Simultaneous Determination of Metformin and Three Gliptins in Pharmaceutical Formulations Using RP HPLC: Application to Stability Studies on Linagliptin Tablet Formulation

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ABSTRACT
Objective: A simple, rapid and validated liquid chromatographic method has been developed for the simultaneous determination of three novel Gliptins namely Vildagliptin (VLD), Sitagliptin (SIT) and Linagliptin (LIN) in their binary mixture with Metformin (MET).

Methodology: The separation was performed on fast monolithic column using isocratic, mobile phase consisting of mixture of sodium dihydrogen phosphate, Sodium dedosyl sulphate and acetonitrile. The flow rate was 2.5 mL/min and UV detection for MET, VLD and SIT was conducted at 208 nm, whereas, that of MET and LIN was at 228 nm. The incipiently developed method was subjected for validation according to ICH guidelines.

Results: The calibration curves of analytes showed good correlation (r²>0.999) over a concentration range 10–100 μg/mL and 50–400 μg/mL for Metformin, 1–10 μg/mL for Vildagliptin & Sitagliptin and 0.25–2.0 μg/mL for Linagliptin.

Conclusion: All three tablet formulations were assayed with accuracy and precision and without interference from excipients. The method is also stability indicating with respect to Linagliptin.

Keywords: Metformin, Gliptins, Simultaneous determination, HPLC, degradation.

INTRODUCTION
Since the discovery of Gliptins, a dipeptidyl peptidase-4 (DDP-4) inhibitors, a number of structural modifications have been made in order to increase their antidiabetic activity. Studies have shown that DDP-4 inhibitors (Vildagliptin, Sitagliptin and Linagliptin) showed better glycemic control when given along with Metformin.1–4

Vildagliptin [(S)-1-[N-(3-hydroxy-1-adamantyl) glycyl]pyrrolidine-2-carbonitrile VLD; Figure 1(A)] is a potent oral hypoglycemic agent. Sitagliptin (SIT) 7-[(3R)-3-Amino-1-oxo-4-(2,4,5-trifluorophenyl) butyl]-5,6,7,8-tetrahydro-3-(trifluoro methyl)-1,2,4-triazolo [4,3-a]pyrazine phosphate (1:1) monohydrate [Figure 1(B)] and Linagliptin (LIN), 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 [Figure 1(C)] are novel antidiabetic agents. Gliptins acts by inhibiting dipeptidyl peptidase-4 (DPP-4) and increase levels of active peptide hormones, such as glucagon like peptide-I (GLP-I) and glucose dependent insulinitropic peptide (GIP)1 and displays better glycemic control in patients with type 2 diabetes.1–4

Metformin HCl [N,N-dimethylimidodicarbonimidic diamide hydrochloride, (MET) Figure 1(D)] is frequently used as standard first-line pharmacotherapy to treat diabetic patients whose condition is uncontrolled by diet and who fail to respond to therapy with
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sulfonylurea.\textsuperscript{5} MET acts primarily by suppressing pancreatic $\beta$-cell function. Type 2 diabetes is progressive in nature and glycemic control become difficult in most patients, requiring either increased Metformin dosage or supplementary (add-on) therapy.\textsuperscript{6}

A survey of the literature revealed some HPLC and LC-MS/MS methods for analysis of MET alone\textsuperscript{7–13} and in combination with other drugs. However, quite a few HPLC methods have been reported for simultaneous determination of MET with SIT\textsuperscript{14–22} or VLD\textsuperscript{23–28} or LIN\textsuperscript{29,30} in formulations, but, no simple and rapid method is yet established for simultaneous determination of Metformin, Sitagliptin, Vildagliptin and Linagliptin using single analytical procedure. Development of single analytical procedure has an advantage of analyzing all drugs alone or in combination using same column and single mobile phase. Hence, for this study we report a rapid, sensitive and reliable HPLC method for simultaneous estimation of Metformin and three Gliptins (Vildagliptin, Sitagliptin and Linagliptin) in combined formulations.

Instrumentation and Chromatographic Conditions
Shimadzu High prominence liquid chromatographic system consisting of a 20 AT pump, coupled with UV detector, column oven and a shimadzu software lab solutions was used for this study. Liquid chromatographic separation was performed on chromolith RP-HPLC (Merk, Germany) (50 X 4.6 mm, i.d. 5µm particle size) column. The final mobile phase consisted of a mixture of 10 mm sodium dihydrogen phosphate and 10 mm SDS 64% and acetonitrile 36% (pH 5.5). The flow rate was fixed at 2.5 ml/min and the analytes were detected at UV wavelength of 208 nm for MET, VLD and SIT and 228 nm for MET and LIN at 30°C.

Reagents: Working standards of Metformin (98.9%) was donated by Kannika Parameshwari Pharmaceuticals (Belgaum, India), whereas other standards Vildagliptin (99.2%), Sitagliptin (98.7%) and Linagliptin (99.1%) were purchased from Biochemix, Hyderabad, India. The branded solid dosage forms containing 500 mg Metformin and 50 mg Vildagliptin, 500 mg Metformin and 50 mg Sitagliptin and 500 mg Metformin and 2.5 mg Linagliptin were purchased from the pharmacy. HPLC grade solvents acetonitrile and methanol and analytical grade sodium dedocyl sulphate, sodium dihydrogen phosphate and orthophosphoric acid were purchased from Sigma Aldrich.

Figure 1: Chemical Structure of Vildagliptin (A), Sitagliptin (B), Linagliptin (C) and Metformin HCl (D)
Preparation of Stock Solution:

Stock solutions of Metformin and Gliptins were prepared by weighing 100 mg each and dissolving in 100 ml of methanol in volumetric flask (100 ml) to get a working concentration 1 mg/ml. Thereafter 10 ml of Sitagliptin, Vildaglipitin each and 1 ml of Linagliptin from the working concentration were transferred into separate volumetric flasks (100 ml) and volume was adjusted up to the mark with mobile phase.

Calibration Standards

Metformin solution was diluted appropriately with mobile phase to get a concentration in the range of 10 μg/mL to 400 μg/mL, whereas Sitagliptin and Vildaglipitin were diluted to get in the range of 1 μg/ml to 10 μg/mL. The calibration standard solution of Linagliptin was prepared in the range of 0.25 to 2.0 μg/mL with mobile phase.

Preparation of Sample Solutions

Ten tablets of Metformin-Sitagliptin, Metformin-Vildaglipitin and Metformin-Linagliptin were weighed separately and crushed. The powder equivalent of 50 mg Metformin and 5 mg Sitagliptin and Vildaglipitin respectively were weighed and transferred in to 100 ml volumetric flask and treated with 25 ml of methanol to dissolve the analytes. Volumetric flasks were sonicated for 15 min and volume was adjusted up to the mark with methanol and filtered through 0.45-micron membrane filter paper. The resulting solutions were further diluted with mobile phase to get the final concentration of 50 μg/mL for Metformin and 5 μg/mL for Sitagliptin and Vildaglipitin respectively.

Similarly, Metformin-Linagliptin sample solutions were prepared to get the final concentration of 200 μg/mL for Metformin and 1 μg/ml for Linagliptin.

Method Validation

The newly developed method was validated in terms of linearity, limits of quantitation, recovery studies, specificity, accuracy and precision, robustness and stability studies according to International Conference on Harmonization (ICH) guidance.31

Accuracy:

To determine the accuracy of the method recovery study was performed by standard addition method. To the preanalyzed sample, standard analytes were added at three different levels (50, 100 and 150%) and analyzed. Thereafter the mean recovery and its RSD were calculated.

Limit of Quantitation (LOQ) and Limit of Determination (LOD)

LOD and LOQ were calculated to establish the sensitivity of the method and to monitor the stability of Linagliptin. LOD values for each compound was calculated based on the S/N=3 criterion and the Limits of quantitation of the assay were evaluated also based on the S/N=10 criterion.

Robustness

The robustness of the proposed method was determined by varying chromatographic conditions. Parameters such as flow rate, pH, and wavelength were varied by ± 0.1 ml/min, ± 0.1, and ± 2 nm, respectively, and changes in the resolution and peak symmetry were recorded.

Accelerated Stability Studies of Linagliptin

Linagliptin was partially degraded by exposing the drug to the various forced degradation conditions in order to study the interference of any degradation products with the analysis of Linagliptin.32 The samples for the degradation study were prepared by dissolving Linagliptin tablet (5 mg/tablet) in methanol and then diluted with water, 0.1N hydrochloric acid, 0.1N sodium hydroxide or 0.3% hydrogen peroxide solution in water to get a concentration of 25 μg/mL. These solutions were subjected to forced degradation studies and later mobile phase was added to get its concentration in the range of the calibration curve.

Acid-Base Hydrolysis:

Separately 0.1N hydrochloric acid or 0.1N sodium hydroxide solution was added to Linagliptin stock solutions for acid and base degradation studies and stored at 40°C for 72 hours. Then the samples were neutralized, diluted with the mobile phase and then analyzed using the optimized HPLC method.

Thermal Stress Study:

Solution for thermal stress study was prepared by heating the Linagliptin solution in water for 24 h at 80°C. Later the sample was slowly cooled to room temperature, filtered using syringe filter and analyzed.
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**Chemical Oxidation Study:**
Solution for oxidative stress was prepared by mixing 10 ml of 0.3% hydrogen peroxide solution with Linagliptin solution and heated at 40°C for 72 h. The samples were filtered using syringe filter and diluted with mobile phase and analyzed.

**Photo Degradation Study:**
The Linagliptin solution was exposed to UV light at 254 nm directly for 72 h. Then, the solution was filtered using syringe filter and diluted with the mobile phase and analyzed.

**RESULTS AND DISCUSSION**
During past two decades monolithic columns were extensively used for separation and quantification of pharmaceuticals in formulation. Hence, using the chromolith RP-HPLC columns various chromatographic conditions were tried to optimize the mobile phase composition. Previous reports of MET analysis by reverse-phase HPLC reported the use of SDS to increase the retention time of MET, thus, we decided to use SDS along with sodium hydrogen phosphate in our method to help fine tune the chromatographic separation. Several mobile phase compositions containing different type and percent of organic solvent, sodium hydrogen phosphate and SDS were attempted. Other factors that were varied to fine tune the chromatographic separation were the concentration of SDS, buffer, and the pH of the mobile phase. Finally, mobile phase consisted of 10 mm sodium dihydrogen phosphate (32%), 10 mM Sodium Dodecyl Sulphate (32%) and acetonitrile (36%) at pH 4.5 were found to be optimal for good separation of analytes with equally high sensitivity. Similarly, a flow rate of 2.5 mL/min was chosen because it allowed good separation of analytes with symmetric peaks at a reasonable time.

**METHOD VALIDATION**
Newly proposed method was subjected to validation process to satisfy the requirements of ICH guidelines. Freshly prepared stock solutions were used to establish system suitability tests. The variation in selectivity, retention time, resolution, and peak asymmetry were well within the acceptable ranges for analytes. The analytes had good resolution of 1.69 resolution factor between MET and VLD, 7.68 between MET and SIT and 5.4 min between MET and LIN. The tailing factors were well within the acceptable range, showing 1.15, 1.05, 1.12 and 1.10 for MET, VLD, SIT and LIN respectively.

The drug concentrations and peak area were plotted to construct a calibration curve. Better linearity was established with excellent correlations (>0.999) for all analytes were observed. Calibration curve data has shown in (Table 1). The LOQ and LOD were determined for all the analytes and were also recorded (Table 1). The low detection and quantification concentrations of the analytes reflect the good sensitivity of the reported procedure.

The mean percentage recovery was calculated to assess the accuracy of the newly developed method. The mean recoveries were from 99.16% to 100.40% for the added analytes from the formulations (Table 2), representing good accuracy of the method.

The precision of the proposed method was assessed in terms of intermediate precision. Intraday and interday precision were undertaken to determine the reproducibility of the process. The % RSD values for the interday and intra-day measurements for analytes were less than 2%, the results listed in (Table 1) show that the proposed procedure is precise.

Comparison of chromatograms of blank and pure samples with those of formulation showed that the tablet excipients did not interfere with the retention times of the analytes. Representative chromatograms acquired from formulations containing of MET with VLD, SIT and LIN are presented in (Figures 2, 3, and 4).

The robustness of the suggested method was confirmed by performing the analysis with modifications to the flow rate of the mobile phase, mobile phase pH, and detection wavelength, and resolution and tailing factors were recorded. The resolution factors obtained were 1.55–1.79, 7.65–7.93, and 5.34–5.88 for MET-VLD, MET-SIT and MET-LIN respectively and tailing factors were 1.1–1.2, 1.05–1.24, 1.02–1.2 and 1.09–1.18 for MET, VLD, SIT and LIN respectively. The results showed that slight modifications did not affect the resolution and tailing factor, indicating good robustness of the developed HPLC method.

**Degradation Behavior**
Forced degradation studies were conducted on Linagliptin under several conditions and the consequent chromatograms are as illustrated in Figure 5. Linagliptin proved to be relatively stable under acidic condition resulting in only minor degradation; however when LIN was subjected to basic condition approximately 25% degradation was observed. LIN was unstable to oxidative stress, resulting in more than 40% degradation using 0.3% hydrogen peroxide. When LIN was exposed to direct UV light the recovery was 98.7% and it was 97.8% when exposed...
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Table 1: Chromatography Parameters Results and Results of HPLC Analysis of Metformin with Gliptins.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MET</th>
<th>VLD</th>
<th>SIT</th>
<th>MET</th>
<th>LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (min)</td>
<td>0.78</td>
<td>1.18</td>
<td>3.83</td>
<td>0.76</td>
<td>2.65</td>
</tr>
<tr>
<td>Wavelength of detection</td>
<td>208 nm</td>
<td>208 nm</td>
<td>208 nm</td>
<td>228 nm</td>
<td>228 nm</td>
</tr>
<tr>
<td>Linearity range (μg/mL)</td>
<td>10-100</td>
<td>1-10</td>
<td>1-10</td>
<td>50-400</td>
<td>0.25-2</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-2424.83</td>
<td>-3372.82</td>
<td>-3815.96</td>
<td>85417.72</td>
<td>369.17</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>33726.91</td>
<td>9768.48</td>
<td>19933.09</td>
<td>42206.51</td>
<td>197193.92</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
<td>0.9996</td>
<td>0.9997</td>
<td>0.9996</td>
<td>0.9994</td>
</tr>
<tr>
<td>LOD (μg/mL)</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
<td>0.09</td>
<td>0.02</td>
</tr>
<tr>
<td>LOQ (μg/mL)</td>
<td>0.04</td>
<td>0.097</td>
<td>0.064</td>
<td>0.29</td>
<td>0.067</td>
</tr>
<tr>
<td>Inter-day(%RSD)</td>
<td>1.28</td>
<td>0.97</td>
<td>1.22</td>
<td>1.89</td>
<td>1.07</td>
</tr>
<tr>
<td>Intra-day(%RSD)</td>
<td>1.79</td>
<td>0.86</td>
<td>1.19</td>
<td>1.59</td>
<td>1.78</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug in dosage form</th>
<th>MET-VLD Tablet</th>
<th>MET-SIT Tablet</th>
<th>MET-LIN Tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET-VLD Tablet</td>
<td>99.61 ± 1.15</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MET-SIT Tablet</td>
<td>99.03 ± 0.98</td>
<td>100.88 ± 1.31</td>
<td>--</td>
</tr>
<tr>
<td>MET-LIN Tablet</td>
<td>--</td>
<td>--</td>
<td>100.83 ± 0.99</td>
</tr>
</tbody>
</table>

% RSD: Percent Relative Standard Deviation

to wet thermal degradation indicating the high stability towards water and photo degradation. Fortunately, the major degraded products found with basic and hydrogen peroxide along with other products formed in the forced degradation studies, did not interfere with its peak (Table 3).

Application of the Proposed Method

The validated analytical method was used to estimate MET, VLD, SIT and LIN in their solid dosage forms. In general, appropriate recovery was achieved using the proposed HPLC method for drugs in their formulations (99.03–101.14 %) (Table 1).

CONCLUSION

The proposed analytical method can be used for simultaneous determination of Metformin and Gliptins using the same mobile phase, which reduced the time required for changing the mobile phase and conditioning of the reverse-phase HPLC column. The validated analytical method is simple, rapid, specific, and robust and can be adopted for simultaneous determination of MET, with VLD, SIT or LIN in pharmaceutical formulations. Forced degradation studies on Linagliptin tablets showed that HPLC method is stability indicating method with respect to Linagliptin.
Table 2. Absolute Recoveries of MET and Gliptins from Pharmaceutical Formulation

<table>
<thead>
<tr>
<th>Amount Added (µg/mL)</th>
<th>% Recovery*</th>
<th>Amount Added (µg/mL)</th>
<th>% Recovery*</th>
<th>Amount Added (µg/mL)</th>
<th>% Recovery*</th>
<th>Amount Added (µg/mL)</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET</td>
<td>VLD/SIT</td>
<td>VLD</td>
<td>SIT</td>
<td>MET</td>
<td>LIN</td>
<td>MET</td>
<td>LIN</td>
</tr>
<tr>
<td>20</td>
<td>100.6</td>
<td>2</td>
<td>98.4</td>
<td>99.1</td>
<td>50</td>
<td>101.3</td>
<td>0.25</td>
</tr>
<tr>
<td>40</td>
<td>98.4</td>
<td>4</td>
<td>101.2</td>
<td>100.5</td>
<td>100</td>
<td>98.6</td>
<td>0.5</td>
</tr>
<tr>
<td>60</td>
<td>98.5</td>
<td>6</td>
<td>98.93</td>
<td>101.8</td>
<td>150</td>
<td>99.1</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean</td>
<td>99.16</td>
<td>99.51</td>
<td>100.46</td>
<td>99.66</td>
<td>100.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.D</td>
<td>1.24</td>
<td>1.48</td>
<td>1.35</td>
<td>1.43</td>
<td>1.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R.S.D</td>
<td>1.25</td>
<td>1.49</td>
<td>1.34</td>
<td>1.44</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average of two injections.

Figure 2. A Typical Chromatogram of Sample Solution Containing Metformin Hydrochloride and Vildagliptin
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Figure 3. A Typical Chromatogram of Sample Solution Containing Metformin Hydrochloride and Sitagliptin

Figure 4. A Typical Chromatogram of Sample Solution Containing Metformin Hydrochloride and Linagliptin
Table 3. Analysis of Linagliptin Under Various Stress Conditions.

<table>
<thead>
<tr>
<th>Stress Conditions</th>
<th>Time (h)</th>
<th>% Recovery of Linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (0.1 M HCl, stored at 40°C)</td>
<td>72</td>
<td>94.5%</td>
</tr>
<tr>
<td>Base (0.1 M NaOH, Stored at 40°C)</td>
<td>72</td>
<td>76.8%</td>
</tr>
<tr>
<td>Hydrogen peroxide (0.3%, Stored at 40°C)</td>
<td>72</td>
<td>57.9%</td>
</tr>
<tr>
<td>Water (Stored at 80°C)</td>
<td>24</td>
<td>97.8%</td>
</tr>
<tr>
<td>Exposure to UV light (254 nm)</td>
<td>72</td>
<td>98.7%</td>
</tr>
</tbody>
</table>

Figure 5. Chromatograms of forced degradation of Linagliptin with 0.1N hydrochloric acid (1), 0.1N sodium hydroxide (2), UV light (3) and 0.3% hydrogen peroxide (4).

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CONFLICT OF INTEREST

Authors have no conflict of interest.

REFERENCES


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