STABILITY INDICATING METHOD OF CARBIDOPA AND LEVODOPA ASSAY IN CARBIDOPA, LEVODOPA AND ENTACAPONE FILM COATED TABLETS BY RP- HPLC

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ABSTRACT: The present work describes high sensitive and reproducible method of Carbidopa and Levodopa assay in Carbidopa, Entacapone and Levodopa film coated tablet. It was developed by using kromosil C18, 125mm x 4.0mm x 5µm column, mobile phase (A) phosphate buffer pH 3.8 and, mobile phase (B) methanol and water with gradient method. Retention time of carbidopa and levodopa is 3.83 and 1.80 minutes respectively. It was evaluated for identification and quantification by using high performance liquid chromatography their separation being dependent on the pH of mobile phase. Critical resolutions between carbidopa and 3-Methoxy DL-Tyrosine (Levodopa impurity) and Levodopa impurity and Levodopa in assay method, it has been validated according to ICH Guidelines. Precision study was obtained results average of 12 sample % RSD Carbidopa 0.88 and Levodopa 0.71. And linearity was found to be (Carbidopa R2 = 0.9991 and Levodopa 0.9990). Recovery was found to be (97% to 103%). Robustness, solution stability and degradation impurities are determined by this method. Based on validation results, it is a stability indicating method.

INTRODUCTION: Carbidopa and Levodopa molecules were used for treatment of Parkinson’s disease, one of the most common progressive, neurodegenerative disorders, characterized by the depletion of dopamine in the dopaminergic neurons of the striatum of the brain 1. Carbidopa is a noncompetitive decarboxylase inhibitor used in combination with levodopa for the treatment of Parkinson's disease 2. Levodopa (L-dopa) is used to replace dopamine lost in Parkinson's disease because dopamine itself cannot cross the blood-brain barrier where its precursor can. However, L-DOPA is converted to dopamine in the periphery as well as in the CNS, so it is administered with a peripheral DDC (dopamine decarboxylase) inhibitor such as Carbidopa, without which 90% is metabolised in the gut wall, and with a COMT inhibitor if possible; this prevents about a 5% loss.

The form given therapeutically is therefore a prodrug which avoids decarboxylation in the stomach and periphery, can cross the blood-brain barrier, and in the brain it is converted to the neurotransmitter dopamine by the enzyme aromatic-L-amino-acid decarboxylase 4. Fig.1: gives the structures of Carbidopa IUPAC name: (2S) – 3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methyl propionic acid, Levodopa IUPAC name: (-)-L-α-amino-β-(3,4-dihydroxybenzene) propionic acid and Entacapone IUPAC name: (2E)-2-cyano-
3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl prop-2-enamide \(^5,6,7\) respectively.

Various works related to the determination of Carbidopa and levodopa was carried out using HPLC technique.\(^8,9,10\) Essentially the present work describes estimation of carbidopa and levodopa in carbidopa, entacapone and levodopa film coated tablets by using HPLC. These three compounds are well separated within 15 minutes, as well as identify the degradation impurity 3, 4 dihydroxy phenyl acetone a metabolic impurity. Critical resolution between Carbidopa and 3-Methoxy DL-Tyrosine (Levodopa impurity) separated by using kromosil Column, also depends on the pH of mobile phase. In validation all critical parameters as well as force degradation study are performed. Based on validation results obtained it proved stability indicating method and it may be used as stability indicating method for carrying out routine analysis is quality control department.

**FIG.1: STRUCTURES OF (a) CARBIDOPA (b) LEVODOPA (c) ENTACAPONE**

**MATERIALS AND METHODS:**

**MATERIALS:**
Carbidopa, levodopa are active pharmaceutical ingredients (API's), Carbidopa and levodopa working standards, and impurities of cyclohexylidene Carbidopa methyl ester (CCM Ester), methyldopa methyl ester (MM Ester), 3,4 dihydroxy phenyl acetone, L-Tyrosine, 3-Acetyl L-Tyrosine, Methyldopa, provided by Devi’s Laboratories Ltd, (Visakhapatnam, India). Entacapone (API) was provided by Aurobindo pharma Ltd (Hyderabad). 3-O methyl Carbidopa, related compound A (Levodopa Impurity) provided by USP Pharmacopeia, 3-Methoxy DL-Tyrosine impurity was provided by EUROPIAN Pharmacopeia. Carbidopa, Entacapone and Levodopa film coated tablets were provided by AET Laboratories Pvt Ltd, India (Formulation Research & Development center). EMPARTA grade Potassium di hydrogen orthophosphate (KH\(_2\)PO\(_4\)), EMPARTA grade orthophosphoric acid (88%) (H\(_3\)PO\(_4\)), EMPARTA grade Hydrochloric acid (37%) (HCl), HPLC Grade Methanol (CH\(_3\)OH) and Water Mill-Q grade purified, Sodium Hydroxide (NaOH) EMPARTA Grade, Hydrogen Peroxide (H2O2) all chemicals were purchased from Merck.

HPLC (High Performance Liquid Chromatography) is from Water’s India Ltd, model Alliance e2695 separations module, Detector UV 2489, Empower 2 (Software), and Photo Diode Array (PDA) model 2996. pH meter, make (Polmon), Analytical Balance model AX205, make: Metler Toledo, Sonicator make: ENERTECH, Rotary shaker make: REMI, Model: RS – 24 BL.

**METHODS:**
Carbidopa and Levodopa assay method was developed by using high performance liquid chromatography, column C18, 125mm × 4.0mm, 5µm particle and flow rate 1.0 mL/min, UV wavelength 280nm, Injection Volume 10 µL, run time was 15minutes, determined a gradient program initial time start from 0.0 to 5.0min in between time mobile phase composition mobile phase A was 95% to 75% and mobile phase B was 5% to 25%, next start from 5.0 to 7.0minutes mobile phase A was 75% to 10% and mobile phase B was 25% to 90%, next 7.0 to 10.0minutes mobile phase A was 10% and mobile phase B 90% same composition continued, then 10 to 12 minutes mobile phase A was 10% to 95% and mobile phase B was 90% to 5%, end of the composition 12 to 15 minutes mobile phase A was 95% and mobile phase B was 5% same composition continued

**Preparation of mobile phase solutions, diluent and needle wash solutions:** Mobile phase (a) was prepared using 20 mM concentration of phosphate
buffer (pH 3.8 adjusted with diluted H$_3$PO$_4$) solution prepared and filtered through 0.45µm Nylon filter as well as mobile phase (b) was prepared (CH$_3$OH:H$_2$O) (60:40) (v/v). Diluent was mixed well solution that is (0.1N HCl: CH$_3$OH) (90:10) (v/v). Needle wash solution containing 0.1 N hydrochloric acid and methanol in the ratio of 50:50 (v/v) was used to avoid the carry over problem.

**Preparation of working standard solution:**

Accurately weighed 25.0 mg Carbidopa (0.25 mg/mL) and 100.0 mg Levodopa (1.0 mg/mL) in to 100 ml volume metric flask, 60ml diluent was added, then sonicated for 2 minutes to dissolve drug in the diluent, after that made up to volume with diluent. For further dilution, 5.0 ml was taken from stock solution in to 25 ml volume metric flask, made up to volume with the diluent. Final standard concentration of carbidopa is 0.05 mg/ml and levodopa is 0.2 mg/mL

**Preparation of sample solution:**

100 mg of Carbidopa equivalent sample taken in to 200 ml volume metric flask, 160 ml diluent added, it was shaked for 15 minutes at 180 rpm on rotary shaker and sonicated for 10 minutes, after that made up to volume with diluent, filtered and sample was collected 10ml by using mill pore PVDF filter 0.45µm in to test tube. Further dilution 5 ml taken from stock solution in to 50 ml volume metric flask made up with diluent. Final sample concentration of carbidopa 0.05 mg/mL and levodopa is 0.2 mg/mL

**Method development of the analytical assay method**

a) Carbidopa and Levodopa both are acidic molecules, pKa value of carbidopa is 2.32, 11, 13 and levodopa is pKa1 2.31, pKa2 8.72, pKa3 9.74, pKa4 13.40 at (25°c). 12, 13 Solubility of the carbidopa and levodopa molecules both are in 0.1N Hydrochloric acid, carbidopa molecular weight is 244.24, 2, 14 and levodopa molecular weight is 197.19. 2, 15 Observed in the molecules structure (acidic group, hydroxyl group, amine group) based on this data evaluated the development of the assay method.

b) Mobile phase A (0.1% OPA) 16 buffer and Mobile phase B (100% CH3OH) both were used to assay method and linear gradient flow was optimized. Because of acidic molecules for compatibility and suitable. But 3-Methoxy DL- Tyrosine (Levodopa impurity C) and Carbidopa peak interference occurred

c) Mobile phase A (100% Phosphate buffer pH 3.0). 6 and Mobile phase B (100% CH3OH) were used to method and linear gradient flow was optimized. Obtained result Carbidopa and 3- Methoxy DL tyrosine (Levodopa impurity C) came at same retention time.

d) Mobile phase A (100% Phosphate buffer pH 3.5) and Mobile phase B (80:20) (CH$_3$OH: H$_2$O) were used to method and linear gradient flow was optimized. Resolution came 1.4 between Carbidopa and 3- Methoxy DL tyrosine impurity

e) Mobile phase A (100% 20 mM concentration phosphate buffer pH 3.8) 8 and Mobile phase B (60:40) (CH$_3$OH:H$_2$O) were used to method and up to 5 minutes linear gradient flow than mobile phase B composition ratio increased, because of Entacapone peak was eluted early. After 10 minutes again initial gradient, after 12 minutes up to 15 minutes column equilibration purpose based on column volume. Therefore total runtime was 15 minutes. Obtained system suitability parameters like (symmetry factor: levodopa 1.13 and Carbidopa 1.04, EP Plate count: levodopa 4950 and Carbidopa 12263, five replicate injections of standard % RSD levodopa 0.17 and Carbidopa 0.16). Critical resolutions achieved between related compound A (Levodopa impurity A) and Levodopa 2.51 and second one between Carbidopa and 3- Methoxy DL- Tyrosine (Levodopa Impurity C) 1.68.

f) Critical resolutions separated by pH 3.8 phosphate buffers; it was developed by Kromasil 125 mm × 4.0 × 5µm, C18 column. All impurities were spiked 1% level in the assay method, separated all known impurities and also peak purity passed. Critical parameters performed precision, linearity,
RESULTS AND DISCUSSION:

Precision study: For performing precision study six samples were same concentration (0.05 mg/ml of Carbidopa and 0.2 mg/ml of Levodopa) injected in the liquid chromatography as well as next day intermediate precision study also performed same way. Results were given below Table 1.

TABLE 1: CARBIDOPA AND LEVODOPA PRECISION RESULTS

<table>
<thead>
<tr>
<th>S. No</th>
<th>Method precision results</th>
<th>Intermediate precision results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbidopa</td>
<td>Levodopa</td>
</tr>
<tr>
<td>1</td>
<td>101.1</td>
<td>102.0</td>
</tr>
<tr>
<td>2</td>
<td>100.4</td>
<td>100.7</td>
</tr>
<tr>
<td>3</td>
<td>100.1</td>
<td>101.6</td>
</tr>
<tr>
<td>4</td>
<td>100.0</td>
<td>100.1</td>
</tr>
<tr>
<td>5</td>
<td>101.2</td>
<td>102.0</td>
</tr>
<tr>
<td>6</td>
<td>101.3</td>
<td>101.7</td>
</tr>
<tr>
<td>Aver</td>
<td>100.7</td>
<td>101.3</td>
</tr>
<tr>
<td>SD</td>
<td>0.60</td>
<td>0.77</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.60</td>
<td>0.76</td>
</tr>
<tr>
<td>Average of 12 sample preparations</td>
<td><strong>100.0</strong></td>
<td><strong>100.9</strong></td>
</tr>
<tr>
<td>Average of S D</td>
<td><strong>0.88</strong></td>
<td><strong>0.72</strong></td>
</tr>
<tr>
<td>Average of % RSD</td>
<td><strong>0.88</strong></td>
<td><strong>0.71</strong></td>
</tr>
</tbody>
</table>

Linearity:
Linearity parameter is used to estimated method range by using chromatography technique (RP-HPLC). Solutions were prepared six levels 50% level, 75% level, 100% level, 125% level, 150% level and 200% level. Six concentrations performed in the assay method results were given below and from the graphs estimated the Carbidopa, Levodopa method sensitivity and range.

Accuracy and Precision:
Recovery test was performed for four levels 50% level, 100% level, 150% level and 200% level injected in the assay method. Obtained experimental values were much close to true value. Essentially method was estimated % of recovery from low level to high level concentrations. Results were given below Table 2, Recovery limit (95% to 105%).
**Specificity study:**
Prepared the test concentration refers to (preparation of sample solution) there was no interference in the Carbidopa and Levodopa main peaks and peak purity was passed. All impurities were spiked 1.0% level with respective to test concentration in the test sample (Carbidopa impurity’s all spiked with respective Carbidopa active test concentration and Levodopa impurity’s all spiked with respective Levodopa active test concentration as well as Z-Isomer (Impurity of Entacapone) [1.0% level spiked with respective to Carbidopa test concentration]. Whichever this impurity was used in this assay method, because of cross specificity study established. As well as placebo and blank also injected in this assay method.

**Forced degradation study:**
Forced degradation study was carried out for Carbidopa and Levodopa.

**Acid degradation:**
1.0 N HCl solution, 20ml added in to sample volumetric flask (200ml), kept on the bench top for 1hr, after that 140ml diluent is added then sample is kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes, after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent. Degradation is not observed as both are acidic molecules as seen in initial point (a) method development and Fig 5 and results are given Table 3.

**TABLE 3: SUMMARY OF STRESS DEGRADATION OF CARBIDOPA AND LEVODOPA**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Condition</th>
<th>Carbidopa drug recovered</th>
<th>Levodopa drug recovered</th>
<th>Carbidopa drug decomposed</th>
<th>Levodopa drug decomposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Untreated sample</td>
<td>101.5</td>
<td>100.6</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>Acid Degradation</td>
<td>100.9</td>
<td>100.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>Base Degradation</td>
<td>89.9</td>
<td>100.0</td>
<td>11.6</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>Peroxide Degradation</td>
<td>96.9</td>
<td>99.8</td>
<td>4.6</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>Water Degradation</td>
<td>101.2</td>
<td>101.3</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>Thermal Degradation</td>
<td>100.6</td>
<td>101.1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
<td>Humidity Degradation</td>
<td>98.6</td>
<td>99.5</td>
<td>2.9</td>
<td>--</td>
</tr>
<tr>
<td>8</td>
<td>UV Degradation</td>
<td>101.6</td>
<td>102.1</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Base degradation:
0.1 N NaOH solution, 20ml added in to sample volumetric flask (200ml), kept on the bench top for 30 minutes, after that 0.1 N HCl 20ml added for neutralized purpose then 120ml diluent added then sample kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes, after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent. Degradation impurities were observed in alkali sample, as seen in Fig – 6, because of Carbodopa has acidic pKa value, so it might be ionized. Levodopa has four pKa values seen in initial point (a) in method development and also one chiral center is available so levodopa was not ionized, method was identified degradation impurities, results are given Table – 3.

Oxidation degradation:
3% H2O2 Solution 30 ml added in to sample volumetric flask (200ml), kept on the bench top for 30 minutes, after that 130ml diluent added then sample kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes, after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent. Degradation impurities were observed in the peroxide sample, carbodopa molecule was degraded might be oxidation reaction occurred and as seen in Fig – 7, results are given Table – 3.

Water degradation:
Water 30ml is added in to sample volumetric flask (200ml), kept on the bench top for 30 minutes, after that 130ml diluent added then sample is kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes. After that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent. Degradation is not observed in water sample, as observed in Fig – 8 results are given Table–3.

Humidity degradation:
(80% RH for one week) kept on the sample and placebo. 100mg Carbidopa equivalent sample taken in to 200ml volumetric flask, 160 ml diluent added sample kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes, after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent, Degradation is observed in the humidity sample but negligible, as seen in Fig – 9 and results are given Table – 3.

**FIG. 9: CHROMATOGRAM OF HUMIDITY DEGRADATION SAMPLE**

**Thermal degradation:**
(Not exceeding 70°C for one week) kept on the sample and placebo 100mg Carbidopa equivalent sample taken in to 200ml volumetric flask, 160 ml diluent added sample kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent, Degradation is not observed in thermal sample, as seen in Fig – 10 and results are given Table–3.

**FIG. 10: CHROMATOGRAM OF THERMAL DEGRADATION SAMPLE**

**Photolytic degradation:**
(UV light (NLT 200 watt hours/square meter) and visible light (NLT 1.2 million lux hour’s intensity) kept on the sample and placebo100mg Carbidopa equivalent sample taken in to 200 ml volumetric flask, 160 ml diluent added sample kept on the rotatory shaker instrument for 15 minutes at 180 rpm and sonicated for 10 minutes after that made up to volume with diluent then filtered and second dilution 5ml taken from stock in to 50ml volumetric flask with diluent. Degradation is not observed in the photolytic sample, results are given Table – 3.

**FIG. 11: CHROMATOGRAM OF PHOTOLYTIC DEGRADATION SAMPLE**

**Note:** This method was identified degradation known impurities, unknown impurities and well separated all peaks as well as how much quantification in the drug product also find out. Based on the results, method is stability indicating method.

**Solution stability:**
Standard solution and sample solution kept in room temperature, these samples were injected at 12hr, 16hr and 40hr. Calculation: % difference between initial area and each established hour obtained area. Specification limits (NMT 2.0%). Results were given Table – 4.

**TABLE 4: CARBIDOPA AND LEVODOPA SOLUTION STABILITY RESULTS.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Assay Solution</th>
<th>After 40Hr (%) Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carbidopa standard solution</td>
<td>1.44</td>
</tr>
<tr>
<td>2</td>
<td>Levodopa standard solution</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>Carbidopa sample solution</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>Levodopa sample solution</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Robustness:
This parameter was evaluated by HPLC. Small deliberate variations in the mobile phase A & B compositions and flow rate filter study, wave lengths changed, if it was any deliberate small changed in the method no quality impact was observed in the drug product. All results were given Table - 5.

**TABLE 5: CARBIDOPA AND LEVODOPA ROBUSTNESS RESULTS**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Robustness parameters</th>
<th>Carbidopa results</th>
<th>Levodopa results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mobil phase A pH 3.6</td>
<td>98.0</td>
<td>100.5</td>
</tr>
<tr>
<td>2</td>
<td>Mobil phase A pH 4.0</td>
<td>97.9</td>
<td>100.4</td>
</tr>
<tr>
<td>3</td>
<td>Mobil phase B (55:45)</td>
<td>98.4</td>
<td>100.3</td>
</tr>
<tr>
<td>4</td>
<td>Mobil phase B (65:35)</td>
<td>98.2</td>
<td>100.2</td>
</tr>
<tr>
<td>5</td>
<td>Flow rate 1.1mL/min</td>
<td>100.6</td>
<td>101.9</td>
</tr>
<tr>
<td>6</td>
<td>Flow rate 0.9mL/min</td>
<td>100.0</td>
<td>101.7</td>
</tr>
<tr>
<td>7</td>
<td>Filter Mille pore PVDF 0.45µm</td>
<td>100.4</td>
<td>101.5</td>
</tr>
<tr>
<td>8</td>
<td>Filter Axiva PVDF 0.45µm</td>
<td>100.0</td>
<td>101.5</td>
</tr>
<tr>
<td>9</td>
<td>Wave length 278nm</td>
<td>101.1</td>
<td>100.4</td>
</tr>
<tr>
<td>10</td>
<td>Wave length 282nm</td>
<td>102.0</td>
<td>100.7</td>
</tr>
</tbody>
</table>

CONCLUSION: This method highly sensitive, reproducible and identified the degradation impurities and also estimated quantity of drug substances of Carbidopa and Levodopa in the Carbidopa, Entacapone and Levodopa film coated tablets. It is high accuracy and precise, robustness method. The method is economical and may be used.

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REFERENCES:
1. Monica Colamartino, Luca Padua, Thomason Cornetta1, Antonella Testa, Renate Cozzi1. Recent advances in pharmacological therapy of Parkinson’s: Levodopa and Carbidopa protective effects against DNA Oxidative damage, Health, 2012, Vol.4, Special issue, 1191-1199
2. SINEMETCRURL:Packageinserts.bms.com/pi/pi_sinemet_cr.pdf.
3. URL:www.drugbank.ca/drugs/DB00190.
4. URL:www.drugbank.ca/drugs/DB01235
10. S. Suresh Kumar, Dr. K. Natraj, Asadulla Khan, B. Kalyan Kumar, Dr. J. venkateswara Rao, (Development and Validation of RP-HPLC Method for Simultaneous Estimation of Levodopa and Carbidopa in Pharmaceutical Dosage Forms), jpr, 2011, Volume – 4 No.11
15. http://www.pharmacopeia.cn/v29240/usp29nf24s0_m44720.html
18. ICH Impurities in New Drug Products Q3B (R2), June 2006, International Conference on Harmonisation - Quality