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Selective and sensitive quantification of glucagon in human plasma using microflow LC/Q-TOF MS

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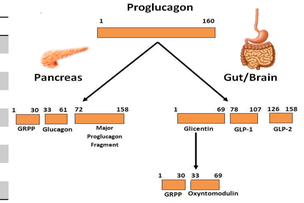
1. Overview

In this study, we developed both a simplified protocol of sample pretreatment and a sensitive quantitation method for plasma glucagon using a microflow LC and a high resolution accurate mass spectrometer with microflow ESI.

2. Introduction

Impaired secretion of endogenous bioactive peptides such as peptide hormones and cytokines is associated with the development and pathophysiology of various diseases. Glucagon is a peptide hormone associated with diabetes and known to increase blood glucose levels. There are several peptide hormones generated from the same precursor protein as glucagon (Figure 1) . These glucagon-related peptides hamper specific detection of glucagon by a conventional immune assay due to its resembled structure. To selectively quantify glucagon in human plasma, we developed a sensitive method using a microflow LC/Q-TOF.

Source	Peptide hormones
Pancreas	Glucagon
	Major proglucagon fragment
	GRPP
Gut & Brain	Glicentin 1
	Glicentin 2
	GLP-1
	GLP-2
	GRPP
	Oxyntomodulin



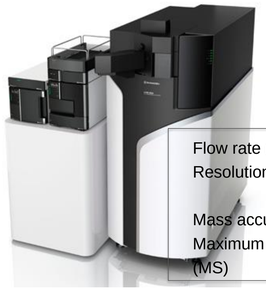
Structural Homology between Glucagon-related Hormones

Glucagon		HSQGTFTSDYSKYLDSRR	AQDFVQWLMNT	
Mini-glucagon			AQDFVQWLMNT	
Oxyntomodulin		HSQGTFTSDYSKYLDSRR	AQDFVQWLMNT	KRNRNNIA
Glicentin (1)	RSLQDTEEKSRSFSASQADPLSDPDQMNEDKR	HSQGTFTSDYSKYLDSRR	AQDFVQWLMNT	KRNRNNIA
Glicentin (2)	RSLQDTEEKSRSFSASQADPLSDPDQMNEDKR	HSQGTFTSDYSKYLDSRR	AQDFVQWLMNT	

Figure 1 Glucagon-related holmones

3. Methods

Intact glucagon was analyzed using a quadrupole time of flight mass spectrometer (LCMS-9030; Shimadzu, Japan) coupled with microflow liquid chromatography (Nexera Mikros; Shimadzu). The LC separation was carried out with binary gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile using a combination of an analytical column (HALO C18 2.7 µm, 0.2 mmID x 100 mmL, Advanced Materials Technologies) and a trap column (L-Column2 C8, 5 µm, 0.2 mmID x 100 mmL, CERI). The absolute concentration of each peptide was calculated from the calibration curve using the peak area of external standard. 500 µl of human plasma was diluted with an equal volume of 5% ammonium hydroxide in water and pretreated by solid phase extraction using EVOLUTE EXPRESS AX 30 mg (Biotage, Sweden).



Flow rate (LC)	: 0.1 – 500 µL/min
Resolution power (MS)	: > 30,000 FWHM at m/z 1,972 / 1,626
Mass accuracy (MS)	: 1 ppm
Maximum acquisition rate (MS)	: 100 Hz

Figure 2 Micro-flow LC-Q-TOF System

Table 1 Analytical condition

LC condition (Nexera Mikros)

Trap column	L-Column2 C8, 5 µm, 0.2 mmID x 100 mmL
Flow rate (Trap)	20 µL/min
Mobile phase (Trap)	0.05 % TFA / water
Analytical column	HALO C18 2.7 µm, 0.2 mmID x 100 mmL
Gradient	Binary gradient
Mobile phase	A. 0.1 % formic acid / water B. 0.1 % formic acid / water
Flow rate	3 µL/min
Column oven	40 degree C
Injection vol.	0.2 (STD) or 10 µL (Plasma)

MS condition (LCMS-9030 with microflow ESI)

Ionization	ESI (Positive)
Ion source	Grass ESI capillary
Analytical mode	MS1 scan
CID gas	230 kPa
IF voltage	-1.8 kV
TOF range(m/z)	500.0000-1300.0000
Event time	0.100 s
Neb gas	0.1 L/min
Heating gas	Off
Drying gas	Off
IF temp.	Off
DL temp.	250 ºC
HB temp	500 ºC

4. Results

4-1. Development of analytical method for intact peptide hormones using a microflow LC-QTOF system.

Intact glucagon is detected as multiple charged ions. Mainly observed charge states are 3+ to 5+ (Figure 2). There is no significant difference in charge-state distribution between semi-microflow LC and microflow LC (Figure 3. *left*). The sensitivity of microflow-LC ESI was 10-fold higher than that of semi-microflow ESI.

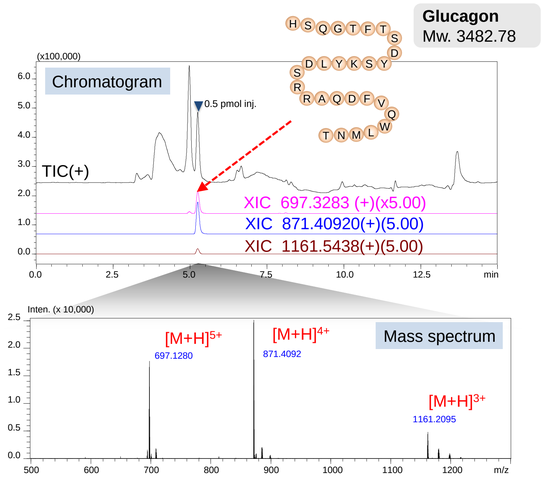


Figure 3. Evaluation of analytical method using standard sample.

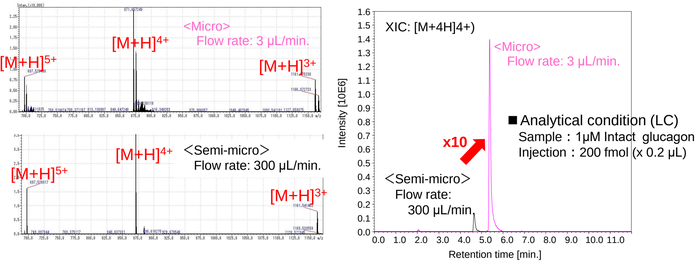


Figure 4. Comparison of charge-state distribution and sensitivity between semi-microflow LC and microflow LC

4-2. Quantitative analysis of plasma sample spiked with glucagon

To prevent degradation of glucagon by endogenous proteases, samples were collected using a blood collection tube containing a protease inhibitor cocktail. HPLC and ESI conditions were optimized by measuring intact peptides. Sensitivity, reproducibility, and accuracy of each peptide measurement were evaluated using standard peptides spiked into human pooled plasma. As a result, the lower limits of detection for glucagon were estimated as lower than 10 pM (Figure 6). According to previous reports using conventional immunoassay, the normal level of plasma glucagon is approximately 10-50 pM. Thus, our results indicate that the method described here is potentially useful for quantification of endogenous glucagon.

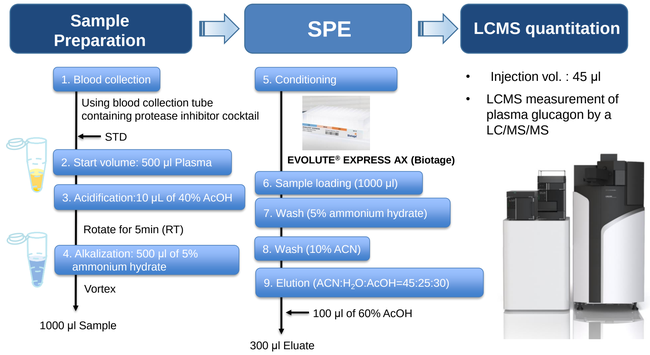
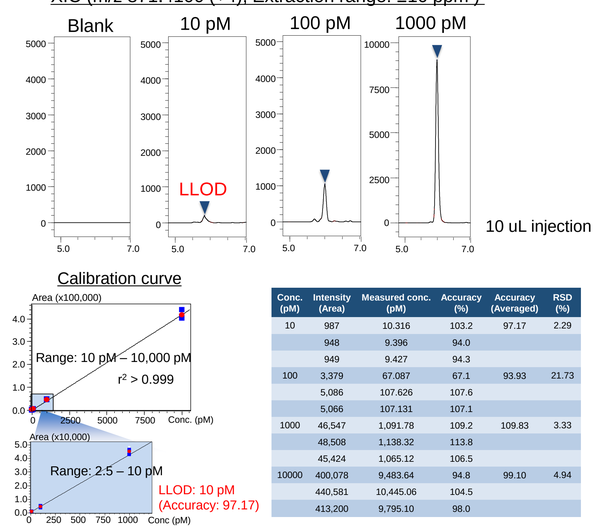


Figure 5. Procedure of sample preparation for glucagon in plasma sample



Conc. (pM)	Intensity (Area)	Measured conc. (pM)	Accuracy (%)	Accuracy (Averaged)	RSD (%)
10	987	10.316	103.2	97.17	2.29
	948	9.396	94.0		
	949	9.427	94.3		
100	3,379	67.087	67.1	93.93	21.73
	5,086	107.626	107.6		
	5,066	107.131	107.1		
1000	46,547	1,091.78	109.2	109.83	3.33
	48,508	1,138.32	113.8		
	45,424	1,065.12	106.5		
10000	400,078	9,483.64	94.8	99.10	4.94
	440,581	10,445.06	104.5		
	413,200	9,795.10	98.0		

Figure 6. Quantitative result of glucagon in plasma sample

5. Conclusions

- The sensitivity of microflow-LC ESI was 10-fold higher than that of semi-microflow ESI.
- Plasma glucagon was successfully quantified at endogenous conc. level by the optimized sample preparation protocol and the sensitivity of the developed method using microflow-LC Q-TOF.