A Sensitive and Robust Method for the Determination of Glucagon in Human Plasma by UHPLC-MS/MS

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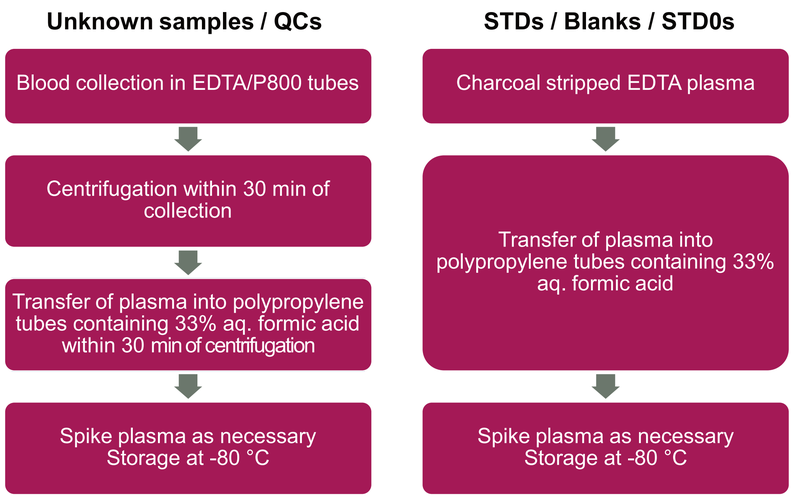
Introduction

The peptide hormone glucagon plays an important role in homeostasis of glucose concentrations in the blood and serves as a biomarker for pathologies such as diabetes or pancreatic cancer. Reliable assays for determination of glucagon levels in biological samples are therefore important in order to gain a better understanding of the pathology and treatment of such diseases. Over the years there have been numerous glucagon assays available that mainly employ an immunochemical approach. However, these methods may suffer from cross-reactivity, lack of specificity and selectivity for determination of low glucagon concentration levels. Recent advances in MS instrumentation and the amenability of glucagon for analysis by LC-MS/MS have brought these new methods to the forefront to support quantification of endogenous glucagon levels in the low pg/mL range. Here we present a sensitive and robust fully automated LCMS/MS method that was developed and validated according to FDA and EMA guidelines allowing for quantification of glucagon from 20.0 – 2000 pg/mL in human plasma.

Sample Collection and Standard (STD) / Quality Control (QC) Sample Preparation

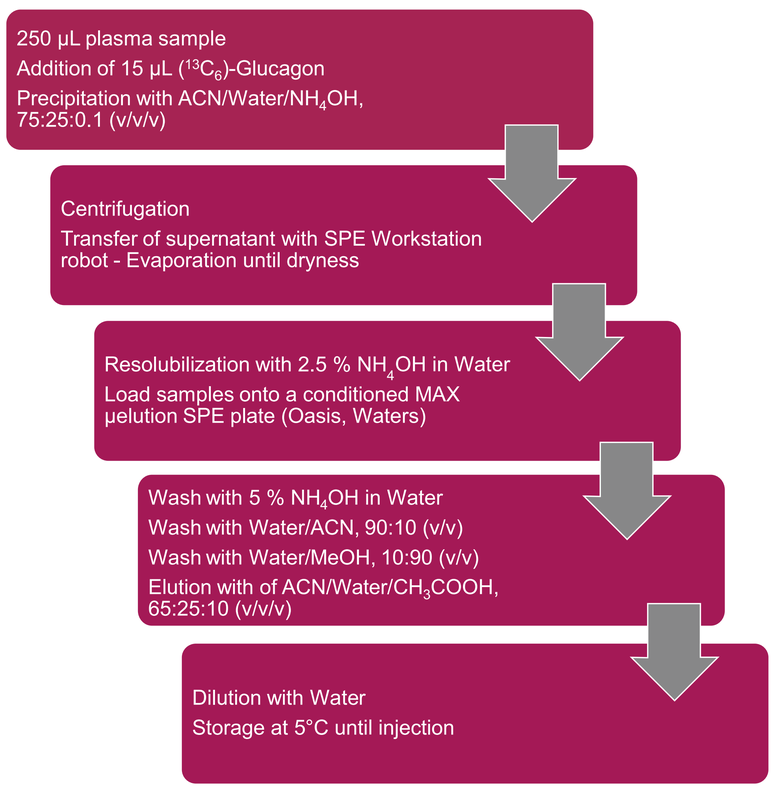
Figure 1: Workflow for clinical sample collection and QC, STD sample preparation.



Glucagon can rapidly get enzymatically degraded in plasma. Therefore, blood collection was done in the presence of protease inhibitors (P800 tubes from BD) and plasma samples were acidified. QC Low, Mid and High samples were prepared by determining endogenous glucagon levels in human plasma samples. Several samples were pooled in order to obtain an appropriate basal glucagon level (QC Low). Next, exact determination of the basal level in the pool (3 runs with n=6 replicates of the pool) was performed. QC Mid and High samples were prepared by spiking human glucagon on top of the basal level. QC LLOQ, STD, Blank and STD0 samples were prepared in acidified charcoal stripped plasma.

Extraction procedure

Figure 2: Detailed extraction procedure combining protein precipitation and solid phase extraction (SPE).



The extraction was automated allowing a higher throughput and better extraction reproducibility. For this purpose, the SPE Workstation robot from Fornax Technologies was programmed to transfer the supernatant of the protein precipitation and to perform the entire SPE work-up.

UHPLC-MS/MS conditions

Table 1: Chromatographic conditions and MS/MS parameters using Sciex TQ 6500 and 6500+ instruments.

Chromatographic conditions		
Autosampler UPLC Pumps	Flow through needle UPLC from Waters Binary solvent manager from Waters	
Analytical column Pre-filter	Supelco Ascentis Express C18, 50 × 2.1 mm, 2.7 µm Krudkatcher from Phenomenex, 0.5 µm porosity	
Mobile phase A Mobile phase B	Water/Formic acid, 0.1% ACN/Formic acid, 0.1%	
Flow rate Column temperature Injection volume	0.6 mL/min 50 °C 45 µL	
Gradient	Loading on the column at 5% B and then elution by increasing % B from 5 to 35% within 6.0 min Wash step at 95% B and re-equilibration at 5% B	
MS/MS conditions		
Mass spectrometer	SCIEX TQ 6500	SCIEX TQ 6500+
Source/Polarity	ESI / Positive	
Followed MRM transitions (unit resolution)	Glucagon: m/z 697.6 → m/z 813.4 ($^{13}C_6$)-Glucagon: m/z 698.7 → m/z 814.8	
DP (V)	45	45
CE (V), CAD (units)	21, 12	21, 12
Ion spray voltage (V)	5500	5500
Source temperature (°C)	600	450
CUR, GS1, GS2 (units)	30, 60, 40	40, 40, 40

Results

Figure 3: Typical chromatograms of the LLOQ in charcoal stripped plasma (blue) and QC Low in P800 plasma (red).

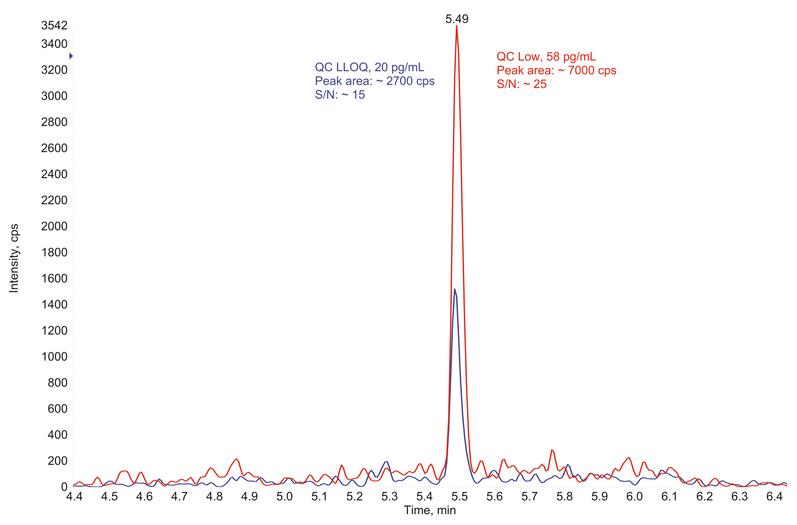


Figure 4: Typical chromatograms of QC High in P800 plasma (red) and Standard ULOQ in charcoal stripped plasma (blue).

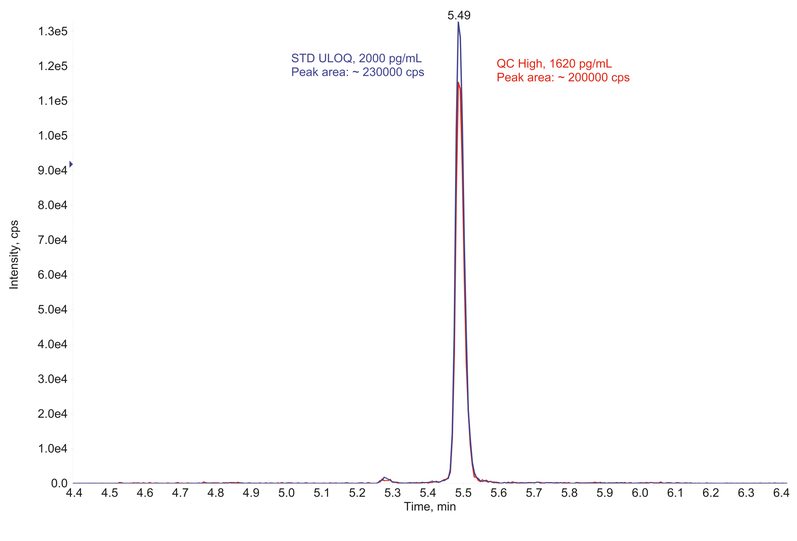


Figure 5: Calibration curve from 20.0 to 2000 pg/mL, linear regression, 1/x weighting factor. Carry-over in the first blank sample following an ULOQ sample was acceptable (approximately 14.0% of the LLOQ response).

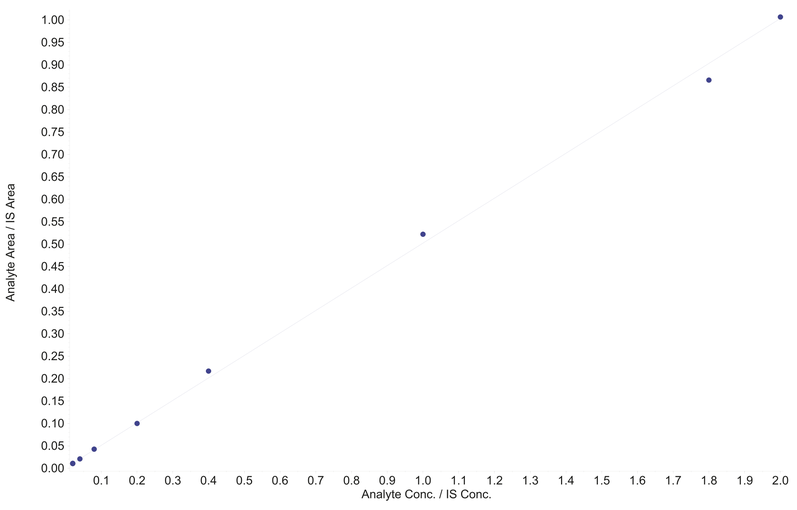


Table 2: Intra- and inter-run precision and accuracy of three independent runs measured on Sciex TQ 6500 and 6500+ instruments.

Intra-batch and inter-batch precision and accuracy results					
		QC LLOQ 20.0 pg/mL	QC Low 58.3 pg/mL	QC Mid 203 pg/mL	QC High 1620 pg/mL
Run 1 (TQ 6500)	Accuracy (%) CV (%) N	107.9 9.9 6	104.9 4.7 6	100.2 4.8 6	103.7 2.3 6
Run 2 (TQ 6500)	Accuracy (%) CV (%) N	91.0 3.9 6	107.5 7.9 6	103.3 4.9 6	109.7 2.1 6
Run 3 (TQ 6500+)	Accuracy (%) CV (%) N	99.0 11.4 6	103.3 5.4 6	99.3 3.8 6	101.5 1.7 6
Inter-batch Precision and Accuracy	**Accuracy (%) CV (%) N**	**99.3 8.5 18**	**105.2 2.0 18**	**100.9 2.1 18**	**105.0 4.0 18**

Table 3: Cumulated freeze/thaw and bench top stability results could be shown in P800 plasma. In addition stability in the autosampler was shown for at least 96 hours at 5°C.

Cumulated freeze/thaw (3 cycles) and bench top (9 hours) stability in P800 plasma		
	QC Low 58.3 pg/mL	QC High 1620 pg/mL
Mean measured (pg/mL) Accuracy (%) CV (%) N	61.9 106.2 6.3 6	1660 102.5 1.4 6

Table 4: Long-term stability at -80°C in charcoal stripped EDTA plasma and in P800 plasma.

Long-term stability in P800 plasma and charcoal stripped plasma (EDTA) at -80 °C		
P800 plasma 158 days	QC Low 55.9 pg/mL	QC High 1630 pg/mL
Mean measured (pg/mL) Accuracy (%) CV (%) N	59.9 107.2 10.3 6	1630 100.0 3.8 6
Charcoal stripped plasma 60 days	QC 40 40.0 pg/mL	STD 1800 1800 pg/mL
Mean measured (pg/mL) Accuracy (%) CV (%) N	39.7 99.2 5.5 6	1720 95.4 5.0 6

Table 5: Matrix effect determination at QC Low level.

Matrix effect assessment at QC Low level in individual P800 matrices				
Individual	Endogenous concentration in Individuals (pg/mL)	Measured concentration after spike of 60.0 pg/mL	Difference between spiked and endogenous concentration (pg/mL)	Bias (%) compared to nominal spiked concentration of 60.0 pg/mL
1	28.7	79.4	50.7	**-15.5**
2	32.0	84.4	52.5	**-12.6**
3	21.9	83.8	61.8	**3.1**
4	25.9	84.1	58.2	**-3.0**
5	25.4	88.6	63.3	**5.4**
6	47.8	108.7	60.9	**1.5**
7_Hemolyzed	25.9	85.1	59.2	**-1.4**
8_Hemolyzed	23.7	75.6	51.9	**-13.5**
9_Hemolyzed	48.3	114.9	66.6	**11.0**
Mean (pg/mL) Accuracy (%) CV (%) N			**58.3 97.2 9.5 9**	

Table 6: Matrix effect determination at QC High level.

Matrix effect assessment at QC High level in individual P800 matrices				
Individual	Endogenous concentration in Individuals (pg/mL)	Measured concentration after spike of 1600 pg/mL	Difference between spiked and endogenous concentration (pg/mL)	Bias (%) compared to nominal spiked concentration of 1600 pg/mL
1	28.7	1502	1473	**-7.9**
2	32.0	1660	1628	**1.8**
3	21.9	1587	1565	**-2.2**
4	25.9	1668	1642	**2.6**
5	25.4	1582	1557	**-2.7**
6	47.8	1651	1603	**0.2**
7_Hemolyzed	25.9	1704	1704	**6.5**
8_Hemolyzed	23.7	1633	1633	**2.1**
9_Hemolyzed	48.3	1721	1721	**7.6**
Mean (pg/mL) Accuracy (%) CV (%) N			**1614 100.9 4.7 9**	

Conclusion

- The validated dynamic range of this method allows for quantification of endogenous as well as dosed glucagon in human plasma.
- Sample stability could be shown during the entire bioanalytical process.
- The robustness of the developed method allowed for analysis of more than 2000 clinical samples with a run success rate of 93%. Incurred sample reanalysis (ISR) was successful with 94% matching rate.

Poster presentation at EBF 2018