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Polypeptide LC-MS Bioanalysis Strategies in Biological Matrices: Glucagon and Plasma Analogs

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Abstract:

Glucagon and its analogs are polypeptide pharmaceuticals designed for the management of metabolic disorders, including hypoglycemia, diabetes, and obesity. Owing to their significant potency, these medicines are often found at low quantities in plasma during pharmacokinetic (PK) investigations, necessitating a very sensitive bioanalytical technique. This study delineates a fast and sensitive LC-MS/MS technique using protein precipitation (PPT) with acidified acetonitrile for sample preparation. Analytes were separated on an ACE C18 column using a linear gradient of water and acetonitrile containing 0.1% acetic acid. Detection was conducted on an AB Sciex mass spectrometer using positive electrospray ionization mode, employing the 5+ charge state as the precursor ion. Selective MS/MS fragment ion monitoring enhanced the signal-to-noise ratio and reduced endogenous interference. The test exhibited a linear range of 0.5–500 ng/mL, characterized by great accuracy, precision, and durability. It has increased sensitivity, selectivity, minimal matrix effects, and is appropriate for regular pharmacokinetic analysis with high throughput and low expense.

Keywords: LC-MS/MS, Polypeptide bioanalysis, Glucagon, GLP-1, GLP-2, Plasma matrix.

INTRODUCTION

Polypeptides and proteins are widely used as therapeutic agents owing to their capacity to substitute or augment endogenous proteins and to trigger innovative therapeutic pathways. In recent decades, the biopharmaceutical sector has concentrated significantly on the development of these compounds. In comparison to small-molecule medications, therapeutic peptides and proteins provide enhanced selectivity, often necessitating reduced dosages and resulting in fewer adverse effects. Nevertheless, their specific characteristics need delicate bioanalytical techniques to evaluate pharmacokinetics, bioavailability, and pharmacodynamics at minimal doses.[1]

Materials and Equipment

Standard materials of glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) were acquired from Sigma–Aldrich Inc. (St. Louis, MO, USA). Acetic acid, formic acid, and DMSO were procured from Sigma. HPLC-grade organic solvents, including methanol, ethanol, isopropanol (IPA), and acetonitrile, were acquired from J.T. Baker Inc. (Philipsburg, NJ, USA). Protease Inhibitor Cocktail Complete™ ULTRA Tablets were procured from Roche Life Science. Deionized water used for HPLC and solution preparation was purified internally using a Milli-Q Academic Gradient A10 system (Millipore, Billerica, MA, USA).

Blank control rat EDTA plasma was acquired from Bioreclamation, Inc. (Hicksville, NY, USA) for the preparation of calibration standards, quality control samples, and control blanks. Polypropylene sample processing vials (0.5, 1.5, or 2.0 mL with lids), LoBind™ vials, and LoBind™ 96-deep-well plates were acquired from Eppendorf NA (Hauppauge, NY, USA). A Tomtec Quadra 3 automated liquid-handling station was acquired from Tomtec, Inc. (Hamden, CT, USA). The TurboVap 96 evaporator originated from Zymark Corporation, located in Hopkinton, Massachusetts, USA. The centrifuges used were the Eppendorf model 5415C (equipped with a tube rotor) and model 5810R (designed for 96-well plates) from Eppendorf (Brinkmann Inst., Inc., Westbury, NY, USA). A syringe pump was obtained from Harvard Apparatus (Holliston, MA, USA). A Hamilton liquid handler (model Microlab Starlet) was acquired from Hamilton Robotics Inc. (Reno, NV, USA).

Liquid Chromatographic Conditions[2-4]

The HPLC system included an Agilent 1200-series quaternary pump (Agilent Technologies, Waldbronn, Germany) and an HTS PAL autosampler including enclosed cooling plate trays (CTC Analytics AG, Zwingen, Switzerland).

The mobile phase A (MPA) consisted of 0.1% acetic acid in water, whereas mobile phase B (MPB) included 0.1% acetic acid in acetonitrile. Flow rates varied between 0.8 and 1.4 mL/min during technique development. The used columns comprised:

- ACE column, 30 × 2.1 mm, 3 µm, 300 Å (MAC-MOD Analytical Inc., Chadds Ford, PA)
- Ascentis Express Peptide ES-C18 column, 30 × 2.1 mm, 3 µm, 160 Å (Sigma, Supelco, St. Louis, MO, USA)
- Waters Acquity™ BEH C18 column, 50 × 2.1 mm (Waters, Milford, MA)

Column temperatures were set at 22°C (room temp), 40°C, and 60°C. The HTS PAL autosampler was equipped with a 50 µL sample loop on a six-port injection valve. The sample needle and valve were washed between injections using:

- Wash 1: methanol/water (50:50, v/v)
- Wash 2: ethanol/IPA/acetonitrile (40:30:30, v/v/v)

Sample injection volume was approximately 25 µL, using a 50 µL Hamilton glass syringe.

Mass Spectrometric Conditions[5]

Analyte identification was conducted via AB Sciex API 4000 and API 5000 triple quadrupole mass spectrometers (Applied Biosystems, Concord, Ontario, Canada), each outfitted with TurboIonSpray™ sources.

The devices functioned in multiple reaction monitoring (MRM) mode using positive ionization. Dwell periods were 100 milliseconds per analyte channel and 50 milliseconds for internal standards. Each analyte was optimized separately.

Optimized global MS parameters included:

- TurboIonSpray temperature (TEM): 650°C
- Nebulizer gas: 50
- Desolvation gas: 60
- Curtain gas (CUR): 15
- Spray voltage: 5500 V
- Entrance potential (EP): 10 V

Each peptide was refined for Q1 selection, fragmentation, and Q3 selection with designated declustering potential (DP) and collision energy (CE). The DP values were often lower on the API 4000 compared to the API 5000, however both instruments exhibited identical optimal CE values (as shown in Table 1).

Table 1 The optimized MRM transitions and analyte-dependent parameters of AB Sciex 4000 and 5000 Mass Spectrometers for analytes glucagon, GLP-1 and GLP-2.

Peptide Analytes	Q1 (m/z)	Q3 (m/z)	DP(4000MS) (V)	DP(5000MS) (V)	CE (V)
Glucagon	697(5+)	694(5+)	70	150	25
GLP-1	671(5+)	668(5+)	75	160	22
GLP-2	785(5+)	782(5+)	75	165	23
IS	969(2+)	961(5+)	85	155	30

Q1: The selected precursor ion and its charge state
Q3: The selected product ion and its charge state
DP: Declustering potential
CE: Collision energy
M/z: Analyte mass charge ratio

Preparation of Standards and Quality Controls[6]

Peptide analytes were produced as individual stock solutions in 100 mM acetic acid at a concentration of 1.0 mg/mL and kept at -20ºC. The analyte stock solution was diluted in a mixed solvent comprising 20% acetonitrile, 80% 100 mM acetic acid, and 0.5% albumin to provide a secondary working solution at a concentration of 10 µg/mL.

Calibration standards (STDs) at concentrations of 0.5, 1.0, 2.5, 10, 25, 100, 250, and 500 ng/mL were newly generated each day by serial dilution in control plasma treated with 0.5% acetic acid and protease inhibitors, using a Hamilton Microlab liquid handler (Hamilton Company, Reno, NV, USA). Quality control (QC) samples in plasma were generated at concentrations of 2, 40, and 400 ng/mL in a similar manner.

An internal standard (IS), a proprietary chemical, was formulated in a solution of 99.5% acetonitrile and 0.5% acetic acid at a concentration of 150 ng/mL. Standards and quality controls for the concurrent quantification of glucagon, GLP-1, and GLP-2 were developed to include all three analytes.

Sample Extraction Procedure

Plasma samples were thawed, brought to room temperature, and then kept on wet ice prior to analysis. All samples were treated with 0.5% acetic acid and protease inhibitors to stabilize peptide analytes, and vortexed for 20 seconds.

Protein Precipitation Method[7]:

100 µL of plasma was transferred to LoBind™ centrifuge tubes (1 mL, polypropylene). Then, 150 µL of acetonitrile with 1% acetic acid and IS was slowly added. After 1 minute of vortexing and centrifugation at 10,000 rpm for 5 minutes, 220 µL of supernatant was transferred to a clean LoBind™ 96-DW plate and diluted with 110 µL of water. A 25 µL aliquot was injected for LC-MS/MS analysis.

Evaporation Option:

To enhance sensitivity, the supernatant was evaporated under nitrogen using a TurboVap 96 (Zymark, MA, USA), and reconstituted with 100 µL of 40% MP-B / 60% MP-A solution (v/v). A 25 µL aliquot was again injected for analysis.

Solid Phase Extraction (SPE) Method[8]:

Used Waters Oasis® HLB 96-well plates (10 mg, 30 µm). 100 µL of plasma with analytes was mixed with 150 µL of 0.5% acetic acid containing IS. SPE plate was conditioned with 1.0 mL methanol and equilibrated with 1.0 mL water. The sample was loaded slowly with increasing vacuum, washed twice with 1.0 mL of 5% methanol in water, then dried. Elution was done with 3 × 50 µL of acetonitrile/water/formic acid (90/10/0.5, v/v/v). The eluate was either diluted with 100 µL water or dried and reconstituted in mobile phase.

Sensitivity and Selectivity

Infusion solutions (2.5 µg/mL) of each peptide were prepared in acetonitrile/water (50/50, v/v) with 0.1% acetic acid or formic acid. These were infused using a Harvard Apparatus syringe pump through a "T" connector into the HPLC system (0.2 mL/min flow rate) using 0.1% acidified mobile phase.

Mid-QC level samples were analyzed to identify optimal MRM transitions. Plasma matrix extract samples were used to assess matrix interference, and selectivity was evaluated using control plasma to detect any LC-MS/MS signals near the analyte retention time.

Assay Precision and Accuracy

Intra- and inter-day precision and accuracy were assessed using eight standard concentrations and three QC levels in duplicate. Precision was expressed as relative standard deviation (RSD, %), and accuracy as relative error (RE, %), based on deviation from nominal concentrations.

Calibration curves were constructed using peak area ratios and analyzed with weighted ($1/x^2$) linear least squares regression.

Results and Discussion

Mass Spectrometric Method Development

The goal was to develop a sensitive LC-MS method for detecting LLOQs of glucagon, GLP-1, and GLP-2 by optimizing signal-to-noise (S/N) ratios from extracted ion chromatograms (XICs).

Polypeptides often show multiple charged states in MS with no dominant product ion, making MRM selection challenging. Glucagon and its analogs fall into this category, complicating their bioanalysis.

While some literature describes GLP-1 detection using ion trap MS [82] and calcitonin using Q-TOF HRMS [83], ion trap MS lacks sensitivity. Compared to Orbitrap and HRMS systems, triple quadrupole MS in MRM mode offers better sensitivity, specificity, and efficiency, with minimal data storage needs.

MS optimization involves selecting the best charge state of the precursor ion and identifying its most intense, interference-free product ion for MRM.

Optimization of Analyte Precursor Ions

Optimization was first performed on the AB Sciex API 4000 MS with individual analyte infusion, and then validated on the API 5000. The declustering potential (DP), typically ~5 kV, was varied to observe effects on charge state distribution and ion intensity.

Using acetonitrile/water (50/50, v/v) with either 0.1% formic acid (FA, pH ~2.8) or 0.1% acetic acid (AA, pH ~4.0), different ion intensities were observed. For glucagon on the API 4000:

- $[M+3H]^{3+}$ at m/z 1162
- $[M+4H]^{4+}$ at m/z 872
- $[M+5H]^{5+}$ at m/z 697

At 90–110 V DP, the 4+ ion was strongest; at 50–60 V DP, the 5+ ion was optimal. On the API 5000, optimal DPs were 150 V (glucagon), 160 V (GLP-1), and 165 V (GLP-2).

Mobile Phase Effect:

With 0.1% FA (pH ~2.8): 5+ < 4+ < 3+With 0.1% AA (pH ~4.0): 5+ > 4+ > 3+

Since pH ~4.0 is below the pI of glucagon (5.8), it favors 5+ ion formation. The 5+ ion

was ultimately selected for all three peptides.

Similar results were obtained using the API 4000 Qtrap and Thermo LTQ Orbitrap HDMS systems. GLP-1 and GLP-2 also exhibited 3+, 4+, and 5+ charge states. Infusion results with MS/MS spectra for GLP-1 and GLP-2 are shown in Figure 1.

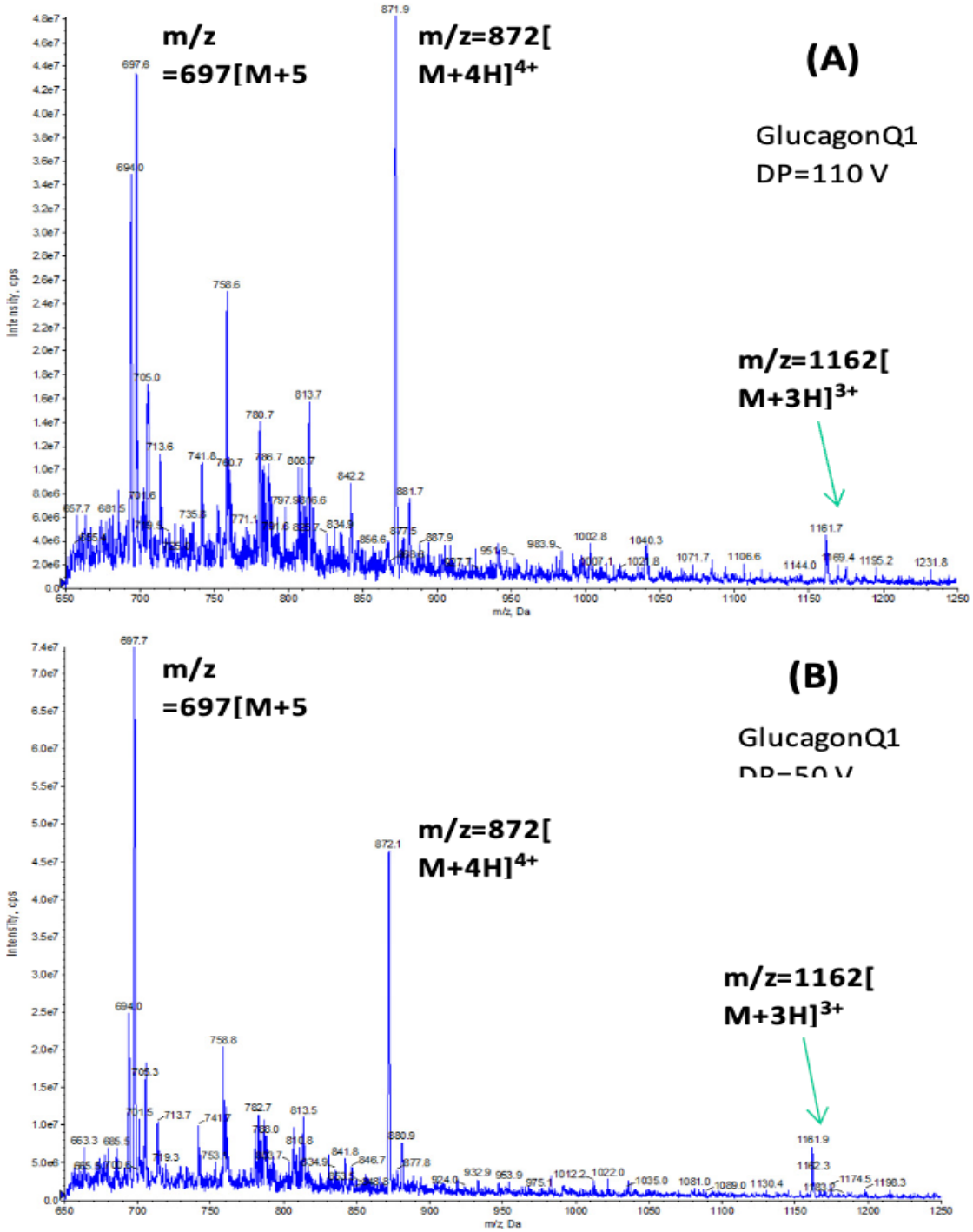

Figure 1: Precursor ion full-scan spectra of glucagon showing the effort of declustering potential on its charge distribution and ion intensity.

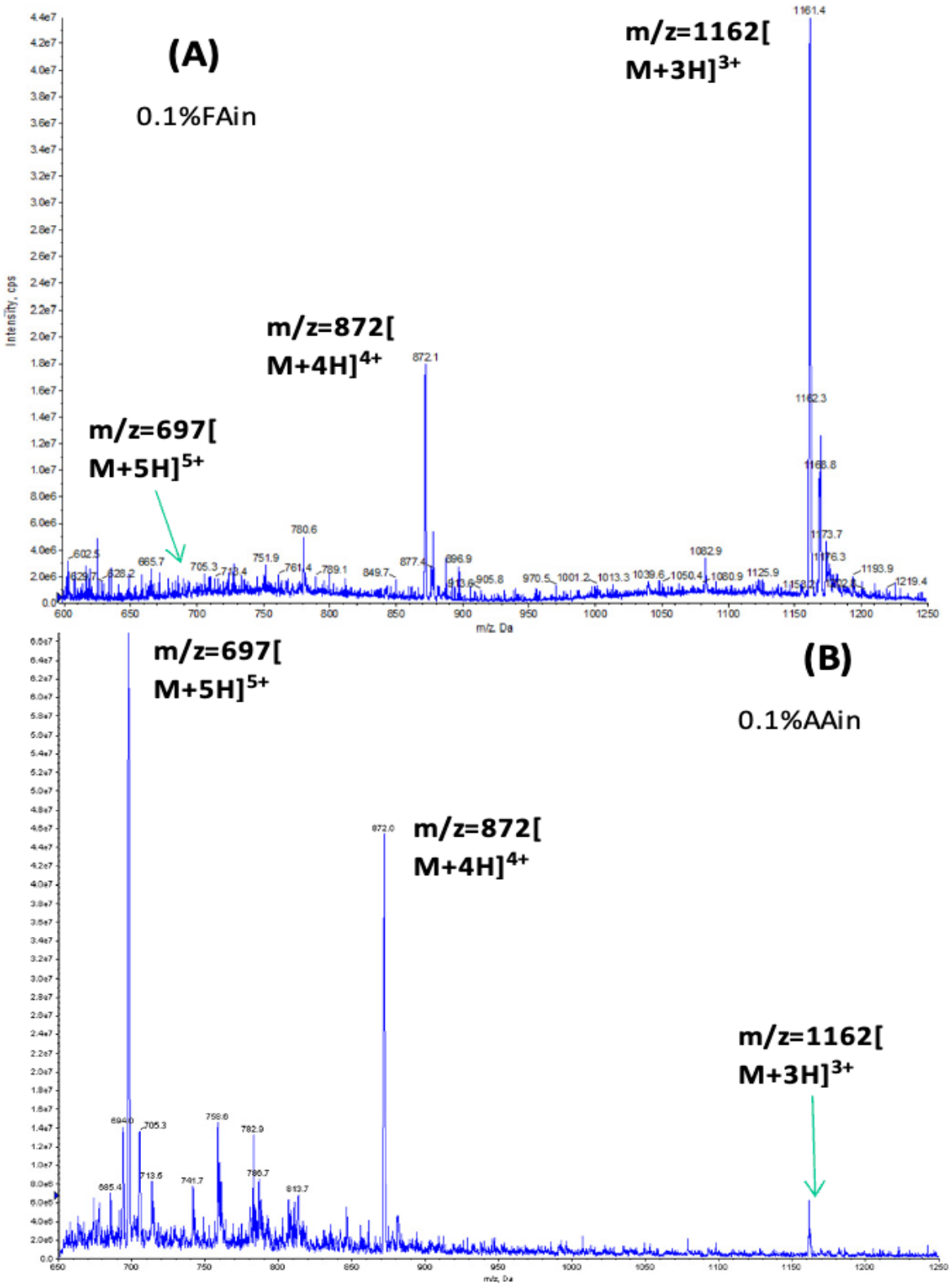

Figure 2: Precursor ion full-scan spectra of glucagon showing the effort of mobile phase pH conditions on precursor ion intensity

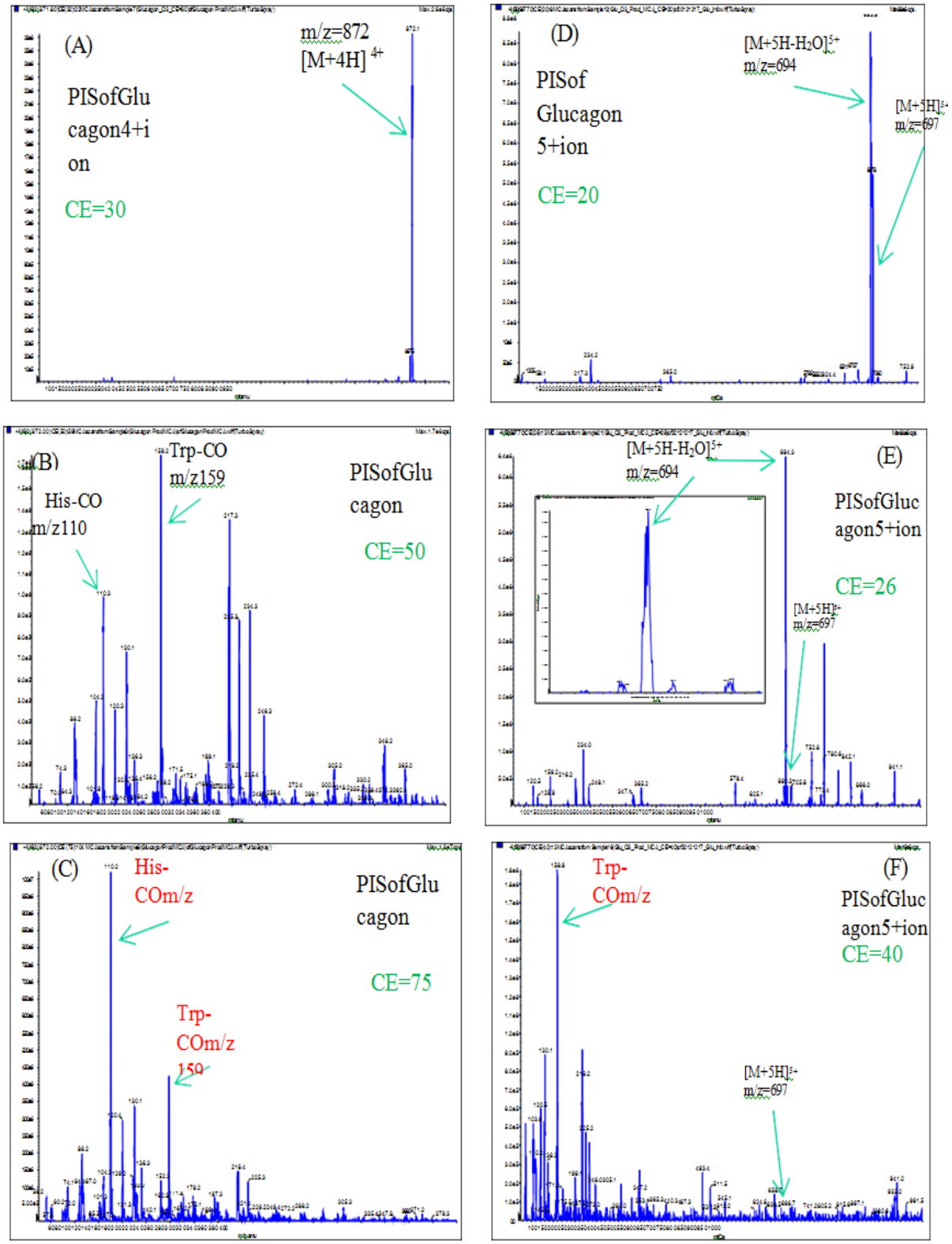

Figure 3: Production MS/MS scan spectra of glucagon $[M+4H]^{4+}$and$[M+5H]^{5+}$ions.

The effect of the charge state of the precursor ions and collision energy changes on the fragmentation pattern and the product ion intensity.Infusion of 1 µg/mL of glucagon by T in with an MP flow rate of 0.2 mL/min.

(A-C) Product ions from the $[M+4H]^{4+}$ion, m/z 872;at CE 30, no PI ; at CE 50, many PI with m/z 110 and 159 derived from His and Trp, respectively; at CE 75, m/z 110.(D-E) Product ions from the $[M+5H]^{5+}$ion, m/z 697; at CE 20, m/z 694 and m/z 697 comparable intensities; at CE 26, m/z 694 (-NH_3) PI dominated; at CE>40, m/z 110 wasonly the major product ion.

Conclusion:

This study focused on the thorough selection, optimization, and equilibrium of mass spectrometry, liquid chromatography, and sample preparation/enrichment techniques to enhance the signal-to-noise (S/N) ratios of analytes.

For MS detection on the API-5000, it is essential to pick the correct charge state of the precursor ion (5+ for all three analytes) and the suitable product ion (usually the loss of H_2O from the precursor ion) to get the optimal S/N ratio amongst a plasma matrix. Sensitivity may be further improved by using modern equipment like the API-6500 or API-5500 mass spectrometers.

In liquid chromatography separation, evaluating suitable peptide columns is crucial for attaining requisite chromatographic resolution and isolating analytes from endogenous interferences. The optimization of mobile phase composition and regulation of column temperature improved signal-to-noise ratios, resulting in a lower limit of quantification (LLOQ) of 0.5 ng/mL in plasma.

Sample preparation challenges concerning process adsorption and matrix stability were mitigated or eradicated by using LoBind® products, regulating pH, and utilizing a protease inhibitor cocktail, yielding relative error (RE) levels below 12%.

These analytical techniques are widely relevant to the intact bioanalysis of various polypeptide analytes in biopharmaceutical research. A fast and precise LC-MS/MS bioanalytical technique has been established for the concurrent quantification of polypeptide glucagon and its analogs in a plasma matrix. This assay exhibits excellent precision, accuracy, sensitivity, little matrix interference, and rapid analytical cycle time, making it extremely appropriate for pharmacokinetic (PK) research.

References:

1. Lau JL, Dunn MK. Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorganic & medicinal chemistry. 2018 Jun 1;26(10):2700-7.
2. Bristow PA, Knox JH. Standardization of test conditions for high performance liquid chromatography columns. Chromatographia. 1977 Jun;10:279-89.
3. Bressolle F, Bromet-Petit M, Audran M. Validation of liquid chromatographic and gas chromatographic methods Applications to pharmacokinetics. Journal of Chromatography B: Biomedical Sciences and Applications. 1996 Nov 8;686(1):3-10.
4. Macko T, Hunkeler D. Liquid chromatography under critical and limiting conditions: a survey of experimental systems for synthetic polymers. Liquid Chromatography/FTIR Microspectroscopy/Microwave Assisted Synthesis. 2003:62-136.
5. Wu C, Dill AL, Eberlin LS, Cooks RG, Ifa DR. Mass spectrometry imaging under ambient conditions. Mass spectrometry reviews. 2013 May;32(3):218-43.
6. Pietersz RN. Quality assurance and quality control in component

preparation. Vox sanguinis. 1994 Mar 1;67(3):197-200.

7. Burgess RR. Protein precipitation techniques. Methods in enzymology. 2009 Jan 1;463:331-42.
8. Ötles S, Kartal C. Solid-Phase Extraction (SPE): Principles and applications in food samples. Acta Scientiarum Polonorum Technologia Alimentaria. 2016 Mar 30;15(1):5-15.