International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.15, No.02, pp 48-57, 2022

Development and Validation of a Bioanalytical Method for determination of Teneligliptin in Human Plasma by RP-HPLC

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Abstract: A simple, reliable, precise, accurate, sensitive and selective RP-HPLC method was developed and validated for estimation of Teneligliptin in Human plasma using protein precipitation extraction method. Teneligliptin is an antidiabetic drug from the class called Dipeptidyl peptidase-4 (DPP-4) inhibitor. The chromatographic separation was carry out using AGILENT C18 column (250mm x 4.6ID) as stationary phase and mobile phase of Methanol and 0.05% orthophosphoric acid solution in ratio of 70:30 v/v with pH of 2.7 at flow rate of 0.7 ml/min. Detection was carry out at 245nm using DAD detector. The injection volume was 20µl. The run time was 8min. The retention time of Teneligliptin was shorter i.e. 3.5 min. The overall recovery of teneligliptin was 97.83%. The calibration curve was linear over the concentration range of 5-25 µg /ml. Accuracy ranges from 98.82% to103.28 % with the precision 1.41% to 3.06% in intra-day method. In inter-day method the accuracy ranges from 99.80% to 103.33% with the precision 2.12% to 5.29%. The Lower limit of quantification (LLOQ) and the Limit of Detection (LOD) for Teneligliptin were found to be 2.09µg/ml and 0.69µg/ml respectively. The method developed can be used in therapeutic drug monitoring units, bioequivalence and bioavailability studies, pharmacokinetic and toxicology studies of Teneligliptin.

Keywords : Teneligliptin, Bioanalytical method, Bioanalytical validation, RP-HPLC, Human Plasma.

DOI: http://dx.doi.org/10.20902/IJPTR.2022.150204

INTRODUCTION

Bioanalysis means the quantitative determination of drugs and their metabolites in biological fluids (blood, plasma, urine etc.). Bioanalysis is used very early in the drug development process and support to drug discovery programs on the metabolic fate as well as pharmacokinetics of chemicals in living cells and in humans.[1]

Bioanalytical method employed for the quantitative estimation of drugs and their metabolites in biological media or fluid and plays an important role in estimation and interpretation of pharmacokinetic, bioequivalence and toxicokinetic studies. Biological media or fluid used in bioanalysis can be the major bioanalytical role is method development, method validation, and sample analysis. Each step in the method must be investigated to decide the extent to which environment, matrix, or procedural variables can interfere the estimation of analyte in the matrix from the time of set up to the time of analysis. Chromatoghraphic method such as high pressure liquid chromatography (HPLC), Gas chromatoghraphy (GC) and liquid chromatography coupled with double mass spectrometry (LCMS-MS) can be used for the bioanalysis of drugs in biological fluid. Various parameters such as linearity, accuracy, precision, selectivity, sensitivity, reproducibility and stability were evaluated during bioanalysis. Bioanalytical method development includes various steps that is, sample collection and preparation, extraction of analyte from biological sample, evalution of various parameters, etc.[2]

Teneligliptin (Fig 1) is the dipeptidyl peptidase-4 inhibitors, also called gliptins. They increase the levels of incretins in plasma (GLP-1 and GIP), leading to insulin increase and decrease in blood glucose.[3]

Determination of Teneligliptin in biological fluid was carried out earlier by LCMS/MS[4,5] in human plasma and by using RP-HPLC in rabbit plasma[6]. A liquid chromatographic method that is LC-ESI-MS/MS[7] and also a hydrophilic interaction liquid chromatography-MS/MS[8] was reported for simultaneous determination of teneligliptin and metformin in human plasma. A LC-MS technique for simultaneous determination of teneligliptin and its active metabolite teneligliptin sulfoxide in human plasma was reported earlier[9]. Number of analytical method were reported for teneligliptin using spectrophotometric techniques as well as HPLC technique[10]. Since sufficient bioanalytical methods have not been reported for The quantitative estimation of teneligliptin using HPLC, there is a necessity for investigation of selective and sensitive new methods for quantitative estimation of teneligliptin in human plasma. Although LC-MS/MS is a versatile tool, the development of HPLC based separation methods makes it more economical and simpler both in terms of maintenance and data interpretation. The present article describes a simple and sensitive RP-HPLC method with

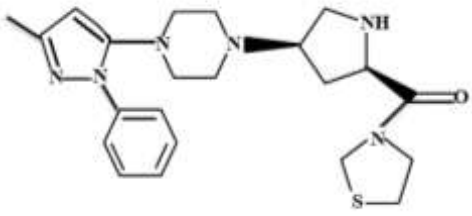

Figure 1: Structure of Teneligliptin

a low LLOQ for UV detection of Teneligliptin eluted under isocratic mode which can be directly applied to the successful estimation of Teneligliptin in a bioequivalence study and to validate the developed method according to ICH guidelines[11].

Drug Name: Teneligliptin[12]

IUPAC name :{(2S,4S)-4-[4-(3-Methyl-1-phenyl-1H-pyrazol-5-yl)-1-piperazinyl] 2pyrrolidinyl}(1,3-thiazolidin-3-yl)methanone, hydrobromide hydrate

Category: Antidiabetic drug (Gliptin)

Chemical structure: 'J-shaped' structure of five consecutive rings.

Molecular Formula: $C_{22}H_{30}N_6OS$.

Molecular Weight: 426.6 g/mol.

Description: An off white to cream coloured powder.

Melting point: 211°C

pKa value: 8.7

λ max: 245 nm

Oral bioavailability: 43-74%

Biological Half-life: 26.9 hr.

t_{max}: 1.3 hr.

C_{max}: 220ng/mL

Plasma protein binding: 78-80%

Volume of distribution: 8.9 L/kg (High tissue distribution)

Apparent plasma clearance: 13.11 L/hr.

Solubility: Teneligliptin is soluble in methanol, water and solubility of teneligliptin in ethanol is approx. 2 mg/ml, solubility in Dimethyl sulfoxide (DMSO) as well as Dimethyl formamide (DMF) is approx. 30 mg/ml. It is insoluble in cyclohexane

Mechanism of action: Teneligliptin inhibits human plasma Dipeptidyl peptidase-4 (DPP-4) activity.

Metabolism: It is metabolized by cytochrome P450 (CYP3A4) and flavin-containing monooxygenase 3 (FMO_3).

Extraction: About 34.4% of teneligliptin is extracted from kidney in an unchanged form and remaining 65.4 % of teneligliptin is metabolized and eliminated via renal and hepatic excretion.

EXPERIMETAL WORK

Chemicals:

Teneligliptin was gifted from Ami Lifesciences Pvt. Ltd., Vadodara, Gujarat (India). Methanol and Water (HPLC grades), Ortho-phosphoric acid and Acetonitrile (analytical grades) were purchased from E. Merck Ltd., Mumbai (India). All other chemicals and reagents were of analytical grade.

Instrumentation (Specification of HPLC):

Analysis was performed using Agilent liquid chromatography with a pump series 1100, Auto sampler, Agilent DAD detector at 245nm. Chromatographic separations were accomplished using a reverse phase system was used consisting of C_{18} column (Agilent column, 250 x 4.6 mm, 5µm).

Chromatographic Conditions: (Table 1)

The separation was carried out using mobile phase of Methanol and 0.05% Orthophosphoric acid buffer in ratio of 70:30 v/v, pH 2.7. Agilent C18 (250 x 4.6 mm, 5µm) column was used as stationary phase. It was eluted at flow rate of 0.7 ml/min with injection volume of 20 µL. The effluent was monitored at wavelength 245 nm with run time of 8 min.

Table 1: chromatographic conditions

Parameters	Chromatographic conditions
Mobile phase	Methanol:0.05% Orthophosphoric acid buffer (70:30v/v)
Stationary phase	Agilent C18 column (250 x 4.6 mm,5µm)
pH	2.7
Flow rate	0.7 ml/min
Wavelength	245nm
Injection volume	20 µL
Run time	8 min

Preparation of standard drug solutions:

Stock solution of Teneligliptin 1000 µg/ml were prepared by dissolving 10 mg of each drug in 10 ml volumetric flasks and the volume was made up to 10 ml with mobile phase. Furthermore, freshly prepared sample solution was sonicated for 10-20 minutes and filtered through 0.5µ nylon filters. From the stock solution 1 ml was taken and diluted to 10 ml (100µg/ml). From this 0.5ml, 1.0 ml, 1.5 ml, 2.0 ml, 2.5 ml was pipette out and transfer separately to 10ml volumetric flasks and make up the volume using mobile phase to make concentration of 5, 10, 15, 20 and 25µg/ml respectively.

System suitability parameters

Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, tailing factor, are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The % RSD for retention time and response was calculated. The results of the system suitability studies were shown in table no.5. These parameters were shown to be within specified limits. The obtained values are less than 2% RSD, which shows the suitability of the system for the analysis of Teneligliptin drug.

Preparation of quality control samples:

Three quality control (QC) plasma samples at concentrations of 5, 15 and 25 µg/ml were prepared by spiking 0.1mL blank plasma with appropriate working standard drug solutions.

Preparation of Standard plasma sample:

Standard plasma samples ranged from 5 to 25µg/mL were prepared by spiking 0.1mL blank human plasma with suitable working standard drug solutions.

Preparation of calibration standards and curve

To 0.1ml of blank plasma and 0.05ml of working standard solution of Teneligliptin were added to get concentration of 5-25 µg/ml respectively. To these calibration standards 0.2ml precipitating agent acetonitrile was added and then centrifuged for 15 min at 5000 rpm. After centrifugation the clear supernatant liquid was collected and a quantity of 100µL was injected into the HPLC column and chromatograms were recorded. Standard calibration graph was plotted using ratio of peak area of Teneligliptin to its concentration.

METHOD VALIDATION:

After developing a method its validation is necessary to prove the suitability of the method for the intended purpose. The proposed method was validated as with respect to linearity, precision, accuracy, robustness, limit of quantification (LOQ) and Limit of detection (LOD). The quality control sample at concentrations of 5, 15 and 25µg/ml was used to determine the precision and accuracy, robustness of assay method.

***A.* Precision and Accuracy:**

Intraday and interday precision studies were conducted. Intra-day precision and accuracy were determined by duplicate analysis of five sets of samples spiked with three different concentrations of teneligliptin at low, medium, high quality control samples within a day. Similarly interday precision and accuracy over a two week

period time was evaluated. . In intraday precision and accuracy plasma sample containing drug at three different concentrations were injected and chromatogram was recorded. For present precision study, samples were prepared at three concentration levels: low (LQC), medium (MQC) and high (HQC) quality controls corresponding to 5, 15 and 25µg/ml.

Acceptance criteria: RSD of the mean concentration of five readings should be less than 15% for bioanalytical method.

B. Linearity and Range:

Linearity and range were estimated by using calibration curve. By using calibration standards prepared by spiking plasma with Teneligliptin at different concentrations like 5 to 25 µg/ml the calibration graph was plotted taking concentration of spiked plasma on x-axis and peak area on y-axis. The linearity is determined from 50% to 150% of the proposed concentration.

Acceptance Criteria: Coefficient of correlation of the calibration should be not less than 0.99

C. Robustness:

The robustness of the method was studied by changing the chromatographic conditions slightly. The standard solutions were injected in these changed chromatographic conditions.

- ± 1 % difference in the ratio of methanol in the mobile phase.
- ± 0.5 difference in units of pH of the buffer.
- ± 1 % difference in flow rate of the mobile phase.

In these changed conditions the separation factor, retention time and peak symmetry was calculated.

Acceptance Criteria: Deviation in results from original run should be less than 2%.

D. Limit of quantification (LOQ):

The limit of quantification was determined as the lowest concentration of Teneligliptin in the standard calibration curve that could be quantified with a value of bias below 20% and a signal-to-noise ratio of at least 10.

$$LOQ = \frac{10 \text{ X Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

E. Limit of Detection (LOD):

The limit of detection is the lowest concentration of analyte in the sample which can be detected but not quantified under given experimental conditions. The lowest concentration which can be distinguished from the background noise with a certain degree of confidence is defined as limit of detection.

$$LOD = \frac{3.3 \text{ X Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

RESULTS:

Method Development:

Selection of Wavelength: The sensitivity of a HPLC method depends upon the proper selection of the wavelength. From the UV spectrum, wavelength of **245 nm** was selected. At this wavelength the drug showed good absorbance.

Chromatographic Optimization

A RP-HPLC method was developed for teneligliptin, which can be conveniently employed for routine analysis in biological fluids. The chromatographic conditions were optimized in order to provide a good performance of the assay. The mobile phase for drug was selected based on its polarity. Different trials were taken and the final chromatographic condition selected is shown in table 1**.** Teneligliptin was eluted at the retention time of 3.46 min. Peaks were also well resolved and symmetric with perfect peak properties.
A typical chromatogram of Teneligliptin is shown in figure 2.

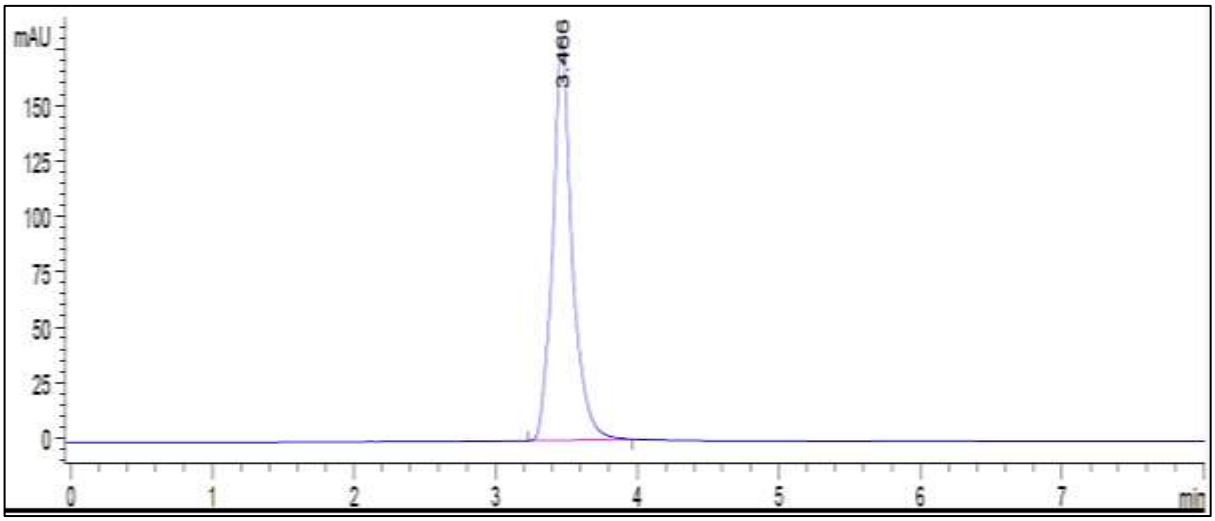

Figure 2: A Typical Chromatogram of Teneligliptin

Chromatographic separation of Teneligliptin in biological fluid:

Chromatogram of blank plasma was recorded at fixed chromatographic conditions and shown in figure (Figure 3).

Various precipitating agents were used for extraction of Teneligliptin in human plasma. But out of all precipitating agents Methanol was proved to be good because of its maximum percentage recovery. Chromatogram of the precipitating agent was recorded and the percentage recoveries were calculated and shown in table (Table 3). The extracted retention time for Teneligliptin was 3.528 min. as shown in figure 4. The peak was symmetric with straight baseline. The extraction method used for the present study was simple and newer than previous method used for extraction of drug from plasma was mostly liquid-liquid extraction but for the first time protein precipitation method used in present study. And it is most simple method and show sufficient recovery which is advantageous.

A typical chromatogram of Teneligliptin in human plasma is shown in figure 4.

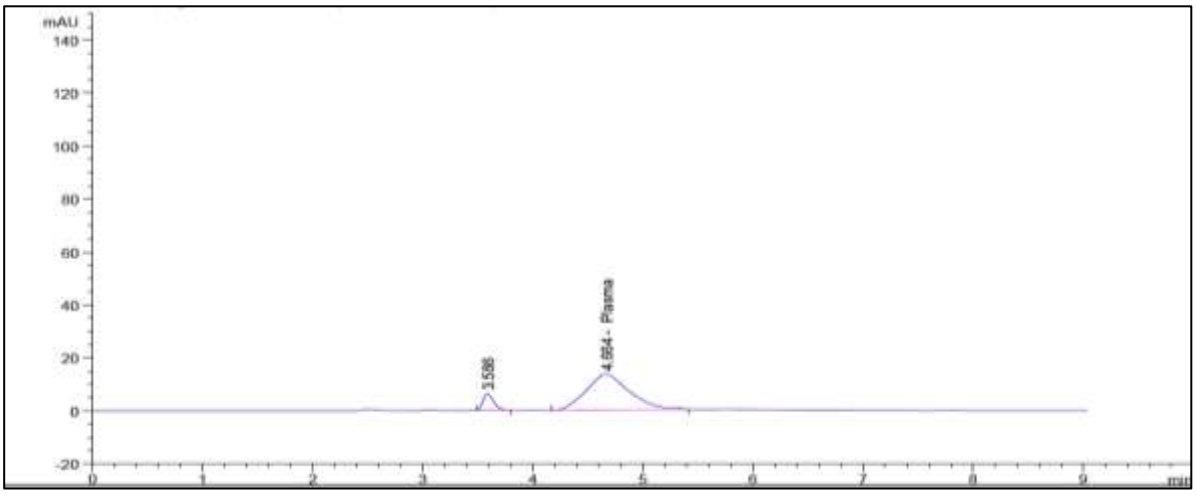

Figure 3: Chromatogram of blank Plasma

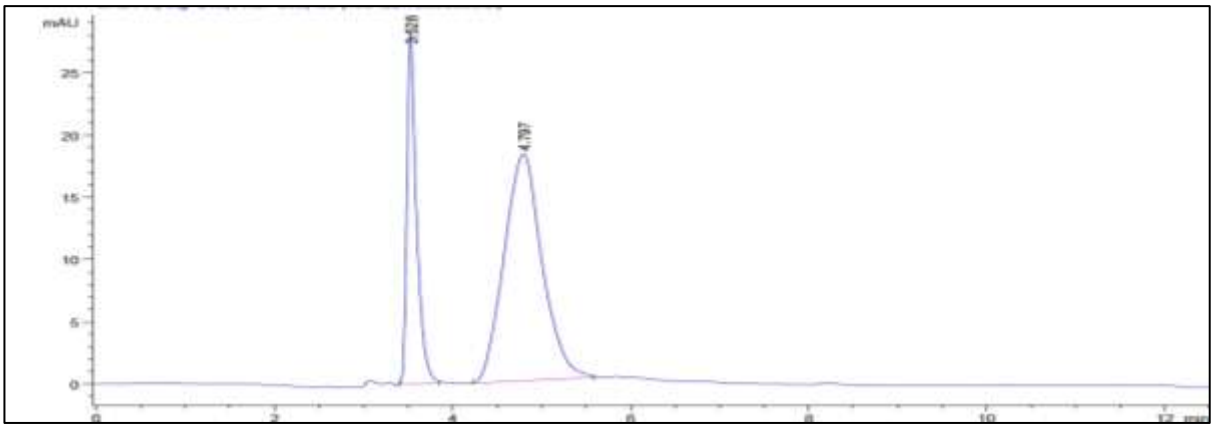

Figure 4: Chromatogram of Teneligliptin in Human Plasma

Method Validation:

A. Accuracy and precision:

For precision and accuracy studies, samples were prepared at three concentration levels: low (LQC), medium (MQC) and high (HQC) quality controls, corresponding to 5, 15 and 25µg/ml of Teneligliptin, respectively, with five replicates each. At two levels these accuracy and precision studies were conducted i.e. intraday and interday. In this the present developed method, shown the good accuracy and precision. Accuracy ranges from 98.82% to103.28 % with the precision 1.41% to 3.06% in intra-day method. In inter-day method the accuracy ranges from 99.80% to 103.33% with the precision 2.12% to 5.29%. Finally the data obtained here, was found to be within limits as per ICH guidelines and method was accurate.

Intra-day studies: In this plasma concentration 5, 15 and 25 µg/ml were injected into HPLC five times and mean peak area was calculated separately for each concentration and from that accuracy and precision percentage RSD values were calculated and shown in table **(Table 2).**

Inter-day studies: In this the plasma concentrations of 5, 15 and 25 µg/ml were injected into HPLC five times in three different days and mean peak areas were calculated and from that accuracy and precision percentage RSD were calculated and shown in table **(Table 2).**

Table 2a: Accuracy And Precision Studies Of Teneligliptin (Intraday)

Sr. no.	Conc. of Drug (µg/ml)	Ratio of Area (Mean)	Conc. Found (µg/ml)	Accuracy (%)	Precision (%RSD)
1	5(LQC)	139.25	5.13	103.28	3.06
2	15(MQC)	457.14	14.96	101.34	1.41
3	25(HQC)	789.08	25.23	98.82	2.68

***Average of five replicate**

Table 2b: Accuracy and Precision Studies Of Teneligliptin (Interday)

Sr. no.	Conc. of Drug (µg/ml)	Ratio of Area (Mean)	Conc. Found (µg/ml)	Accuracy (%)	Precision (% RSD)
1	5(LQC)	142.09	5.22	101.92	5.29
2	15(MQC)	455.63	14.92	103.33	3.22
3	25(HQC)	791.18	25.29	99.80	2.12

***Average of five replicate**

B. Linearity and range:

The linearity graph of average peak area at each level against the concentration in µg/ml is plotted and found to be straight line graph. This method proved to be linear between µg/ml of Teneligliptin in human plasma, with a typical calibration curve of correlation equation y = 32.34x - 26.9, correlation coefficient > 0.999 shown in table **(Table 3)**

The chromatograms of the plasma calibration standards with concentrations 5, 10, 15, 20 and 25 µg/ml were recorded and shown in figures **(Fig 2)** and their peak areas of drug was noted. The calibration curve for Teneligliptin was plotted as peak Area vs. concentration of the Teneligliptin calibration standards in plasma.
The correlation coefficient of Teneligliptin shown was 0.9997 which was within limits. This calibration curve plotted was linear and showed that the method had adequate sensitivity to the concentration (5-25 µg/ml) of the drug. Finally the data obtained, in this was within limits. Coefficient of correlation of Teneligliptin was found to be more than 0.99.

Table 3: Calibration Standards Peak Area

Concentration (µg/mL)	Peak Area of Teneligliptin
5	140.59412
10	290.4433
15	458.86707
20	614.09021
25	787.39685
Regression Equation	y = 32.34x - 26.9
coefficient of correlation	0.9997

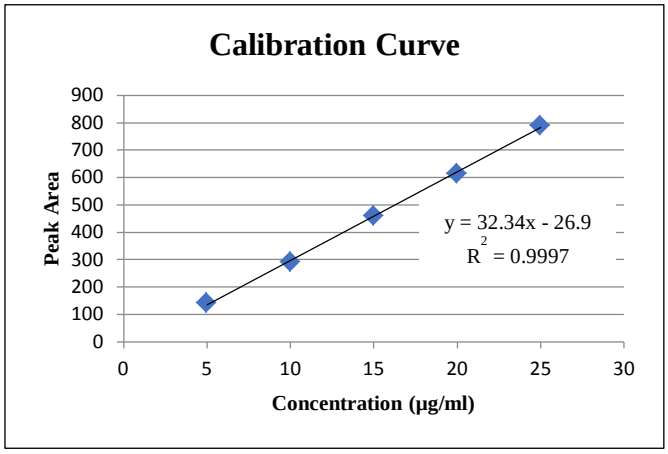

Figure 5: Calibration curve for Teneligliptin

C. Robustness:

Robustness was attempted by deliberately changing the chromatographic conditions to evaluate the difference in resolution, capacity factor, peak height and peak width (tailing factor). Robustness was studied for Teneligliptin, results obtained was displayed in following Table 4.

Table 4: Robustness Study Of Teneligliptin

Variables	Peak Area ± SD	%RSD
Standard	604.09 ±6.33	1.05
Flow rate (+0.1 ml/min)	595.13 ±6.21	1.02
Flow rate (-0.1 ml/min)	612.88 ±4.78	0.78
Methanol (+1%)	610.85 ±11.32	1.84
Methanol (-1%)	620.10 ±0.42	0.07
Wavelength (+1nm)	604.69 ±0.49	0.08
Wavelength (-1nm)	603.39 ±6.33	1.05

D. Lower Limit of Quantification and Limit of Detection:

The Lower limit of quantification (LLOQ) and the Limit of Detection (LOD) for Teneligliptin were separately determined and reported, based on the calibration curve for spiked plasma solutions was found to be 2.09µg/ml and 0.69µg/ml respectively.

E. System suitability:

These parameters were shown to be within specified limits. Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, HETP, tailing factor, LLOQ are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table **(Table 5).** These parameters were shown to be within specified limits.

Table 5: System Suitability Studies

	Retention time (min) (n=5)		Peak area (n=5)		Theoretical Plate Number	Tailing Factor
	Mean ±SD	%RSD	Mean ±SD	%RSD		
Mean	3.50 ± 0.02	0.104	140.2 ± 2.89	2.061	6229	0.1053

DISCUSSION:

The developed RP-HPLC method in human plasma using protein precipitation extraction for sample preparation was found to be very simple, reliable, precise, accurate, sensitive and selective analytical method for the estimation of Teneligliptin. The peaks obtained for the drug of interest by the present method was symmetrical in nature with acceptable tailing factor and from the plasma endogenous proteins by Protein precipitation Extraction. The retention time of Teneligliptin was shorter and proves that the method is rapid. The Protein precipitation Extraction method demonstrated relative recoveries with acceptable relative standard deviation. The method is suitable for routine quantitative analysis in pharmaceutical dosage forms. The method developed can be used in therapeutic drug monitoring units, bioequivalence and bioavailability studies, pharmacokinetic and toxicology studies of Teneligliptin.

ACKNOWLEDGEMENT:

Authors are thankful to Ami Lifesciences Pvt. Ltd for providing drug as gift sample.

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