Postprandial Plasma Glucagon Kinetics in Type 2 Diabetes Mellitus: Comparison of Immunoassay and Mass Spectrometry

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Context: Accurate glucagon level measurements are necessary for investigation of mechanisms for postprandial hyperglycemia in type 2 diabetes.

Objective: To evaluate the accuracy of postprandial glucagon level measurements using a sandwich ELISA vs a recently established liquid chromatography-high resolution mass spectrometry (LC-HRMS) method in type 2 diabetes mellitus.

Design and Participants: Twenty patients with type 2 diabetes treated with insulin underwent a meal test before and after administration of the dipeptidyl peptidase-4 inhibitor anagliptin for 4 weeks. Blood samples were taken serially after the meal, and glucagon levels were measured using both ELISA and LC-HRMS. We compared the change from baseline to 4 weeks (D0–4W) using the area under the curve for plasma glucagon during the meal test [area under the curve (AUC)0–3h] measured using ELISA and LC-HRMS.

Results: ELISA-based glucagon AUC0–3h was higher than LC-HRMS–based AUC0–3h at baseline and 4 weeks. However, differences in D0–4W-AUC0–3h measured using ELISA and LC-HRMS were not statistically significant. Additionally, D0–4W-AUC0–3h measured using ELISA and LC-HRMS were strongly correlated (r = 0.87, P < 0.001).

Conclusions: Plasma glucagon levels during a meal test in patients with type 2 diabetes measured using ELISA were consistently higher than those measured using LC-HRMS. However, given that the changes in glucagon levels measured using ELISA before and after dipeptidyl peptidase-4 inhibitor therapy were similar to those based on LC-HRMS, this ELISA seems to be useful for evaluating the effect of the drug interventions on postprandial glucagon levels.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; AUC, area under the curve; BMI, body mass index; DPP-4, dipeptidyl peptidase-4; ESI, electro-spray ionization; GIP, glucose-dependent insulinotropic polypeptide; GLP, glucagon-like peptide; HbA1c, glycosylated hemoglobin; LC-HRMS, liquid chromatography-high resolution mass spectrometry; MS, mass spectrometry; PRM, parallel reaction monitoring.
Glucagon, which is secreted by pancreatic α-cells, is an important hormone that works with insulin to tightly control blood glucose levels [1]. Glucagon counteracts the effects of insulin, especially in terms of gluconeogenesis and glycogenolysis in the liver [2]. In addition, glucagon plays important roles in enhanced adiposity [3] and gastric emptying [4]. Furthermore, glucagon has been reported to play various roles in pancreatic development and lipid metabolism [5]. A common feature of type 2 diabetes is an inappropriately high plasma glucagon to insulin ratio, suggesting the existence of pancreatic β-cell and α-cell dysfunction [6–8]. Dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide (GLP) -1 receptor agonists, which have excellent glucose-lowering effects at least in part via inhibition of glucagon secretion, are available in the clinical setting [9–11]. Thus, the various pathophysiological roles of glucagon in type 2 diabetes have attracted great interest in the research and clinical settings.

Glucagon is a 29-amino acid peptide hormone that is secreted from the stomach and intestines, in addition to pancreatic α-cells [12]. Glucagon (1-29) is cleaved from proglucagon by prohormone convertase 2, mainly in pancreatic α-cells. GLP-1 and GLP-2 are cleaved from proglucagon by prohormone convertase 1, mainly in intestinal L-cells [13]. This tissue-specific posttranslational processing leads to the production of various proglucagon fragments such as glicentin, oxyntomodulin, glicentin-related pancreatic peptide, and major proglucagon fragment. The existence of multiple glucagon-related peptides makes it difficult to establish an assay system to measure plasma glucagon levels accurately.

An ELISA kit (Mercodia Glucagon, Uppsala, Sweden) using monoclonal antibodies against both the C- and N-terminal regions of glucagon was recently developed [14]. This commercially available ELISA kit can measure plasma glucagon levels with lower cross-reactivity against proglucagon fragments and higher specificity than a conventional radioimmunoassay kit [15]. On the other hand, the ELISA kit was reported to show modest cross-reactivity against glicentin and oxyntomodulin [15]. Miyachi et al. [16] recently established a method to measure plasma glucagon levels using liquid chromatography-high resolution mass spectrometry (LC-HRMS), which is a highly accurate quantitative assay for plasma glucagon levels. In a previous study, when they compared plasma glucagon levels during oral glucose and meal tolerance tests in healthy volunteers measured using ELISA vs LC-HRMS, they found that plasma glucagon levels measured using these two methods were highly correlated. On the other hand, type 2 diabetes, especially with reduced insulin secretion capacity, is accompanied by pancreatic islet dysfunction. Thus, there is no guarantee that plasma glucagon levels measured using ELISA are well correlated with levels measured using LC-HRMS in patients with type 2 diabetes.

In this context, to evaluate the usefulness of this ELISA kit further, we investigated the relation of postprandial glucagon levels determined using the ELISA kit to levels measured using LC-HRMS before and after treatment with anagliptin, a DPP-4 inhibitor, in patients with type 2 diabetes taking insulin.

1. Materials and Methods

A. Participants

This prospective, 4-week, single-center, open-label, single arm study was conducted at the outpatient clinic of Juntendo University Hospital between July 2016 and June 2017. The
inclusion criteria at study registration included: (i) age ≥20 years; (ii) 7.0 ≤ glycosylated hemoglobin (HbA1c) < 11.0%; (iii) insulin therapy; and (iv) therapy with a maximum of three drugs from the biguanide, sulfonylurea, thiazolidine, glinide, sodium-dependent glucose cotransporter-2 inhibitor and α-glucosidase inhibitor classes. Patients were excluded from the study if any of the following conditions was diagnosed at registration: (i) type 1 diabetes; (ii) serious kidney disease (serum creatinine level ≥1.5 mg/dL); (iii) serious liver disease [liver cirrhosis or aspartate aminotransferase (AST) and aspartate transaminase (ALT) ≥100 IU/L]; (iv) serious heart disease (i.e., heart failure or unstable angina pectoris); (v) body mass index (BMI) ≥30 kg/m²; (vi) history of gastrointestinal surgery; (vii) malignancy; and (viii) unsuitability for the study (e.g., irregular visits to the hospital). The study protocol was approved by the Human Ethics Committee of Juntendo University and was conducted in compliance with the Declaration of Helsinki and legal regulations in Japan at the time. Written informed consent was obtained from each patient before enrollment in the study. This study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN 000023414), which is a nonprofit organization in Japan that meets International Committee of Medical Journal Editors requirements.

B. Study Design

A previous study compared the differences in responses of glucagon (1-29) to oral glucose loading using ELISA and radioimmunoassay kits in patients with type 2 diabetes in 17 patients [15]. As a result, we sought to register 20 patients with type 2 diabetes, allowing for the possibility of several dropouts. In all patients, anagliptin (100 mg twice daily) was added to their existing treatment regimen. A meal test was performed at baseline and 4 weeks after anagliptin administration. The meal test was performed using the standard meal (Recovery® Amino 400 mL, NUTRI Co., Ltd., Mie, Japan). The total energy content of the standard meal was 400 kcal with 61.8 g of carbohydrates, 10.8 g of fat, and 20.0 g of protein. The patients visited the hospital at 9:00 AM after a 16-hour fast. They were instructed to consume the entire meal within 5 minutes and to remain seated and at rest throughout testing. An IV line was inserted into an antebrachial vein before the meal and kept patent using 0.9% NaCl for repeated blood sampling. Blood samples for the meal test were collected at 0 hour (immediately before the meal), and 0.5 hour, 1 hour, 2 hours, and 3 hours after the start of the meal. Anagliptin (100 mg) and other antidiabetes drugs, including insulin, were administered immediately before the meal test. Plasma glucose, C-peptide, active GLP-1, active glucose-dependent insulinitropic polypeptide (GIP), and glucagon levels were measured at each time point. The area under the curve (AUC) from the start of the meal test to 3 hours (AUC0–3h) was calculated based on values at 0 hour, 0.5-hour, 1-hour, 2-hour, and 3-hour values using the trapezoidal method [17]. Incremental AUCs of 0–3h were calculated based on values (each measurement value minus baseline one).

The primary outcome of this study was the change from baseline to 4 weeks in the (AUC) for plasma glucagon during the meal test (Δ0–4W-AUC0–3h) for LC-HRMS and ELISA. The secondary outcomes were: (i) correlation between glucagon levels at each measurement point during the meal test at baseline and 4 weeks after anagliptin administration for the two methods; (ii) changes in blood glucose; C-peptide, active GLP-1, and active GIP levels from baseline to 4 weeks; and (iii) adverse events such as hypoglycemia.

C. Plasma Glucagon Measurement Using LC-HRMS and an ELISA Kit

Plasma concentrations of glucagon (1-29) were quantified using the recently developed LC-HRMS method with parallel reaction monitoring (PRM) [16] as well as a commercially available sandwich ELISA kit (10-1271-01, Mercodia, Uppsala, Sweden) [18]. For mass spectrometry (MS)-based quantification of glucagon (1-29), an automated nanoLC-HRMS system consisting of an Ultimate 3000 Series nanoLC system and a Q Exactive Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, GmbH, Bremen, Germany) equipped with a nano-electro spray ionization (ESI) interface and Black XYZ ion source (AMR Inc.,
Tokyo, Japan) was used. For quantification of glucagon levels in plasma samples, stable isotope-labeled glucagon (1-29) was added as an internal standard (IS) to every 200 μL of plasma. Protein precipitation followed by solid phase extraction was used to deplete plasma proteins and unnecessary peptides. PRM was performed with monitoring of the summed peak area of five fragment ions in the +4 charge state for glucagon (1-29) (m/z 871.66), and stable isotope-labeled-glucagon (1-29) (m/z 873.17).

D. Biochemical Assays

Blood glucose, HbA1c (NGSP), and C-peptide levels were measured using standard techniques. Active GLP-1 [GLP-1 (7-36) and (7-37) amide] levels were measured using the Glucagon-Like Peptide-1 (Active) ELISA kit (Merck Millipore, Bedford, MA) [19]. Active GIP levels were measured using LC-MS/MS/MS as described previously [20].

E. Statistical Analysis

All statistical analyses were performed by a private company (Soiken, Inc., Osaka, Japan) with SAS software version 9.3 (SAS Institute, Cary, NC). Data are expressed as means ± SD. Comparisons of data before and after anagliptin administration and between the ELISA kit and LC-HRMS were performed using the nonparametric Wilcoxon signed-rank test. Pearson’s correlation analysis was used to determine the association between plasma glucagon levels determined by ELISA vs LC-HRMS. Bland-Altman plots were also used to display the distribution of the data and investigate the discrepancies between the ELISA kit and LC-HRMS. 

2. Results

All 20 patients completed the 4-week study. Clinical characteristics of the study patients at baseline and 4 weeks after anagliptin administration are shown in Table 1. Study

| Table 1. Characteristics of Study Participants at Baseline and 4 Weeks |
|--------------------|-----------------|
| Baseline | 4 wk (n = 20) |
| Sex, male/female | 15/5 |
| Age, y | 68.8 ± 9.1 |
| Duration of diabetes, y | 22.6 ± 9.7 |
| BMI, kg/m² | 24.6 ± 2.9 |
| Systolic blood pressure, mm Hg | 148.9 ± 17.3 |
| Diastolic blood pressure, mm Hg | 81.3 ± 11.2 |
| HbA1c, % | 8.4 ± 0.9 |
| Fasting blood glucose, mg/dL | 183.9 ± 40.6 |
| Fasting C-peptide, nmol/L | 0.4 ± 0.2 |
| Fasting active GLP-1, pmol/L | 2.0 ± 1.3 |
| Fasting active GIP, pmol/L | 7.0 ± 4.1 |
| Medication for diabetes | |
| Insulin dosage, U/d | 23.9 ± 10.9 |
| Oral therapy | |
| Sulfonylurea | 0 |
| Metformin | 6 |
| Thiazolidine | 3 |
| Glinide | 2 |
| SGLT2 inhibitor | 4 |
| α-Glucosidase inhibitor | 2 |

Data are means ± SD.
SGLT2, sodium-dependent glucose cotransporter-2.

*P < 0.01.
participants were aged 68.8 ± 9.1 years; five were women. As is typical for Asian patients with type 2 diabetes on insulin treatment, mean BMI was not high (24.6 ± 2.9 kg/m²) and fasting C-peptide levels were low (0.4 ± 0.2 nmol/L). After anagliptin treatment, fasting blood glucose and HbA1c levels were substantially lower, and both fasting active GLP-1 and GIP levels were higher. None of the patients experienced hypoglycemia during the study period, therefore, the dosages of insulin and oral antidiabetic drugs were not changed.

Table 2 presents the comparisons of incremental glucagon AUC0–3h and glucagon AUC0–3h measured using ELISA vs LC-HRMS during the meal test at baseline and 4 weeks. ELISA-based incremental glucagon AUC0–3h was significantly higher than LC-HRMS-based incremental glucagon AUC0–3h both at baseline and 4 weeks after anagliptin administration (baseline, $P = 0.036$ and 4 weeks, $P < 0.001$). In addition, glucagon AUC0–3h showed the same results (baseline and 4 weeks, $P < 0.001$). However, Δ0–4W-AUC0–3h measured with ELISA and LC-HRMS were similar both in incremental and glucagon AUC0–3h ($P = 0.39$ and $P = 0.25$, respectively). Regarding plasma glucagon levels at each time point after the meal test, plasma glucagon levels at 0.5 hour, 1 hour, and 2 hours after the meal were decreased substantially after anagliptin treatment as shown in Fig. 1A (ELISA) and 1C (LC-HRMS). Additionally, glucagon AUC0–3h measured with both assays decreased substantially after anagliptin treatment (Fig. 1B and 1D). In Supplemental Table 1, ELISA-based values were significantly higher than LC-HRMS-based values. The change in glucagon levels from 0 to 4 weeks was similar for the two methods, except at 2 hours after the meal ($P = 0.048$).

To assess the relation between Δ0–4W-AUC0–3h measured with ELISA and LC-HRMS, we calculated the correlation between the two methods at baseline (Fig. 2A), 4 weeks (Fig. 2B), and change from baseline to 4 weeks in each measurement point (Fig. 2C) and found that the correlations between plasma glucagon levels obtained by the two methods were strong. For additional analysis of Fig. 2A and 2B, Bland-Altman plots were presented in supplemental Fig. 1A and 1B, respectively, and we found that several glucagon values at 0.5 hours after the meal were plotted over 95% limit. Next, Δ0–4W-incremental Glucagon AUC0–3h measured with ELISA was well correlated with values measured with LC-HRMS ($r = 0.87$, $P < 0.001$, Fig. 2D).

Regarding the effect of anagliptin, blood glucose levels at 1 hour and 2 hours after the meal were substantially lower after anagliptin treatment, and incremental AUC0–3h for blood glucose was also substantially lower (Supplemental Fig. 2A and 2B, respectively). On the other hand, plasma C-peptide levels at 2 hours and 3 hours after the meal were substantially higher after anagliptin treatment, and incremental AUC0–3h for C-peptide was also higher (Supplemental Fig. 2C and 2D, respectively). Active GLP and GIP levels at 0.5 hour, 1 hour, 2 hours, and 3 hours were substantially higher (Supplemental Fig. 2E and 2G, respectively) and incremental AUC0–3h for both active GLP-1 and GIP were also substantially higher after anagliptin treatment (Supplemental Fig. 2F and 2H, respectively). The median values of these parameters during the meal test are presented in Supplemental Table 2.

### Table 2. Comparison of AUC0–3h for Plasma Glucagon Measured Using ELISA vs LC-HRMS

|                      | ELISA (n = 20)       | LC-HRMS (n = 20)     | $P$   |
|----------------------|----------------------|----------------------|-------|
| Incremental glucagon |                      |                      |       |
| AUC0–3h (pmol·h/L)   | 12.3 ± 18.7          | 6.7 ± 13.5           | 0.036 |
| Baseline (pmol·h/L)  | 4 weeks (pmol·h/L)   | 0.4 ± 19.2           | -4.2 ± 16.0 | <0.001 |
| Δ0–4W-AUC0–3h (pmol·h/L) | -12.0 ± 24.9       | -10.9 ± 19.0         | 0.39  |
| Glucagon AUC0–3h     |                      |                      |       |
| Baseline (pmol·h/L)  | 68.4 ± 40.9          | 54.5 ± 36.7          | <0.001 |
| 4 weeks (pmol·h/L)   | 57.4 ± 35.5          | 45.4 ± 30.7          | <0.001 |
| Δ0–4W-AUC0–3h (pmol·h/L) | -11.0 ± 14.7        | -9.0 ± 17.5          | 0.25  |

Data are means ± SD.
3. Discussion

This study investigates glucagon kinetics during a meal test before and after DPP-4 inhibitor administration that compared results from a commercially available ELISA kit with a LC-HRMS method. Recently, MS technology has advanced considerably and there has been an increasing interest in the use of liquid chromatography-mass spectrometry (LC-MS) for peptide quantification because of its high specificity, sensitivity, and relatively short developing time. In addition, LC-MS can distinguish modified peptides, including oxidized peptides, from intact peptides. However, MS-based methods have not yet achieved the same widespread usage as immunoassay-based techniques, because they do not offer the same sensitivity. The MS-based assay used in this study is one of only a few practical methods to quantify endogenous peptide levels. The PRM approach [21–23] in a high-resolution mass spectrometer instead of the selected reaction monitoring [24–26] approach in a triple quadrupole mass spectrometer makes it possible to achieve high specificity and sensitivity. Using the LC-HRMS PRM method, glucagon concentrations can be quantified to a lower limit of 0.5 pmol/L (less than \(2 \times 10^{-17}\) mol on the column), which is lower than the lower limit for a sandwich ELISA (1.5 pmol/L), leading to the precise analysis of plasma glucagon levels in the current study.

In the current study, AUC0–3h values for glucagon measured using ELISA at baseline and 4 weeks after anagliptin administration were substantially higher than those measured using LC-HRMS. It should be noted that monoclonal antibodies in the ELISA kit show a weak
cross-reactivity to the end of the N-terminal region in oxyntomodulin and the end of the C-terminal region in glicentin (1–61), which might have affected the accuracy of ELISA results. This limitation of the immunoassay-based glucagon assay has been observed by several groups [14, 15]. Miyachi et al. [16] demonstrated that plasma glucagon levels measured using ELISA during oral glucose and meal tests were lower than those measured using LC-HRMS in healthy controls. In addition, postprandial levels of oxyntomodulin and glicentin were reported to be lower in patients with type 2 diabetes compared with healthy control [27, 28]. Thus, theoretically, postprandial glucagon levels measured using ELISA could be even lower than those measured using LC-HRMS in patients with type 2 diabetes compared with healthy controls. However, our study showed the opposite result, suggesting the possibility of factors other than oxyntomodulin and glicentin affecting the measurement accuracy of ELISA in patients with type 2 diabetes. Therefore, further study is necessary to clarify this point.

Our study has some limitations. First, plasma glicentin and oxyntomodulin levels after the meal were not measured in our study. Therefore, we could not accurately evaluate the effects of monoclonal antibody cross-reactivity with oxyntomodulin and glicentin on the difference in plasma glucagon levels between LC-HRMS and ELISA. Second, patients with type 2 diabetes on insulin were recruited into this study. Because insulin secreted from β-cells inhibits glucagon secretion in a paracrine manner, we aimed to recruit patients with reduced

Figure 2. Correlations between each data point for plasma glucagon at 0 h, 0.5 h, 1 h, 2 h, and 3 h after the meal determined using ELISA and LC-HRMS at (A) baseline and (B) 4 wk after anagliptin treatment. (C) Correlation between Δ0-4W each measurement point determined using ELISA and LC-HRMS. (D) Δ0-4W-AUC0–3h indicates the change from baseline to 4 weeks in the AUC for plasma glucagon calculated based on value (each measurement value—baseline one). Each value is shown with a white circle in (D) and dots in various colors in (A–C).
endogenous insulin secretion to minimize the effects of endogenous insulin on glucagon secretion. However, AUC0–3h for C-peptide was substantially higher after anagliptin treatment. Therefore, we could not exclude this effect completely, although the increase in AUC0–3h for C-peptide was modest.

In conclusion, the change in the area under the curve for plasma glucagon during the meal test before and after anagliptin treatment measured using LC-HRMS and ELISA was similar. Therefore, the performance of a commercially available ELISA kit was equivalent to LC-HRMS for the analysis of postprandial glucagon kinetics before and after anagliptin treatment.

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