EXPLORING QUANTIFICATION OF PEPTIDES: MEASUREMENT OF GLUCAGON IN HUMAN PLASMA BY LC-MS/MS

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INTRODUCTION

The measurement of peptides in biological matrices presents an array of challenges many of which cannot be resolved with a generalized approach. Analyte stability, solubility, extraction recovery and sample collection need to be carefully investigated on a case by case basis. Analytical sensitivity may also represent a significant obstacle for LC-MS/MS of large peptides, however the ability of a mass spectrometer to discriminate between masses of intact peptides versus their enzymatic products allows for high method specificity. This feature is not shared by most currently available methodologies for peptide quantification such as immunochemically-based assays.

A robust LC-MS/MS method for the measurement of glucagon in human plasma for bioequivalence studies was validated in our laboratory as an example of the applicability of this methodology. The calibration range for the method is from 100 to 10,000 pg/mL. The validated method employs commercially available des-(Thr_7)-glucagon as an internal standard. The method could be optimized to measure endogenous glucagon levels at low pg/mL concentrations.

Figure 1: Amino acid sequence of glucagon and des-(Thr7)-glucagon (IS)

A 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

Molecular Weight: 3482.8 Da

B NH_2-His-Ser-Gln-Gly-Thr-Phe-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-COOH

Molecular Weight: 3381.7 Da

METHOD OVERVIEW

Aliquots of human plasma (0.250 mL) were spiked with an internal standard and diluted with glycine buffer. Samples were loaded to a 96-well ion-exchange plate and washed with glycine buffer followed by organic solvents. Elution was performed by ammonia hydroxide in organic solvent. After drying, the samples were reconstituted in 25% ACN with formic acid and analyzed using AB/ Sciex API 4000 tandem mass spectrometer in the multiple-reaction-monitoring mode. An Agilent Technologies Zorbax Rapid Resolution 300SB-C18, 50 x 2.1 mm, 3.5 µm analytical column with a mobile phase containing 30% ACN with formic acid was used for LC-MS/MS separation.

RESULTS

Validation Summary

Sample volume	0.250 mL
Standard Curve Range	100-10,000 pg/mL
Dilution integrity	up to 25,000 pg/mL
Short-term matrix stability	14 hours on ice water bath
Freeze/thaw stability	6 cycles at -80 °C
Processed sample integrity	128 hours at 5 °C
Post-preparative stability	129 hours at 5 °C
Sample collection stability (whole blood)	2 hours on ice water bath

Table 1: Inter-Batch Precision and Accuracy for Glucagon in Human Plasma (EDTA)

Batch	LLOQ QC 100 pg/mL	QC A 300 pg/mL	QC B 1000 pg/mL	QC C 7500 pg/mL
Inter-Batch Mean	103	318	1060	7890
Inter-Batch SD	11.0	20.8	23.2	262
Inter-Batch % CV	10.7	6.5	2.2	3.3
Inter-Batch % Bias	3.0	6.0	6.0	5.2
n	18	18	18	18

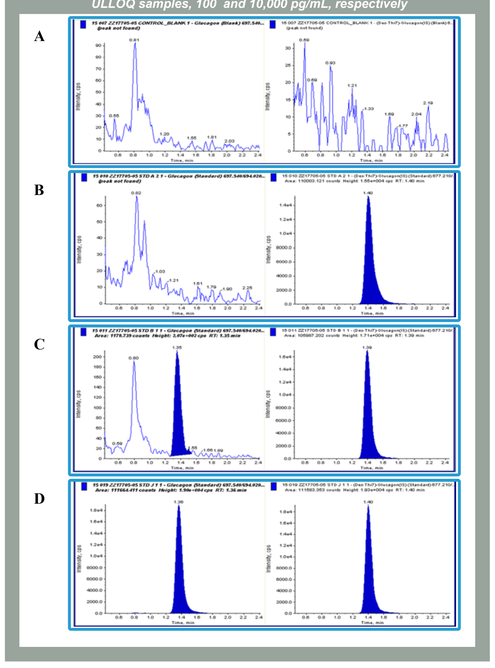
Table 2: Matrix Effect for Glucagon in Human Plasma (EDTA)

Batch	Lot#	LLOQ 100 pg/mL	% Bias	High 7500 pg/mL	% Bias
	1	101	+1.0	8100	+8.0
	2	109	+9.0	8480	+13.1
	3	95.8	-4.2	8370	+11.6
	4	118	+18.0	8330	+11.1
	5	111	+11.0	8680	+15.7
	6	96.7	-3.3	8240	+9.9
Inter-Batch Mean		105		8370	
Inter-Batch % CV		8.4		2.4	
Inter-Batch % Bias		+5.0		+11.6	
n		6		6	

Table 3: Recovery Data of Glucagon from Human Plasma (EDTA)

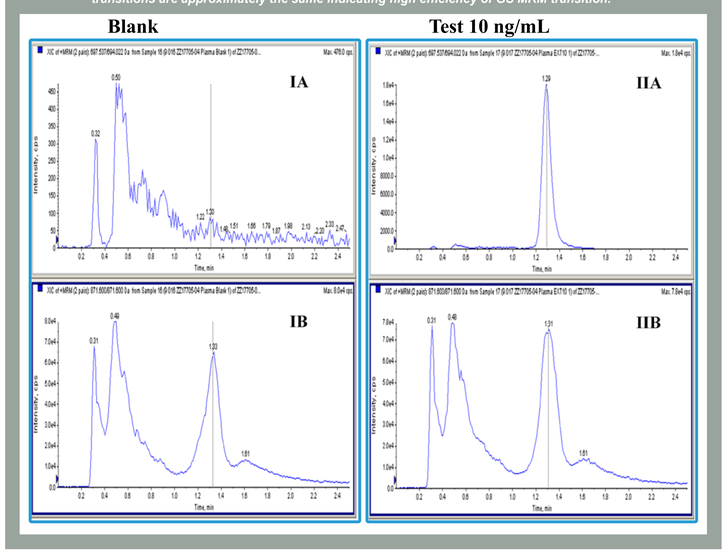
Theoretical Concentration:	300 pg/mL Peak Area		1000 pg/mL Peak Area		7500 pg/mL Peak Area	
	Extracted	Unextracted	Extracted	Unextracted	Extracted	Unextracted
	3395	7231	9557	21829	81065	173225
	2855	6964	10452	24084	76302	160634
	3701	6736	10136	22627	86073	178188
	3048	6593	10705	23077	77406	155176
	3219	7327	9860	20508	88088	160092
	2735	6447	11168	22759	82284	155780
Mean	3159	6883	10313	22481	81870	163849
% CV	11.3	5.1	5.7	5.4	5.7	5.8
% Recovery	46		46		50	
n	6	6	6	6	6	6

Figure 2: A, extracted blank plasma; B, extracted plasma spiked with internal standard only; C and D, extracted LLOQ and ULLOQ samples, 100 and 10,000 pg/mL, respectively



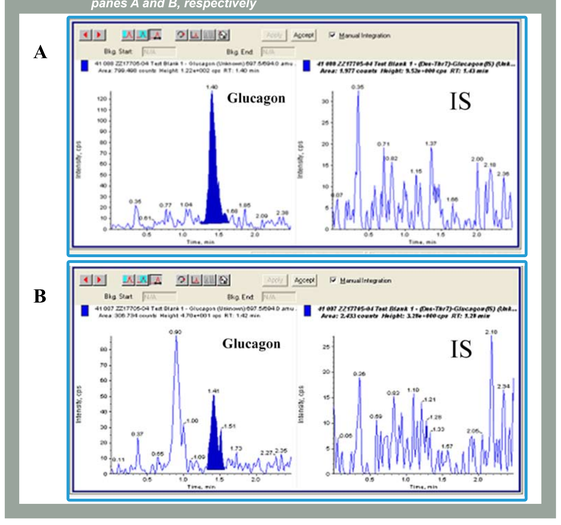
SELECTIVITY OF GLUCAGON DETECTION

Figure 3: Comparison of MRM selectivity of glucagon detection. A selective fragmentation of multiply-charged glucagon ions (glucagon selective MRM, GS MRM) was established complementary to the developed SPE. Chromatograms of the unspiked blank (left panels) and spiked (10 ng/mL) extracted human plasma (right panels) for GS MRM (A) and M+4→M+4 (B) transitions, top and bottom panels, respectively. Note the relative increase of the analyte response in spiked samples for the both transitions are approximately the same indicating high efficiency of GS MRM transition.



EVALUATION OF GLUCAGON ENDOGENOUS LEVEL

Figure 4: Examples of extracted unspiked human plasma (API4000): extrapolated endogenous level approximately 55 and 25 pg/mL, panes A and B, respectively



ENHANCEMENT OF GLUCAGON STABILITY IN PLASMA

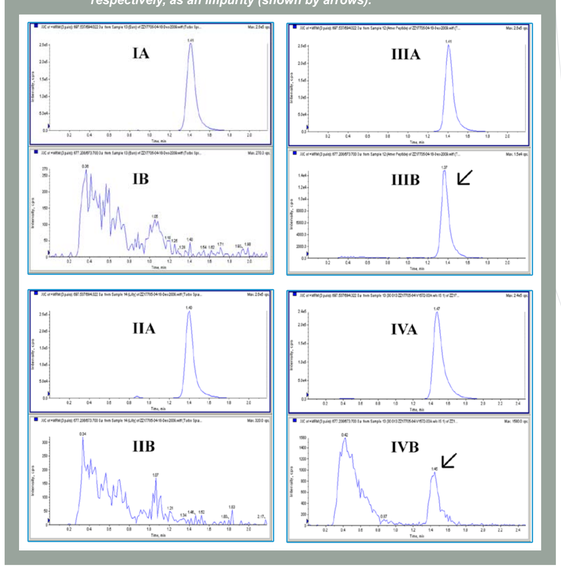
Table 4: Glucagon stability in the presence of inhibitor cocktail vs stability in the presence of aprotinin only

Inhibitor	Aprotinin 250 KIU/mL		Inhibitor Cocktail	
QC	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
% Control				
n		36.9		97.7

QC samples (6000 pg/mL) were prepared in human plasma (EDTA) containing aprotinin (250 KIU/mL) or inhibitor cocktail. Control QC samples were thawed on an ice water bath for 2.5 hours, short-term stability (STS) QC were incubated on an ice water bath for 17 hours

CHARACTERIZATION OF GLUCAGON REFERENCE MATERIAL

Figure 5: Comparison of glucagon reference material from several commercial sources (I-IV). The top panels (A) of each chromatogram are glucagon channels; the bottom panels (B) are chromatograms monitoring des-(Thr_{29})-glucagon impurities in the samples. I and II are chromatograms of European Pharmacopeia Reference Standard and glucagon from commercially available Eli Lilly Glucagon Emergency Kit, respectively (both recombinant peptides). Synthetic glucagon from manufacturer III and IV have approximately 5.4 and 0. 4% of des-(Thr_3)-glucagon, respectively, as an impurity (shown by arrows).



CONCLUSION

A sensitive, accurate and reproducible method for glucagon was developed and validated with improved selectivity as compared to currently available immunochemical methods.
The method incorporates a modified sample collection procedure to enhance the analyte stability in plasma. The method could be potentially optimized to measure endogenous glucagon levels at low pg/mL concentrations using a more sensitive mass spectrometry platform.

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