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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SITAGLIPTIN PHOSPHATE BY RP-HPLC AND ITS APPLICATION TO PHARMACOKINETIC STUDY

ANIL DUBALA[1*], RIZWANBASHA KHATWAL[1], JAYASANKAR KOSARAJU[2], VENKAT MEDA[3], MALAY K. SAMANTA[1]

[1]Department of Pharmaceutical Biotechnology, [2]Department of Pharmacology, [3] Department of Pharmacy Practice,J.S.S.College of Pharmacy, Ooty, Tamilnadu, India. Email: anildubala@gmail.com

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ABSTRACT

A simple, rapid and selective HPLC method was developed for the estimation of Sitaliptin phosphate (SP) in human plasma by protein precipitation technique. The chromatographic separation was carried out on a reverse phase Phenomenex C_{18} (250 × 4.6mm, 5μ) column. A mixture of 0.5% v/v of Triethylamine solution and acetonitrile (77:23 v/v) was used as mobile phase. The pH of 0.5% v/v Triethylamine was adjusted to 6.8 using ortho-phosphoric acid. The flow rate of mobile phase was set at 1.0 ml/min and the detection of SP was carried out at 267 nm by an UV detector. The retention time of Sitagliptin phosphate and internal standard was 6.1 and 7.7 min, respectively. The method was validated and found to be linear in the range of 10-1000ng/ml. the limit of detection (LOD) and limit of quantification (LOQ) were 1 ng/ml and 10 ng/ml. The results indicate the bio analytical method is linear, precise and accurate. The developed method was validated and found suitable for application in designing pharmacokinetic studies with simplified solvent system.

Keywords: Sitagliptin, RP-HPLC, and Pharmacokinetics

INTRODUCTION

Sitagliptin phosphate (SP)(*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl) butan-2-amine is an oral antihyperglycemic of the dipeptidyl peptidase-4 (DPP-4) inhibitor class(Fig.1).

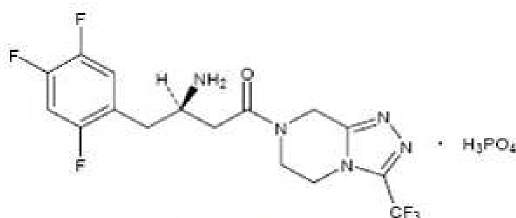

Fig. 1: Structure of Sitagliptin phosphate

This enzyme-inhibiting drug is used either alone or in combination with other oral antihyperglycemic agents (such as metformin or a thiazolidinedione) for treatment of diabetes mellitus type 2. Sitagliptin works to competitively inhibit the enzyme DPP-4. This enzyme breaks down the incretins GLP-1 and GIP, gastrointestinal hormones released in response to a meal [1]. By preventing GLP-1 and GIP inactivation, they are able to increase the secretion of insulin and suppress the release of glucagon by the pancreas. This drives blood glucose levels towards normal. Several analytical methods based on UV [2-5], RP-HPLC [6-8], LC-MS/MS [9-13] was reported for the determination of sitagliptin phosphate in plasma and urine of humans, rats and dogs.

Most of the methods sited in the literatures for estimation of SP was found to be cumbersome. Though the LC-MS/MS was highly sensitive, the cost and availability have made application of HPLC with UV detection for quantification of SP. The available HPLC bioanalytical techniques for estimation of SP were found to be tedious. In the present study a simple, sensitive, accurate and reproducible RP-HPLC method with very low detection range for estimation of SP in human plasma was developed and validated. Based on the degradation studies, the method was also tested for its stability indicating ability.

MATERIALS AND METHODS

Instrumentation

Shimadzu Prominence HPLC system was equipped with model series LC- 20AT pump, Rheodyne 7752i injector with 20µl loop and SPD-20A UV/VIS detector. Separation and quantification were made on the Phenomenex C_{18} column (250 x 4.6 mm i.d., particle size 5 µm). Spinchrom Chromatography station software performed the data acquisition. ACN and 0.5% v/v TEA (pH 6.8) at a ratio of 23:77 v/v was applied as mobile phase with flow rate of 1.0 ml/min. The detection was performed at 267 nm.

Chemicals and reagents

ACN and Methanol of HPLC Grade were procured from S.D. Fine Chemicals, Mumbai, India. Ortho-phosphoric acid, Perchloric acid AR graded, Zinc Sulphate LR, and Triethylamine AR grade was purchased from Qualigens Fine Chemicals, India. Water of HPLC grade was obtained from Milli-Q RO system. Working standards of SP was gift sample from the Matrix Pvt Ltd, Hyderabad, India. Blood was collected from healthy human volunteers in an anticoagulant (EDTA) coated vials and plasma was separated by centrifugation. The pooled plasma was stored at -20 ± 2°C until further analysis.

Calibration standards

The bio analytical curve of SP was developed by spiking 0.5ml of SP into a mixture of 0.5ml of IS (Rosiglitazone), 0.5ml of plasma and 0.5ml of methanol (protein precipitating agent). The spiking was done in such a way that the test samples produce a concentration of 1000, 800, 600, 400, 200, 100, 50, 20 and 10 ng/ml remove. The concentration of IS was maintained at 500 µg/ml. These solutions were labeled and stored at –20 ± 2°C until analysis.

Quality control standards

Lowest quality control standards (LQC), median quality control standards (MQC) & highest quality control standards (HQC) of SP were prepared by spiking the working standard solutions into a pool of drug free human plasma to produce a concentration of 25, 500 and 850ng/ml remove. These solutions were labeled and stored at –20 ± 2°C till the time of analysis.

Validation

The validation parameter like selectivity of the method for SP was checked for interference with plasma. The standard curve consisting of nine points ranging from 10.0 to 1000 ng/ml was developed. Quality control samples of LQC (25 ng/ml), MQC (500 ng/ml) and HQC (850 ng/ml) were used to determine the intra and inter-day precision and accuracy of the assay. Peak area ratio's of SP to IS were fixed to linear equation Y = mX ± C. Where, Y symbolizes the response factor, m the slope with X indicating the concentration in nanograms. C denotes the Y intercept when X is equivalent to zero.

RESULTS AND DISCUSSION

During the process of validation the solid phase extraction, liquid-liquid extraction and protein precipitation technique were applied to determine the limit of detection for SP. The solid phase extraction technique reported the limit of detection for SP as 1.0 ng/ml. The protein precipitation technique also reported the same limits when methanol was used as a protein precipitating agent. The liquid-liquid extraction technique accounts much higher limit of detection for SP. Thus the further validation of method was carried by protein precipitation technique since the solid phase extraction is time consuming with multi purification steps. A flow rate of 1.0 ml/min, the mobile phase of 23:77 ratio reported the runtime of 10 min with retention time of 6.1 and 7.7 min respectively for SP and IS. Increase in percentage of ACN show a faster elution with poor resolution. The broad peaks were observed with enhanced run time with a decrease in the ratio of ACN. SP was highly sensitive to the wavelength of 267 nm.

Validation

The Quality control samples of LQC, MQC and HQC were used to determine the intra and inter-day precision and accuracy of the assay. The percentage recovery of the drug molecule in the above conditions was determined by correlation with standard curves. The equation of linearity for SP with IS was Y = 0. 0001X + 0.0045. The drug concentration in control samples along with the same day standard curve samples were calculated using the above equation. For all the curves the correlation coefficient (r^2) was never lower than 0.999.

Selectivity

Selectivity of the method was investigated by screening six different batches of human plasma. Under the proposed assay conditions the blank plasma samples did not report any peaks at retention time of 6.1 and 7.7 min as observed for SP and IS respectively. The rest of the peaks observed in HPLC chromatogram were due to the plasma components. The specificity of a method can be defined as the extent to which the analyte can be estimated without the intervention of other components. SP and IS were very well resolved under the proposed chromatographic conditions. None of the drug free plasma samples studied in this assay yielded endogenous interference at the retention time observed for drug. Figure 2 and 3 represents the standard HPLC chromatogram of blank and spiked plasma.

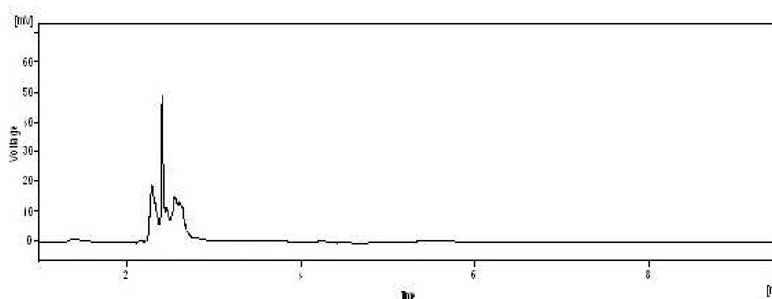

Fig. 2: Standard HPLC Chromatogram of Blank plasma

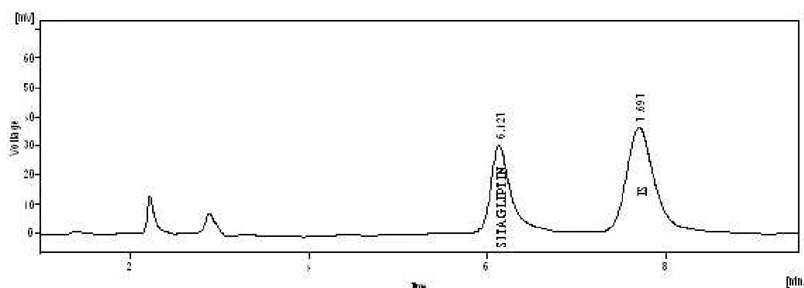

Fig. 3: Standard HPLC Chromatogram of Spiked plasma containing Sitagliptin and IS

Recovery

The mean percent recovery values of SP ranged from 91.67 to 92.73%. The coefficient of variation of these values was less than 5 % (Table 1).

Accuracy/ Precision

Intra and inter-day precision (%CV) of SP was 3.8, 1.3, 2.3 and 3.8, 3.8, 2.5 for the spiked concentration at 25.0, 500.0 and 850.0ng/ml respectively. Intra and inter-day % accuracy of SP was 91.67, 92.27, 90.75 and 83.33, 85.45, 86.75 for the spiked concentration at 25.0, 500.0 and 850.0 ng/ml respectively (Table 2). According to the obtained results, a good precision and accuracy were observed by this method. The plasma components did not interfere in the analysis of SP.

Linearity

The linearity of each calibration curve was determined by plotting response factor and concentration of the standard solution. The linearity range for SP was observed at 1000, 800, 600, 400, 200, 100, 50, 20 and 10 ng/ml and their slope (k) and the intercept value (B) were 0.0001 and 0.0045 respectively (Fig.4). The calibration curve was constructed on six different days over a period of two weeks to determine the variability of slopes and intercepts. (Table 3). The high correlation coefficient validated the linearity of the calibration curves of SP with intercept, which was not different from zero. The range of linearity was set up through consideration of each concentration present in the pharmaceutical product to provide precise and accurate results with minimized dilution factors.

Table 1: Recovery Studies of Sitagliptin Phosphate (n=6)

Sample Concentration (ng/ml)	Concentration found (mean±S.D) (ng/ml)	% Recovery	% CV
25.0	22.92±1.12	91.67	4.89
500.0	463.65±1.34	92.73	0.29
850.0	784.13±5.33	92.25	0.68

Table 2: Accuracy and Precision Studies of Sitagliptin Phosphate (n=6)

Statistical variables	Intra-batch (ng/ml)			Inter-batch (ng/ml)		
	25.0	500.0	850.0	25.0	500.0	850.0
Mean	22.92	461.35	771.38	20.83	427.25	737.38
Accuracy (%)	91.67	92.27	90.75	83.33	85.45	86.75
Precision (C.V.%)	3.8	1.3	2.3	3.8	3.8	2.5

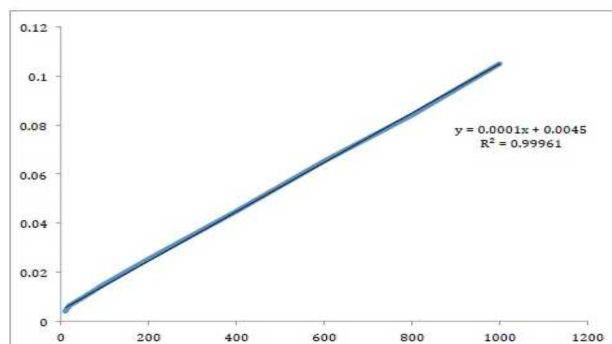

Fig. 4: Calibration curve for Sitagliptin phosphate

Table 3: Linearity and Range of Sitagliptin Phosphate

Sitagliptin Phosphate Concentration (ng/ml)	Internal standard Concentration (ng/ml)	Response Factor
10	100	0.0041
20	100	0.0065
50	100	0.0096
100	100	0.0152
200	100	0.0255
400	100	0.0449
600	100	0.0654
800	100	0.0841
1000	100	0.1051

Stability study

The freeze thaw, short-term and long-term stability studies were conducted for plasma samples, whereas the stability study for the stock solution was carried at the room temperature and refrigerated condition. The percentage degradation of the quality control samples were compared to the theoretical concentrations. Short-term and long-term stock solution stability when evaluated showed no deviation from normal value when stored at -20± 2°C. The stability of SP in plasma was determined by measuring percentage of degradation in quality control samples over a period. Stability was tested by subjecting the quality controls to three freeze-thaw cycles and compared with freshly prepared quality control samples. As shown in Table 4, the percentage degradation of SP in quality control samples did not change within the time period under the indicated storage conditions. Long-term stability studies results indicate that SP was stable in the plasma matrix at least for three months when stored at -20± 2°C.

Table 4: Stability of HPLC assay method for Sitagliptin Phosphate in human plasma (n = 6)

Statistical variables	Freeze thaw stability (ng/ml)			Short term Stability (ng/ml)			Long term Stability (ng/ml)			Standard Stock solution stability (ng/ml)		
	(25.0)	(500.0)	(850.0)	(25.0)	(500.0)	(850.0)	(25.0)	(500.0)	(850.0)	(25.0)	(500.0)	(850.0)
Mean	22.9	478.9	836.7	23.8	489.2	834.4	21.6	458.4	786.5	24.1	489.6	839.7
CV (%)	3.8	2.5	1.4	3.8	1.2	2.8	3.8	3.8	2.3	3.8	2.5	2.9
%Degradation	8.4	4.2	1.6	4.8	2.2	1.8	13.6	8.3	7.5	3.6	2.2	1.2

CONCLUSION

HPLC based analytical method for quantification of SP in human plasma was validated by protein precipitation technique. Among the protein precipitation reagents (ACN, $ZnSO_4$, Methanol, Tungstic acid and $HClO_4$) the methanol provided the better intensity with more sensitivity to solid phase extraction technique.

Thus, we conclude the method was sensitive and simple and can be further applied in design of pharmacokinetic studies compared to the earlier reported cumbersome techniques. The method was also reproducible with robust stability of component in the matrix of plasma.

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