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FULL PAPER

Validated Stability Indicating and Assay Method Development of Linagliptin in Formulation by RP-HPLC Using Quality by Design

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Abstract:

Quality by Design (QbD) approach was used to facilitate stability indicating HPLC method development of linagliptin (LIN) in tablet dosage form. The method was developed using the PrimesilC18, 250 mm x 4.6 mm, 5µ column using the mobile phase consisting of 0.3% TEA: methanol. (60:40 v/v) pH 4.5 adjusted with o-phosphoric acid. Design of experiment tools was used for optimization of the chromatographic conditions. A three-level Box-Behnken design was employed and statistical analysis of the experimental data showed the significant influential factor of chromatographic conditions. The design space suggested that the current center point parameters could be further modified results with better acceptability for the response parameters. The performance of the optimized method was validated according to ICH guidelines. Linagliptin was exposed to different stress conditions (acid, base, neutral, oxidative, thermal and photolytic) and chromatograms recorded at 292 nm. The degradation of linagliptin followed zero order kinetics for acidic, oxidative and neutral hydrolysis whereas for basic hydrolysis first-order kinetics under experimental conditions. Peak purity plots were evaluated for the degraded sample. The results obtained suggest that the method can be adopted for its analysis and is stability indicating as well. The three-level design helps in understanding the interaction among factors rather than one time one variation as carried out in routine method development.

Keywords: assay; Box-Behnken design; linagliptin; stability indicating; validation

1. Introduction

Linagliptin [1] (LIN) is a Type 2 Anti diabetic drug. LIN (Figure 1), 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl)methyl]-3,7-dihydro-1H-purine-2, 6-dione, acts by blocking the action of DPP-4, an enzyme that destroys the hormone GLP-1, which helps the body to provide more insulin when it is needed. It is a yellowish white amorphous Powder which is highly soluble in water at pH 7.4 and readily soluble in methanol. LIN is a basic drug having pKa of 8.6.

In the modern analytical laboratory, there is always a need for significant stability-indicating methods of pharmaceutical formulation. Environmental factors, such as temperature, pH, buffer species, ionic strength, light, oxygen, moisture, additives and excipients, can play an important role in the stability of drug substances. Stress testing can help in identifying degradation products and provide important information about the intrinsic stability of drug substances [2]. With the advent of the International Conference on Harmonization (ICH) guidelines [ICH guideline Q1A (R2)2003], requirements for the establishment of stability-indicating methods have become more clearly mandated. The guidelines clearly require the conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc.

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and separation of drug from degradation products. The method is supposed to allow analysis of individual degradation products. Moreover, kinetic studies on the decomposition of drugs using stability testing techniques are essential for their quality control and to predict the expiry date of pharmaceutical products.

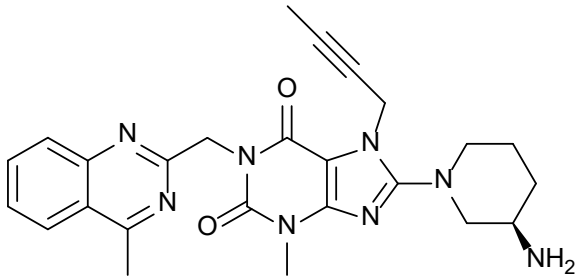

Figure 1. Structure of Linagliptin.

To identify the optimum conditions for analysis during method development response surface methodology approached can be used. The iterative procedure used during studies includes performing experiments in the region of the best-known solution, fitting a response model to the experimental data and then optimizing the estimated response model. The conventional practice of modification of a single factor at a time may result in poor optimization as other factors are maintained at constant levels that do not depict the combined effect of all the factors involved in a separation. This approach is also time consuming and requires a vast number of experiments to establish optimum levels. These limitations can be eliminated by collectively optimizing all parameters using response surface methodology. Compared with the traditional optimization method, response surface methodology has distinct advantages such as the use of minimum number of experiments, shorter time of operation and feasibility of generating data that may be analyzed statistically to provide valuable information on the interactions among experimental parameters. These designs require three levels for each factor [3]. A stability-indicating assay accurately measures the active ingredients without interferences from degradation products. There are various methods available for the estimation of linagliptin in the tablet formulation. The methods are reported for its estimation like HPTLC [4] and HPLC [5-10] for its determination. The reported methods lack stability indicating ability, higher run time, lesser theoretical plates. One such method for estimation of linagliptin based on design of experiments approach was reported [5], but no efforts were made to analyze the critical method attributes as well as to establish that method is stability indicating assay method. Hence the objective of present work was to develop a stability indicating method for determination of linagliptin using design of experiments approach and degradation kinetics study of exposed samples to various stress conditions.

2. Results and Discussion

2.1. Method development and optimization

Design of experiments optimized method has been proved to be a promising tool for quantification of Linagliptin and its utilization for stability indicating assay method. Linagliptin was well separated with good peak shape. No interfering peaks were observed in blank. Based on Linagliptin solubility, methanol was selected as organic phase, Reversed-phase HPLC (C18) columns was used. Mobile phase finalized was 0.3% TEA: methanol (60:40) pH 4.5 adjusted with o-phosphoric acid. Optimum detection wavelength selected was 292 nm

For risk assessment of the process six independent variables (i.e. Mobile phase aq, Mobile Phase org, Flow Rate, Wavelength and pH) and five dummy factors were selected as a possible cause of change in the method development. The Pareto charts were prepared to examine the relationship in the independent parameters which is given in Figure 2a-c respectively. From the Pareto charts it was observed that the Plackett-Burman design shows that the factors have significant effect on retention time.

After satisfactory method optimization it was subjected to method validation as per ICH guideline. The method was validated to demonstrate that it is suitable for its intended purpose. The result of system suitability parameter was found to be complying with acceptance criteria: relative standard deviation standard area of replicate injection is not more than 2.0%.

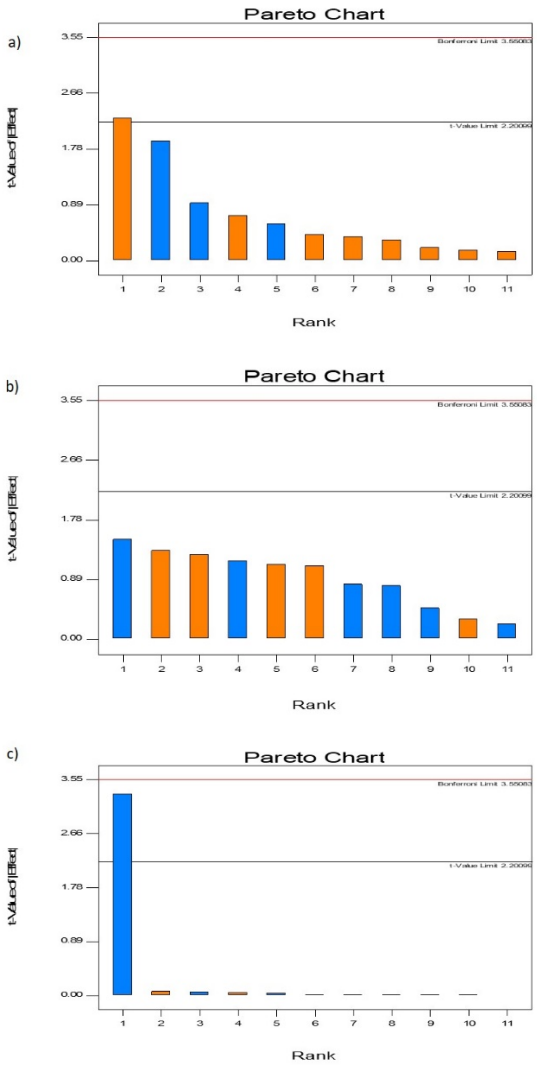

Figure 2. Pareto charts of dependent variables. a) Theoretical Plates; b) Tailing factor; c) Retention time.

2.2. Method validation

The % assay of Sample was found to be 99.03% which states that the proposed method is suitable for analysis of the commercial formulation. The recovery of drug was observed very close to 100% at the selected levels representing the accuracy of the method and also shows that excipients have no interference in the estimation are within the acceptable limits. In Intraday and Interday study, results obtained by proposed method shows that drug is stable in solution form. The relative standard deviation was found to be within limits. The results of estimation of Linagliptin by different analysts were very much reproducible, indicating the ruggedness of the method in the hands of different analysts (Table 1).

The Percent relative standard deviation for system and method precision was found to be 1.12 and 0.71 respectively. Replicate estimation of Linagliptin Standard and Sample analyzed by proposed method yielded quite concurrent results indicating that method is precise. The relative standard deviation of system and method precision was less than 5%. So, the method was found to be precise. The plot of percent label claim Vs area showed a linear relationship with correlation coefficient very close to 1 (i.e. 0.9975). The Range is indicative of accurate estimation of drug in tablet over range of at least 80-120 % of label claim. The detection limit and quantitation limit obtained were found to be 0.5370µg and 1.6274µg, respectively (Table 1).

Table 1. Summary of Method Validation.

System suitability* (RSD)	Linearity (r^2)	Assay# (%label claim)	Precision# (RSD)		%Recovery (accuracy)	Rugged-ness* (RSD)	Intermediate precision (RSD)		Range (r^2)	DL (µg)	QL (µg)
			System	Method			Intra day	Inter day			
0.85	0.9983	99.034	1.12	0.71	100.71	1.31	0.75	0.82	0.9975	0.5370	1.6274

*mean of 6 observations, # mean of 5 observations

2.3 Stability-indicating property

For acid hydrolysis, after 1h the drug was found to be slowly degrading upto 5h. The drug was degraded to around 28% in exposed standard and around 33.85% degradation in sample. In the case of alkaline hydrolysis, the drug was found to be degraded around 10% in case of sample and lesser degradation was observed in standard to around 6% than sample. The standard drug was found to be degraded around 2% whereas sample was found to be degraded to around 10% when exposed to oxidative hydrolytic conditions. The difference in the degradation between standard/sample is may be due to drug excipient induced degradation. From the observation for neutral hydrolysis it was observed that the standard drug was degraded to

around 1% and sample was found to be degraded to 10%. No additional peaks were generated after 5 h.

Linagliptin under acidic condition showed more degradation than that for basic, neutral and oxidative study. The drug was found to be more liable to degradation in the acidic medium. Forced degradation chromatograms are shown in Figure 3a-3d for acid, alkali, oxidizing reagent and distill water respectively.

The humidity study was carried out on sample for period of 30 days. From the results, it was revealed that the sample was found to be un-degraded to around 94.80% after 30 days. Similarly, the drug was found be degraded around 19% in both dry and wet heat degradation (60°C) after 5 h. the effect could be due higher temperature and incase of humidity study the formulation with its packing strip was exposed. From above observations it was seen that the drug is more degraded in case of dry and wet heat degradation than humidity study (75%RH/40°C). No additional peak(s) were found after exposing Linagliptin to degradation conditions. Figure 3e-3f for dry & wet degradation studies and Figure 3g for humidity exposed sample respectively. The data of degradation studies are shown in Table 2.

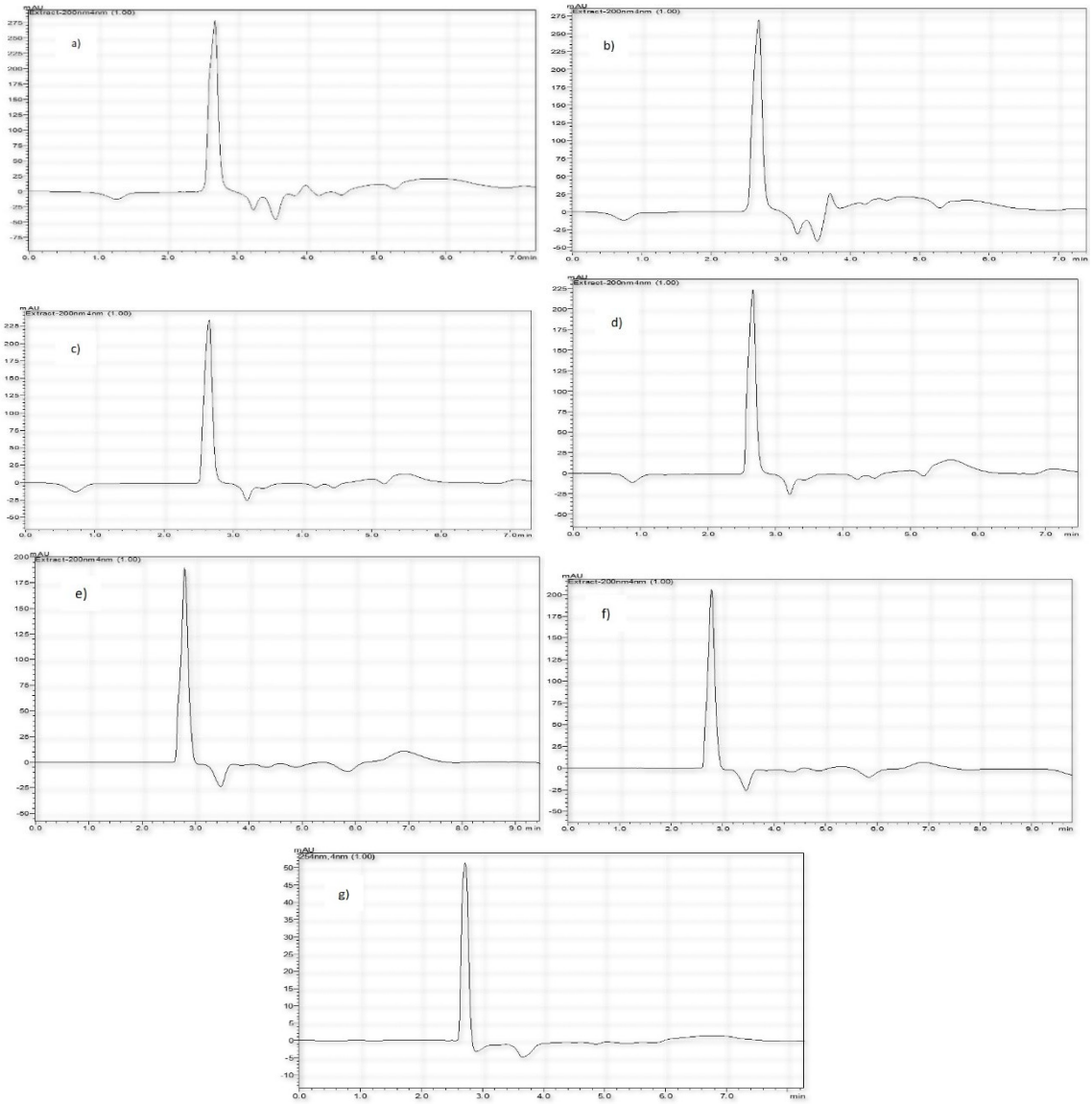

Figure 3. Chromatograms of linagliptin under a) acid hydrolysis for exposed sample; b) alkali hydrolysis for exposed sample; c) oxidative hydrolysis for exposed sample; d) neutral hydrolysis for exposed sample; e) sample for dry degradation study; f) sample for wet degradation study; and g) humidity study for sample after 30 days.

Table 2. Degradation of Linagliptin under various stress conditions.

% Un degraded													
Acid Hydrolysis		Basic hydrolysis		Oxidative hydrolysis		Neutral hydrolysis		Humidity (40ºC±2ºC / 75%RH)				Wet heat	Dry heat
Std (5h)	sample (5h)	Std (5h)	Sample (5h)	Std (5h)	sample (5h)	Std (5h)	sample (5h)	Std 15days	sample 15days	Std 30days	Sample 30days	(Std 5h)	(Std 5h)
71.50	66.15	94.88	90.27	98.91	90.33	99.49	90.33	106	99.89	92.47	94.80	81.81	81.48

2.4 Peak purity study

The integrity of drug un-degraded was studied using peak purity data. Peak purity data for all the hydrolytic conditions are shown Table 3. The peak purity index and threshold indicates that the peak obtained is solely due to the intact Linagliptin and no impurity /degradation peak was merged with it. The result of specificity study ascertained the separation of Linagliptin peak and the spectral purity of all exposed samples were found spectrally pure.

2.5 Kinetics of Degradation Studies (Solution State)

The treatment of linagliptin under specified stress condition resulted in gradual decomposition of Linagliptin. The kinetics of degradation for all hydrolytic conditions were studied (Table 4) and observed that the drug followed zero order kinetics for acidic, oxidative and neutral hydrolysis while the alkaline hydrolysis followed first order kinetics (Figure 4a-4b).

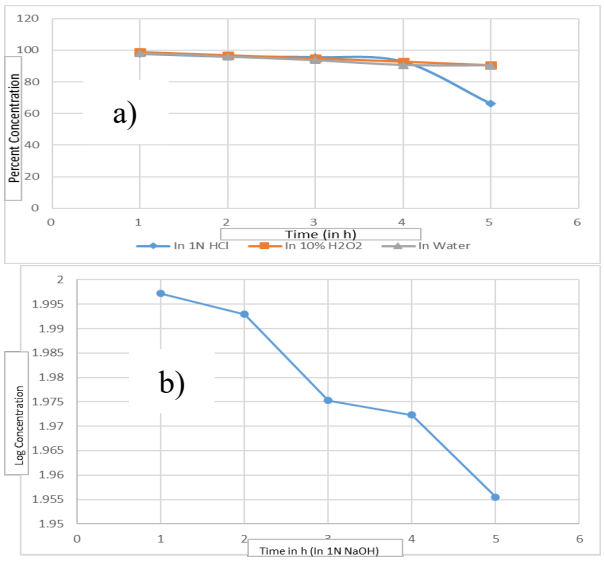

Figure 4. Graphical plot for a) First Order Kinetic Study and b) Zero Order Kinetic Study.

Table 3. Peak Purity Analysis.

Conditions	Peak purity index	Peak threshold
Assay sample	0.999978	0.999362
Acid hydrolysis	0.999885	0.999653
Basic hydrolysis	0.999931	0.999712
Oxidative hydrolysis	1.000000	0.999574
Neutral hydrolysis	0.999998	0.999590

Table 4. Kinetics of Solution State Degradation Study.

Degradation Medium	Conditions (At 50ºC for 5h)	Value of "R^2"	Order of Reaction
Acidic	1N HCL	0.6276	Zero
Alkaline	1N NaOH	0.9665	First
Oxidative	10% H_2O_2	0.9993	Zero
Neutral	Water	0.8881	Zero

3. Material and Methods

3.1. Chemicals, reagents and solutions

Pharmaceutical grade Linagliptin was gifted by Glenmark Pharmaceuticals, Mumbai. Methanol (HPLC grade) was purchased from Merck Chemical Company (India). Triethylamine,o-phosphoric acid, hydrochloric acid, sodium hydroxide, and 10% hydrogen peroxide used were of GR grade. 0.3% TEA water was prepared by dissolving 0.3 mL and upto 100mL with double distilled water.

3.2 Determination of Wavelength of Absorption Maximum

A working stock solution of linagliptin was prepared in methanol and scanned in the over the range 400-200nm against solvent blank. The absorption maximum in the ultraviolet range was found to be 292 nm. Figure 5 shows the absorption spectrum of Linagliptin.

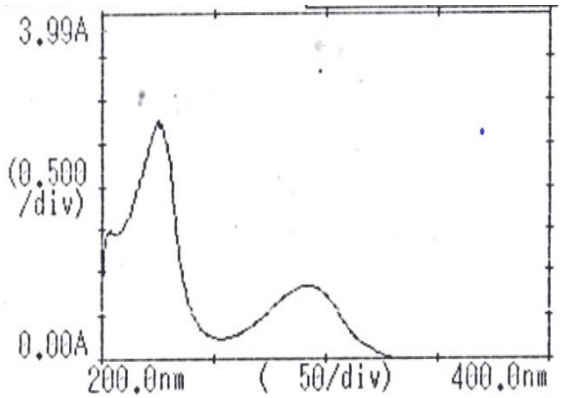

Figure 5. UV spectra of Linagliptin standard.

3.3. HPLC instrumentation and chromatographic conditions

The HPLC system Shimadzu, SPD-10A VP was used, a manual injector with 20 µL capacity per injection. Column used was Primesil C18, 250 mm x 4.6 mm, 5µ. Chromatographic separation of linagliptin was achieved at ambient temperature using the mobile phase consisted of 0.3% TEA: Methanol (60:40 v/v) pH 4.5 adjusted with o-phosphoric acid at a flow rate of 1.0 mL/min. Before use, the mobile phase was filtered through a 0.45µ membrane filter and sonicated for 15 min. Injection volume was 20µL, and the optimum wavelength selected for quantification was 292 nm using PDA detector**.**

3.4. Construction of the calibration curve

Standard stock solution of linagliptin was prepared in methanol at a concentration of 1mg/mL and further diluted with the diluent to furnish the working standard stock solution of 100µg/mL. The working standard stock solution was diluted with the diluent to prepare calibration samples in the concentration range of 1–10µg/mL. Peak areas were plotted against the corresponding concentration to obtain the calibration curve.

3.5. Analytical Quality by Design

Quality by design incorporates planning, developing formulations and manufacturing procedures which ensures predefined product specifications. In 2002, the FDA announced a new initiative (cGMP or the 21st Century: A Risk based Approach). The use of Quality by Design concepts results in a well-understood product and process that consistently delivers its intended performance. The knowledge acquired during development may support the formation of a design space and determines suitable process controls. These same quality by design principles have been applied to the development of analytical methods, and are termed “Analytical Quality by Design” (AQbD). Analogous to process quality by design, the result of Analytical quality by design is a well understood, fit for purpose, and robust method that consistently delivers the intended performance throughout its lifecycle. The broad knowledge obtained from this process is used to establish a method operable design region (MODR), a multidimensional space based on the method factors and settings that provide suitable method performance. Analytical quality by design helps in development of a robust and cost effective analytical method and facilitate the regulatory flexibility in analytical method i.e. the choice to change method parameters within a method’s design space, referred to as the method operable design region (MODR).

3.6. Method Design

3.6.1. Technique Selection

Linagliptin having chromophore group, so HPLC connected with PDA detector as analytical technique has been selected.

3.6.2. Analytical Target Profile

Analytical Target Profile is way for method development, or it is simply a tool for method development and has been mentioned in the ICH Q8R (2) guidelines. It defines the method requirements which are expected to be measured that direct the method development process i.e.it is combination of all performance criteria required for the proposed analytical application.

An example analytical target profile is provided below for the stability indicating assay of a tablet formulation.

3.6.3. Assay

The procedure must be able to accurately quantify the active pharmaceutical ingredient in tablet over the range 80% - 120% of the nominal

concentration with specificity, linearity, accuracy, and precision such that measurements fall within ±3.0% of, the true value with a 95% probability.

3.6.4. Selection of Draft Method Conditions

Linagliptin is a base (pKa= 8.6) with the partition coefficient of the free base of log P = 1.7. Based on literature, initial experiments were carried out using weakly basic pH buffer, BDS column, methanol (Low UV cutoff) with an isocratic flow. To avoid linagliptin pKa, Initial experiments were started with pH 7.4 phosphate buffer and methanol as Mobile phase B, by using ACE and primesil columns, Mobile phase buffer changed from phosphate buffer to TEA solution pH adjusted to 4.5. Isocratic program was optimized and performed method robustness parameters was carried out by Plackett-Burman design to check the method impacting factors. The Plackett-Burman design, a statistical method was successfully employed for the risk assessment of method development process for Linagliptin. According to this design, total 12 runs were taken. For investigating the effect, each independent variable was studied at two levels namely, “High” and “Low” which indicated the upper limit and lower limit of the range covered by each variable.

3.7. Method Evaluation

3.7.1. Risk Assessment of Method Parameter

Risk assessment is a critical step in the analytical quality by design method development. Risk Assessment of the Method parameters was performed to evaluate the impact on Critical Method Attributes. The relative risk of Critical Method Parameters on Critical methods Attributes was ranked as High, Medium and Low. The high risk parameters require necessarily further investigation whereas the low risk attributes required no further investigation. The medium risk is considered acceptable based on current knowledge. Further investigation for medium risk may be needed in order to reduce the risk. Based upon the initial method development trails, the risk assessment of method attributes is given in Table 5. Linagliptin is highly soluble in methanol and water pH 7.4. Methanol as diluent is suitable to get sufficient recovery in tablet dosage form.

Table 5. Risk assessment of method attributes.

Sr.No	MP org	Flow rate	pH	MPaq	Λ	Conc	Th.Pl	Tailing factor	RT	Area
1	44	1.2	4.7	54	287	0.1	7070	0.93	2.15	117590
2	44	1.2	4.7	66	297	0.3	9501	0.9	2.14	190573
3	44	1.2	4.3	54	287	0.3	7966	1.06	2.1	178585
4	44	0.8	4.7	66	297	0.1	7933	0.81	3.34	216028
5	36	0.8	4.3	54	287	0.1	8930	0.89	3.33	350963
6	44	0.8	4.7	66	287	0.3	10923	0.88	3.34	238628
7	44	0.8	4.3	54	297	0.1	5525	0.93	3.3	285760
8	36	1.2	4.7	54	297	0.3	9331	1.01	2.16	128997
9	36	1.2	4.3	66	297	0.1	16042	1.19	2.18	323539
10	36	1.2	4.7	66	287	0.1	15392	1.1	2.18	137512
11	36	0.8	4.3	66	287	0.3	18553	1.18	3.33	315997
12	36	0.8	4.7	54	297	0.3	7536	0.84	3.32	189155

3.7.2. Summarized Quality Attributes (QA)

Critical quality attribute can differ from one analytical technique to another. Critical quality attribute for HPLC (UV or RID) are buffers used in mobile phase, pH of mobile phase, diluent, column selection, organic modifier and elution method. Critical method attributes is important for method performance that should be measured to assess whether the method is capable of producing fit-for-purpose data. However, the significantly varied response/s be fit as critical method attributes. Assuring to sustain the methods predefined analytical target profiles, the responses for the peak of Linagliptin are:

i. Resolution from closely eluting peak
ii. Theoretical plates (USP plate count)
iii. Tailing factor (USP tailing)
iv. Area

3.8. Multifactor Experimental Design

3.8.1 Chromatography Selection

Based on method design, following method conditions are selected. Mobile phase consists of 0.3% TEA solution pH 4.5 and consists of methanol. Flow rate 1.0 mL/min with primesil ODS 250 mm × 4.6 mm and 5 µm column. The HPLC isocratic program was 60:40 v/v. Detection was carried out at 292nm using PDA detector.

3.8.2. Factor and Response Selection

Critical Method parameters were selected and Method attributes (responses) were identified from the Risk Assessment. Low and high values were set, factor limits for experimentation along with acceptance limits for attributes. The factors and responses are presented in Table 6a and Table 6b along with experimental ranges investigated.

Table 6a. Acceptance criteria for responses.

Code	Response	Acceptance Criteria
A	Theoretical Plate	Not less than 5000
B	Tailing factor	Not more than 1.5
C	Retention Time	---
D	Area	---

3.8.3. Design Selection and Design Layout

A statistical design was selected and generated for the factors that allowed the determination of important main effects and four-factor interactions using an appropriate statistical software package Design-Expert. The number of factors being 4, a Box Behnken design was proposed. A Box Behnken design apart from being a simple design would also facilitate in obtaining maximum information regarding factors, and Factor interaction effect on the responses in as less as 24 experimental runs. The entire experimental run sequence designed to provide a means to test for a systematic time effect that may have occurred during the experiment.

3.8.4. Design of Experiment HPLC Analysis

Instrument methods were generated in Lab solutions software to support factor variations for each of the design points. Samples were evaluated under each design point and response results gathered and summarized for statistical analysis.

Table 6b. Factors and levels selected in Box-Behnken design.

Factors	Level +1	Level 0	Level -1
Proportion of Organic solvent	42	40	38
Flow rate (mL/min)	1.2	1	0.8
pH	4.7	4.5	4.3
Concentration of Aq. phase	0.4	0.3	0.2

3.8.5. Design of Experiments Evaluation

The design studied was 3-level Box Behnken design with 4 factors. This implies 24 runs (not counting the 5 center points). The complete augmented design with responses is presented in Table 7. Each of the responses was analyzed using Design Expert 8 software.

3.8.6. Design of Experiments Statistical Response Analysis

The design model evaluation was studied using ANOVA, Lack of Fit and Residual Plots on dependent factors.

3.8.7a. Theoretical plates

The 2D counter plots are presented in Figure 6a and are very useful in studying the interaction effects of factors on theoretical plates. The normal plot of residuals for theoretical plates is shown in Figure 7a. Close inspection of the residuals reveals that they generally fall on a straight line which indicates that the errors are normally distributed, thus supporting the fact that the model fits the data adequately.

Figure 8a shows the Surface Response plot for Theoretical plates which is linear in nature and states that, “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable and the ratio obtained after performing the experiment was 6.928 which indicates the adequate signal. So, this model can be used to navigate the design space.

Table 7. Design of Experiment and observed responses.

Std. Runs	Sr. No.	Conc of org solvent	Flow Rate (mL/min)	pH	Aq phase %	Theoretical Plates	Tailing factor	R.T.	Area
20	1	42	1	4.7	0.3	11701.1	2.55	2.823	1733200
9	2	38	1	4.5	0.2	24695.7	1.3	2.821	742597
10	3	42	1	4.5	0.2	14687	1.02	2.82	1122792
28	4	40	1	4.5	0.3	14731.4	1.15	2.837	630394
13	5	40	0.8	4.3	0.3	21652.9	1.25	2.826	673150
26	6	40	1	4.5	0.3	14731.4	1.15	2.837	630394
6	7	40	1	4.7	0.2	16441.4	1.09	2.768	1018956
29	8	40	1	4.5	0.3	14731.4	1.15	2.837	630394
11	9	38	1	4.5	0.4	22783.6	1.78	2.786	499043
24	10	40	1	4.5	0.4	10470.7	1.29	2.46	505953
21	11	40	0.8	4.5	0.2	24463.3	1.35	3.495	1181593
2	12	42	0.8	4.5	0.3	14417.5	1.08	3.517	907551
8	13	40	1	4.7	0.4	17245.5	1.43	2.758	677446
25	14	40	1	4.5	0.3	14731.4	1.15	2.837	630394
5	15	40	1	4.3	0.2	14545.8	1.14	2.745	1203472
17	16	38	1	4.3	0.3	19612.2	1.201	2.924	985440
1	17	38	0.8	4.5	0.3	11813.5	1.14	3.484	318963
16	18	40	1.2	4.7	0.3	14379.1	1.03	2.826	177787
23	19	40	0.8	4.5	0.4	11187.5	0	3.451	909579
27	20	40	1	4.5	0.3	14731.4	1.15	2.837	630394
3	21	38	1.2	4.5	0.3	14143.5	1.49	2.297	166282
22	22	40	1.2	4.5	0.2	13979.7	1.01	2.278	846167
12	23	42	1	4.5	0.4	14660.8	0.92	2.8	382233
19	24	38	1	4.7	0.3	14189.9	1.12	2.801	213997
14	25	40	1	4.3	0.3	10390.4	1.11	2.289	241683
18	26	42	1	4.3	0.3	7319.35	0	2.798	630063
7	27	40	1	4.3	0.4	20057.3	0.96	2.799	334716
4	28	42	1.2	4.5	0.3	6391.68	0	2.301	382659
15	29	40	0.8	4.7	0.3	14465.2	0.947	3.488	315228

3.8.7b. Tailing factor

The 2D counter plots are presented in Figure 6b and are very useful in studying the interaction effects of factors on asymmetry. The normal plot of residuals for tailing factor is shown in Figure 7b.

Figure 8b shows the Surface Response plot for tailing factor which is 2FI and states that, “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable and the ratio obtained after performing the experiment was 7.383 which indicates the adequate signal.

3.8.7c. Retention Time

The 2D counter plot is presented in Figure 6c and is very useful in studying the interaction effects of factors on retention time. The normal plot of residuals for retention time is shown in Figure 7c.

Figure 8c shows the Surface Response plot for Retention time which is linear and states that, “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable and the ratio obtained after performing the experiment was 17.953 which indicates the adequate signal.

3.8.7d. Area

The 2D counter plot is presented in Figure 6d and is very useful in studying the interaction effects of factors on area. The normal plot of residuals for area is shown in Figure 7d.

Each of the responses was analyzed using Design Expert 8 software. The p values from the corresponding ANOVA (adjusted for curvature) for each response are presented in (Table 8a and 8b). The level of significance was determined based on the p values. A response term with p value less than 0.05 was considered significant and for a response term with p value more than 0.05 was considered not significant. Factor terms and 2 factor interaction terms with p value less than 0.05 are significant.

Lack of fit was also observed to be not significant for all the response. This further

assures the suitability of model for optimization use.

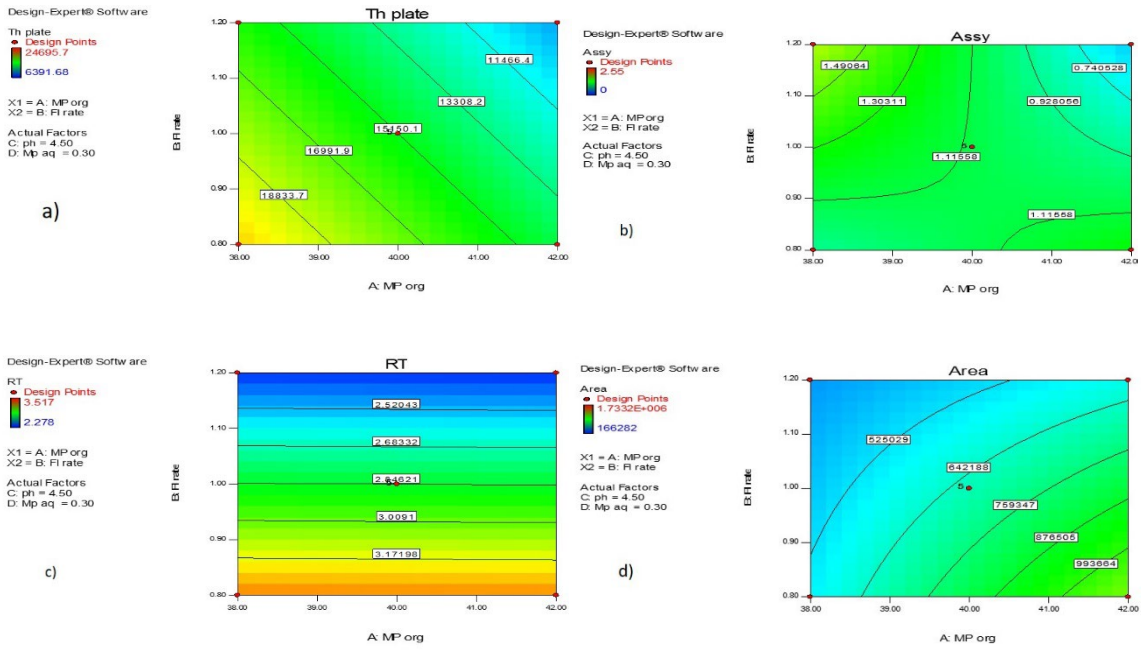

Figure 6. Counter Plot for a) Response for Theoretical plates (Y1); b) Response for Tailing Factor (Y2); c) Response for Retention Time (Y3); and d) Response for Area (Y4).

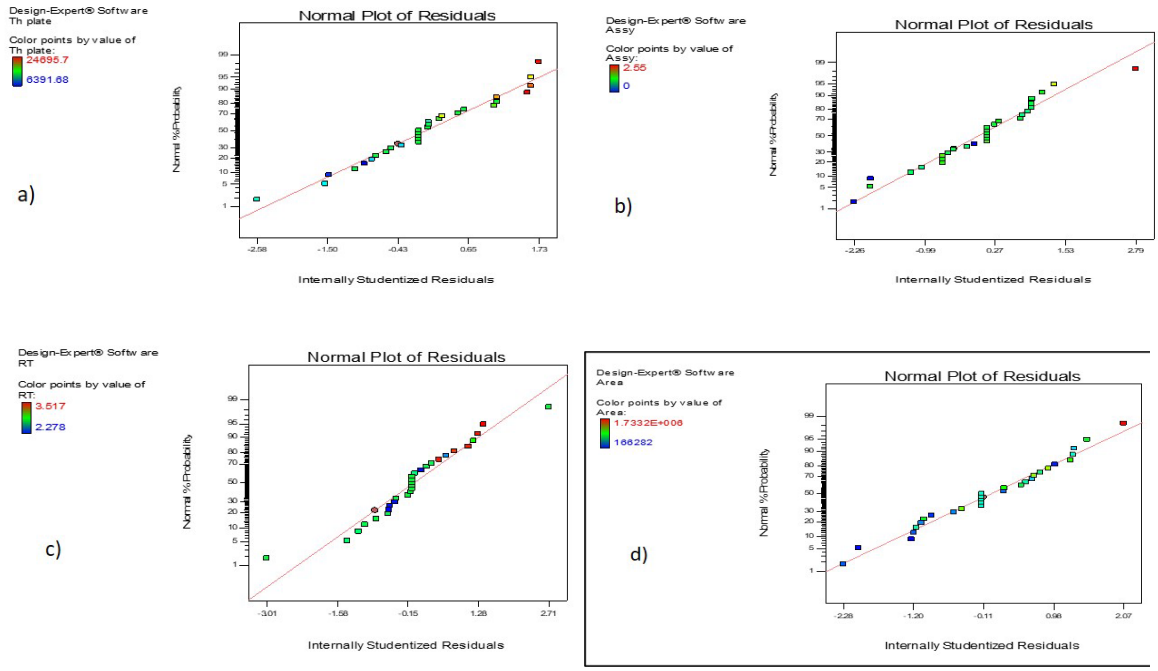

Figure 7. Normal Plot of Residuals for Responses a) Theoretical plates; b) Tailing factor; c) Retention Time; and d) Area.

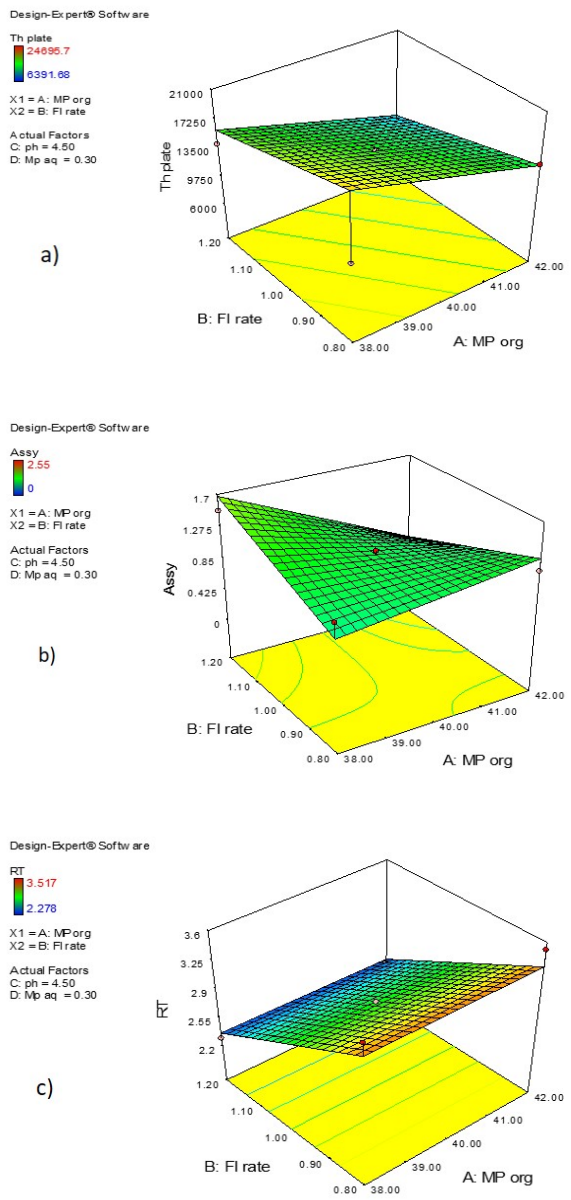

Figure 8. Surface Response Curve for a) Y1 Response (Theoretical plates); b) Y2 Response (Tailing factor); and c) Y3 Response (Retention Time).

3.9. Design of Experiment Optimization

The desirability function was utilized in the proposed method; it reflects the desirable ranges for each response. The desirable ranges are from zero to one (least to most desirable, respectively).From the above observations it was evident, that among the four factors studied; most significant factors were the Mobile phase organic portion and flow rate of mobile phase , whereas pH of mobile phase and mobile phase (aqueous phase concentration) appeared to be of lesser significance. Hence during optimization study, pH was fixed at 4.5, the acceptance criteria for all the response were set in the software. It shows increase in mobile phase organic portion and the flow rate of mobile phase, increases desirability, which indicates improvement in the acceptability of the responses measured.

Table 8a. p-value for responses Y1 and Y3.

Sr. No.	Source	p-Value Prob>F	
		Y1	Y3
1	Model Significant	0.0237	< 0.0001
2	A-MP	0.0086	0.9203
3	B-Flow Rate	0.0443	< 0.0001
4	C-Ph	0.7018	0.0537
5	D-MP aq	0.3604	0.8140

Table 8b. p-value for responses Y2 and Y4.

Sr. No.	Source	p-Value Prob>F	
		Y2	Y4
1	Model Significant	0.0260	0.0100
2	A- MP org	0.0787	0.0257
3	B- Flow Rate	0.9031	0.0443
4	C- pH	0.0738	0.9417
5	D-MP aq	0.6928	0.0068
6	AB	0.0770	0.4916
7	AC	0.0028	0.0024
8	AD	0.4566	0.3610
9	BC	0.7732	0.5860
10	BD	0.0465	0.8991
11	CD	0.5038	0.3332

3.10. Method Control

3.10.1. Determination of Method Operable Design Region

Method Operable Design Region is defined by the actual linear equations for the critical responses set to their respective acceptance limit, with respect to the method factor input values. A chromatogram of the final set point conditions is shown in Figure 9a and 9b. From the data given in Table 9, excellent agreement between the response models was obtained. Predictions and the actual results obtained using the final method conditions, indicated that the model is capable of navigating and accurately predicting the multivariate design space.

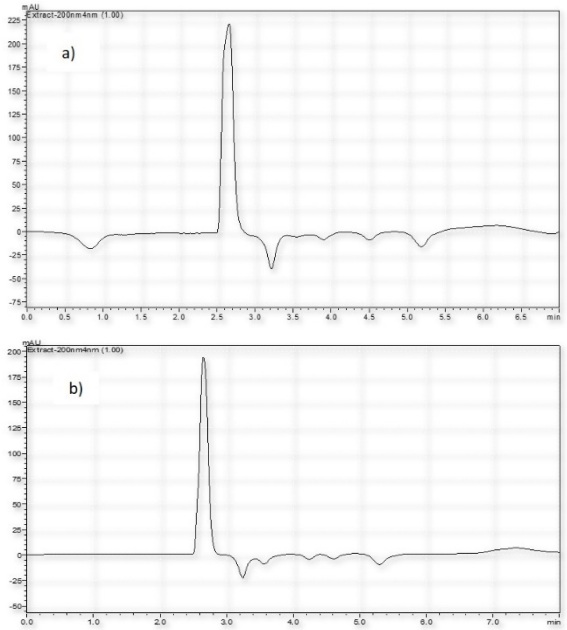

Figure 9. Chromatogram recorded of a) Standard Linagliptin and b) Sample Linagliptin.

3.10.2. Method Validation

To confirm the suitability of the method for its intended purpose, the method was validated in accordance with the ICH guidelines for system suitability, linearity, limits of detection and quantification, accuracy, intra- day and inter-day precision, specificity and robustness.

3.10.3. System suitability

System-suitability test was an integral part of method development and has been used to ensure adequate performance of the chromatographic system. Retention time (Rt), Number of theoretical plates (N) and Tailing factor (T), were evaluated for six replicate injections of the drug.

Table 9. Response model verification.

Sr.No	Responses	Values Predicted	Responses	Actual values	Model
1	Theoretical plates	15353	Theoretical plates	14986	Linear
2	Asymmetry	1.03804	Asymmetry	1.15	2FI
3	RT	2.81573	RT	2.7	Linear
4	Area	680733	Area	666293.793	2FI

3.10.4. Linearity

Linearity of the proposed method was evaluated according to the ICH guidelines. Linagliptin showed linearity in the concentration range of 1–10 µg/mL, (r^2 =0.9983). The regression equation obtained was Y=46099x+18830, where Y is peak area and X is concentration of linagliptin. This equation was used to determine the amount of linagliptin present in the stability samples.

3.10.5. Accuracy

Accuracy of the proposed method was determined by performing the recovery experiments. Known amount of the standard at 50%, 100% and 150% levels were fortified to the pre-analysed sample.

The amount of standard drug added recovered was estimated.

3.10.6. Ruggedness

The studies were carried out for two different parameters i.e. Days (Intraday and Interday) and Analyst to analyst.

3.10.7. System Precision and Method Precision

The six replicate injections of linagliptin standard and sample solution were chromatographed, the results for system precision as well as method precision in terms of percent relative standard deviation was calculated.

3.10.8. Range

For the drug in the formulation 80-120% of label claim was weighed and appropriately diluted. The chromatograms were recorded for each sample and area noted. A plot of Percent label claims v Area under curve was constructed.

3.10.9. Limits of detection and quantification

The limit of detection (LOD) was defined as the lowest concentration of linagliptin and limit of quantification (LOQ) was calculated from the standard error of regression line and slope of the calibration curve.

3.10.10. Assay

An accurately weighed quantity of tablet powder equivalent to 1 mg linagliptin was transferred in 10 mL of volumetric flask, 5 mL of diluent was added, sonicated for 30 min. and volume made up to the mark with diluent (S1). This solution was filtered through nylon filter. From the stock standard solution (S1), accurately pipetted out 1mL was transferred to 10 mL volumetric flask and dilute upto the mark with diluent (S2). From the above solution 3 mL was transferred to 10 mL of volumetric flask and volume made up to the mark with diluent (3µg/mL) (S3). Each sample solution was injected into the system under optimised conditions and chromatograms recorded.

3.10.11. Forced degradation of LIN

3.10.11.1 Hydrolytic conditions: acid, alkali and water induced degradation

An accurately weighed tablet powder equivalent to 1 mg linagliptin was transferred to a series of 10 mL volumetric flasks. To each flask 1 mL of reagent (acid, alkali, oxidizing reagent and distilled water) was added. The flasks were placed in oven at 50 ºC. The standard drug was also weighed and subjected to similar conditions, was withdrawn at end of 5h while the samples were withdrawn at an interval of 1h, 2h, 3h, 4 h and 5h and the volume was made up to the mark with the diluent.

3.10.11.2 Thermal conditions: Dry Heat and Moist Heat Induced Degradation

An accurately weighed tablet powder equivalent to linagliptin 10 mg was transferred to a series of 10 mL volumetric flasks. The flasks were placed in oven at 50 ºC and the samples were withdrawn at 1h, 2 h, 3h, 4h, 5h and volume made up with diluent. Each final diluted solution was injected into the system.

3.10.11.3. Humidity studies

Tablet powder of linagliptin was spread on petri dish and placed in humidity chamber at 40ºC, 75% Relative humidity for a period of 30 days. After this period, an accurately weighed amount of sample linagliptin was transferred to 10 mL volumetric flask and diluted upto the mark with diluent. The final diluted solution was injected into the system.

3.10.12. Method Control Strategy

Establishing a control strategy is of utmost importance while ensuring that the method is performing as intended on a routine basis as goals described in analytical target profile. Basically, it's a planned set of controls aimed at minimizing the variability in the process.

3.10.13. Life Cycle Management

Analytical quality by design for a particular analytical method the key steps that ensure fitness of the method for its intended use includes the method validation, verification and transfer. Combining all together is termed as 'lifecycle management of analytical procedure', which commence with establishment of analytical target profile and continues till the methods are in use.

4. Conclusions

A novel simple, fast and robust RP-HPLC analytical method of linagliptin was successfully developed by employing quality by design approach (Box Behnken Design) and further validated according to ICH guidelines. After performing the Robustness study using Plackett-Burman design it is observed that, there is no significant effect on the dependent parameters so, the method can be used in the future point of view. The Quality by Design principle was applied to assay method development of Linagliptin by the use of design of experiments approach where, Box-Behnken Design was used to analyse the various Analytical Target Profiles. The automated quality by design method development approach provided a better performing and more robust method in less time as compared to the manual method development. Moreover, this RP-HPLC method is acceptable for concurrently determining

the best possible condition and the robustness that along with its effectiveness strongly indicates a powerful strategy that can be used to estimate the drug in different pharmaceutical dosage forms. The proposed method was applied to assay and to degradation (stability analysis) of Linagliptin. It was found that linagliptin rapidly degraded under acidic conditions as compared to basic, oxidative and neutral condition. The analyte followed zero order degradation kinetics for acidic, hydrolytic and neutral hydrolysis. Hence the design of experiment-based method is said to be stability indicating and suitable for analysis of linagliptin in formulation.

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