Quantitative LC-MS/MS Analysis of Glucagon

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Agenda

- Comparison with small molecule LC-MS/MS
- LC-MS/MS sensitivity of peptides detection
- Stability: neat vs. matrix solutions
- Method: extraction & LC-MS/MS
- Reference material
- Conclusions

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Examples for Improvement of Peptides MS/MS Sensitivity

- Negative ESI MS/MS with loss of water
- β-amyloid peptides, 100 pg/mL
- Sequence specific fragmentation
- cleavage of peptide bonds involving Pro residues
- Chemical modification
- Cys residues derivatization with iodoacetamide (terlipressin) increases MRM response by ~ 5-fold

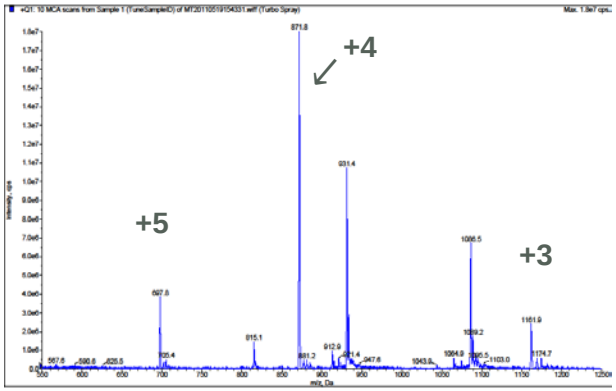
Glucagon MS/MS

NH_2-His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-

Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-

Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-

Met-Asn-Thr-COOH



- Multiple-charged species in ESI mass spectra
- Unique highly efficient fragmentation of M^{+5} ions with loss of ammonia
  - Other peptides with N-terminal His share this fragmentation feature, including glucagon analogs missing Thr^5 and Thr^7

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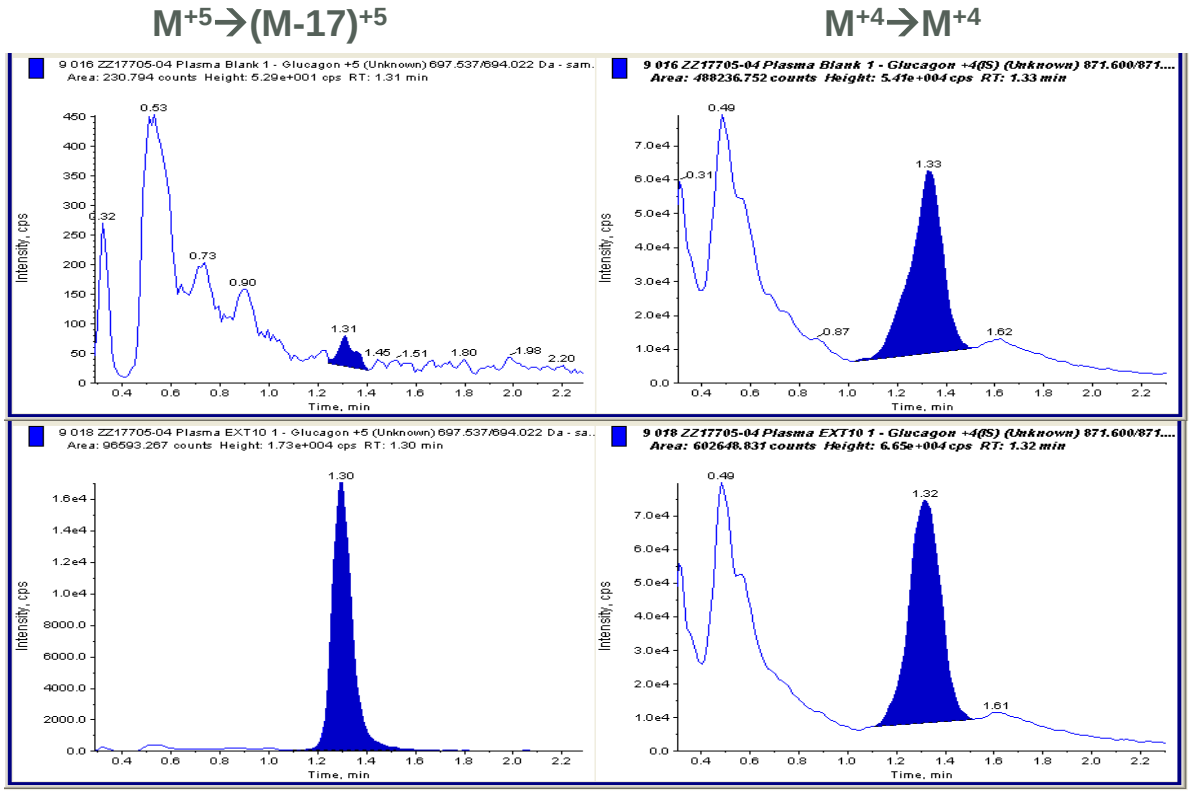
Selectivity/Efficiency of Glucagon MRM



Selectivity: distinct difference between blank and spiked samples
MRM Efficiency: relative increase of the analyte response in spiked samples vs blanks for both transitions is approximately the same
[peak area counts: 103,000 (M^{+5} MRM) vs. 114,000 (M^{+4} MRM)]

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Glucagon Stability (Neat Solutions)

- Soluble in acidic (pH < 3) and basic (pH >9.5) solutions
- Chemically stable: decomposition of Trp, Met oxidation, deamidation of Asn/Gln, or peptide bonds hydrolysis only significant in relatively harsh conditions
- Prevention of adsorption: coating of polypropylene tubes with BSA
  - Glucagon solution below 50 µg/mL: addition of “keeper” peptides
    - Choice of “keeper” peptide/compound: lack of interferences, compatibility with method/analyte
- Extracted samples: no adsorptions (96 hours, 5°C)

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Glucagon Stability (Plasma)

- Proteolysis rate is matrix lot-dependent
- In some lots of human plasma, aprotinin alone (250 KIU/mL) does not provide sufficient glucagon stability
- Cocktail of inhibitors was developed to enhance glucagon stability in human plasma and in whole blood
- Proteolysis rates of (des-Thr^{5})- and (des-Thr^{7})-glucagon variants are similar to glucagon degradation rates

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Enhancement of Glucagon Stability in Plasma

- Human: Short-term stability (17 hours) of Test QC samples on an ice water bath

Inhibitor	Aprotinin 250 KIU/mL		Cocktail of inhibitors	
	Control QC	STS QC	Control QC	STS QC
	5910	2180	6030	5830
	5930	2090	6050	5800
	5570	2160	6010	6050
Mean	5800	2140	6030	5890
% CV	3.5	2.2	0.3	2.3
Stability (% of Control)		36.9		97.7

- Rat: Acidification of plasma is also required to provide sample integrity along with addition of protease inhibitor cocktail

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Glucagon Method: Extraction

- Ion-exchange 96-well plates
- Sample incubation with detergent & acetonitrile
  - Minimize protein binding
  - Improve accuracy of quantitation in matrix from multiple donors
- SPE washes with several organic solvents
  - Ensure consistency of the analyte/IS recovery
  - Lack of matrix effect
- Internal standard: (des-Thr^{7})-glucagon

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Glucagon Method: LC-MS/MS

- Parallel-column system (Agilent Zorbax 300SB-C18, 3.5 µm, 50x2.1 mm)
  - Isocratic elution on column 1 (analysis)
  - Gradient regeneration on column 2

- Advantages:
  - Stability of LC-MS/MS system (ratio & response)
  - Lack of carry-over
  - Run time < 4 minutes

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Glucagon (Human Plasma) Method Parameters

- Analytical range: 100-10,000 pg/mL
- Dilution integrity: up to 25,000 pg/mL
- Sample volume: 0.250 mL
- Sample collection and handling stability: 2 hours (5°C)
- Short-term stability in matrix: 14 hours (5°C)

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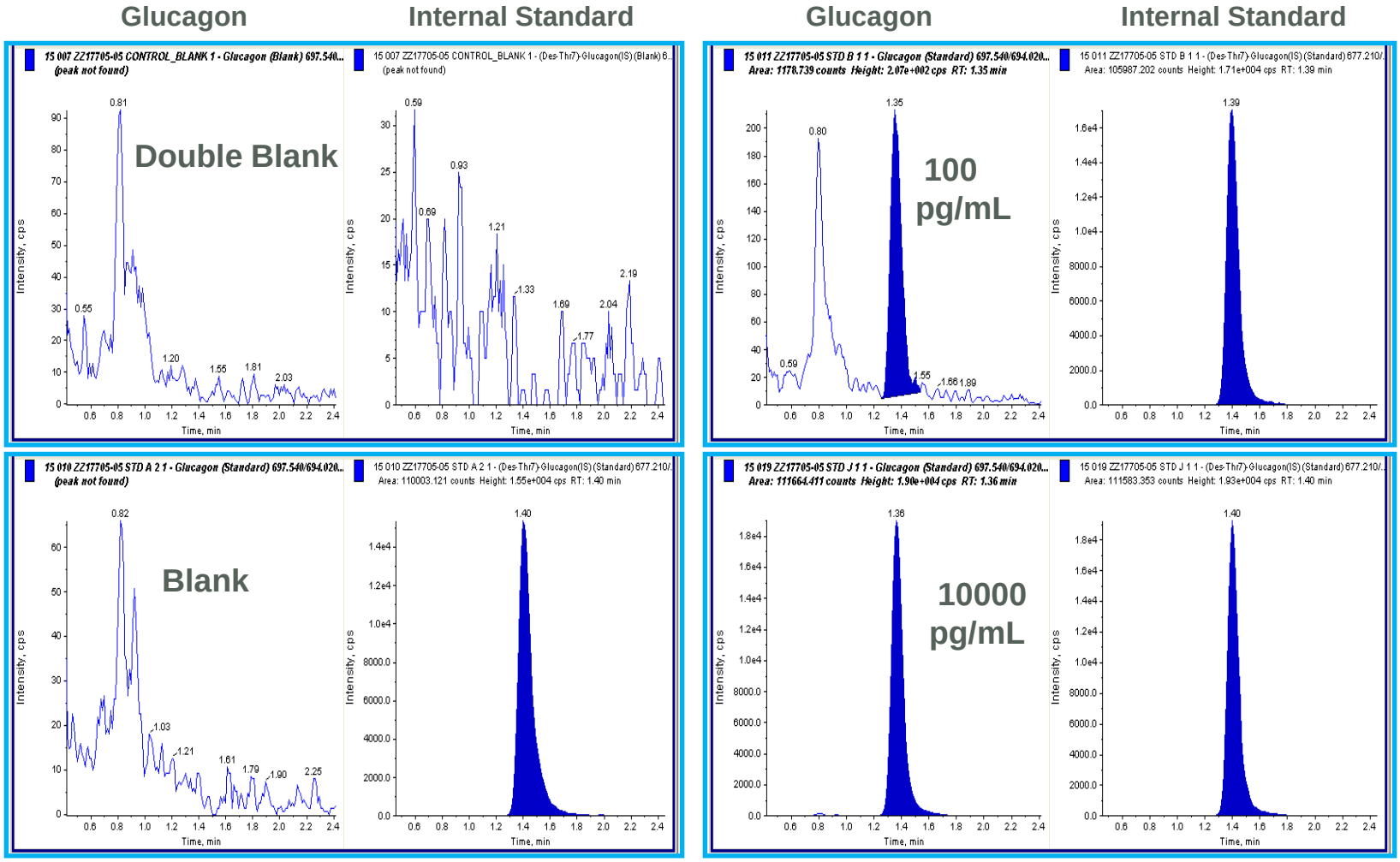
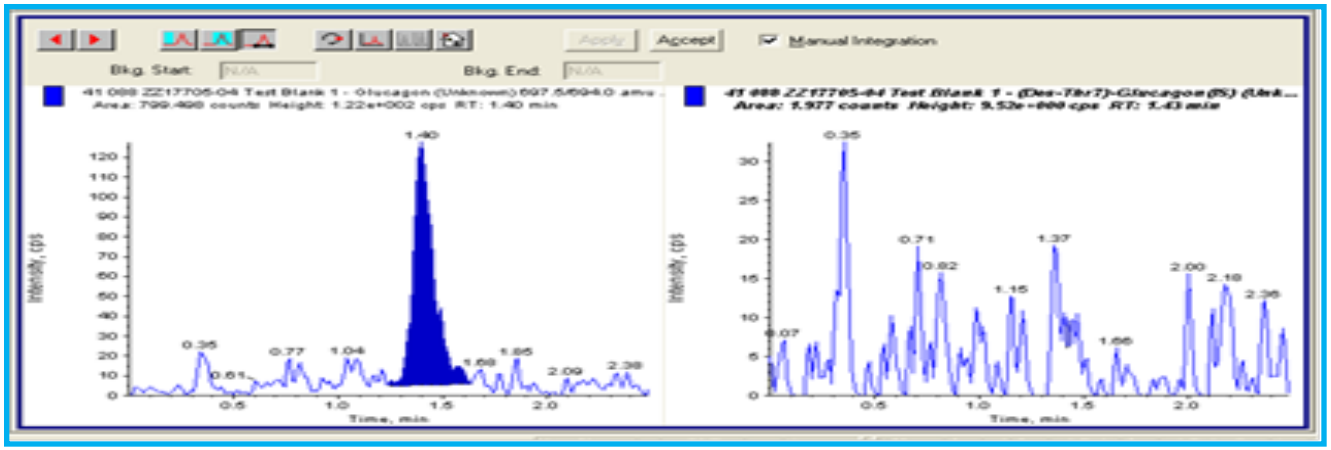
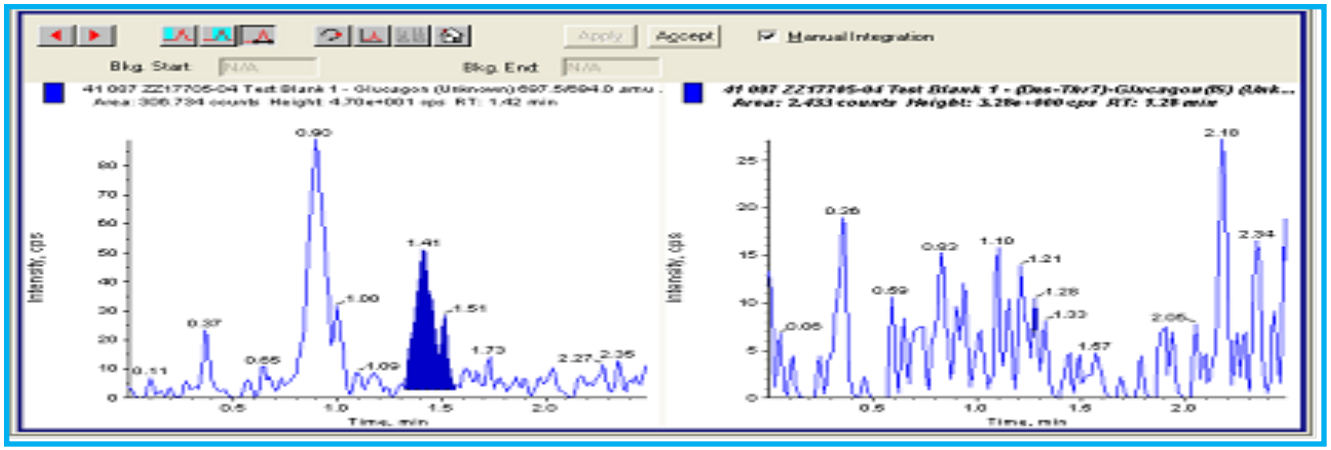
Glucagon Extracted Samples



Evaluation of Glucagon Endogenous Level

	Glucagon	Internal Standard
Lot 1 (~55 pg/mL)		
Lot 2 (~25 pg/mL)		

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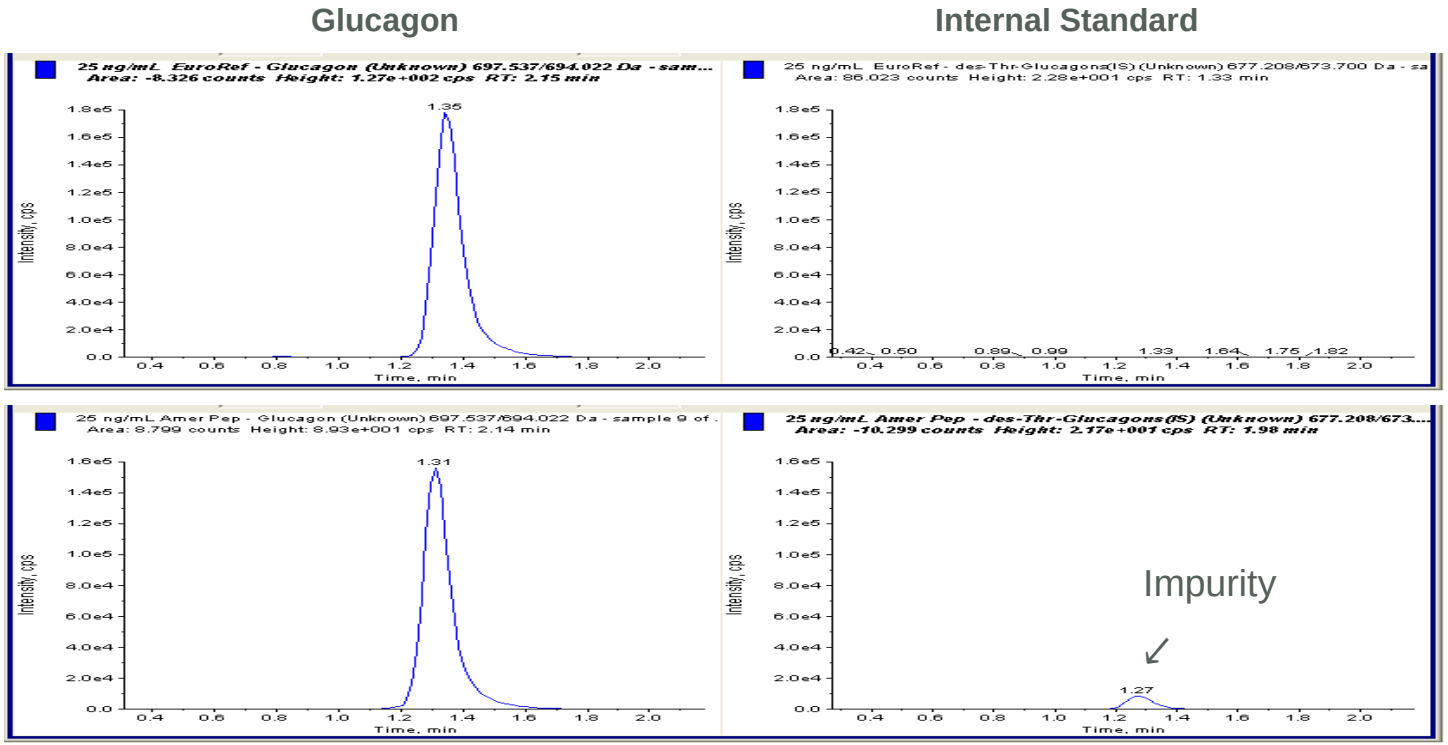
Glucagon Reference Standards



- Concentrations of glucagon as European Pharmacopeia Reference Standard and glucagon from commercially available Eli Lilly Glucagon Emergency Kit matched well
- Some synthetic preparations may contain a significant amount of peptide impurities not shown in Certificate of Analysis

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LC-MS/MS vs Immunochemical Methods (Selectivity)

- Glucagon (RIA vs LC-MS/MS)
  - Low concentration quality control samples (140 pg/mL, RIA method) had no detectable intact glucagon in LC-MS/MS method
- 13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$ (ELISA vs. LC-MS/MS)
  - Analytical samples: up to 200-fold difference in concentrations between methods

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Conclusions

- Some challenges in LC-MS/MS with peptides similar to those with small molecules
- Glucagon MRM transition with loss of ammonia provides an easy LC-MS/MS solution
- Several inhibitors are required to ensure glucagon stability in human plasma
- LC-MS/MS glucagon method advantages
  - More selective than immunochemical
  - Lack of matrix effect
  - Large linear range

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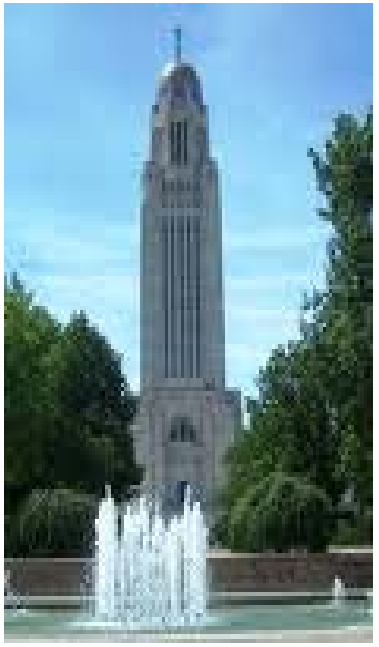

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