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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TENELIGLIPTIN USING RP-HPLC IN RABBIT PLASMA

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Abstract:

A simple, highly sensitive, precise and accurate high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of teneligliptin in rabbit plasma samples. The chromatographic separation was achieved with a reverse phase column thermo C18 (4.6×100 mm, 5µ) and the mobile phase consisted of methanol and 5mm potassium phosphate buffer (60:40 v/v) at a flow rate of 1mL/min. Sitagliptin was used as an internal standard. The retention time of Teneligliptin and sitagliptin were found to be 3.9and 2.2min respectively. The calibration curve was linear (r2 > or = 0.99) ranging from 7.20 to 470ng/ml and the lower limit of quantification was 7.20 ng/mL. Interday precision were lower than 5% (CV) and accuracy ranged from 90 to 110% in terms of percent accuracy. Mean extraction recovery was found to be above 82%. The method was successfully developed and validated in rabbit plasma for excellent selectivity, accuracy, precision, recovery and stability.

Keywords: *HPLC; Teneligliptin; internal standard, Rabbit plasma; Validation*

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INTRODUCTION:

Teneligliptin has the molecular formula $C_{22}H_{30}N_6OS$ and chemical name{(2S,4S)-4-[4-(3-Methyl-1-phenyl-1H-pyrazol-5-yl)-1-piperazinyl]-2- pyrrolidinyl}(1,3-thiazolidin-3-yl)methanone with a molecular mass of 426.583 g/mol. and absorption maxima around 244 nm. Teneligliptin belong to class of Antidiabetic drugs known as dipeptidyl peptidase inhibitors or gliptins.Teneliglitpin got FDA approval in 19 mainly for the treatment of diabetic. The mode of action of the teneligliptin **is** the glucagon-like peptide-1 (GLP-1) is secreted from alimentary canal in response to meal that promotes insulin secretion from pancreas and regulates blood sugar post meal by controlling glucagon secretion [1-5]. Teneligliptin exhibits a hypoglycemic effect by controlling the degradation of GLP-1 by inhibiting dipeptidyl peptidase-4 (DPP-4) activity and thereby increasing blood concentration of active GLP-1.

Pharmacokinetics:

After oral administration of a single 20 mg and 40 mg dose to healthy subjects, teneligliptin was rapidly absorbed, with peak plasma concentrations (mean T max) occurring at 1.8 hours and 1 hour post dose. Plasma AUC of teneligliptin increased in a dose-proportional manner. Following a single oral 20 mg and 40 mg dose to healthy volunteers, mean plasma AUC of teneligliptin was 2028.9 and 3705.1 ng*hr/ml, Cmax was 187.2 and 382.4 ng/ml, and apparent terminal halflife (t1/2) was 24.2 and 20.8 hours. Plasma AUC of teneligliptin increased following 20 mg doses at steady-state compared to the first dose. Coadministration with food reduces the Cmax by 20%, increases the Tmax from 1.1 to 2.6 hours but does not affect the AUC of teneligliptin as compared to that in the fasting state. The plasma protein binding rate is 77.6 – 82.2%. Following a 20 mg single oral dose of [14C] teneligliptin, 5 metabolites M1, M2, M3, M4 and M5 were observed. In vitro studies indicated that CYP3A4 and flavin-containing monooxygenase (FMO1 and FMO3) are involved in the metabolism of teneligliptin. Teneligliptin does not inhibit CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C8/9, CYP2C19, CYP2E1, is a weak inhibitor of CYP2D6, CYP3A4, and FMO (IC50 value ：489.4, 197.5 and 467.2 μmol/l) and does not induce CYP3A4 and CYP1A2. Following a 20 mg single oral dose of [14C] teneligliptin, 45.4% of administered radioactivity was excreted in urine and 46.5% in faeces till 216 hours after dose. The cumulative urinary excretion rates for upto 120 hours for un-metabolized, M1, M2, and M3 were 14.8%, 17.7%, 1.4% and 1.9% respectively while the cumulative faecal excretion rates for un-metabolized, M1, M3, M4 and M5 were 26.1%, 4.0%, 1.6%, 0.3% and 1.3% respectively. The single administration of teneligliptin at 20 mg in patients with renal impairment revealed no remarkable changes in Cmax and t1/2 corresponding to the level of renal impairment. Compared with healthy adult subjects, the AUC0–∞ of subjects with mild renal impairment (50 ≤ creatinine clearance [Ccr] ≤ 80 mL/minute), moderate renal impairment (30 ≤ Ccr < 50 mL/minute), and severe renal impairment (Ccr < 30 mL/minute) was approximately 1.25 times, 1.68 times, and 1.49 times higher than that of healthy adult subjects, respectively. A single administration of teneligliptin 20 mg in patients with hepatic impairment revealed that the Cmax of subjects with mild hepatic impairment (Child–Pugh classification: total score 5–6) and moderate hepatic impairment (Child–Pugh classification: total score 7–9) was approximately 1.25 times and 1.38 times that of healthy adult subjects, respectively. Compared to healthy adult subjects, the AUC0–∞ of subjects with mild and moderate hepatic impairments was approximately 1.46 times and 1.59 times higher than that of healthy adult subjects, respectively. There have been no previous clinical studies using teneligliptin in patients with severe hepatic impairment (Child–Pugh classification: total score was greater than 9).

Adverse effects:

The most common adverse reactions reported with teneligliptin are hypoglycemia and constipation. Other adverse reactions reported with teneligliptin are: Gastrointestinal Disorders: Intestinal obstruction, abdominal bloating, abdominal discomfort, nausea, abdominal pain, flatulence, stomatitis, gastric polyps, colon polyps, duodenal ulcer, reflux esophagitis, diarrhea, loss of appetite, increased amylase, lipase increased, acute pancreatitis [6-10]. Kidney and Urinary system: Proteinuria, urine ketone-positive. Skin and Subcutaneous Tissue Disorders: Eczema, rash, itching, allergic dermatitis. Investigations: Increase in AST, ALT, γ-GTP and ALP. Others: Increased CPK, increased serum potassium, fatigue, allergic rhinitis, elevation of serum uric acid.

Over Dosage:

In the event of an overdose, it is reasonable to employ the usual supportive measures, e.g., remove unabsorbed material from the gastrointestinal tract, employ clinical monitoring (including obtaining an electrocardiogram), and institute supportive therapy as dictated by the patient's clinical status.

Warnings and Precautions:

Teneligliptin should be administered carefully in the following: Patients with advanced liver failure (safety has not been established),Patients with congestive heart failure (NYHA category III-IV) (safety has not been established), Patients with

pituitary insufficiency or adrenal insufficiency, poor nutritional state, starvation, an irregular dietary intake, or debilitating condition, intense muscle movement or excessive alcohol intake (may cause low blood sugar), Patients with history of abdominal surgery or with a history of bowel obstruction (may cause bowel obstruction), Patients with arrhythmia, severe bradycardia or its history, patients with heart disease such as congestive heart failure or patients with low serum potassium, congenital prolonged QT syndrome, history of Torsades de pointes or patients using antiarrhythmic drugs (may cause QT prolongation), Patients using an insulin secretagogue (e.g., sulfonylurea) (risk of severe hypoglycaemia).

Drug name: Teneligliptin

Chemical Formula: $C_{22}H_{30}N_6OS$

Chemical Structure:{(2S,4S)-4-[4-(3-Methyl-1-phenyl-1H-pyrazol-5-yl)-1-piperazinyl]-2-pyrrolidinyl}(1,3-thiazolidin-3-yl)methanone.

Molecular Weight: 426.22 g/mol

Solubility: in water, DMSO,methanol

PKa: 8.7

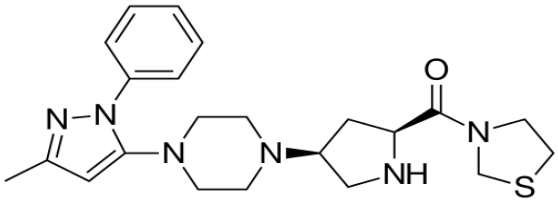

Fig 1 : chemical structure of Teneliglitpin

Drug name: Sitagliptin

Chemical Formula: $C_{16}H_{15}F_6N_5O$

Chemical Structure: (3R)-3-amino-1-3(trifluoromethyl)-5H,6H,7H,8H-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one

Molecular Weight: 407.32 g/mol

Solubility: in water, 179.2mg/L at 25^0 C

LogP : 1.5

PKa : 8.78

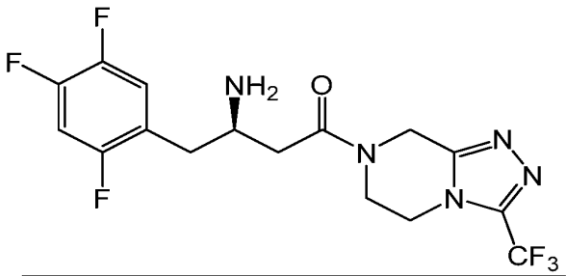

Fig 2: chemical structure of sitagliptin

Literature survey

Literature survey has revealed that there is only single method is reported for the determination of Teneligliptin in human plasma by LCMS/MS with the chromatographic conditions of run time 1.50 min run time, separation was achieved on a Hypersil Gold C18 column using a mobile phase composed of 0.1% formic acid in Milli-Q water/0.1% formic acid in acetonitrile in gradient elution mode. The quantification of teneligliptin was performed in a positive electro spray ionization mode and multiple reaction monitoring (MRM) Run time:10mins,Hypersil gold column,mobile phase :0.1% formic acid in Milli-Q water/0.1% formic acid in acetonitrile. Methods reported in the another is that the estimation of Teneligliptin in bulk and pharmaceutical dosage forms by RP-HPLC with the chromatographic conditions of and another Method reported in the literature for the estimation of Teneligliptin in bulk and pharmaceutical dosage forms by UV spectroscopy and HPTLC of conditions are solvent system consisted of toluene: chloroform: ethanol: diethylamine in the proportion of 4:4:1:1, v/v/v/v. Methods reported in the another is that the estimation of Teneligliptin in bulk and degradation forms by RP-HPLC with the chromatographic conditions for Separation was achieved on a Shisedo C18column, 5µm, 250mm × 4.6 mm i.e. column using a mobile phase consisting of Acetonitrile:Methanol: Water (30:40: 30 % v/v/v) at a flow rate of 1.0ml/min and UV detection at 246nm. Estimation of Anti-Diabetic Teneligliptin Hydrobromide Hydrate by Rp-Hplc and Derivative Spectroscopic Method with the Isocratic elution at the flow rate of 1.0 ml/min was employed on a Kromasil 100-5-C8 column at ambient temperature. The mobile phase consisted of Methanol: 0.025M phosphate buffer pH adjusted to 3 with o-phosphoric acid (60:40 v/v) and the detection wavelength was at 254nm[11].

MATERIALS AND METHODS:

Chemicals and reagents

Teneligliptin (Figure 1) of the highest quality has been purchased from Microlabs and sitagliptin (Internal Standard) (Figure 2) was donated by MSD. HPLC grade Methanol 99.8% was obtained from Merck chemicals; Hplc grade water and ethylacetate were of analytical reagent grade supplied by Merck Chemicals.

Instrumentation

WATERS HPLC, Model: Aglient 2695, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software.

Chromatographic conditions

Chromatographic separations were achieved by using Thermo C18 column (250×4.6 mm, 5 µm). The mobile phase consisted of methanol and 5 mm potassium hydrogen phosphate buffer in the ratio of 60:40 v/v. runtime – 7miuntes and flow rate of 1 mL/min and the injection volume was 20 µl. the column oven was kept at 25°C throughout the analysis. The eluent was detected by PDA detector.

Preparation of stock and standard solutions

Teneligliptin was weighed and dissolved in methanol and concentrations made to 1000µg/ml and futher samples are prepared of eight different standard concentrations of 7.20, 20.24, 47.060, 117.660, 213.940, 305.620, and 470.00ng/ml. The QC samples are prepared at four different levels are HQC, MQC, LQC and LLOQ. All the standard concentration and QC samples are prepared by using methanol and water (80:20) as diluents.

Extraction method

Extraction of teneligliptin from rabbit plasma sample was carried out by using liquid-liquid extraction. Each standard concentration sample and QC sample are individually taken and pipette out 0.25ml of sample and 0.1ml of internal standard into centrifuge tubes and add plasma & to the tube add 3ml of ethylacetate and centrifuge for 15minutes (2500rpm).then evaporate the contents and reconstitute the tube with mobile phase and filled in the HPLC vials and run. The representative chromatograms were shown in Figure 2.

Bioanalytical method validation

Preparation of calibration curve: the linearity of the method was evaluated by a calibration curve in the range of 7.20 – 490ng/ mL of teneligliptin. The calibration curve was achieved by plotting the peak area ratios of teneligliptin versus the concentration of teneligliptin by least-squares linear regression analysis. The calibration curve requires a correlation coefficient (R2) of >0.999. The acceptance criteria for each back-calculated standard concentration should be within 15% of the nominal concentration, except it should not exceed 20% for the LLOQ.(table-1)

Accuracy and precision:

Intra-day accuracy and precision were determined by duplicate analysis of six sets of samples spiked with four different concentrations of teneligliptin at low, medium, high quality control samples (18.000,211.680,376.000ng/mL) including LLOQ (7.200 ng/mL) within a day or on 6 consecutive days. For acceptance criteria for intra and inter-day precision, accuracy should be within 85–115% of the nominal concentration and coefficient of variation (%CV) values should be <15% over the calibration range, except at the LLOQ, where accuracy should be between 80 – 120% and %CV should not be more than 20%.(table-2)

Selectivity:

The selectivity of the assay methodology was established using a minimum of six independent sources of the same matrix. There were no interferences from the endogenous material at the retention time for both teneligliptin and internal standard (sitagliptin). The representative chromatogram is shown in Figure 3.

Recovery:

Recovery of teneligliptin was evaluated by comparing the mean peak areas of three extracted low, medium and high quality control samples to mean peak areas of three neat reference solutions (un-extracted). Recovery of internal standard was evaluated at a concentration of 210 ng/mL and corresponding mean peak area of the extracted samples compared to the mean peak areas of neat reference solutions. Recovery of the analyte need not be 100%, but the extent of recovery for analyte (teneligliptin) and internal standard (sitagliptin) should be consistent and reproducible.

Stability:

In order to find out the stability of teneligliptin in rabbit plasma, bench top stability, freeze thaw stability, and long term stability studies were carried out by using six replicates of the low and high plasma quality control samples. For the bench top stability, frozen plasma samples were kept at room temperature for 24 hr before sample preparation. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 hr and refrozen for 12–24 hr for each cycle. Freshly prepared solutions are taken and analysed after 24hours and Long term stability of teneligliptin in rabbit plasma was tested after 3 days. For the acceptance criteria of stability, the deviation compared to the freshly prepared standard should be within ± 15% of the nominal concentration.

Matrix effect:
The matrix effect was performed in 6 different lots of rabbit plasma by taking aqueous samples and spiked plasma samples at HQC and LQC level (6replicates of each).Matrix suppression or enhancement was calculated as follows: 100×mean peak area of post extracted sample/mean peak area of neat standard solution. The acceptance criteria for matrix effect implied that the %CV should be less than 15% of matrices tested and at least 80% of matrices should meet the above criteria. the results obtained were displayed in Table 4.

Table 1: Calibration table

Concentration(ng/ml)	Area Ratio	back.cal.con	% concentration
7.080	0.018	7.377	104.2
20.240	0.050	20.497	101.3
47.060	0.117	47.277	100.5
117.660	0.293	117.883	100.2
213.940	0.529	212.514	99.3
305.620	0.760	305.080	99.8
399.500	0.997	399.930	100.1
470.000	1.173	470.541	100.1

Table 2: precision and accuracy (n=6).

QC Sample	Teneligliptin			
	LLOQ	LQC	MQC	HQC
Theoretical concentration (ng/mL)	7.200	18.000	211.680	376.000
precision and accuracy				
Mean estimated concentration (ng/mL)± SD	7.107±0.04	17.210±0.05	199.478±0.07	354.871±0.40
Precision (CV, %)	0.17	0.2	0.150	0.644
Accuracy (%)	98.7	95.63	95.10	94.36

Table 3: Extraction Recovery (n=6).

teneligliptin	LQC %	MQC%	HQC%
Extraction recovery of six different aliquots of rabbit plasma	101.4745	101.521	100.00
	97.12576	102.8593	107.7903
	103.0403	102.7362	101.3169
	103.0584	101.952	107.0072
	101.3758	103.6097	103.8113
	89.14724	103.3616	114.2069
Mean of extraction recovery	99.20366	102.6733	105.6888
Sitagliptin (IS)		99.05	

Table 4: Matrix effect (n=6).

Teneligliptin	Extracted area	Unextracted area
HQC	1007715	935722
	1007623	934799
	1006654	993570
	1005683	939827
	1004378	967504
	1073892	940304
Average area	1017657.5	951954.3
M.E at HQC (%)	106.9019242	

Teneligliptin	Extracted area	Unextracted area
LQC	48241	47540
	47241	48639
	48567	47134
	49872	48392
	47895	47245
	42098	47223
Average area	47319.0	47695.5
M.E at LQC (%)	99.21061735	

Table 5: Stability details of teneligliptin in rabbit plasma sample (n=6)

Sample name	Mean concentration ±SD	%cv
Freeze thaw stability		
LQC	17.16±0.05	O.29
HQC	354±0.46	0.12
Short term stability		
LQC	17.14±0.11	0.64
HQC	356±0.12	0.31
Long term stability		
LQC	17.14±0.12	0.70
HQC	353±0.11	0.31

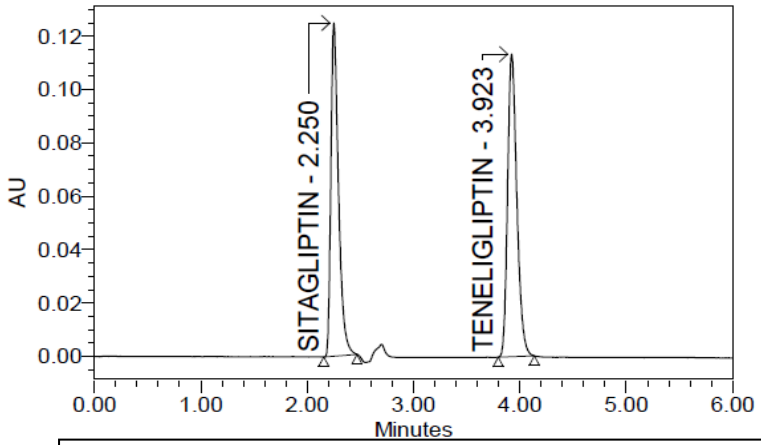

Fig 3 : Representative chromatogram of sitagliptin and Teneligliptin in rabbit plasma.

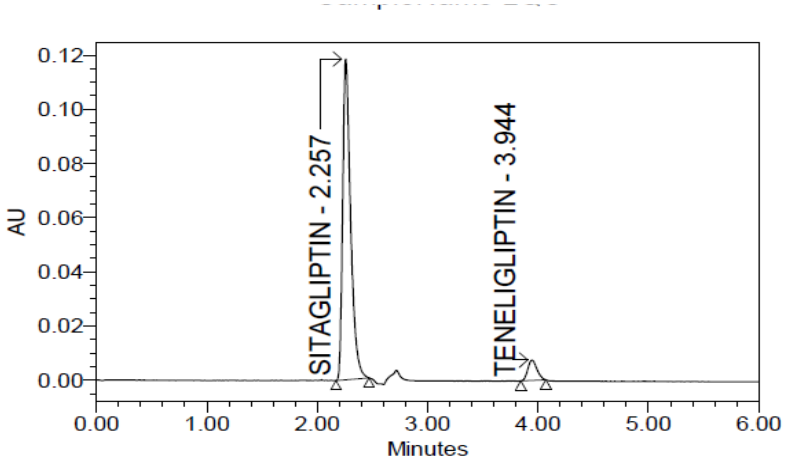

Fig 4: Representative chromatogram of Teneligliptin and sitagliptin at lower limit of quantification in rabbit plasma.

RESULTS:

Selectivity and optimization of chromatographic conditions

Plasma matrices were obtained from six different sources and assayed to evaluate the selectivity of the method and the detection of interference. Teneligliptin and sitagliptin (internal standard) were well separated from the co-extracted material under the described chromatographic conditions at retention times of 3.9and 2.2min respectively. No endogenous peak from plasma was found to interfere with the elution of either the drug or the internal standard. The LLOQ which could be measured with acceptable accuracy and precision for the analyte 7.20ng/mL was established (Figure 4). It indicates that the proposed method is highly selective and specific.

Calibration curve

Linear detector response for the peak-area ratios of the teneligliptin was observed in the concentration range between 7.20-470 ng/mL with a mean correlation coefficient of 0.999. the reason for choosing a wide calibration range for teneligliptin is to analyze samples of higher and lower dose concentration for route of administration like oral route where the Cmax concentration , best it for the calibration curve could be achieved with the linear equation Y = mx + c. where Y was the peak area ratio of the analyte and x was the concentration of the analyte. the results were given in the Table 1.

Accuracy and precision

The intra-day accuracy and precision ranged between 94.36-98-7%, and 0.17-0.64%, respectively. The accuracy and precision at the LLOQ and at LQC, MQC, HQC control samples of teneligliptin in plasma were within acceptable limits (N = 6). The results of the method validation studies presented in Table2.

Matrix effect

The sample solutions are prepared at two QC levels that is HQC and LQC one is of aqueous format and other is of spiked plasma. matrix effect is calculated that is average area observed at QC level in matrix sample by average area observed at QC level in aqueous sample * 100.the matrix effect studies presented in table 4.

Recovery

The recovery of teneligliptin in plasma was calculated at three QC levels. the response (extracted) compared to that of unextracted samples of the reference solution. The percentage recovery of teneligliptin and sitagliptin (internal standard) were found to be above 85%. Results are displayed in the Table 3.

Stability

Stock solutions of teneligliptin (1 mg/mL) and internal standard (1 mg/mL) were separately prepared and diluted at two levels LQC and HQC. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. All stability results were summarized in Table 5. the results of three freeze– thaw cycles and when the spiked samples were kept at room temperature indicated that teneligliptin was stable in rabbit plasma under these conditions. Analyte spiked QC samples were stable .

Application of the method

The method had been successfully employed for the quantitative estimation of teneligliptin in rabbit plasma samples. This Bioanalytical method has selectivity, sensitivity and reproducible.

DISCUSSION:

RP-HPLC optimization

The RP-HPLC method for the detection of Teneligliptin in rabbit plasma was investigated. Teneligliptin was dissolved in methanol to obtain the primary stock solution followed by subsequent dilution in methanol: water (80:20) and was directly introduced into the HPLC and various conditions are maintained and checked out for the response.

During the early stages of method development, attempts were made to optimize the response in a perfect manner. And. When standard working stock solutions of the analyte were directly injected into the HPLC system, it was observed that the response for the analyte was linear over the mentioned calibrated range. The linearity was found to be best it for the calibration range when the compound was dissolved in methanol: water (80:20) for preparing the working stock solution. the buffer pH at 4 helped in increasing the linear response by influencing the column properties like retention time, peak shape and peak response with relative variation of 15% for the analyte. It is accepted as a fact that a non linear range would not be adequate to the pharmacokinetic study. But by controlling the variable parameters like solubility, pH, column, temperature, ionization, less injection volume, obtaining a huge linear range with less possible relative error is possible. As per this method, flow rate and injection volume was set at 1 mL/min and 10 µL injection volume. Generally split ratio tends to change with changes in back pressure and expensive in terms of high solvent consumption. But, in this method, no split employed and that possibly decreases solvent consumption, improves reproducibility, increase column life and simultaneously declines chemical noise. It is also important for selection of column , column dimensions (250 mm x 4.6 mm), indicating fast elution with shorter run time. Hence, this method is validated for the linearity range from 7.20 – 470 ng/mL. During an early phase of method development attempts were made to choose the right column, since the columns like waters C18, Supelco C18, Zodiac C18 showed that the analyte eluted within the void volume with bad peak shape and poor area response, but as we tried to improve retention time through C18 columns, even though the area response and peak shape was improved considerably. The best C18 column, chosen based on separation, reproducibility, and response. Taking into account the non polarity nature of the teneligliptin, liquid liquid extraction had been proven to be an effective technique in the published methods. However, liquid liquid extraction was found to be LLE provides efficient removal of analyte with desired specificity/selectivity required for intended bioanalysis. Employing a methanol and ethylacetate for the extraction and constituted with mobile phase. Since, the reported bioanalytical methods for the estimation of teneligliptin by LC-MS/MS in human plasma. But, the present method is determination of Teneligliptin in rabbit biological samples by RP-HPLC.In this proposed method, no interfering peaks were observed at the elution times of Teneligliptin and sitagliptin (IS). The method also had sufficient selectivity, specificity, precision and accuracy over the concentration range of 7.20–490 ng/mL. This method had a quantification limit of 7.20 ng/mL, which was adequate enough to quantify the drug in rabbit plasma.

CONCLUSION:

Since merits of HPLC compare to other techniques are well recognized, a highly sensitive, specific, less cost compared to LCMS/MS and reproducible HPLC method is more valuable. In addition, along with method development, the method is also validated to quantify the concentration range of 7.20 – 470 ng/mL of Teneligliptin in rabbit plasma samples. The HPLC method presented here fulfils the criteria generally required for the Bioanalytical assays. This Bioanalytical method has sensitivity, selectivity and recovery above 85%.

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Abbreviations:

CV: Coefficient Variation; HPLC: High Performance Liquid Chromatography; IS: Internal Standard; LC: Liquid Chromatography; LC-MS/MS: Liquid Chromatography –Mass Spectrometry / Mass Spectrometry; LLOQ: Lower Limit of Quantitation; LQC: Low Quality Control; MQC: Medium Quality Control; HQC: High Quality Control; PK: Pharmacokinetics; QC: Quality Control; RP: Reverse Phase; TK: Toxicokinetic studies; BCS: Biopharmaceutics Classiication System; UV: Ultraviolet spectrophotometry; C_{max}: the maximum plasma concentration of the drug; psi: Pounds per square inch.