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Research Article

Bioanalytical Method Development and Validation and forced degradation of Sitagliptin and determination of Pharmacokinetic application study in Human Plasma by RP-HPLC method

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Abstract

In the present investigation, an attempt was made for the development, validation, forced degradation and pharmacokinetic application of sitagliptin in the human plasma spiking studies by UV - HPLC method. The experimentation was developed based on the extensive literature survey and ascertained by the statistical parameters of the sampling. A simplified, accurate method was created by the liquid chromatographic system from Shimadazu LC 20AD consisting of manual injection. The optimized chromatogram was obtained with acetonitrile in the isocratic mobile phase method at a 1.0 mL/min flow rate. A thermo C-8 column (4.6X250mm,5µm) was used as a stationary phase, and 265.0nm was selected as the detection wavelength with the aid of a UV-Vis detector. The proposed method was validated as per ICH guidelines. The technique was linear in the range of 10-50µg/mL with correlation coefficient R2 = 0.9746, respectively. Recovery studies postulated that % RSD 19.14, 3 & 9.95 respectively. Injection repeatability values were found to be % RSD 17 & 10.63 for intraday and interday, respectively. Stress degradation studies revealed that sitagliptin degrades more rapidly when subjected to 0.1 NaoH. Human plasma spiking studies reported 3.02 ng/mL at 3.02+/-60 min of C and T max, respectively.

Keywords: Sitagliptin, Method development, HPLC, Validation, Stress degradation studies, Human plasma spiking studies

INTRODUCTION

Sitagliptin (SGL) was the first dipeptidyl peptidase-4 (DPP-4) inhibitor approved by United Stated Food and Drug Administration in 2006 for the treatment of diabetes mellitus.[1] It is chemically known as (3R)-3-amino-1-(3-(trifluoro methyl)-6,8- dihydro -5H- (1,2,4) triazolo (4,3a) pyrazin- 7yl) - 4- (2,4,5- trifluorophenyl) - butan-1-one [2]. A review of literature expressed that the selective HPLC method by protein precipitation technique [3] using a mixture of triethylamine and acetonitrile (50:50v/v), determined the long-term effect on cardiovascular effects by randomized double- blinded study,[4] Lavanya *et al* [5] developed an RP-HPLC method, The chromatographic elution was performed by using mobile phase consisting of 0.01M potassium hydrogen phosphate and methanol 50: 50 v/v and the pH adjusted with 0.2 % orthophosphoric acid. Ping *et al.* [6] evaluated the pharmacokinetics and bioequivalence between Sitagliptin phosphate/metformin hydrochloride tablets at a single dose of 50/850mg in healthy Chinese subjects. The chromatographic separation had been achieved by LC-MS tandem mass spectroscopy with UV detection at 248 nm. In the present study, the development of a stress-stabilized liquid chromatographic separation method for the evaluation of Sitagliptin and to determine its pharmacokinetic behaviour in the human plasma in healthy subjects

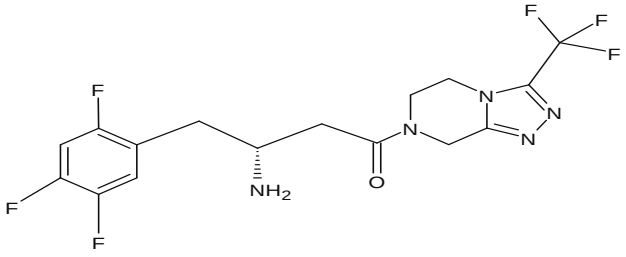

Figure 1: Structure of Sitagliptin

MATERIALS AND METHODS

Instrumentation

The liquid chromatographic system was achieved by Shimadzu model LC 20AD equipped with LC 2010 AD solvent delivery system, comprising a rheodyne manual injector with a binary pump for constant flow and constant pressure delivery and UV- Visible detector connected to software LC solutions for controlling the instrumentation and processing the data. Weighing was done on a digital Shimadazu microbalance (TX2022 L) manufactured by Shimadazu Limited.

Reagents and chemicals

Sitagliptin (S G L) was procured from Yarrow chem products, Mumbai. Methanol (HPLC), Acetonitrile (HPLC), and Potassium hydrogen phosphate (AR Grade) were procured from UV Scientifics, Hyderabad. Triple distilled water was utilized for the whole experiment, which was generated using the in-house method. All other related chemicals for the experiment were analytical grade and purchased from UV Scientifics, Hyderabad.

Selection of Mobile phase Preparation of the Phosphate buffer [7]

Weigh 2.950g of potassium dihydrogen phosphate and 540mg dipotassium hydrogen orthophosphate into a 1000 mL beaker. The solution mixture was prepared and diluted to 1000mL with HPLC water. The pH was found to be 6.26. HPLC grade acetonitrile is taken and filtered in 0.45µm and sonicate to degas it.

Preparation of stock solution [8]

Accurately weighing, 100mg of Sitagliptin (SGL) was weighed and dissolved in 100mL of acetonitrile (1mg/mL). Dilute 1mL of the stock solution to 10mL acetonitrile to achieve 100µg/mL.

Study Population [9]

Healthy non -smoking both gender subjects aged 35 - 45 y with BMI 32-45 kg/m2 were eligible to participate in the trial study who were possessing diabetes. Until the trial duration (3 months), participants were prohibited from consuming any other medications except Sitagliptin (SGL) in order to avoid drug interferences. To prevent the confounding effects of Sitagliptin (SGL), subjects agreed to restrict their utilization of fruit juices and caffeine products.

Study design [10]

A randomized, open-label two sequences, single dose cross bio equivalence study under fasting conditions.

Blood sampling [11]

Twenty blood samples were collected in 5mL K2 EDTA vacutainers via an indwelling catheter placed in the radial vein. Blood samples will also be collected by direct vein puncture during ambulatory blood sampling visits. A pre-dose blood sample will be collected taken 1 hour before the start of the drug administration. The post-dose blood samples were collected at 0,30,60,120,150 mins. The sample collected tubes were centrifuged at 2000 rpm for 10 mins, and the plasma was collected. The collected plasma samples were processed per the extraction procedure by adding 1.0mL methanol in the ratio of 1:1 and then centrifuged for 10 min at 2,000 rpm. The supernatant was filtered through a 0.45µm pure membrane filter. The obtained supernatant liquid was analyzed using the RP-HPLC -UV method using LC software 8.01 version.

Method development [12]

It is critical to develop a simple, rapid, reproducible quantified method for Sitagliptin (SGL) with improved demonstrated sensitivity utilizing the ODS C8 (4.6x250mm, 5µm) using acetonitrile: Phosphate buffer (pH6.26). The optimized chromatogram is achieved in the ratio of 95:5v/v at 265nm.

Validation of the developed method [13]

System suitability: The chromatographic system's suitability was evaluated before each validation stage. The placebo was assessed from five replicate injections.

Linearity: The linearity of a procedure is its ability to obtain tests that are directly proportional to the area of analyte in the sample. The calibration plot was constructed after analysis of five different concentrations (10µg-50µg/mL for Sitagliptin), and areas for each concentration were recorded in triplicate, and the mean area was calculated. The response factor was found by dividing the AUC with respective concentrations.

Accuracy: Recovery of the method was evaluated at three different concentration levels of the standard drug (80,100, and 120%) by adding known amounts of the standard to placebo preparation. Three sets were prepared and injected five times for each concentration level, and then their recovery was analysed.

Precision

Repeatability

The repeatability was performed for five replicas at five concentrations in the linearity range of 10,20,30,40, and 50 µg/mL for Sitagliptin under the same operating condition over a short time interval.

Intermediate Precision

Day to day Precision

Intermediate precision was also performed with laboratory variation on different days and different analysts in five replicas at five concentrations.

Robustness

The method's robustness was assessed by assaying the test solutions under different analytical conditions that were deliberately changed from the original. As per the ICH norms, minor but deliberate variations in the mobile phase concentration were made to check the method's capacity to remain unaffected. The ratio of acetonitrile showed a 5% increase and a 5 % decrease and changes in the flow rate1.0, 0.9 & 1.1 mL, respectively.

Detection of Limit and Quantification Limit

The LOD and LOQ of the developed method were calculated based on the standard deviation response and slope of the linearity curve.

Stress degradation studies [14]

Stress testing of the drug substance can help identify the likely degradation products, which can aid to perform the degradation pathways and the intrinsic stability of the molecule and develop and validate the stability, indicating the power of the procedures used. Stress studies were performed on API using 0.5mg/ml solutions. Considering the excellent solubility of Sitagliptin phosphate, all stress study solutions

were prepared in water. Samples were withdrawn from stress study samples at particular intervals and diluted with water: methanol (1:1) to achieve solubility of water-insoluble impurities and obtain 0.2mg/mL final concentration. All samples were filtered through 1µm membrane filters before subjecting to LC analysis for monitoring degradation behaviour by injecting 15µl of each sample.

Acid Degradation studies

Solutions for acid degradation studies were prepared in 0.1N HCl and stored in an oven at 55ºC for 4hr. A sufficient amount of samples was withdrawn at regular intervals to examine the degradation of the analyte.

Alkali Degradation studies

Solutions for alkali degradation studies were prepared in 0.1N NaOH. When stored at 55ºC, rapid degradation was observed; hence, alkali-forced degradation study samples were stored at laboratory temperature (25ºC), and a sufficient quantum of samples were withdrawn at particular intervals for monitoring the degradation of the analyte.

Oxidation studies

Solutions for oxidation studies were prepared in 5% H2O2 and kept in the oven at 55ºC for two weeks. A sufficient amount of samples was withdrawn at regular intervals to examine the degradation of the analyte.

Thermal studies

Thermal studies were performed on a solid Sitagliptin powder sample at 80ºC for three days.

Photostability studies

Drug substance and tablet powder were exposed to light as a layer (1mm thickness) and spread in a Petri dish using a photostability chamber for three days. After removing the samples from the light cabinet, 0.2mg/ml concentration. Samples were prepared for HPLC analysis using methanol: water (1:1) as a diluent.

Analysis of both the API and tablet sample

Twenty tables were accurately weighed, and their mean value was established. The tablets were pulverized to fine powder, and an accurately weighed quantum of powder equivalent to 100mg of SGL was transferred to a 10mL volumetric flask containing Acetonitrile. The mixture was sonicated for 30 minutes, and the final volume was made using the mobile phase. The mixture was then filtered with a aid of 0.45µm filter. The stock solution was diluted sufficiently with Acetonitrile to get a sample solution of a drug concentration of 10µg/mL. The amounts of SGL in tablet formulation were calculated by extrapolating the area value from the calibration curve. The Analysis procedure was repeated in triplicate with formulation.

Pharmacokinetic and Linearity studies in drug spiking in human plasma[15]

The meticulous chromatographic conditions were meticulously adhered to in order to determine the precise amount of sitagliptin in pooled human plasma. Multiple blood samples (10 ml) were meticulously collected in evacuated glass tubes through an indwelling cannula placed in the forearm veins or directly from the vein. The blood was then gently shaken and centrifuged at 10,000 rpm for 10 min, and the plasma was meticulously separated.

To 1.0mL plasma, 1.0ml of methanol was carefully added, and the mixture was separated for 1 min and then proceeded to centrifuge for 10 min at 2,000rpm. The supernatant was filtered through a 0.45µm pure membrane filter. The plasma thus obtained was spiked on the ratio of 1:1 with drug solutions, ensuring the desired drug concentration in plasma. These solutions were stored at -20° C until analysis. The amount of drug bound in the plasma was calculated based on the area obtained from the HPLC.

The Ethical Committee (PIMS/IEC/2023/023) approved the protocol, and the volunteers provided informed written consent. The post-blood samples were collected at 0,30, 60,120,150 mins. The sample collected tubes were centrifuged at 2000 rpm for 10 mins. The collected plasma was extracted with 0.1mL of methanol in the ratio of 1:1, and the supernatant was filtered through a 0.45µm membrane filter. The obtained supernatant drug substance and tablet powder were exposed to light as a layer (1mm thickness) and spread in a Petri dish using a photostability chamber for three days. After removing the samples from the light cabinet, 0.2mg/ml concentration. Samples were prepared for HPLC analysis using methanol: water (1:1) as a diluent. The liquid was analysed utilising the LC solutions; C max and T max are calculated based on the mean drug concentration and time profile. The HPLC analysis was performed using a [specific HPLC system] with [specific column and mobile phase]. These parameters were selected for their ability to separate and detect sitagliptin accurately.

RESULTS AND DISCUSSION

The simplified, optimized chromatographic conditions are used to develop and easily reproduce the proposed method, which can abide by the criteria of the objective of the research work. The detection wavelength had been stabilized at 265 nm, resulting in good response and good linearity. The ƛ max of the UV spectra has been reported in Figure 2

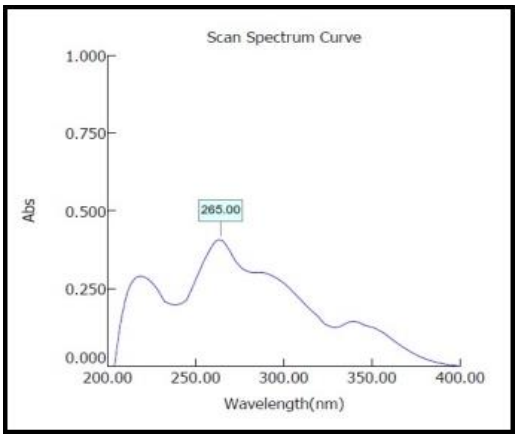

Figure 2: UV spectra showing the ƛmax of Sitagliiptin

System Suitability

The separation variables were set, and the mobile phase was allowed to saturate the column at 1.00 mL /min. After saturation of the column, five replicates of the working standard of SGL 100µg/mL were. The result of the system suitability parameter is tabulated in table 1

Table 1: Results of system suitability Parameters

Parameters	Sitagliptin
	% Mean +/- SD
Peak Area	18875627
Retention time	3.322
Tailing factor	0.93
USP – Theoretical Plate	100.23
Resolution	1.063

Linearity

The linearity of the analytical method was carried out to analyze its ability to elicit test results, which are proportional to the analyte concentration in a sample within a given range. Varied levels of concentration of standard solutions in the range of 10-50µg/mL were prepared and injected into the HPLC, and the chromatogram was recorded. The results of linearity are reported in table 2; Figure 3-4

Table 2: Results of linear studies of Sitagliptin

Analyte	*Concentration (µg/Ml)*	*Concentration level*	*RT value*	*Average peak area*	*Regression equation*
Sitagliptin	10	25	2.765	429699	***R² = 0.9746***
	20	50	2.765	476745	***Y=72683x+349899***
	30	75	2.765	567950	
	40	100	2.765	667731	
	50	125	2.765	697623	

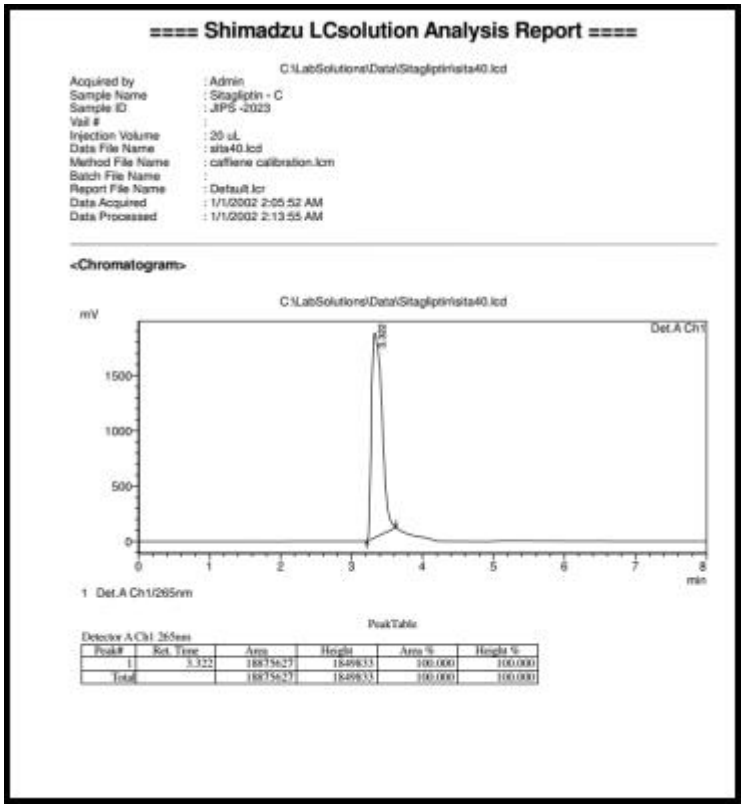

Figure 3: Chromatogram of Sitagliptin

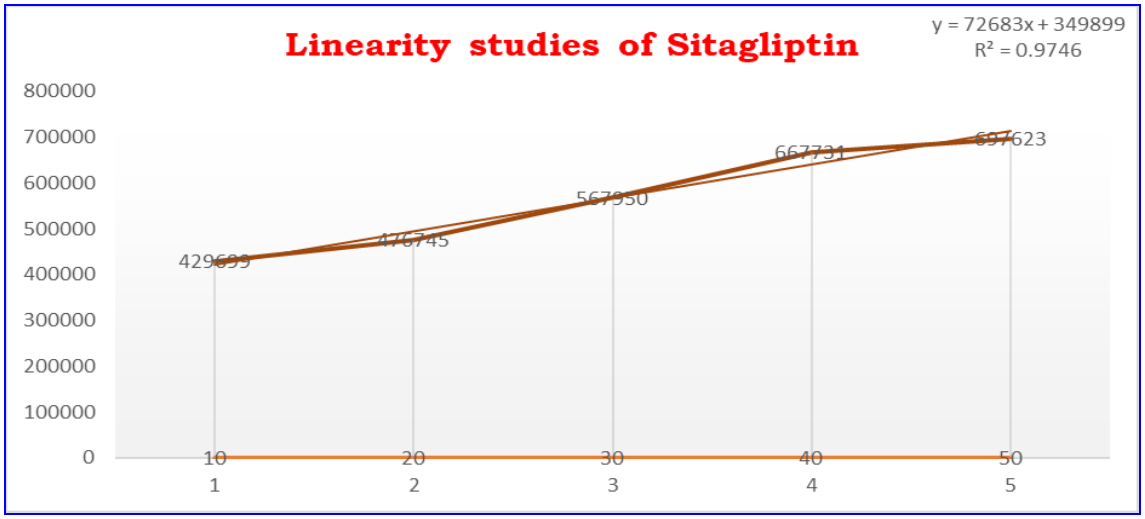

Figure 4: Calibration curve of Sitagliptin

Accuracy

Recovery studies evaluated the Validity and reliability of the proposed methods. The recovery of added standards (80%, 100% and 120%) was found at three replicate and three concentration levels. The value of % means just close to 100, and SD and % RSD are less than 2, indicating the method's accuracy. The results of the recovery study are shown in Table 3.

Table 3: Results of recovery study

Amount (%) of drug added to analyte	Weight Taken (mg)	RT value	Sample added (mg)	peak area	Average Peak area	Sample recovery	Recovery		
							% of recovery	Standard deviation	% RSD
80	80	2.523	80	196544	178271.3	0.90703	102.6	19.65	19.14%
	80	2.804	80	138750		1.284838			
	80	2.812	80	199520		0.893501			
100	100	2.823	100	1096758	722408.3	0.6587	100.0	3	3%
	100	2.744	100	1070467		0.6749			
	100	2.759	100	1046272		0.6352			
120	120	3.570	120	886907	921743.7	1.039279	101.0	10.01	9.95%
	120	3.418	120	1025501		0.898823			
	120	3.196	120	852823		1.0808			

Precision

The repeatability and intermediate precision of the drug determined precision. The repeatability result indicates the precision under the same conditions over a short period interval. The intermediate precision study is expressed within laboratory variation on different days. The SD and % RSD values are should not be less than 2, indicating the method's precision. The result of precision is shown in table 4 & 5.

Table 4: Results of Intra day - precision studies of Sitagliptin

S.No	Peak Name	Retention time	Area	Height	USP plate count	USP tailing
1	Sitagliptin	3.568	783315	93857	0.231	1.372
2	Sitagliptin	3.767	660089	83604	0.221	1.258
3	Sitagliptin	3.616	671966	79435	0.237	1.259
4	Sitagliptin	3.472	846224	96189	0.241	1.419
5	Sitagliptin	4.132	720404	89294	0.225	1.366
Mean			736,399.6			
Standard deviation			78,251.69			
% RSD			**10.63%**			

Table 5: Results of Inter day - precision studies of Sitagliptin

S.No	Peak Name	Retention time	Area	Height	USP plate count	USP tailing
1	Sitagliptin	6.663	824260	89660	0.267	1.168
2	Sitagliptin	6.115	976205	109084	0.257	1.147
3	Sitagliptin	6.107	961138	103547	0.265	1.216
4	Sitagliptin	6.067	1023056	114956	0.250	1.227
5	Sitagliptin	6.423	1295171	98488	0.356	1.252
Mean			1,015,966			
Standard deviation			172,730.95			
% RSD			**17%**			

Robustness

The method's robustness was assessed by assaying the test solutions under different analytical conditions that were deliberately changed from the original. The standard and test solutions were prepared separately for each analytical condition. The test solution's assay result was not affected by varying conditions and was by the actual value. The system suitability data were also found to be satisfactory during variation of the analytical conditions. The analytical method, therefore, remained unaffected by slight but deliberate changes in the analytical conditions. Hence, the proposed method is robust. The result of robustness is shown in Table 6.

Table 6: Results of Robustness

Parameter used for sample analysis	Peak Area	Retention	USP width	Tailing factor
Actual Flow rate of 1.0 mL/min	759464	3.395	0.194	1.326
Less Flow rate of 0.9 mL/min	918703	3.065	0.214	1.276
More Flow rate of 1.1 mL/min	650736	3.214	0.206	1.321
More organic phase	438149	3.029	0.218	1.798
Less organic phase	422777	3.011	0.204	2.001

LOD and LOQ

The LOD and LOQ of the developed method were calculated based on the standard deviation of response and slope of the linearity of the curve. Results of LOD and LOQ were tabulated in table 7

Table 7: Results of LOD and LOQ.

Name	LOD (µg/mL)	LOQ (µg/mL)
Sitagliptin	0.74	2.24

Stress/Forced degradation studies

Sitagliptin's highest levels of degradation were observed in 0.1 N sodium hydroxide (75%) and thermal degradation (65%), respectively. In the following study, (45%) degradation was observed when Sitagliptin was subjected to 0.1N Hydrochloric acid and 5% hydrogen peroxide. 38% degradation had been observed when subjected to UV at 254nm. In the present study, the stability of Sitagliptin in acidic and basic pH buffers is examined. The degradation studies of Sitagliptin are shown in figure 5.

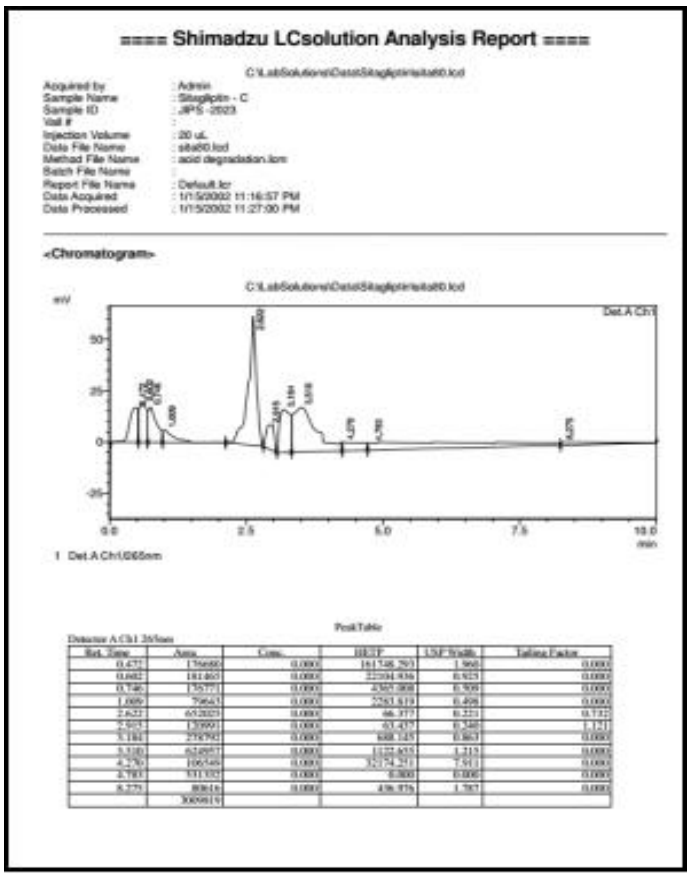

Figure 5: Forced degradation studies of Sitagliptin

Analysis of both the API and tablet sample

The results of the analysis of API and tablet formulation were reported. The assay value of the drug was found to be 99.32% w/w, and SD and % RSD were found to be 0.89, which indicates there is no interference of excipient in the estimation of the drug.

Pharmacokinetic and Linearity studies in drug spiking in human plasma

The plasma concentration - time profiles for Sitagliptin under fasting conditions are shown respectively in table 8 and figure 6.

Table 8: Results of pharmacokinetic parameters of Sitagliptin

S.No	Drug	Time (Min)	RT value	Area	C_{max} (ng/mL)	T_{max}
1	Sitagliptin (100mg) -Tablet	0	3.666	70927	0.35	60 mins
2		30	4.078	231746	1.15	
3		60	3.051	603723	3.01	
4		90	4.739	107051	0.53	
5		120	4.454	36355	0.18	
6		150	4.238	38160	0.16	
Mean				**781327**		
Standard deviation				**200074.6**		

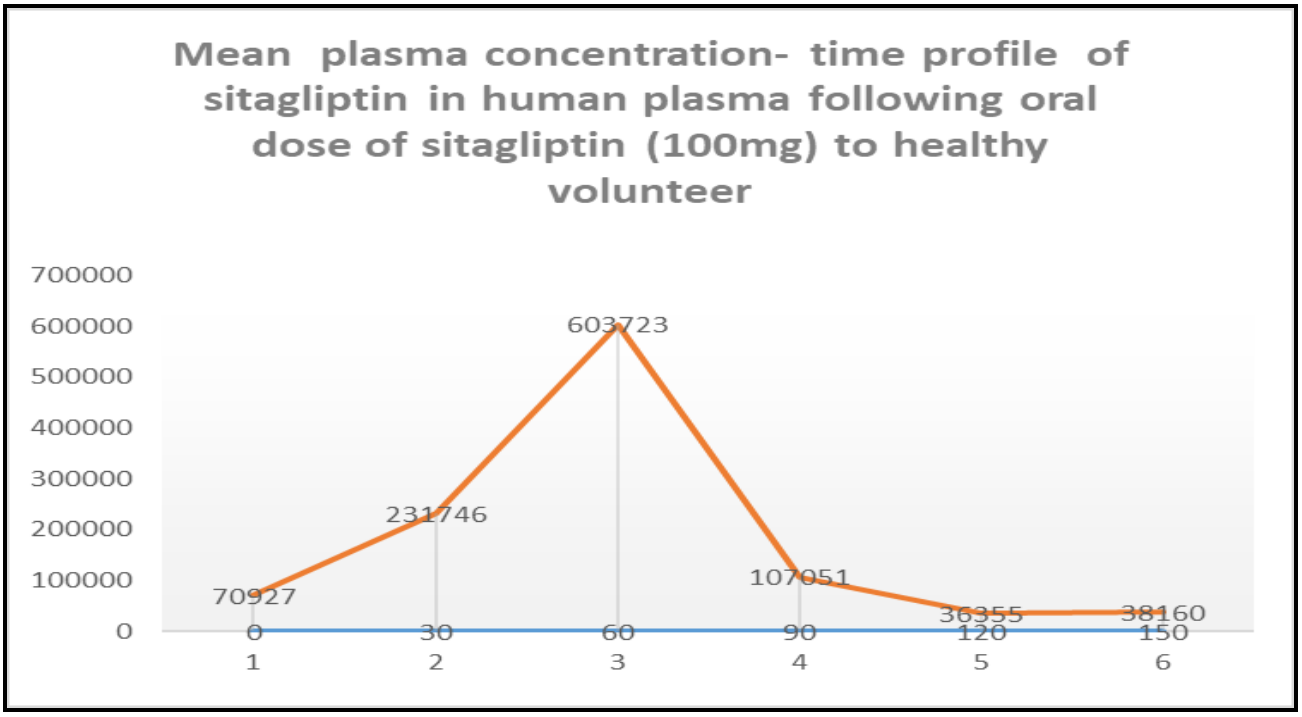

Figure 6: Mean plasma concentration- time profile of Sitagliptin in human plasma

CONCLUSION

Our study evaluated the developed method: cost-effective, rapid (short retention time), simple, accurate, precise and stabilized under forced conditions in the bulk drug and the formulation. Further, we evaluated Sitagliptin's pharmacokinetic behaviour, which shows good tolerability with a similar safety profile.

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