〈Research Article〉

Measurement of Dipeptidyl Peptidase IV Activity Using a Biochemical Autoanalyzer

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Summary Dipeptidyl peptidase IV (DPP-IV, CD26, EC 3.4.14.5) is a 110 kDa glycoprotein that is ubiquitously expressed not only in plasma, but also on the surface of various cells such as endothelial cells, lymphocytes, monocytes, kidney, liver, and adipocytes. DPP-IV selectively cleaves N-terminal dipeptides from various substrates, including incretin hormones. Recent studies have suggested that increased circulating DPP-IV activity and elevated expression in each tissue are associated with metabolic diseases, such as inflammation, diabetes, obesity, cardiovascular disease, and non-alcoholic fatty liver disease. Manual methods such as enzyme immunoassays, enzyme-linked immunosorbent assays, fluorescence, and chemiluminescence are used to measure DPP-IV activity; however, this leads to a considerable influence of the procedure on the results. Moreover, depending on the method, measurement times can be long. Therefore, we attempted to develop an automatic assay system that can measure DPP-IV activity easily and with high accuracy over a short period. We used an automatic analyzer for measurement and found that it had good reproducibility, with a coefficient of variation of less than 1.5%. Moreover, DPP-IV activity can be measured within 10 min. We also used this method to examine DPP-IV activity levels in patients with diabetes.

Key words: DPP-Ⅳ activity, DPP-Ⅳ inhibitor, Automation

1. Introduction

Recent years have witnessed an increase in the use of dipeptidyl peptidase-IV (DPP-IV) inhibitors such as sitagliptin for the treatment of diabetes[1]. When administered alone, DPP-IV inhibitors not only mitigate hyperglycemia without causing obesity and hypoglycemia but they also likely have a protective effect on pancreatic β cells[2-3]. Therefore, it has become one of the first-choice drugs for the treatment of type 2 diabetes. DPP-IV belongs to the serine protease family. If a peptide has a proline or alanine amino acid in the second position from the N-terminus, DPP-IV cleaves the amino acids, which often results in the loss of physiological activity of the peptide. DPP-IV is an enzyme that degrades gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are gastrointestinal hormones, and suppresses insulin secretion[4]. It is a 110 kDa glycoprotein that is ubiquitously expressed on the surface of various cells, such as endothelial cells, lymphocytes, monocytes, kidney cells, liver cells, and adipocytes. Furthermore, circulating DPP-IV is also present in the plasma, and its activity level is associated

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with metabolic diseases such as diabetes, obesity, cardiovascular disease, and non-alcoholic fatty liver disease[5-8]. However, DPP-IV activity levels are rarely measured clinically. This is likely because DPP-IV has not been proven to be associated with metabolic diseases, and the main measurement methods of DPP-IV activity are manual methods, such as enzyme immunoassay[9-10]. In the present study, we investigated a method to measure DPP-IV activity in the plasma using an automatic biochemical analyzer.

2. Materials and Methods

Measurement principle

DPP-IV (CD26, EC 3.4.14.5) is a serine-dipeptidyl peptidase that cleaves alanine or proline at the second N-terminal residue of target polypeptides such as chemokines and peptide hormones. When DPP-IV reacts with the substrate, Gly-Pro-*p*NA, it cleaves the two amino acid residues at the N-terminal and generates p-nitroaniline (*p*NA). Although *p*NA has maximum absorption near 405 nm, it can be measured at 404 nm based on the equipment used. The activity of DPP-IV was determined by measuring the rate of increase in absorbance. In the present method, we used two wavelengths (404 nm and 700 nm) (Fig. 1A, Fig. 1B).

Equipment

A biochemical automatic analyzer, TBA-120FR Sora Edition (Canon Medical Systems Inc., Tochigi, Japan), was used in this study. A plate reader MTP-310Lab (Corona Denki Co., Ltd., Ibaraki, Japan) was used for manual measurements.

Sampling

The residual specimens of 19 students at the Kagawa Prefectural University of Health Sciences and Kinashi Obayashi Hospital, as well as those of 38 diabetic patients at the Kinashi Obayashi Hospital (including 21 who were receiving DPP-IV inhibitor treatment), were used as analytical samples. Nineteen students gave their consent to participate in the study. This study was approved by the Ethics Review Committee of the Kagawa Prefectural Health Sciences and Kinashi Obayashi Hospital. (Kagawa Prefectural University of Health Sciences Ethics Review Committee Approval No. 188, Kinashi Obayashi Hospital Ethics Review Committee Approval No. 29/2)

Chemicals

Buffer

A homemade 50 mmol/L Tris-HCl buffer (pH 7.5) prepared from tris (hydroxymethyl) aminomethane (Tris, FUJIFILM Wako Chemicals, Osaka, Japan) was used as the buffer.

Substrate

Gly-Pro-*p*NA (GP-*p*NA) (Enzo Life Sciences, New York, USA) was used as the substrate in the present study[11]. Its concentration was adjusted to 10 mmol/L using a buffer solution, and the resulting solution was used as the substrate solution.

DPP-IV inhibitor

P32/98 (3-*N*-[((*2S,3S*)-2-Amino-3-methylpentanoyl]-1,3-thiazolidine. hemifumarate) was used as a DPP-IV inhibitor (Enzo Life Sciences, New York, USA)[12].

DPP-IV standard substance

Recombinant human DPP-IV protein (R&D Systems, Minneapolis, MN, USA) was used as a standard.

Examination of coexisting substances

Interference Check A Plus (Sysmex Corporation, Hyogo, Japan) and Intralipos (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) were used to test the effects of coexisting substances.

DPP-IV measurement kit

The DPP-IV Drug Discovery Kit (Enzo Life Sciences, New York, NY, USA) was used for manual measurement of DPP-IV activity[13-14].

Conditions

We added 5 μL of the sample to 150 μL of buffer solution (first reagent). Five min later, 50 μL of the substrate solution (second reagent) was added. After

adding the second reagent, the change in absorbance at the dominant wavelength of 404 nm and sub-wavelength of 700 nm was measured over a period of 5 min to determine the peptidase activity value (Fig. 1B, upper row ①). Peptidase activity values were obtained using a buffer solution containing P32/98 under the same conditions (Fig. 1B bottom row ②). The DPP-IV activity value was obtained by subtracting ② from ①. (Fig. 1B)

Examination of substrate-agent concentration

We diluted a 10 mmol/L GP-*p*NA substrate solution with buffer to obtain six different concentrations of the substrate (0.25, 0.5, 1, 2, 2.5, and 5 mmol/L). Standards were measured at each substrate concentration. The Km (Michaelis-Menten constant) value was calculated using the Lineweaver-Burk plot. The substrate concentration was then determined as 10 times the Km value.

Examination of inhibitor concentration

P32/98 adjusted to 1 mmol/L with Tris-HCl buffer was further diluted with the buffer, and to produce eleven different concentrations (0, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 300, and 400 μmol/L), and the inhibition rate of P32/98 was examined.

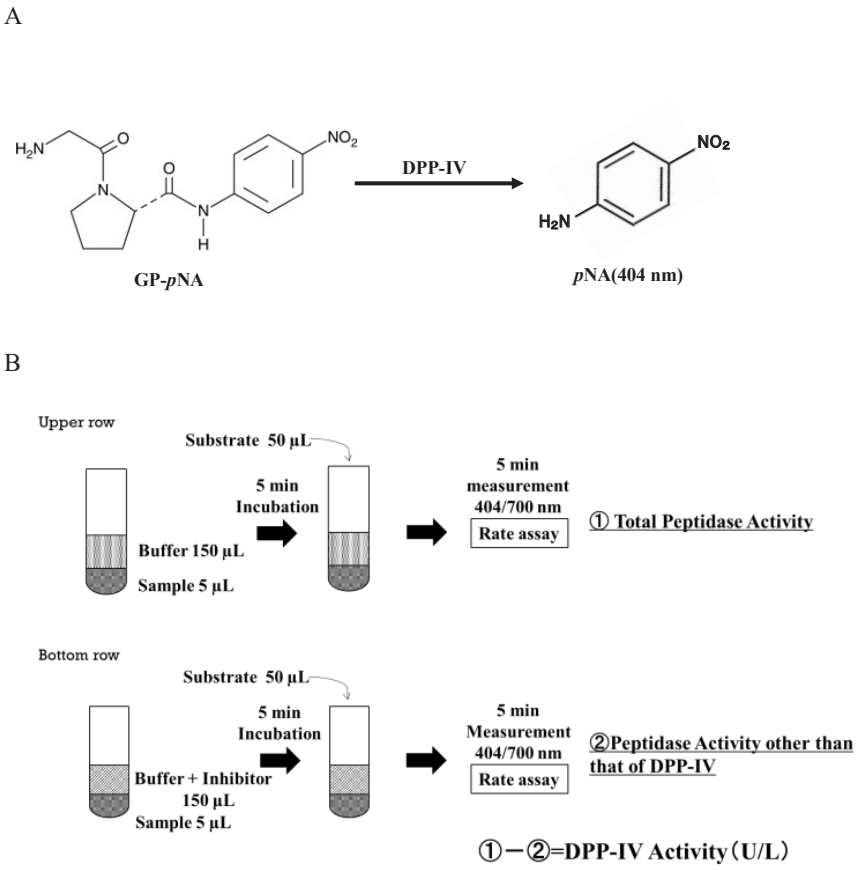

Fig. 1 A: Reaction principle. DPP-IV converts the substrate agent GP-*p*NA to *p*NA. The increase in absorbance (404 nm) was measured for *p*NA. GP-*p*NA: H-Gly-Pro-*p*-nitroanilide, *p*NA: *p*-nitroanilide, DPP-IV: Dipeptidyl peptidase-IV. B: Outline of the measurement method. Upper row: Reaction of the buffer solution without inhibitors. Bottom row: Reaction of the buffer solution containing DPP-IV inhibitor. The DPP-IV activity was calculated by subtracting ② from ①. Buffer:50 mmol/L Tris-HCl buffer (pH 7.5); substrate:4 mmol/L GP-*p*NA ; Inhibitor:300 μmol/L P32/98.

Examination of the calibration factor (K factor)

The activity value of an enzyme is expressed by multiplying the amount of change in absorbance per minute of reaction by a constant coefficient. This constant factor is called the calibration factor (K factor). When the enzyme activity value is expressed in international units U/L, at a measurement temperature of 37 °C, a formula including the K factor is used[15]. The enzyme activity was calculated using the following formula:

$$U/L, t℃ = \frac{\Delta A}{min} \times \frac{1}{\varepsilon} \times \frac{1}{L} \times \frac{Sv + Rv}{Sv} \times 10^6$$

where U/L is the enzyme required to catalyze 1 mol/L substrate per min, t °C is the measurement temperature, ΔA/min is the absorbance change per min, ε is the molecular extinction coefficient, L is the length (mm), Sv is the sample volume (μL), and Rv is the reagent volume (μL).

Examination of measurement time

To confirm the state of the reaction, changes in absorbance were measured for 10 min after the addition of the first reagent and for up to 5 min after the addition of the second reagent.

Examination of repeatability

Two types of samples with different concentrations (8.42 U/L and 34.62 U/L) were measured 10 times, and the standard deviation (SD) and coefficient of variation (CV%) of each were calculated.

Examination of coexisting substances

Interference Check A Plus and Intralipos were added to the appropriate specimens to investigate the effects of various coexisting substances. Ascorbic acid, conjugated and unconjugated bilirubin up to 20 mg/dL, hemoglobin up to 500 mg/dL, chyle up to 3,000 FTU, formazin turbidity, and Intralipos up to 2% were assessed.

Correlation with manual techniques

The DPP-IV Drug Discovery Kit, which uses the same principle, buffer, substrate, and inhibitor as this method was used for manual measurements, and measurements were performed using a plate reader (MTP-310Lab). However, the attached reagent was used for the measurement.

Measurement of the patient samples

Using the reported method, DPP-IV activity was measured in 38 patients with diabetes (21 of whom were receiving DPP-IV inhibitor treatment). Subjects (2 males and 17 females) who were not obese (BMI: 20.9 ± 2.3), had no abnormal liver or renal function, and had no history of diabetes were used as healthy volunteers.

Statistical analysis

Statistical analysis used Statcel-the Useful Addin Forms on Excel-2nd ed (OMS publishers, Tokyo, Japan) , the correlation between our method and the manual method was determined using Pearson's correlation coefficient, and non-correlation was calculated using the determined correlation coefficient. A significant difference test for the comparison of patient specimens was performed using the Student's t-test.

3. Results

Substrate concentration

Using a six-step dilution process, the concentration of the substrate ware set to 0.25, 0.5, 1, 2, 2.5, and 5 mmol/L, and the DPP-IV activity of a standard substance (recombinant human DPP-IV protein) with a known concentration was measured at each concentration of the substrate agent. The Km value was 0.4 mmol/L, which was calculated using a Lineweaver-Burk plot diagram plotted using the absorbance values (Fig. 2). In enzymatic reactions, the substrate concentration is 10 to 20 times higher than the Km value in the zero-order reaction region; hence, the substrate concentration used in this method was set to 4 mmol/L, which was 10 times higher than the calculated Km value.

Inhibitor concentration

After adjusting 1 mmol/L of inhibitor by an 11-step serial dilution, buffer solutions containing inhibitors at 11 different concentrations were used, and

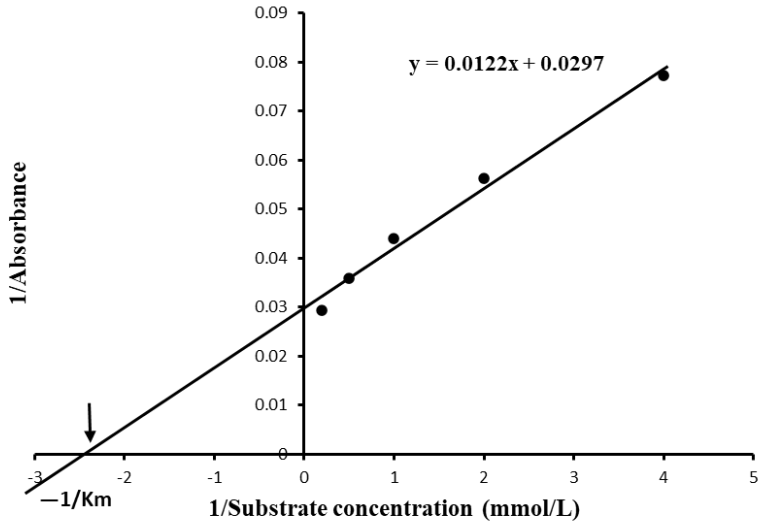

Fig. 2 Calculation of Km value using the Lineweaver–Burk plot. A Lineweaver–Burk plot was constructed by measuring the absorbance of the standard substance that was serially diluted. The intersection of the plotted line and the X-axis (arrow) is -1/Km. Km : Michaelis-Menten constant.

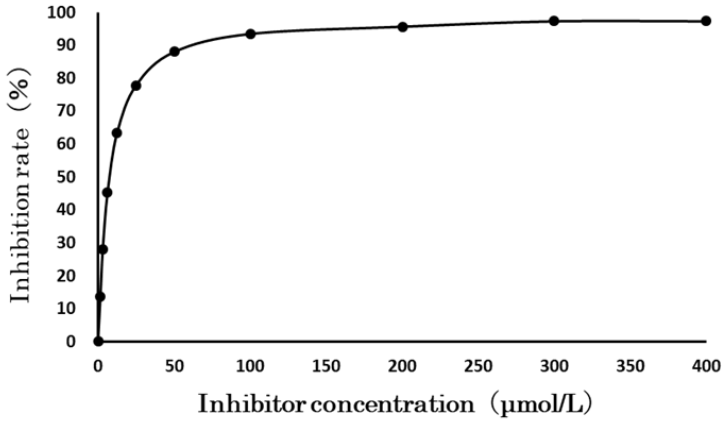

Fig. 3 Examination of the inhibition rate at different inhibitor concentrations. Different DPP-IV inhibitor concentrations were prepared by serially diluting the inhibitor in the buffer. A nearly 100% inhibition rate was observed at a DPP-IV inhibitor concentration of 300 μmol/L.

a standard solution of a known concentration was measured against 4 mmol/L of substrate agent. The relationship between the inhibition rate and inhibitor concentration is shown in Fig. 3. The inhibitor concentration was 300 μmol/L or higher and the inhibition rate was 98% or higher. To compare these values with those obtained using the DPP-IV measurement kit, we used the same inhibitor.

Calibration factor (K factor)

The calibration factor (K factor) was calculated in the enzyme activity formula, when the enzyme activity value was expressed in U/L at a measurement temperature of 37 °C, as follows:

$$\frac{1}{\varepsilon} \times \frac{1}{L} \times \frac{Sv+Rv}{Sv} \times 10^6 = \text{K factor}$$

Two methods were used to calculate the molar

extinction coefficient of *p*NA. In the first method, a 1 μmol/L *p*NA standard was measured at 404 nm, whereas in the second method, it was calculated using the activity of the DPP-IV standard. The molar extinction coefficients obtained from both methods were similar and comparable to the values obtained in previous studies[16]. In this method, the molar extinction coefficient of *p*NA was set to 10,000. This value was substituted into the formula to calculate the K-factor. This calibration coefficient obtained was then set in the automatic biochemical analyzer.

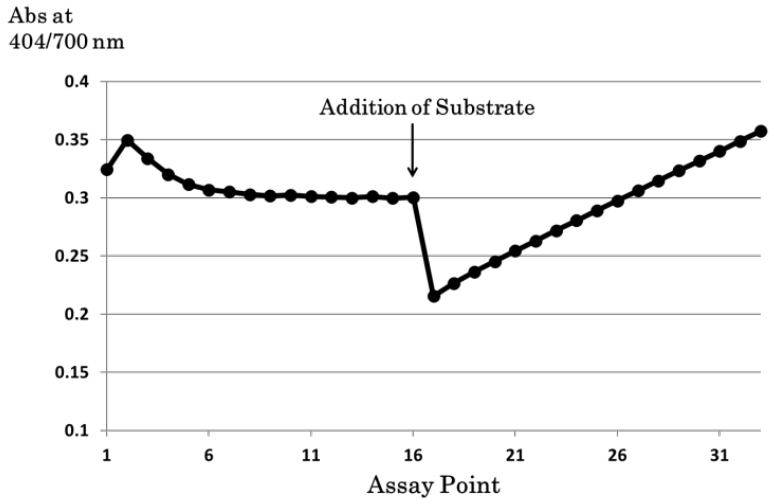

Fig. 4 Change in absorbance over time after the addition of the substrate The substrate was added 5 min after the addition of the first reagent (buffer) and the sample (assay point 16). The changes in absorbance were measured for 5 min after adding the substrate.

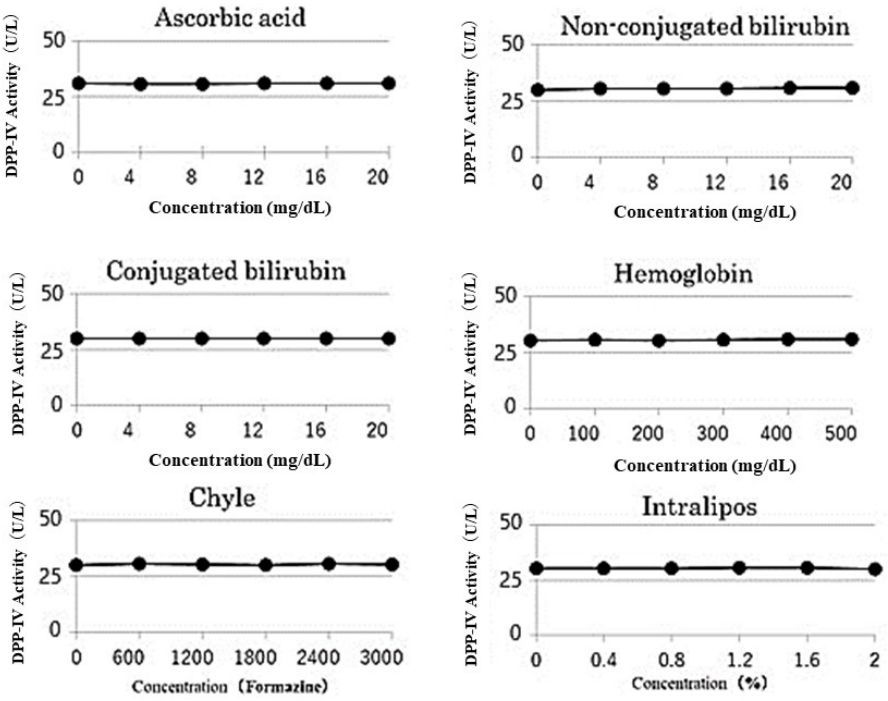

Fig. 5 Effects of six coexisting substances on the automatic assay system. No effect was observed on the measurement results of the system due to the six coexisting substances: ascorbic acid, bilirubin, hemoglobin, chyle, and Intrapolis.

Temporal changes

A buffer solution (pH 7.5), 4 mmol/L substrate agent, 300 μmol/L inhibitor, and a standard substance of known concentration were used to measure the changes with respect to time using a biochemical automatic analyzer. A linear increase in absorbance was observed for approximately 5 min after the addition of the substrate agent (Fig. 4).

Repeatability

Two samples with different concentrations were used, and each DPP-IV activity value was measured 10 times. SD was 0.12 and CV was 1.41% at the low DPP-IV activity value, whereas SD was 0.34 and CV was 0.97% at the high DPP-IV activity value. Intermediate precision and reproducibility were not examined in this study.

Effects of coexisting substances

Ascorbic acid, conjugated and unconjugated bilirubin, hemoglobin, chyle, and Intralipos did not affect this method (Fig. 5). Hence, we developed a reaction system that is less affected by coexisting substances.

Correlation with manual techniques

The correlation coefficient between our method and the manual DPP-IV Drug Discovery Kit was 0.81 ($p < 0.01$) (Fig. 6).

DPP-IV activity value of clinical samples

A significant increase in the activity of DPP-IV in clinical specimens was observed in patients with diabetes compared with that in healthy subjects ($p<0.05$). In patients with diabetes who were treated with DPP-IV inhibitors, we observed a significant decrease in the activity value compared with that in patients with diabetes who did not use DPP-IV inhibitors ($p < 0.01$), and the activity level in the former was similar to that in healthy subjects (Fig. 7).

4. Discussion

In addition to DPP-IV, serine protease family enzymes, such as DPP-II, DPP-VIII, and DPP-IX[17] generate *p*NA. These enzymes also react with the substrate agents GP-*p*NA , thrombin (a serine protease in the coagulation fibrinolytic system[18], tissue plasminogen activators[19], and plasmin[20]. To eliminate these influences, peptidase activity was measured in a buffer containing a DPP-IV inhibitor, and subtracted to obtain the accurate DPP-IV activity value. The substrate concentration used for enzymatic activity is typically 10–20 times the Km value. The Lineweaver–Burk plot showed that the Km value was 0.4 mmol/L, and the

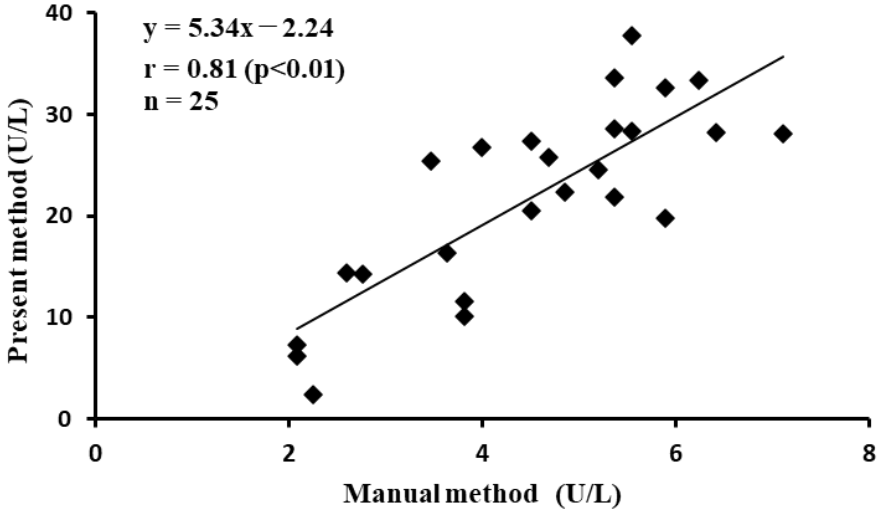

Fig. 6 Correlation between automated analytical methods and manual methods. Comparison of this method and manual method. The correlation coefficient (r) is Pearson's product-moment correlation coefficient. P<0.01 was considered significant.

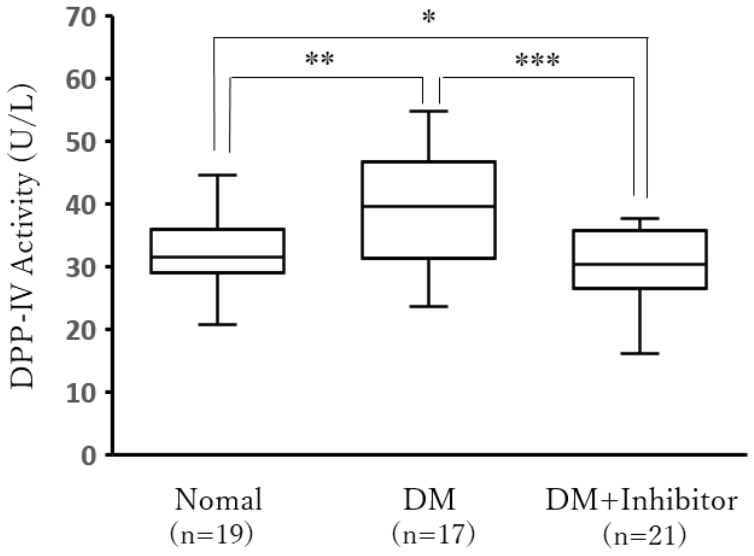

Fig. 7 Comparison of DPP-IV activity levels in diabetic patients. The Normal, DM, and DM + Inhibitor groups consisted of healthy subjects, diabetic patients, and diabetic patients undergoing inhibitor treatment, respectively. The DM group had significantly higher values than the normal group. No significant differences were observed between the normal and DM + Inhibitor groups. The DM + Inhibitor group had significantly lower values than the DM group. *: n.s (not significant), **: $p<0.05$, ***: $p<0.01$

substrate concentration used in this method was 4 mmol/L, which was ten times higher than the Km value (Fig. 2). The inhibitor concentration used in this method was 300 μmol/L because the inhibition rate was 98% or higher at this concentration (or higher) (Fig. 3).

The calibration factor (K factor) was calculated using the K factor formula using the amount of change in absorbance due to the liberation of 1 μmol of *p*NA per minute and was input as a parameter according to the specifications of the automatic biochemical analyzer. In this study, a linear increase in absorbance was observed for 5 min after the addition of the substrate; hence, the reaction was considered stable.

Moreover, the results were highly reproducible and showed little variation at both low and high DPP-IV activity levels. Therefore, a highly accurate reaction system was developed. The CV (%) of the manual kit used in this study was not specified by the manufacturer. However, the CV of similar manual kits has been reported to range between 5% and 10%. Hence, we constructed a measurement system with high reproducibility compared to manual methods. However, further studies are required to determine the measurement limits.

In this study, the reaction system was not affected by coexisting substances. Substances such as ascorbic acid, conjugated/unconjugated bilirubin, hemoglobin, chyle, and Intralipos did not affect the measured values.

A relatively good correlation (correlation coefficient = 0.81) was obtained between the manual kit and our method, although a difference was observed in the activity values measured using the two kits. This difference is likely due to the differences in the measurement temperature and measurement accuracy. In the manual method, the substrate was added and allowed to react at 37 °C. Two absorbance measurements were recorded at a time interval of 10 min after the addition of the substrate. Measurements were performed using a plate reader, and the change in absorbance per minute was determined by measuring the change in absorbance. As the plate reader was used at room temperature, this suggests that the reaction during the measurement time was also performed at room temperature. In contrast, the new method described in this study uses an automatic analyzer at 37 °C, which could likely be the

reason behind the differences in the activity values calculated using the two methods.

We also measured the clinical specimens using our method. DPP-IV activity levels increased in patients with diabetes, and for patients who used DPP-IV inhibitors, they decreased to levels that were comparable with those of healthy subjects. Our method accurately reflects changes in DPP-IV activity levels in vivo. Future studies can investigate the clinical uses of this method; for example, it can be used to test the drug intake status of patients or determine the therapeutic effects of drugs.

DPP-IV is associated with various metabolic diseases; however, its clinical significance has not yet been established. In cases where DPP-IV inhibitors are used in patients with metabolic diseases, such as diabetes, the reported method can be used to examine the clinical significance of DPP-IV activity levels. However, the size of the data used in the present study was small, and hence, future studies must consider using a larger data set.

5. Conclusion

The measurement of DPP-IV activity levels can be beneficial, as they can indicate the presence of several metabolic diseases. In this study, we describe a method that can be used to measure DPP-IV activity. We constructed a rapid and highly accurate measurement method using an automatic analyzer. By measuring clinical specimens using this method, we confirmed the effect of DDP-IV inhibitor treatment in patients with diabetes. In the future, this method can be used to measure DPP-IV activity in other metabolic diseases. In the future, this method can be used to measure DPP-IV activity in other metabolic diseases, and contribute to the treatment and prevention of diseases.

Conflicts of interest

Conflicts of interest of the authors related to the content of this paper: None.

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