

Optimization and Validation of a Fluorescent Kinetic Analysis for the Measurement of an Enzymatic Activity of Plasma DPP4

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Running Title: Validation for an Enzymatic Assay of Plasma DPP4
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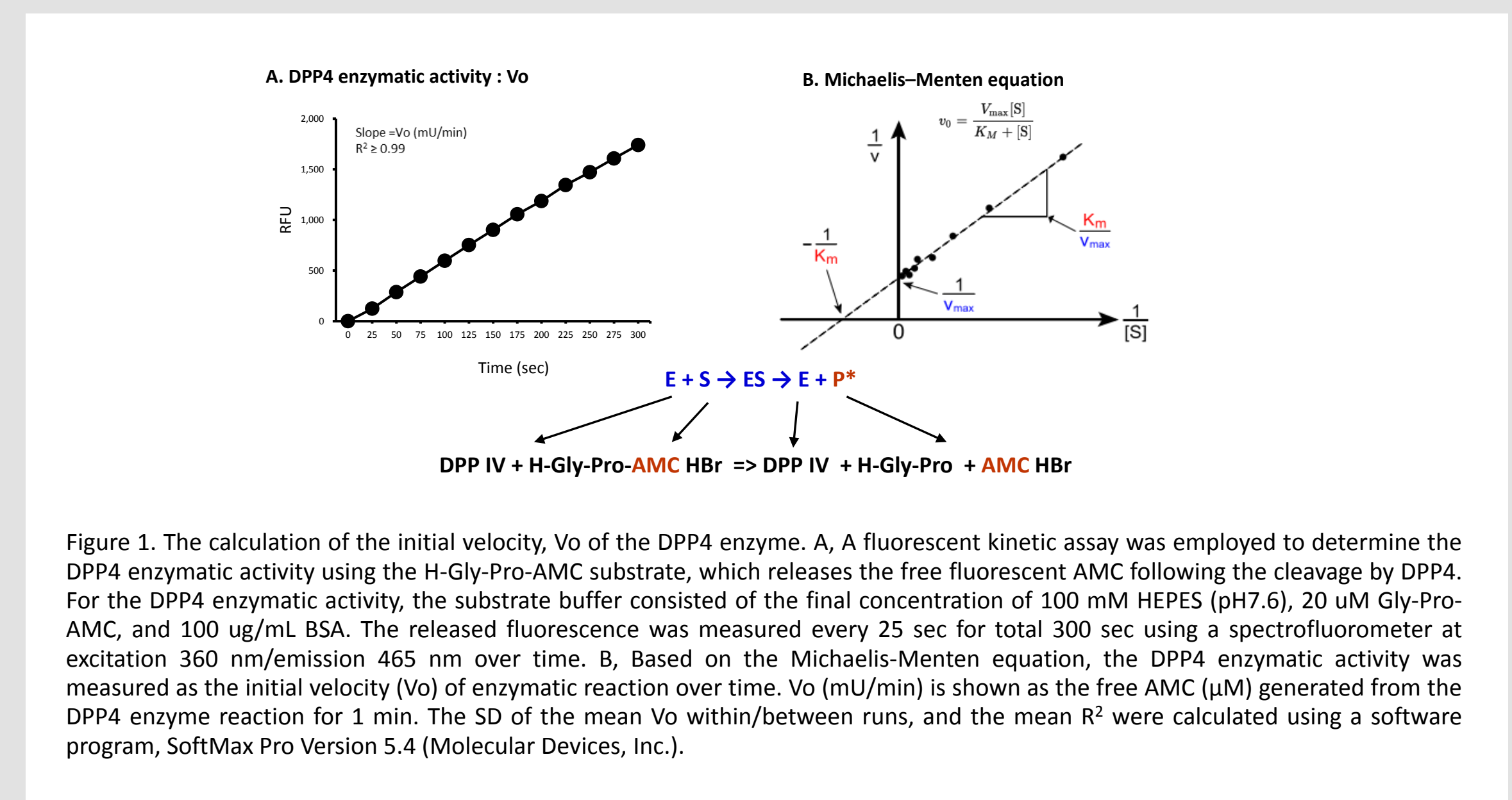
Abstract

In the course of a clinical study developing evogliptin (DA-1229) tartrate, a specific dipeptidyl peptidase 4 (DPP4) inhibitor for the treatment of type 2 diabetes, an analytical method of fluorescent kinetic assay was optimized and verified to determine an enzymatic activity of soluble DPP4 in human plasma using a spectrofluorometer. The validation was performed for the parameters including the accuracy, the precision, the limit of detection (LOD), the linearity, the dynamic range, the short/long-term stability, the freezing-thawing stability, the *Km* constant, the dilution effect, and the recovery efficiency. The plasma DPP4 enzymatic activity (mU/min) was measured as the initial velocity (*V₀*) of enzymatic reaction over time. After the reaction, the deviation of the mean from the nominal value, the coefficient of variation (CV) within/between runs, and the relative determinant constant (*R*²) were calculated. Accuracy and precision were within the deviation of the mean ≤ 15%, CV ≤ 15%, *R*² > 0.99 except for LOD, where it did not exceed the deviation of the mean ≤ 20%, CV ≤ 15%, *R*² > 0.95, respectively. The linearity of *V₀* and the dynamic range of DPP4 values were reliable in the range of 6.06 × 10³ - 5.13 × 10⁵ mU/min and 62.5 - 1,500 ng/mL, respectively. Plasma DPP4 was stable under the various temperatures and even after three cycles of the freezing-thawing. The *Km* constant of plasma DPP4 was similar to that of the recombinant DPP4. Evogliptin (DA-1229) tartrate effectively inhibited the DPP4 enzymatic activity in a dose-dependent manner without the dilution effect of sample. Due to the limited recovery efficiency of DPP4 in sample larger than 10 μL, the volume of sample was determined to be 10 μL for reliable assays. The optimized and validated analysis method of the DPP4 activity was successfully set up and employed for the measurement of the DPP4 activity in human plasma.

Introduction

Dipeptidyl peptidase 4 (DPP4/CD26) is a representative serine protease to cleave two N-terminal dipeptide with alanine or proline, thereby regulating the immunological activity of a variety of substrates. In addition, sDPP4 is abundant in plasma and membrane DPP4 (mDPP4) is expressed in most of organs including kidney and various cell types such as epithelial cells and T lymphocytes. DPP4 enzymatic activity regulates incretin hormone GLP-1 and GIP-1, which promotes glucose homeostasis in blood by the stimulation of insulin synthesis from pancreatic β-cells. This study was performed to validate the analytical method for the measurement of the DPP4 activity in human plasma which were collected from Dong A-ST clinical study relating evogliptin (DA-1229) tartrate, a specific DPP4 inhibitor for the treatment of type 2 diabetes mellitus.

Methods



Results

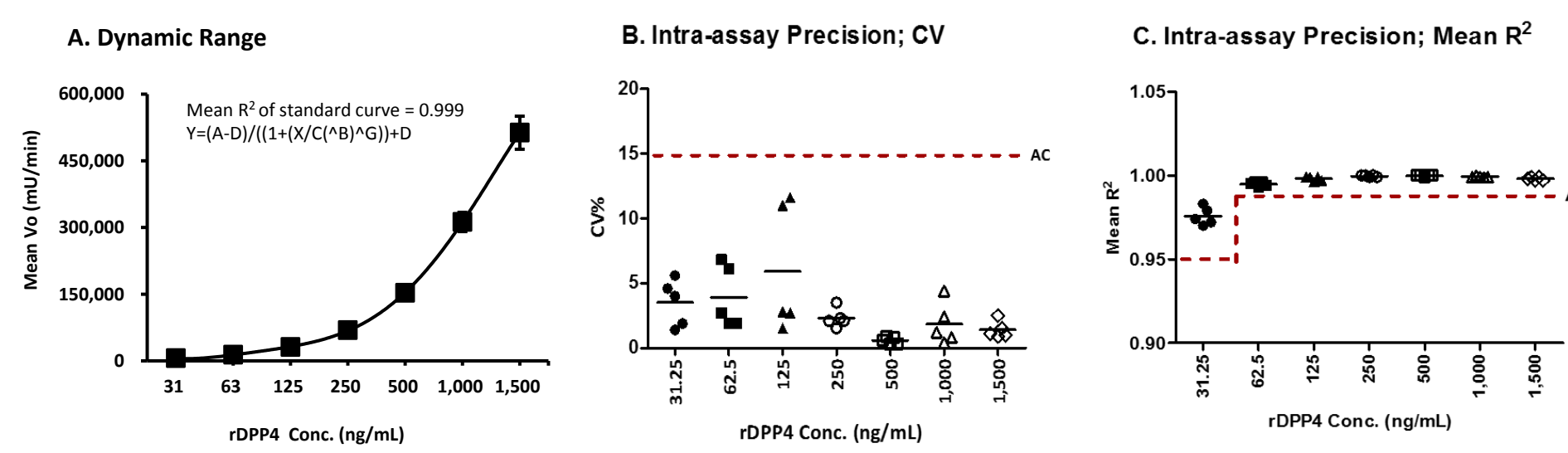
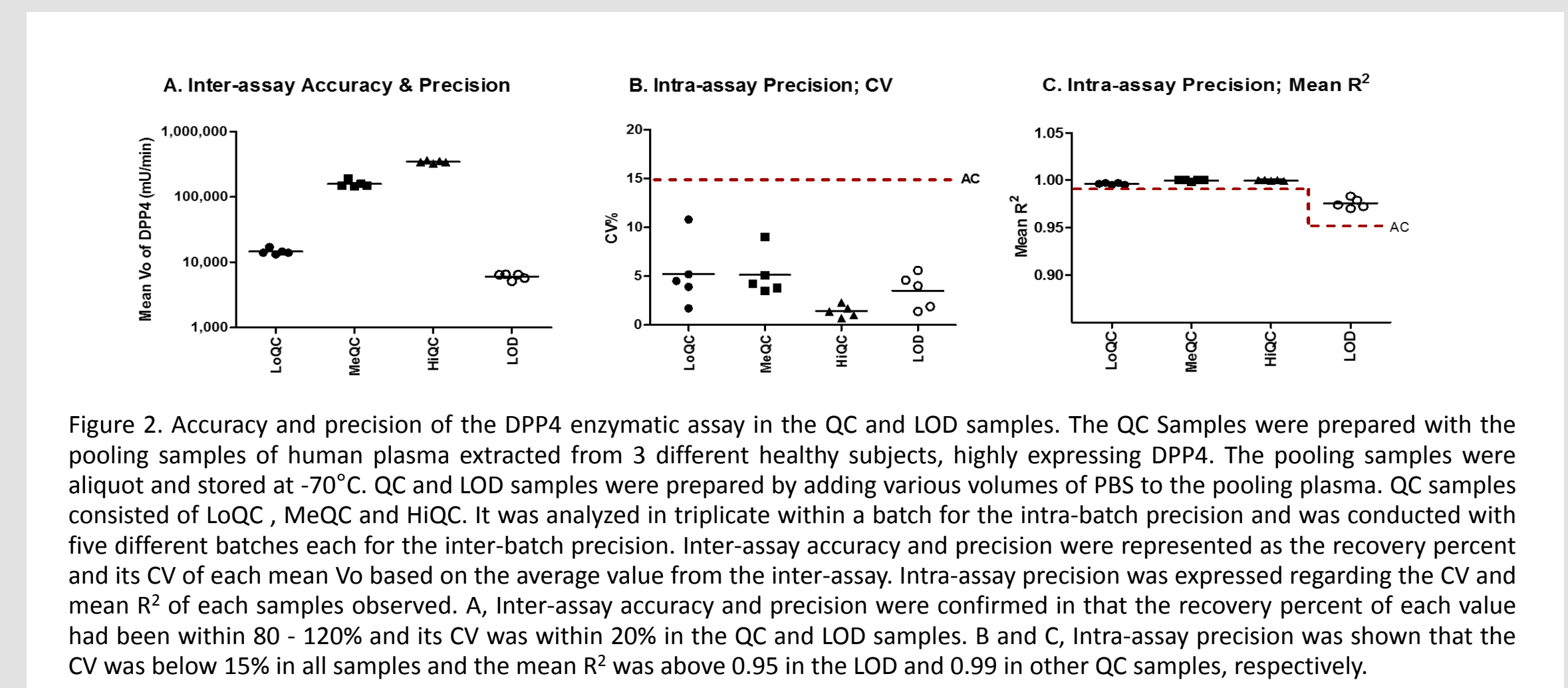


Figure 3. Dynamic range and precision of DPP4 enzymatic assay in the standard samples. The recombinant DPP4 (rDPP4) was diluted serially with PBS from 1,500 ng/mL to 31.25 ng/mL was freshly prepared and analyzed to estimate the mean *V₀* of DPP4 enzyme. To determine the concentration of DPP4 enzyme in an unknown plasma sample, a standard curve of DPP4 was drawn with varying concentrations of rDPP4 versus its mean *V₀*. The linearity of *V₀* and the reliability of a dynamic range of DPP4 values were shown by the mean *R*² of the standard curve generated. Also, intra/inter-batch precision was performed repeatedly as in the analysis of the QC samples. A, Reliable dynamic range for mean *V₀* of DPP4 was from LLOQ (31.25 ng/mL) to ULOQ (1,500 ng/mL) and the mean *R*² for the standard curve was 0.999. B and C, Intra-assay precision was confirmed in that the CV was below 15% in all standard samples and the mean *R*² was above 0.95 in the LLOQ (31.25 ng/mL) and 0.99 in other standard samples, respectively. Taken together, the linearity of *V₀* and the dynamic range of DPP4 values were reliable in the range of 6.06 × 10³ - 5.13 × 10⁵ mU/min and 62.5 - 1,500 ng/mL, respectively.

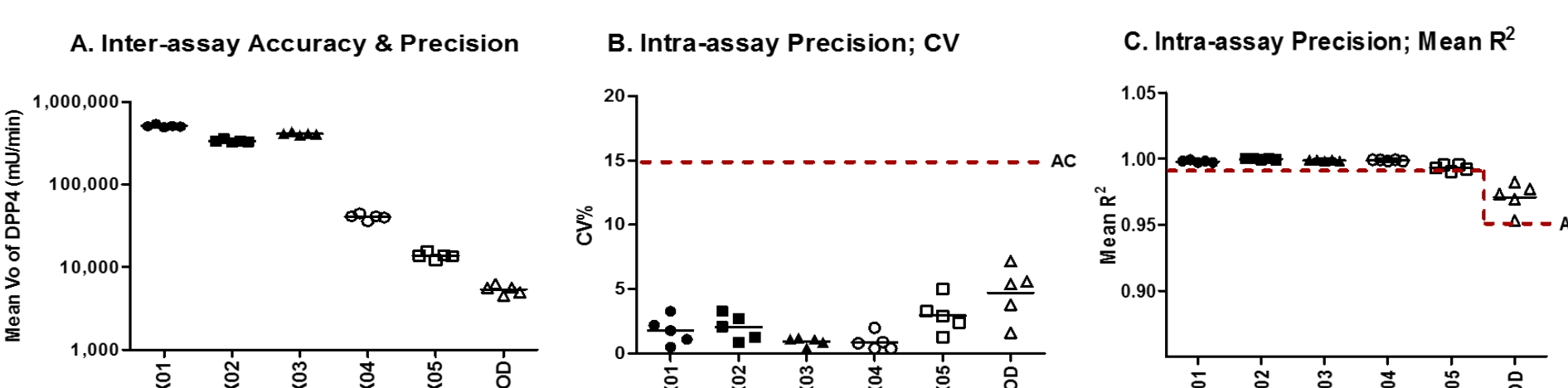


Figure 4. Accuracy and precision of the DPP4 enzymatic assay in human plasma samples. Plasma samples were obtained from 3 different healthy subjects (X01 - X03), highly expressing DPP4 enzymatic activity and other 3 different healthy subjects (X04 - XLOD) that were treated a DPP4 inhibitor (Sitagliptin 100 mg/tablet) to reduce its enzymatic activity. The samples were aliquot and stored at -70°C until the analysis. Also, intra/inter-batch precision conducted as in the analysis of the QC samples. A, Inter-assay accuracy and precision were confirmed in that the recovery percent of each value had been within 85 - 115% and its CV was within 15% in the LOD and plasma samples. B and C, Intra-assay precision was shown that the CV was below 15% in all samples and the mean *R*² was above 0.95 in the LOD and 0.99 in other plasma samples, respectively.

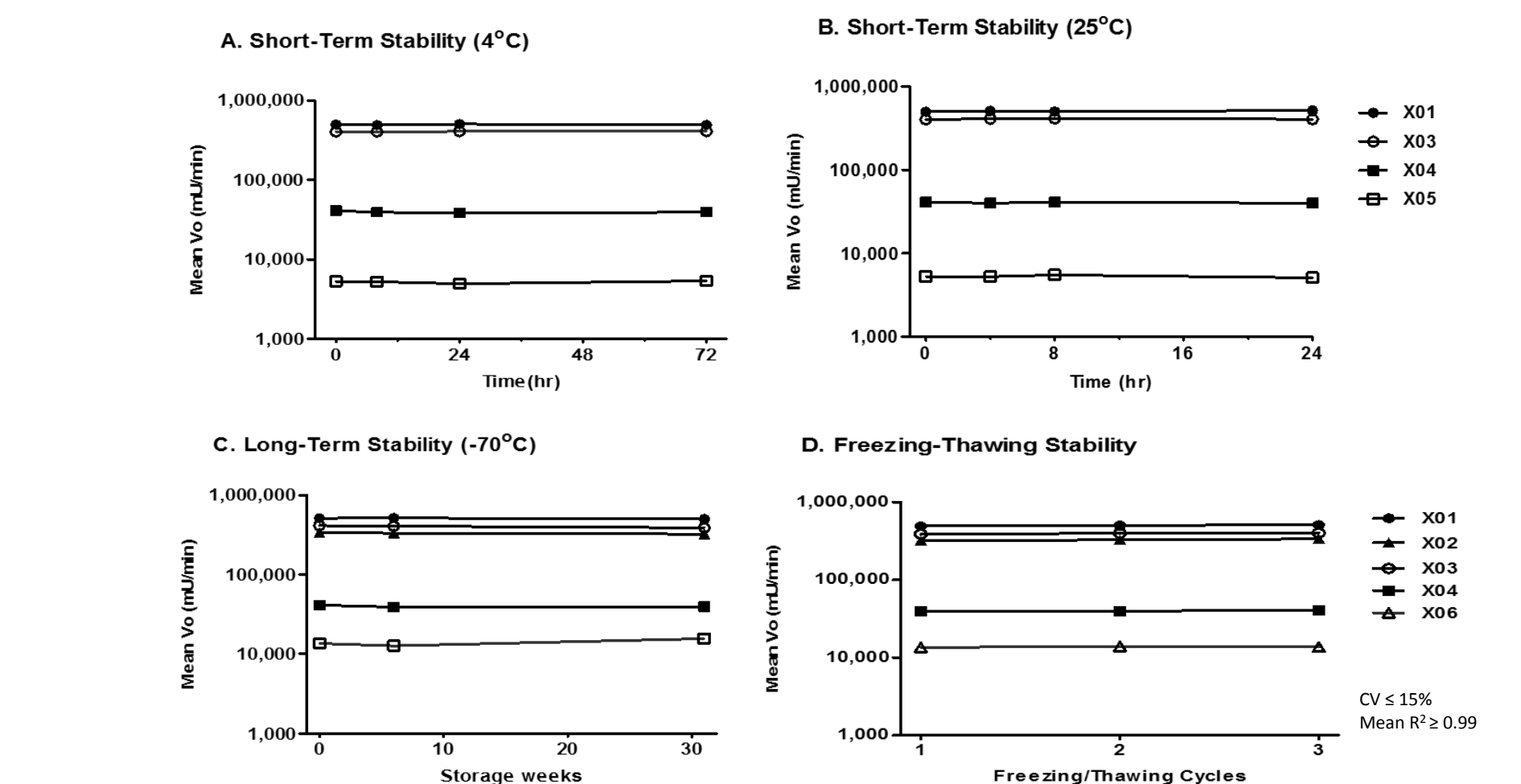


Figure 5. Stability of the DPP4 enzymatic activity in the plasma samples. Plasma samples (n=4-5) were prepared to evaluate thermal and freezing/thawing stability of the DPP4 enzyme. The storage conditions or freezing/thawing cycles were determined considering a storage period and subsequent use of the samples. To evaluate the short-term stability at 4°C and RT (25°C) or long-term stability at -70°C, the DPP4 enzymatic activities were measured 3-4 times while these samples were incubated under the storage conditions. To validate freezing/thawing stability, its enzyme activity was measured after 1 to 3 cycles of freezing/thawing. A and B, The DPP4 enzymatic activities were not changed at 4°C over 72 hours and RT over 24 hours. C, It was also consistent at -70°C over 31 weeks. D, It did not decrease at all even after 3 cycles of the freezing-thawing.

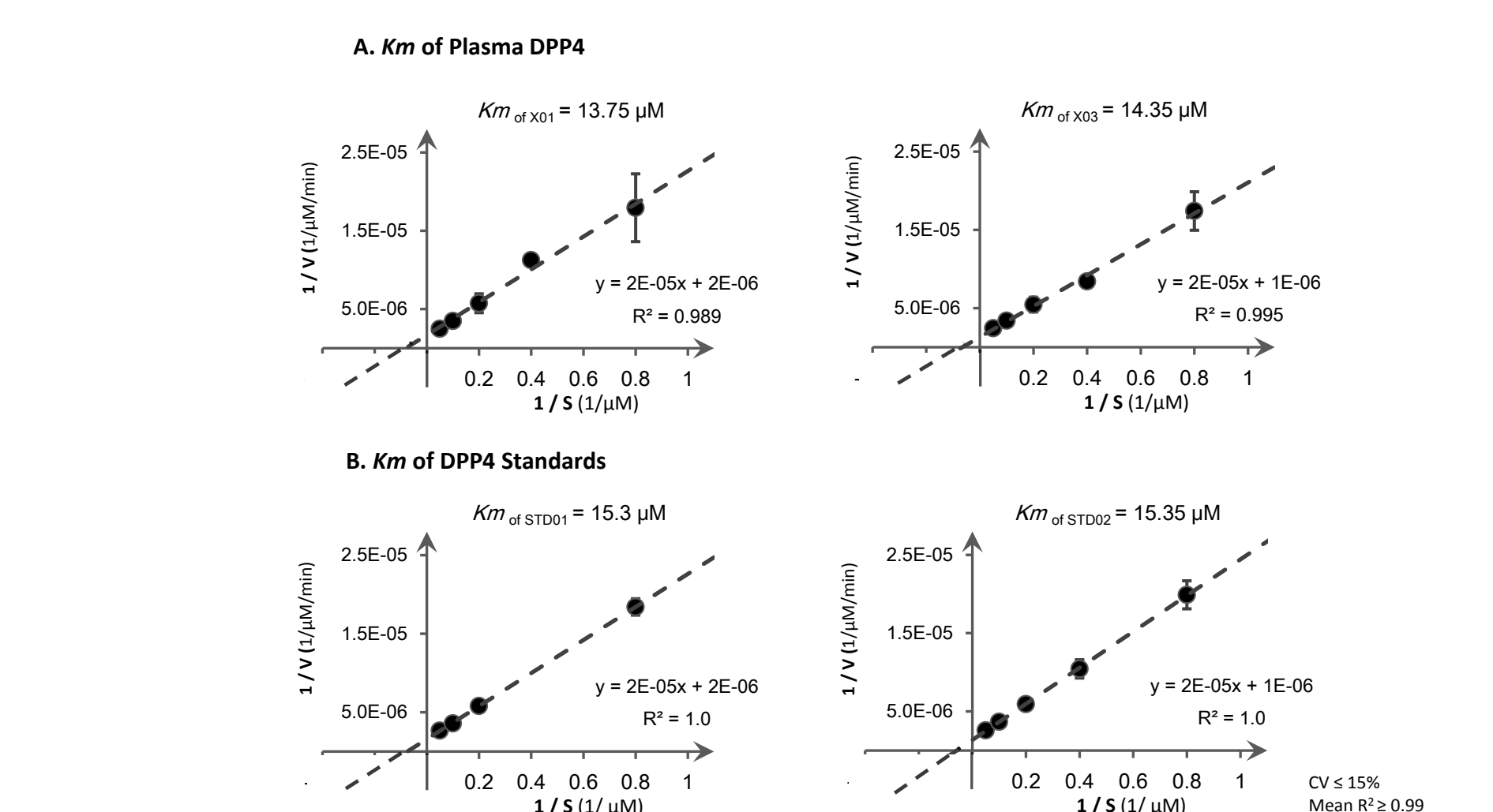


Figure 6. *Km* constants of DPP4 enzymatic activity in the plasma and recombinant standard samples. The *Km* value is defined as the substrate concentration [S] at 1/2 the maximum velocity [*V_{max}*] using Michaelis-Menten equation ($V_0 = \frac{V_{max}[S]}{K_m + [S]}$) and Lineweaver-Burk equation ($1/V_0 = (K_m/V_{max})(1/S) + 1/V_{max}$). It was determined by the DPP4 enzymatic reaction of the plasma (X01, X03) and recombinant standard samples with 4-5 different concentrations of substrate and plotted as a graph of the mean *V₀* against the concentration of substrate [S]. It was analyzed in triplicate within a batch for the intra-batch precision and was conducted 2 different batches each for the inter-batch precision. A, *Km* constants of the plasma samples were 13.75 - 14.35 μM. B, *Km* constants of the standard sample were 15.3 - 15.35 μM.

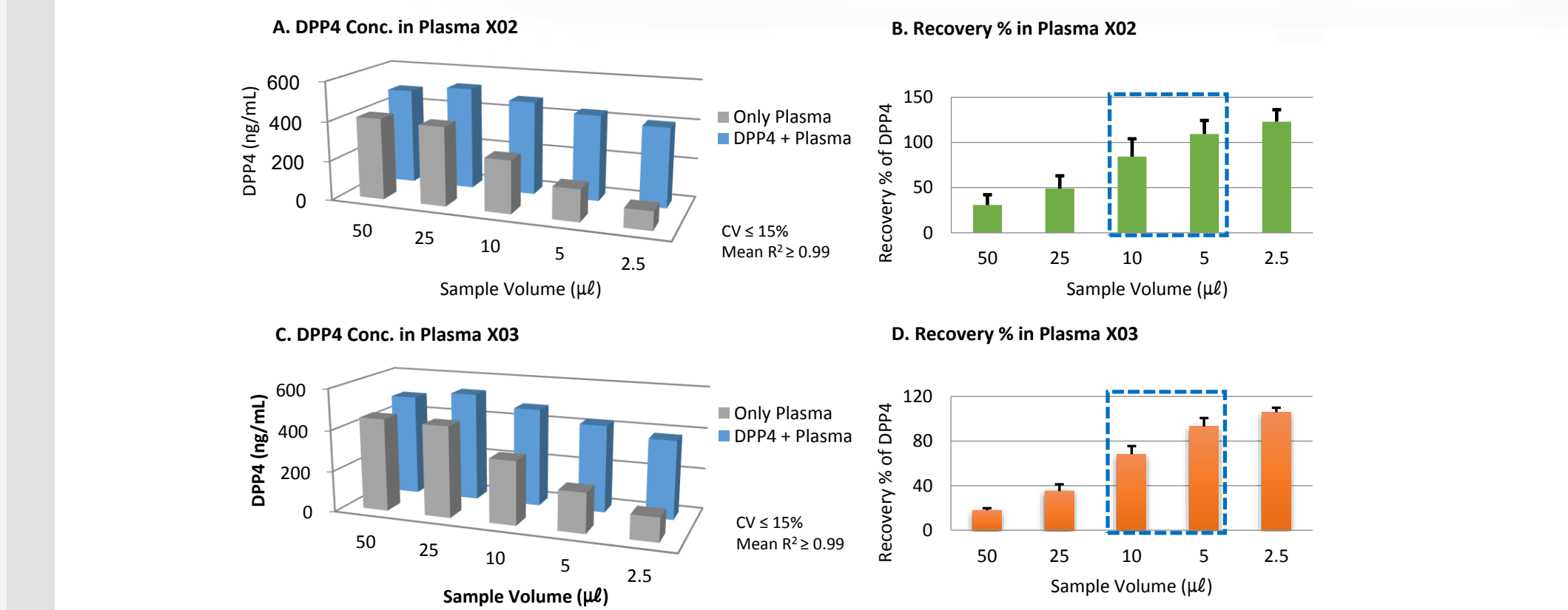


Figure 7. Recovery effect of the DPP4 enzyme in the plasma samples. The standards and the test samples including only the plasma and recombinant DPP4 (250 ng/mL) treated plasma samples (X02, X03) were freshly prepared. Plasma samples of different volumes and standard were added in the substrate buffer, and the DPP4 enzymatic activity was measured. The concentration of DPP4 enzyme was quantified from the standard calibration curve. Also, intra/inter-batch precision was analyzed repeatedly as described in Figure 6. Due to the limited recovery efficiency of DPP4 in sample larger than 10 μL, the volume of sample was determined to be 10 μL for the reliable assays. The recovery percent was calculated as follows : equation described below

$$\text{Recovery \%} = \frac{(B - A) \times 100\%}{\text{Treated DPP4 Conc. (250 ng/mL)}}$$

A = DPP4 Conc. of only plasma sample
B = DPP4 Conc. of plasma sample + rDPP4 (250 ng/mL)

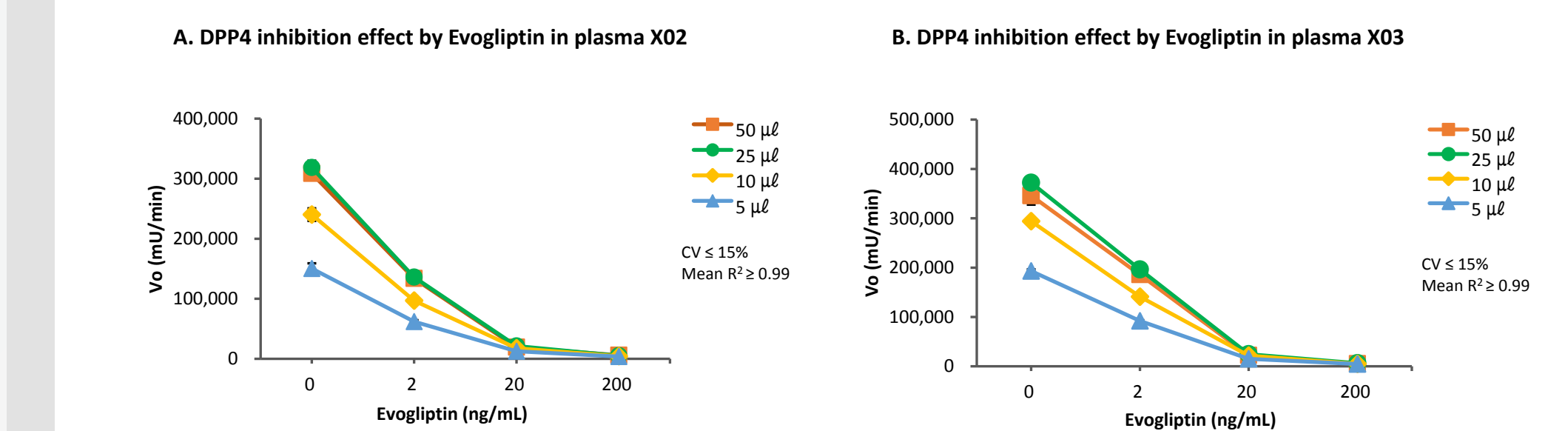


Figure 8. DPP4 inhibition effect by evogliptin (DA-1229) tartrate in the plasma samples. The plasma samples (X02, X03) and 40X stock concentrations (8 - 80,000 μg/mL) of evogliptin (DA-1229) tartrate were freshly prepared. Plasma samples of different volumes and a final 1X concentration (2 - 200 ng/mL) of evogliptin (DA-1229) tartrate were treated in the substrate buffer, and the DPP4 enzymatic activity was measured. In addition, intra/inter-batch precision was analyzed repeatedly as described in Figure 6. Evogliptin (DA-1229) tartrate effectively inhibited the DPP4 enzymatic activity in a dose-dependent manner without the dilution effect of a sample.

Conclusions (Validation summary)

As the results of validation fulfilled the acceptance criteria, it was concluded that the method has been established for the analysis of the plasma samples collected from the clinical study.

Parameters	Test samples	Acceptance criteria	Results
• Accuracy & Precision	QC, Plasma	Recovery: 85 - 115% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Results of QC samples were acceptable • Results of plasma samples were acceptable
• LOD	Standard, Plasma	Recovery: 80 - 120% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.95 Mean <i>R</i> ² for Standard curve ≥ 0.995	• LOD of standard; 6,058 ± LOD > 3,066 mU/min • LOD of plasma; approximately 5,399 mU/min
• Linearity of Standard curve • Dynamic range of DPP4 Conc. (ng/mL) • Dynamic range of Mean <i>V₀</i> (mU/min)	Standard	Recovery : 80 - 120% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Mean <i>R</i> ² for Standard curve ≥ 0.995 • Dynamic range of DPP4 Conc.: 62.5 - 1,500 ng/mL • Dynamic range of mean <i>V₀</i> : 6.06 × 10 ³ - 5.13 × 10 ⁵ mU/min
• Short-term stability • Long-term stability • Freezing-thawing stability	Plasma	Recovery: 85 - 115% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Stable after 3 cycles of the freezing-thawing • Stable for 72 hours at 4°C and for 24 hours at 25°C • Stable for 31 weeks at -70°C
• Km constant	Standard, Plasma	Recovery: 85 - 115% CV ≤ 20% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Km of standard = 15.3 - 15.35 • Km of plasma = 13.75 - 14.35
• Recovery efficiency	Standard, Plasma	Recovery: 85-115% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Recovery of sample vol. (10 μL) = 62.9 - 98.2%
• Sample dilution effect on the DPP4 inhibition by evogliptin (DA-1229) tartrate	Plasma	Recovery: 85 - 115% CV ≤ 15% Mean <i>R</i> ² for <i>V₀</i> ≥ 0.99	• Evogliptin (DA-1229) tartrate effectively inhibited the DPP4 enzymatic activity without the dilution effect of sample.

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