Development and Validation of A Stability Indicating Method for Simultaneous Determination of Metformin Hydrochloride and Linagliptin in A Formulation by RP-HPLC

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ABSTRACT
Objective: Present study was aimed at the development of a simple, rapid and validated liquid chromatographic method for the simultaneous determination of Linagliptin and Metformin hydrochloride in a fixed dose combination. Methods: The method was developed on a LiChrosphere 100 RP 18e (125×4.0 mm, 5 μm) column with the mobile phase composed of 70:30 (v/v) mixture of methanol and 0.05 M potassium dihydrogen orthophosphate (pH 4.6 adjusted with o-phosphoric acid). Absorption of the elution was measured at 267 nm. The developed method was subjected to validation according to ICH guidelines. Results: The retention time of Linagliptin and Metformin hydrochloride were found to be 8.3 and 4.6 min, respectively. The calibration curves of two drugs showed a good correlation coefficient (>0.990) over a concentration range 1-6 µg/mL for Linagliptin and 200-1200 µg/mL for Metformin hydrochloride. The method was found to be stability indicating for both MET and LIN that separated the stress degradation impurities from the respective parent peak. Conclusion: The method was validated for linearity, accuracy and precision were found to be acceptable over the linear range of the Linagliptin and Metformin hydrochloride. The method was found suitable for the routine analysis of Linagliptin and Metformin hydrochloride in pharmaceutical dosage forms.

Keywords: Chromatography, linagliptin, metformin, RP-HPLC, simultaneous estimation

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INTRODUCTION
Type 2 diabetes mellitus is a metabolic disorder characterized by enhanced blood-glucose levels as an effect of various reasons that includes decreased pancreatic insulin secretion, increased peripheral insulin resistance, increased hepatic glucose production, impaired lipolysis, gastrointestinal incretin deficiency/resistance, α-cell hyperglucagonemia, increased renal glucose reabsorption, and neurotransmitter dysfunction [1]. The treatment of diabetes is mainly targeted towards achieving the normoglycemic levels. Lifestyle modification and Metformin hydrochloride (MET) is majorly used as first-line therapy for the treatment of diabetes [2]. However, the long term usage of the drugs for diabetes like chronic disease necessitates the addition of another drug or drugs. Dipeptidyl peptidase (DPP)-4 inhibitors represent a new therapeutic approach to the treatment of type 2 diabetes that functions to stimulate glucose-dependent insulin release and reduce glucagon levels [3]. Recently, DPP-4 inhibitors have been recommended for the treatment of diabetes mellitus to improve glycemic control [4]. DPP-4 inhibitors are the preferred drug class to use with metformin due to the advantages like different glucose-lowering mechanism and advantages like low incidence of hypoglycemia and weight gain [4]. Among the available DPP-4 inhibitors, Linagliptin (LIN) is the drug of choice to be used with MET as a fixed dose combination for better glycemic control [1]. A survey of the literature revealed some HPLC and LC-MS/MS methods for analysis of MET alone and in combination with other drugs [5-23].
However, quite a few HPLC methods have been reported for simultaneous determination of MET with LIN in formulations [24-25]. The development of a stability indicating analytical method for multiple active components in a fixed dose combination has been ever in demand. In view of this, there was a need to develop simple, precise, accurate and stability indicating RPHPLC method for LIN and MET that can be employed for the simultaneous estimation of the studied drugs in their pharmaceutical dosage forms. Hence, the objective of the current study was to develop a validated stability indicating RPHPLC method for the simultaneous estimation of LIN and MET in their dosage form.

**MATERIALS AND METHODS**

**Chemicals and reagents**
LIN (USP) was a generous gift from MSN Pharmachem Pvt. Ltd, Hyderabad, India. MET (USP) was obtained as a gift from Aurobindo Pharmaceuticals Ltd, Hyderabad, India. Methanol (HPLC) and acetonitrile (HPLC) were purchased from Sigma Aldrich Ltd, Bengaluru, India. Water (HPLC) was obtained in-house from a deionization system coupled with Synergy185 system (Millipore Billerica, MA, USA). All the other chemicals used were of analytical grade purchased from Merck Chemicals, Mumbai, India. Fixed-dose combination tablet containing 2.5 mg of LIN and 500 mg of MET (Jentadueto®) was obtained from Eli Lily, USA.

**Instrumentation and chromatographic conditions**

The HPLC system consisted of a Shimadzu LC-10AT pump, a Rheodyne 7725i sample injector with a 20 μL loop and a Shimadzu SPD-M10Amp diode array detector. The data acquisition was performed by processing software “LC Solution” (Shimadzu Corp., Kyoto, Japan). The method was developed on a LiChrosphere 100 RP 18e (125 × 4.0 mm, 5 μm) column maintained at an ambient temperature. The mobile phase was 70:30 (v/v) mixture of methanol and 0.05 M potassium dihydrogen orthophosphate (pH 4.6 adjusted with orthophosphoric acid) delivered at a flow rate of 0.6 mL/min. The column was maintained at 25°C and the absorption of the elution was measured at 267 nm. The injection volume was kept at 20 μL.

**Preparation of standard and sample solutions**

**Working standard solution**

The stock solution of LIN and MET was prepared by dissolving accurately weighed amount of drugs in 10 mL methanol, followed by sonication for 5 minutes to obtain a final concentration of 250 μg/mL LIN and 50,000 μg/mL MET and further dilutions were prepared in methanol to obtain working standards of different concentrations.

**Sample solutions**

Ten tablets, Jentadueto (Boehringer Ingelheim and marketed by Eli-lilly, USA) containing 2.5 mg of LIN and 500 mg of MET were weighed and finely powdered. A quantity of powder equivalent to 2.5 mg of LIN and 500 mg of MET was weighed and transferred to a standard flask, from this stock solution the drug solutions of 250 μg/mL LIN and 50,000 μg/mL of MET was prepared. Working standard solution of LIN (1-6 μg/mL) and MET (200-1200 μg/mL) were prepared from the stock solution.

**Specificity (Stability studies)**

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. It can be performed by various methods and among them one is analyzing the sample after subjecting to degradation studies, which results in to analyte degradation. The Forced Degradation studies were performed by exposing the drug solution to stress conditions like acidic (0.1 N HCl, 1 N HCl), basic (0.1 N NaOH, 1 N NaOH), peroxide (3% H₂O₂ v/v) and photo induced degradation (exposure to direct sunlight). The resulting solutions were analyzed for analyte peak and unknown degradants generated by stress induced degradation.

**Acid induced degradation**

Stock solution (two mL) was added separately to two ml of hydrochloric acid (0.1 N, 1N) in 10 mL volumetric flasks. The resultant solution was refluxed for 1 h at 80 ºC. These samples were cooled to room temperature, neutralized and diluted to the
volume with methanol. From this, the samples equivalent to 6 μg/mL and 1200 μg/mL of LIN and MET were analyzed.

**Alkali induced degradation**

Stock solution (two mL) was added separately to two ml of sodium hydroxide (0.1 N, 1N) in 10-mL volumetric flasks. The resultant solution was refluxed for 1 h at 80 °C. The samples were cooled to room temperature, neutralized and diluted to the volume with methanol. From this, the samples equivalent to 6 μg/mL and 1200 μg/mL of LIN and MET were analyzed.

**Peroxide induced oxidative degradation**

Stock solution (two mL) was added to two mL of hydrogen peroxide (3% v/v) in a 10-mL volumetric flask. The resultant solution was refluxed for 30 minutes at 80 °C. Volume was made up to 10 mL with methanol. From this, the samples equivalent to 6 μg/mL and 1200 μg/mL of LIN and MET were analyzed.

**Photolytic degradation**

Stock solution (two mL) was added to two mL of methanol in a 10-mL volumetric flask, and the resultant solution was exposed to direct sunlight for 24hrs. The samples equivalent to 6 μg/mL and 1200 μg/mL of LIN and MET were analyzed.

**RESULTS AND DISCUSSION**

After conducting various trials by using different mobile phase containing buffers of various pH, better peak shapes for LIN and MET were observed with the mobile phase that composed of 70:30 (v/v) mixture of methanol and 0.05 M KH2PO4 (pH adjusted to 4.6 with o-phosphoric acid). The flow rate of the mobile phase was kept at 0.6 mL/min. The retention times of LIN and MET were 8.3 minutes and 4.6 minutes, respectively (Fig. 1).

![Figure 1: Standard chromatogram of MET (4.628 min) and LIN (8.393 min).](image)

**Method validation**

The developed method was validated for precision, accuracy, specificity, linearity, limit of detection (LOD) and limit of quantitation (LOQ), and robustness. Validation of the proposed method was carried in accordance with the International Conference on Harmonization (ICH) guidelines [26].

**Linearity**

The analytical methods are tested for linearity, as it indicates its ability (within a given range) to obtain test results, which are in proportion to the concentration of the analyte in the testing sample. The method linearity was tested by plotting peak area against concentration of solutions. The plot of peak area versus the respective concentrations of LIN and MET were found to be linear over the concentration range of 1-6 μg/mL and 200-1200 μg/mL, respectively. The regression equation for LIN was $y=25179x - 1061$ with a coefficient of correlation ($R^2$) of 0.99. The regression equation for MET was $y=864.3x - 11662$ with a coefficient of correlation ($R^2$) of 0.99. In the tested concentration ranges, the linearity data has shown an excellent correlation between the peak area and concentration of analytes. The linearity concentration range of the present method was superior to the already reported methods Kavithaat al [25], Thakare et al [27] and Swamy et al [28] and. The calibration curves were shown in (Fig. 2) (for LIN) and (Fig. 3) (for MET).
Limit of Quantitation (LOQ) and Limit of Determination (LOD)

Limit of detection (LOD) and limit of quantitation (LOQ) values were calculated by analyzing the standard drug sample at various concentrations. LOD values for each compound was calculated as the signal to noise ratio equals to three (S/N=3) and the LOQ of the assay were evaluated as signal to noise ratio equals to ten (S/N=10). LOD values for LIN and MET were found to be 0.069µg/mL and 0.025µg/mL, respectively. Further, LOQ values were found to be 0.211µg/mL and 0.077 µg/mL for LIN and MET, respectively.

Precision

The precision of the analytical method was determined by replicating the process in six times at three different concentrations of LIN and MET (combination) and chromatograms were recorded. Precision was calculated as a percentage relative standard deviation. The % relative standard deviation of LIN (2, 3, 4 µg/ml) and MET (400, 600, 800 µg/ml) was found less than 2%, which is in accordance with ICH guidelines. Thus, the method was found precise (Table 1 & 2).

Table 1: Precision study of proposed stability-indicating HPLC method for LIN

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Intra-day Precision (n = 6)</th>
<th>Intermediate precision (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean. ± SD</td>
<td>% RSD</td>
</tr>
<tr>
<td>2</td>
<td>52430.3 ±428.93</td>
<td>0.81</td>
</tr>
<tr>
<td>3</td>
<td>76377±438.94</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>101611 ± 678.61</td>
<td>0.66</td>
</tr>
</tbody>
</table>
Table 2: Precision study of proposed stability-indicating HPLC method for MET

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Intra-day Precision (n = 6)</th>
<th>Intermediate precision (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>% RSD</td>
</tr>
<tr>
<td>2</td>
<td>352618 ± 3151.5</td>
<td>0.94</td>
</tr>
<tr>
<td>3</td>
<td>506912 ± 7654.10</td>
<td>1.50</td>
</tr>
<tr>
<td>4</td>
<td>695541.3 ± 8613.58</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Accuracy (recovery)

Accuracy of the developed method was performed by the standard addition method by adding known concentration standard solutions to the pre analyzed samples at different levels, i.e., 50, 100 and 150%. The mean recovery and RSD values were calculated. Recoveries of LIN (Table 3) and MET (Table 4) were in between 98-102%. This is in accordance with ICH guidelines. Thus, the method was found to be accurate.

Table 3: Accuracy of LIN in term of % recovery (n = 3)

<table>
<thead>
<tr>
<th>Spiked amount (µg/ml)</th>
<th>Amount detected ± SD</th>
<th>Mean % recovered</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (50%)</td>
<td>2.98 ± 0.03</td>
<td>99.43</td>
<td>1.04</td>
</tr>
<tr>
<td>2 (100%)</td>
<td>4.02 ± 0.03</td>
<td>100.36</td>
<td>0.76</td>
</tr>
<tr>
<td>3 (150%)</td>
<td>5.01 ± 0.06</td>
<td>100.26</td>
<td>1.13</td>
</tr>
</tbody>
</table>

Robustness

The robustness of the method was studied by deliberate changes in the method like alteration in, flow rate, percentage organic content, pH of the mobile phase and changes in the wavelength. It was observed that there were no distinct changes in the chromatograms demonstrating that the robustness of the developed HPLC method.

Table 4: Accuracy of MET in term of % recovery (n = 3)

<table>
<thead>
<tr>
<th>Spiked amount (µg/ml)</th>
<th>Amount detected ± SD</th>
<th>Mean % recovered</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 (50%)</td>
<td>600.43 ± 0.85</td>
<td>100.05</td>
<td>0.15</td>
</tr>
<tr>
<td>400 (100%)</td>
<td>800.20 ± 1.76</td>
<td>99.94</td>
<td>0.21</td>
</tr>
<tr>
<td>600 (150%)</td>
<td>1004.23 ± 9.35</td>
<td>100.42</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Specificity

Under various conditions the stability studies for LIN and MET were conducted. The results of the stress degradation studies were shown in (Table 5). The LIN was found relatively stable than MET under acidic and basic degradation conditions, whereas MET found more stable than LIN in peroxide and photolytic degradation studies. Though both the drugs were degraded under acidic and basic conditions, the drugs were proven to be relatively more stable under acidic conditions than the basic conditions. The proposed method was proven to be specific for the analytes, in view of the fact that none of the degradants peak from stability studies, and formulation excipients have affected analyte peaks. This proves that the method was found suitable and selective in the routine analysis.

Table 5: Stress induced degradation studies (n = 3)

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>Time (h)</th>
<th>Mean % recovered ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl (0.1 N)</td>
<td>1</td>
<td>93.23 ± 2.12</td>
</tr>
<tr>
<td>HCl (1.0 N)</td>
<td>1</td>
<td>75.52 ± 1.49</td>
</tr>
<tr>
<td>NaOH (0.1 M)</td>
<td>1</td>
<td>85.86 ± 1.26</td>
</tr>
<tr>
<td>NaOH (1.0 M)</td>
<td>1</td>
<td>62.19 ± 1.97</td>
</tr>
<tr>
<td>H2O2 (3% v/v)</td>
<td>1</td>
<td>30.87 ± 0.75</td>
</tr>
<tr>
<td>Daylight</td>
<td>24.0</td>
<td>6.24 ± 0.38</td>
</tr>
</tbody>
</table>

LIN MET
CONCLUSION
The developed HPLC method is simple, accurate, precise and reliable for the simultaneous estimation of LIN and MET in combined dosage form. Further, the noninterference from analyte degradants and formulation excipients showed that the method is suitable for routine quantitative simultaneous estimation of both the drugs in fixed dose combination of pharmaceutical preparation.

ACKNOWLEDGEMENT
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CONFLICT OF INTEREST
The authors do not have any conflict of interest.

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