Analytical method development for simultaneous estimation for drug content and release of Levodopa, Carbidopa and Entacapone in combined dosage form by RP-HPLC

Alka N Choudhary, Ajay Chaudhary and Kamlesh K Dutta

Abstract

Objective: The present study was designed to develop a RP-HPLC method which is capable of estimating the content and release of Levodopa, Carbidopa and Entacapone simultaneously in combined dosage form.

Methods: Selection of diluents for a clean sample was based on the solubility to provide sink condition for the target analytes in common. C18 column was selected as the stationary phase. Gradient Scouting Technique was opted to screen whether isocratic or gradient method is the possibility with different mobile phases. Detection wavelength was selected with help of PDA detector screening over the wavelength 200 – 400 nm. Strength & pH of buffer, column temperature, flow rate and injection volume were optimized by performing trials at different buffer strengths & pHs, column temperatures, flow rates and injection volumes respectively.

Results: Simultaneous separation of Levodopa, Carbidopa and Entacapone was achieved on a Sunshell Fused Core C18 column (150 mm x 4.6 mm x 2.6 µm) as stationary phase with combination of mobile phases; Phosphate Buffer, pH 3.0 and Methanol in gradient mode at flow rate of 1.0 ml/min, column temperature kept at 35ºC, PDA/UV detector at 280 nm and injection volume 5 µL. The diluents used for sample preparation was Phosphate Buffer, pH 5.5. The retention time of Levodopa, Carbidopa and Entacapone were found to be 2.27, 3.43 and 11.77 minutes respectively with tailing factor ≤ 1.5, resolution > 2 and NTP > 2000 for each analyte peak.

Conclusion: Simple, economical and rapid method was developed and further validated successfully as per ICH: Q2 (R1).

Keywords: Parkinson’s disease, Levodopa, Carbidopa, Entacapone, RP-HPLC, method development, validation, ICH: Q2 (R1)

1. Introduction

Parkinson’s disease (PD) is a type of movement disorder that can affect the ability to perform common, daily activities. The disease develops as cell loss occurs in a very specific region of the brain called substantia nigra. The nerve cells, or neurons, in this region produce a specific type of neurotransmitter (a chemical messenger that allows neurons to communicate) called dopamine. The neurotransmitter dopamine helps to regulate movement \([1,2]\). Symptoms of Parkinson’s disease are related to depletition of dopamine. But administration of dopamine is ineffective in the treatment of Parkinson’s disease. This is because it does not cross 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 \([3]\). Hence, Levodopa is used in the treatment of Parkinson’s disease. Levodopa is the most effective medicine for relieving symptoms of Parkinson’s disease. However, Levodopa, is extensively metabolized to various metabolites and only small portion of a given dose is transported unchanged to the central nervous system. Two major pathways of metabolism are decarboxylation by dopa decarboxylase (DDC) and O-methylation by catechol-O-methyltransferase (COMT) \([4]\). Levodopa when administered concomitantly with Carbidopa and Entacapone, plasma levels of Levodopa are greater and more sustained than after administered of Levodopa alone \([5]\). Carbidopa is an inhibitor of dopa decarboxylase (DDC), and Entacapone is an inhibitor of catechol-O-methyltransferase (COMT) preventing decarboxylation and O-methylation of Levodopa outside of the central nervous system respectively providing greater and sustained plasma level of Levodopa available to cross...
blood-brain barrier and get converted to dopamine in the brain [6, 7]. Carbidopa and Entacapone have no antiparkinson’s actions by itself alone [8]. Hence, Levodopa, Carbidopa and Entacapone in combined dosage form is an effective medication for Parkinson’s disease.

**Levodopa**

![Molecular Structure of Levodopa](image)

Chemical Name: (2S)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid [10]
Molecular Formula: C₉H₁₁NO₄ [9]
Molecular Weight: 197.19 g/mol [9]

**Carbidopa**

![Molecular Structure of Carbidopa](image)

Chemical Name: (2S)-3-(3, 4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid hydrate [10]
Molecular Formula: C₁₀H₁₄N₂O₄·H₂O [11]
Molecular Weight: 244.24 g/mol [11]

**Entacapone**

![Molecular Structure of Entacapone](image)

Chemical Name: (2E)-2-cyano-3-(3, 4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide [10]
Molecular Formula: C₁₄H₁₅N₃O₅ [12]
Molecular Weight: 305.286 g/mol [12]

There is no pharmacopoeial or literature reference of a suitable single method for simultaneous estimation of drug content and release for Levodopa, Carbidopa and Entacapone in combined dosage form. This aspect caters the need for the study.

2. Materials and Methods

The research work was carried out at Analytical Development Laboratory, Quest Pharmaceuticals Pvt. Ltd. All the required materials like APIs, excipients, reagents, chemicals, instruments and other analytical accessories were facilitated from the company.

2.1 Reagents and Chemicals

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<td>Fisher Scientific</td>
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<td>4.</td>
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<td>5.</td>
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<td>6.</td>
<td>Ortho Phosphoric Acid</td>
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2.2 Active Pharmaceutical Ingredients

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<td>3.</td>
<td>Entacapone</td>
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</table>

2.3 Instrument

Method development was performed on a modular type Shimadzu HPLC System, Model - UFLCXR, equipped with binary pump, automatic sample injector, column thermostat and PDA detector. Data acquisition and interpretation were performed using Lab Solutions software.

2.4 Method

Method development goals and strategy were designed and performed as follows:

2.4.1 Separation Goal

Separation goals for the desired method was targeted as shown in the Table 3.

<table>
<thead>
<tr>
<th>S. No</th>
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<td>Resolution(R)</td>
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<td>Run Time</td>
<td>≤ 20 Minutes</td>
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<td>Number of Theoretical Plates(NTP)</td>
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<td>5.</td>
<td>Tailing Factor(Aₜ)</td>
<td>≤ 2</td>
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2.4.2 Sample Preparation
Selection of diluents for a clean sample was based on the solubility of the target analytes Levodopa, Carbidopa and Entacapone. It was targeted to choose diluent for sample preparation such that it works both for assay and dissolution study.

2.4.3 Selection of Stationary Phase/Column
Target analytes Levodopa, Carbidopa and Entacapone being the polar compounds, the commonly used and most non-polar fused core stationary phase C18 (among C18, C8, C4, Cyano, Phenyl and Amino column) was selected for RP-HPLC method development [16].

Shorter column length, 150 mm was chosen for speedy analysis with short retention time of analytes. Smaller particle size of stationary phase, 2.6 µm was selected to achieve better efficiency and resolution of target analyte peaks. So, considering all above discussed aspects, Sunshell, Fused Core C18, 150mm x 4.6 mm x 2.6 µm column was selected for development of the analytical method.

2.4.4 Gradient Scouting
Gradient Scouting Technique was opted to screen whether isocratic or gradient method is the possibility. Screening study with different mobile phases like combination of Acetonitrile, Methanol, and Tetrahydrofuran with water (or an aqueous buffer) was performed for selection of mobile phase.

2.4.5 Selection of Detection Wavelength
Detection wavelength was selected with help of PDA detector screening over the wavelength 200 – 400 nm.

2.4.6 Optimization of Buffer Strength and pH
Strength and pH of the buffer was optimized by performing trials at different pHs (like 4.0, 3.0, 2.5, 2.1, etc) and strengths (like 0.01 M, 0.05 M, etc).

2.4.7 Selection of Column Temperature
Column temperature was optimized by performing trials at different column temperatures (like 30, 35, 40 & 45°C).

2.4.8 Selection of Flow Rate
Flow rate was optimized by performing trials at different flow rates (like 0.5, 0.8, 1.0 ml/min, etc).

2.4.9 Selection of Injection Volume
Injection volume was optimized by performing trials at different injection volumes (like 5, 10 µL, etc).

3. Results and Discussion
3.1 Sample Preparation
Different diluents like 0.1N HCl, Acetate Buffer (pH 4.5), Phosphate Buffer (pH 5.5), and Phosphate Buffer (pH 6.8) were opted. Highest dose (i.e 200 mg Levodopa, 50 mg Carbidopa and 200 mg Entacapone) was dissolved in 500 ml each of the four diluents [17, 18]. Phosphate Buffer, pH 5.5 was selected as diluent as it only provides sink condition for all the three target analytes Levodopa, Carbidopa & Entacapone. Hence, same diluents (Phosphate Buffer, pH 5.5) was used as dissolution medium for dissolution study.

Preparation of Phosphate Buffer, pH 5.5: 6.8 g/L of Monobasic Potassium Phosphate, KH2PO4 in water, adjust pH to 5.5 with 1M Sodium Hydroxide. It is recommended to sonicate the stock solution for 15 minutes in sufficient solvent prior to volume make up and finally filter the final solution through 0.2 µm membrane filter prior to injection.

3.2 Gradient Scouting
A common test solution containing, Levodopa - 100 µg/ml, Carbidopa (Anhydrous) - 100 µg/ml and Entacapone - 100 µg/ml was used for gradient scouting. Mobile phases like Acetate and Phosphate Buffers were tried. Among Acetate and Phosphate Buffers, Phosphate Buffer showed better peak symmetry for Levodopa and Carbidopa at low strength.

For elution of Entacapone at desired capacity factor Methanol was required. Successful separation was achieved with solvent system: Buffer and Methanol. The initial condition of successful separation of all the three target analytes (Figure 4) was as follows.

Column: C18, 150mm x 4.6mm x 2.6 µm
Mobile Phase: Phosphate Buffer, pH 2.5 and Methanol
Gradient Run: 0 – 90 % of methanol (A) in 0 - 20 minutes (or 100 – 10 % Buffer (B) in 0 – 20 minutes)
Flow Rate: 0.8 ml/min
Injection Volume: 5 µL
Column Temperature: Ambient
Separation of all the three target analyte were achieved with first peak (Levodopa) at 3.02 mins, second peak (Carbidopa) at 4.63 mins and third peak (Entacapone) at 15.67 mins (Figure 4). Here, Tg, the time over which the solvent composition is changed is 20 mins. The difference between the first and last desired peaks is 15.67 – 3.02 = 12.65 mins. 
Now, Te /Tg = 12.65/20 = 0.63
(For Isocratic Mode, Te /Tg <0.25 & For Gradient Mode, Te /Tg >0.45) [15]
Hence, gradient mode was selected for method development.

3.3 Selection of Detection Wavelength
A common solution of Levodopa, Carbidopa, & Entacapone in selected diluent was injected with selected mobile phase combination and column. Spectra of all three analytes over 200-400 nm was obtained with PDA detector and overlaid to select a single & common detection wavelength for all three analytes. 280 nm was selected as detection wavelength (Figure 5).

3.4 Optimization of Buffer Strength and pH
pH and strength of the buffer was optimized by performing trials at different pH (2.1, 2.5, 3.0, & 4.0) and strength (0.01M & 0.05M) with a common test solution containing, Levodopa - 240 µg/ml, Carbidopa (Anhydrous) - 60 µg/ml and Entacapone - 240 µg/ml (Figure 6).
**0.01M Phosphate Buffer, pH 2.1**

![Graph of 0.01M Phosphate Buffer, pH 2.1](image)

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<th>Height</th>
<th>Area%</th>
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<th>Tailing</th>
<th>NTP</th>
<th>(k)</th>
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**0.01M Phosphate Buffer, pH 2.5**

![Graph of 0.01M Phosphate Buffer, pH 2.5](image)

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</table>
- Increase in pH of the buffer shows decrease in retention time for all the target analytes.
- Increase in strength of the buffer shows increase in the retention time for all the target analytes.
- Resolution and Tailing factor for all the analytes at all pHs and strengths was >2 and ≤ 1.5 respectively.
- NTP was found optimum considered for all the analytes at pH 3.0.

Finally, the buffer was optimized as 0.01M Monobasic Sodium Phosphate (NaH₂PO₄·2H₂O) Buffer, pH 3.00 to target short analysis time and optimum NTP.

**Preparation of 0.01M Monobasic Sodium Phosphate (NaH₂PO₄·2H₂O) Buffer, PH 3.00:** 1.56 g/L Monobasic Sodium Phosphate, NaH₂PO₄·2H₂O in water, adjust pH to 3.0 with 10% Ortho-Phosphoric Acid.

### 3.5 Selection of Column Temperature

Column temperature was optimized by performing trials at different column temperature (30, 35, 40 & 45°C) with a common test solution containing: Levodopa - 240 μg/ml, Carbidopa (Anhydrous) - 60 μg/ml and Entacapone - 240 μg/ml (Figure 7).
### Column Temp. 35 °C

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</table>
Increase in column temperature shows slight decrease in retention time for all the target analytes.
Resolution and Tailing factor for all the analytes at all column temperature was >2 and ≤ 1.5 respectively.
NTP was found optimum considered for all the analytes at column temperature 35 °C.

Finally, the column temperature was optimized as 35°C to target optimum NTP.

### 3.6 Selection of Flow Rate

Flow rate was optimized by performing trials at different flow rate (0.5, 0.8 & 1.0 ml/min) with a common test solution containing, Levodopa - 240 µg/ml, Carbidopa(Anhydrous) - 60 µg/ml and Entacapone - 240 µg/ml (Figure 8).
Increase in flow rate shows significant decrease in retention time for all the target analytes.
Resolution and Tailing factor for all the analytes at all flow rate was >2 and ≤ 1.5 respectively.
NTP was found optimum considered for all the analytes at all flow rate.
Finally, the flow rate was optimized as 1.0 ml/min to target short analysis time.

3.7 Selection of Injection Volume
Injection volume was optimized by performing trials at different injection volume (5 & 10 µL) with a common test solution containing, Levodopa - 240 µg/ml, Carbidopa (Anhydrous) - 60 µg/ml and Entacapone - 240 µg/ml (Figure 9).
• No significant differences found between two injection volumes in respect of retention time, resolution, tailing factor and NTF for all the analytes. Finally, the injection volume was optimized as 5µL to just target low sample load in the column.

Fig 9: Chromatogram and its data at different Injection Volumes
3.8 Summary of Optimized Method

The retention time of Levodopa, Carbidopa and Entacapone is about 2.27, 3.43 and 11.77 mins respectively with tailing factor ≤ 1.5, resolution > 2 and NTP > 2000 for all the target analytes (Figure 10).

Diluent: Phosphate Buffer (KH$_2$PO$_4$), pH 5.5
Mobile Phase: A - Phosphate Buffer (NaH$_2$PO$_4$·2H$_2$O), pH 3.0
B - Methanol
Mode: Gradient (Table 4)

![Figure 10: Chromatogram and its data at Optimized Chromatographic Condition](image)

<table>
<thead>
<tr>
<th>Time (Mins)</th>
<th>A – Buffer (%)</th>
<th>B – Methanol (%)</th>
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</thead>
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<td>2</td>
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<td>98</td>
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</table>

Column: C18, 150 mm x 4.6 mm x 2.6 µm
Detection Wavelength: 280 nm
Column Temperature: 35°C
Flow Rate: 1.0 ml/min
Injection Volume: 5 µL

4. Conclusion
A simple, economical and rapid RP-HPLC method that is capable of estimating the content and release of Levodopa, Carbidopa and Entacapone simultaneously in combined dosage form was developed. The method is developed in gradient mode and has run time of 20 minutes. The retention time of Levodopa, Carbidopa and Entacapone were found to be 2.27, 3.43 and 11.77 minutes respectively with tailing factor ≤ 1.5, resolution > 2 and NTP > 2000 for each analyte peak. The developed method was validated successfully as per ICH: Q2 (R1) for transfer to laboratory for routine analysis.[19]

5. Conflict of interest
The authors declare no conflict of interest, financial or otherwise.

6. Acknowledgements
The authors express sincere gratitude to Analytical Development Laboratory of Quest Pharmaceuticals Pvt. Ltd, Nepal for facilitating the research work.

7. List of abbreviations
RP-HPLC: Reverse Phase High Performance Liquid Chromatography
HPLC: High Performance Liquid Chromatography
SQ: Special Quality
UV: Ultraviolet
PDA: Photo Diode Array
R: Resolution
k: Capacity Factor
NTP: Numbers of Theoretical Plates
A$_S$: Tailing Factor/ Symmetry Factor
$\lambda_{max}$: Wavelength at Maximum Absorbance
API: Active Pharmaceutical Ingredient
ICH: International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human
References


