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# LC-MS based assay method for DPP-IV inhibitor screening and substrate discovery

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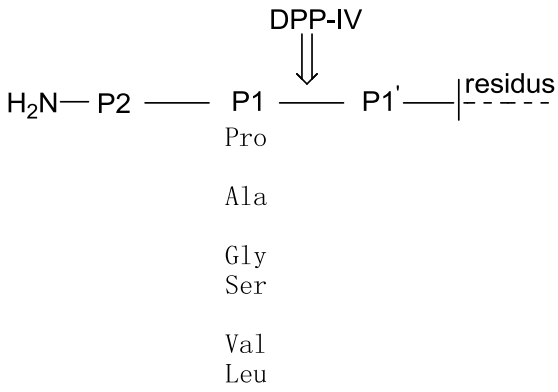
Abbreviations: LC-MS: liquid chromatography-mass spectrometry; DPPIV: Dipeptidyl peptidase IV; GLP-1: glucagon like peptide-1; Ala: Alanine; Gly: Glycine; Pro: Proline; HTS: high-throughput screening; ESI: electrospray ionization; APCI: Atmospheric Pressure Chemical Ionization; pNA: p-nitroanilide; Asp: Aspartic acid; Thr: Threonine; Met: Methionine; Phe: Phenylalanine; Leu: Leucine; Ser: Serine; HOBT: Hydroxybenzotriazole ; EDCI: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Me:methyl

**Abstract:** The traditional methods for dipeptidyl peptidase IV (DPP-IV) inhibitor screening using fluorescence and chromogenic detections have a number of limitations. Interference with assay readout may occur if compounds have strong absorption at the wavelength region used for the detection. Some commonly used fluorescent and chromogenic DPP-IV substrates have poor aqueous solubility and require organic solvents such as DMSO for solubilization. The use of organic co-solvents in enzymatic assays for DPP-IV may lead to unreliable results. Furthermore, some fluorescent and chromogenic DPP-IV substrates are unstable in aqueous solution and undergo hydrolysis in the absence of DPP-IV. We have developed an LC-MS based method for DPP-IV activity assay which allowed the use of wider selections of substrates than the fluorescent and chromogenic methods. The LC-MS method was validated with several known DPP-IV inhibitors in comparison with a chromogenic assay method and was used to test library compounds to discover new inhibitors of DPP-IV. In addition, being a more universal detection technology, LC-MS method facilitates the discovery of new substrates. A mini-library of tripeptides was synthesized and screened for the discovery of new substrates with improved properties for DPP-IV assay.

**Keywords:** LC-MS, bioassay, DPP-IV, substrate, tripeptide

## 1. Introduction

Of the many enzymatic activities that are involved in peptide metabolism, proteolysis is one of the most important. Proteolytic enzymes such as peptidases and proteases are intimately involved in the production [1] degradation [2] and signaling of peptides, including peptide hormones that control many physiological processes. Dipeptidyl peptidase IV (DPP-IV) was discovered in 1966 by Hopsu-Havu and Glenner [3] and it has been known under different names, such as ‘‘Dipeptidyl aminopeptidase IV’’, ‘‘Postproline dipeptidyl aminopeptidase IV’’, ‘‘X-Pro dipeptidyl aminopeptidase’’ or ‘‘Gly-Pro naphthylamidase’’ [4]. The enzyme was for many years believed to be the unique cell membrane protease cleaving X-Pro dipeptides from the N-terminal end of peptides and proteins [5]. Many biologically active peptides contain an evolutionary conserved proline residue as a proteolytic-processing regulatory element and therefore proline-specific proteases could be seen as important `check-points' controls [6]. Whilst proline and alanine are almost exclusively present at the P1 (scheme 1) position of DPP-IV substrates, peptides containing hydroxyproline, dehydroxyproline, serine, glycine, and valine at this position are also hydrolysed by DPP-IV, although at greatly reduced rates [7]. In the P2 position any amino acid residue is accepted, provided it has a protonated amino group [8].




Scheme 1: Mode of DPP-IV substrate catalysis

**Scheme 1.** Mode of DPP-IV substrate catalysis.

DPP-IV is an enzyme of considerable biomedical interest because it is upregulated in certain diseases [9]. It plays an important role in glucose homeostasis through proteolytic inactivation of incretin hormones, primarily glucagon like peptide-1 (GLP-1) which is critical in sugar balance[10]. Other known effects of GLP-1 are slowed gastric emptying, reduced appetite, and increased insulin-sensitivity in β-cells [11]. GLP-1 has a short lifetime due to rapid degradation by the DPP-IV enzyme, which cleaves the two N-terminal amino acids to give the inactive GLP-1 amide [12]. Thus, inhibition of DPP-IV, which leads to increases in the circulating level of GLP-1, is an important approach for the treatment of type 2 diabetes.

In vitro, the activity of DPP-IV is often assayed using chromogenic substrates, such as glycylproline b-naphthylamide [13] and glycylproline p-nitroanilide [14,] or fluorescent probes, such as (Ala-Pro)2-cresyl violet and (Ala-Pro)2-Rhod110 [15]. Although, widely used in practice, there are a number of limitations with these assay detection methods. First, if the compounds to be tested have absorbance at

the same wavelength region as the substrates, interference with assay readout may occur. For example, some herbs have absorption at 405nm, the same as glycylproline p-nitroanilide used for chromogenic methods. Second, although these chromogenic or fluorescent substrates are widely available, they have poor aqueous solubility and frequently require organic solvents such as DMSO for solubilization. The use of organic co-solvents in enzymatic assays for DPP-IV may lead to unreliable results. For example, DPP-IV activity has been reported to be modulated by DMSO, with 1% DMSO reducing activity by 50% [16]. Third, both (Ala-Pro)2-cresyl violet and (Ala-Pro)2-Rhod110 are unstable in aqueous solution and undergo hydrolysis in the absence of DPP-IV[15]. Thus there is a need for new DPP-IV assay methods and substrates that can improve the above aspects of the traditional methods.

Mass spectrometry (MS) is one of the most powerful analytical techniques and it plays a key role in nearly every stage of the drug development process [17]. Recent advances in MS instrument design, especially the development of electrospray ionization (ESI), have extended the reach of MS to the lead discovery stages of drug development as well. In some screening formats, the selectivity achieved by MS enables direct analysis of compound mixtures such as combinatorial libraries and unpurified natural products extracts, an advantage that is not easily achieved using other analytical methods. MS-based screening is primarily implemented in two ways: by monitoring the functional output of a protein-dependent biochemical

reaction, or by using affinity-based methods that directly assess binding of a candidate molecule to its target protein.

Recently, the combination of HPLC and mass spectrometry applying atmospheric pressure chemical ionization (APCI) or ESI as an ionization process, has permitted low level quantitation of pharmaceuticals and biochemicals [18].

Liquid chromatography–mass spectrometry (LC–MS) has the advantages of very short analysis time, higher sensitivity, selectivity and feasibility of direct use of assay samples containing buffers and salts. More importantly, LC–MS is a much more general detection technique that can be applied to wider classes of compounds than chrogenic and fluorogenic techniques. It is routinely used in the analysis of drugs with poor UV absorbing properties such as propranolol and phenytoin and compounds otherwise can interfere with fluorescent detection.

We have developed an LC-MS based method for the DPP-IV activity assay using the well established chromogenic method as reference. We took advantage of the LC-MS capability of detecting wider range of structures without the need of labeling. We focus on LC-MS detection of dipeptide Gly-Pro which is the product of the cleavage by DPP-IV of commonly used substrates. This method can be used for any substrates containing N-terminal dipeptide Gly-Pro. The LC-MS method was used to test library compounds to discover new inhibitors of DPP-IV. In addition, being a more universal detection technology, LC-MS method facilitates the discovery of new substrates. We synthesized a mini-library of tripeptides containing N-terminal Gly-Pro and used the LC-MS method to

screen for substrates that have improved properties for DPP-IV assay such as more water-soluble, more stable in aqueous solution, and have different Km and kcat properties than the fluorescent and chromogenic substrates.

## 2. Materials and methods

***Materials*** Recombinant human DPP-IV enzyme was purchased from Sigma (St. Louis, MO, USA, Cat. No. D4943), Gly-Pro p-nitroanilide hydrochloride (Gly-Pro-pNA) was purchased from Sigma (Cat. No. G0513), 96-well ELISA plate was purchased from Corning (Shanghai, China, Cat. No.3370). Dipeptide Gly-Pro and other chemicals were purchased from Sigma with the highest quality. RP-HPLC C8 column was from Agilent (Shanghai, China) or Themo (Shanghai, China) or Bio Rad (Shanghai, China). HPLC-grade LiChrosolv acetonitrile and HPLC-grade LiChrosolv methanol were purchased from Merck (Shanghai, China). The rest of the reagents were of analytical grade.

***LC-MS instrument and conditions*** The LC-MS equipment used in this work was an Agilent 1200 (Agilent, Shanghai, China) HPLC coupled with Waters LCT-TOFMS (Waters, USA). The chromatography was performed using Thermo HYPURITY C18, 50×2.1mm for monitoring Gly-Pro formed from substrate tripeptides and Gly-Pro-pNA and Waters Diol 120A 50×1mm for tripeptides, at 30 $^{o}$C temperature. For the analysis of Gly-Pro, the mobile phase A was aqueous solution containing 10mM ammonium acetate and mobile phase B was acetonitrile, gradient flow at 0.8 mL/min with a split ratio of load to waste of 20:80 with an effective flow rate of 0.16 mL/min going through the column. For

the analysis of the tripeptides, the mobile phase A was aqueous solution containing 0.1% formic acid and mobile phase B was acetonitrile, gradient flow at 0.3 mL/min.

Mass spectrometric detection was done with Waters LCT-TOFMS. Typical source conditions were as follows: positive ion electrospray ionization mode, desolvation temperature was set at 350 $^{o}$C, source temperature was set at 90 $^{o}$C, and the capillary voltage was set at 3500 V. The target ions monitored were m/z 173 for [Gly-Pro+H] $^{+}$ and corresponding protonated ions for each of the tripeptides.

**Methods**

We have used both chromogenic and LC-MS methods to characterize the activity of DPP-IV enzyme using the same substrate Gly-Pro-pNA and verified the performance of the LC-MS assay method by comparing the $IC_{50}$ values of reference compounds obtained from LC-MS method with those from the chromogenic method.


**2.1 LC-MS assay**

We monitored the change of Gly-Pro (m/z 173 in positive ion mode) from proteolysis of Gly-Pro-pNA and other tripeptide substrates containing N-terminal Gly-Pro, catalyzed by DPP-IV by LC-MS. All experiments were performed at 37 $^{o}$C in a 50 mM Tris-HCl buffer. The total reaction volume is 50 μL. Samples for

LC-MS analysis were prepared in the following condition:2 μL assay solution was diluted in 8 μL acetonitrile and the injection volume was 5 μL.

***DPP-IV activity***

The incubation mixture contained different concentration of substrate, DPP-IV and 50 mM Tris-HCl (pH 8.0) buffer in a final volume of 200 μL. The reaction took place at 37 $^{o}$C. We quantified the amount of Gly-Pro every 5 minutes in 1 hour time to obtain the standard curve of the Gly-Pro concentration by LC-MS.

***Enzymatic Characterization of DPP-IV and measurement of substrate Km***

The enzymatic activity of DPP-IV was assayed by measuring the formation of Gly-Pro by LC-MS. All the reactions were initiated by addition of Gly-Pro-pNA. The reaction mixture without enzyme was used as control sample.

During the investigation of the Km value of Gly-Pro-pNA, the concentrations of Gly-Pro-pNA varied from 50 to 3000 μM. And the reaction was initiated by addition of fixed concentration of DPP-IV of 475 ng/mL. For all assays, the reaction mixture without enzyme was used as background.

***$IC_{50}$ determination of positive reference compounds***

To validate the recombinant human DPP-IV enzymatic reaction system, two commonly used positive control compounds, vildaglitin and sitaglitin were used. The optimized standard reaction mixture contained 475 ng/mL DPP-IV and 691.9 μM Gly-Pro-pNA in total volume of 50 μL. For $IC_{50}$ determination of the two compounds, eight concentrations of each compound ranging from 0.01 to 5000

μM were used. Compounds were pre-incubated with DPP-IV at room temperature for 30 min, and then started the enzymatic reaction by adding Gly-Pro-pNA. Enzymatic reaction sample without reference compound was used as control, and compound without enzyme was used as blank. All the data were analyzed by GraphPad Prism 5.0 software, Km was calculated by fitting the initial velocity data to the Michaelis–Menten hyperbola mode.

## 2.2 Chromogenic assay

### *DPP-IV activity*

DPP-IV activity was measured as described [19], using the substrate Gly-Pro-pNA. We measured absorbance at 405 nm at each 1 minute increment during 60 minutes using an Ultrospec 4000 spectrometer.

### *Enzymatic Characterization of DPP-IV and measurement of substrate Km*

The enzymatic activity of DPP-IV was assayed by measuring the formation of pNA by recording the absorbance at OD405. All the reactions were initiated by addition of Gly-Pro-pNA. The reaction mixture without enzyme was used as blank.

In the Km determination of DPP-IV, series concentrations of DPP-IV ranged from 0.0003 to 100 μg/mL were reacted with fixed Gly-Pro-pNA concentration of 500 μM. The enzymatic reaction was started by addition of Gly-Pro-pNA. During the investigation of the Km value of Gly-Pro-pNA, the concentrations of Gly-Pro-pNA varied from 0.001to 2000 μM. And the reaction was initiated by

addition of fixed concentration of DPP-IV of 475 ng/mL. For all assays, the reaction mixture without enzyme was used as background.

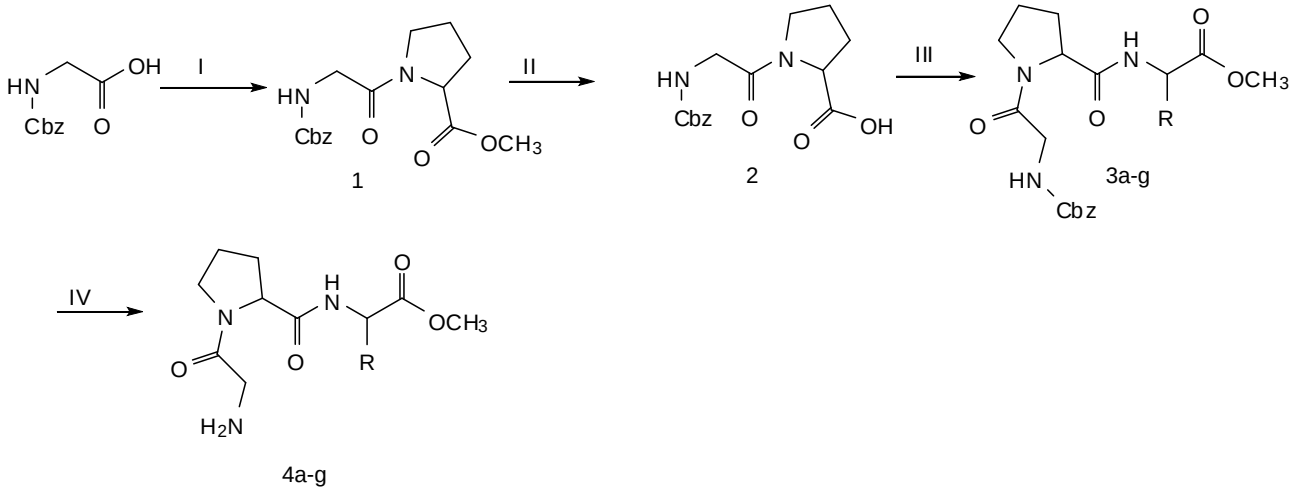
***IC50 determination of positive reference compounds***

To validate the recombinant human DPP-IV enzymatic reaction system, two commonly used reference compounds, vildaglitin and sitaglitin were used. The optimized standard reaction mixture contained 475 ng/mL DPP-IV and 691.9 μM Gly-Pro-pNA in total volume of 50 μL. For determination the IC50 of the two compounds, eight concentrations of each compound ranging from 0.01 to 2000 μM were used. Compounds were pre-incubated with DPP-IV at room temperature for 30 min before starting the enzymatic reaction by adding Gly-Pro-pNA. Enzyme without compound sample was used as control, and compound without enzyme sample was used as blank. All the data were analyzed by GraphPad Prism 5.0 software, $IC_{50}$ was calculated by fitting data to the Does-response-Inhibition mode.

## 2.3 Chemistry

The protected amino acids with L-configuration and amino-acid ester were purchased from TCI. All coupling and deprotective reactions were carried out under anhydrous conditions. Chromatography was performed on Qingdao silica gel H. The purities of the intermediates and the products were confirmed by TLC (Merck silica gel plates of type 60 F254, 0.25 mm layer thickness) and HPLC (Waters, C18 column 4.6 ×150 mm). The molecular weight and structure of

products were confirmed by Waters LCT-TOFMS and NMR. The procedure of synthesis can be seen in scheme 2.




**Scheme 2.** Synthesis of the tripeptide analogues. Reagents and conditions. （Ⅰ) HOBT, EDCI, $Et_3N$, DMF (Ⅱ) LiOH , MeOH (III) $H_2NCH(R)COOCH_3$, $ClCO_2i$-Bu, $CH_2Cl_2$ (IV) hydrogen, Pd/C. In a**: R=$CH_2CH(CH_3)_2$; b: R=$CH_2CH_2SCH_3$; c: R=$CH_2Ph$; d:=$CH_3$; e: R=$CH_2OH$; f: R=$CH_2COOCH_3$; g: R=$CHOHCH_3$.**

***methyl 1-(2-(benzyloxycarbonylamino)acetyl)pyrrolidine-2-carboxylate (1)***

At 0 $^oC$ , to a solution of 2.0 g (9.57 mmol) of 2-(benzyloxycarbonylamino)acetic acid in 30 mL of anhydrous DMF, 1.94 g (14.35 mmol) of HOBt was added. After stirring for 10 min, 2.74 g (14.35 mmol) of EDCI and 2 mL triethylamine was added. The suspension of 9.57 mmol of HCl methyl pyrrolidine-2-carboxylate and 1.33 mL triethylamine in 3 mL of anhydrous DMF were added and stirred at room temperature for additional 20 min. This suspension was then added to the solution of above, and the reaction mixture was continued stirring at 0 $^oC$ for 2 h and then at room temperature for 16 h. The mixture was dissolved in 30 mL of ethyl acetate. The solution was washed extensively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride, and the organic phase was separated and dried over

anhydrous sodium sulfate and concentrated under reduced pressure to give crude materials. Chromatographic purification of the crude materials over silica gel gave the title compound 1.

***1-(2-(benzyloxycarbonylamino)acetyl)pyrrolidine-2-carboxylic acid***

To a solution of 1.0 g (3.12 mmol) compound 1 in 20 mL alcohol, 0.112 g LiOH and two drop of water was added. Then the solution was stirred at 70 ℃ for 7 hours. The mixture was adjust with hydrochloride to PH 3-4, then removed the alcohol under vacuum, the residue was diluted in ethyl acetate, washed with water for triple times. The organic part was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give crude materials of compound 2.

***General procedure for preparation of 3a–g***

Compound 2 and triethylamine were disolved in dichloromethane, isobutyl chloroformate was added over 30min at 5 ℃. Then the mixture was stirred at 0-5 ℃ for 30min. Amino-acid ester hydrochloride and triethylamine were stirred in another flask for 30 min, then the mixture was added to the mixture of compound 2 over 2 h. After that, the mixture was stirred at room temperature for 16 h. The orgnic solution was washed with water and brine, dried over anhydrous sodium sulfate and concentrated under reduced pressure to give crude materials. Chromatographic purification of the crude materials over silica gel gave the title compound 3a–g.

***General procedure for preparation of 4a–g***

Asolution of compound 3a–g in MeOH was vigorously stirred overnight under an atmosphere of hydrogen in presence of Pd/C (10%) at room temperature. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give the compound 4a–g.

# 3. Results and discussion

### 3.1. LC-MS assay method development

We used DPP-IV substrate Gly-Pro-pNA for our LC-MS based assay method development and in comparison with the chromogenic assay method using the same substrate. Using LC-MS detection we can monitor the substrate Gly-Pro-pNA or the reaction products Gly-Pro or pNA. We selected to monitor the reaction product dipeptide Gly-Pro because Gly-Pro is also a common product for many other chromogenic and fluorescent substrates and the tripeptide substrates we have examined in this work. In addition, Gly-Pro showed better LC-MS sensitivity than the substrate and the other reaction product pNA in this work. The m/z value 173 is lower than most of the compounds that will be used for screening and therefore less probability of assay interference due to overlapping of m/z and LC-MS retention time of Gly-Pro with screening compounds. Figure 1 shows the relationship between LC-MS signal of Gly-Pro and concentration. Under the conditions used in this work the LC-MS method showed good linear range of 2 μM to 4 mM, sufficient to cover the nomally used concentration range of the substrate. The limit of quantitation for Gly-Pro is 1 μM. Injection to injection cycle time of each sample was 5 minutes.

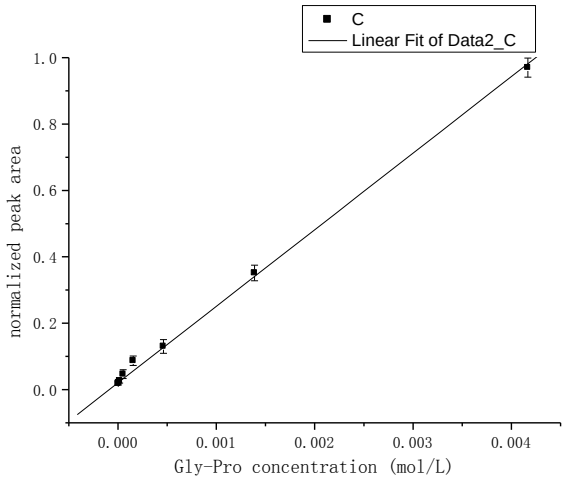
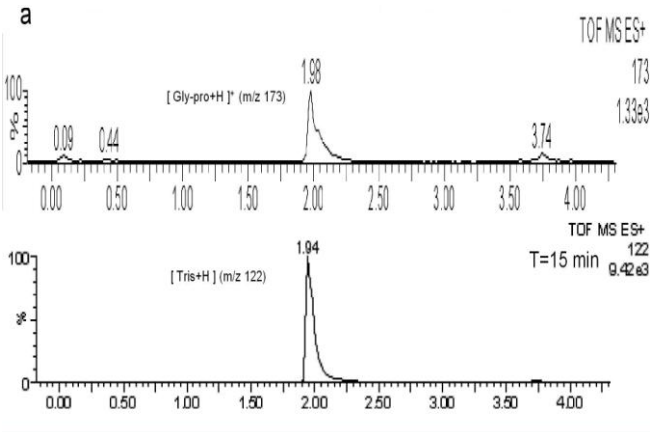



**Fig. 1.** Plot of normalized LC-MS peak area of Gly-Pro vs. concentration. Data are represented as means (n = 3).

The buffer component tris (m/z 122 for protonated tris) was used as internal standard for LC-MS quantitation. The m/z 122 peak was observed at similar retention time as Gly-Pro(1.94, 1.98 respectively)( Figure 2). The LC-MS data for Gly-Pro is presented as normalized to the signal of tris.



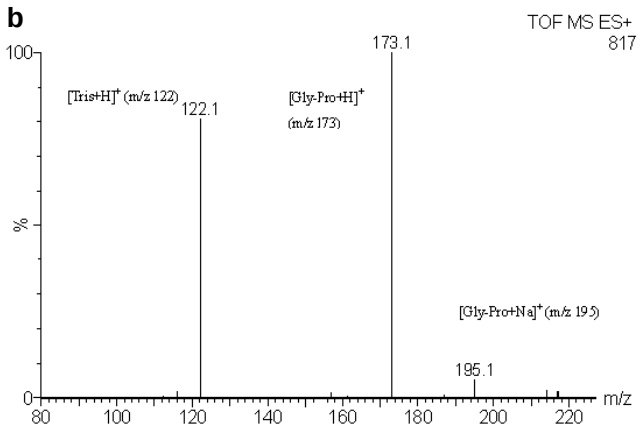



**Fig. 2.** LC-MS chromatogram (a) and mass spectrum (b) for the DPP-IV assay.

### *3.2 DPP-IV activity measurement by LC-MS and chromatography method*

We carried out DPP-IV enzyme activity measurement using the LC-MS method we have developed. Figure 3 shows the time course of the reaction measured by LC-MS at different concentrations of DPP-IV. Concentration of the substrate Gly-Pro-pNA used was 1000 μM. The active unit of DPP-IV measured by LC-MS method is 0.054 mg.

The enzyme activity was also measured by chromatography method [19], the active unit of DPP-IV measured by chromatography method is 0.034 mg.

DPP-IV enzyme activity assay demonstrate that the DPP-IV enzymatic reaction measured by LC-MS and chromatography method showed similar overall characteristics. This observation also showed that the performance of the LC-MS assay method is similar to that of the chromogenic method.

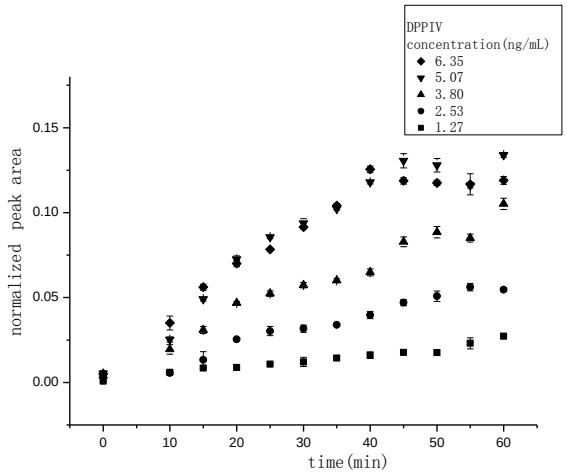



**Fig. 3.** The increment of Gly-Pro normalized peak area by time of different DPP-IV concentration. 6.35, 5.07, 3.80, 2.53, 1.27 ng/mL DPP-IV were test in 60 minutes. Data are represented as means (n = 3).

### *3.3 Enzymatic Characterization of DPP-IV by LC-MS and chromatography method*

The DPP-IV enzyme used in this work was characterized using the LC-MS method. Under fixed substrate concentration and reaction time, different concentration of DPP-IV showed different proteolytic ability. It was found that 475 ng/mL DPP-IV enzyme was preferred to be used in this work because at that enzyme concentration the amount of hydrolysis product is about 80% of the maximum (Figure 4). The Km measurement was done by varying the concentration of the substrate Gly-Pro-pNA. It was found that the Km value is 691.9 μM (Figure 5a).

The preferred DPP-IV enzyme concentration and the Km value of Gly-Pro-pNA measured by chromatography method was 449 ng/mL ( Figure 6 ) and 265.5 μM ( Figure 5b).

The Km value of DPP-IV substrate and preferred DPP-IV concentration measured by two methods were all in the same order of magnitude. There were no significant differences between the results measured by two methods.

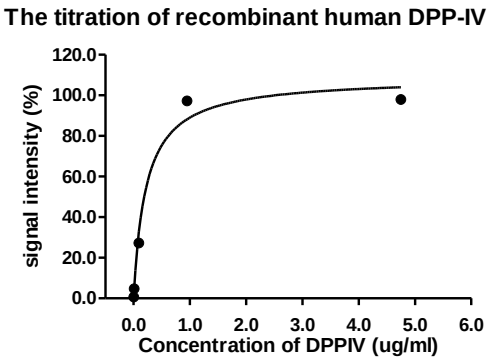



**Fig. 4.** The titration of recombinant human DPP-IV enzyme. The experiment was carried out at seven concentrations of recombinant human DPP-IV with fixed Gly-Pro-pNA concentration of 1000 μM at 37 $^{o}$C.

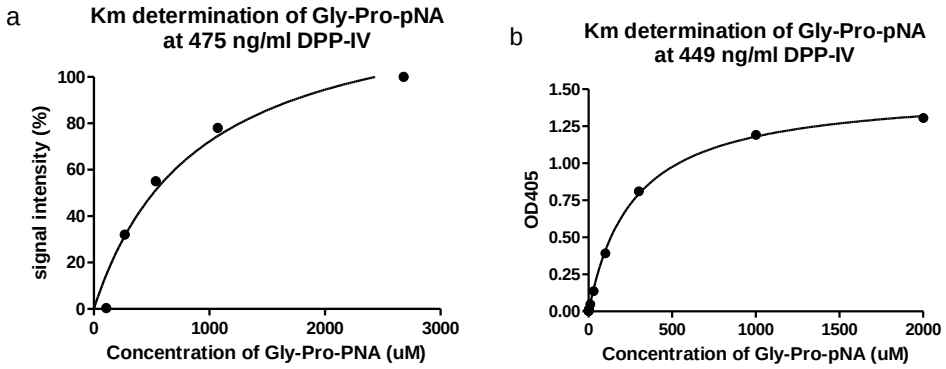



**Fig. 5.** Km determination of Gly-Pro-pNA by (a) LC-MS method and (b)Chromatography method. (a) LC-MS method .The experiment was carried out at 6 concentrations of Gly-Pro-pNA with fixed DPP-IV concentration of 475 ng/mL at 37 $^{o}$C. Calculated Km value of Gly-Pro-pNA is 691.9 μM. (b)Chromatography method. The experiment was carried out at 7 concentrations of Gly-Pro-pNA with fixed DPP-IV concentration of 449 ng/mL at 37 $^{o}$C. Calculated from the figure, the Km value of Gly-Pro-pNA is 265.5 μM.

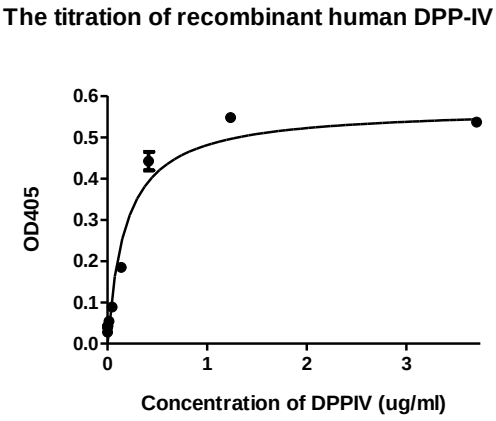



**Fig. 6.** The titration of recombinant human DPP-IV enzyme. The experiment was carried out at nine concentrations of recombinant human DPP-IV with fixed Gly-Pro-pNA concentration of 500 μM at 37 $^{o}$C.

### *3.4 $IC_{50}$ determination of positive control compounds* by LC-MS and chromatography method

To validate the LC-MS assay method developed in this work, we used the LC-MS assay method to determine the $IC_{50}$ values of two known inhibitors of DPP-IV, vildagliptin and sitaglitin. As previously reported, vildagliptin is a slow-binding inhibitor; therefore, we pre-incubated the compounds with enzyme at room temperature for 30 min. The rest of the enzymatic reaction was carried out as described in Methods. From Figure 7 (A, C), it was seen that the $IC_{50}$ values of vildagliptin and sitaglitin were 20.82 nM and 63.07 nM, respectively. The $IC_{50}$ values of vildagliptin and sitaglitin measured by chromatogenic method were 11.45 nM and 20.49 nM respectively, Figure 7 (B,D). The results are shown in Table 1 with the reported $IC_{50}$ values. $IC_{50}$ values obtained with the chromogenic method showed upward deviation from the reported values. The $IC_{50}$ values

obtained with the LC-MS method showed similar trend, although the magnitude of the deviation is slightly higher than the chromogenic method. These results indicate that the LC-MS method can be used for assay of DPP-IV inhibition and for the screening of compounds.

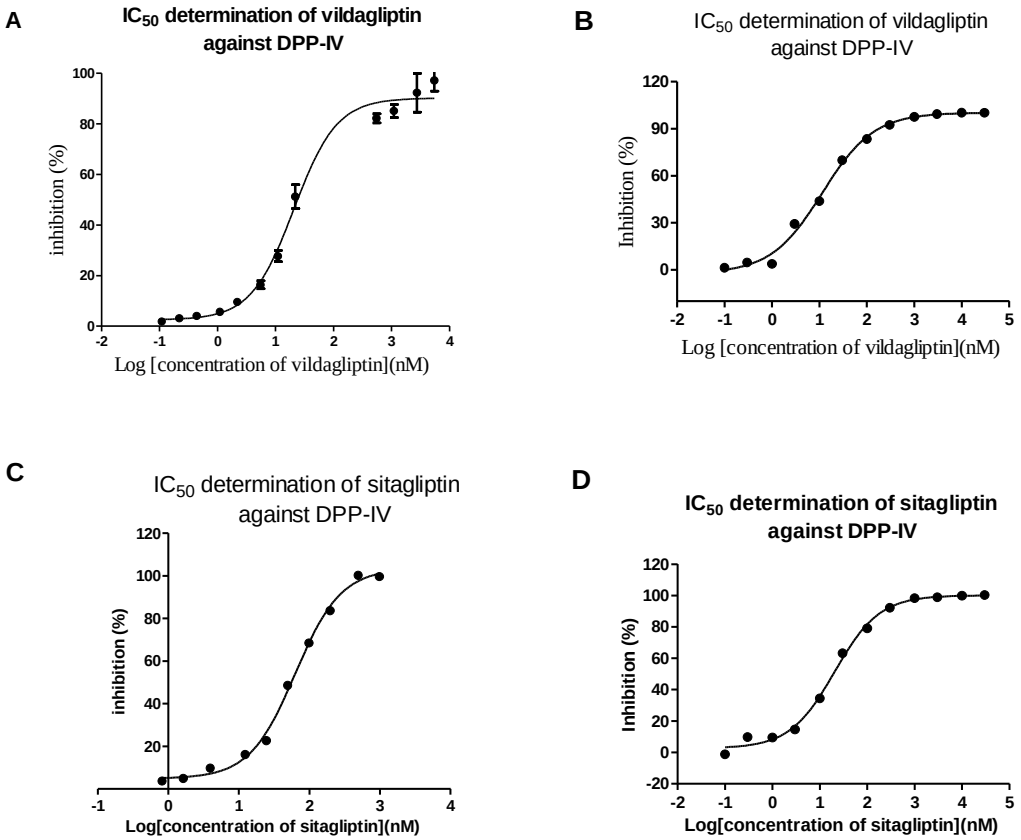



**Fig. 7.** $IC_{50}$ determination of positive control compounds. (A) $IC_{50}$ value of vildagliptin measured by LC-MS method. (B) $IC_{50}$ value of vildagliptin measured by chromogenic method. (C) $IC_{50}$ value of sitagliptin measured by LC-MS method. (D) $IC_{50}$ value of sitagliptin measured by chromogenic method. The $IC_{50}$ values of vildagliptin were 20.82 nM measured by LC-MS method and 11.45 nM measured by chromogenic method, respectively. The $IC_{50}$ values of sitagliptin were 63.07 nM measured by LC-MS method and 20.49 nM measured by chromogenic method, respectively.

**Table 1.** IC50 of reference compounds

| Compound name | $IC_{50}$(nM) Detected by LC-MS | $IC_{50}$(nM) Detected by ELIASA | Reported $IC_{50}$ (nM) |
|---|---|---|---|

| | | | |
|---|---|---|---|
| **Sitaglitin** | **63.07** | **20.49** | **20** |
| **Vildagplitin** | **20.82** | **11.45** | **3.5** |

### 3.5 Search for superior substrates by using LC-MS

LC–MS technique provides an useful approach to identifying new DPP-IV substrates for development of new assay methods. We used a combinatorial synthesis to get a mixture of seven tripeptide, and used the LC-MS technique to discover new substrates form the mixture tripeptides that showing improved properties than the traditionally used substrates. We are interested in discovering new substrates with faster reaction time with DPP-IV for improved assay throughput, with different Km value than Gly-Pro-pNA to accommodate different assay conditions (substrate concentration), and with good stability under bioassay conditions. In addition, the tripeptides synthesized do not contain strongly hydrophobic groups and are expected to have better aqueous solubility.

#### *3.5.1 General test of the tripeptide esters mixture*

The 7 tripeptides synthesized in this work all have different molecular weights. The signal change of these peptides can be monitored simultaneously using LC-MS technique in the mixture format. From the results (Figure 8) we can see that the concentration of tripeptide ester all decreased in the presence of DPP-IV. Among the tripeptides showing fast reaction kinetics, the tripeptide Gly-Pro-AspOMe can be cleaved by DPP-IV in 15 minutes, much faster than the chromogenic substrate Gly-Pro-pNA. The tripeptide Gly-Pro-LeuOMe showed similar reaction kinetics compared to Gly-Pro-pNA. All 7 tripeptides showed

good aqueous solubility and stability. We synthetized the two tripeptide esters Gly-Pro-AspOMe and Gly-Pro-LeuOMe separately, and tested their kinetic parameters.

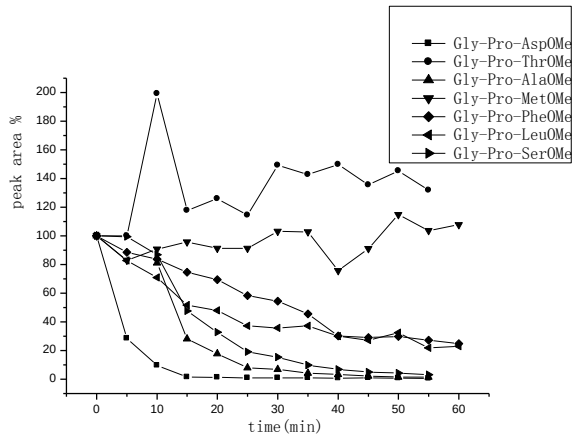


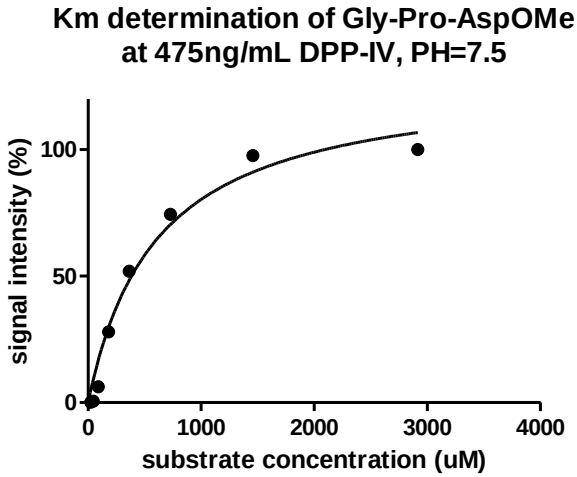

**Fig. 8.** The decrease of tripeptides at the existence of DPP-IV. (A) The content of Gly-Pro-AspOMe, Gly-Pro-ThrOMe, Gly-Pro-AlaOMe, Gly-Pro-PheOMe, Gly-Pro-MetOMe, Gly-Pro-LeuOMe, Gly-Pro-SerOMe monitored by LC-MS in 60 minutes at the existence of DPP-IV. The experiment was carried out with fixed DPP-IV concentration of 475ng/mL at 37 $^{o}$C. The content of tripeptide esters at 0 minute was defined as 100%.

### *3.5.2 Km study of Gly-Pro-AspOMe*

The enzymatic reaction was started by addition of Gly-Pro-AspOMe. During the investigation of the Km value of Gly-Pro- AspOMe, the concentrations of Gly-Pro-AspOMe varied from 20 to 3000 μM. And the reaction was initiated by addition of fixed concentration of DPP-IV of 475 ng/mL. For all assays, the reaction mixture without enzyme was used as background, and the reactions were done twice.

We could see that the enzymatic activity of the enzyme follows a substrate concentration dependent mode, and the Km value of Gly-Pro-AspOMe was 606.2

μM which was slightly less than Gly-Pro-pNA in Figure 7, Km=691.9μM with 475 ng/mL enzyme( Figure 9).




| Michaelis-Menten | |
|---|---|
| Best-fit values | |
| VMAX | 129.0 |
| KM | 606.2 |
| Std. Error | |
| VMAX | 11.88 |
| KM | 151.9 |

**Figure 9.** Km determination of Gly-Pro-AspOMe at 475ng/mL DPP-IV. The reaction was done at 37 $^{o}$C. Calculated from the figure, the Km of substrate (Gly-Pro-AspOMe) is 606.2 μM.

***3.5.3 Kinetic study of Gly-Pro-AspOMe***

Figure 14 shows the concentration change of Gly-Pro-AspOMe in the presence and absence (blank) of DPP-IV. The concentration of DPP-IV used was 475 ng/mL , and the concentration of Gly-Pro-AspOMe used was 606.2μM. Each data point was tested twice. The concentration of Gly-Pro-AspOMe was monitored by LC-MS every 5 minutes. As can be seen from Figure10 that the concentration of Gly-Pro-AspOMe decreased rapidly and the reaction is essentially complete in lesss than 30 minutes about two times quicker than Gly-Pro-pNA.

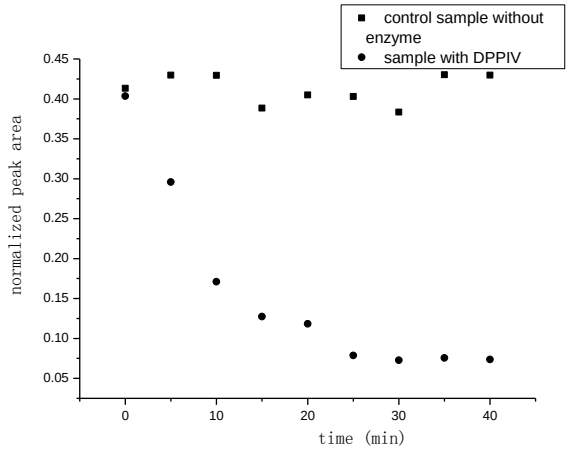
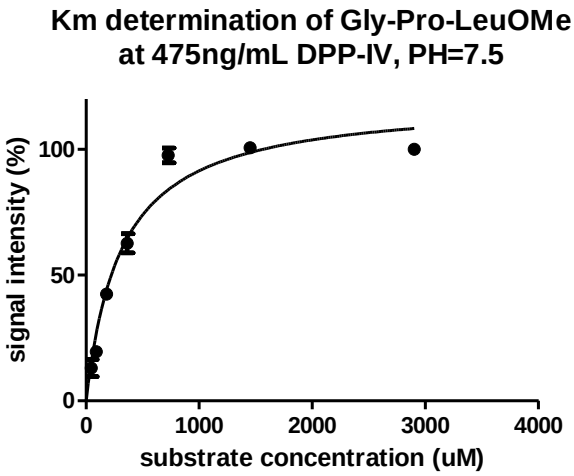



**Fig. 10.** The decrese of Gly-Pro-SspOMe signal at substrate concentration of 606.2μM and 475 ng/mL DPP-IV. The reaction was done at 37 $^{o}$C.

***3.5.4 Km study of Gly-Pro-LeuOMe***

The enzymatic reaction was started by addition of Gly-Pro-LeuOMe. During the investigation of the Km value of Gly-Pro-Leu*OMe*, the concentrations of Gly-Pro-LeuOMe varied from 40 to 3000 μM. And the reaction was initiated by addition of fixed concentration of DPP-IV of 475ng/mL. For all assays, the reaction mixture without enzyme was used as background, and the reactions were done twice.

We could see that the enzymatic activity of the enzyme follows a substrate concentration dependent mode, and the Km value of Gly-Pro-Leu*OMe* was 311.0 μM only a half to Gly-Pro-pNA in Figure 7 , Km=691.9 μM with 475 ng/mL enzyme. (Figure 11.)




| Michaelis-Menten | |
|---|---|
| Best-fit values | |
| VMAX | 119.9 |
| KM | 311.0 |

**Fig. 11.** Km determination of Gly-Pro-LeuOMe at 475ng/mL DPP-IV. The reaction was done at 37 °C. Calculated from the figure, the Km of substrate (Gly-Pro-LeuOMe) is 311.0 μM. Data are represented as means (n = 3).

### *3.5.5 Kinetic study of Gly-Pro-LeuOMe*

The kinetic study of Gly-Pro-leuOMe aimed to find the linear reaction time period of Gly-Pro-leuOMe (Figure 12). The concentration of DPP-IV used was 475 ng/mL , and the concentration of its substrate Gly-Pro-leuOMe used was 311.0μM. Each data point was tested twice. The product was monitored by LC-MS every 10 minutes. The total reaction time is 60 min. As figure 16 shows the linear reaction time ranged from 15 minute to 50 minute similar to Gly-Pro-pNA but only half substrate concentration needed.

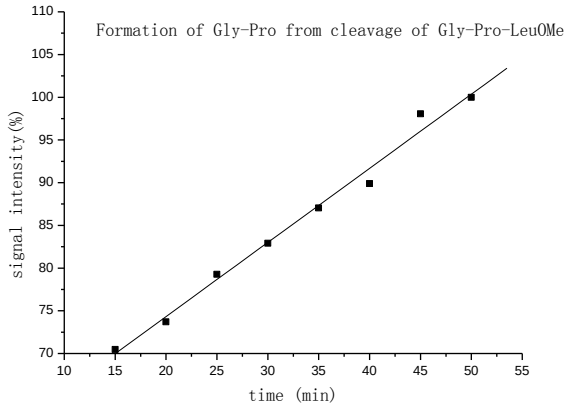
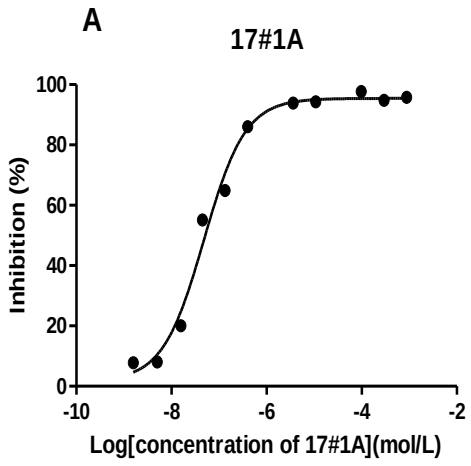


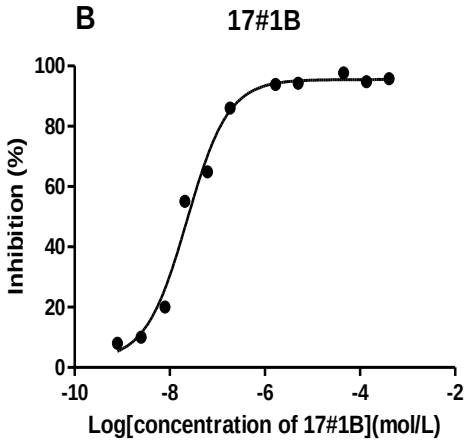

**Fig. 12.** Kinetic study of Gly-Pro-LeuOMe. 311.0 μM substrate (Gly-Pro-LeuOMe) reacted with 475ng/mL DPP-IV at 37 °C. Calculated from the figure, the linear reaction time period of DPP-IV is 50min.

### 3.6. Compound screening

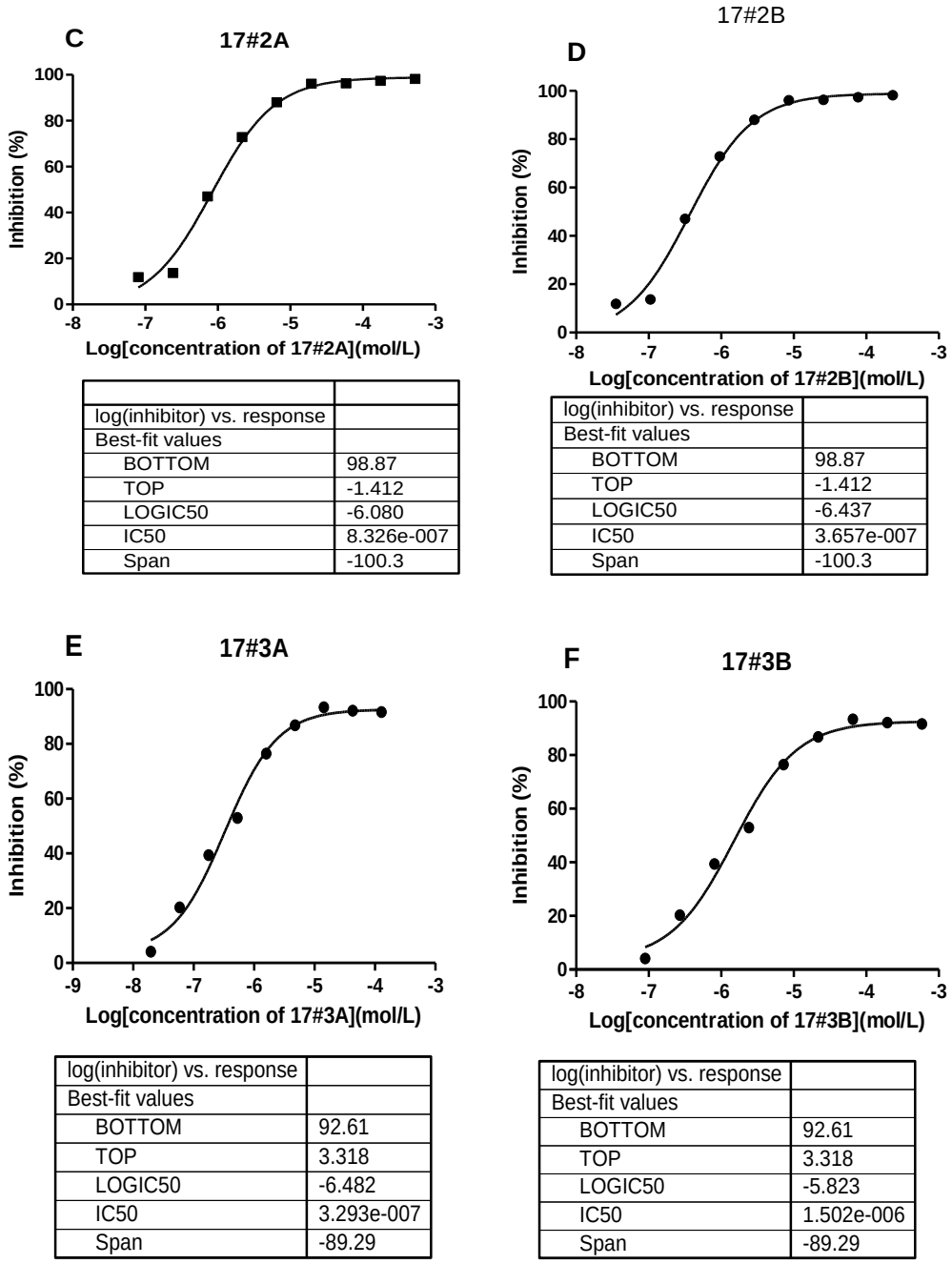
In the present work, to demonstrate the utility of the LC-MS based DPP-IV assay method we have developed, selected compounds from a DPP-IV drug discovery project were screened for inhibitory activity of DPP-IV. Some of those compounds showed good inhibitory activity ( Figure 13).




| | |
|---|---|
| Best-fit values | |
| BOTTOM | 95.44 |
| TOP | 1.666 |
| LOGIC50 | -7.317 |
| IC50 | 4.820e-008 |
| Span | -93.77 |




| log(inhibitor) vs. response | |
|---|---|
| Best-fit values | |
| BOTTOM | 95.51 |
| TOP | 2.266 |
| LOGIC50 | -7.636 |
| IC50 | 2.313e-008 |
| Span | -93.24 |




| | |
|---|---|
| log(inhibitor) vs. response | |
| Best-fit values | |
| BOTTOM | 98.87 |
| TOP | -1.412 |
| LOGIC50 | -6.080 |
| IC50 | 8.326e-007 |
| Span | -100.3 |

| | |
|---|---|
| log(inhibitor) vs. response | |
| Best-fit values | |
| BOTTOM | 98.87 |
| TOP | -1.412 |
| LOGIC50 | -6.437 |
| IC50 | 3.657e-007 |
| Span | -100.3 |

| | |
|---|---|
| log(inhibitor) vs. response | |
| Best-fit values | |
| BOTTOM | 92.61 |
| TOP | 3.318 |
| LOGIC50 | -6.482 |
| IC50 | 3.293e-007 |
| Span | -89.29 |

| | |
|---|---|
| log(inhibitor) vs. response | |
| Best-fit values | |
| BOTTOM | 92.61 |
| TOP | 3.318 |
| LOGIC50 | -5.823 |
| IC50 | 1.502e-006 |
| Span | -89.29 |

**Fig. 13.** The $IC_{50}$ of compounds (A, B, C, D, E, F) tested for DPP-IV inhibitory acivity.

The enzymatic reaction was carried out after pre-incubating the compounds with enzyme at room temperature for 30 min with 691.9 μM substrate (Gly-Pro-pNA) and 475ng/mL DPP-IV at 37 °C.

Among the compounds tested, some showed excellent inhibitory activity against

DPP-IV. Compounds 17#1A and 17#1B showed $IC_{50}$ in the low range, significantly more active than the reference compounds vildagliptin and sitaglitin used in this work.

The LC-MS based method we developed for DPP-IV activity assay allowed the use of wider selections of substrates than the fluorescent and chromogenic methods. The method is not affected by the presence of fluorescent and chromogenic compounds. This LC-MS method monitors the m/z value of Gly-Pro as part of the substrates, so any compound containing such N-terminal dipeptide structure may use this method. Similarly, for peptides with a different N-terminal fragment, corresponding ion of the N-terminal fragment of the substrate can be monitored and a corresponding LC-MS based assay method can be developed. LC-MS method also facilitates the discovery of new substrates having improved properties as demonstrated in this work. Some of these properties include faster reaction time for better assay throughput, more aqueous soluble, more stable under assay conditions, and different Km values to accommodate the need for different substrate concentrations in different situations.

## 4. Conclusion

An LC-MS based DPP-IV assay method has been developed. The method was validated using a chromogenic method as reference. The LC-MS method monitors the Gly-Pro fragment part of the substrate in the assay readout based on m/z value and HPLC retention time. This method of readout has high specificity and is not affected by screening compounds with chromophore or fluorophore. Any

compounds containing Gly-Pro segment can be used as potential substrates, the presence of chromogenic or fluorescent group is not required. Similar LC-MS methods can be developed for potential substrates containing other LC-MS sensitive segments. The utility of this LC-MS based assay method was demonstrated by testing compounds from a DPP-IV drug discovery project and several highly active compounds were discovered. In addition, we have taken advantage of the generality of the LC-MS detection technique to find new substrates with improved properties. A seven-member tripepetide library with good aqueous solubility and stability was synthesized and was screened as a mixture. Two members of the mixture showing interesting properties were synthesized and tested as individual compound. Gly-Pro-AspOMe showed much faster reaction kinetics and can potentially be used for assays with better throughput. Gly-Pro-LeuOMe has similar reaction time as Gly-Pro-pNA but has lower Km value which can potentially allow running activity assay where lower substrate concentration is beneficial.

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