‘HOW LOW CAN YOU GO: DRIVING DOWN LIMITS OF QUANTITATION FOR PEPTIDE BIOMOLECULES BY HYBRID IA-LC/MS’

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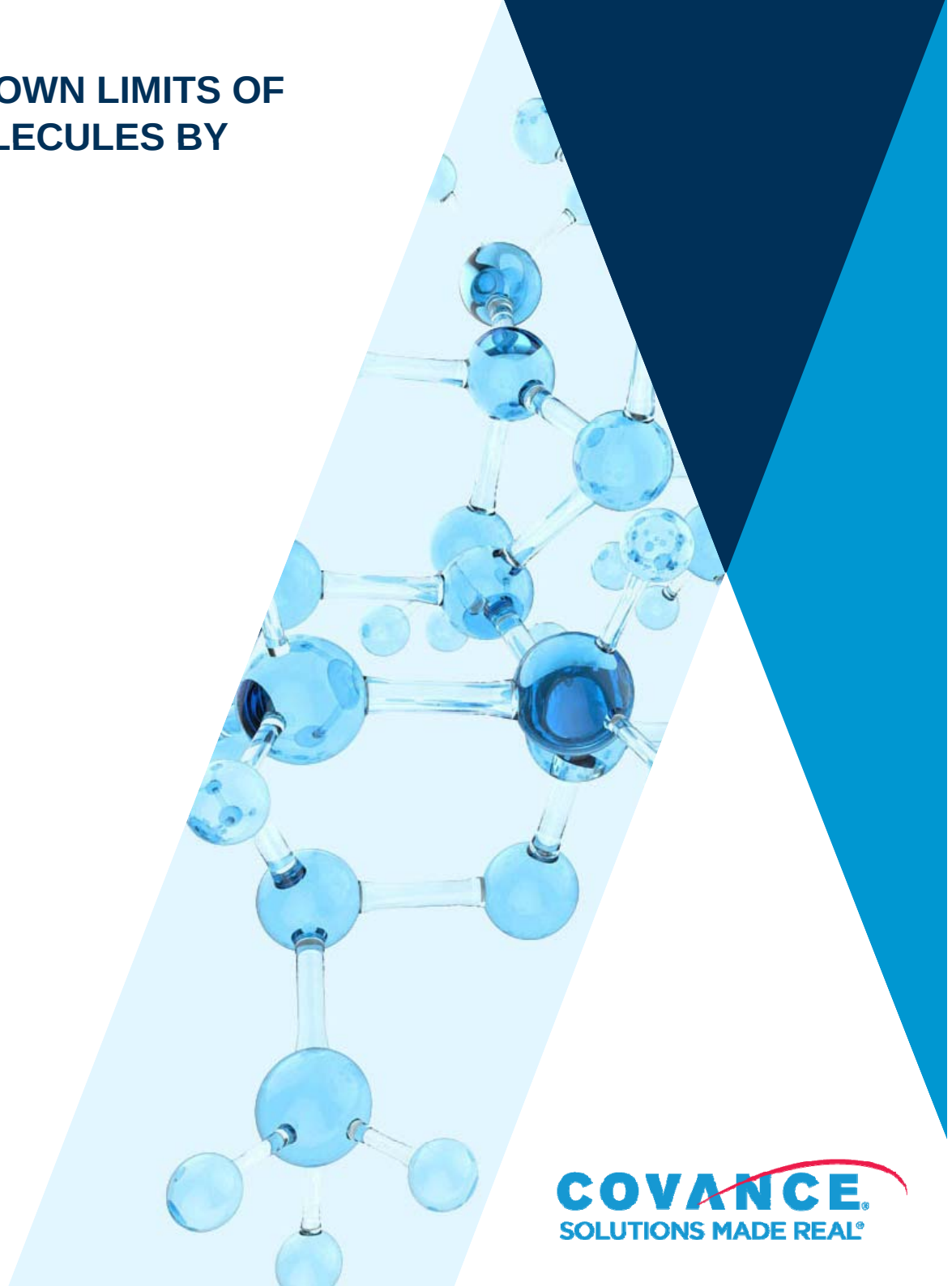

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CONTENTS

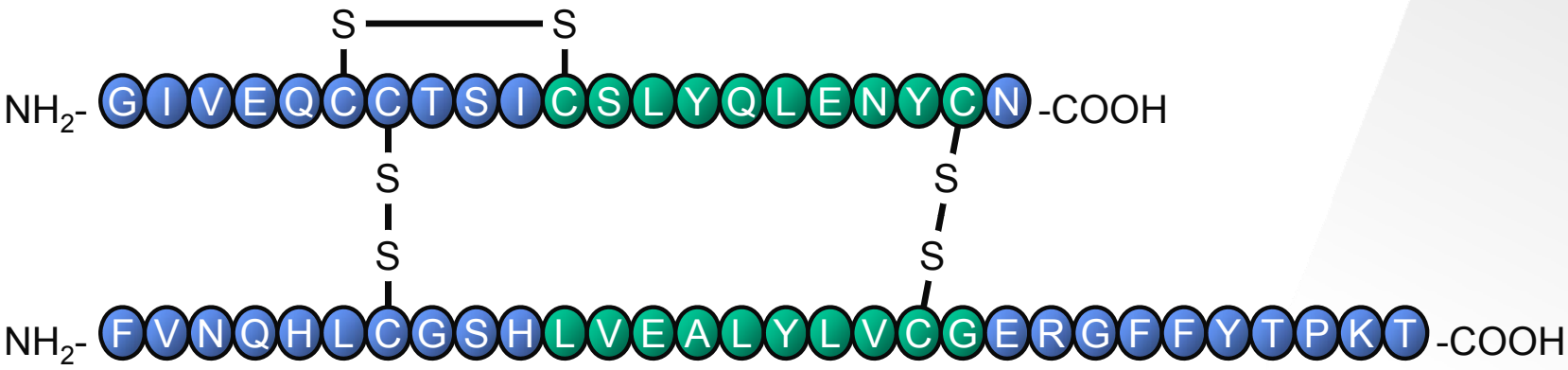
INSULINS: the perfect peptides

Glargine, glulisine, lispro, aspart etc, are modified insulins, also known as insulin analogues (IAs).

IAs have a modified isoelectric point (pI) which reduces solubility at physiological pH.

Changing pI changes adsorption into body: get fast & slow-acting insulins

Structure of Human Insulin: 51 amino acids, a large peptide



1955: Sanger sequences insulin, Cambridge
1982: Marketing of synthetic insulin (Humilin, Eli Lilly and Company)
1996: First insulin analogue (Lispro, Eli Lilly and Company)
2015: High annual growth rate, entry of biosimilars, new analogues & combination therapies

INSULIN Q1 MASS SPECTRA: manipulation of charge state

Select Parent & Product ions carefully: Q1 MS Charge state envelope can be to some extent controlled

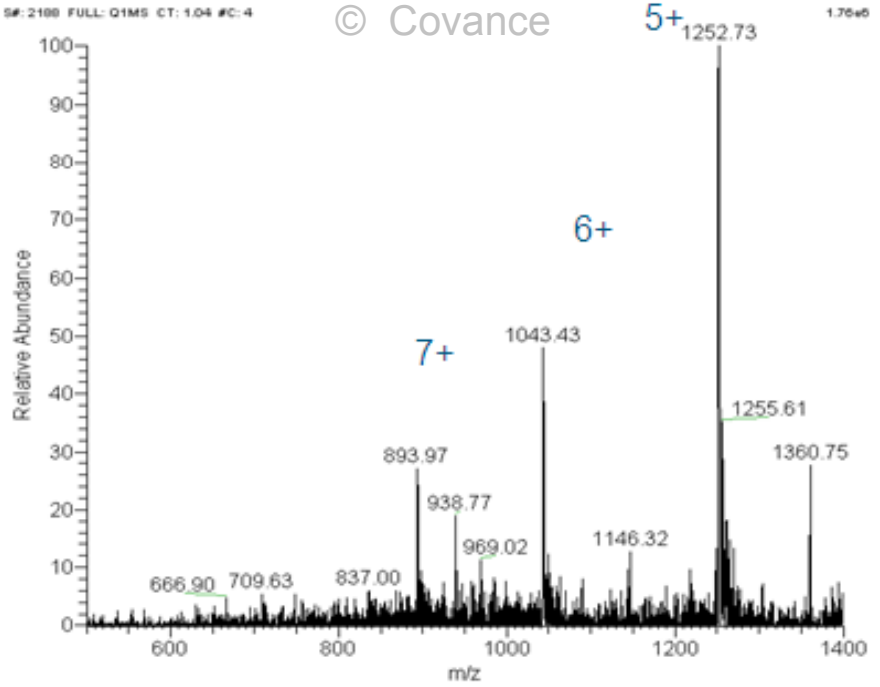
- Using mobile phase composition, buffer, methanol vs acetonitrile
- Addition of acid will increase charging eg formic acid, acetic acid

Also, some difference observed between different source types for same mobile phase

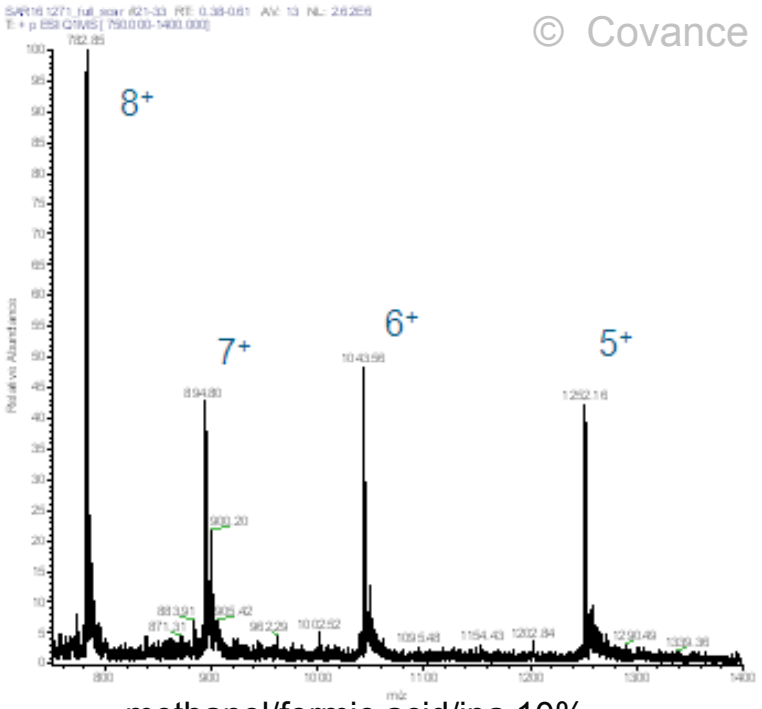
- Waters Z spray, Thermo HESI , also Sciex ® API5000 Ionspray

Can control specificity of assay through selection of MS/MS transition

THERMO QUANTUM ULTRA HESI I



20mM ammonium formate/acetonitrile



methanol/formic acid/ipa 10%

LIMITS OF QUANTITATION

The American Diabetes Society recommends that bioanalytical assays for insulins should be able to achieve an LLOQ of 70 pg/mL

►Endogenous levels are between 90 to 3000 pg/mL in human serum

►2009: Original limit of quantitation for first Sanofi study 200 pg/mL based on previously accepted work (insulin becomes pharmacologically active around or even below 200 pg/mL).

►Therefore continuous push to reduce LLOQ to 100 pg/mL and below to support new drug development studies

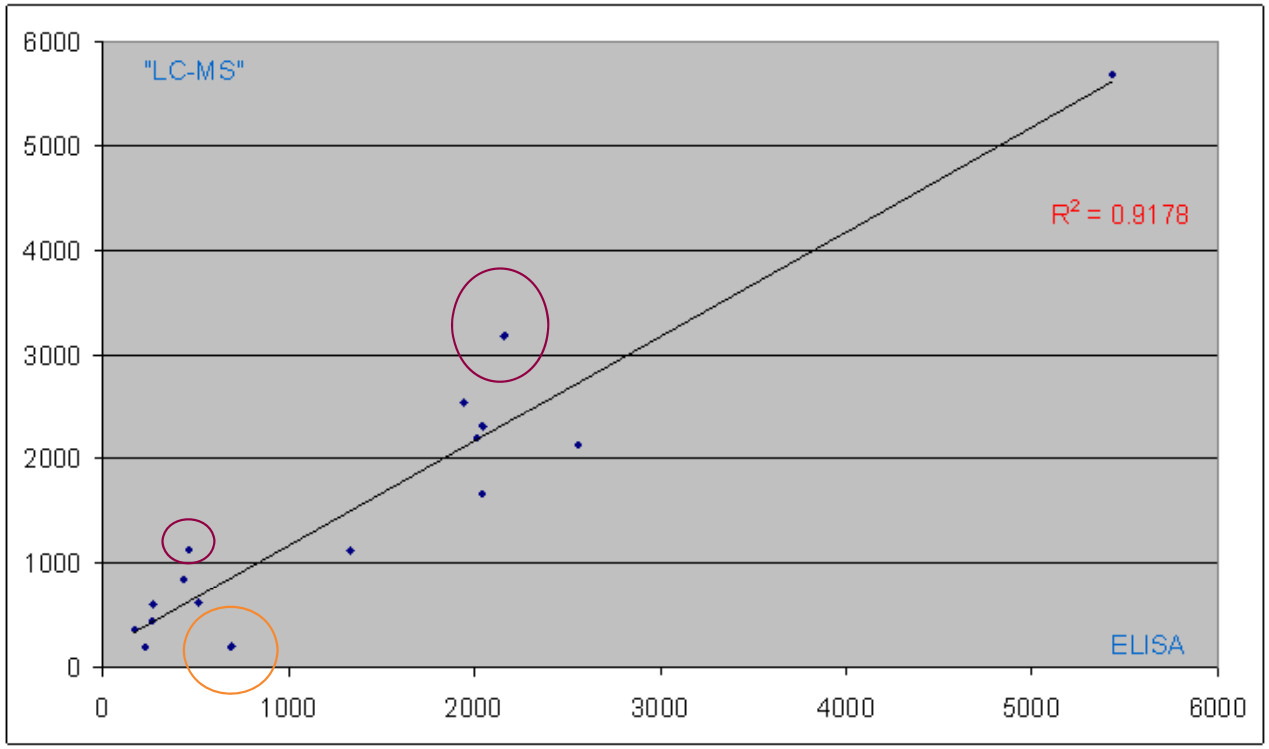
Reported levels of insulin in different matrices

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

CORRELATION OF LCMS vs ELISA ASSAYS

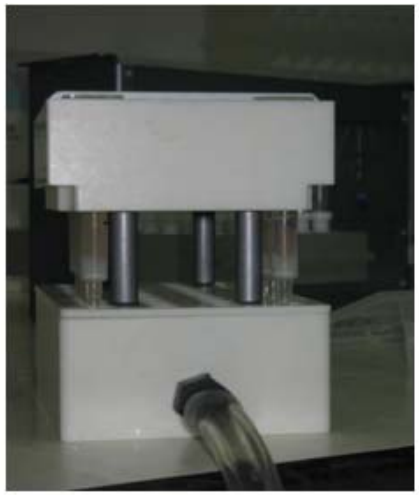
- ELISA: measures total insulin activity
- LCMS: measures single specific analyte

- IAC-LC-FAIMS-MS vs ELISA values for 16 samples from discovery study (discovery LC-MS assay)



Both axes are insulin analogue concentration in pg/mL

PHYSICOCHEM. vs IMMUNOAFFINITY EXTRACTION



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Covance have extracted insulins using SPE (Oasis HLB) on a robot;

'Home made' immunoaffinity extraction columns prepared from antibody gel supplied by client

Commercially supplied tips containing insulin antibody (MSIA) and magnetic beads

PROS and CONS FOR SPE vs.Immuno-affinity (IA)

√ SPE is relatively cheap and we are familiar with it

√ SPE is robust and we have different chemistry options

X SPE is non selective; we extract interfering peptides and proteins

√ IA is highly specific, based upon an insulin epitope

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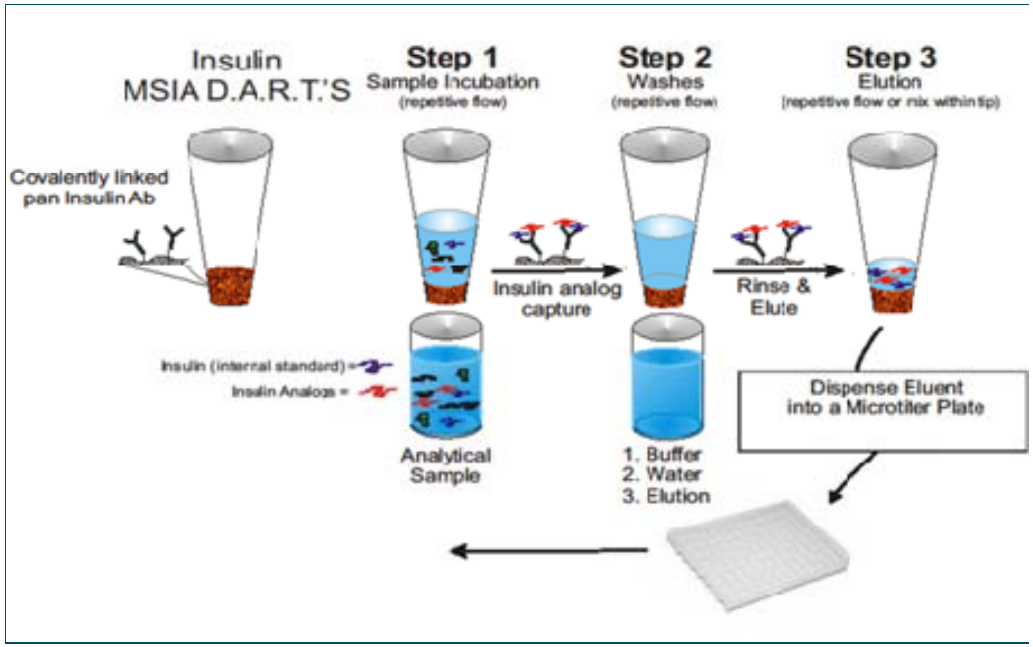
IMPROVEMENT OF LLOQ USING MSIA ASSAY

- Improvement of Immunoaffinity extraction technique
  - Home-made columns were non-uniform, extraction is a manual, possibly extraction not optimised (no wash), Abs have to be bound to gel
- Testing of MSIA (**M**ass **S**pectrometric **I**mmuno**A**ssay) tips
  - Anti-insulin antibodies loaded into pipette tips. D.A.R.T.S (**D**isposable **A**utomation **R**esearch **T**ips)

Aim to improve LLOQ by using a cleaner extraction procedure



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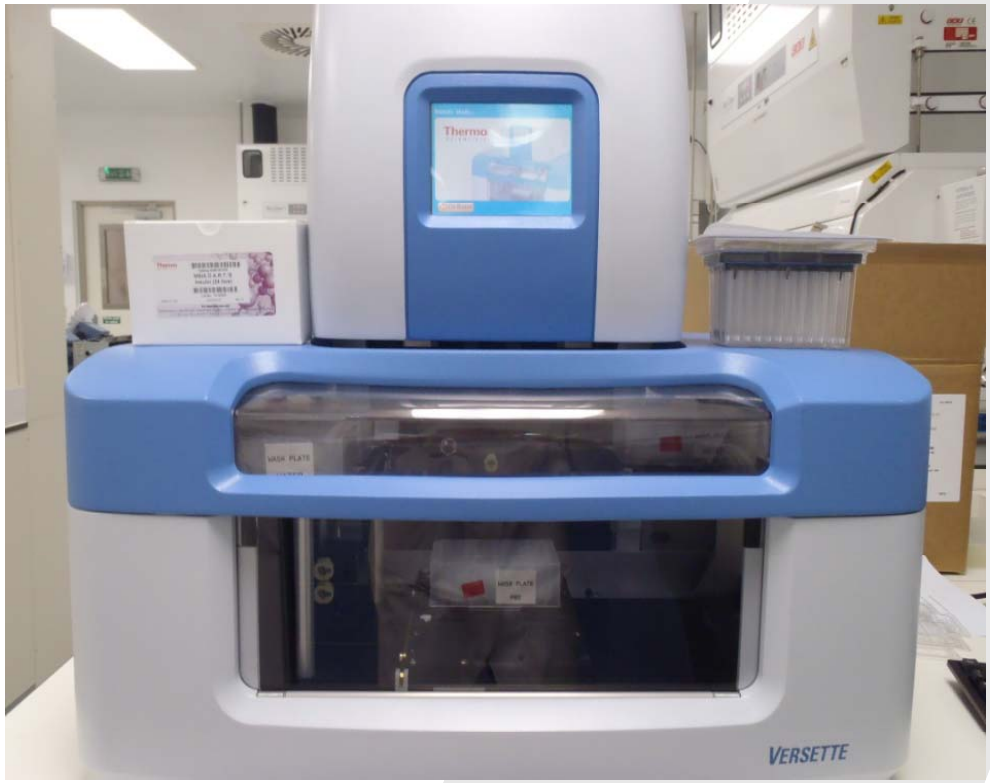
Use an automated pipette or robot to load, wash and elute the tips.

Each tip is used once.

Extraction time is about 2h per batch

ROBOT FOR AUTOMATED IMMUNOAFFINITY EXTRACTION



Single MSIA tip containing monoclonal pan-insulin Ab (tips are single use)

Extraction of up to 96 samples simultaneously:
Load wash & elution solvents into 96 well plates,
Press go!

Can also use an automated pipette for this extraction - Finnpipette (8 or 12 samples at a time)

COMPARISON OF LLOQ: SPE vs MSIA

- **Aim:** to compare SPE and MSIA techniques using exactly same samples & analytical conditions.

- Application of MSIA technique to glargine, lispro, aspart assay
  - Used Thermo MSIA workflow & reagents, slightly modified volumes. 300uL human plasma. 50uL injection volume.

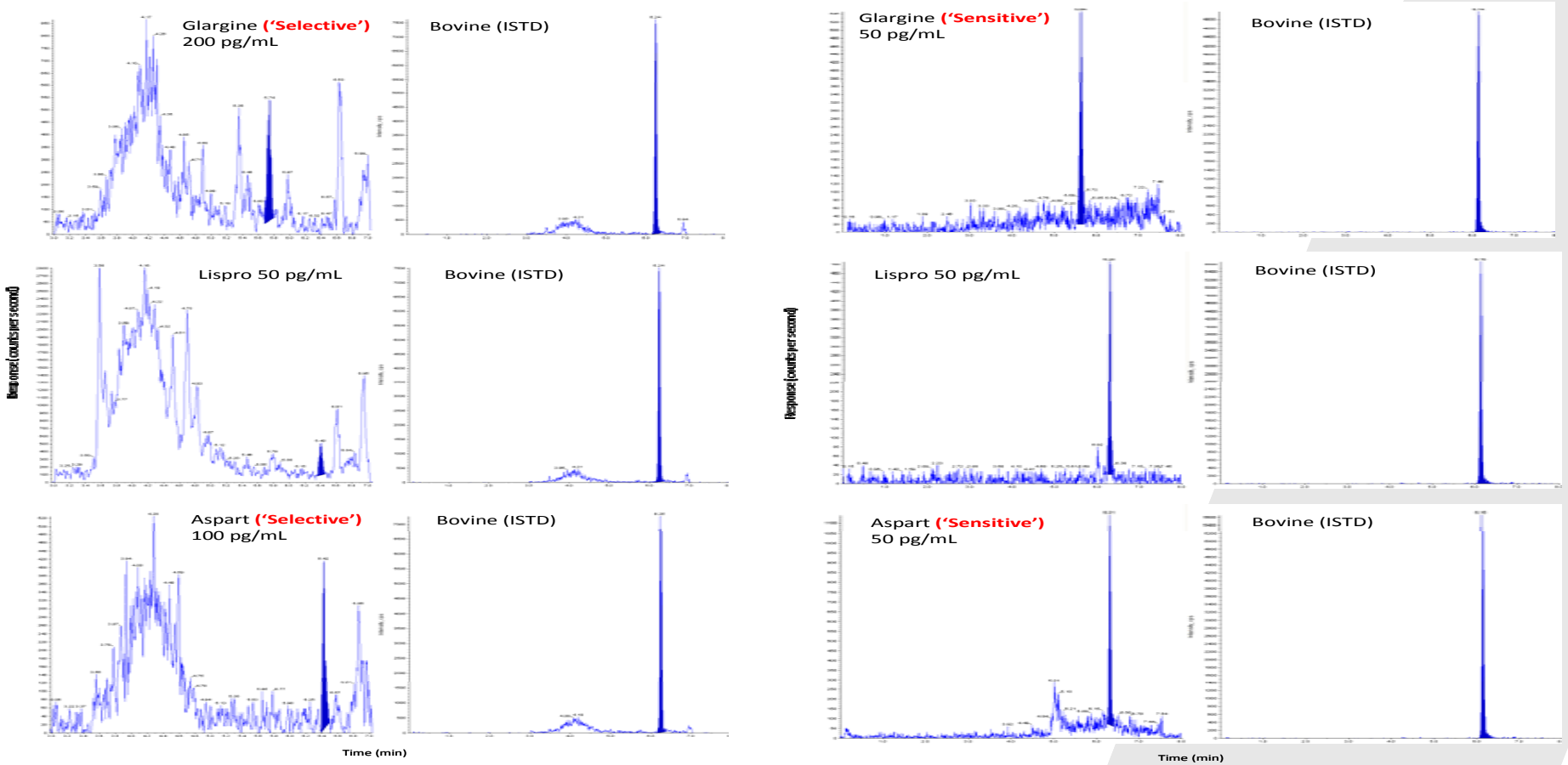
- Comparison of MSIA extraction to same plasma volume extracted by SPE (developed at Covance Harrogate, LLOQ 200pg/mL glargine)
  - MSIA extraction at Alnwick using Finnpipette & standard MSIA workflow adapted to match SPE method.
  - LC-MS at Harrogate using Acquity-API5000
  - Same spiked standards used throughout

- Elution into 96 well plate. Samples stored overnight, analysed the following day.

- **Comparators**: Signal to noise and overall signal for each compound

- Potential to use different SRM transitions according to background noise

COMPARISON OF LLOQ: SPE vs MSIA



SPE **MSIA**

- **CONSIDERABLE IMPROVEMENT IN SIGNAL TO NOISE FOR EACH COMPOUND**
  - Potential to use less selective transitions with MSIA

COMPARISON OF LLOQ SPE VS MSIA : results

- MSIA extracts are cleaner than SPE extracts. Are cleaner than original IA extracts using home made columns

Compound	Calibration line R-value (linear $1/x^2$)	50 pg/mL QC sample accuracy (%RSD) (n=3)	100 pg/mL QC sample accuracy (%RSD) (n=3)
Glargine	0.9995	113.3% (13.0%)	105.2% (4.5%)
Lispro	0.9997	98.6% (10.9%)	106.5% (7.4%)
Aspart	0.9999	101.0% (15.0%)	95.3% (6.4%)

- Application of MSIA technique results in improved LLOQ even with 'ordinary' MS instrumentation
- More sensitive less selective MS transitions may be used with immunoAssays (subject to matrix assessment)

MS/MS Parameter	Details	
System	AB Sciex API 5000 in positive Turbo Ion Spray	
Transitions	Glargine – **'Selective'**	867.2 → 984.4
	Glargine – **'Sensitive'**	867.2 → 136.0
	Lispro	968.9 → 217.1
	Aspart – **'Selective'**	971.9 → 1139.8
	Aspart – **'Sensitive'**	971.9 → 136.0
	Bovine (ISTD)	956.5 → 1121.3

- Tested analytical range 50 to 10,000 pg/mL.

ANTIBODY CONSIDERATIONS: MSIA and magnetic beads

► Consider polyclonal vs. monoclonal antibodies: where is the Antibody epitope compared to the insulin modification?

- Ab epitope usually on the B chain; some insulins, e.g. insulin detemir are modified on the B chain and this may affect the extraction efficiency of the antibody.
- Endogenous or simply modified insulins will not be affected

► In this case, consider custom made IA tips or magnetic beads with a custom antibody. The Ab can be bound directly to the tip or a streptavidin tip/bead can be used to capture the biotinylated Ab.

► Streptavidin MSIA tips are available. Where capacity is an issue this may also be the preferred approach.

► The Covance approach is based on automated immunoaffinity extraction using a robot (MSIA). Automated magnetic beads approach is equally valid. Avoid manual preparation as this is very time consuming for large sample numbers.

ACCEPTANCE CRITERIA AND INTERNAL STANDARDS

PRECISION and ACCURACY: Early hybrid LC-MS Assay 2009

Nominal concentration (ng/mL)	Number of samples	Mean calculated concentration (ng/mL)	Mean % Difference	Total %CV
0.2	18	0.198	-1.06	22.2
0.5	18	0.512	2.30	18.8
2	18	2.08	3.83	9.06
5	18	4.81	-3.79	8.37

- For this assay (new technique, hybrid assay) we used LBA acceptance criteria.
- For a later SPE assay 4-6-15 was employed.
- Choose acceptance criteria carefully prior to validation based upon your assay type and the assay performance

- INTERNAL STANDARDS
- Isotopically labelled insulin; the gold standard, but £££
- Iodated Version of analyte: reliable for short term study but risk of degradation; test carefully
- Bovine insulin: very useful and readily available

ANTI DRUG ANTIBODY EFFECTS

IMMUNOGENICITY **-** Any biological therapeutic agent may have an unwanted immune response. This can range from having no effect to a severe hypersensitivity reaction. Immunogenicity can also result in neutralisation of the drug i.e. no clinical benefit, or at least a reduction in response and a change in pharmacokinetics.

- Immune response should be considered when there is more than 7 days of drug exposure. All biotherapeutics need to be assessed for immunogenicity by measuring ADA – anti drug antibodies.

- Differences in insulins across species may well produce ADA effects

- Repeat dose 28 day preclinical study
  - Day One Samples: variability of internal standard around 10-30%, recovery high
  - Day 28 samples internal standard recovery was low (< LLOQ of compound) and highly variable
  - Evidence of an ADa effect, antibodies were also measured directly

- Clinical Study
  - Have also observed this using SPE. Patients who for example have previously received animal insulins. Antibodies in plasma reduce recoveries of ISTD and analyte. Variability is increased.

REDUCTION OF LLOQ: microflow chromatography plus sensitive MS system (Xevo TQ-S)

- Aspart, lispro and glargine in human plasma
- Plasma volume 300uL
- MSIA extraction
- Elution volume 140uL

Trapping Conditions	
Trap Column	Symmetry C18 5 µm, 300µm x 50 mm
Trapping	85:15 Solvent A: Solvent B
Flow rate (µL/min)	25
Time (min)	2.0

MClass Conditions	
iKey	BEH C18 130 1.7µm, 150µm x 100 mm
Solvent A	0.1% Formic Acid
Solvent B	Acetonitrile with 0.1% Formic Acid
Injection volume	10 µL
Column Temp	75 °C
Sample Temp	5 °C

Time (min)	Flow rate (µL/min)	% A	% B
0	2.5	75	25
5		45	55
6		5	95
8		5	95
9		75	25
13.5		75	25

Method conditions are taken from the Waters application note:

Reducing Sample Volume and Increasing Sensitivity for the Quantification of Human Insulin and 5 Analogs in Human Plasma Using ionKey/MS. Erin Chambers and Kenneth Fountain.

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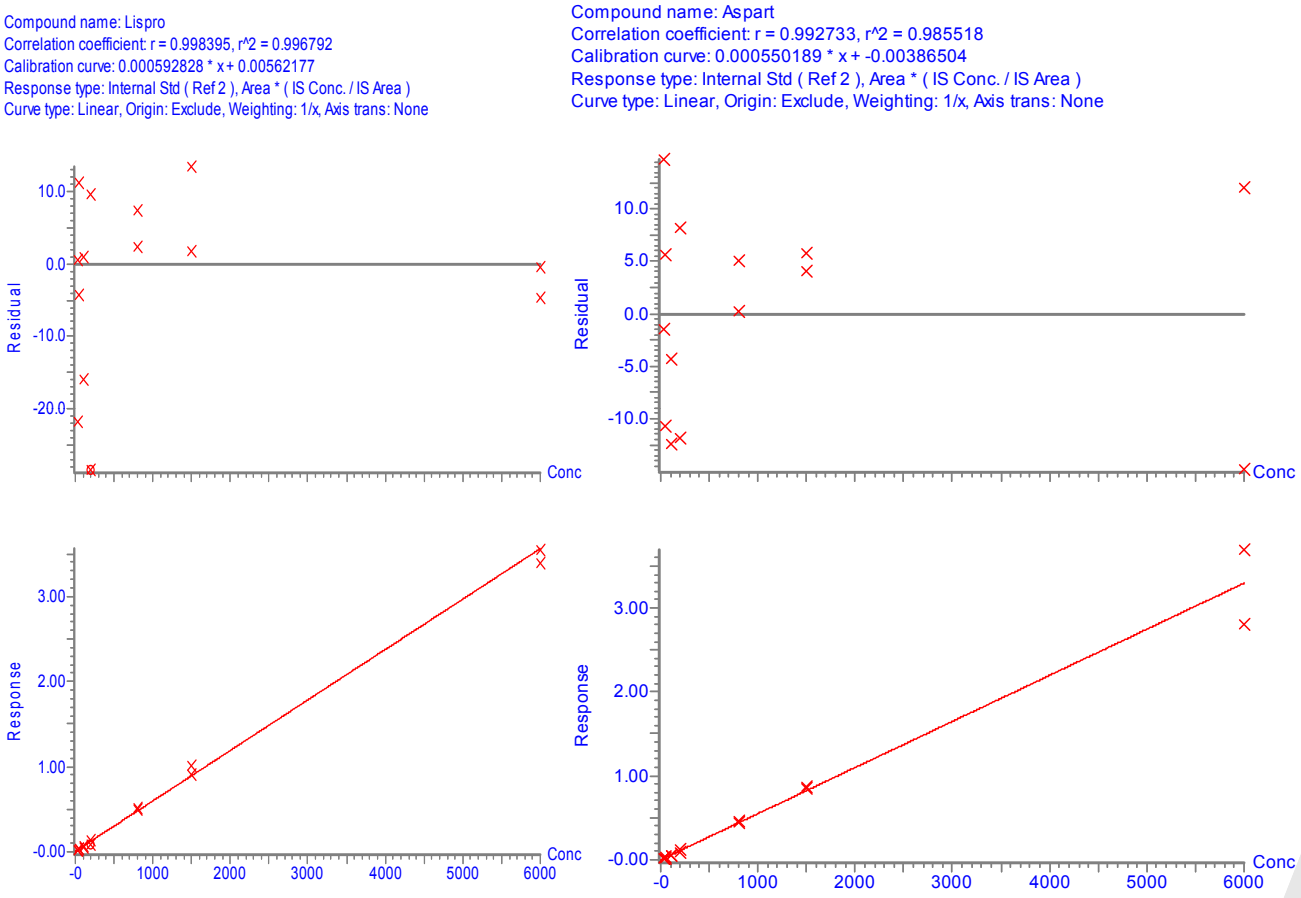
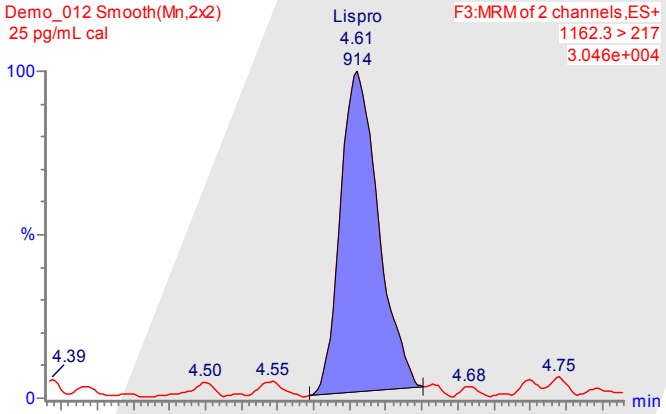
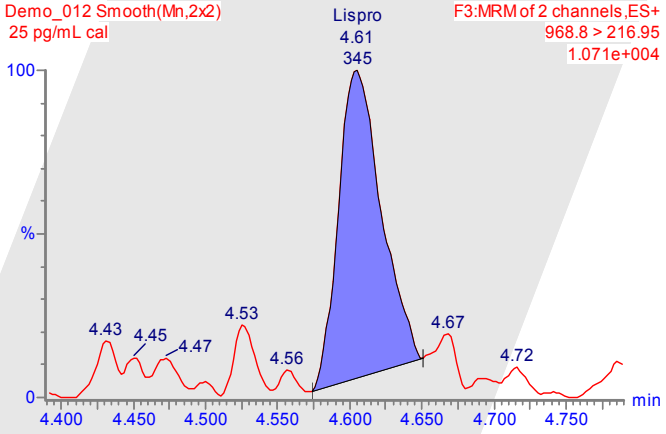
RESULTS OF LLOQ REDUCTION TEST

- Tested calibration range was 10 to 6000 pg/mL
- Lispro, aspart were linear to 25pg/mL – 10pg/mL not detected
- Glargine was only linear to 50pg/mL. Appeared to bind in the plate overnight.
- Extra consideration required to NSB (binding) at very low detection limits.







INSULINS BY LCMS

- LC-MS is increasingly used for insulin analysis – client decision, based on development need: i.e. total insulin activity assay vs. quantitation of specific insulins or metabolites, limits of quantitation and cost, time.
- Covance offer two options for LCMS: Physicochemical (SPE) extraction versus Immunoaffinity extraction (Hybrid LCMS)
  - SPE. Lower cost, robust assay. Higher LLOQ (~ 200 pg/mL @ 300uL plasma).
  - Immunoaffinity extraction. Greater sensitivity (~ 70 pg/mL @ 300uL plasma). Higher cost to pay for tips or gel. Cleaner extract provides greater specificity
  - MSIA tips allow automated immunoaffinity extraction and can be purchased commercially either with insulin monoclonal Ab or streptavidin.
  - Magnetic beads can be used according to client preference.
- Acceptance criteria need careful consideration for these assays
  - Small molecule limits 4-6-15 or ligand binding limits 4-6-20?
- Must consider possible ADA effect multi-dose, particularly across species. So look at 'total' or free insulins? ADA affects both SPE & IA assays
- Use of sensitive instruments and microflow chromatography can reduce LLOQs still further to 50 pg/mL and below. Binding effects are pronounced at very low levels
- Best to use this technology to reduce sample volumes rather than ever lower LLOQs?

SOME USEFUL REFERENCES

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- Clinical significance of insulin antibodies in insulin-treated diabetic patients. Van Haeften, TW, Diabetes Care 12:641-648, 1989
- Work of Thevis and Thomas of course. Magnetics beads IA-LC-MS.
- Advances in Insulin Bioanalysis: A strategy to achieve 50 pg/mL in a high-throughput LC-MS/MS assay. Michael Blackburn, Matthew Ewles, Nicholas Gray, Stuart McDougall and Johannes Stanta. Presented at EBF Open Meeting, Barcelona, Spain November 2014.

http://www.covance.com/content/dam/covance/assetLibrary/posters/Stanta-EwlesEBF14s.pdf

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Matthew Ewles, Covance Harrogate: SPE & LC methodology, challenge of MS transitions. Development of magnetic beads approach.

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Stuart McDougall: leadership of the Alnwick team.

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