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High performance thin layer chromatographic determination of linagliptin in pharmaceutical formulations and in biological samples

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Abstract

A HPTLC method was developed and validated for determination of Linagliptin in tablet formulation and in biological sample. The chromatography was performed on pre-coated silica gel 60 F_{254} plates using methanol: toluene 7:3 (v / v) as mobile phase. The optimized chromatographic conditions gave good peak shape with acceptable R_f value of 0.48 for Linagliptin. Method was validated as per ICH guidelines. The calibration plots were linear between 50–300 ng/ band and between 50-500 ng/ band in pure and in biological sample, respectively. Accuracy of the proposed method was evaluated by recovery studies (% recovery= 100.38% and 99.99% from tablet sample and biological sample, respectively). In stability testing, Linagliptin was found susceptible to acid hydrolysis, alkali hydrolysis and oxidation (3 % H_2O_2). The method was able to selectively quantitative. Linagliptin in presence of degradation products and components likely to be present in the biological matrix.

Keywords: Linagliptin, high performance thin layer chromatography, Method development, Validation, Pharmaceutical formulation, Biological sample.

Introduction

Linagliptin is chemically 8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methyl quinazolin-2-yl) methyl]purine-2,6-dione is an anti-diabetic drug, it belongs to DPP-IV (Dipeptidyl Peptidase IV) inhibitor class.[1,2]

Linagliptin is not official in any pharmacopoeia. Hence, no official method is available for determination of Linagliptin. Literature survey revealed few HPLC[4,5] and spectrophotometric methods[9,10] for determination of Linagliptin in pharmaceutical formulations. Recently there is one HPTLC[11] method reported for determination of Linagliptin in pharmaceutical formulation but not extended for determination in biological sample. Also, the methods reported for estimation of Linagliptin is not evaluated for their stability indicating property. The parent drug stability test guideline requires assay procedure of drug product to be stability-indicating. Even USP has a requirement listed under "Stability Studies in Manufacturing" which says that samples of the products should be assayed for potency by the use of stability indicating assay. In recent times HPTLC has gained importance in pharmaceutical analysis due to its advantage like low solvent consumption, lower detection limit, economical and rapid. The present work describes a stability indicating, selective, accurate, reliable and economical HPTLC method for the determination of Linagliptin in tablets and blood plasma. The proposed HPTLC method was validated in accordance with the criteria given by regulatory standards for pharmaceuticals.

Materials and Methods

Chemicals and reagents: Pure sample of Linagliptin was obtained as gift sample from Glenmark Research Center, Navi Mumbai and marketed formulation was procured from local market. All chemicals including methanol and toluene were of analytical grade purchased from Fischer scientific Ltd, Mumbai, India.

Instruments and chromatographic conditions: Chromatographic separation of drugs was performed on aluminium plates precoated with silica gel 60 F_{254} plates (E.Merck, Darmstadt, Germany; supplied by Merck India, Mumbai, India). The plates were initially prewashed with methanol and dried in oven at for 10 min. Samples were applied on plate as a band with 6 mm width, by using Camag 100 µl syringe (Hamilton, Bonaduz, Switzerland) with a Camag Linomat V sample applicator (Muttenz, Switzerland). A constant application rate of 150 nL s^1 was used. The plates were saturated for 15 min in a twin through glass chamber (for 10 × 10 cm) with the mobile phase of methanol: toluene (7:3 v/v). The plates were then placed in the mobile phase and ascending development was performed to a distance of 80 mm from the point of application at ambient temperature. Subsequent to development, the plates were dried in a current of air with the help of an air drier and a densitometric scanning was performed at 295 nm using Camag TLC scanner III densitometer operated in reflectance–absorbance mode. The scanning speed was 5-100 mm/s. The source of ration used was deuterium lamp, halogen tungsten and mercury vapour emitting continuous UV spectra between 190-800 nm and controlled by WinCATS software (Version 1.4.3.6336).

Preparation of standard stock solution: Standard stock solution of Linagliptin was prepared by

dissolving 5.0 mg of drug in 10.0 mL volumetric flask, dissolved and diluted to the mark with methanol to get concentration of (500 µg/mL).

Selection and optimization of mobile phase: Aliquot portions of standard stock solution, 10 µl, was applied on TLC plates in the form of band (band size: 6 mm) and the plates were run in different solvent systems. In an attempt to achieve the desired Rf value range (0.1-0.8) with a compact band, several trials were made by using different solvent systems containing non-polar solvents and relatively polar solvents at different concentration levels were tried in order to obtain a sharp peak of Linagliptin with minimal tailing.. Among the different mobile phase combinations tested Methanol: Toluene (7:3 v/v) gave compact bands which showed symmetrical peak on chromatogram and desired Rf value.

Method Validation

Linearity and range: Working standard solution of Linagliptin 50, 100, 150, 200, 250, and 300 ng/band were prepared in methanol and 5 µl of each solution was applied and chromatographed under optimum chromatographic conditions. A linear relationship between peak area and concentration was ascertained by making five measurements at six concentration levels over a range of 50-300 ng / band.

Specificity: To ascertain the specificity of the proposed method, blank sample was applied on the TLC plate and Chromatographed under optimum chromatographic conditions. The chromatogram was observed for any peak at the Rf value of Linagliptin.

Limit of detection and limit of quantitation: The limits of detection and quantification of the developed method were calculated using 3a/s and 10a/s phenomena for the limits of detection and quantification, respectively, where a is the standard deviation of the y intercept and S is the slope of calibration curve.

Precision: Intra-day and inter-day precision was determined by analyzing tablet sample solutions at different time intervals on the same day and on three different days, respectively. Precision of method was evaluated by calculating the % RSD of peak areas obtained from each spot of sample.

Accuracy: To ascertain the accuracy of the proposed methods, recovery studies were carried out by standard addition method at 50, 100 and 150 % of the test concentration as per ICH guidelines. To the accurately weighed quantity of tablet powder equivalent to 5 mg of Linagliptin, amount of pure drug 2.5 mg, 5.0 mg, and 7.5 mg was added in solution form and analyzed under optimized chromatographic conditions.

Robustness: Robustness of the proposed method was studied by small but deliberate variations in the optimized method parameters. The effect of changes in the mobile phase composition (± 1 ml), amount of mobile phase (± 1.0 ml), duration of chamber saturation with mobile phase (± 5%), time from spotting to development (5 min, 20 min, and 1 hrs) and time from development to scanning (10 min, 20 min and 1 hrs) on R_f value and peak area of drug were examined.

Stress degradation studies: Amount of tablet powder equivalent to about 5.0 mg of Linagliptin was separately transferred to five different 25.0 ml volumetric flasks (Flask no. 1, 2, 3, 4 and 5), added 3.0 mL of methanol as cosolvant. To flask no. 1, 2 and 3, added 5.0 mL 1.0 M HCl, 0.5 M NaOH and 3 % H_2O_2, respectively. The flask no. 1, 2, and 3 were kept in water bath at 80^0C for 3 hr. Flask no. 4 containing tablet powder was kept at 60^0C for 24 hrs in hot air oven to study the effect of heat on tablet sample (heat degradation). The forced degradation studies were performed in dark to exclude the possible degradative effect of light. Flask no. 5 containing tablet powder was kept under UV chamber for 24 hr. to study the photolytic degradation of tablet sample. All the flasks were removed after stipulated time interval, all the flasks were cooled to room temperature, dissolved and diluted up to the mark with methanol. The solution was then analyzed under optimum chromatographic conditions.

Analysis of marketed formulation: Twenty tablets were weighed and crushed to obtain fine powder and average weight of tablets was calculated. Accurately weighed quantity of tablet powder equivalent to about 5.0 mg of Linagliptin was transferred to 10.0 mL volumetric flask, added 5.0 mL methanol and ultrasonicated for 20 min and volume was then made up to mark with methanol. The solution was mixed and filtered through Whatmann filter paper no. 42, to get a clear solution. The filtrate was appropriately diluted to obtain concentration of 40 µg/mL. The resulting solution was used as a sample solution.

On HPTLC plate, two bands and standard solution (40 µg/mL) and four bands of sample solution were applied and chromatographed under optimum chromatographic conditions. The amount of drug present in mg/tablet and percent label claim was calculated by comparing the peak area of sample band with that of the standard band.

Application of Proposed method for estimation of linagliptin in plasma sample analysis: The proposed method was validated as per FDA guidelines with respect to selectivity, sensitivity, recovery studies and precision.

Collection of Blood plasma: Fresh blood sample of goat was collected in eppendrof tubes. The blood samples were centrifuged at 10,000 rpm for 20 min for separation of plasma. The separated plasma was employed for the study.

Preparation of Spiking stock solution: 20 mg each of Linagliptin was weighed and transferred to a 100.0 mL volumetric flask, dissolved and diluted to the mark with methanol to obtain concentration of 200 µg / mL.

Preparation of Quality control samples by spiking of plasma sample: In 10 mL centrifuge tubes, 1mL of stock solution of Linagliptin (200 ug/mL each) was added to 1.0 mL drug-free plasma. The samples were incubated at RT (28ºC) for 2 hrs. Protein precipitation and extraction was carried out using acetonitrile (2 mL) and chloroform (4 mL) and with vigorous vortex shaking for 2 min and centrifuged at 10,000 rpm for 10 min. The organic phase was recovered and evaporated to dryness at RT. The residual mass was reconstituted with 2.0 mL methanol. Lastly 1.0, 2.0, 3.0, 4.0 µl (Lower Limit of Quantitation LLOQ: 100 ng/band; Low QC sample: 200 ng/band; Middle QC sample: 300 ng/band; High QC sample: 400 ng/band of Linagliptin) from the reconstituted solution was applied on TLC plates to obtain the QC samples and developed under the optimized chromatographic conditions.

Optimization of Extraction method: Different solvents were tried for the extraction of Linagliptin from plasma. Initially hexane, n-butanol were tried for the precipitation of plasma but the sample recovery was very less. Chloroform (4mL) when employed resulted in significant improvement in sample recovery (80-90%). Acetonitrile was also added to increase the precipitation of plasma proteins. Finally a mixture of acetonitrile and chloroform (2:4 mL) was employed as the final solvent for extraction of Linagliptin with good sample recovery.

Method Validation

Study of linearity range: Working standard solution of Linagliptin 10-100 µg/mL were prepared in methanol and 5 µl of each solution was applied and chromatographed under optimum chromatographic condition. A linear relationship between peak area and concentration was ascertained at ten concentration levels over a range of 50-500 ng/ band.

Selectivity: The selectivity of the method was investigated by analyzing blank goat plasma. The blank plasma sample was checked for its interference using the proposed LLE procedure under the optimized HPTLC conditions and compared with spiked sample concentration of Linagliptin at lower limit of quantification (LLOQ) 100 ng/mL in goat plasma.

Sensitivity: Sensitivity was determined by calculating accuracy and precision at LLOQ (100 ng/band) by analyzing 5 replicates.

Precision and Accuracy: Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) samples 100, 200, 300, 400 ng/band (LLOQ, LQC, MQC, HQC) in replicate (n=5).

Extraction efficiency (Recovery): Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of same concentration. Recovery data was determined in triplicate at two concentrations (low and high) as recommended by FDA guidelines.

Results and Discussion

Optimization of mobile phase: After trying several permutations and combinations of solvents with varying polarity, the mobile phase consisting of methanol:toluene (7:3, v/v) gave good peak shape for Linagliptin with acceptable Rf value (in the range of 0.1- 0.8). It gave well defined band when the chamber was saturated for 20 min and hence it was selected for further analysis (Fig. 1).

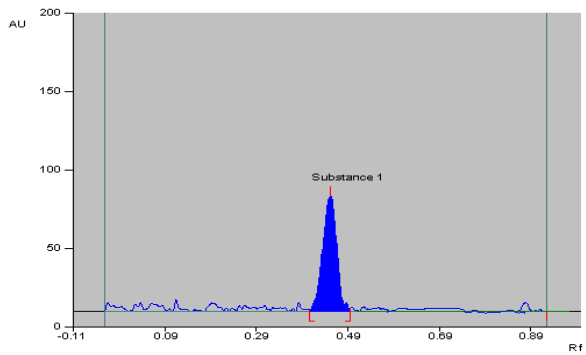

Fig. 1: Typical densitogram of Linagliptin (Rf 0.48 ± 0.5)

Analysis of tablet formulation: Amount of drug present in sample was calculated by comparing the mean peak area of sample with that of the satandard band. The result of marketed formulation is given in Table 1. The percent label claim is around 100 percent indicating that the excipients present in the tablet formulation does not interfere in the analysis.

Table 1: Result of analysis of marketed formulation

Sr. No	Drug	Amount of drug estimated (mg/tablet)	% Label Claim*	S.D.	% R.S.D.

1	Linagliptin	4.99	99.96	1.722	1.720

Method validation

Linearity and range: The linearity of detector response was observed in the concentration range of 50-300 ng/ band for Linagliptin. Coefficient of correlation was r^2= 0.995.

Specificity: Specificity was performed to detect the presence of interfering peak at the retention time of the analyte peak. The chromatogram for blank sample did not show any peak at the retention time of linagliptin indicating that the excipient has no interference in the analysis.

Limit of detection and limit of quantitation: The LOD and LOQ were separately determined based on the standard deviation of response of the calibration curve. LOD and LOQ were calculated by the formula 3.3 σ / s and 10 σ / s, where s is the slope of calibration curve and σ is the standard deviation. The LOD and LOQ for Linagliptin were found to be 2.23 ng/band and 7.45 ng/band. The low LOD and LOQ values indicates the sensitivity of the developed method.

Precision: Precision of the method was studied by performing the analysis of marketed formulation at three different times in a day (intra-day precision) and on three different days (inter-day precision), respectively. The percent RSD was fond to be less than 2% (Table 2) indicating that the method is precise and reproducible.

Table 2: Result of precision study

Drugs	Intra-day Precision			Inter-day Precision		
	% Label claim*	S.D. (±)	R.S.D.	% Label claim*	S.D. (±)	R.S.D.
Linagliptin	100.61	1.078	1.071	101.74	1.242	1.221

* Mean of three determinations

Accuracy: The accuracy of the proposed method recovery was carried out by standard addition method at three different levels (50%, 100%, & 150%) of the standard solution. The samples were chromatographed at three different levels and the percent recovery was calculated. The results of accuracy are shown in Table 3. The percentage recovery was found in the range of 100.3% to 101.2% indicating that the excipients present in tablet formulation did not interfere in the analysis.

Robustness: Robustness of the proposed method was studied by small but deliberate variations in the optimized method parameters. The effect of change in method parameters was not significant as the Rf value for Linagliptin was within ± 0.05 Rf units and the percent label claim obtained under different method parameters was around 100 percent indicating that there was no significant change in peak area. Results of robustness study are shown in Table 4.

Table 3: Results of accuracy studies

Level of recovery	% Recovery*	S.D. (±)	R.S.D.
50%	101.24	1.167	1.152
100%	100.38	1.073	1.068
150%	100.84	1.079	1.070

*****Mean of three determinations

Table 4: Results of robustness study

Factor	R_f Value	% Label Claim
Mobile phase composition (± 0.1 ml)	0.49	99.16
Amount of Mobile Phase (± 1.0 ml)	0.44	99.27
Duration for chamber saturation (± 5%)	0.49	98.70
Time from spotting to development	0.48	99.82
Time from development to scanning	0.49	99.17

*****Mean of three determinations

Stress degradation studies: Linagliptin was found to degrade under acidic, alkali and oxide stress conditions. The method was able to selectively quantitate Linagliptin in presence of degradation products indicating the stability

indicating property of the method. The chromatogram obtained for tablet sample exposed to acid, alkali and oxide stress condition are shown in **(Fig. 2, 3 and 4)** respectively. The results are summarized in Table 5.

Table 5: Results of stress degradation studies

Stress Condition	Temperature and Time	Percent assay of active substance	R_f Value of degraded product
Acid (0.1M HCl)	80 ºC for 2 hrs	76.35	0.15, 0.45, 0.80
Alkali (0.1M NaOH)	80 ºC for 2 hrs	71.6	0.15, 0.46, 0.88
Oxide (3.0% H_2O_2)	80 ºC for 2 hrs	83.09	0.01, 0.13, 0.28, 0.29, 0.44, 0.80
Neutral	80 ºC for 2 hrs	98.23	-
Heat	60 ºC for 24 hrs	99.35	-
UV-Exposure	254 nm for 24 hrs	98.74	-

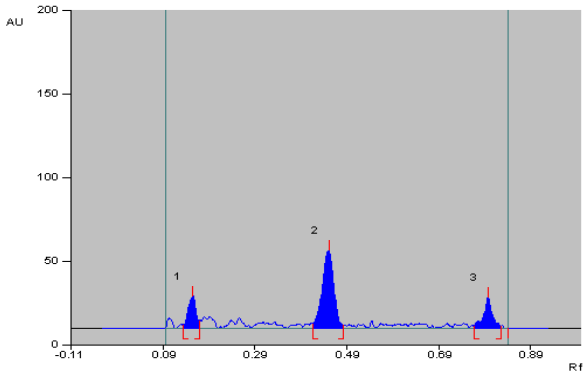

Fig. 2: Densitogram of acid (0.1 M HCl) treated sample

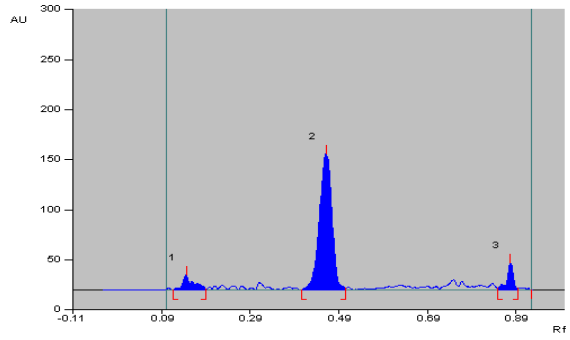

Fig. 3: Densitogram of Alkali (0.1 N NaOH) treated sample

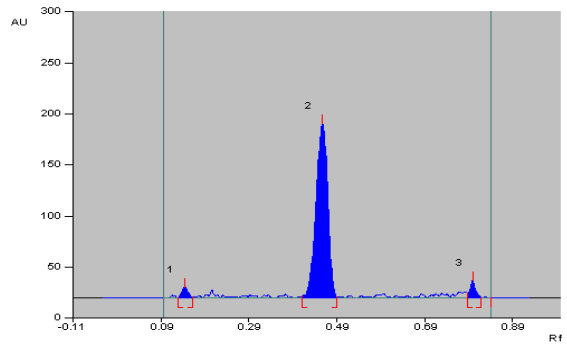

Fig. 4: Densitogram of oxide (3%H_20_2) treated sample

Application of Proposed method for estimation of linagliptin in plasma sample analysis

Optimization of extraction method: Different solvents were tried for the extraction of Linagliptin from plasma. Finally a mixture of acetonitrile and chloroform (2:4 mL) was employed as the final solvent for extraction of Linagliptin with good sample recovery.(Fig. 5)

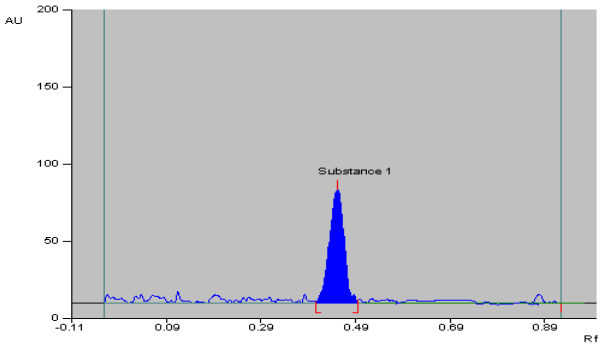

Fig. 5: Typical densitogram of goat plasma spiked with Linagliptin

Study of linearity range: Standard stock solution was applied on the TLC plate in the range of 50-500 ng/band. The plate was then developed and scanned under the optimum chromatographic conditions. Peak area was recorded for each drug concentration and the calibration curve of the concentration verses peak area was constructed. The linearity of detector response was observed in the concentration range under study **(Fig. 6)**. The regression analysis equation was y = 4.913 + 199.9 and the correlation coefficient was $r^2 = 0.997$.

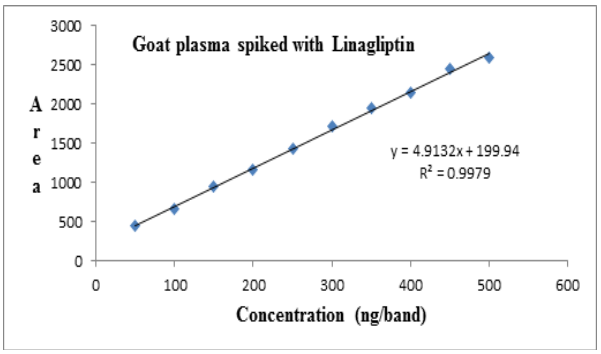

Fig. 6: Standard Calibration curve for goat plasma spiked with Linagliptin

Selectivity: The selectivity of the method was investigated by analyzing blank goat plasma. The blank plasma sample was checked for its interference using the proposed LLE procedure under the optimized HPTLC conditions and compared with spiked sample concentration of Linagliptin at lower limit of quantification (LLOQ) 100 ng/mL in goat plasma. There was no interference of the component of the biological matrix (plasma) in the quantitation of Linagliptin.

Sensitivity: Sensitivity was determined by calculating accuracy and precision at LLOQ (100 ng/band) by analyzing 5 replicates. The lower limit of quantitation was found to be 97.86 ng/band for Linagliptin. The % CV was found to be 0.90% and % Bias was found to be 2.31% for Linagliptin, and is within the acceptable limits.

Precision and Accuracy: Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) samples 100, 200, 300, 400 ng/band (LLOQ, LQC, MQC, HQC) in replicate (n=5). Intra-day and Interday precision (% CV) was found to be 1.61%, 1.24%, 0.93%, 0.76% and 2.39%, 1.46%, 1.83%, 0.77% respectively. The accuracy (% BIAS) was found to be 2.34%, 1.34%, 3.26%, 1.63% and 2.6%, 2.7%, 0.78%, 0.83% respectively. The coefficient of variance (% CV) and percent bias (% BIAS) were within the acceptable limits.

Extraction efficiency (Recovery): Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with standard solutions of same concentration. Recovery data was determined in triplicate at two concentrations (low and high) as recommended by FDA guidelines. The recovery at two concentrations 50 and 100 ng/band was found to be 87.10%, 94.42% for Linagliptin.

Conclusion

Introducing HPTLC in pharmaceutical analysis represents a major step in terms of quality assurance. The proposed HPTLC method gives well resolved peaks for Linagliptin. Based on the results obtained it is concluded that the method is sensitive, accurate, precise, selective, reproducible and less time consuming and can be employed for determination of Linagliptin in pharmaceutical formulation and biological samples without interference from the excipients.

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