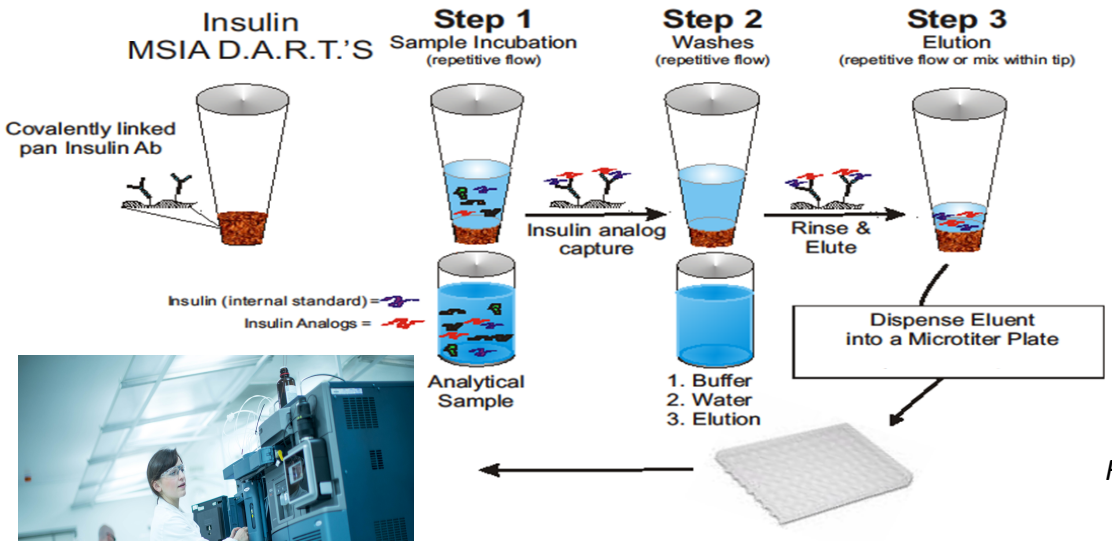

Figure: Thermo

LC-MS/MS

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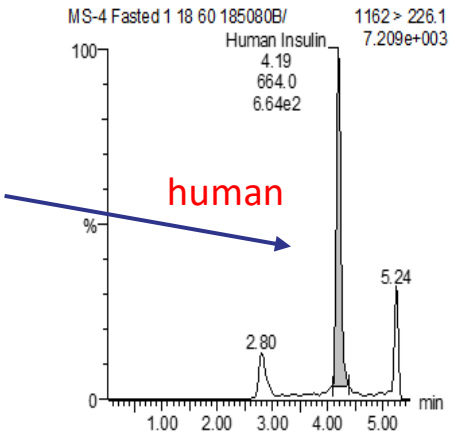
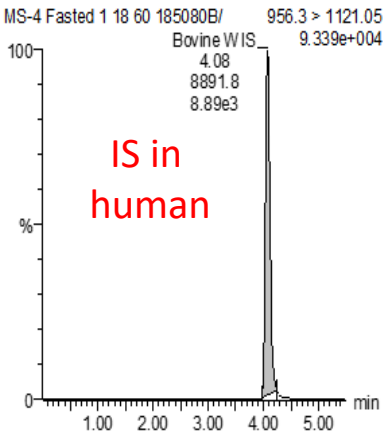
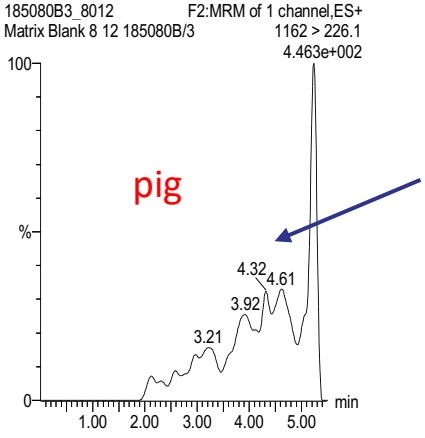
Endogenous assays: calibration line







For analogue assays in human, we can simply spike control human plasma with the analogue

Human insulin: cannot use same matrix for calibration.

Match with a surrogate matrix: we have chosen pig plasma

Method sample volume is 350 µL

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What analytical range?

- The American Diabetes Society recommends that bioanalytical assays for insulins should be able to achieve an LLOQ of **12 pmol/L** = approximately **70 pg/mL** depending on the analogue
- Endogenous levels are between 90 to 3000 pg/mL in human serum
- Insulin becomes pharmacologically active around or even below 200 pg/mL
- Requirement to reduce LLOQ to 100 pg/mL and below to support new drug development studies
- Multiple analytes may need to be monitored simultaneously e.g. active metabolites
- **Sample volumes are limited** – 250 to 400 µL of plasma available for analysis

Reported levels of insulin in different matrices

Species	Matrix	Model	Insulin pg/mL	Insulin µU/mL	Method
Human	Serum	Normal 12h fasting	90 to 1100	2 to 25	NR
Human	Serum	Non-Fasting	200 to 3400	5 to 75	NR
Human	Plasma	Dosed with glargine 1.2U	2000 (glargine M1)	50	IAC-MS
Human	Urine	Average	360	8	NR
Human	Urine	Fasting dependant	80 to 3600	1.7 to 80	NR
Human	serum	C peptide non diabetic*	3000 to 11000	67 to 250	IDA-MS
Human	Serum	Proinsulin	50 to 1000	1 to 20	IDA-MS
Dog	Serum	Fasting dependant	180 to 1300	4 to 28	NR

NR not released
IAC immunoaffinity capture
IDA immunodepletion capture

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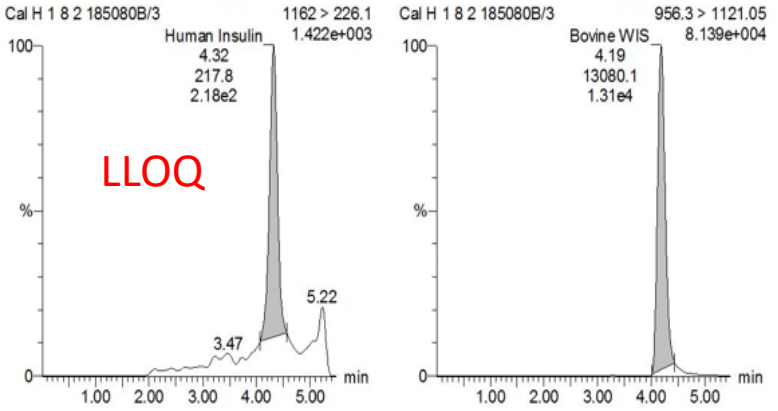
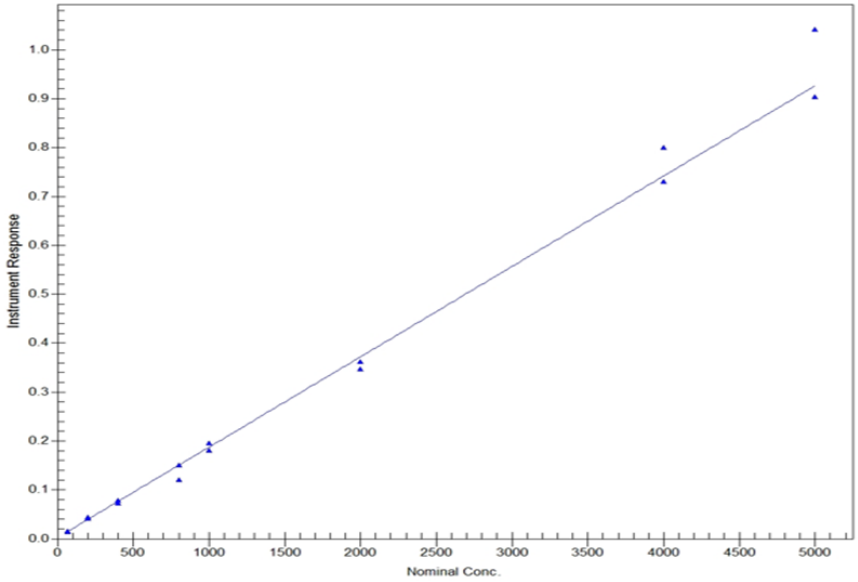
Calibration line in surrogate (pig) plasma





$R^2 = 0.991$

70-5000 pg/mL

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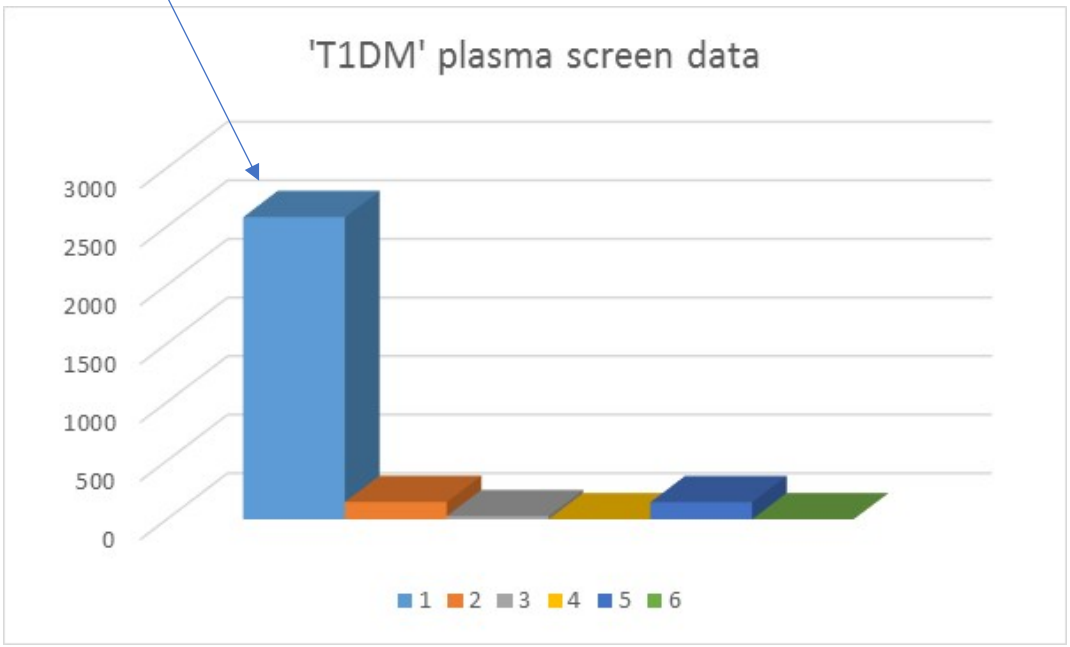
Endogenous assay: QC screen #1: T1DM* plasma

	screen 1	screen 2	screen 3	screen 4	screen 5	screen 6
Measured (pg/mL)						
#1	2750	132	<70	<70	146	<70
#2	2660	125	<70	<70	157	<70
#3	2650	174	<70	<70	153	<70
#4	2540	169	<70	<70	141	<70
#5	2480	141	<70	<70	132	<70
#6	2380	138	<70	<70	143	<70
Mean	2580	147	<70	<70	145	<70
%CV	5.23	13.70	N/A	N/A	6.15	N/A
n	6	6	5	6	6	6

*type one diabetes



Run 6 replicates of 6 different batches plasma

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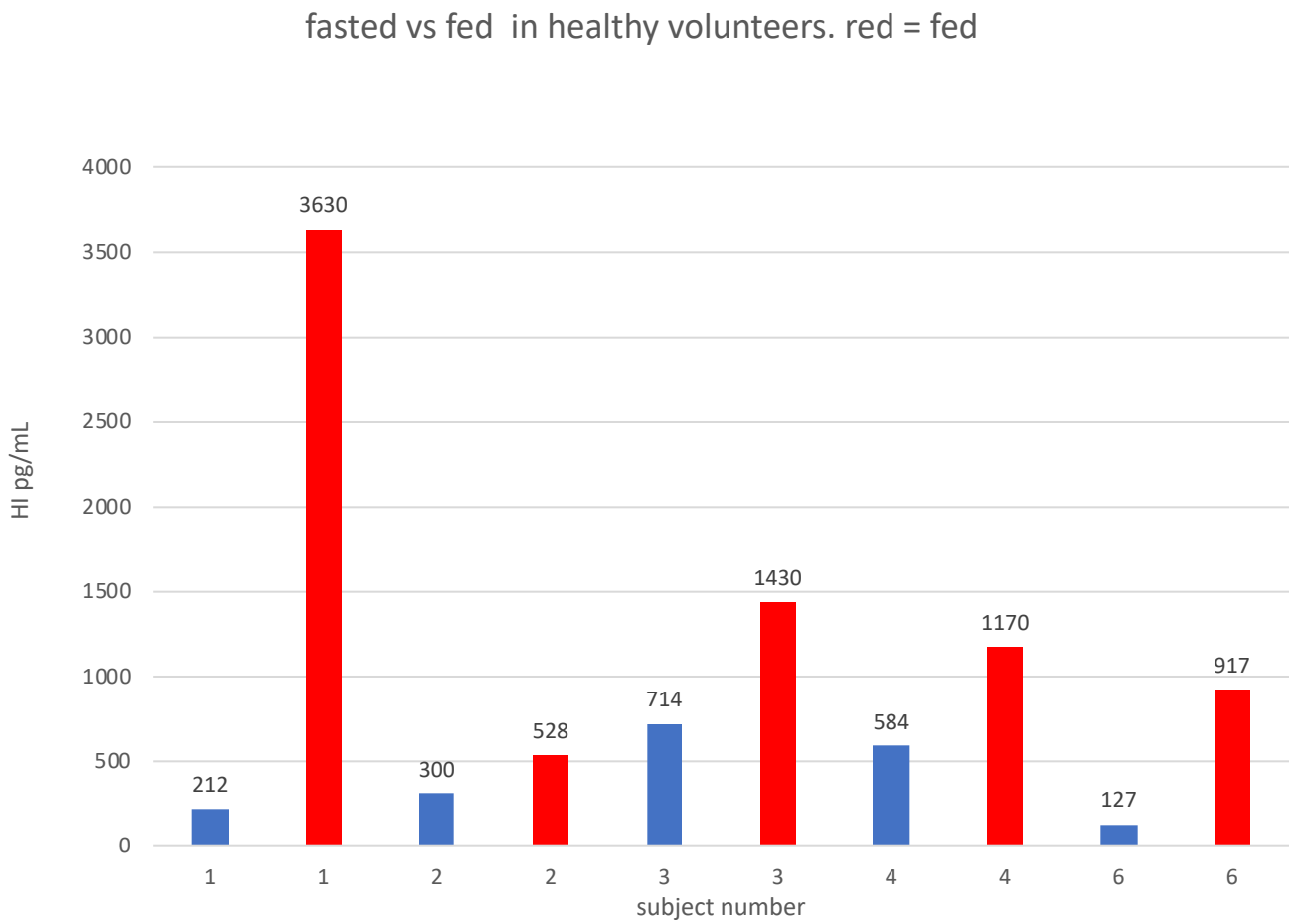
QC screen #2: Human insulin levels in fed and fasted volunteers



Choose plasma from fasted volunteers as QC and dilute to LLOQ with control animal (pig) plasma.

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Performance of the human assay: typical for isoforms also

Quality Control	A&P LLOQ 77.8 pg/mL	A&P QCL 213 pg/mL	A&P QCM 1050 pg/mL	A&P QCH 4010 pg/mL	A&P ULOQ 5230 pg/mL
Measured (pg/mL)	74.6	207	1040	3920	5130
Inter-run %CV	15.7	6.62	6.14	8.93	6.22
Inter-run %Bias	-4.11	-2.82	-0.95	-2.24	-1.91
n	18	18	18	18	18

Applying hybrid 4-6-20 acceptance criteria
Assay is fully validated- matrix effect, freeze-thaw, LTS etc

Jenkins et al, AAPS J, (2015) 17(1) 1-16
Knuttson M, Schmidt R and Timmerman P, Bioanalysis (2013) Sep 5(18):2211-4
Yang Xu et. Al., Journal of Chromatography B1063 (2017) 50-59.

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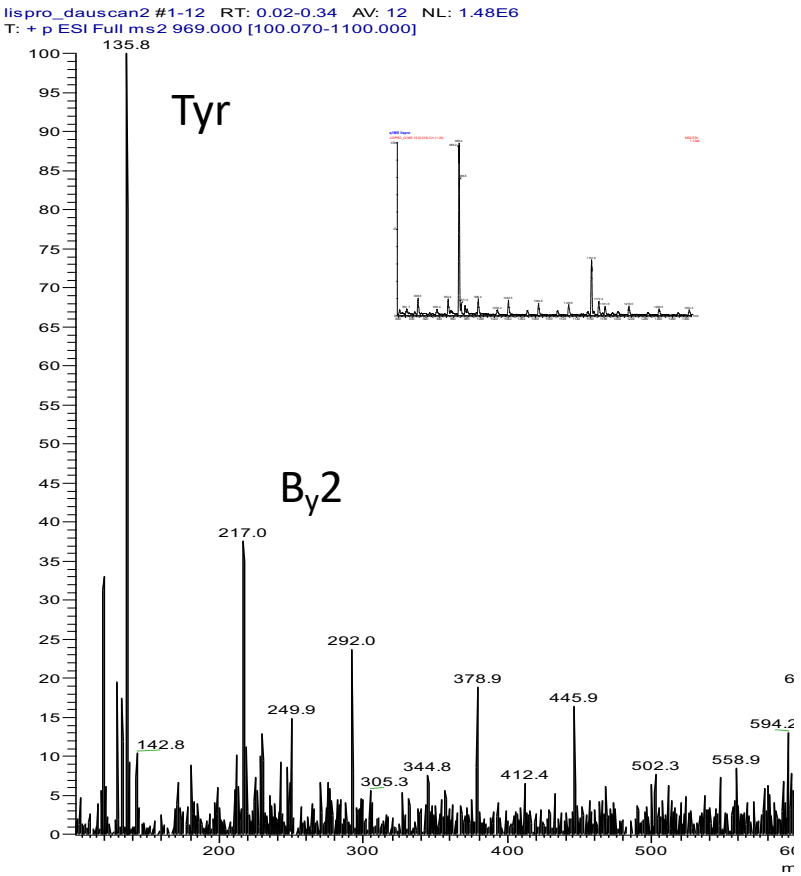
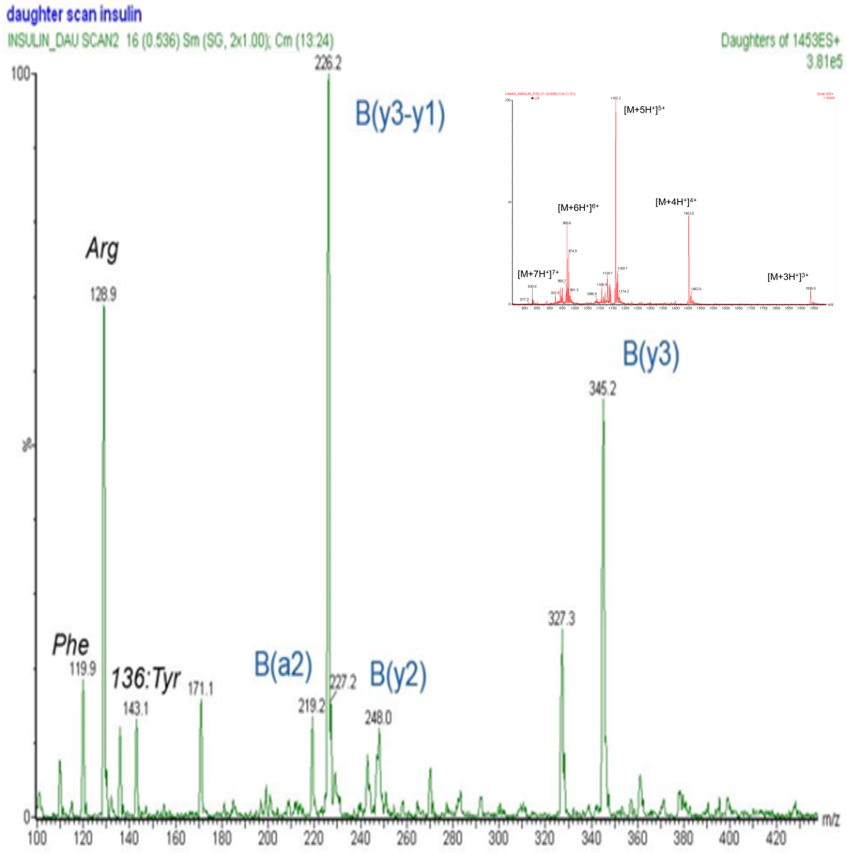
Assay specificity examples

lispro



human



Need to use MS/MS to distinguish; cannot rely on mass of cluster alone

Potential for cross-talk?

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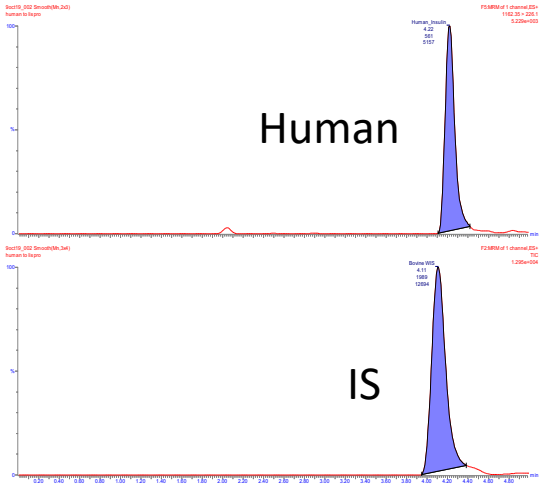
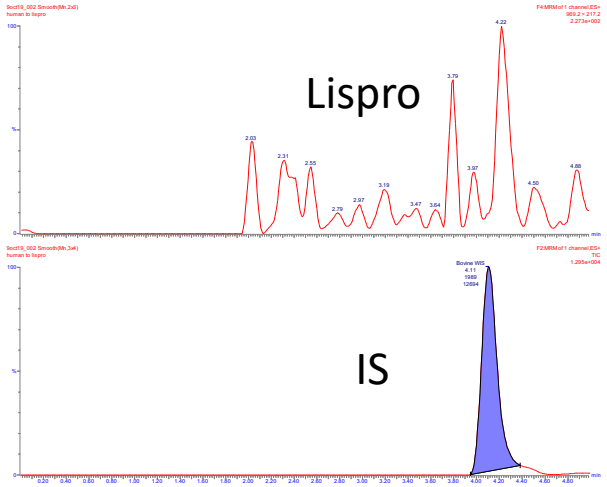
Cross-reactivity experiments

Mass spectrometry provides specificity. Standards spiked into dog plasma and extracted by MSIA. Measuring human insulin and analogues against each other at high spiked levels

n=6





Example of human insulin 5000 pg/mL to lispro: no crossover
e.g. endogenous insulin will not interfere with lispro quantitation

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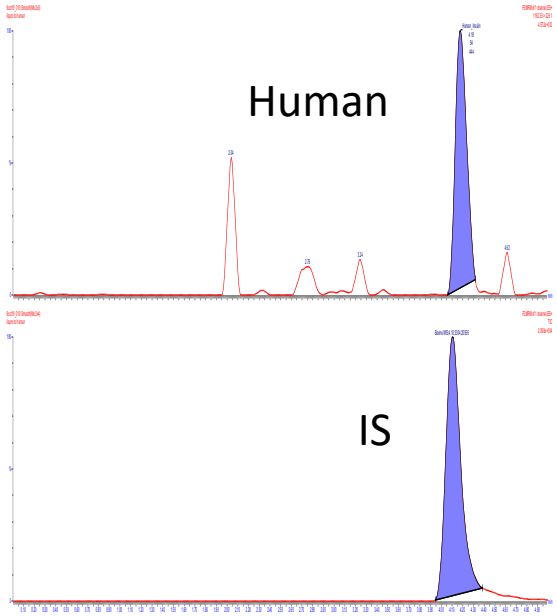
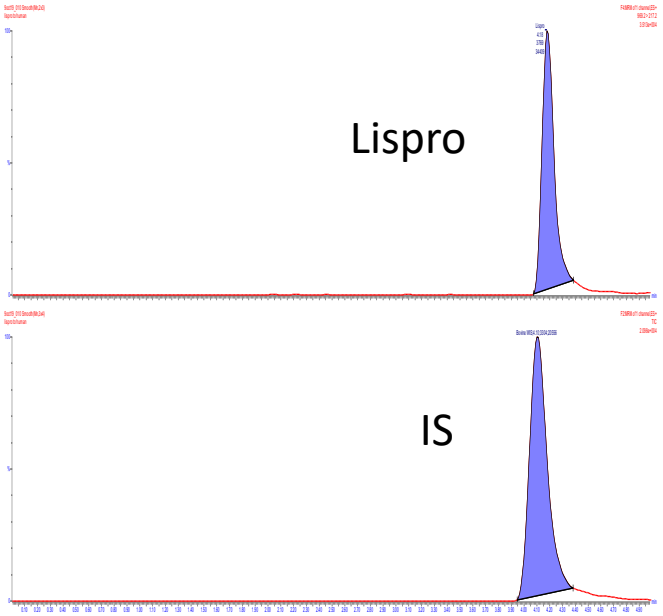
Cross-reactivity experiments

n=6





Lispro to human insulin: minimal crossover. 5000pg/mL lispro gives human signal equivalent to about 50% of human insulin LLOQ.
(ie can quantify endogenous insulin in presence of lispro)

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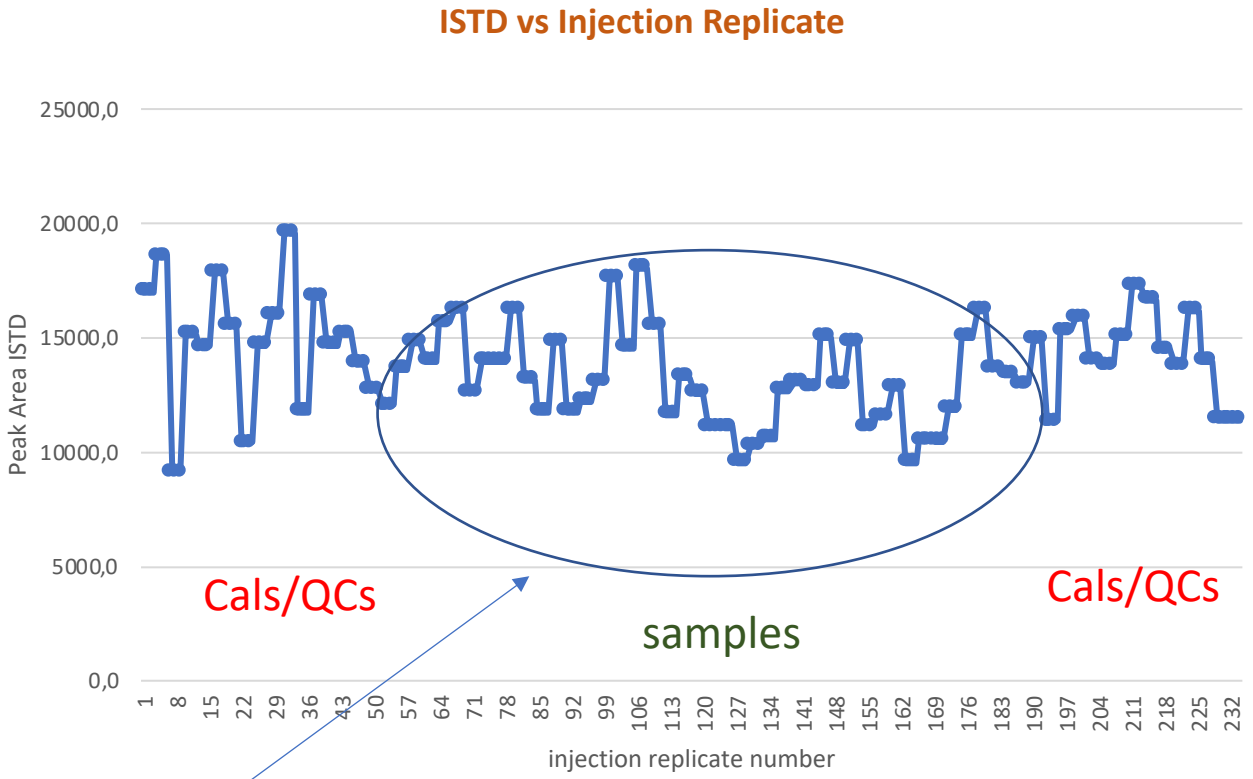
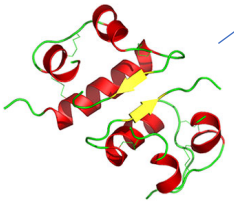
Insulin analogues clinical insulins Study: Effect of induced antibodies (Immunogenicity)



example of a normal result: IS recovery is consistent



free bovine WIS

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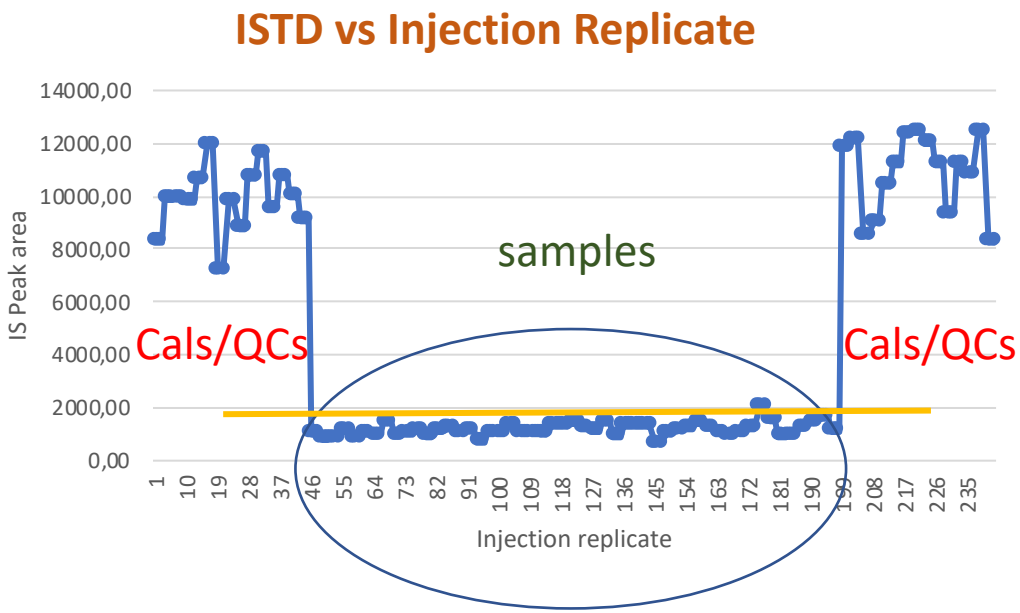
Clinical insulins Study: Effect of induced antibodies



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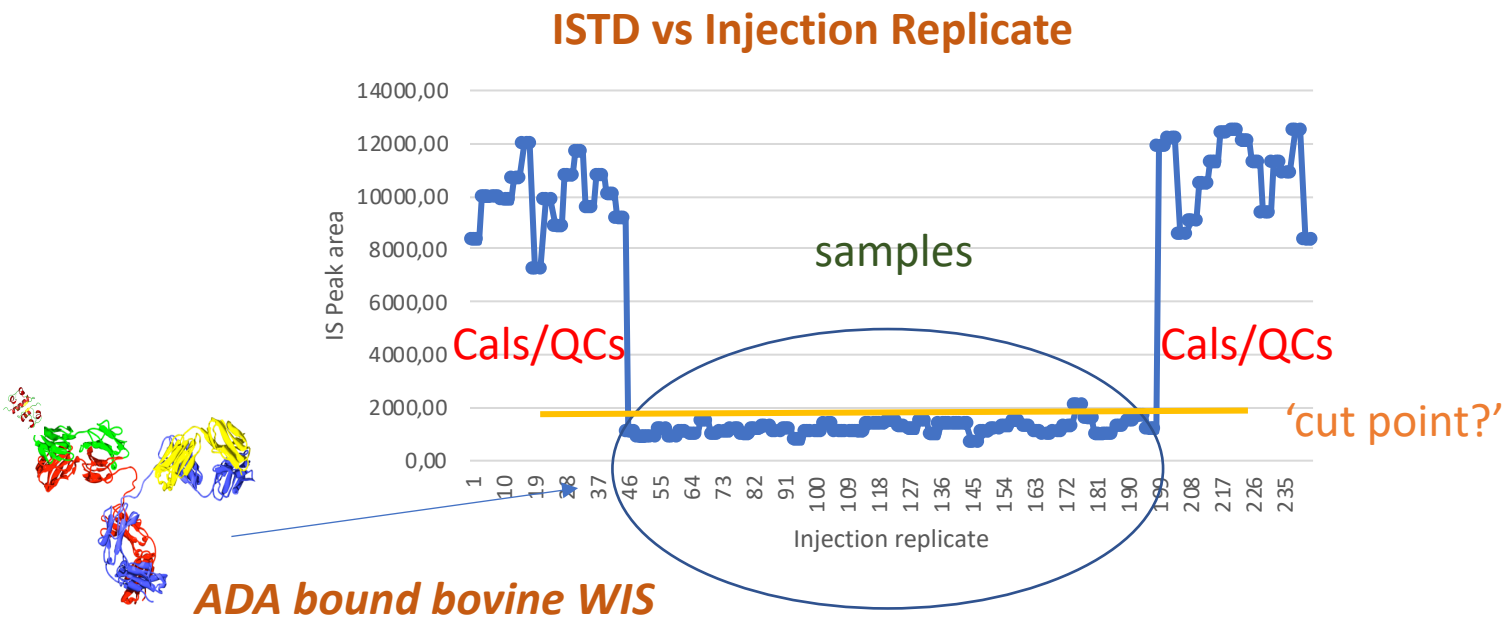
Clinical insulins Study: Effect of induced antibodies



ADA positive subject: Internal standard recovery is heavily compromised for this patient.
This is due to the presence of induced anti-insulin antibodies (ADA) in the sample.

ADA effects can occur in diabetic patients (also affect purely physicochemical assays e.g. SPE), particularly where patients have previously taken animal insulins.

Samples are diluted to reduce effect and re-analysed.

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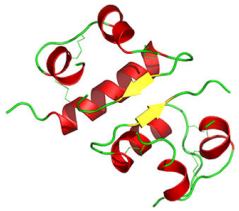

Conclusions

Hybrid IA-LCMS assays are now common for large peptides, especially insulins, due to their specificity and sensitivity, and are frequently expected.

Pig plasma is a useful surrogate matrix for calibration lines for an endogenous human insulin LC-MS/MS assay

QCs can be prepared using plasma from healthy volunteers, diluted or spiked as necessary to create appropriate QC/Val samples

Assay is fully validated and cross-over effects from other insulins are unlikely; but need MS/MS to distinguish lispro versus human insulin

ADA effects have not been observed in healthy volunteers, but can be observed in large trials with diabetic patients: how to respond to this?

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Thankyou
for listening

Stephen Gray, Stuart McDougall, Paige Bellis, Hannah Gent, Elizabeth Linsley, Carolyn Mailer, Polly Wight, Sheryl Atkinson