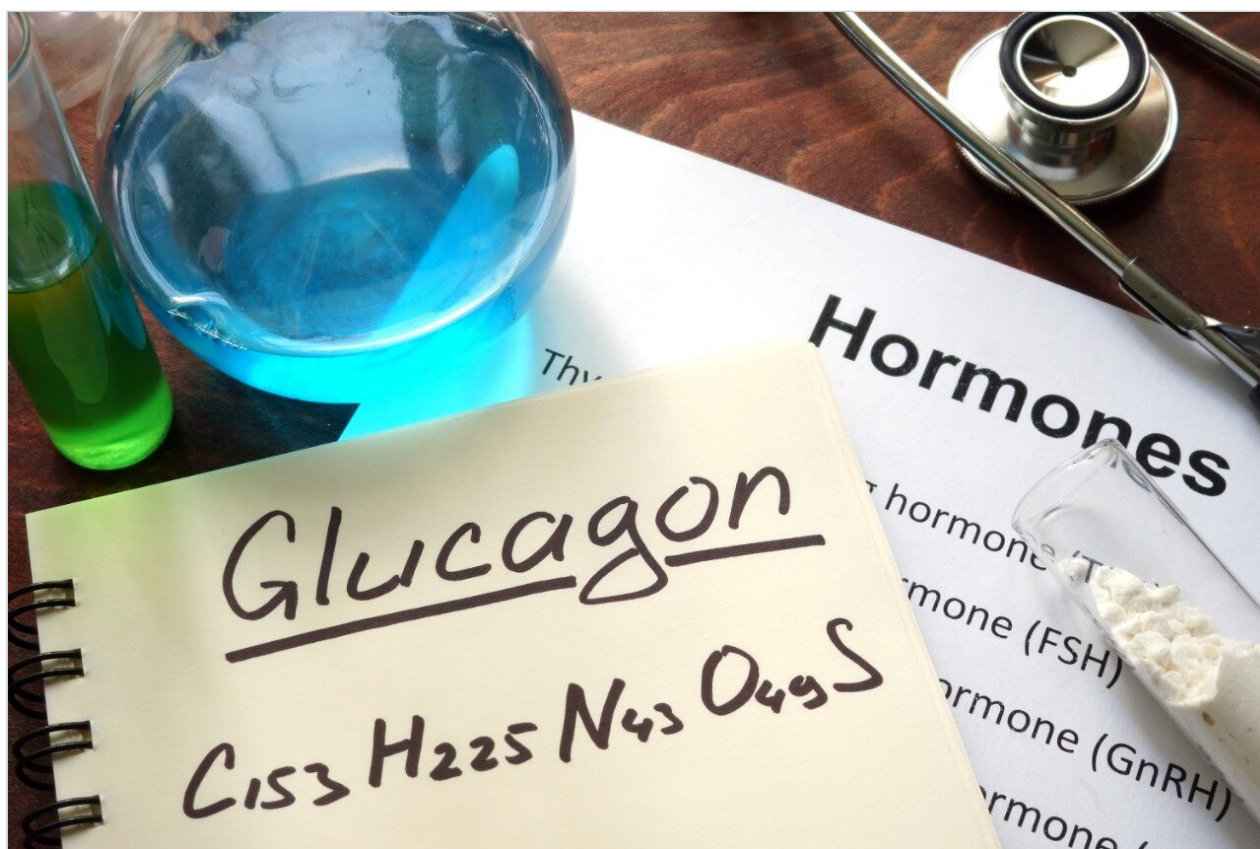


# Development of a High Sensitivity SPE-LC-MS/MS Assay for the Quantification of Glucagon in Human Plasma Using the ionKey/MS System

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## Abstract

The combination of ionKey/MS, mixed-mode  $\mu$ Elution SPE, and higher  $m/z$  b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify low pg/mL concentrations of glucagon in extracted plasma. Use of  $\mu$ Elution format SPE eliminated the need for evaporation, reducing glucagon losses due to adsorption and non-specific binding. The 150  $\mu$ m iKey enabled the development of a highly sensitive, low flow quantitative MRM method for glucagon with an LOD of 12.5 pg/mL and a dynamic range from 12.5–1,000 pg/mL. The current analysis uses 200  $\mu$ L of plasma and provides a significant improvement in sensitivity and S:N over the analytical scale (2.1 mm I.D.) analysis using 1/5th the sample injection volume. Furthermore, an injection of the same volume (5  $\mu$ L) of sample corresponded to a 10X increase in on-column sensitivity allowing for greater confidence in results, as compared to the traditional analytical method for this peptide. In addition, ionKey/MS reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for incurred sample reanalysis (ISR).

## Benefits

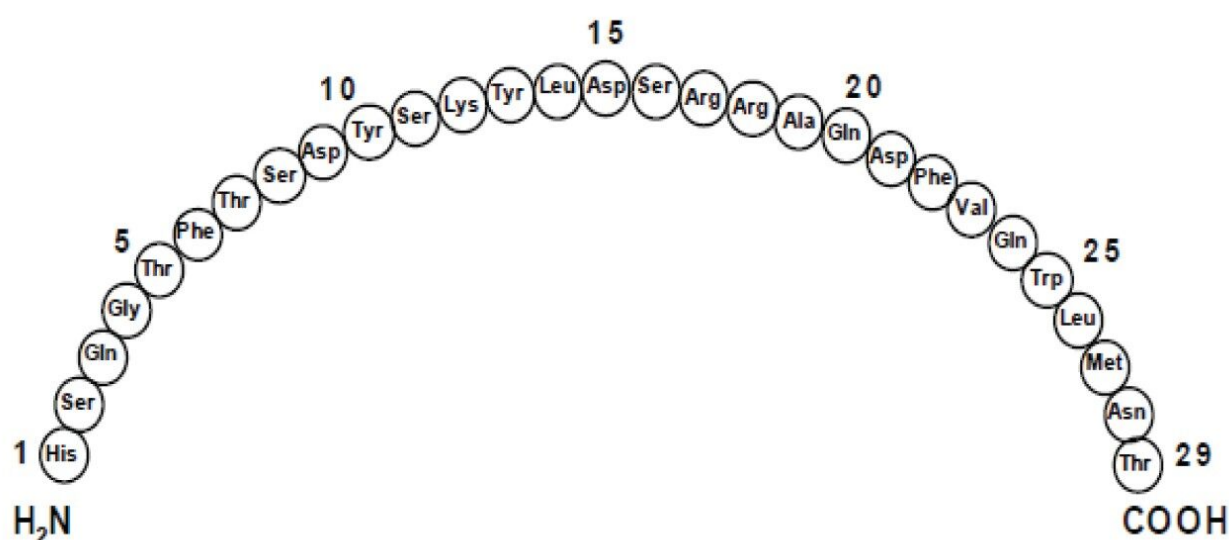
- High sensitivity assay with LOD of 12.5 pg/mL in human plasma.
- Reduced solvent consumption (50X) compared to 2.1 mm scale means significant cost savings.
- Use of mixed-mode solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction.
- 96-well  $\mu$ Elution plate format enables concentration of the sample while maintaining solubility and minimizes peptide loss due to adsorption.
- Selective, fast SPE extraction (<30 minutes) without time-consuming immunoaffinity purification.
- The ionKey/MS System yielded 4X greater S:N and a 10X improvement in sensitivity over 2.1 mm scale allowing for greater confidence in results, more tests per sample, and more injections.

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## Introduction

Glucagon for Injection (rDNA origin) is a polypeptide hormone identical to human glucagon and is used to treat severe hypoglycemia (low blood sugar).<sup>1</sup> It is a single chain polypeptide that contains 29 amino acids

residues with a molecular weight of 3483 (Figure 1). As a research tool, accurate quantification of glucagon from biological matrices can help us to better understand diabetes as a function of disease progression and/or drug treatment. Many assays, using different methodologies exist for glucagon analysis in biological samples.<sup>2-7</sup> Glucagon, like other biologics, has historically been quantified using ligand binding assays (LBAs).<sup>2-5</sup> With advances in MS and chromatography technologies over the past few years there has been a trend toward the analysis of large molecules by LC-MS/MS. This is, in part, driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. Additionally, LC-MS/MS also has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites or endogenous interferences. Large peptides, such as glucagon, are particularly difficult to analyze by LC-MS/MS as MS sensitivity is low due to poor transfer into the gas phase and poor fragmentation. In addition, glucagon suffers from significant non-specific binding, poor solubility, and must be properly stabilized in biological matrices during collection and sample preparation,<sup>6-8</sup> making LC and sample preparation method development challenging.



*Figure 1. Representative amino acid sequence of glucagon.*

The pharmacokinetic profile of administered exogenous glucagon is characterized by a rapid absorption and elimination with a half-life of <20 minutes, resulting in a total duration of exposure to the peptide of ~2 hours. At the practical clinical dose, 0.25–2.0 ng/mL, maximum glucagon levels of ~8 ng/mL are reached in 20 minutes. Endogenous glucagon in plasma is present in low pg/mL levels (<100 pg/mL), which makes detection by LC-MS/MS even more difficult.

The work described herein uses a combination of selective  $\mu$ Elution mixed-mode SPE sample preparation, optimal MS precursor and fragment choice, and the ionKey/MS System (Figure 2) for the highly selective and sensitive quantification of glucagon in human plasma. Detection limits of 12.5 pg/mL using only 200  $\mu$ L of plasma were achieved with a linear dynamic range from 12.5 to 1,000 pg/mL. This work also capitalizes on the attributes of the ionKey/MS System enabling a 5X reduction in injection volume, a 10X increase in sensitivity, and 4X increase in signal-to-noise (S:N) compared to 2.1 mm I.D. analytical scale method.



Figure 2. ionKey Source.

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## Experimental

### Sample preparation

### Step 1: Pretreatment

Commercially available plasma was treated with a protease inhibitor cocktail (1:100). Plasma was then spiked with various concentrations of glucagon and mixed. These samples (200 µL) were acidified with acetic acid (0.5% final concentration) and vortexed, followed by dilution with 200 µL 5% ammonium hydroxide in water.

### Step 2: SPE using a Oasis MAX µElution 96-well Plate (p/n 186001829)

|                                                                                            |
|--------------------------------------------------------------------------------------------|
| CONDITION: 200 µL methanol                                                                 |
| EQUILIBRATE: 200 µL water                                                                  |
| LOAD SAMPLE: Entire diluted plasma sample<br>(400 µL) was loaded onto the extraction plate |
| WASH 1: 200 µL 5% ammonium hydroxide in water                                              |
| WASH 2: 200 µL 10% acetonitrile in water                                                   |
| ELUTE: 2 X 25 µL 65:25:10 acetonitrile:water:<br>acetic acid                               |
| DILUTE: 50 µL water                                                                        |
| INJECT: 5 µL                                                                               |

### Method conditions

#### UPLC conditions

|                    |                                                                                                       |
|--------------------|-------------------------------------------------------------------------------------------------------|
| LC System:         | ACQUITY UPLC M-Class, configured for trap and back-flush elution                                      |
| Separation device: | iKey Peptide BEH C <sub>18</sub> , 130Å, 1.7 µm, 150 µm x 100 mm (p/n 186006766)                      |
| Trap column:       | ACQUITY UPLC M-Class Symmetry C <sub>18</sub> Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186007498) |
| Mobile phase A:    | 0.1% formic acid in water                                                                             |

|                      |                                                                                                                                             |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------------|
| Mobile phase B:      | 0.1% formic acid in acetonitrile                                                                                                            |
| Loading solvent:     | 85:15 mobile phase A:B, 25 µL/min for first two minutes, reverse valve                                                                      |
| Valve position:      | Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column) |
| Analytical gradient: | see Table 1                                                                                                                                 |
| Elution flow rate:   | 2.0 µL/min                                                                                                                                  |
| Column temp.:        | 75 °C                                                                                                                                       |
| Sample temp.:        | 15 °C                                                                                                                                       |
| Injection volume:    | 5 µL                                                                                                                                        |
| Total run time:      | 14.5 min                                                                                                                                    |
| Collection plates:   | Waters 1 mL Collection Plates                                                                                                               |

### Gradient:

| Time(min) | Profile |    | Curve |
|-----------|---------|----|-------|
|           | %A      | %B |       |
| 0         | 85      | 15 | 6     |
| 6         | 55      | 45 | 6     |
| 6.5       | 15      | 85 | 6     |

|     |    |    |   |
|-----|----|----|---|
| 8.5 | 15 | 85 | 6 |
| 9.5 | 85 | 15 | 6 |

---

*Table 1. LC gradient conditions.*

## MS conditions

|                          |                                     |
|--------------------------|-------------------------------------|
| MS system:               | Xevo TQ-S                           |
| Ionization mode:         | ESI positive                        |
| Capillary voltage:       | 3.8 kV                              |
| Source temp.:            | 120 °C                              |
| Cone gas flow:           | 100 L/hr                            |
| Collision cell pressure: | $5.5 \times 10^{(-3)}$ mbar         |
| Collision energy:        | Optimized by component, see Table 2 |
| Cone voltage:            | Optimized by component, see Table 2 |

## Data management

|                          |              |
|--------------------------|--------------|
| Chromatography software: | MassLynx 4.1 |
| Quantification software: | TargetLynx   |

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## Results and Discussion



## Mass spectrometry

The 4+ ( $m/z$  871.5) and 5+ ( $m/z$  697.1) multiply charged precursors were observed for glucagon; MSMS spectra for these precursors, obtained at their optimal collision energies, are shown in Figure 3. The fragments at  $m/z$  693.5 and 940.2 of the 5+ precursor, and 1040.2 of the 4+ precursor were chosen for quantification (Table 2). Although many peptides produce intense fragments below  $m/z$  200, these ions (often immonium ions) result in high background in extracted samples due to their lack of specificity. In this assay, the use of highly specific b/y fragments yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

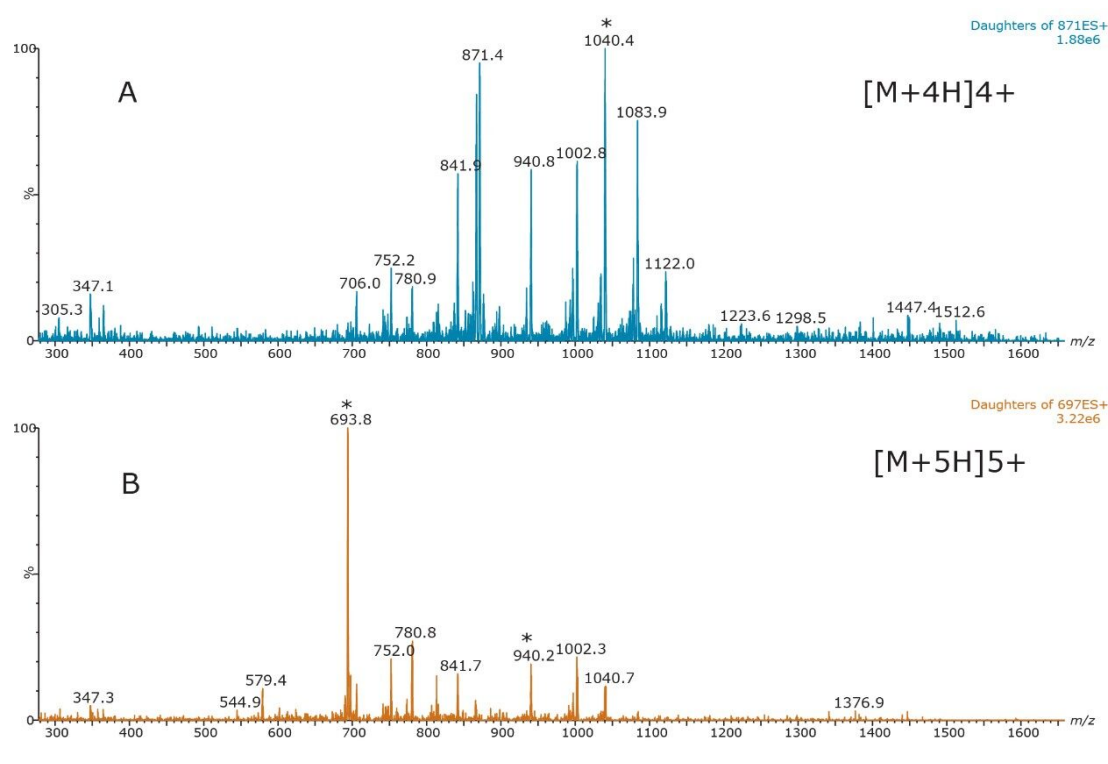


Figure 3. MSMS spectra of the 4+ (A) and 5+ (B) precursors of glucagon. Fragments chosen for quantitation are indicated by asterisks (\*).



| Precursor            | MRM transition | Cone voltage (V) | Collision energy (eV) | Product ion type |
|----------------------|----------------|------------------|-----------------------|------------------|
| [M+5H] <sup>5+</sup> | 697.1>693.5    | 40               | 12                    | Ammonia loss(5+) |
| [M+5H] <sup>5+</sup> | 697.1>940.2    | 40               | 20                    | b24(3+)          |
| [M+4H] <sup>4+</sup> | 871.5>1040.2   | 40               | 26                    | b26(3+)          |

Table 2. MS Conditions for glucagon.

## Liquid chromatography

Chromatographic separation of glucagon was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device (Figure 4) is packed with UPLC-grade sub-2- $\mu$ m particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume, are avoided. Use of the iKey Separation Device provided excellent peak shape, narrow peak widths (<4.0 secs at base), and resolution from endogenous matrix interferences.



Figure 4. iKey Separations Device.

Glucagon was eluted using a linear gradient from 15–45% B over 6 minutes. Representative chromatograms are shown in Figure 5. The use of multidimensional chromatography, specifically a trap and back-flush elution strategy, provided further sample cleanup and facilitated the loading of 5  $\mu$ L of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte breakthrough. Additionally, the ability to inject sample volumes typical for analytical scale LC analysis on the iKey

Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptides and proteins in complex matrices.

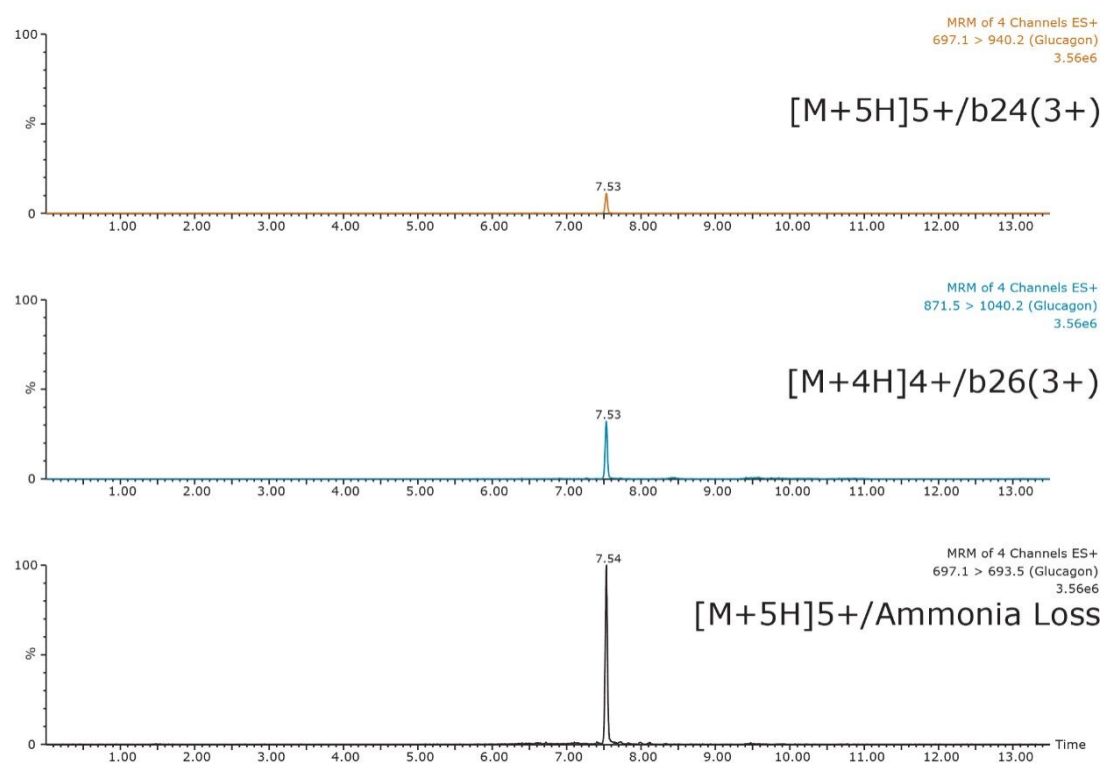


Figure 5. UPLC separation of glucagon from extracted plasma, using the iKey Peptide BEH  $C_{18}$ , 130Å 1.7  $\mu$ m, 150  $\mu$ m x 100 mm (p/n 186006766).

## Enhanced sensitivity with the use of the ionKey/MS System

Versus analytical scale (2.1 mm I.D.), the ionKey/MS System generally offers increased sensitivity, making it ideal for glucagon analysis. This also facilitates the use of smaller sample volumes whilst maintaining or improving sensitivity. For a 250 pg/mL plasma extracted sample, the same injection volume (5  $\mu$ L) on the ionKey/MS System yielded 4X greater S:N and a 10X improvement in sensitivity versus 2.1 mm scale (Figure 6). Using the 150  $\mu$ m iKey Separation Device, low pg/mL levels of glucagon were detected in extracted plasma. Figure 7 demonstrates the improvements the ionKey/MS System provides for a 25 pg/mL extracted plasma sample. Versus 2.1 mm scale (25  $\mu$ L injection), a 5  $\mu$ L injection on the ionKey/MS System yields 5X greater sensitivity and a 3X improvement in S:N. Ultimately, the use of the ionKey/MS System enabled the development of a low flow quantitative MRM method for glucagon that achieved detection limits of 12.5 pg/mL from only 200  $\mu$ L of plasma.

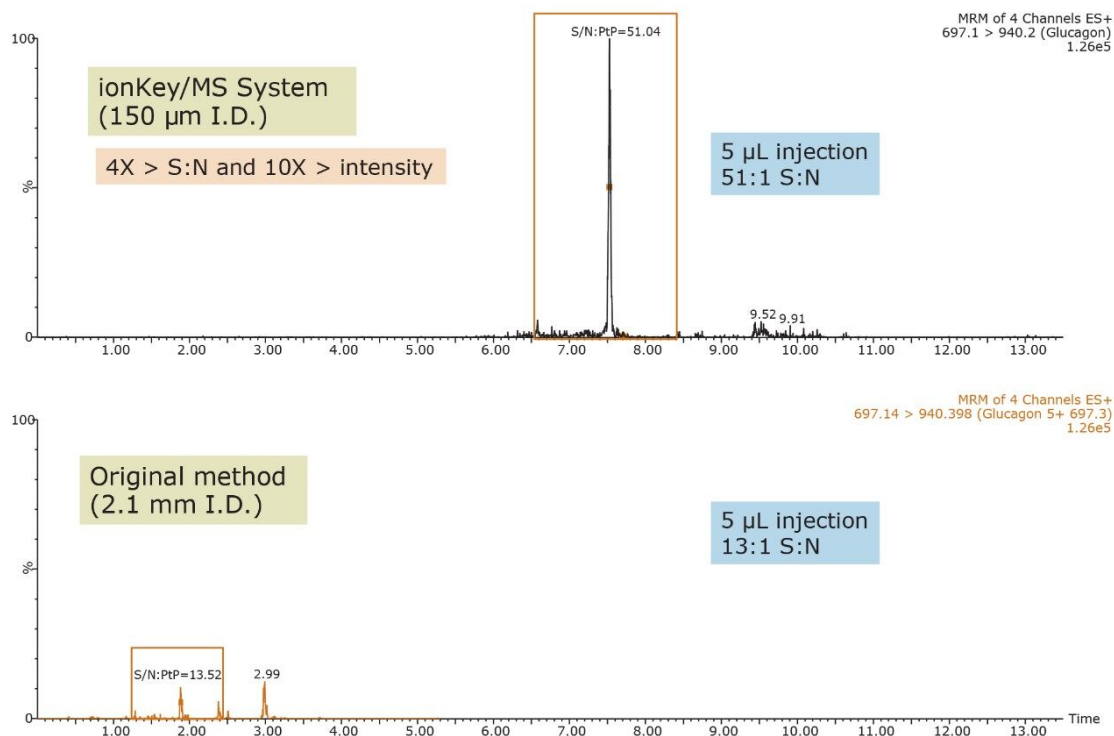


Figure 6. Comparison of 250 pg/mL glucagon extracted from human plasma (200 µL): iKey Separation Device (150 µm I.D.) vs. traditional analytical flow (2.1 mm I.D.).

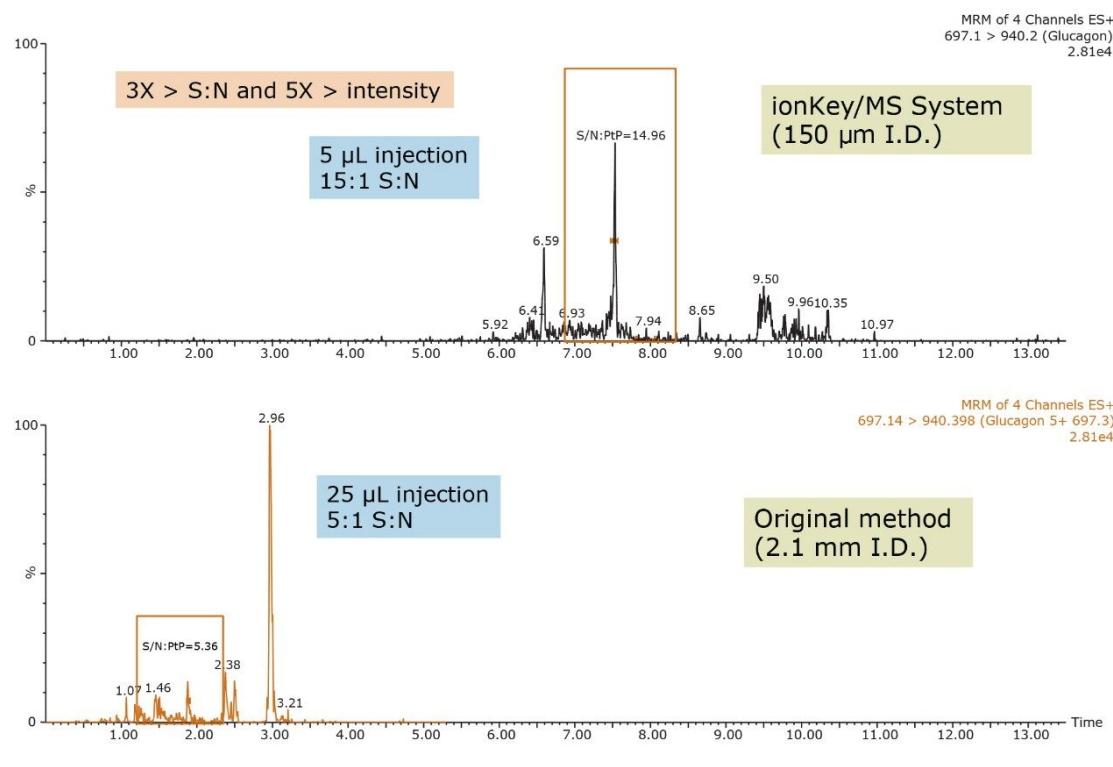


Figure 7. Comparison of 25 pg/mL glucagon extracted from human plasma: iKey Separation Device (5 µL injection) vs. traditional analytical flow (25 µL injection).

## Sample preparation

Development of this assay was challenging due to a high degree of non-specific binding (NSB) and difficulty maintaining peptide solubility throughout the SPE extraction and elution process. SPE was performed using Oasis MAX, a mixed-mode sorbent, to enhance selectivity. To ensure glucagon stability during sample preparation and extraction, human plasma was treated with protease inhibitor cocktail. Glucagon was then spiked at various concentrations into the plasma and mixed. These samples were then acidified with acetic acid (0.5% final concentration). Acidification helped disrupt protein binding. Plasma samples were then pre-treated with 5% NH<sub>4</sub>OH in water to adjust pH prior to SPE. The diluted plasma (pH >10) samples were then applied to conditioned SPE plates. Glucagon was well retained on the SPE sorbent during the load step, with no break-through occurring. At this basic pH, glucagon will carry a net negative charge, putting it in the proper charge state to bind to Oasis MAX (quaternary amine) by ion exchange. Optimization of the elution solution was critical to maximize recovery, maintain its solubility, and minimize interferences from the plasma matrix. The optimum elution solution was 65% organic, 25% water, with 10% acetic acid.

The enhanced selectivity of the Oasis MAX SPE extraction was imperative to accurately detect and quantify

low pg/mL levels of glucagon in plasma. This is especially important where the use of the less specific 5+ precursor and ammonia loss fragment MRM transition might be necessary to achieve low limits of detection. During method development, use of reversed-phase (RP) only SPE was assessed. RP SPE yielded 10–15% greater recovery than the strong anion-exchange mixed-mode SPE (MAX). However, endogenous background was higher and yielded greater matrix effects (data not shown). In particular, matrix effects were >30% for the ammonia loss MRM transition when RP only extraction was employed. This greatly limited its use for robust quantification. Alternatively, the enhanced selectivity of the Oasis MAX SPE device greatly reduced matrix effects (<15%) and facilitated use of the less specific ammonia loss fragment for accurate quantification. In addition, the 96-well Oasis  $\mu$ Elution Plate can be processed manually in under 30 minutes and is compatible with most liquid-handling robotic systems for automation to meet sample throughput requirements. This format also provides the ability to elute in very small sample volumes, minimizes the potential for adsorptive peptide losses and chemical instability, as well as concentrates the sample for increased sensitivity.

### Specificity vs. sensitivity

Triple quadrupole mass spectrometers, when operated in MRM mode, offer a unique combination of sensitivity, specificity, and dynamic range. However, in biological matrices, the task of achieving analyte specificity can be difficult, particularly with large peptides due to the high abundance of other endogenous proteins and peptides. Thus, the challenge of improving specificity lies not only in the sample preparation, and chromatography, but also selective choice of MRM transition for analysis. In the case of glucagon, the 697.1/693.5 MRM transition, corresponding to the 5+ precursor and ammonia loss fragment, resulted in a 10X greater signal than any of the other selective precursor/fragment transitions when tested in neat standard solution (data not shown) and in extracted plasma. However, in extracted plasma samples it was not as specific and resulted in higher background noise. Figure 8 shows a 500 pg/mL extracted plasma sample, and demonstrates the increased sensitivity and lack of specificity of the ammonia loss transition compared to the selective b-ion transitions.

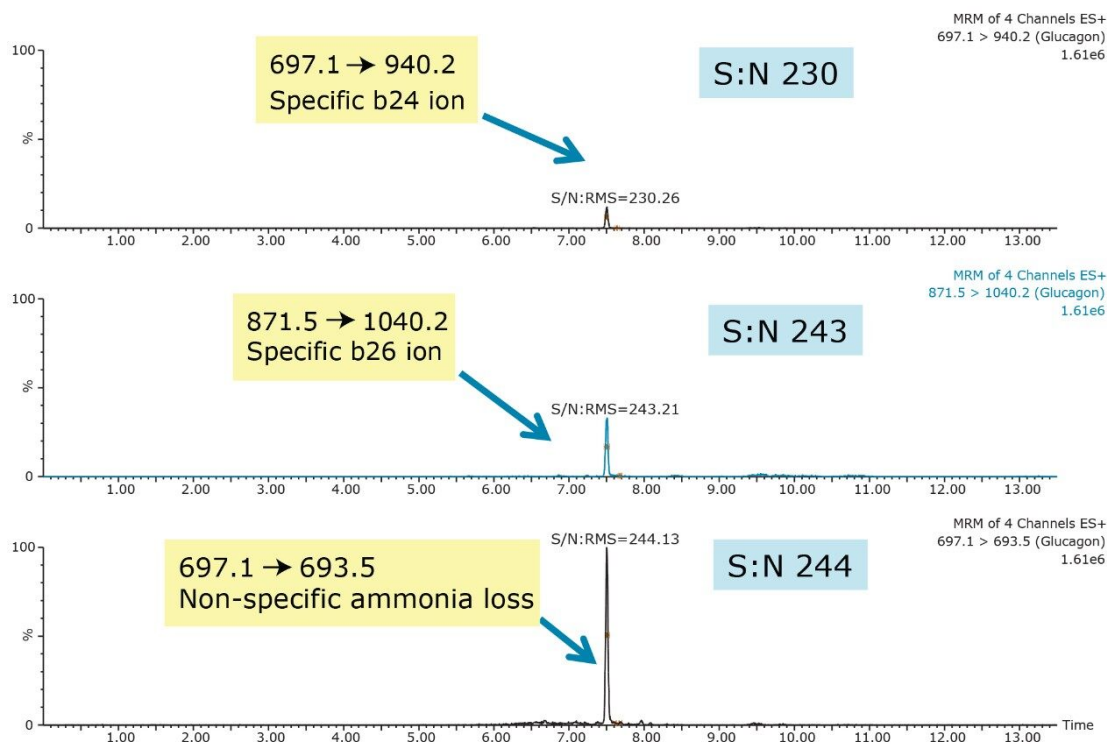


Figure 8. Comparison of glucagon MRM transition sensitivity and specificity in a 500 pg/mL extracted human plasma sample.

Although the intensity using the 697.1/ 693.5 MRM transition is 10X greater than the b-specific ion transitions (697.1>940.2 and 871.5 >1040.2), its sensitivity is mitigated by the accompanying higher background signal, as is demonstrated by the equivalent S:N ratios of all 3 MRM transitions. Additionally, measured matrix effects for the b-specific ion transitions were less than 10% in plasma, while the ammonia loss transition resulted in matrix effects between 10–15%. A summary of standard curve performance is shown in Table 3, and is illustrated in Figure 9. Using a 1/X regression, glucagon was linear from 12.5–1,000.0 pg/mL with  $R^2$  values of >0.99 for all 3 MRM transitions monitored. Representative chromatograms for extracted glucagon plasma standard samples are shown in Figure 10.

| Std. conc<br>(pg/mL) | Area  | Calc. conc.<br>(pg/mL) | %Dev  | Accuracy |
|----------------------|-------|------------------------|-------|----------|
| Blank                | —     | —                      | —     | —        |
| Blank                | —     | —                      | —     | —        |
| 12.5                 | 469   | 13.8                   | 10.5  | 89.5     |
| 12.5                 | 461   | 13.6                   | 9.0   | 91.0     |
| 25                   | 982   | 25.8                   | 3.3   | 96.7     |
| 25                   | 959   | 25.3                   | 1.1   | 98.9     |
| 50                   | 2005  | 49.8                   | -0.4  | 100.4    |
| 50                   | 2080  | 53.5                   | 6.9   | 93.1     |
| 100                  | 3958  | 95.6                   | -4.4  | 104.4    |
| 100                  | 3733  | 90.3                   | -9.7  | 109.7    |
| 250                  | 10142 | 240.5                  | -3.8  | 103.8    |
| 250                  | 9481  | 225.0                  | -10.0 | 110.0    |
| 500                  | 20893 | 492.4                  | -1.5  | 101.5    |
| 500                  | 20184 | 475.8                  | -4.8  | 104.8    |
| 1000                 | 44244 | 1039.5                 | 4.0   | 96.0     |
| 1000                 | 44094 | 1036.0                 | 3.6   | 96.4     |

Table 3. Glucagon standard curve summary statistics from 12.5–1,000.0 pg/mL extracted from human plasma.

Compound name: Glucagon 1040  
Correlation coefficient:  $r = 0.998699$ ,  $r^2 = 0.997400$   
Calibration curve:  $42.6771 * x + -120.577$   
Response type: External Std, Area  
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

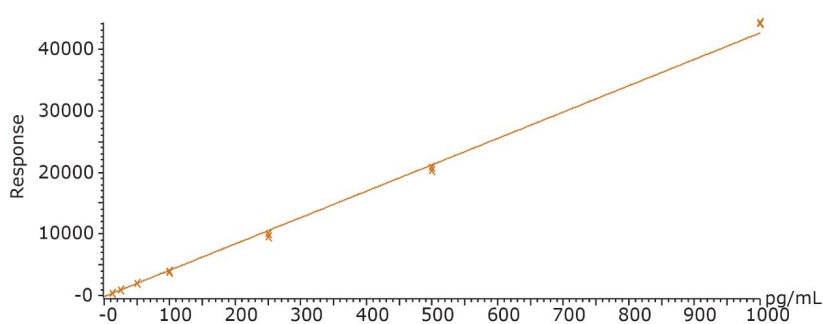


Figure 9. Representative standard curve in human plasma, from 12.5–1,000.0 pg/mL.



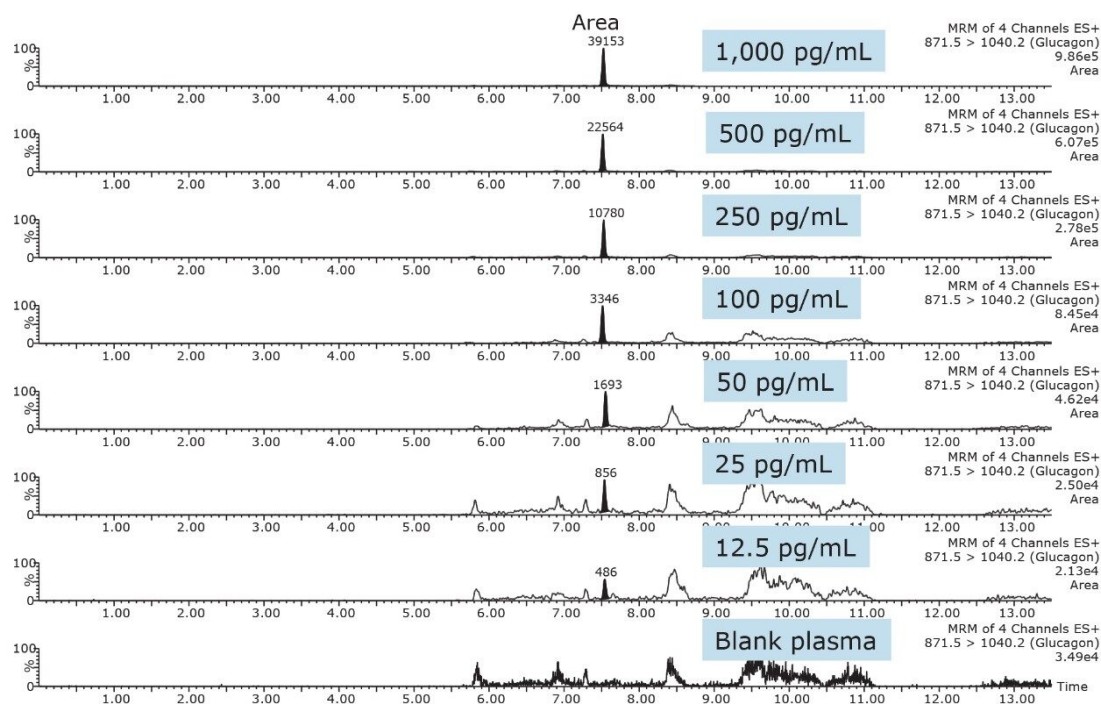


Figure 10. Representative chromatograms from glucagon extracted from plasma at 12.5, 25, 50, 100, 250, 500, and 1,000 pg/mL, compared to blank plasma.

## Conclusion

The combination of the ionKey/MS System, mixed-mode  $\mu$ Elution SPE, and higher  $m/z$  b or y ion MS fragments provided the level of selectivity and sensitivity necessary to accurately quantify low pg/mL concentrations of glucagon in extracted plasma. Use of  $\mu$ Elution format SPE eliminated the need for evaporation, reducing glucagon losses due to adsorption and non-specific binding. The 150  $\mu$ m iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method for glucagon with an LOD of 12.5 pg/mL and a dynamic range from 12.5–1,000.0 pg/mL. The current analysis uses 200  $\mu$ L of plasma and provides a significant improvement in sensitivity and S:N over the analytical scale (2.1 mm I.D.) analysis using 1/5th the sample injection volume. Furthermore, an injection of the same volume (5  $\mu$ L) of sample corresponded to a 10X increase in on-column sensitivity allowing for greater confidence in results, as compared to the traditional analytical method for this peptide. In addition, the ionKey/MS System reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for incurred sample reanalysis (ISR).

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