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Bioanalysis Strategies of Polypeptide LC-MS in Biological Matrices – A Case Analysis of Glucagon and Its Analogs in Plasma

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Abstract:

Glucagon and its analogs are polypeptide drugs developed for treating metabolic conditions such as hypoglycemia, diabetes, and obesity. Due to their high potency, these drugs are typically present at low concentrations in plasma during pharmacokinetic (PK) studies, requiring a highly sensitive bioanalytical method. This work describes a rapid and sensitive LC-MS/MS method 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 with 0.1% acetic acid. Detection was performed on an AB Sciex mass spectrometer in positive electrospray ionization mode, using the 5+ charge state as the precursor ion. Selective MS/MS fragment ion monitoring improved signal-to-noise ratio and minimized endogenous interference. The assay demonstrated a linear range of 0.5–500 ng/mL with high accuracy, precision, and robustness. It offers high sensitivity, selectivity, low matrix effects, and is suitable for routine PK analysis with high throughput and low cost.

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

Introduction

Polypeptides and proteins are increasingly used as therapeutic agents due to their ability to replace or enhance endogenous proteins and activate novel therapeutic pathways. In recent decades, the biopharmaceutical industry has focused heavily on developing these molecules. Compared to small-molecule drugs, therapeutic peptides and proteins offer higher specificity, often requiring lower doses and causing fewer side effects. However, their precise nature demands sensitive bioanalytical methods to assess pharmacokinetics, bioavailability, and pharmacodynamics at very low concentrations. [1]

Materials and Equipment

Glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) standard materials were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Acetic acid, formic acid, and DMSO were also obtained from Sigma. HPLC-grade organic solvents — methanol, ethanol, isopropanol (IPA), and acetonitrile — were purchased from J.T. Baker Inc. (Philipsburg, NJ, USA).

Protease inhibitor cocktail complete™ ULTRA Tablets were ordered from Roche Life Science. Deionized water used for HPLC and solution preparation was purified

in-house using a Milli-Q Academic Gradient A10 system (Millipore, Billerica, MA, USA).

Blank control rat EDTA plasma was purchased from Bioreclamation, Inc. (Hicksville, NY, USA) and used to prepare calibration standards, QC samples, and control blanks. Polypropylene sample processing vials (0.5, 1.5, or 2.0 mL with caps), LoBind™ vials, and LoBind™ 96-deep-well plates were purchased from Eppendorf NA (Hauppauge, NY, USA).

A Tomtec Quadra 3 automatic liquid-handling station was obtained from Tomtec, Inc. (Hamden, CT, USA). The TurboVap 96 evaporator was from Zymark Corp. (Hopkinton, MA, USA). Centrifuges used were the Eppendorf model 5415C (with tube rotor) and model 5810R (for 96-well plates) from Eppendorf (Brinkmann Inst., Inc, Westbury, NY, USA). A syringe pump was from Harvard Apparatus (Holliston, MA, USA). A Hamilton liquid handler (model Microlab Starlet) was obtained from Hamilton Robotics Inc. (Reno, NV, USA).

Liquid Chromatographic Conditions [2-4]

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

Mobile phase A (MPA) was 0.1% acetic acid in water, and mobile phase B (MPB) was 0.1% acetic acid in acetonitrile. Flow rates ranged from 0.8 to 1.4 mL/min during method development. Columns used included:

- 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 detection was performed using AB Sciex API 4000 and API 5000 triple quadrupole mass spectrometers (Applied Biosystems, Concord, Ontario, Canada), both equipped with TurboIonSpray™ sources.

The systems operated in multiple reaction monitoring (MRM) mode in positive ionization. Dwell times were 100 ms per analyte channel and 50 ms for internal standards. Each analyte was optimized individually.

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 optimized for Q1 selection, fragmentation, and Q3 selection using specific declustering potential (DP) and collision energy (CE). The DP values were generally lower on the API 4000 than on the API 5000, while both instruments

shared the same optimized CE values (as listed 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]

Individual stock solutions of peptide analytes were prepared in 100 mM acetic acid at a concentration of 1.0 mg/mL and stored at -20ºC. The analyte stock solution was diluted into a mixed solvent of 20% acetonitrile / 80% 100 mM acetic acid / 0.5% albumin to create a secondary working solution at 10 µg/mL.

Calibration standards (STDs) at 0.5, 1.0, 2.5, 10, 25, 100, 250, and 500 ng/mL were freshly prepared daily 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 at 2, 40, and 400 ng/mL were prepared similarly.

An internal standard (IS), a proprietary compound, was prepared in 99.5% acetonitrile / 0.5% acetic acid at 150 ng/mL. STDs and QCs for the simultaneous determination of glucagon, GLP-1, and GLP-2 were prepared 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 were 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.

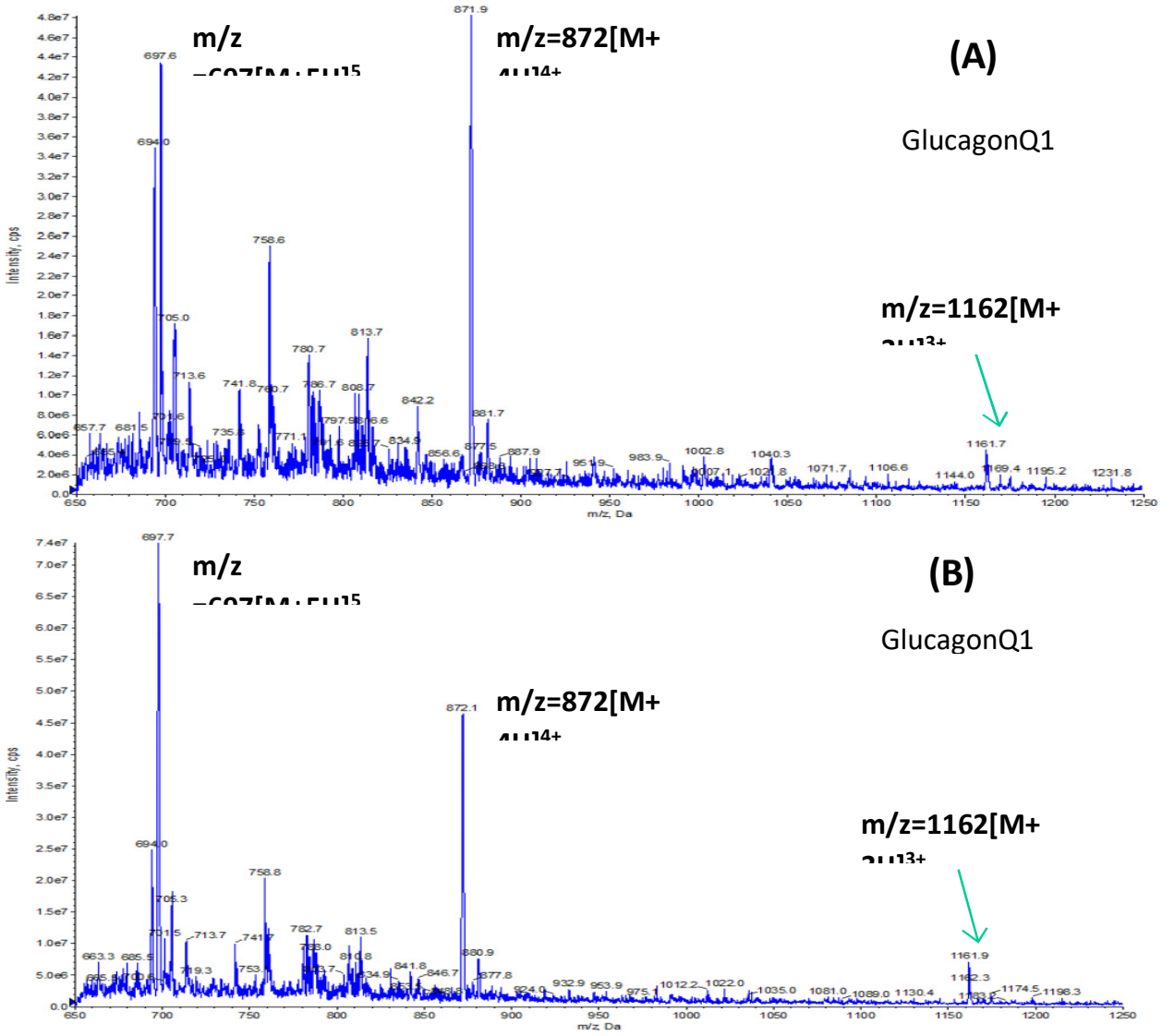

Figure 1: Precursor ion full-scan spectra of glucagon showing the effort of declustering potential on its charge distribution and ion intensity

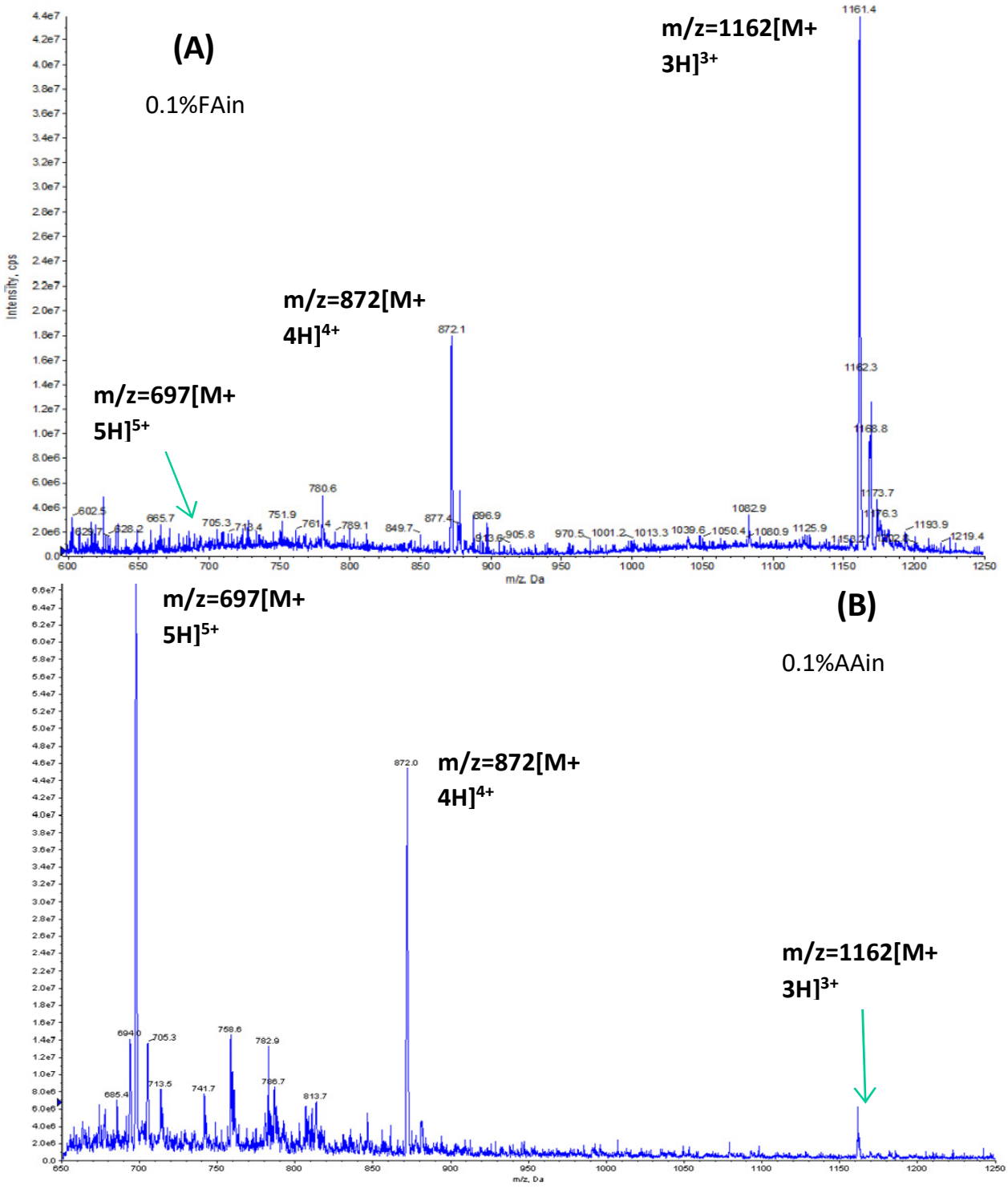

Figure 2: Precursor ion full-scan spectra of glucagon showing the effort of mobile phase pH conditions on precursor ion intensity

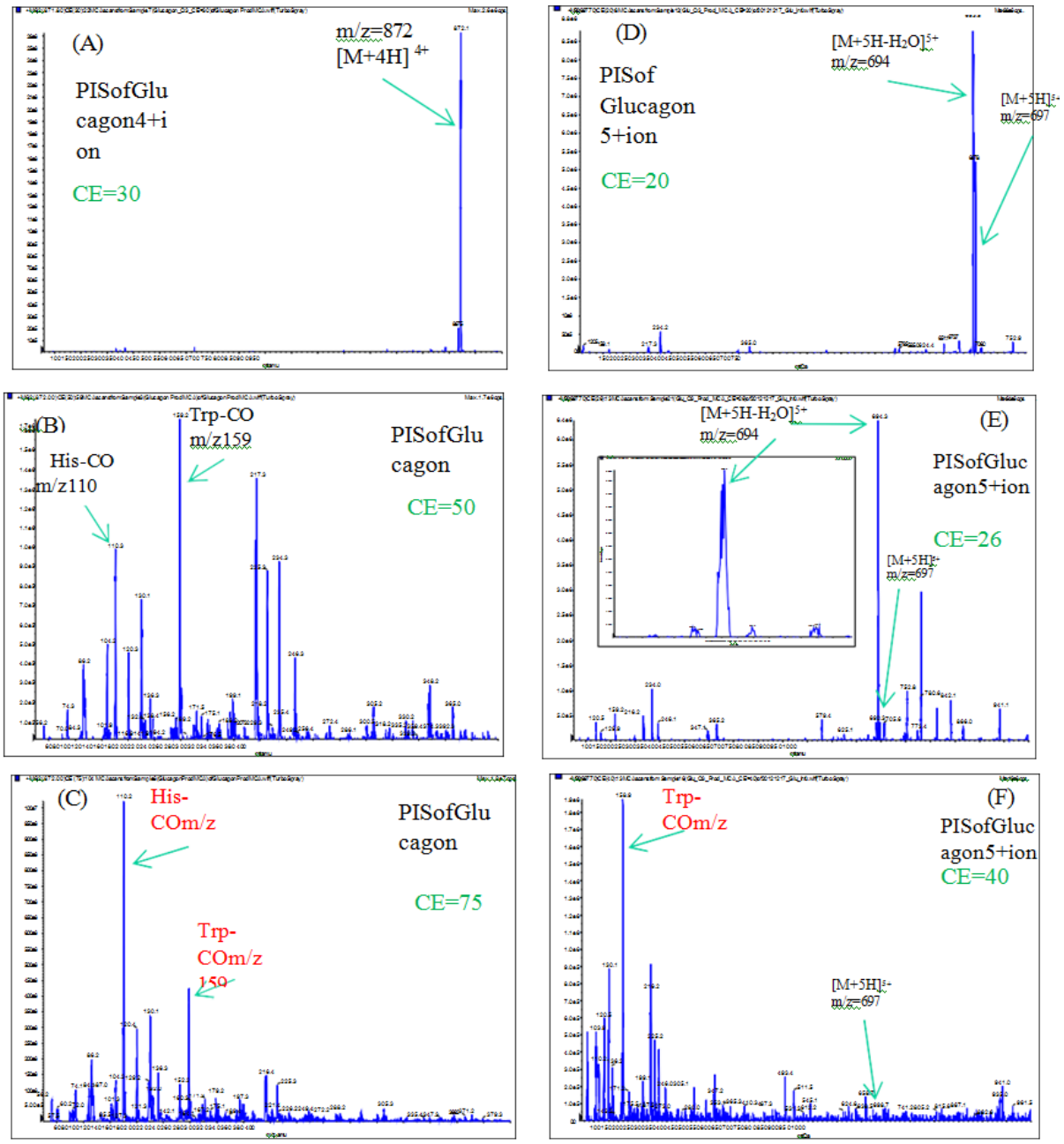

Figure 3: Production MS/MS scan spectra of glucagon $[M+4H]^{4+}$and$[M+5H]^{5+}$ions.

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:

In this work, the investigation of bioanalysis strategies involved the comprehensive selection, optimization, and balance of mass spectrometry, liquid chromatography, and sample preparation/enrichment conditions to maximize the signal-to-noise (S/N) ratios of analytes.

For MS detection on the API-5000, selecting the proper charge state of the precursor ion (5+ for all three analytes) and the appropriate product ion (typically the loss of H_2O from the precursor ion) is crucial to achieving the highest S/N ratio in the presence of a plasma matrix. Sensitivity may be further enhanced by using advanced instruments such as the API-6500 or API-5500 MS.

For LC separation, screening potential peptide columns is important to achieve necessary chromatographic resolution and to separate analytes from endogenous interferences. Optimization of the mobile phase composition and column temperature control also contributed to enhanced S/N ratios, enabling a lower limit of quantification (LLOQ) of 0.5 ng/mL in plasma.

In terms of sample preparation, issues related to process adsorption and matrix stability were minimized or eliminated through the use of LoBind® products, pH control, and protease inhibitor cocktail treatment, resulting in relative error (RE) values of less than 12%.

These analytical strategies are broadly applicable to intact bioanalysis of other polypeptide analytes in biopharmaceutical research. A rapid and sensitive LC-MS/MS-based bioanalytical method has been developed for the simultaneous determination of polypeptide glucagon and its analogs in a plasma matrix. This assay demonstrates good precision, accuracy, sensitivity, minimal matrix effect, and fast analytical cycle time, making it highly suitable for pharmacokinetic (PK) studies.

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