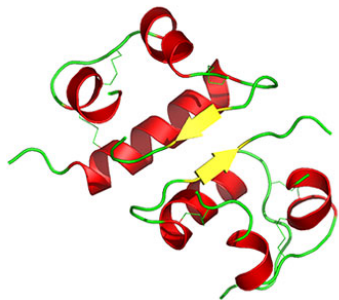


Quantitation of a Therapeutic Insulin Analogue by Immuno-Affinity Extraction LC-MS/MS

*(or running a real peptide project with a hybrid assay:
questions and some solutions?)*



5th EBF Open Symposium
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Barcelona, Spain

*Michael Blackburn
Covance Laboratories Ltd
Alnwick, UK*

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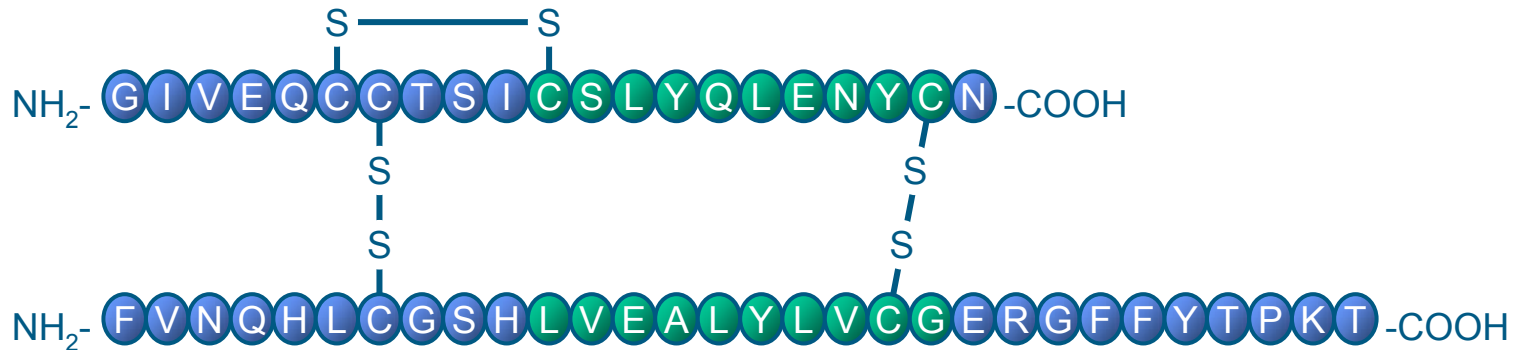
Acknowledgements

- Martin Gerl and his team, Sanofi, Frankfurt : Scientific leadership of this project, supply of IA-gel.
- Dietmar Schmidt, Ronald Schmidt and team, Sanofi, Frankfurt: Dialogue, expertise with insulins and training in Immuno-Affinity extraction.
- Isotope Chemistry Group, Sanofi, Frankfurt: Production of iodated and stable labelled internal standards
- Barbara Bell, Nick Gray and Jennifer Mackie, Alnwick: Method development, sample analysis.
- Stuart McDougall: leadership of the Alnwick team.

Insulin Analogues

- 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

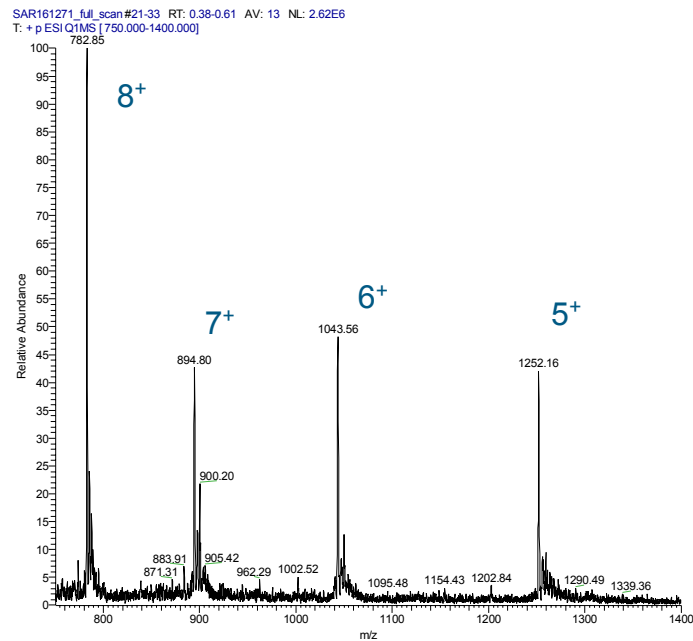
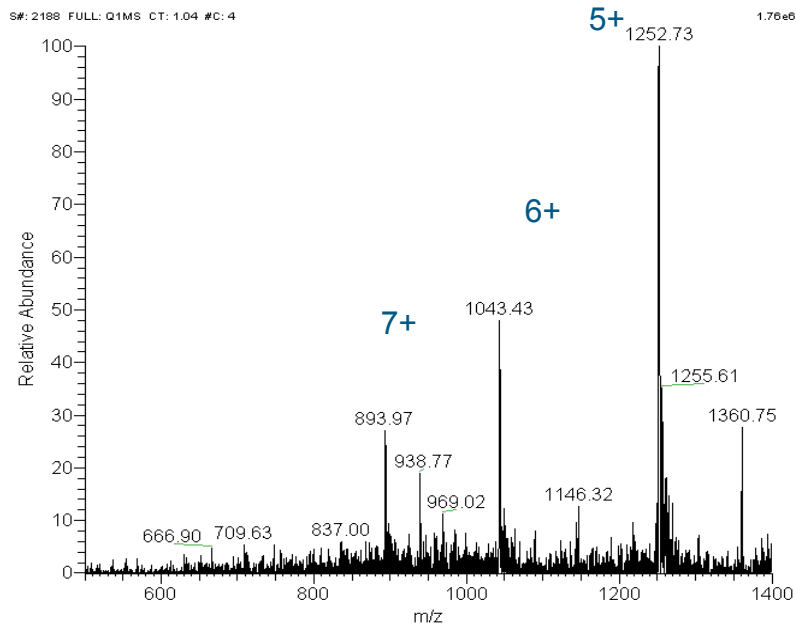


Longer Lasting Insulin Development Compound: LoLA

- Molecular Weight 6.2 kDa approximately: modified at A & B chain
- Requirement for a reliable LC-MS/MS assay in animal & human plasma
- LLOQ requirement of 200 pg/mL in plasma or better

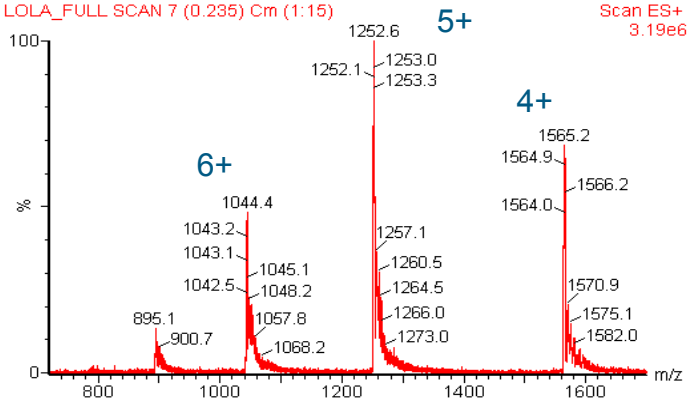
LoLA Q1 Mass Spectra & Manipulation of Charge State

Thermo Quantum Ultra



20mM ammonium formate/acetonitrile

161271 full scan VAA119 16-Jan-2009 11:42:39
LOLA_FULL SCAN 7 (0.235) Cm (1:15) Scan ES+ 3.19e6



Waters Z spray, Quattro Ultima

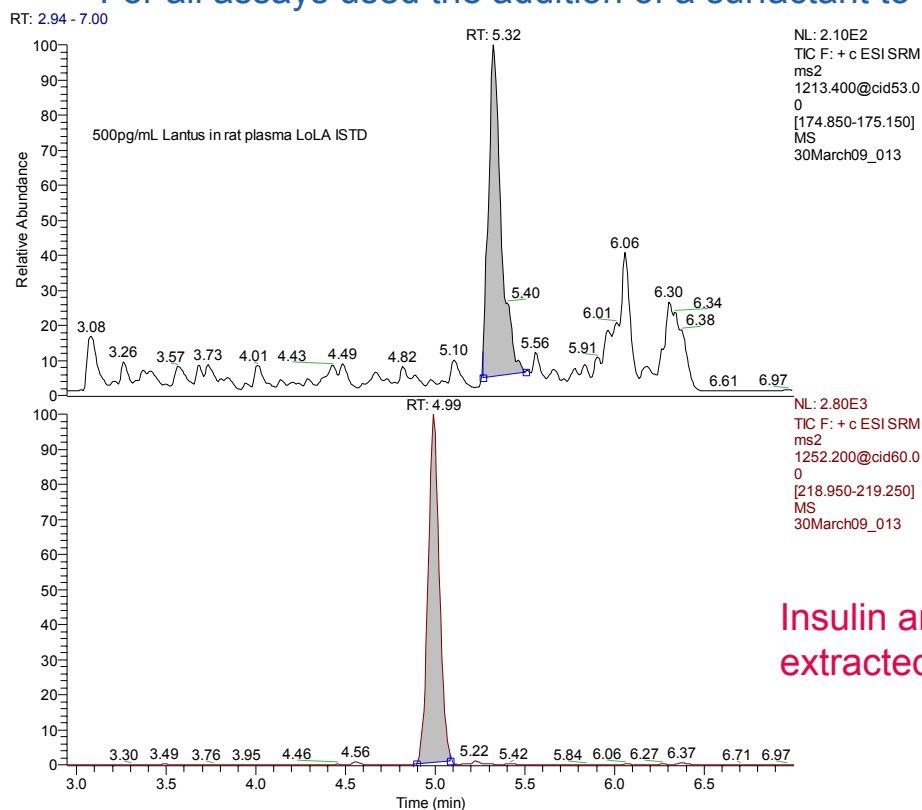
LoLA charge distribution envelope: Methanol/formic acid/ipa 10%

- Charge state envelope can be to some extent controlled using mobile phase composition
- Some difference observed between different source types
 - Waters Z spray, Thermo, also APi5000 at Frankfurt

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Direct Assay (physico-chemical assay) Approaches

- A combination of solid phase extraction of plasma (1mL) combined with LC/MS/MS
- MS/MS transition chosen depended on cluster chosen for fragmentation
- Initially used 1252 > 219
- Extraction by Waters Oasis HLB
- Limit of quantitation using a direct assay was 500-1000 pg/mL LoLA from 1mL plasma
- For all assays used the addition of a surfactant to stop binding of standard solutions



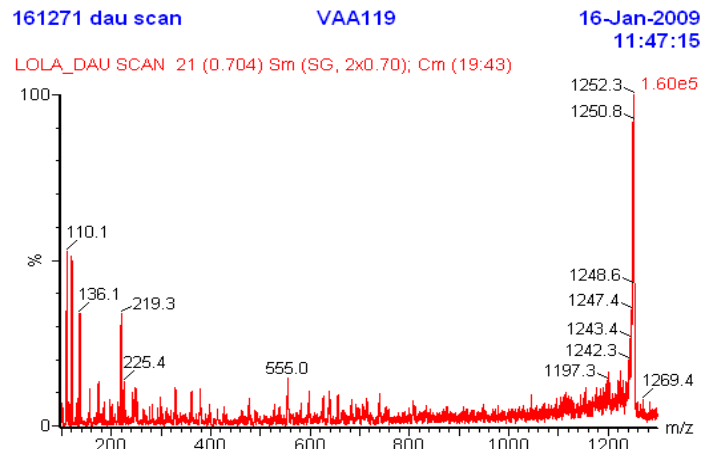
Direct physico-chemical assay did not give the required sensitivity

Instrumentation: TSQ Quantum Ultra

Insulin analogue spiked into rat plasma & extracted

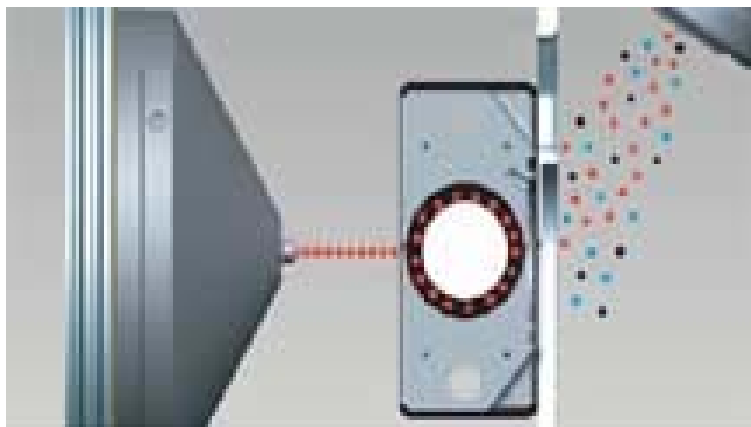
Sensitivity Enhancement 1: Use of FAIMS-pSRM

Useful for clusters which fragment poorly



In this example fragmentation is relatively poor;
at higher collision energy the molecule 'unzips'

i.e. produces lots of low intensity fragments



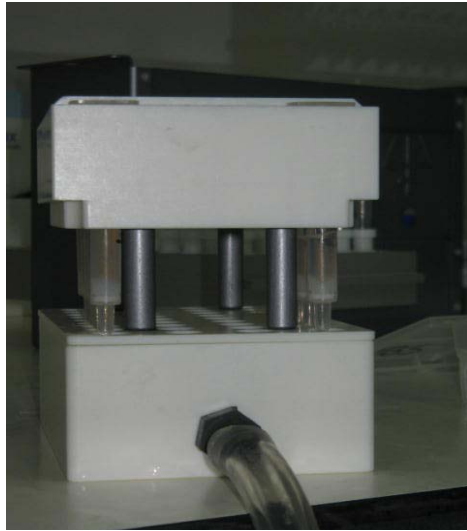
Combine a specific, high molecular weight
Cluster with ion mobility selection
(with some collision energy)
and 'pseudo' SRM = high energy Q3 SIM

Or can use a 0 constant neutral loss experiment
(0 CNL). Gives an alternative specificity for poorly
fragmenting peptides

Can use the full signal from the precursor ion for best
sensitivity and improve selectivity using an alternative
technique: *Ion mobility spectrometry*

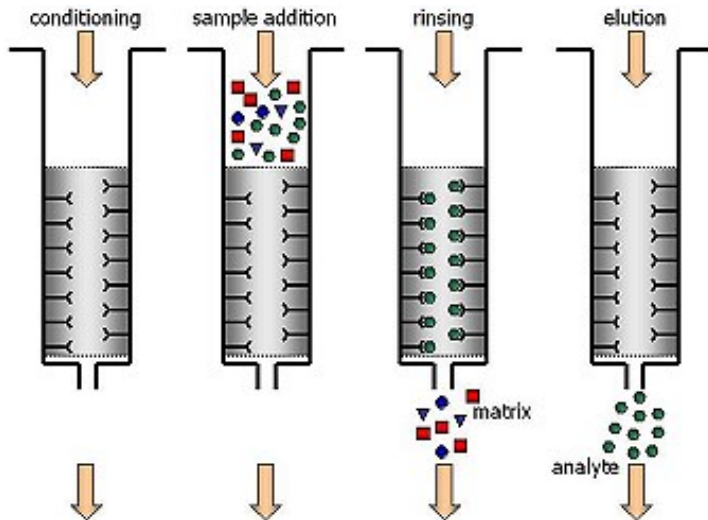
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Enhancement 2: Immuno-affinity Extraction



Procedure adapted to use multi-channel pipettes to enhance throughput

Immunoaffinity columns (filled with 100 μL of 10 mg/mL Zentech[®] anti-insulin mouse antibody coupled to CNBr-activated Sepharose 4 Fast Flow)



Cleaner extract than SPE

Columns can be re-used: >20 x



Expensive, a little slower

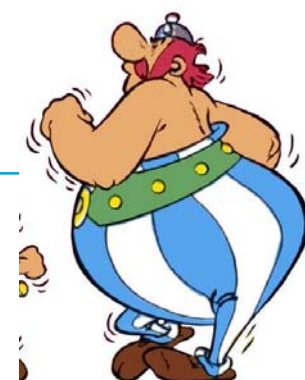
Throughput = 1 plate/analyst per day

Immunoaffinity image: services.leatherheadfood.com

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SPE vs IAC (or small & large)



Copyright (c) 2001 Lexipol, Inc. All Rights Reserved. (01/01/01)

SPE

- Commercially prepared columns
- Used once
- Can be automated

Procedure

- Condition column solvent
- Equilibrate column aqueous
- Load sample
- Apply wash (aqueous solvent)
- Elute Compound
- Discard column/plate



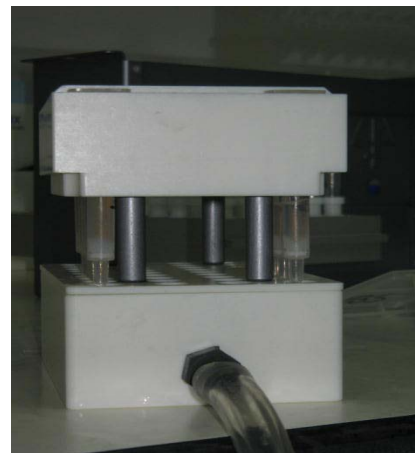
We used Oasis HLB for initial method development in a vacuum manifold.

IAC

- Columns prepared in the lab
- Columns are re-used
- Probably best to run manually
 - but can use multi-channel pipettes

Procedure

- Wash column with elution buffer
- Wash column with buffer
- Slowly apply sample to column
- Wash with buffer
- Elute with elution buffer (acid/ethanol)
- Wash & prepare for storage



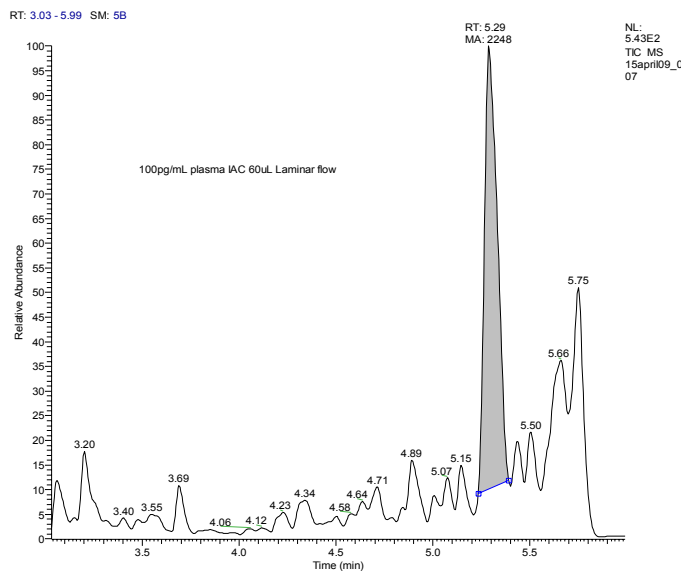
Very Good Tip:

Don't put IAC gels in the freezer

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Result: Hybrid Immunocapture – LC-MS Approaches

- IAC extracts were cleaner, with absolute recovery approximately 80-90%
- Limit of quantitation using immuno-capture assay was 200 pg/mL LoLA from 250uL spiked plasma. Immuno-capture gives a more sensitive assay.
- This was true whether using FAIMS-pSRM on a Thermo system or MS/MS on the API5000 system.
- Enzyme digestion (Edman) was investigated as a back-up: did not improve signal vs ‘top down’ approach



100pg/mL LoLA from spiked plasma

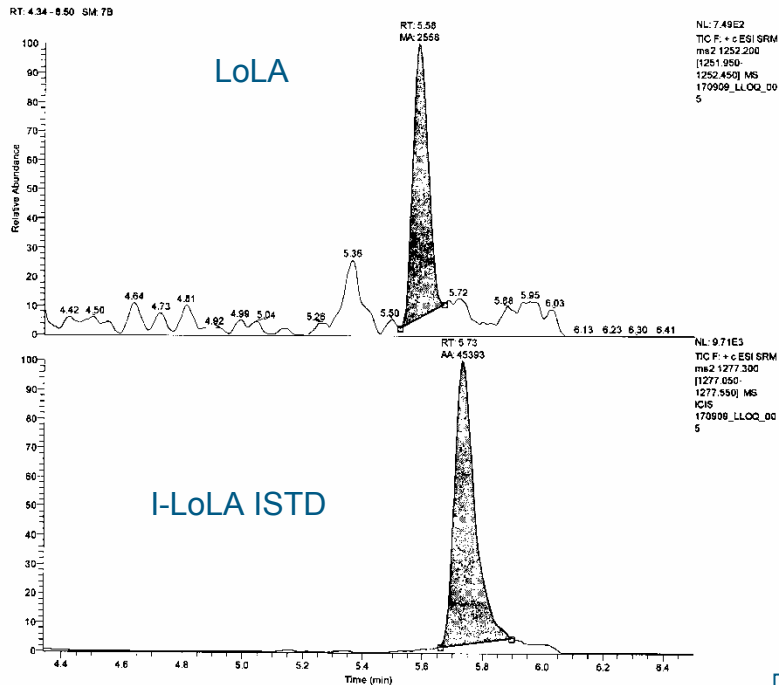
Decision: ‘Hybrid’ IA- LC-MS approach selected for longer lasting insulin project

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Internal Standards

- Initial method development used an analogue ISTD (e.g. bovine insulin, insulin glargine)
- For early studies used iodated compound
 - This ISTD performed well and we did not see evidence of loss of iodine and conversion to parent compound during the period of use. Recommend regular checking of this.
- GLP Studies used ^{15}N isotopically labelled compound: all nitrogens labelled
- Concentration ISTD 2ng/mL (assay range: 0.2 – 5 ng/mL)

FAIMS method LLOQ & LC conditions



LLOQ in animal plasma (0.2 ng/mL)



TSQ Vantage with Turboflow system & FAIMS unit

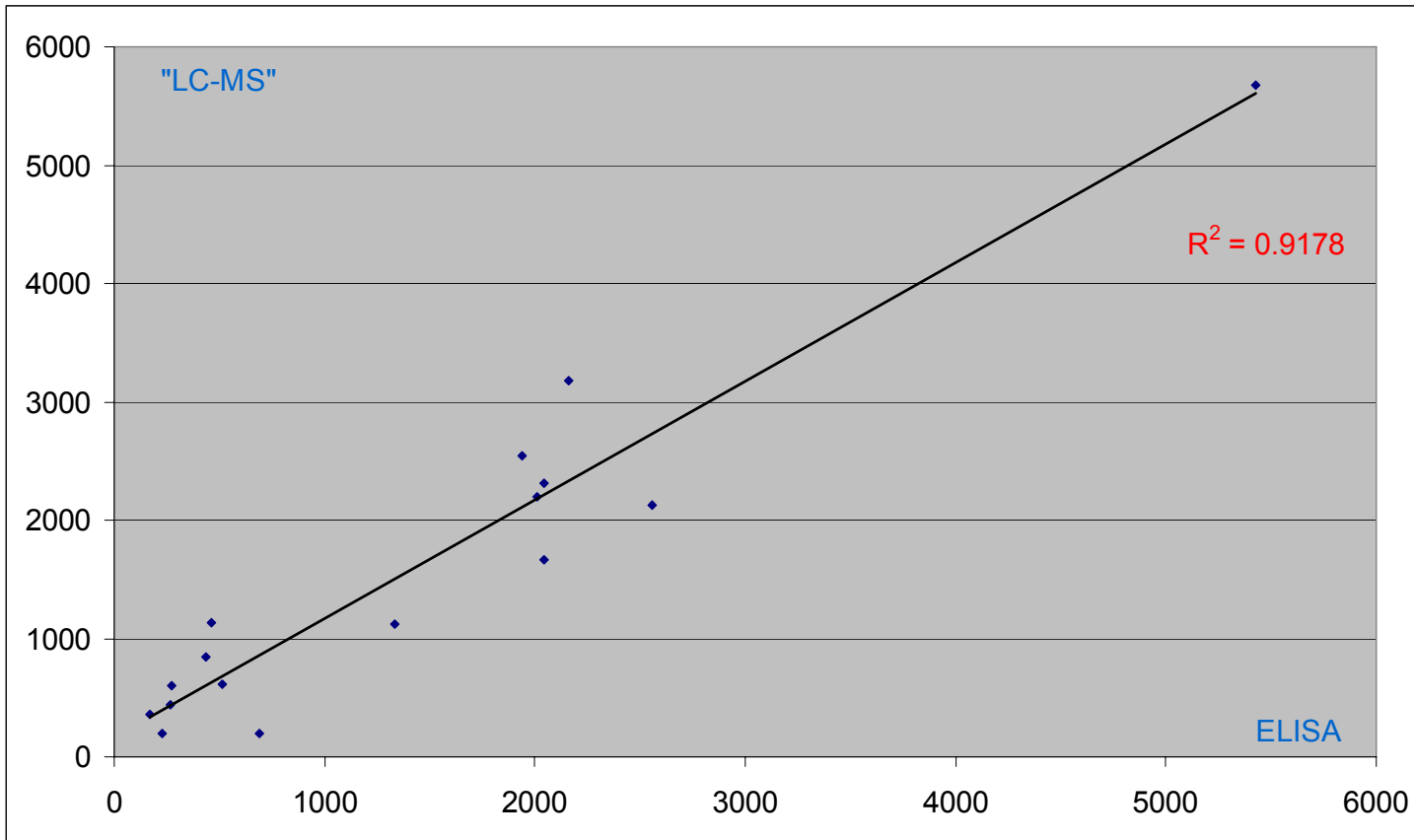
NOTE: 0.2 ng/mL is still a rather high LLOQ for this work. Some low dose levels were not properly covered. The ideal LLOQ was 100 pg/mL or lower

- Load 100uL of extract onto an Oasis on line extraction column HLB 2.1 x 20mm
- Elute onto Hichrom Ace 3 C18, 50 x 2.1 mm, 3 µm
- Mob Phase A: 20mM ammonium formate pH 3
- Mob Phase B: acetonitrile/0.1% formic acid flow rate: 0.4 mL/min
- Sample volume 250 uL; injection volume 100 uL

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Correlation of MS with LBA Assay

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



Both x & y axes are LoLA concentration pg/mL

Acceptance Criteria

Precision and Accuracy Data for first GLP Study (IAE-LC-FAIMS-pSRM).

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

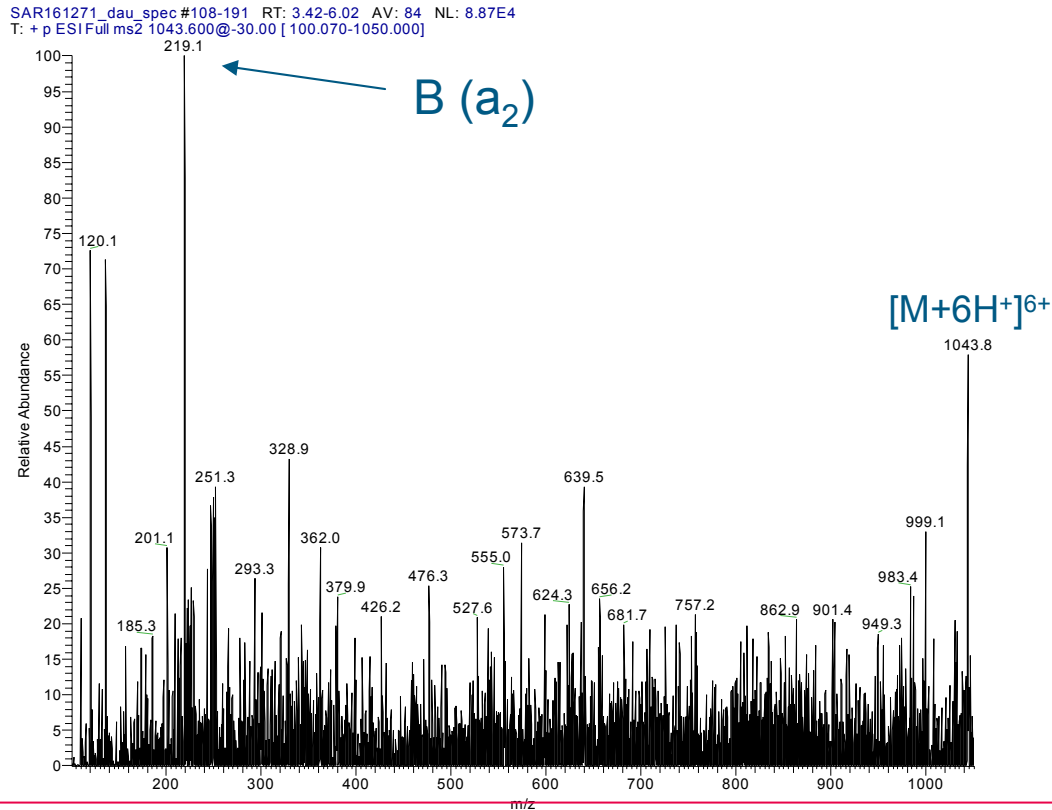
- As this was a hybrid assay, method validated using 20/25 'biologicals' criteria
 - Decision based on assay complexity (immuno-capture extraction)
 - Also we are at the limits of instrument performance due to the size of analyte
- Same criteria used at Frankfurt. These were inspected and accepted by FDA
- Analytical Range 0.2 – 5ng/mL
 - LoLA concentrations rarely higher than 2 ng/mL
- As part of method development/validation we checked analytical cross-over between LoLA and other insulin analogues

IA-LC-FAIMS pSRM method was used for discovery and GLP pre-clinical studies.

Need to move to a clinical assay. Some concerns about matrix variability in human plasma with pSRM method.

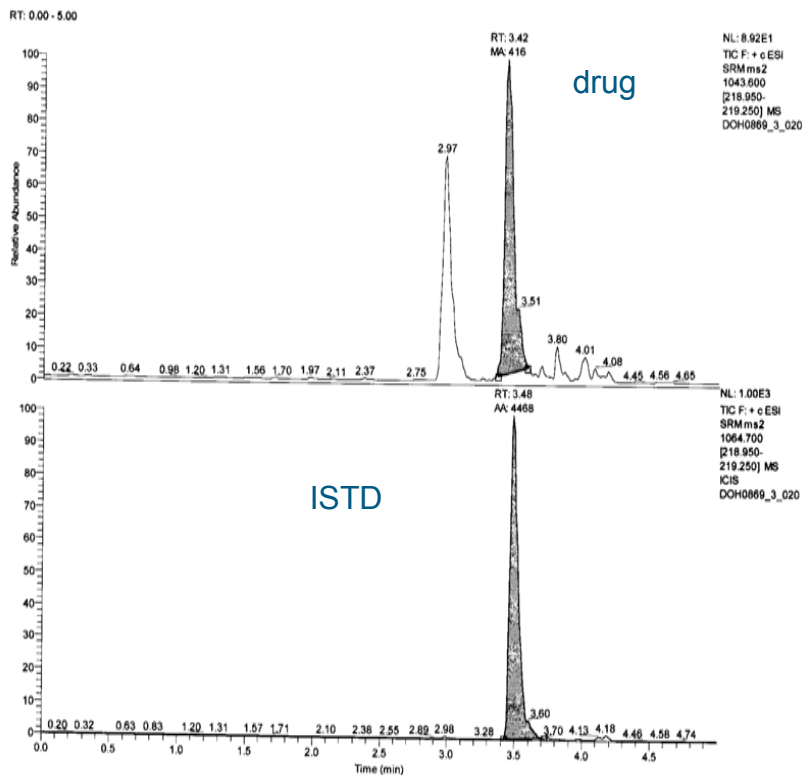
Product Ion Mass Spectra for long lasting insulin

B chain fragmentations Reference: Thevis and Schanzer Analyst 2007, 132, p287-291



Non-specific immonium ions should be avoided where possible: eg. 120 Phe
Specific ions from B chain a & b series are available for insulins (Thevis & Schanzer, 2007).
Lower charged clusters eg 1252 5^+ did not fragment so well, 6^+ have higher internal energy and fragment more readily. Need to compromise specificity vs ease of fragmentation.

Clinical Studies: LC-MS/MS Method



- Changed mobile phase to boost ratio of 6⁺ charge state & increase signal
- Used diagnostic 219 product ion (possible because of clean IAC extract)
- ¹⁵N labelled internal standard available

Mob. Phase B: Methanol/Iso-propanol/formic acid 0.5%
Mobile Phase A: 0.5% Formic acid
Column Thermo Hypersil Gold 1.9um 20 x 2.1mm
Agilent 1200, Stainless steel LC tubing
Semi-UPLC method: Pressure approx 5000psi/360bar
Flow rate 0.3 mL/min
500 uL sample volume; 50 uL injection volume

LLOQ in plasma (0.2 ng/mL)

NOTE: 0.2 ng/mL is still a rather high LLOQ for this work
Some low dose levels were not properly covered,
particularly moving into clinical studies.
Ideally LLOQ should be 100 pg/mL or lower

Anti-Drug Antibody Effects: Repeat Dose Study

- **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 ie no clinical benefit, or at least a reduction in response and a change in pharmacokinetics.
 - Immune response must be monitored 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
 - LoLA: 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 LoLA) and highly variable
 - Evidence of an ADa effect, antibodies were also measured directly

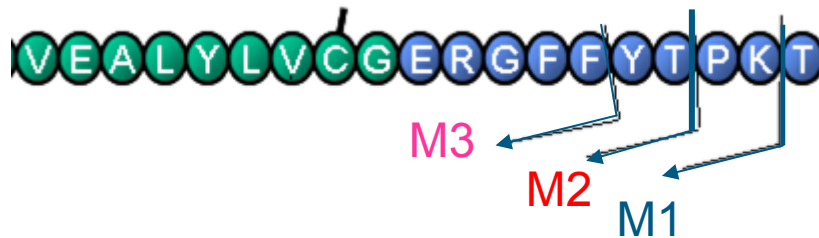
Effect of ADa in multi-dose studies

- Antibodies to LoLA formed from 2-3 weeks exposure depending on dose
- These bind to drug & internal standard (iodated LoLA in pre-clinical studies)
- Equilibrium between free (active) and total insulins
- Request for a 'total' method for bound & unbound insulin
- Ab complex is dissociated by acidification with HCl
- Leave 1 hour followed by neutralisation and then IAC
- Total method has an LLOQ of 1 ng/mL, HLOQ 100 ng/mL
- Pre-clinical multi-dose studies used 'Total' method
 - 'free' LoLA could be assessed by filtration or addition of excess insulin to bind AD-antibodies

Metabolite Assays

- Much of insulin activity can be attributed to metabolites of the compound
- Peptides have ‘catabolism’ not metabolism
 - Step-wise loss of amino acids from B chain preserves insulin type activity

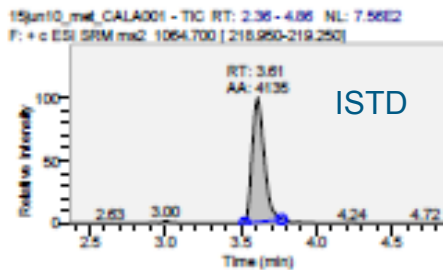
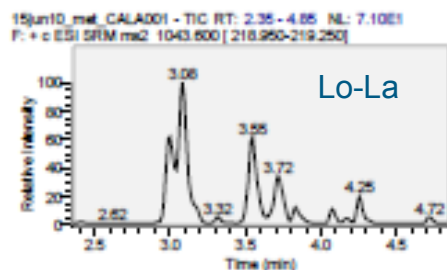
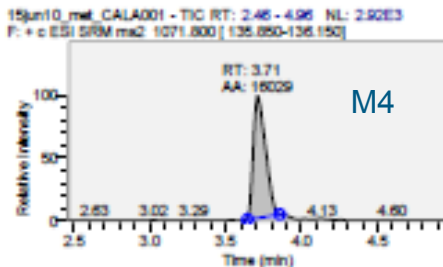
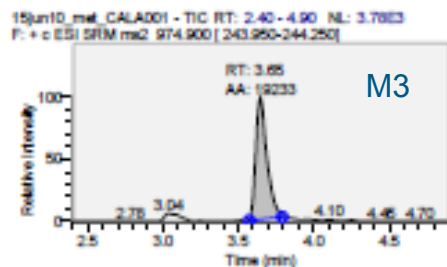
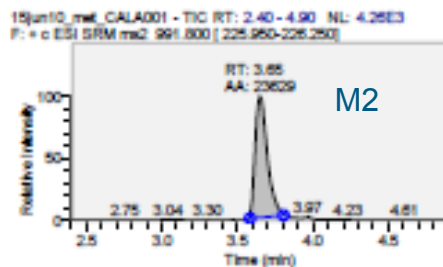
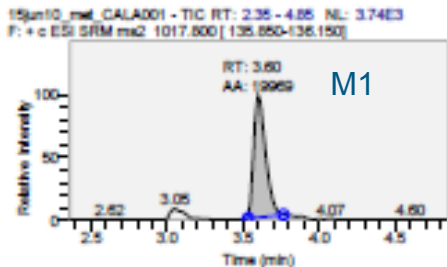
example:



- IAC antibody extracts based on insulin epitope on the B chain
 - will also extract metabolites therefore & we can quantify these and gain extra information regarding compound activity.

Exploratory Metabolite Assay

M1 – M4 5ng/mL, spiked into human plasma, LoLA blank



- These were synthesised in Frankfurt
- Used same LC conditions and extraction procedure as clinical method
- Added MS/MS transitions for major metabolites
- Note for this sample there is no cross-over of Metabolites > LoLA
- Ran selected samples at higher concentrations to give extra information regarding activity & safety

Considerations for LC-MS Assays for Insulins

- No waiting required for development of specific antibodies for immunoassays – get information faster
- Greater assay specificity relative to LBA gives confidence and can be used to generate new information
 - Levels of different metabolites give insight into action of the analogue
 - No cross reactivity with other insulin analogues (tested glulisine and 4 other insulin analogues for reactivity) or endogenous insulin (tested at 50 ng/mL)
 - Improved instrumentation (sensitivity, increasing mass range) allows us to reach required LLOQs
 - Used halogenated & stable labelled internal standard
- IAC gives clean extracts to allow lower LLOQs for insulins and is surprisingly quick and robust
 - Must consider possible ADa effect multi-dose, esp. across species. So look at 'total' or free insulins?
- Acceptance criteria need careful consideration for these assays
 - Small molecule limits may not be appropriate or achievable
- **Peptide and small protein analysis (<8KDa?) by LC-MS/MS is becoming the standard**

References

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- POSTER: Quantitation of a Therapeutic Insulin Analogue by Immuno-Affinity Extraction LC-MS/MS. M Blackburn et al., EBF, Barcelona 2012
- Mass spectrometric identification of peptide hormones in doping control analysis. Thevis and Schanzer., *Analyst*, 132, 287-291 (2007).
- Quantitative Analysis of Biomarkers, drugs and toxins in biological samples by immunoaffinity chromatography coupled to mass spectrometry or tandem mass spectrometry: A focused review of recent applications. Tsikas, D.I., *J. Chromatography B.*, (2010).
- FAIMS: GLP-Validated quantitation method for a peptide in biological matrix. Klaasen, T., and Romer, A. ASMS Poster Presentation, Denver 2008.
- A Mass Spectrometric Method for quantitation of intact insulin in Blood Samples. Darby, S.M, Miller, M.L., Allen, R.O and LeBeau, M. *Journal of Analytical Toxicology*, Vol. 25, 2001.
- Clinical significance of insulin antibodies in insulin-treated diabetic patients. Van Haefen, TW, *Diabetes Care* 12:641-648, 1989.

THANK YOU FOR LISTENING!