Development and validation of a new analytical RP-HPLC method for simultaneous determination of Glibenclamide and Atenolol in bulk

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ABSTRACT
A new, simple, reliable, fast, sensitive and economical RP-HPLC method was developed and validated for simultaneous estimation of two fixed-dose combinations frequently prescribed in coexisted chronic diseases such as diabetes (GLB) and hypertension (ATN) in bulk for the first time. The mobile phase used for the chromatographic runs consisted of 0.01N potassium dihydrogen ortho phosphate (pH 4.8) and acetonitrile (55:45, v/v). The separation was achieved on column (BDS C18 250 x 2.1mm, 1.6m) using isocratic mode. Drug peaks were well separated and were detected by a UV detector at 235.0 nm. The method was linear at the concentration range 2.5-15 $\mu$g/ml for Glibenclamide (GLB) and 6.25-37.5 $\mu$g/ml for Atenolol (ATN), respectively. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. The method was validated for system suitability, linearity, accuracy, precision, detection, quantification limits and robustness and was found it is acceptable in the range of 2.5–15 $\mu$g/ml for GLB and 6.25–37.5 $\mu$g/ml for ATN. The LOD and LOQ of GLB was found to be 0.48 $\mu$g/ml and 1.47 $\mu$g/ml and for ATN was found to be 0.72 $\mu$g/ml and 2.20 $\mu$g/ml, respectively. The method was applied to drug interaction studies of GLB with ATN to illustrate the scope and application of the methods to manage two different therapeutic classes of drugs, as they may co-administered in concurrent diseases.

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INTRODUCTION

Diabetes mellitus (DM) is by defects in insulin activity, insulin secretion or both which is a metabolic disorder characterized by hyperglycemia (International Diabetes Federation, 2015; Boyle et al., 2010). The reason for the disease may be not enough insulin is produced in Type I DM (T1DM), glucose is not moved out into cells Type 2 DM (T2DM) (Bilous et al., 2010) and another one is gestational diabetes that may occur during pregnancy. Complications such as stroke, coronary heart diseases, nephropathy, neuropathy and retinopathy make great contributions to mortality (Roglic and Unwin, 2010).

Diabetes mellitus have two to fourfold higher death rate due to cardiovascular diseases than others (Sowers et al., 2001). Various treatment approaches for hypertension to reduce the risk of such complications (Mogensen et al., 1991; Fuller et al., 1983) in patients with T2DM. Stringent control over
To achieve these aspirations in the majority cases, two to three drugs from different categories need to be prescribed (Munger, 2010). The chief oral antidiabetic drugs for diabetes mellitus are Metformin alone or in combination with the second-generation sulfonylureas viz. Glibenclamide (GLB), Gliclazide, Glimepiride, and Glipizide (Haupt et al., 1991). As compared to other sulfonylurea’s GLB provides convenient, effective and better tolerance (Giachetti et al., 1997).

Beta-blockers/calcium channel blocker with a renin inhibitor (Bell, 2009) are prime agents for control of hypertension. When compared with the individual use, the combined form provides better control of hyperglycemia, insulin resistance and blood pressure. However, when they are combined the in vivo safety of the drugs may get altered. Therefore, the potential drug interactions of the combination of the Atenolol (ATN) and GLB in terms of pharmacokinetic perspective need to be studied before going further. There are numerous validated methods available for analysis of ATN (Davidson et al., 2004; Aburuz et al., 2005; Georgita et al., 2007) and GLB (Mistri et al., 2007; Johnson and Lewis, 2006) Robert and Russell 2006) alone. There was no reported validated method for their simultaneous quantization in any type of biological sample.

The concept of combining agents with manifestly different indications has become a certainty of treatment in patients with a chronic disease where it requires treatment for several different pharmaceuticals (Porwal and Talele, 2017). As a primary treatment to diabetic hypertensive subjects, generally, a fixed-dose of GLB and ATN or combination of drugs is commonly prescribed (Sengupta et al., 2017). Fixed-dose combination of GLB and ATN shows effective control on glucose balance and better BP control in a diabetic hypertensive patient which are not tested literally. Given this background, the present study was envisaged to develop and validate the HPLC method as per ICH guidelines. There is no analytical method available to simultaneously quantitate ATN and GLB in a single run till date.

**EXPERIMENTAL PART**

**Chemicals and reagents**

GLB obtained from Sri Raghavendra Chemicals and Suppliers, Bangalore. ATN obtained from Indian Drugs, Hyderabad. Analytical/HPLC grade chemicals and solvents were obtained from Rankem (Hyderabad, India).

**Chromatographic apparatus and Condition**

A high-performance liquid chromatographic system (Water 2690) composed of PDA-2996 detector. Chromatographic integration, data acquisition and recording were performed by Empower 2 software. Optimization of the chromatographic condition is by using different mobile phase combination, columns and organic phases. The mobile phase flows with a flow rate of 1.0 ml/min. For retention of GLB & ATN (Figure 1) and a combination of actives, various HPLC methods were optimized and validated. Optimum separation conditions were obtained with BDS C18 250 x 2.1mm, 1.6m column, mobile phase taken in the ratio 55:45 consisting of 0.01N potassium dihydrogen ortho phosphate (pH 4.8) and acetonitrile (ACN) in gradient mode with an injection volume 1.0 ml with column oven temperature maintained at 30°C and elution monitored by a detector wavelength at 235.0 nm. Various methods have been described for the individual drug determination (El-Saharty, 2003; Sultana et al., 2008), but for the simultaneous analysis of these drugs, no method is reported till date.

**Preparation of standard solution**

2.5 mg of GLB & 6.25 mg of ATN were accurately weighed and diluted to 25 ml and add 10 ml of diluents. After 10min of sonication, the solution is diluted with diluents (100 μg/ml GLB & 250 μg/ml ATN). 1ml of the standard solution was diluted to 10ml with diluents (10μg/ml GLB & 25μg/ml ATN).

**Method validation**

According to ICH Guidelines, for ATN and GLB assay, the RP-HPLC method was validated by linearity, accuracy, precision, LOD, LOQ, and robustness.

**Accuracy**

Nearness between the expected value and the obtain value (value found) gives the analytical method accuracy. Accuracy of the developed method is by successive analysis (n = 3) of three different concentrations (50%, 100% and 150%) of standard drugs solution. Percent recovery (R %) of an analyte recovered is obtained. The data of the results were statistically analyzed.

**Precision**

When the method is applied repetitively in three different occasion’s precision of a technique is the degree of agreement among individual tests. By analyzing the calibration curves of different concentrations of drugs on three different days and within the
same day respectively in six replicates, the inter-day precision and intra-day precision was determined. The relative standard deviation (%RSD) of the method was expressed as total precision, where % RSD ≤ 2% was accepted.

**Linearity**

Linearity is the ability to obtain results of the experiment is directly proportional to the analyte concentration. Linearity was determined by a minimum of six calibration levels (25, 50, 75, 100, 125 and 150%). Coefficient of correlation, slope and intercept were used to estimate the linearity by using the calibration curve. The correlation coefficient ($r^2$) > 0.998 value is accepted for the data to the regression line.

**LOD and LOQ**

Limit of detection (LOD) and limit of quantification (LOQ) were considered based on the signal-to-noise ratio, which is 3 & 10, respectively. Actually, it is established by the minimum concentration at which
the analyte can be reliably detected and quantified by comparing blank samples with a known low concentration of an analyte.

**Robustness**

For robustness, the chromatographic conditions were changed deliberately using HPLC method. Reliability during normal usage is measured by its capacity to remain unaffected by small, but deliberate variations in method parameters.

**Degradation studies**

Stress factors suggested for common forced degradation studies such as acid degradation, base degradation, oxidation, photolysis, thermal degradation (FDA, 2000; Blessy et al., 2014).

**Sample Preparation**

10mg of GLB and 25mg of ATN powder were accurately weighed and diluted to 50 ml, by adding diluents and sonicated for 25 min, and filtered by HPLC filters (200μg/ml GLB & 500μg/ml ATN). Finally, filtered sample solution of 0.5ml was diluted to 10ml with diluents (10μg/ml GLB & 25μg/ml ATN).

**Acid Degradation Studies**

1ml of 5N Hydrochloric acid was added to 1 ml of sample solution GLB & ATN, and at 60°C it is refluxed for 30mins. The final solution was diluted to obtain a 10ppm, & 25ppm solution and injected (10 μL) solution into the system. The stability of the sample solution was determined by analysing chromatograms (Singh and Bakshi, 2000).

**Alkali Degradation Studies**

5N Sodium hydroxide was added to 1 ml of sample solution GLB & ATN, and at 60°C it is refluxed for 30 min. 10 μL of resulting solution (10ppm & 25ppm) was injected into the system and evaluate the stability of the sample by recorded chromatograms (Alsante et al., 2007).

**Peroxide degradation**

30% hydrogen peroxide (H₂O₂) was added to 1 ml

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*Figure 4: Chromatograms at 50% Recovery level (Triplicate)*
Figure 5: Chromatograms at 100% Recovery level (Triplicate)

Table 1: System suitability Results for HPLC.

<table>
<thead>
<tr>
<th>System suitability Parameters</th>
<th>GLB</th>
<th>ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>2.339</td>
<td>3.302</td>
</tr>
<tr>
<td>Area</td>
<td>408922</td>
<td>1041734</td>
</tr>
<tr>
<td>USP Plate Count</td>
<td>3595.0</td>
<td>5584.1</td>
</tr>
<tr>
<td>USP Tailing</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Resolution</td>
<td>-</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Figure 6: Chromatograms at 150% Recovery level (Triplicate)
Table 2: Recovery studies of GLB and ATN.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>% Level of recovery</th>
<th>Mean % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLB</td>
<td>50</td>
<td>99.15±0.12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>99.94±0.87</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>99.77±0.44</td>
</tr>
<tr>
<td>ATN</td>
<td>50</td>
<td>99.24±0.88</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.10±0.69</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>99.97±0.76</td>
</tr>
</tbody>
</table>

Table 3: System Precision studies of GLB and ATN

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Precision</th>
<th>GLB</th>
<th>ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% RSD</td>
<td>% RSD</td>
</tr>
<tr>
<td>1</td>
<td>Repeatability</td>
<td>406510 ± 3604.4</td>
<td>1067155±4525.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
<td>394228 ± 3959.0</td>
<td>984912±4210.8</td>
</tr>
<tr>
<td></td>
<td>Precision</td>
<td>± 1.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 4: Linearity studies of GLB and ATN

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Concentration of GLB (ppm)</th>
<th>AUC for GLB</th>
<th>Concentration of ATN (ppm)</th>
<th>AUC for ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.5</td>
<td>107857.6</td>
<td>6.25</td>
<td>256152.3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>204184.6667</td>
<td>12.5</td>
<td>506781</td>
</tr>
<tr>
<td>4</td>
<td>7.5</td>
<td>315382</td>
<td>18.75</td>
<td>754455</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>404592.6667</td>
<td>25</td>
<td>1040950</td>
</tr>
<tr>
<td>6</td>
<td>12.5</td>
<td>515066.3333</td>
<td>31.25</td>
<td>1265461</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>611057.3333</td>
<td>37.5</td>
<td>1503674</td>
</tr>
<tr>
<td>8</td>
<td>Slope</td>
<td>40685.7</td>
<td>Slope</td>
<td>40364.7</td>
</tr>
<tr>
<td>9</td>
<td>Y-Intercept</td>
<td>3162.66</td>
<td>Y-Intercept</td>
<td>4230.7</td>
</tr>
<tr>
<td>10</td>
<td>R2</td>
<td>0.999</td>
<td>R2</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Table 5: Robustness results for both GLB and ATN (acceptance limit RSD% < 2)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Robustness Parameter</th>
<th>% RSD for GLB</th>
<th>% RSD for ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flow rate (0.9 ml/min)</td>
<td>0.4</td>
<td>0.91</td>
</tr>
<tr>
<td>2</td>
<td>Flow rate (1.1ml/min)</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>Temperature 25°C</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>Temperature 35°C</td>
<td>0.79</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>Mobile phase (50B:50 A)</td>
<td>0.73</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>Mobile phase (60B:40A)</td>
<td>0.77</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 7: Linearity curves for GLB and ATN

Figure 8: Chromatogram for Acid degradation

Figure 9: Chromatogram for Base degradation
Table 6: Forced Degradation Studies of GLB and ATN

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>GLB</th>
<th>ATN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative Amount (%)</td>
<td>% Degradation</td>
</tr>
<tr>
<td>Acid</td>
<td>94.61</td>
<td>5.39</td>
</tr>
<tr>
<td>Alkali</td>
<td>95.36</td>
<td>4.64</td>
</tr>
<tr>
<td>Peroxide</td>
<td>91.32</td>
<td>8.68</td>
</tr>
<tr>
<td>Thermal</td>
<td>97.30</td>
<td>2.70</td>
</tr>
<tr>
<td>UV</td>
<td>98.33</td>
<td>1.67</td>
</tr>
<tr>
<td>Water</td>
<td>98.66</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Figure 10: Chromatogram for Peroxide degradation

Figure 11: Chromatogram for Thermal degradation

Photo Stability studies
The sample solution was kept for 7 days in UV Light chamber or in photo stability chamber at 200 Watt h/m² for studying photo stability studies. For assessing the stability of the sample solution, the resultant diluted solution (10 ppm & 25 ppm) of 10μL was injected into the system (Ahuja and Scypinski, 2001).

Neutral Degradation Studies
Neutral degradation studies were studied by drug refluxing in water for 6hrs at a temperature of 60 ºC. The...
resultant diluted solution (10 ppm & 25ppm), 10 μL of it were injected into the system for assessment of the stability of the sample (Blessy et al., 2014).

RESULTS AND DISCUSSION

Development and optimization of HPLC method

An isocratic LC method, coupled with PDA detection, was developed for the simultaneous determination of ATN and GLB. Chromatogram A and chromatogram B represents the blank mobile phase and an average retention time of 2.322 min for GLB and 3.260 min for ATN, and with no interfering peaks respectively (Figure 2&Figure 3), the suitability parameters were given Table 1, which indicates the specificity of the method. By using BDS C18 250 x 2.1mm, 1.6μm column and gradient elution technique using a mobile phase composed of Buffer: ACN pH 4.8 in a ratio of 55:45 and the most suitable separation with the highest resolution between peaks of GLB and ATN was achieved. However, drugs peaks are obtained at shorter retention times at a temperature of 30°C compared to those at 25°C. So, 30°C was chosen for the chromatographic measurement. According to the buffer capacity of phosphate, the pH of the buffer was selected, which leads to less variation in retention time and also resisted to pH changes.

According to ICH guidelines (International Council for Harmonisation), this method was validated. The following validation characteristics were addressed.

Accuracy

The developed HPLC method was assessed for accuracy in a laboratory at three different concentration levels (50%, 100% and 150%) within the working linearity range of both drugs of a standard mixture of ATN and GLB. Accuracy was expressed as the recovery% (ICH, 2005) and standard error for GLB and ATN. Table 2 and Figures 4, 5 and 6 shows the method accuracy for both drugs with satisfactory recovery%. These results reveal that a developed method was found to be accurate.

Precision

Information on the random errors was given by the
precision of a given method. The nearness of values obtained from multiple sampling of the same homogenous sample under prescribed condition (ICH, 2005) between a series of measurements. Both repeatability (intra-day precision) and intermediate precision (inter-day precision) were obtained. The results for precision were found to be within permissible limits (1% & 2% respectively) Table 3.

**Repeatability (intra-day)**

The Repeatability of the method was obtained by using the same analytical procedure, within the same laboratory on the same day, using the same equipment, by the same analyst between a series of measurements. Repeatability is by RSD% values obtained by repeating the assay (Table 3).

**Intermediate precision (Inter-day)**

The Intermediate precision was carried out as described under repeatability but on three successive days, each of two determinations (n = 6) (Table 3).

The standard for intra-day and inter-day precisions requires a RSD less than 2%.

**Linearity and Range**

The linearity of the method was assessed at a range of 2.5–15 ppm GLB and 6.25-37.5 ppm ATN. Calibration curves were plotted by taking peak areas against concentrations (Table 4 and Figure 7).

**LOD and LOQ**

LOD is the minimum concentration which can be detected, not quantified and LOQ is the minimum concentration with satisfactory precision and accuracy in a ratio of 3:1 and 10:1 respectively. The LOD and LOQ of GLB was found to be 0.48 μg/ml and 1.47 μg/ml and for ATN was found to be 0.72 μg/ml and 2.20 μg/ml respectively.

**Robustness**

One can determine the considerable or significant influence of factors on analytical results by performing a set of experiments and combining changes in conditions (see Table 5). No significant effect in resolution between two drugs peaks when there are small variations in column temperature, chromatographic flow and mobile phase composition (% acetonitrile) which was confirmed by the Standard means and averages.

**Degradation Studies**

Degradation Studies was carried out to exhibit explicitly the method developed to measure the changes in concentration of two drugs. For validation of chromatographic assays, between 5% and 20% has been accepted (Szepesi et al., 1991; Carr and Wahlich, 1990). For analytical validation, 10% degradation is optimal (Jenke, 1996; Blessy et al., 2014). The chromatograms of two drugs degradation are shown in Figures 8, 9, 10, 11, 12 and 13. For quantitative analysis of the drugs under various stress, the condition was done by the proposed HPLC method. The contents of stress testing are shown in Table 6. Under our developed auto-injector device, the combination drugs solution was stable and within the acceptance criteria in all degradation condition.

**CONCLUSIONS**

For simultaneous determination of ATN and GLB the developed method is suitable and valid because of small retention time separation for both peaks and good resolution. It can be used for routine analysis of GLB and ATN in combination products which reduces the multiple medical regimens. This method is simple, specific, linear, accurate and precise. Has ability to separate the two drugs which are usually found in the serum of diabetic hypertensive patients. The results of the present study benefits in the studies of drug interaction and also very profitable for pharmaceutical companies.

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