Stability Indicating HPLC Method for Simultaneous Estimation of Entacapone, Levodopa and Carbidopa in Pharmaceutical Formulation

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Abstract

In pharmaceutical industry, researchers aim at catering to the need of robust analytical methods for analysis of generic drug products. The paper deals with the pharmaceutical formulation - Entacapone, Levodopa and Carbidopa tablets for the treatment of Parkinson’s disease. The paper presents a simple and efficient stability indicating HPLC method that has been developed in a multi component drug formulation for simultaneous estimation of Entacapone, Levodopa and Carbidopa in presence of their related impurities. This HPLC method uses Cosmosol PE 150 × 4.6 mm, 5 µ HPLC column, phosphate buffer pH 2.5 and Methanol as mobile phase in gradient mode with UV detection at 280 nm. The method was validated and found to be precise, robust, accurate, linear (in range 0.05 to 0.15 mg/ml, 0.012 to 0.15 mg/ml and 0.003 to 0.038 mg/ml of Entacapone, Levodopa and Carbidopa respectively), and specific for 15 known impurities ensuring suitability of the method for quantitative determination of Entacapone, Levodopa and Carbidopa.

Keywords: Pharmaceutical formulation; HPLC method; Simultaneous estimation; Assay test; Multi component drug formulation; Parkinson

Introduction

Parkinson’s disease is a progressive, neurodegenerative disorder of the extrapyramidal nervous system affecting the mobility and control of the skeletal muscular system. Symptoms of Parkinson’s disease are related to depletion of dopamine. But administration of dopamine is ineffective in the treatment of Parkinson’s disease. This is because it does not cross the blood-brain barrier. However, levodopa, the metabolic precursor of dopamine, does cross the blood-brain barrier, and presumably is converted to dopamine in the brain. Carbidopa inhibits the decarboxylation of peripheral levodopa, making more levodopa available for transport to the brain. Entacapone is a selective and reversible inhibitor of catechol-O-methyltransferase (COMT). When entacapone is given in conjunction with levodopa and carbidopa, plasma levels of levodopa are greater and more sustained than after administration of levodopa and carbidopa alone.

It is very difficult to develop a stability indicating method for such triple combination drug products that is capable of analyzing each active ingredient in presence of their related impurities.

Literature survey revealed few methods for individual or combination product analysis. Spectroscopic methods for simultaneous estimation of Levodopa and Carbidopa [1], and LC estimation of Entacapone in tablets [2]. A method for in-vitro release of drugs is also found [3-5]. Publications were found on LC method for estimation using electrochemical detector [6] and LC method for estimation of levodopa and carbidopa using fluorescence detector [7-18]. Few official pharmacopical monographs for single and dual drug combinations [19-24] were also found. In the present study, we propose a rapid and stability indicating HPLC method for simultaneous estimation of Levodopa [(2S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid], Carbidopa [(2S)-3-(3,4-dihydroxyphenyl)-2-hydrizinio-2-methylpropanoic acid] and Entacapone [(2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide] in presence of their 15 process related or degradation impurities [25-29].

Materials and Methods

Reagents and materials

All analytical reagent grade (AR Grade) reagents were used for method development purpose. Acetonitrile (Merck) and Tetrahydrofuran (Merck) were used for standard and sample solution preparation. Orthophosphoric acid (Rankem) and Potassium dihydrogen orthophosphate (Merck) were used for mobile preparation. Milli-Q water (HPLC grade) was used for all solution preparations. Impurities and working standards of Entacapone, Levodopa and Carbidopa were obtained from Macleods Pharmaceuticals Limited, Mumbai, India.

Chromatographic system and conditions

Development study was performed on Shimadzu HPLC, consisting of UV-Visible, photodiode array detector and a quaternary gradient pump. Sample loop in the system was of 100 µl capacity. Cosmosol 5PE 150 × 4.6 mm, 5 µ (Nacalai Tesque, USA) HPLC column was used for chromatographic separation. Mobile phase consisted of phosphate buffer and methanol in gradient mode. Buffer consisted of 10mM potassium dihydrogen orthophosphate solution with pH adjusted to 2.5 using orthophosphoric acid. Flow rate was 1.0 mL/min and detection was carried out at 280 nm based on there wavelength maxima as per UV spectrum. Labsolutions software was used for data collection. For intermediate precision study, Agilent HPLC system

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Selection of mobile phase: Due to difference in acidity of levodopa/carbidopa and entacapone, low pH was selected to achieve optimum separation of all the peaks. Looking at the pH range of HPLC column, pH 2.5 was evaluated and found to be optimum.

Selection of HPLC column: Entacapone elute late on a C18 column even with 60% solvent in mobile phase (Figure 3). Levodopa and Carbidopa are polar in nature which makes them elute early on a non polar octadecyl (Inertsil ODS 250 mm × 4.6 mm, 5 µ) phase. In order to elute Entacapone early, a more polar phase was evaluated and selected for method development. Cosmosil PE, 150 × 4.6 mm, 5 µ was the column of choice. Phenyl phase is polar in nature but do not last long at low pH due to its weak bonding. Cosmosil PE column has an ethyl group attached to phenyl group which makes this column a rugged stationary phase with better column life than phenyl column. A 150 mm column was chosen to achieve a shorter run time.

Results and Discussion

Preliminary studies

There is no pharmacopoeial or literature reference of a suitable stability indicating assay test for the proposed triple combination formulation. The development of Assay method was initiated from USP method for Levodopa tablets which use a simple HPLC method including octadecyl stationary phase and phosphate buffer pH 2.0/ acetonitrile for mobile phase.

Selection of wavelength: Wavelength was selected based on absorbance maxima of three drugs as per UV spectrum. 280 nm was optimum for all the active ingredients (Figure 2).
Selection of HPLC pump mode: Entacapone do not elute early with a low solvent mobile phase. Hence, gradient mode was chosen and optimized for separation of active ingredients with a flow rate of 1 ml/min and run time of 17 minutes (Table 1).

Selection of diluent: The difference in solubility of the active ingredients makes it difficult to finalize an optimum diluent. Levodopa and Carbidopa dissolves in acidic and aqueous condition whereas entacapone dissolves in less polar solvent like acetonitrile. Looking at the difference in solubility, a combination of 1% orthophosphoric acid and acetonitrile in the ratio 60:40 (diluent 1) was suggested for stock solution preparation. But entacapone has a tendency to precipitate on standing with acetonitrile and methanol in diluent. For better solubility of entacapone and stability of solutions with improved peak shapes, second dilution was performed in 1% orthophosphoric acid and tetrahydrofuran in the ratio 80:20 (diluent 2). Higher percent of tetrahydrofuran is not recommended due to its corrosive nature.

Solution preparation

Standard preparation: About 50 mg of Entacapone and about 31.25 mg of Levodopa was accurately weighed and dissolved in 50 ml of Diluent 1 (Solution A). About 31.25 mg of Carbidopa was dissolved in 100 ml of diluent 2 (Solution B). Further, 10 ml of (Solution A) and 5 ml of (Solution B) was diluted to 100 ml with diluent 2.

Sample Preparation: To prepare the sample, 5 intact tablets were transferred to a volumetric flask of 500 mL; 250 ml of diluent (1) was added to it and was sonicated for 30 minutes to dissolve. It was then cooled to room temperature and made up to mark with the same diluent. Filtered through 0.45 µ nylon filter, and further diluted 5 ml of the above solution to 100 mL with diluent 2.

Method validation

Once optimum separation conditions are achieved, method is validated to ensure its suitability and reliability for routine use in estimation of % content of active ingredients in a pharmaceutical formulation. Validation parameters adopted are as follows:

Specificity: Specificity for blank, placebo, and known impurities was established by injecting known concentration of impurity solutions.
Specificity for unknown impurities was established by performing forced degradation study on tablet formulation as shown in Table 2. Peaks of interest were subjected to peak purity assessment using photodiode detector. All the peaks were found to be spectrally pure and no co-elution of any impurity was observed.

As shown in Table 3, no interference from blank, placebo and known impurities was observed at retention times of Entacapone, Levodopa and Carbidopa peaks.

**Solution stability:** Solution stability was evaluated by storing solutions at 25°C and 10°C. Carbidopa degraded by 2% at 25°C, whereas, solutions were found to be stable till 24 h when stored at 10°C (Tables 4 and 5).

**Accuracy:** Since Entacapone, Levodopa, and Carbidopa tablets have 7 strengths [(200+200+50), (200+175+43.75), (200+150+37.5), (200+125+31.25), (200+100+25), (200+75+18.75), and (200+50+12.5)], accuracy study was performed at 50% of the lowest concentration and 150% of the highest concentration of individual active ingredient. Recovery solutions were prepared by spiking Entacapone; Levodopa and Carbidopa API to placebo powder to obtain solutions of desired concentration (Table 6).

**Linearity:** A series of solutions were prepared by quantitative dilutions of the stock solution of standard to obtain solutions as mentioned in the following table. Each solution was injected and the peak area was recorded. Slope, Y-intercept and Correlation coefficient of the regression line were calculated (Table 7). In above, 200+200+50 mg strength was taken into consideration. By establishing linearity in entire working range, samples of all the 7 strengths can be analyzed against a single standard corresponding to any strength.

**Precision**

**Repeatability:** Six sample preparations were prepared and injected. The mean and relative standard deviation of the results was calculated. The results obtained for assay are tabulated in Table 8.

**Intermediate precision:** For intermediate precision analysis was carried out different day, using a different HPLC and different column. The absolute difference between the mean assay results obtained in repeatability and intermediate precision was calculated (Figure 5).

The obtained results for % assay and overall comparative data presented in the following Table 9.

The absolute difference between the mean assay results obtained in repeatability and intermediate precision is within the acceptance criteria of not more than 2.0. Hence, the method is precise.

**Robustness:** The Assay method was carried out as described
Forced Degradation Condition | % Degradation | % Impurity
--- | --- | ---
Acid Hydrolysis: Exposure to acidic condition with 5M hydrochloric acid | About 5.7% Degradation of carbidopa observed | Methyldopa: About 1.16% DHP: About 1.16% Total Impurity: 5.34%
Base Hydrolysis: Exposure to basic condition with 5M sodium hydroxide | About 5.5% Degradation of carbidopa observed | Methyldopa: About 1.06% DHP: About 3.18% Total Impurity: 4.24%
Oxidative Degradation: Exposure to Oxidative condition with 3% hydrogen peroxide | About 9.5% Degradation of carbidopa observed | Methyldopa: About 1.20% DHP: About 2.24% Unknown Impurity about 6.5% Total Impurity: 9.94%
Thermal Degradation: Exposure to 80°C for 24 hrs | About 2.02% Degradation of carbidopa observed | Methyldopa: About 2.83% Total Impurity: 2.83%
Photostability: Exposure to UV Radiation NLT 1.2 million lux hours | About 1.6% Degradation of carbidopa observed | Methyldopa: About 2.13% Total Impurity: 2.13%
Humidity Degradation: Exposure to 40°C temperature and 75% Relative humidity | About 5.6% Degradation of carbidopa observed | Methyldopa: About 2.7% DHP: About 3.8% Total Impurity: 6.5%

Table 3: Observations of forced degradation study.

Table 4: Chromatograms of Forced Degradation.
### Table 5: Observation of solution stability.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Entacapone</th>
<th>Levodopa</th>
<th>Carbidopa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>AT 25°C 3163365</td>
<td>AT 10°C 3208318</td>
<td>AT 25°C 24 hours 3161080</td>
</tr>
<tr>
<td></td>
<td>% Difference w.r.t. Initial 0.07</td>
<td>-</td>
<td>0.26</td>
</tr>
</tbody>
</table>

### Table 6: Accuracy results of the proposed method.

<table>
<thead>
<tr>
<th>% Level</th>
<th>Entacapone</th>
<th>Levodopa</th>
<th>Carbidopa</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>99.2</td>
<td>100.0</td>
<td>98.7</td>
</tr>
<tr>
<td>100%</td>
<td>99.4</td>
<td>100.2</td>
<td>99.0</td>
</tr>
<tr>
<td>150%</td>
<td>99.6</td>
<td>98.5</td>
<td>99.2</td>
</tr>
</tbody>
</table>

### Table 7: Linearity results of the proposed method.

<table>
<thead>
<tr>
<th>% Assay</th>
<th>Entacapone</th>
<th>Levodopa</th>
<th>Carbidopa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample-1</td>
<td>98.8</td>
<td>100.9</td>
<td>95.6</td>
</tr>
<tr>
<td>Sample-2</td>
<td>99.4</td>
<td>101.0</td>
<td>96.3</td>
</tr>
<tr>
<td>Sample-3</td>
<td>99.3</td>
<td>101.2</td>
<td>95.5</td>
</tr>
<tr>
<td>Sample-4</td>
<td>98.8</td>
<td>101.1</td>
<td>95.7</td>
</tr>
<tr>
<td>Sample-5</td>
<td>99.0</td>
<td>101.4</td>
<td>96.4</td>
</tr>
<tr>
<td>Sample-6</td>
<td>98.5</td>
<td>101.3</td>
<td>96.1</td>
</tr>
<tr>
<td>Mean</td>
<td>98.96</td>
<td>101.5</td>
<td>95.9</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

### Conclusion

A simple and efficient stability indicating HPLC method for simultaneous estimation of Entacapone, Levodopa and Carbidopa in...
Table 9: Comparison of precision and intermediate precision results of the proposed method.

<table>
<thead>
<tr>
<th>Altered condition</th>
<th>Retention time (min)</th>
<th>Tailing Factor</th>
<th>Theoretical plates</th>
<th>% RSD</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (unaltered) (Repeatability)</td>
<td>11.6</td>
<td>1.19</td>
<td>44742</td>
<td>0.08</td>
<td>98.9</td>
</tr>
<tr>
<td>Flow rate of mobile phase (0.8 mL/min)</td>
<td>12.94</td>
<td>1.20</td>
<td>43095</td>
<td>0.08</td>
<td>100.5</td>
</tr>
<tr>
<td>Flow rate of mobile phase (1.2 mL/min)</td>
<td>10.61</td>
<td>1.18</td>
<td>47443</td>
<td>0.09</td>
<td>99.4</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.3)</td>
<td>10.30</td>
<td>0.98</td>
<td>71000</td>
<td>0.07</td>
<td>100.0</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.7)</td>
<td>10.30</td>
<td>1.01</td>
<td>69703</td>
<td>0.03</td>
<td>100.8</td>
</tr>
</tbody>
</table>

Table 10: Robustness results of the proposed method (For Entacapone).

<table>
<thead>
<tr>
<th>Altered condition</th>
<th>Retention time (min)</th>
<th>Tailing Factor</th>
<th>Theoretical plates</th>
<th>% RSD</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (unaltered) (Repeatability)</td>
<td>2.60</td>
<td>1.37</td>
<td>5402</td>
<td>0.15</td>
<td>101.15</td>
</tr>
<tr>
<td>Flow rate of mobile phase (0.8 mL/min)</td>
<td>3.22</td>
<td>1.36</td>
<td>5928</td>
<td>0.22</td>
<td>101.9</td>
</tr>
<tr>
<td>Flow rate of mobile phase (1.2 mL/min)</td>
<td>2.18</td>
<td>1.32</td>
<td>4943</td>
<td>0.12</td>
<td>102.3</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.3)</td>
<td>2.37</td>
<td>1.00</td>
<td>7508</td>
<td>0.11</td>
<td>100.4</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.7)</td>
<td>2.33</td>
<td>1.04</td>
<td>7097</td>
<td>0.08</td>
<td>99.4</td>
</tr>
</tbody>
</table>

Table 11: Robustness results of the proposed method (For Levodopa).

<table>
<thead>
<tr>
<th>Altered condition</th>
<th>Retention time (min)</th>
<th>Tailing Factor</th>
<th>Theoretical plates</th>
<th>% RSD</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (unaltered) (Repeatability)</td>
<td>3.62</td>
<td>1.19</td>
<td>6330</td>
<td>0.10</td>
<td>95.9</td>
</tr>
<tr>
<td>Flow rate of mobile phase (0.8 mL/min)</td>
<td>4.51</td>
<td>1.18</td>
<td>6907</td>
<td>0.12</td>
<td>96.9</td>
</tr>
<tr>
<td>Flow rate of mobile phase (1.2 mL/min)</td>
<td>3.04</td>
<td>1.17</td>
<td>5204</td>
<td>0.12</td>
<td>96.6</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.3)</td>
<td>3.27</td>
<td>0.94</td>
<td>6436</td>
<td>0.94</td>
<td>96.0</td>
</tr>
<tr>
<td>pH of buffer of mobile phase (pH=2.7)</td>
<td>3.05</td>
<td>1.01</td>
<td>6201</td>
<td>0.09</td>
<td>96.5</td>
</tr>
</tbody>
</table>

Table 12: Robustness results of the proposed method (For Carbidopa).
presence of 15 impurities has been developed. Method was validated for specificity, accuracy, linearity, precision and robustness ensuring suitability of the method for quantitative analysis. The results indicated that this method is suitable for simultaneous estimation of Entacapone, Levodopa and Carbidopa in a pharmaceutical formulation.

Acknowledgements

The authors express sincere gratitude to the Research & Development Center of Macleods Pharmaceuticals Limited, Mumbai, India, for granting permission to use the Analytical Research and Development section for this research work. The first author is also thankful to his colleagues for their consistent support to carry out this research.

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