A new sensitive isocratic RP HPLC method with its dual application for the simultaneous estimation of Linagliptin and Metformin hydrochloride and for Impurity profiling of Metformin Impurity-A (Dicyandiamide)

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ABSTRACT

A simple, sensitive and rapid reverse phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of Linagliptin and Metformin hydrochloride and their degradants in bulk form and formulations was developed. The separation was carried on by isocratic elution using Enable C18 (250 x 4.6 mm, 5 µm) column and Methanol- Buffer pH 5.5 (60:40, v/v) as the mobile phase. The flow rate was fixed at 1.0ml/min. The analytes and degradants were studied using PDA detector at 228nm. The retention time for Metformin hydrochloride at 2.71 mins and for Linagliptin was at 5.0 mins. Beer-Lambert’s law was obeyed in the range of 0.015 µg/mL to 40 µg/mL for Linagliptin (r = 0.9995) and 0.02 µg/mL to 20 µg/mL for Metformin (r = 0.9995). Percentage recovery for Linagliptin was 99.25% and for Metformin was 99.09%. Forced degradation studies were performed to determine the possible degradants formed and hence to infer the precautionary measures to be taken during the storage the two analytes in bulk form and in formulations. Metformin Impurity-A (Dicyandiamide) was identified as one of the degradants of Metformin formed when the above two analyte combination was exposed to stress conditions. The method was also validated for Impurity profiling of Metformin Impurity-A and it was found to be precise and accurate.

Keywords: Linagliptin, Metformin hydrochloride, RP-HPLC, Forced degradation studies, Dicyandiamide, Impurity profiling.

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INTRODUCTION:
In the present scenario, the entire world is trying to deal with one of the major diseases that is, Diabetes mellitus. Diabetes mellitus is a metabolic disorder in which the carbohydrate intake by the person remains in the blood stream in the form of glucose. It is not taken up by the cells and hence results in Hyperglycemia. The main cause of Type II Diabetes mellitus (T2DM) (Noninsulin- dependent Diabetes mellitus) in adults is the unhealthy lifestyle.\(^1\)
Metformin hydrochloride [Fig. 1(A)] (MET) [3-(diaminomethylidene)-1, 1-dimethylguanidine] is a biguanide antidiabetic drug. It is used as the first-line drug therapy for the management of T2DM. Activation of the AMP dependent protein kinase by Metformin hydrochloride is responsible for its anti-diabetic actions. Metformin hydrochloride suppresses hepatic gluconeogenesis, enhances insulin mediated glucose uptake and its disposal in skeletal muscles and fat.\(^2\) However, a progressive deterioration of the glycaemic control is generally observed over the years in patients with T2DM on Metformin monotherapy.\(^3\) During the course of Diabetes mellitus, beta cell dysfunction and failure is observed and this results in deterioration in the glycaemic control. As insulin is hyposynthesized, the beta cells have to overwork to produce more insulin. And this can be a cause of the beta cell failure.\(^4\) The loss of β-cell function prevents the achievement of normoglycemia in patients on Metformin monotherapy.\(^5\)
Dipeptidylpeptidase-4 (DPP-4) inhibitors (Glitins) offer new options for combined therapy with Metformin hydrochloride. Linagliptin [Fig. 1(B)] (LINA) is 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-metyl-1-[(4-methylquinazolin-2-yl)methyl]-3,7-dihydro-1H-purine-2,6-dione. It increases the incretin levels (GLP-1 and GIP) and thus inhibits glucagon release and increases insulin biosynthesis and its secretion. This is done in a glucose-dependent manner, which explains the minimal risk of hypoglycaemia associated with its use. GLP-1 contributes to appetite regulation and thereby avoids or minimises weight gain despite of glucose control in T2DM patients unlike other hypoglycaemic drugs. Linagliptin as compared to the other available DPP-4 inhibitors is characterized with negligible renal excretion and predominant biliary excretion. Hence it can be used in patients with chronic kidney disease without dose adjustment. Co-administration of Metformin hydrochloride and Linagliptin improves blood glucose control more potently than either of the above monotherapies and also increases patient compliance.\(^3\)

![Fig. 1: (A) Linagliptin and (B) Metformin hydrochloride and (C) Metformin Impurity- A (Dicyandiamide or 1-Cyanoguanidine)](image)

A lot of analytical work on LINA and MET has been reported. Literature review revealed UV spectroscopic, HPTLC and RP-HPLC methods for the analysis of LINA \(^6\)\(^-\)\(^12\) and MET \(^13\)\(^-\)\(^15\) alone. Research
work on LINA along with Empagliflozin has also been done. \(^{[16]}\) Simultaneous analysis of MET with various other anti-diabetic drugs \(^{[17-39]}\), with Atorvastatin calcium \(^{[40]}\) and Fenofibrate \(^{[41]}\) have also been reported. LINA and MET have been simultaneously analysed by analytical methods like HPTLC \(^{[42, 43]}\) and UV spectroscopy \(^{[44, 45]}\). Quantitative estimation of these two drugs by the HPLC method has also been reported. \(^{[46-54]}\) LINA and MET has also been studied by LC-MS method. \(^{[55]}\) Forced degradation studies involve the degradation of the drug substance or drug product under conditions more severe than accelerated conditions. It helps to study the stability of the drug molecules. Further, the degradants formed during these studies can be identified and pathway of degradation can be established. \(^{[56]}\)

Some Stability indicating methods for LINA-MET combination have been developed earlier where in only the interference of the degradants with the analyte peaks of LINA and MET under various stress conditions was studied. \(^{[57-62]}\) However, there is no HPLC method reported till date which is available with which the degradants of Linagliptin and Metformin hydrochloride formed during the Forced degradation studies have been isolated and identified. Hence, the authors planned to develop a rapid, sensitive, accurate and precise RP-HPLC method which could be efficiently used for both simultaneous estimation of LINA and MET as well as to study the related substances of LINA and MET formed during the forced degradation studies in their combined dosage forms.

The aim of our research was to develop and validate a simple and rapid method without compromising on the resolution between the two APIs and their degradants. Forced degradation studies were performed to determine the conditions in which degradants were formed. This would help to decide the storage conditions for LINA and MET bulk APIs as well as their formulations. And to study and identify whether any of these degradants formed under the various stress conditions was MET Impurity-A (Dicyandiamide). The authors also planned to validate the chromatographic method for its use in impurity profiling of MET Impurity-A (Dicyandiamide), in case it was found to be formed during the forced degradation studies.

Metformin Related Compound A USP-Cyanoguanidine also known as Metformin EP Impurity A [Fig. 1(C)] is an impurity of Metformin hydrochloride. It is a guanidine derivative which is also used in the synthesis of barbiturates. \(^{[63]}\) Dicyandiamide is one of the starting material in the manufacture of Metformin hydrochloride. \(^{[64]}\) As per the pharmacopeial standards, the acceptance limit for MET Impurity-A is NMT 0.02 % of the working concentration of MET.

In our research, efforts were focused on making the most of all the inherent advantages offered by reversed-phase HPLC to develop a time saving, economical and practical method which would be of immense assistance to Quality control laboratories to ensure the identity, purity and performance of the drug product.

**MATERIALS AND METHODS:**

**Chemicals, Reagents and Materials:**

Linagliptin API, Metformin hydrochloride API and Dicyandiamide (MET Impurity-A) (Potency: 99.9 \%) were obtained as gift samples from Mylan India Ltd, Bollaram, Hyderabad (India). Jentaduet tablets 2.5 mg/850 mg SR of Glenmark Pharmaceuticals Ltd. were purchased from the local pharmacy. HPLC grade Methanol was purchased from J.T. Baker (Philipsburg, USA). Water for chromatography (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany). DiPotassium hydrogen orthophosphate (K\(_2\)HPO\(_4\)) (AR grade) and Ortho phosphoric acid (AR grade) used for the preparation of Buffer pH 5.5 were purchased from SD fine chemicals (Mumbai, India).

**Instrumentation:**

SHIMADZU 20A LC system with a double reciprocating pump and SPD- M20A PDA detector was the HPLC system used for the research. RP-C18 Enable column (250 x 4.6 mm, 5\(\mu\)m) was the stationary phase used to perform the chromatography. The RP-HPLC system was equipped with software LC Solutions version 1.5 for data processing. Mettler Toledo pH meter and Mettler Toledo analytical and microbalances were used during the research. Ultra-
sonicator (Make: Leelasonic-200) was used to assist the dissolution of the solute in the solvent and to degas the mobile phase. The filtration of the mobile phase was done with Durapore® HVLP 47 mm membrane filters (0.45 µ pore size) using the vacuum filtration system by Microlab scientific. Whatman No. 1 filter paper and Durapore® GVWP 13 mm syringe filters (0.22 µ pore size) were used to filter the sample.

**Preparation of Buffer pH 5.5, Standard and Sample solutions:**

**Preparation of Buffer pH 5.5:**
K₂HPO₄ (1.74 gms) was dissolved in 1000 mL HPLC grade water (10 mM K₂HPO₄ buffer) and adjusted to pH 5.5 with OPA.

**Preparation of the Mobile phase:**
The Mobile phase was prepared by mixing Methanol and Buffer pH 5.5 in the proportion 60 : 40 %v/v. The mobile phase was used as the diluent.

**Preparation of Metformin and Linagliptin Standard and Sample Solutions:**

**Standard solution preparation:**
Stock solutions of Linagliptin (100 µg/ml) and Metformin hydrochloride (100 µg/ml) were prepared. And a mixed standard solution containing 20 µg/mL Linagliptin and 10 µg/mL Metformin was prepared using the above stock solutions of the two analytes.

**Sample solution preparation:**
The sample solutions were prepared using Jentadueto tablets containing 850 mg of Metformin hydrochloride and 2.5 mg of Linagliptin. Because of the vast difference in strength of the two analytes, two sample solutions were prepared.

**Metformin Sample solution:**
Jentadueto tablet powder equivalent to 50 mg of MET was accurately weighed and transferred into a 50 ml volumetric flask. A volume of 30 mL methanol was added. The flask was sonicated for 30 minutes and the volume was made up to the mark with methanol. The solution was filtered through a Whatman filter No. 1 filter paper. A 20 µg/ml LINA solution was prepared from this filtered solution using the diluent. The sample solutions were filtered through a 0.22 µm syringe filter before injecting it into the chromatographic system.

**Chromatographic conditions:**
Reversed Phase Chromatographic technique was used to resolve and quantitate LINA and MET and to resolve and study the different degradants formed in the forced degradation studies. Enable C18 column (4.6× 250 mm, 5µm), Make: Shimadzu was used as the stationary phase and Methanol-Buffer pH 5.5 (60: 40, v/v) was used as the mobile phase. Isocratic mode of elution was used to chromatograph the analytes and their degradants formed during the stress degradation studies. The mobile phase flow rate was maintained at 1 ml/min. The wavelength for detection selected was at 228 nm. Column temperature was maintained at 30°C. The injection volume was set at 20 µl.

**Chromatographic Procedure:**
A volume of 20 µL of the standard and sample were injected into the chromatographic system. The peak areas, retention time and resolution were measured for MET and LINA peaks. The % RSD was calculated for the peak area of each analyte.

**Method Validation:**
The proposed RP-HPLC method was validated as per the ICH Q2 (R1) guidelines.

**System suitability:**
The system suitability was assessed by injecting six replicate injections of mixed standard containing Metformin (10 µg/ml) and Linagliptin (20 µg/ml). Parameters such as USP plate count, USP tailing, resolution which would determine the performance of the chromatographic system were determined. Parameters such as % RSD of the peak areas and the retention times of LINA and MET were also evaluated which would determine the repeatability of the results.
Specifity:
The specificity was evaluated by checking for any interference of the diluent blank and placebo solution at the Rt of MET and LINA peaks. The co-elution of any peaks with the peaks of MET and LINA was checked. Forced degradation studies were performed to check the possible impurities formed when LINA and MET was exposed to oxidation, acid, alkali, high temperature and UV light.

Forced degradation studies:

i) Acid degradation:
In a 25 ml volumetric flask, 12.5 ml LINA stock solution and 1.25 ml of MET stock solution were pipetted out. To this 0.5 ml of 0.1 N HCl was added and the volume was made up to the mark with the diluent. The above was placed in a water bath at 60 °C for 1 hour. The solution was cooled and 1 ml was pipetted out into a 10 ml volumetric flask. The volume was made up to the mark with the diluent. Similarly MET and LINA stock solutions were individually subjected to acid degradation and diluted to produce a solution containing 10 µg/mL of MET and 20 µg/mL of LINA respectively.

ii) Alkaline degradation:
In a 25 ml volumetric flask, 12.5 ml LINA stock solution and 1.25 ml of MET stock solution were pipetted out. To this 0.5 ml of 0.1 N NaOH was added and the volume was made up to the mark with the diluent. The above was placed in a water bath at 60 °C for 1 hour. The solution was cooled and 1 ml was pipetted out into a 10 ml volumetric flask. The volume was made up to the mark with the diluent. Similarly MET and LINA stock solutions were individually subjected to alkaline degradation and diluted to produce a solution containing 10 µg/mL of MET and 20 µg/mL of LINA respectively.

iii) Oxidative degradation:
In a 25 ml volumetric flask, 12.5 ml LINA stock solution and 1.25 ml of MET stock solution were pipetted out. To this 0.5 ml 3% H_2O_2 of was added and the volume was made up to the mark with the diluent. The above was placed in a water bath at 60 °C for 1 hour. The solution was cooled and 1 ml was pipetted out into a 10 ml volumetric flask. The volume was made up to the mark with the diluent. Similarly MET and LINA stock solutions were individually subjected to oxidative degradation and diluted to produce a solution containing 10 µg/mL of MET and 20 µg/mL of LINA respectively.

iv) Thermal degradation:
The LINA API and the MET API were subjected to 100 °C in a hot air oven for 1 hour. These APIs were used to prepare 20 µg/ml solution of Linagliptin and 10 µg/ml solution of Metformin. A mixed solution containing 20 µg/ml Linagliptin and 10 µg/ml Metformin was also prepared using the above LINA API and MET API.

v) Photolytic degradation:
In a 25 mL volumetric flask, 12.5 mL Linagliptin stock solution and 1.25 mL of Metformin stock solution was pipetted out. The volume was made up to the mark with the diluent. This solution was subjected to 1.2 million lux hours and 200 Watt h/m2 UV light for 8 hours. In a 10 mL volumetric flask, 1 mL of the above solution was pipetted out and made up to the mark with the diluent. Similarly MET and LINA stock solutions were individually subjected to photolytic degradation and diluted to produce a solution containing 20 µg/mL of LINA and 10 µg/mL of MET.

Limit of detection (LOD) and Limit of Quantitation (LOQ):
The LOD and LOQ were determined by calculating the signal to noise (S/N) ratio.

Limit of detection (LOD):
From the Metformin stock solution (100 µg/ml) and Linagliptin stock solution (100 µg/ml) many respective solutions with very dilute concentrations were prepared. The S/N ratio was determined. The concentration which gave a S/N ratio between 3 or 2:1 was noted.

Limit of Quantitation (LOQ):
Similarly, many dilute solutions were prepared from the Metformin stock solution (100 µg/ml) and Linagliptin stock solution (100 µg/ml) and the concentration of MET and LINA giving a S/N ratio of 10:1 was determined.

Linearity:
A range of concentrations from 0.02 ppm (LOQ) to 20 ppm for MET were prepared from 100 µg/ml MET standard solution. Similarly, solutions of concentrations ranging from 0.015 ppm (LOQ) to 40 ppm for LINA were prepared from 100 µg/ml LINA standard solution.
ppm of LINA were prepared using 100 µg/ml LINA standard solution. These solutions were analyzed by the developed method. The peak areas for the two analytes were integrated. A calibration curve of Concentration versus Peak area was plotted for MET and LINA. The coefficient of correlation was estimated to determine the linearity of the method.

**Accuracy:**
Accuracy was calculated with respect to 80 %, 100 % and 120 % of the target assay concentration. The accuracy of the method was demonstrated through recovery studies. Because of the vast difference in the strengths of MET and LINA, two accuracy solutions were prepared. One for MET and the other one for LINA. Accuracy was evaluated by the standard addition method.

The standard solution, Accuracy-80%, Accuracy-100 % and Accuracy-120% solutions were filtered through a 0.22 µm filter and each solution is injected thrice. The amount found was calculated and depending on the amount added, the individual recovery and the mean recovery values were calculated.

**Precision:**
The precision of the method was demonstrated through three parameters which are injection reproducibility (system precision), method precision and intermediate precision (Ruggedness).

For injection reproducibility, mixed standard solution containing 20 µg/ml Linagliptin and 10 µg/ml Metformin was injected for 6 times. The % RSD for the peak areas and retention time for LINA and MET were calculated. For method precision, 6 samples were prepared using the tablet samples and 2 injections for each sample were made and the % RSD for peak areas and retention times were calculated. For intermediate precision, the above procedure was repeated on a different day by a different analyst. The %RSD for all should not be more than 2.

**Robustness:**
The robustness study was done by making small deliberate changes in the optimized method. Variations in the parameters like ± 0.2 change in the buffer pH, ± 10 % in the Organic phase of the Mobile phase, ± 2 °C change in column temperature, change in the flow rate to 0.9 mL/min and 1.1 mL/min were made to evaluate the impact on the results.

**Stability of the solution:**
Mixed standard solution containing 20 µg/ml of LINA and 10 µg/ml of MET was prepared and stored at R.T. and at 10 °C. These were analyzed at time intervals of 0, 24 and 48 hours. Chromatograms were checked for any extra peaks, splitting of peaks or decrease in the peak area.

**Stability of the Mobile phase:**
To evaluate the stability of the mobile phase, the assay of MET and LINA standard was done at the time interval of 0 hour, 24 hours and 48 hours. The system suitability parameters were also checked at each of these time intervals.

**Assay of marketed preparation**
Ten Jentadueto tablets were weighed and crushed to fine powder. The sample solutions were prepared as per the procedure given above using the tablet powder. Assay standard was also prepared as per the procedure as mentioned above. A 20 µl of the standard solution was injected six times and each sample solution was injected twice into the sample injector under chromatographic conditions as described above. Area of each peak was measured at 228 nm. The amount of LINA and MET present in the Jentadueto tablet samples were calculated using equation (1). The Percent label claim and Standard deviation (SD) were calculated.

\[
\text{% Assay} = \frac{\text{Sample area}}{\text{Std. area}} \times \frac{\text{Wt. of Std.}}{\text{Dilution of Std.}} \times \frac{\text{Dilution of Sample}}{\text{Wt. of Sample}} \times \frac{\text{% Purity of Std.}}{100} \times \frac{\text{Avg. Wt. Label claim}}{x 100} \quad (1)
\]

**Application of the method for Impurity Profiling of Metformin Impurity-A (Dicyandiamide):**

In this study, the above developed method was applied to identify whether any of the impurities formed during the forced degradation studies was MET.
Impurity A (Dicyandiamide also called as 1-Cyanoguanidine). The impurity profiling of MET Impurity-A in bulk drugs and Jentadueto tablets was done as per ICH Q3A (R2) and ICH Q3B (R2) guidelines.\[66, 67\]

Procedure:
The Solution of MET Impurity-A, blank, placebo, MET standard solution, MET standard solution (100 µg/mL) spiked with MET Impurity A and MET sample solution (100 µg/mL) spiked with MET Impurity-A were injected into the chromatographic system and run as per our developed method. The chromatograms of these were compared with the chromatograms obtained during the Forced degradation studies. The RRT of the impurities of MET were compared with RRT of MET Impurity- A (Dicyandiamide).

During this study it was identified that, MET Impurity-A and Impurity M1 [Relative Retention time (RRT) 1.54] of MET formed during the Forced degradation study was the same. Hence it was decided to validate our developed method for the Impurity profiling of MET Impurity-A.

The method was validated for the simultaneous analysis of MET Impurity A and MET as per the ICH Q2 (R1) guidelines for Specificity, Precision, Accuracy, Linearity, Range, Robustness and Solution stability.

Specificity:
Specificity of the method was evaluated by analyzing solutions of MET, MET Impurity-A, blank, placebo, MET standard solution spiked with MET Impurity-A and MET sample solution spiked with MET Impurity-A. The co-elution of any peak at the Retention time (Rt) of MET and MET Impurity-A was checked by determining the peak purity for MET Impurity-A and MET in individual and spiked solutions.

Limit of Detection and Limit of Quantitation (LOD and LOQ):
The LOD and LOQ were established by analyzing diluted solutions of MET Impurity-A and MET solution. The Signal to Noise (S/N) ratio for each was determined. LOD and LOQ of the analyte would be the concentration with S/N ratio equal to 3:1 and 10:1 respectively.

Precision at LOQ level:
Solution containing MET and MET Impurity-A at LOQ level was injected six times. The % RSD for the peak areas of MET and MET Impurity-A were calculated.

Accuracy at LOQ level:
TriPLICATE solutions of MET (conc. 100 µg/mL) spiked with MET Impurity-A (conc. at LOQ level) was prepared and analyzed. Percentage recovery of the spiked MET Impurity A at LOQ level was determined.

Linearity and Range:
A series of linearity solutions of MET Impurity-A of concentrations at LOQ level, 60 %, 75 %, 90 %, 100 %, 250 %, 375 % and 500 % w.r.t. the specification level were prepared.

A series of linearity solutions of MET of concentrations at LOQ level, 0.025 %, 0.05 %, 0.075 %, 0.10 %, 0.15 %, 0.20 % and 0.30 % w.r.t. the working concentration were prepared.

All of the above solutions were analyzed and Linearity curve of Concentration versus Peak area was plotted for MET and MET Impurity- A. The coefficient of correlation and slope was determined for both the graphs. From the slopes of the above two graphs, the Relative Response factor (RRF) value was calculated using equation (2).

\[
RRF = \frac{\text{Slope of Calibration curve of Metformin Impurity} - A}{\text{Slope of Calibration curve of Metformin hydrochloride}}
\] ………………………………………… (2)

Accuracy:
MET (100 µg/mL) solution was spiked with MET Impurity-A at different concentration levels, i.e. 60 %, 75 %, 250 %, 375 % and 500 % w.r.t. its specification limit. Triplicate solutions were prepared for each concentration level and analyzed. Percentage recovery of MET Impurity-A was determined.
Precision:

*Method Precision:*
Six separate solutions of MET (100 µg/mL) spiked with MET Impurity-A at specification level were prepared and analyzed twice each. The Peak area for MET Impurity-A was recorded and the %RSD was calculated.

*Intermediate Precision:*
Intermediate Precision was determined by analyzing six separate solutions of MET (100 µg/mL) spiked with MET Impurity-A at specification level. These were analyzed on different days, by different analysts, by using different column. The peak area of MET Impurity-A was integrated and the %RSD was calculated.

*Robustness:*
Robustness was determined by making small deliberate alterations in the method conditions. Alterations in the column temperature, Buffer pH and flow rate of the mobile phase was done. Effect of the changes incorporated in the chromatographic method on the RRT of MET Impurity-A w.r.t. MET, Tailing factor, Theoretical plates and the %RSD for Peak area was determined.

*Stability of Metformin hydrochloride spiked with Impurity-A at RT and at 10 ºC:*
Metformin hydrochloride solution (100 µg/mL) spiked with MET Impurity-A at specification level (0.02 %) was prepared and kept at room temperature and at 10 ºC. These solutions stored at R.T. and at 10 ºC were analyzed at 0, 24 and 48 hour time intervals.

*Assay of Metformin Impurity-A in Jentadueto tablet (2.5 mg/ 850 mg) marketed sample:*
The sample solution was prepared as per the above procedure containing 100 µg/mL of MET. The sample was filtered through 0.22 µ filter and chromatographed as per our developed method. The chromatograms were evaluated for the presence of MET Impurity-A and the amount of MET Impurity A calculated.

RESULTS AND DISCUSSION

*Method development:*

*Selection of Wavelength:*
An absorption maximum (λ max) for LINA was observed at 225 nm whereas for MET it was observed at 235 nm. Considering the sensitivity of the method, it was observed that a good absorbance was obtained for both LINA and MET at 228 nm. Hence 228 nm was chosen as the wavelength was detection.

*Column and Column temperature:*
LINA has a log P of 2.8 and the log P of MET is – 1.43. Since LINA and MET are polar in nature, Reversed Phase Chromatography was selected. Various brands of C8 and C18 columns like Inertsil, Cromosil, Zorbax, Enable of various dimensions were experimented with. Enable C18 (250 x 4.6 mm, 5 µm), make: Shimadzu was finalized as this gave symmetrical peak shape and a high theoretical plate count for both LINA and MET. A 250 mm long column would give good resolution between the two analytes and their probable degradants. The column temperature selected was 30 ºC as it helped in the elution of the two analytes and their degradants. A combination of high resolution, short retention times and sharp symmetrical peaks for LINA, MET and their respective degradants was obtained at this temperature.

*Optimization of the Mobile phase:*
Metformin hydrochloride is highly soluble in water, while it is practically insoluble in acetone, ether and chloroform. It has a pKa of 12.4. Linagliptin is soluble in methanol, sparingly soluble in ethanol and very slightly soluble in isopropanol and acetone. It has a pKa of 8.6 and occurs as a cation at between pH 5 to 9. Many trials were carried out using different solvents like acetonitrile, methanol, and water. Buffers of various pH (4.5, 5.5, 6.5, 7.5 and 8.0) were also trailed with. Different combinations of these solvents in various proportions were used so as to get shorter retention time, sharp symmetrical peak shape and high resolution between the peaks. The trials showed results like broaden peaks, tailing of peaks, blank peak interference with an API peak or less resolution between the two API peaks.
The optimized mobile phase which gave the desired results was Methanol-Buffer pH 5.5 (60:40, v/v). Sharp symmetrical peak shapes for both the drugs with a retention time of 2.71 minutes for MET and 5.0 for LINA were obtained. A high resolution of 13.12 was obtained in between the peaks of MET and LINA. This would accommodate the impurity peaks of the two drugs during their forced degradation studies and prevent the interference of the impurity peaks with the main analyte peaks of MET and LINA. A representative chromatogram of MET and LINA using the developed method is given in Fig. 2.

**Method Validation:**
To assure the accuracy and precision of results, integration between the HPLC system, electronic configuration, LC solutions (version 1.5) software and the developed method is required. This was established through the system suitability test. The resolution between MET and LINA API peaks was 13.12. And the resolution between the main API peaks of LINA, MET and their degradants obtained during the stress degradation studies were also more than 2. The USP Plate count for the two analytes was found to be more than 2000, the USP tailing was less than 1.5 and the % RSD for the peak areas and the retention time for LINA and MET was less than 2 %. These factors proved the system suitability of our method. No peak interference of the blank or the placebo was observed at the retention times of LINA and MET. The peaks of the impurities formed during the forced degradation studies were also well resolved from the peaks of LINA and MET APIs. The peak purity study of the two analytes and the all the degradants using the PDA detector confirmed no co-elution of peaks. Hence the method is specific for LINA and MET and is also suitable for analyzing the degradants of the combination of LINA and MET.

The forced degradation studies indicated that the extent of degradation of LINA due to all the stress conditions is much higher than that of MET. Results of the Forced degradation study of MET and LINA are given in Table 1. Four impurities of Linagliptin were formed during the forced degradation studies namely LINA Imp I (L1) (RRT 0.60), LINA Imp II (L2) (RRT 0.76), LINA Imp III (L3) (RRT 0.91) and LINA Imp IV (L4) (RRT 1.21). It was observed that all these were formed on Photo degradation of LINA API and LINA and MET combination. On thermal degradation of LINA API and in the combination LINA Imp I (0.60), LINA Imp II (0.76) and LINA Imp III (RRT 0.91) were formed. Oxidative degradation and Acid degradation of LINA resulted in formation of LINA Imp I (RRT 0.60) and LINA Imp II (RRT 0.76). While Alkaline degradation resulted in the formation of LINA Imp I (RRT 0.60) and LINA Imp III (RRT 0.91). MET was seen to be prone to photolysis. It was observed that when MET individually and in combination with LINA was exposed to Photolytic degradation, MET Imp I (M1) (RRT 1.54) and MET Imp II (M2) (RRT 2.54) were formed. However, under basic and oxidative conditions MET showed slight degradation of about 1 % by formation of MET Imp I (RRT 1.54) only. MET molecule was observed
to be stable at 100 °C and under acidic conditions. The number of degradants formed of MET and LINA during their Forced degradation studies and the extent to which these were formed is given in Table 2. Chromatograms depicting the degradants formed when LINA and MET were exposed to various stress conditions in the Forced degradation studies are given in Fig. 3(a), Fig. 3(b), Fig. 3(c), Fig. 3(d) and Fig. 3(e). Results of the forced degradation studies show that the LINA and MET have to be stored in containers protected from light and oxygen.

![Fig. 3(a): Thermal degradation of Linagliptin and Metformin](image1)

![Fig. 3(b): Photolytic degradation of Linagliptin and Metformin](image2)

![Fig. 3(c): Acid degradation of Linagliptin and Metformin](image3)
Table 1: Results of Forced degradation study of Metformin hydrochloride and Linagliptin

<table>
<thead>
<tr>
<th>STRESS CONDITION</th>
<th>METFORMIN HYDROCHLORIDE</th>
<th>LINAGLIFTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Degraded</td>
<td>Purity angle</td>
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<tr>
<td>Alkaline hydrolysis</td>
<td>1.00</td>
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<td>Acid hydrolysis</td>
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<td>Photolytic degradation</td>
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</tr>
<tr>
<td>Thermal degradation</td>
<td>----</td>
<td>0.065</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>1.32</td>
<td>0.097</td>
</tr>
</tbody>
</table>
Table 2: Degradants formed during the Forced degradation studies

<table>
<thead>
<tr>
<th>DEGRADATION CONDITION</th>
<th>METFORMIN HCl</th>
<th>LINAGLIPTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M 1</td>
<td>M 2</td>
</tr>
<tr>
<td>Imp RRT</td>
<td>1.54</td>
<td>2.54</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>0.95 %</td>
<td>-</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Photolytic degradation</td>
<td>3.33 %</td>
<td>7.93 %</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>1.25 %</td>
<td>-</td>
</tr>
</tbody>
</table>

The method was found to be very sensitive. The LOD and LOQ of Metformin was found to be 6 ng/ml and 20 ng/ml respectively. While the LOD and LOQ of Linagliptin was found to be 5 ng/ml and 15 ng/ml respectively.

Linearity for the method was proved for MET in the concentration range of 0.02 µg/ml to 20 µg/ml and for LINA in the concentration range of 0.015 µg/ml to 40 µg/ml. The correlation coefficient (‘r’ value) for both LINA and MET was found to be 0.9995. The calibration curves for MET and LINA are given in Fig. 4(a) and Fig. 4(b) respectively. The method was found to be accurate for both LINA and MET with a mean recovery of 99.25% and 99.09% for LINA and MET respectively.

The method was found to be precise with % RSD of System precision of 0.012 for MET and 0.015 for LINA. The % RSD of Method precision was found to be 0.746 for MET and 0.888 for LINA and the % RSD of Intermediate precision was found to be 0.63 for MET and 0.996 for LINA. The method was rugged since the % RSD of intermediate precision was less than 2%.

![Linearity of Metformin](image1)

![Linearity of Linagliptin](image2)

**Fig. 4:** Calibration curves of (a) Metformin hydrochloride and (b) Linagliptin

The chromatographic method proved to be robust when subjected to various deliberate changes in the pH of the buffer, change in organic phase composition, flow rate and the column temperature. It was observed that all the system suitability factors under these varied conditions were within the
acceptance limit. The mixed standard solution was found to be stable for 48 hours under refrigeration (10 °C) and at RT as there were no extra peaks, splitting of peaks or decrease in the peak area seen in the chromatograms.

**Assay of the marketed preparation:**
The developed method was applied for performing the Assay of Jentadueto tablets (2.5 mg/ 850 mg). The amount of Metformin hydrochloride and Linagliptin present in the formulation was calculated as per the formula (1). The % assay of MET and LINA in the marketed product of Jentadueto tablets (2.5 mg/ 850 mg) was found to be 99.16 % and 101.42 % respectively.

**Impurity Profiling of MET Impurity-A (Dicyandiamide):**
The developed RP-HPLC method was also applied to identify whether MET Impurity-A (Dicyandiamide) was formed during the forced degradation studies. It was observed that when MET standard solution (100 µg/mL) spiked with MET Impurity-A was analyzed, it was observed that MET Impurity-A had a RRT of 1.54. When compared with the chromatograms of the Forced degradation studies, it was noted that in the chromatograms of Photo degradation, Alkaline degradation and Oxidative degradation of individual MET API and LINA and MET combination, Impurity MI was formed with RRT 1.54. Hence it can be concluded that MET Impurity-A was formed as a degradants of MET during its Photo degradation, Alkaline degradation and Oxidative degradation. Thus, it was decided to validate our isocratic RP-HPLC method for the Impurity profiling of MET Impurity-A as per ICH Q2 (R1) guidelines. The test for Specificity was performed to check any peak interference at the retention time of Metformin hydrochloride and MET Impurity-A. Metformin and MET Impurity–A individual solutions, MET standard solution (100 µg/mL) spiked with MET Impurity-A and Jentadueto sample solution containing MET (100 µg/mL) spiked with MET Impurity-A solution were analyzed by the developed method. It was observed that the Rt of MET in MET individual solution and spiked Standard and sample solution was 2.71 mins. While the Rt of MET Impurity-A in MET Impurity-A individual solution and spiked Standard and sample solution was 4.22. The RRT of MET Impurity-A with respect to MET in the spiked solutions was 1.54. The resolution between MET peak and MET Impurity-A peak was found to be 8.51. Fig. 5(a) denotes the chromatogram of Metformin solution (LOQ conc) spiked with Metformin Impurity-A (LOQ conc). Fig. 5(b) denotes the chromatogram of Metformin standard solution (100 µg/mL) spiked with Metformin Impurity-A solution (0.02 µg/mL ). And Fig. 5(c) denotes chromatogram of Metformin sample solution (100 µg/mL) spiked with Metformin Impurity-A solution (0.02 µg/mL ).

No co-elution of other peaks was observed at the Rt of MET and MET Impurity-A as the Peak purity was noted to be less than the Purity threshold at these Rts.

**Fig. 5(a) Metformin solution (LOQ conc) spiked with Metformin Impurity-A solution (LOQ conc)**
The LOD and LOQ of MET Impurity-A was determined to be 0.003 µg/mL (S/N ratio = 3.2) and 0.01 µg/mL (S/N ratio = 10.4) respectively. While the LOD and LOQ of MET was determined to be 0.006 µg/mL (S/N ratio = 3.1) and 0.02 µg/mL (S/N ratio = 10.6) respectively.

The method was found to be precise at the LOQ level of MET Impurity-A and MET with a % RSD of 0.73 and 1.04 respectively. Accuracy was also proved for MET Impurity-A at the LOQ level when it was spiked in three sample solutions of MET (Conc. 100 µg/mL) with a mean % Recovery of 94.90%.

The method proved to obey the Beer-Lambert’s law in the concentration range of 0.02 µg/mL (LOQ level) to 0.3 µg/mL (0.3 % w.r.t. the working concentration) for MET and 0.01 µg/mL (LOQ level) to 0.1 µg/mL (500 % of specification) for MET Impurity-A. The Fig. 6(a) and Fig. 6(b) shows the Calibration curves of Metformin (LOQ to 0.3 % of working conc.) and Metformin Impurity-A (LOQ to 500% of specification level) respectively. The coefficient of correlation for the MET and MET Impurity-A was 0.9995. The RRF was calculated from the ratio of the slopes of the Calibration curve of MET and the Calibration curve of MET Impurity-A and it was found to be 0.38.
The method proved to be accurate at different concentrations of spiking levels with respect to the specification concentration (0.02 % of the working concentration of MET) with a mean % Recovery of 99.69 %. Results for the Accuracy test for Impurity profiling of Metformin Impurity-A are given in Table 3. The % RSD obtained for method precision, intermediate precision test and the overall % RSD for method and intermediate precision was found to be NMT 10.0 %. The overall % RSD calculated for the content of MET Impurity-A spiked in MET sample solution (100 µg/mL) was 2.23, hence the method was found to be precise.

Table 3: Accuracy test for Impurity profiling of Metformin Impurity-A

<table>
<thead>
<tr>
<th>Spiking level (w.r.t.specification)</th>
<th>Sample no.</th>
<th>Amount added (µg/mL)</th>
<th>Amount obtained (µg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 %</td>
<td>1</td>
<td>0.012</td>
<td>0.01193</td>
<td>99.42 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.01185</td>
<td>98.75 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.01176</td>
<td>98.00 %</td>
</tr>
<tr>
<td>75 %</td>
<td>1</td>
<td>0.015</td>
<td>0.01482</td>
<td>98.8 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.01495</td>
<td>99.67 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.01513</td>
<td>100.87 %</td>
</tr>
<tr>
<td>250 %</td>
<td>1</td>
<td>0.05</td>
<td>0.04936</td>
<td>98.72 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.05042</td>
<td>100.84 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.04987</td>
<td>99.74 %</td>
</tr>
<tr>
<td>375 %</td>
<td>1</td>
<td>0.075</td>
<td>0.07413</td>
<td>98.84 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.07519</td>
<td>100.25 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.07474</td>
<td>99.65 %</td>
</tr>
<tr>
<td>500 %</td>
<td>1</td>
<td>0.1</td>
<td>0.10112</td>
<td>101.12 %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.10084</td>
<td>100.84 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.09979</td>
<td>99.79 %</td>
</tr>
<tr>
<td>Mean % Recovery</td>
<td></td>
<td></td>
<td></td>
<td>99.69 %</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>0.952</td>
</tr>
<tr>
<td>RSD</td>
<td></td>
<td></td>
<td></td>
<td>0.955</td>
</tr>
</tbody>
</table>
With deliberate changes in the column temperature, Buffer pH and flow rate of the mobile phase the system suitability parameters such as tailing factor and theoretical plates were within the acceptance limits. The RRT of MET Impurity-A w.r.t. MET and % RSD for Peak area of MET Impurity-A were also within the acceptance limits. Hence, the method was found to be robust.

The solution stability of MET (100µg/mL) spiked with MET Impurity-A at specification level (0.02 %) was proved at 10 °C and at RT for 48 hours.

**Assay of Jentadueto Tablets (2.5 mg/ 850 mg) for Metformin Impurity-A:**

No presence of MET Impurity-A was found in the Jentadueto Tablet (2.5 mg/ 850 mg) sample.

**CONCLUSION:**

The proposed isocratic RP-HPLC method having a dual application for the simultaneous estimation of Linagliptin and Metformin hydrochloride and impurity profiling of Metformin Impurity-A proved to be very efficient. It is a very sensitive, rapid, simple, accurate and precise method. Here the analytes and their degradants were resolved and eluted on Enable C18 (250x 4.6 mm, 5 µm) column using Methanol-Buffer pH 5.5 (60:40, v/v) as mobile phase. The flow rate of 1ml/min was maintained. The peaks were analyzed at 228 nm wavelength. The peaks of the analytes and the degradants were well resolved and no co-elution of peaks was observed. Beer-Lambert’s law was obeyed in the concentration range of 0.015 - 40 µg/ml for Linagliptin and 0.02 - 20 µg/ml for Metformin. The correlation coefficient (‘r ’ value) for both Linagliptin and Metformin was 0.9995. The %RSD of the precision studies and tablet analysis is less than 2% and the percentage recoveries were obtained within the limit 98 to 102%, indicating high degree of precision and accuracy. The method also proved to be robust.

Research using the same RP-HPLC method, also aided the identification of the Metformin degradant formed during the forced degradation studies having RRT 1.54 as Metformin Impurity-A (Dicyandiamide also called as 1- Cyanoguanidine). The method was validated using the ICH Q2 (R1) guidelines for its application of Impurity profiling of Metformin Impurity-A.
The sensitivity, rapidness as well as its ability to resolve and identity Metformin Impurity-A makes our developed RP-HPLC method novel and more efficient than any other previously reported analytical methods.

This research would benefit Pharmaceutical manufactures to decide the storage conditions for Linagliptin and Metformin hydrochloride in bulk drugs as well as in combined dosage form. The proposed analytical work could be used in Quality control departments not only to assay the above two analytes in bulk form and in multicomponent formulations. Additionally, our method can be applied to do a quality check of the product to study the related substances of Linagliptin and Metformin hydrochloride formed during the Stability studies. Hence our analytical method would be beneficial for the vendor qualification of Linagliptin and Metformin hydrochloride APIs and well as for Product formulation of the two analyte combination.

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