Development of a rapid resolution HPLC method for the quantitative determination of sitagliptin in Human plasma

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Abstract: A new high performance liquid chromatography (HPLC) method for the quantitative determination of sitagliptin in human plasma was developed and validated for pharmacokinetics study. The plasma was spiked with the internal standard (Salbutamol, IS), extracted with trichloro acetic acid. The extracted analyte was injected into a Symmetry® ODS C18 column (250mm×4.5mm, 5m) and the flourometric detector was operated at 267nm for excitation and 575nm for emission. The mobile phase consisting of Potassium dihydrogen phosphate buffer pH (4.9)-Acetonitrile-Methanol (30:50:20 v/v) at flow rate of 1.0mL/min. The method showed high specificity. Calibration curves of the peak area ratio of each analyte/IS versus sitagliptin concentration were linear in the range of 0.122–31.25µg/mL (r>0.989) for plasma and 0.012-25ug/ml for QC solution(r>0.995). The lower limit of quantification (LLOQ) was 0.122µg/mL in plasma and 0.012 in QC solution. The intraday and interday coefficient of variation was lower than 10%. The accuracy (relative recovery) at three levels was 100.95%, 101.03% and 97.79% respectively. The extraction recovery was 97.6%, 92.2% and 91.96% at the concentrations of 6.25, 25 and 100µg/mL, respectively. Short term and long term, freeze thaw stability of standard solutions and plasma samples were satisfactory. The optimized HPLC method was validated and proved to be specific, robust and accurate for determination of Sitagliptin in human plasma.

Keywords: Sitagliptin, HPLC, human plasma, pharmacokinetics.

INTRODUCTION

Dipeptidylpeptidase IV (DPP4) inhibitors are a novel class of antidiabetic drugs, which include sitagliptin (Herman *et al*., 2007), vildagliptin (Mastoshi *et al*., 2009), and saxagliptin (Ahren, 2009). They enhance insulin release and decline glucagon levels by preventing the inactivation of incretin hormones, glucagon like peptide1 and glucose dependent insulinotropic polypeptide, to treat the type 2 diabetes (Tina and Eunice, 2007; James *et al*., 2009). Sitagliptin, is the first DPP4 inhibitor approved by the Food and Drug Administration (FDA) in October 2006 (Pham *et al*., 2008) , it has been proven to be effective in reducing the levels of glycosylated hemoglobin (HbA1c), fasting plasma glucose, and two hour postprandial glucose in patients with type 2 diabetes (Giuseppe,2010) . To support the pharmacokinetic study of sitagliptin, an effective and sensitive method to determine its concentration in plasma was needed. (Zeng *et al*., 2008) reported a HTLCMS/ MS method to determine the sitagliptin concentration in human urine and hemodialysate in 2007, and an LCMS method for the quantification of sitagliptin in human plasma was validated by Nirogi (Nirogi *et al*., 2008). Because the LCMS method is expensive, a cost effective HPLC method is still needed. The HPLC method reported by Sun *et al*. was not practical because of the high lower limit of quantification (2.0 µg/mL) and the narrow linear range (5-500µg/mL, r = 0.9993) (Sun *et al.*, 2009). The aim of this study was to develop a simple, sensitive, specific, robust and accurate HPLC method for the quantitative determination of sitagliptin in QC solution and human plasma.

MATERIALS AND METHODS

Reagents and chemicals

Sitagliptin phosphate (with a purity of 96.38%) was donated by Hilton Pharmaceuticals Pakistan. HPLC grade methanol of RCI Abscan limited and HPLC grade Acetonitrile of RCI Abscan limited was purchased from the market. Chromatographic grade water was prepared at University Lab through Distillation plant WDA/4 R & M (England). Analytical grade potassium dihydrogen phosphate, Phosphoric acid, trichloro acetic acid, sodium hydroxide, and ethyl acetate were purchased from market. Salbutamol was also obtained from the Hilton Pharmaceuticals Pakistan.

Chromatographic conditions

Agilent 1200 HPLC system, consisting of pump and a FLD detector (Agilent Technologies was used for the analysis. The chromatography was performed on an analytical Symmetry ODS C18 Column (250 mm×4.5 mm, 5µm,). The mobile phase was composed of Potassium dihydrogen phosphate buffer pH (4.9)-Acetonitrile-Methanol (30:50:20 v/v) at flow rate of 1.0 mL/min. The column temperature was kept ambient. The

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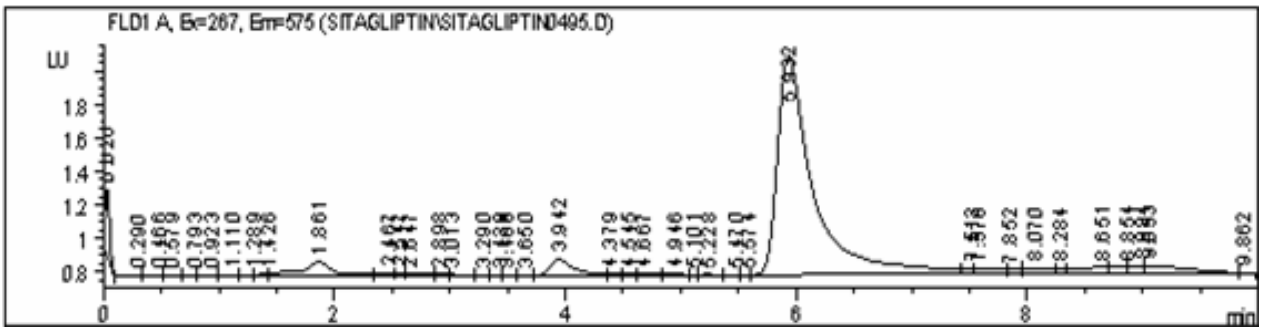

Fig. 1: Rpresentative chromatogram of blank human plasma spiked with sitagliptin phosphate at a concentration of 25µg/mL and salbutamol (IS).

Table 1: Known Plasma Concentrations (µg/ml) of Sitagliptin and its respective Peak height ratio.

S No.	Concentration (ug/ml)	Peak Height Ratio
1	0.122	0.147
2	0.244	0.195
3	0.488	0.073
4	0.976	0.131
5	1.953	0.183
6	3.9	0.417
7	7.81	0.663
8	15.62	1.667
9	31.25	3.667

Table 2: Validation of the intraday assay (n = 5)

Batch No.	High Conc. (HC) µg/mL	Medium Conc. (MC) µg /mL	Low Conc. (LC) µg/mL
sita-01	101.12	12.70	147
	100.23	12.63	154
	100.56	12.67	151
sita-02	101.23	12.60	156
	101.89	12.61	149
	100.92	12.56	157
sita-03	99.78	12.89	147
	100.82	12.80	151
	101.98	12.20	161
Mean	100.95	12.63	153
SD	0.72	0.19	0.05
N	9.00	9.00	9.00
Nominal	100.00	12.50	150
% CV	0.71	1.52	3.15
% accuracy	100.95	101.03	97.79

sample injection volume was 20µL. The detection wavelength was set at 267 for excitation and 575 for emission.

Preparation of standard solutions

A standard stock solution was prepared by dissolving 25.0 mg of sitagliptin phosphate in 25.0mL water, yielding a 1.0mg/mL solution. Then the stock solution was diluted with water into the standard solutions of 25, 12.5, 6.25, 3.125, 1.562, 0,781, 0.390, 0,195, 0,097, 0.048, 0.024, 0.012, 0.006µg/mL. The stock solution of IS was prepared in water at the concentration of 1.0mg/mL. The two stock solutions and standard solutions were stored at 2-8ºC.

Preparation of calibration standards and quality control samples

Blank plasma was pooled from human and used for the preparation of calibration standards and quality control (QC) samples. Calibration standards were prepared by spiking 1mL of standard solution into 1mL of blank human plasma. Liquid-liquid extraction was used for the sample preparation in this study. Three organic solvents,

Table 3: Validation of the interday assay (n = 5)

Batch (Sita-01)	High Conc. Mg/ mL (HC)	Medium Conc. µg /mL (MC)	Low Conc. ng /mL (LC)
01	101.12	12.70	147
02	100.23	12.63	154
03	100.56	12.67	151
04	101.23	12.60	156
05	101.89	12.61	149
06	100.92	12.56	157
Mean	100.99	12.63	152
SD	0.57	0.05	0.04
N	6.00	6.00	6.00
Nominal	100.00	12.50	1.50
%CV	0.57	0.40	2.61
%Accuracy	100.99	101.03	97.65

Table 4: Extraction recovery of sitagliptin (n = 5)

Sitagliptin (actual conc. µg/mL)	Area of Chromatogram	Sitagliptin (observed µg/mL)	Percentage Recovery
100	185	91.96	91.96
25	50	23.05	92.2
6.25	12.9	6.10	97.6

dichloromethane, ethyl acetate, Trichloro acetic acid and their mixtures in different ratios were evaluated. Finally, Trichloroacetic acid was selected because it had the most acceptable specificity and the highest extraction recovery. The final concentrations of calibration standards were 31.25, 15.62, 7.81, 3.90, 1.953, 0.976, 0.488, 0.244, 0.122 µg/mL. The QC samples were prepared by the same method. Lower QC (LQC), medium QC (MQC) and high QC (HQC) samples at 0.122, 1.953 and 31.25µg/mL were prepared, respectively. All QC samples were stored at –20 ºC.

Sample preparation

Stored at -20ºC until used, the plasma samples (500µL) were placed in a 10mL plastic tube, then 25µL of IS solution (1mg/mL) was added and vortexed for 2 min. Then centrifuged at 4000r/min for 5min. The organic layer was transferred to another tube and filtered through 0.45 micron filter using filter assembly and a 20µL aliquot was injected into the chromatographic system.

RESULTS

A new HPLC method developed was validated step by step to ensure the testing of significant method characteristics according to good analytical practice guidelines.

Stability

To investigate the short term and long term stability of sitagliptin in human plasma, the plasma samples at 1.22, 1.953 and 31.25µg/mL (n=5 for each concentration) were stored at an ambient temperature for 24 h and at -20ºC for 72 h, respectively. The freeze thaw stability was evaluated by five freeze thaw cycles (-20ºC for 72h and ambient for 6h). The samples were then processed and the concentrations were determined using the method described above. The stability of standard solution was investigated as follows: after being prepared, the samples at 1.22, 1.953 and 31.25µg/mL (n=5 for each concentration) were stored at ambient temperature for 24 h and analyzed.

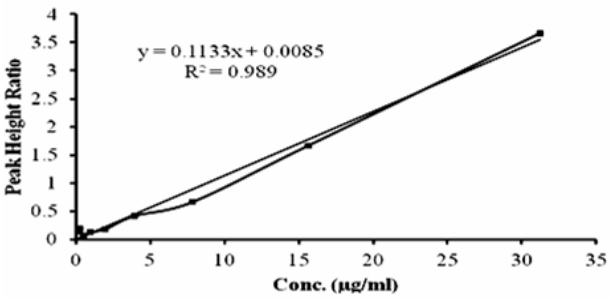

Fig. 2: Standard Curve for known concentration of Sitagliptin (µg/ml) spiked in the human plasma**.**

DISCUSSION

Fig. No.1shows blank plasma spiked with sitagliptin phosphate at a concentration of 25µg/mL and IS. The retention time of sitagliptin and IS was 6.7 min and 3.8 min, respectively which is bit improved the previously reported retention time 6.9min (Parsad *et al*,. 2014). The peaks were sharp and symmetrical with good baseline resolution and minimal tailing. The mobile phase was optimized by changing the composition, the pH of the mobile phase and the amounts of modifier to achieve good resolution and symmetric peak shapes for analyte

and IS, as well as a short run time. Water in the mobile phase was replaced by 10mM potassium dihydrogen phosphate buffer to obtain better baseline stability. The retention time of sitagliptin in the mobile phase was pH dependent and the optimal pH was identified as 4.9 (Pulla *et al*,. 2011). Finally, a mixture of Potassium dihydrogen phosphate buffer pH (4.9)-Acetonitrile-Methanol (30:50:20 v/v) was adopted as the mobile phase. Good linearity was obtained for sitagliptin in the range from 0.122–31.25µg/mL which is far better than the reported ranges as 20-60µg/mL, 10-50µg/mL and 5-30µg/mL respectively (Rananjaneyulu *et al*,. 2013, Parsad *et al*,. 2014, Ramalingam *et al*,. 2014 and Lavanaya *et al*,. 2013). The regression equation of the calibration curve was as follows: y=0.1133x+0.0085 (r>0.989), where y and x represented the peak area ratio and sitagliptin plasma concentration, respectively. Weighted (1/x 2) linear regression was used to perform standard calibration. The LLOQ of sitagliptin phosphate was determined to be 0.122µg/mL, which was less than the LLOQ reported as 2µg/mL and 0.6 simultaneously (Sun *et al.*, 2009 and Lavanaya *et al*,. 2013). In addition, the lowest sitagliptin concentration of 5µg/mL in the calibration curve (5-500µg/mL) reported by Sun is much higher than that of our present work (0.122-31.25µg/mL), indicating our HPLC method with high sensitivity is more suitable than the previously reported methods for pharmacokinetics study. In addition, sitagliptin was extracted by a liquid-liquid extraction method, which increases its concentration in the final analysis sample. However, in the previously reported study, the concentration in the analysis sample is lower than the concentration in plasma because it was diluted 2fold by methanol after protein precipitation (Sun *et al.*, 2009).

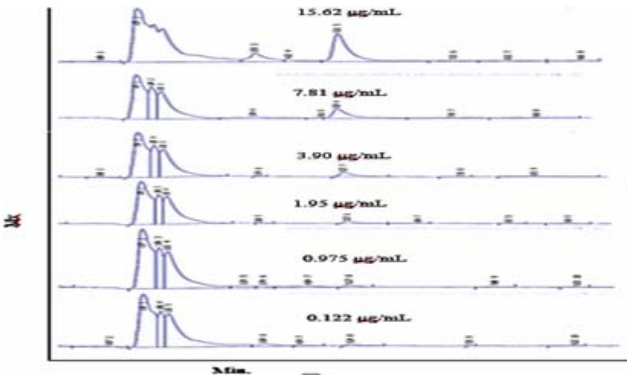

Fig. 3: Linearity chromatograms of Sitagliptin (µg/ml) with internal standard spiked in the human plasma**.**

CONCLUSION

A simple, rapid and reliable HPLC method is developed and validated for the measurement of sitagliptin in human plasma. A linear calibration curve was obtained in the range from 0.122 to 31.25µg/mL. The specificity, sensitivity, accuracy, precision, recovery and stability met the requirement for the quantitative determination of sitagliptin in human plasma. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of Sitagliptin.

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