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Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

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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 known to increase blood glucose levels. Glucagon-like peptide 1 (GLP-1), a peptide hormone generated from the same precursor as glucagon, regulates glucose metabolism by enhancing insulin secretion from pancreatic β-cells.
Because of similarities between amino acid sequences of glucagon and glucagon-related peptides derived from proglucagon (Figure 1), the quantification of glucagon in blood by conventional immunoassay methods have been hampered by cross-reactivity of anti-glucagon antibodies with glucagon-related peptides.
In the present study, to selectively quantify these peptide hormones in human plasma, we developed a sensitive method using a LC/MS/MS.

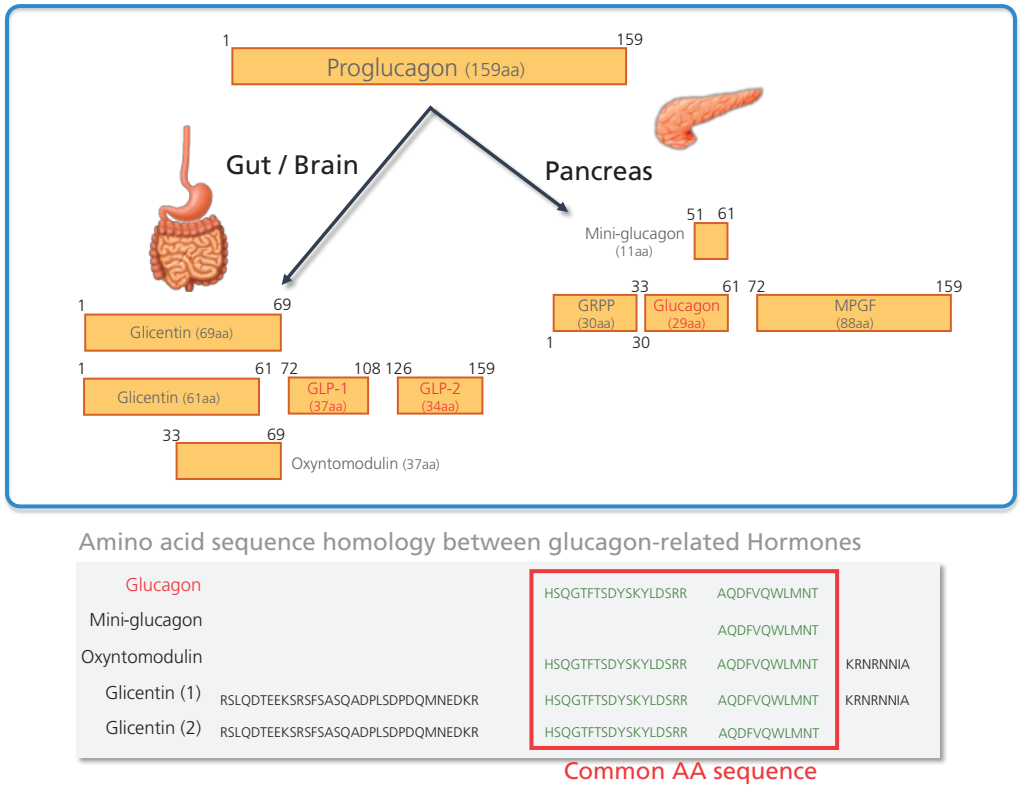

Figure 1. Processing of proglucagon in pancreas and gut / brain.

Methods

Intact peptides (insulin, glucagon, GLP-1 (7-36) amide, GLP-1 (7-37), exenatide, and liraglutide) were analyzed using a triple quadrupole mass spectrometer (LCMS-8060; Shimadzu, Japan) coupled with conventional flow liquid chromatography (Nexera *X2*; Shimadzu). The LC separation was performed using Shim-pack ODS II column (1.6 µm, 2.0 mm × 150 mm, Shimadzu) or Kinetex 2.6u XB-C18 100A (2.1 mm × 100 mm, Phenomenex) with binary gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The absolute concentration of each peptide was calculated from the calibration curve using the peak area of external standard. Plasma samples were collected using a blood collection tube containing protease inhibitors cocktail to prevent degradation of glucagon and glucagon-related peptides, and pretreated by solid phase extraction using EVOLUTE EXPRESS AX 30 mg (Biotage, Sweden).

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Result

Development of analytical method for intact peptide hormones by a LCMS-8060

Peptide hormones are detected as multiple charged ions. Mainly observed charge distribution of glucagon and insulin are 3+ to 5+ and 4+ to 6+, respectively (Figure 2). Sensitivity of measurement was evaluated using standard peptides (Figure 3). HPLC and SRM conditions for simultaneous analysis of insulin, glucagon, GLP-1, and GLP-1 analogues were optimized by measuring each intact peptide (Figure 4).

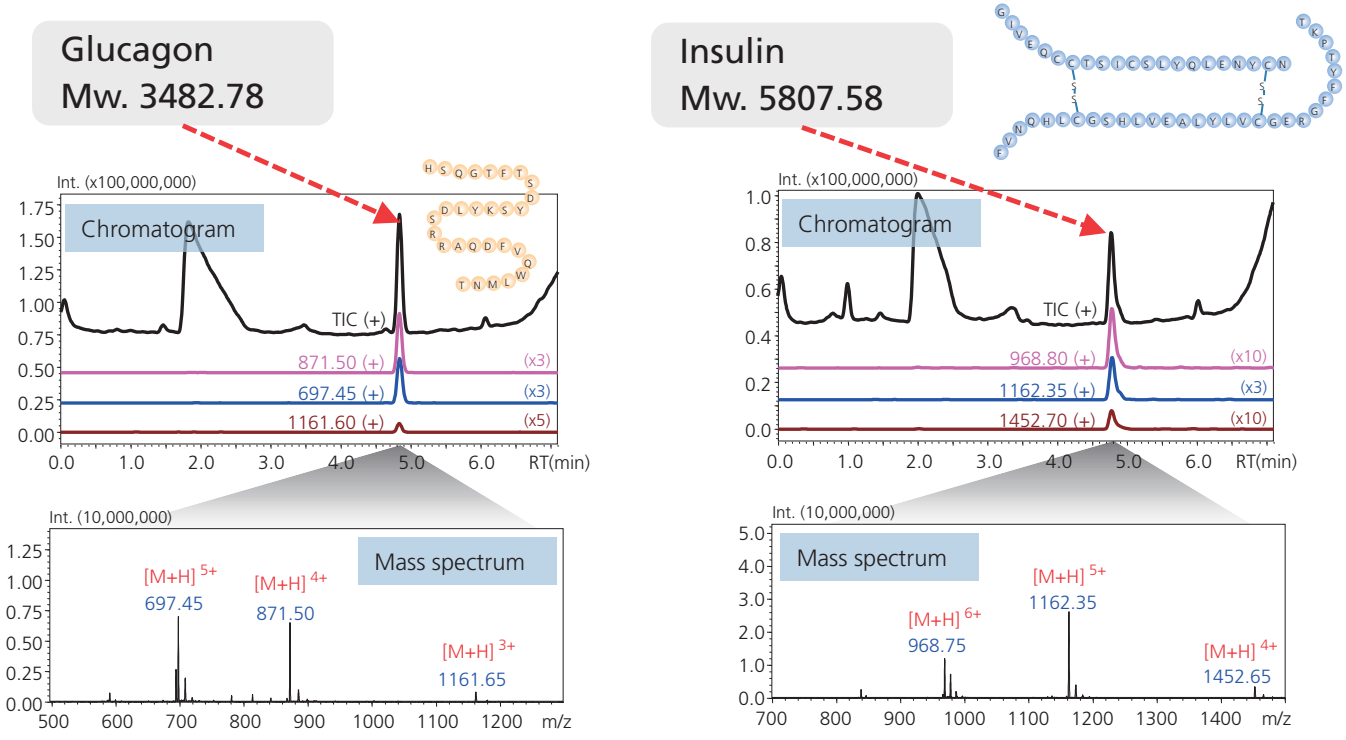

Figure 2. Precursor scan analysis of intact glucagon and insulin.

HPLC conditions (Nexera X2)	
Column	: Shim-Pack ODS II (2.0mmI.d x 150 mm)
Column temperature	: 40 deg. C
Mobile phase A	: 0.1 % formic acid / water
Mobile phase B	: 0.1 % formic acid / acetonitrile
Solvent for sample loading	: 0.1% formic acid / water
Flow rate	: 0.3 mL/min
Total cycle time	: 10 min
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive
Gas flow	: 2.5/ 10/ 5.0 L/min (Neb./ Heating/ Drying)
Temp.	: 350/ 250/ 500 deg. C (IF/ DL/ Heat block)
CID gas	: 350 kPa

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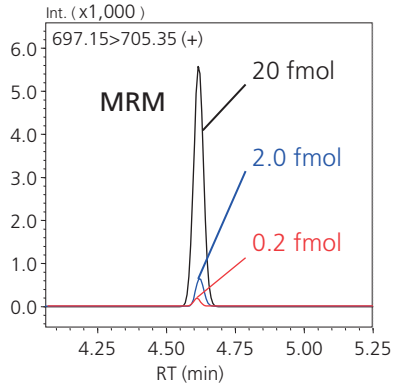
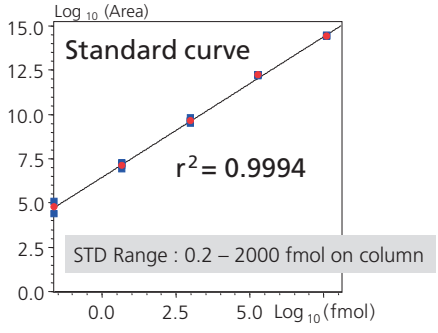
MRM parameter

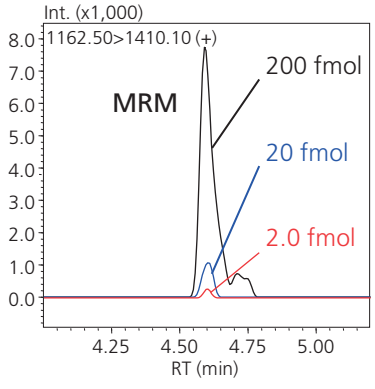
Protein	Charge	Transition	CE	Type
Glucagon	5+	697.15>705.35	-23	target
	4+	871.15>225.10	-40	ref.
	4+	871.15>940.10	-30	ref.
	4+	871.15>1002.15	-29	ref.
	5+	697.15>1002.15	-22	ref.
	5+	697.15>751.85	-19	ref.
Insulin	5+	1162.50>1410.10	-34	target
	5+	1162.50>1129.40	-34	ref.
	5+	1162.50>1158.40	-25	ref.
	6+	968.95>651.85	-24	ref.

Glucagon





Insulin



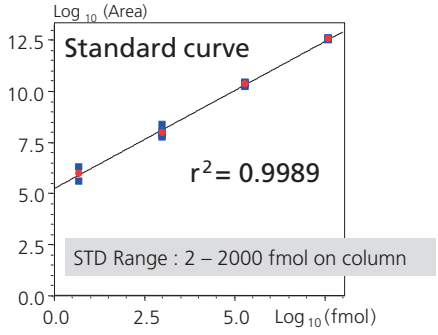


Figure 3. Evaluation of analytical method using standard sample.

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

HPLC conditions (Nexera X2)	
Column: Kinetex 2.6u XB-C18 100A (2.1 mm × 100 mm, Phenomenex)	
Column temperature	: 40 deg. C
Mobile phase A	: 0.1 % formic acid / water
Mobile phase B	: 0.1 % formic acid / acetonitrile
Solvent for sample loading	: 0.1% formic acid / water
Flow rate	: 0.15mL/min
Total cycle time	: 13 min

MS conditions (LCMS-8060)	
Ionization	: ESI, Positive
Gas flow	: 2.5/ 10/ 5.0 L/min (Neb./ Heating/ Drying)
Temp.	: 350/ 250/ 500 deg. C (IF/ DL/ Heat block)
CID gas	: 350 kPa

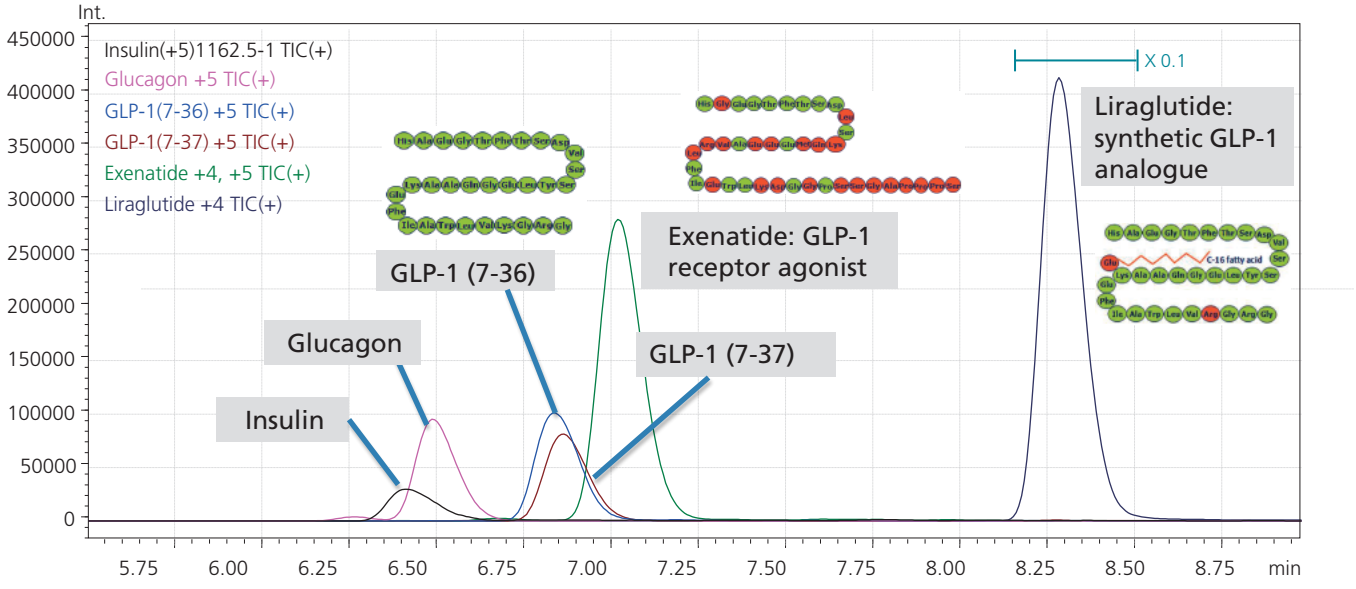


Figure 4. Analytical condition and MRM chromatogram of insulin, glucagon, GLP-1, and GLP-1 analogues (10 fmol of each intact peptides standards).

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Sample preparation for glucagon in plasma sample (Figure 5)

To release a protein-protein interaction of glucagon in blood, 500 µl of plasma collected using blood collection tube containing protease inhibitor cocktail was acidified with 10 µl of 40% acetic acid at once. Then, to alkalize the solutions, 500 µl of 5% ammonium hydrate are added to samples. Total 1000 µl of sample is applied to solid phase extraction. Glucagon is eluted with 200 µl of elution buffer and diluted with 100 µl of 60% acetic acid. Of the 300 µl of eluate, 45 µl is subjected to LC-MS/MS analysis.

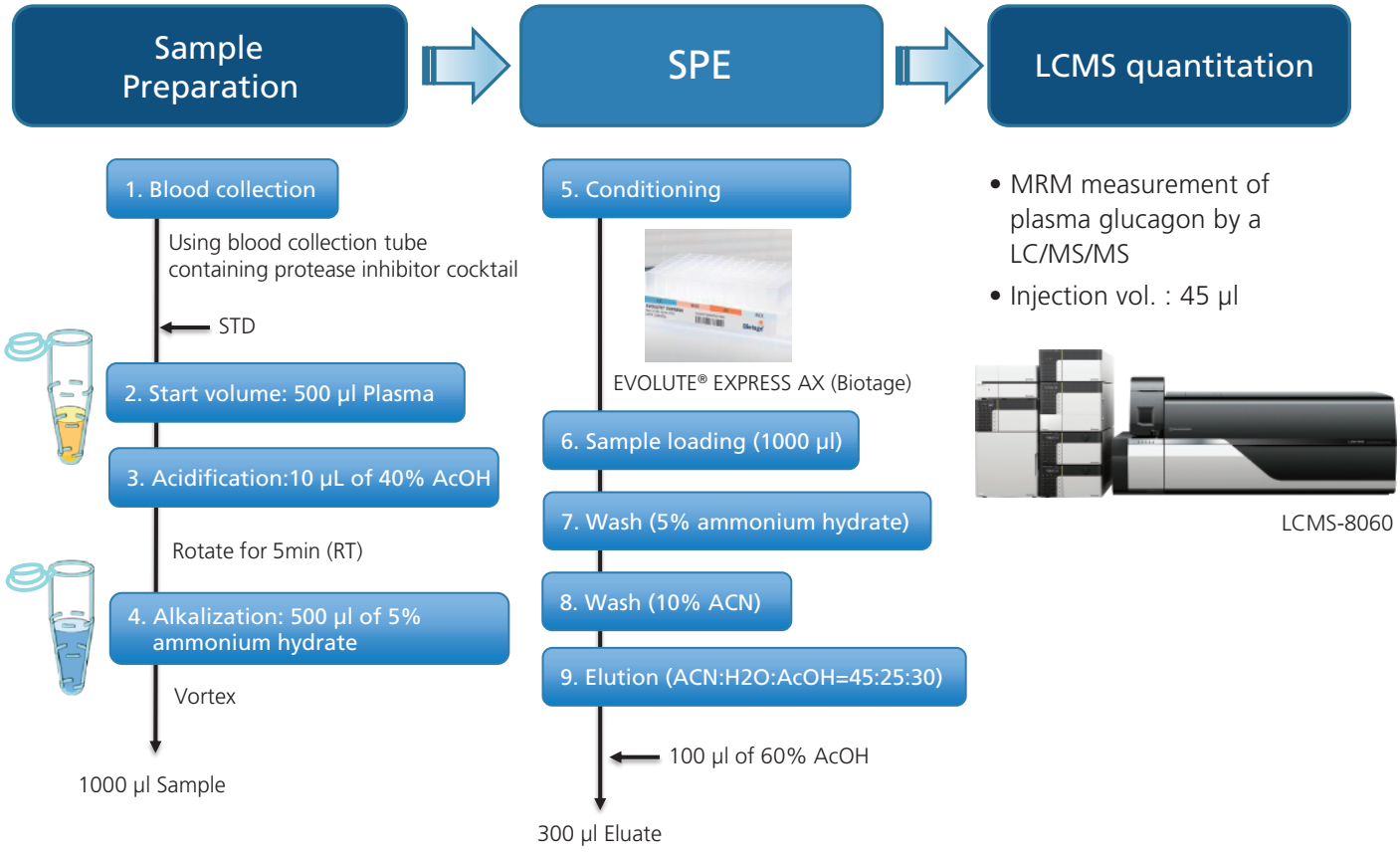

Figure 5. Procedure of sample preparation for glucagon in plasma sample

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Quantitative analysis of plasma sample spiked with glucagon

The lower limit of detection for glucagon was estimated as 2.5 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.

- Sample: Glucagon spiked in pooled plasma
- 45 µL injection (n=2)
- Averaged accuracy was 99.8% (104.9% at 2.5 pM)

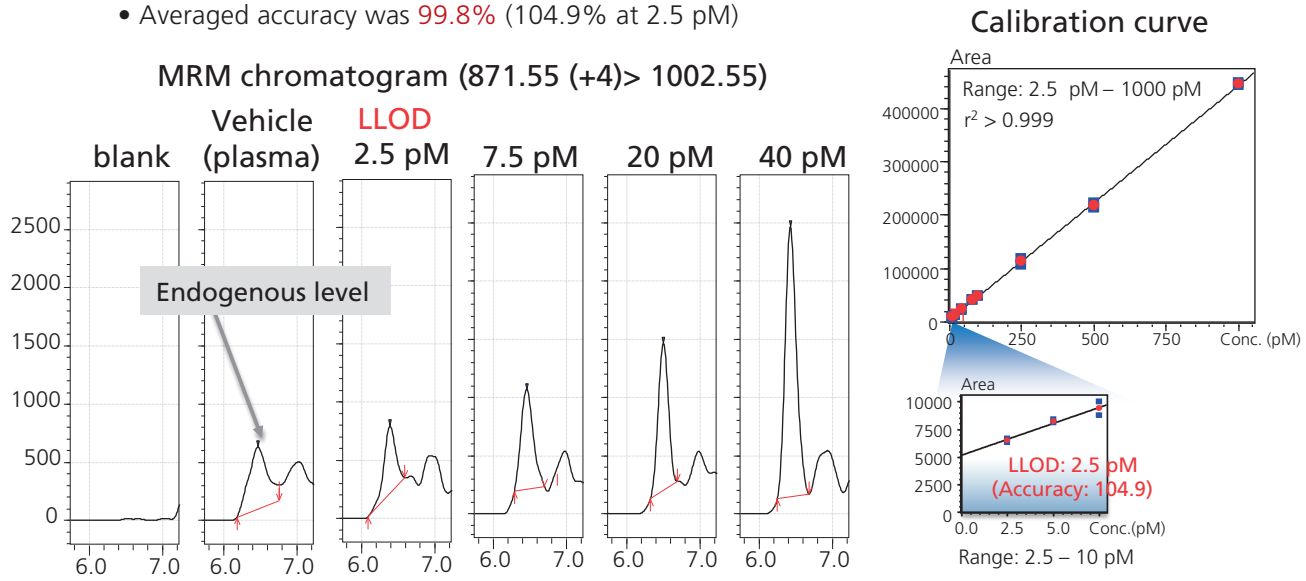

Figure 6. Performance of developed method using plasma sample spiked with glucagon.

Quantitative analysis of endogenous glucagon in healthy volunteers

Blood glucagon levels in fasting plasma is two-folds higher than casual plasma (Figure 7). From this result, enhanced secretion of glucagon under the fasting condition was confirmed since glucagon secretion is reported to upregulated in fasting state of healthy subjects.

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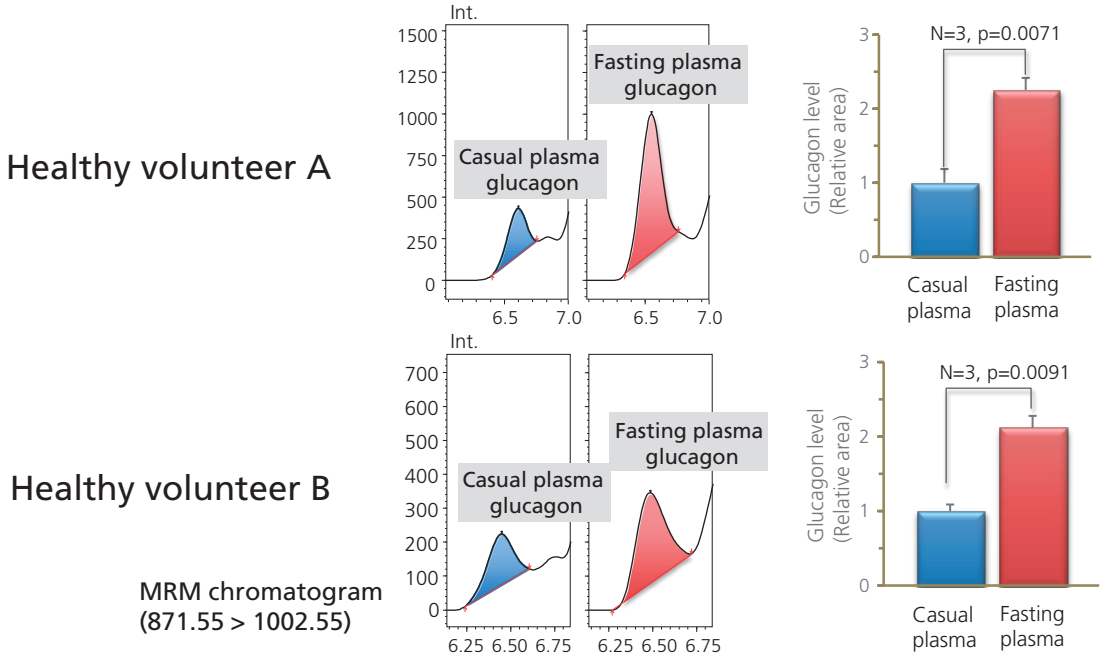

Figure 7. Glucagon levels in casual and fasting blood of healthy volunteers.

Conclusions

- Endogenous glucagon was successfully detected by the optimized sample preparation protocol and the sensitivity of the developed method.
- Under the fasting conditions in healthy subjects, glucagon secretion is known to be increased to maintain the blood glucose level. Developed methods clearly figured out this physiological change.

Disclaimer : LCMS-8060 is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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