Anti-Parkinsonian Drug Estimation by RP-HPLC

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Authors’ contributions

This work was carried out in collaboration between both authors. Author RRN designed the study, wrote the protocol and managed the analyses of the study. Author PA made the literature searches and performed the statistical analysis. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The main aim of the current study is to give best and simple method for the estimation of antiparkinsonian drugs named Carbidopa, levodopa and entacapone.

Study Design: Simultaneous estimation of Carbidopa, levodopa and entacapone was performed by using Quadrupumped (SHIMADZU Prominance-i, LC-2030C) RP-HPLC equipped with PDA detector.

Place and Duration of Study: Chalapathi Drug Testing Laboratory, Chalapathi Institute Of Pharmaceutical Sciences, Lam, Guntur-522034, Andhra Pradesh, India during the period of August 2019 to February 2020.

Methodology: The assets of the study can determined as the process of qualification and quantification was done on SHIMADZU Prominance-i, LC-2030C system equipped with Phenomenex ODS (150 x 4.6 mm, 5μm) column and mobile phase was optimized using combination of acetonitrile and 0.1% ortho phosphoric acid in the ration of 50:50 v/v at a flow rate 1.0 ml/min. The wavelength was set as 270nm at ambient temperature by injecting 20µl of solution and the run time was fixed for 5 min.

Results: Calibration plot shown best regression over the concentration range of 5-160 µg/ml of Carbidopa, Levodopa and Entacapone standard solutions. The LOD and LOQ were found to be 0.85 and 2.54 µg/ml for Entacapone, 0.24 and 0.71 µg/ml for Levodopa, 0.14 and 0.43 µg/ml for Carbidopa respectively. The accuracy of the proposed method was determined by performing recovery studies and was found to be between 98-102%. The repeatability testing for both sample
and standard solutions was found as %RSD<2.0% which is within the acceptable limits showing that the method is precise as well. The proposed method was successfully applied for the marketed formulations of Carbidopa, Levodopa and Entacapone tablets. In addition the main feature of proposed method is economic and eco-friendly with less retention time around 5.0 min.

**Conclusion:** Including all the optimized method parameters and statistical results given it can be concluded as a new, simple, sensitive, precise and accurate economical analytical method was developed and validated by RP-HPLC for the detection and quantification of Carbidopa, Levodopa and Entacapone which can be applied to the marketed formulation where there are no official compendial methods reported for this particular combination. The high sensitivity (LOD), mobile phase utilized and run time (=5) can be determined as an important features for this proposal.

**Keywords:** Carbidopa; levodopa and entacapone; HPLC; UV; method development; validation.

1. **INTRODUCTION**

Parkinson's disease is a progressive disorder of the nervous system that affects movement. Young adults rarely experience Parkinson's disease. It ordinarily begins in middle or late life, and the risk increases with age. People usually develop the disease around their sixties or older. Men are one-and-a-half times more likely to get Parkinson's disease than women. Parkinson's disease is caused by the gradual break down or death of certain nerve cells in the brain. This leads to a reduction in the amount of a chemical called dopamine in the brain. Carbidopa, levodopa and entacapone is the combination of drugs approved by U.S. FDA in June 2003, to treat adults with Parkinson's disease. Levodopa is an immediate precursor to dopamine. Entacapone is a reversible catechol-O-methyl transferase inhibitor which prevents the degradation of levodopa. Carbidopa is a aromatic peripheral L-amino acid decarboxylase inhibitor. Combination of Levodopa, carbidopa & entacapone (Catechols), intended as improved therapy for Parkinson Disease [1-8].

2. **EXPERIMENTAL RESOURCES**

2.1 **Chemical Resources**

Entacapone, levodopa and carbidopa working standards are procured as a gift sample from Aurobindo Pharma Pvt., India. ortho phosphoric acid (OPA), triethyl amine are purchased from LOBA chemical laboratories Pvt. Ltd., HPLC grade water and acetonitrile are purchased from Thermo Fisher Scientific Pvt. Ltd., India.

2.2 **Instrument Resources**

An Quadrupumped (SHIMADZU Prominance-i, LC-2030C) RP-HPLC equipped with PDA detector, micro balance (Teraoka Pvt. Ltd ), pH meter (LAB INDIA), variable range micro pipettes (Cyberpet pro, ANM Amkette Industries), variable size glass bottles, graduated measuring cylinders, volumetric flasks (Borosil), ultrasonic water bath (LOBA Chem Pvt. Ltd., Mumbai), vortexer (Remi equipment Pvt. Ltd.,), deep freezer (-48°C Ilshn lab Co.Ltd.,), refrigerator (Godrej). Pipette tips 10 µL-1000 µL and variable size surgical gloves (Surgicare) are employed in the present investigation.

3. **METHOD DEVELOPMENT**

**Optimized Chromatographic Conditions:** The following conditions were optimized as developed chromatographic conditions.

- **Mobile phase**: Acetonitrile: ortho phosphoric acid (50:50 v/v).
- **Flow rate**: 1.0 ml/min.
- **Column**: Phenomenex ODS (150 X 4.6 mm, 5µm).
- **Detector wave length**: 270 nm.
- **Column temperature**: Ambient
- **Injection volume**: 20 µL
- **Run time**: 5 min.

4. **METHOD VALIDATION**

The analytical method validation was done according to ICH Q2 (R1) guidelines of validation of analytical methods for the parameters of specificity, system suitability, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, precision and robustness were discussed [9-12].

4.1 **Specificity**

Specificity is the ability of the analytical method to produce a response for the analyte in the presence of other components present in the
solution; technically they can be like impurities, degradants or matrix. In this method the specificity is tested for the standard solution and blank and found no interference in the blank injection. Tailing factor and theoretical plates were taken into consideration.

4.2 System Suitability

System suitability was performed for the standard solution and confirmed the method suitability by taking tailing factor, theoretical plates, % RSD and retention time parameters into the consideration [13-16].

4.3 Linearity

The linearity of an validation parameter which confirms the ability of a method (within a given range) to obtain test results which will be directly proportional to the concentration of analyte in the sample. By giving different concentrations of sample solutions it is confirmed that the method is linear in 5-160µg/ml range with 0.999 regression value.

4.4 Limit of Detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The values were determined by calculating from slope and regression line by following the equation [17-18].

\[ \text{LOD} = \frac{3.3 \times \sigma}{S} \]

Fig. 1. Chemical structure of entacapone

Fig. 2. Chemical structure of levodopa

Fig. 3. Chemical structure of carbidopa

Fig. 4. Optimized chromatogram
4.5 Limit of Quantification (LOQ)

LOQ is the parameter which will explain about the detection and quantification of lowest amount. In the method the values of LOQ was determined from the following formula.

\[ \text{LOQ} = \frac{10^* \sigma}{S} \]

4.6 Precision

Precision is an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under prescribed conditions. In the current study the % RSD for the sample solution was found below <2.0 \( [19] \).

4.7 Accuracy

Accuracy can be defined as the closeness of agreement between accepted reference value and the value found. In this study recovery was calculated by standard weighing method for 50%, 100% and 150%.

4.8 Robustness

A robustness method was performed to confirm whether the method is capable of reproducibility during the deliberate changes taken place in the proposed method.

5. RESULTS AND DISCUSSION

5.1 Identification of Wavelength

Approximately 100 mg of Entacapone, Levodopa And Carbidopa is weighed and transferred into 100 ml volumetric flasks individually, to that 70 ml of diluent is added and sonicated to dissolve the compounds, mixed well and made up to the mark with diluent. From those solutions 2 ml is transferred into 3 individual 100 ml volumetric flask, mixed well and made up to the mark with diluent. The prepared solutions are scanned between 200-400 nm to detect the \( \lambda_{\text{max}} \). All the spectra are overlaid and the isobestic point is identified as 270 nm.

6. METHOD VALIDATION

6.1 Specificity

By injecting blank solution it is confirmed that there is no inference found in the standard chromatogram by taking tailing factor and theoretical plates into consideration.

6.2 System Suitability

Six replicate injections of sample were given for the test of system suitability and found % RSD was within limits (<2.0). Results were given in Table 1.

6.3 Precision

The precision of the relative standard deviation of individual area of entacapone and levodopa and carbidopa were found to be within limits.

6.3.1 Intra-day precision

Intraday precision is determined by analyzing same concentration of entacapone, levodopa and carbidopa for six times in the same day.

6.3.2 Inter-day precision

Interday precision is determined by analyzing the same concentration of entacapone, levodopa and carbidopa on different days.

6.4 Limit of Detection and Limit of Quantization

LOD and LOQ of entacapone, levodopa and carbidopa were found be 0.85, 0.24, 0.14 and 2.54, 0.71, 0.43 respectively. They can be calculated as

\[ \text{LOD} = \frac{3.3 \sigma}{S} \]

\[ \text{LOQ} = \frac{10 \sigma}{S} \]

\[ \text{SD} = \text{The standard deviation of Y-intercept 5 calibrations} \]

\[ \text{Slope} = \text{The mean slope of the 5 calibrations} \]

6.5 Linearity

For linearity, Six linear concentrations of entacapone, levodopa and carbidopa (5-160μg/ml) were injected in a triplicate manner. A plot of average peak area versus the concentration in μg/ml or mg/ml is made and from this the correlation coefficient, y-intercept (const. of regression) and slope (coefficient of regression) of the regression line were calculated.
6.6 Accuracy

Accuracy can be defined as the closeness of agreement between accepted reference value and the value found. In this study recovery was calculated by standard weighing method for 50%, 100% and 150%.

6.7 Robustness

Robustness of the method is performed by altering the chromatographic conditions such as pH of the buffer, wavelength, mobile phase composition and observed the variation of the results which should be within the acceptance criteria.

6.8 Assay Result of Marketed Formulation

Assay:

\[
\text{Amount Present} = \frac{\text{At}}{\text{As}} \times \frac{\text{Ws}}{\text{Ds}} \times \frac{\text{Dt}}{\text{Wt}} \times \frac{\text{At}}{\text{As}} \times \frac{\text{Ws}}{\text{Ds}} \times \frac{\text{PA}}{\text{Wt}} \times 100
\]

% Purity = \( \frac{\text{Amount obtained}}{\text{Label Claim}} \) \times 100

Where

At = Area of sample, As = Area of standard
Ws = Weight of standard, Wt = Weight of sample (1.013gms)
Dt = Dilution of sample, Ds = Dilution Of Standard.
PA = Potency

Six replicates of the samples solutions were injected for quantitative analysis. The amounts of entacapne, levodopa and carbidopa estimated were found to be 99.5% and 99.98% and 99.68% respectively. A good separation and resolution of all the drugs indicates that there were no interference from the excipients commonly present in pharmaceutical formulations. This showed that the estimation of dosage form was accurate within given acceptable level of 95% to 105%.

Fig. 5. Chromatogram of blank

Fig. 6. Chromatogram of standard
Table 1. System suitability data

| S. No | Injection number | Peak area for entacapone | Peak area for levodopa | Peak area for carbidopa | Acceptance criteria |
|-------|------------------|--------------------------|------------------------|-------------------------|-------------------|
| 1     | 01               | 24712691                 | 91156709               | 45245759                |                   |
| 2     | 02               | 24463324                 | 91701339               | 44503119                |                   |
| 3     | 03               | 24246704                 | 91987990               | 45054196                |                   |
| 4     | 04               | 24549943                 | 90158178               | 44649392                |                   |
| 5     | 05               | 24711992                 | 91329540               | 45123345                |                   |
| 6     | 06               | 24230160                 | 91947369               | 45049506                |                   |
| Mean  |                  | 24485802                 | 91380187               | 44937552                |                   |
| %RSD  |                  | 0.88                     | 0.75                   | 0.65                    |                   |

System suitability parameters

| Entacapone | Levodopa | Carbidopa | Acceptance criteria |
|------------|----------|-----------|---------------------|
| Tailing for entacapone, levodopa and carbidopa in standard solution | 0.78 | 0.91 | 1.14 | NMT 2.0 |
| Theoretical plates for entacapone, levodopa and carbidopa in standard solution | 2125 | 2338 | 2832 | NLT 2000 |
| Resolution entacapone, levodopa and carbidopa peaks in standard solution | NA | 4.78 | 3.99 | NLT 2.0 |
Table 2. Intra-day precision for entacapone and levodopa and carbidopa

| S.NO | Injection Number | Peak area for entacapone | Peak area for levodopa | Peak area for carbidopa |
|------|------------------|--------------------------|------------------------|-------------------------|
| 1    | Standard 1       | 24478245                 | 91380188               | 44928585                |
| 2    | Standard 2       | 24489724                 | 92151072               | 44852632                |
| 3    | Standard 3       | 24287892                 | 91211254               | 44769889                |
| 4    | Standard 4       | 24645898                 | 91245689               | 44826594                |
| 5    | Standard 5       | 24548561                 | 92015814               | 44915626                |
| 6    | Standard 6       | 24898245                 | 92015489               | 44915623                |
| Mean |                  | 24558094                 | 91669918               | 44868158                |
| %RSD |                  | 0.83                     | 0.47                   | 0.14                    |

Table 3. Inter-day precision for entacapone and levodopa and carbidopa

| S.NO | Injection number | Peak area for entacapone | Peak area for levodopa | Peak area for carbidopa |
|------|------------------|--------------------------|------------------------|-------------------------|
| 1    | Standard 1       | 24878241                 | 91380188               | 44228584                |
| 2    | Standard 2       | 24589724                 | 92151072               | 44752612                |
| 3    | Standard 3       | 24387882                 | 91211254               | 44665889                |
| 4    | Standard 4       | 24445898                 | 91245689               | 44229594                |
| 5    | Standard 5       | 24547561                 | 92015814               | 44815826                |
| 6    | Standard 6       | 24888245                 | 92015489               | 44715823                |
| Mean |                  | 24622925                 | 91669918               | 44568055                |
| %RSD |                  | 0.87                     | 0.47                   | 0.60                    |

Table 4. Report of LOD and LOQ

| S.NO | Drugs      | LOD (µg/ml) | LOQ (µg/ml) |
|------|------------|-------------|-------------|
| 1    | Entacapone | 0.85        | 2.54        |
| 2    | Levodopa  | 0.24        | 0.71        |
| 3    | Carbidopa | 0.14        | 0.43        |

Fig. 7. Calibration curve for entacapone
Table 5. Linearity data

| Standard concentration (µg/ml) | Area of entacapone (µg/ml) | Area of levodopa (µg/ml) | Standard concentration (µg/ml) | Area of carbidopa (µg/ml) |
|-------------------------------|-----------------------------|---------------------------|-------------------------------|---------------------------|
| 5                             | 3060725                     | 22545047                  | 1.2                           | 11034388                  |
| 10                            | 6221451                     | 45690094                  | 2.4                           | 21468776                  |
| 20                            | 12242901                    | 69535141                  | 3.6                           | 33703164                  |
| 40                            | 24485802                    | 91380188                  | 4.8                           | 44937552                  |
| 80                            | 47971604                    | 112225235                | 6                             | 57171941                  |
| 160                           | 98943208                    | 136070282                 | 7.2                           | 67406328                  |

$R^2 = 0.9998$  

$R^2 = 0.9997$  

$R^2 = 0.9996$

Fig. 8. Calibration curve for levodopa

![Calibration curve for levodopa]

$y = 5E+06x + 357143$

$R^2 = 0.9997$

Fig. 9. Calibration curve for carbidopa

![Calibration curve for carbidopa]

$y = 9E+06x - 392857$

$R^2 = 0.9996$
### Table 6. Accuracy data

| Level | Peak area | % recovery | Mean % recovery | Over all recovery |
|-------|-----------|------------|----------------|------------------|
|       | Ent       | Levo       | Car            | Ent              | Levo | Car      | Ent  | Levo | Car  |
| 50    | 12239502  | 45681254   | 23468776       | 99.30            | 98.71 | 99.60    | 99.10| 98.63| 99.06|
|       | 12289021  | 45725689   | 23315687       | 99.36            | 98.47 | 98.61    | 99.10| 98.63| 99.06|
|       | 12212021  | 45894587   | 23425285       | 98.62            | 98.72 | 98.96    | 99.10| 98.63| 99.06|
| 100   | 24432586  | 91661245   | 46906251       | 99.40            | 99.32 | 99.82    | 99.40| 99.47| 99.88|
|       | 24356895  | 91589784   | 46827251       | 99.38            | 99.50 | 99.94    | 99.40| 99.47| 99.88|
|       | 24395986  | 91725865   | 46857251       | 99.41            | 99.55 | 99.88    | 99.40| 99.47| 99.88|
| 150   | 36859254  | 138685213  | 69507252       | 100.22           | 100.43| 98.85    | 99.69| 100.13| 99.07|
|       | 36547821  | 138489113  | 69748695       | 99.21            | 100.13| 99.04    |       |      |      |
|       | 36654578  | 137889123  | 69845625       | 99.64            | 99.84 | 99.32    |       |      |      |

Entacapone and Carbidopa working standard purity: 99.8%
Levodopa working standard purity: 99.20%

### Table 7. Report of robustness – entacapone

| S.No. | Parameter                               | Condition       | System suitability results |
|-------|-----------------------------------------|-----------------|---------------------------|
|       |                                         | % RSD           | USP Tailing               | USP plate count |
| 1     | Flow rate by ± 2%                       | 1.2 ml          | 0.94                      | 0.99           | 2878 |
|       |                                         | 1.0 ml          | 1.05                      | 0.83           | 2695 |
|       |                                         | 1.4 ml          | 1.10                      | 1.01           | 2308 |
| 2     | Column oven temperature by ± 2°C        | 23°C            | 1.00                      | 1.02           | 2603 |
|       |                                         | 25°C            | 0.95                      | 1.11           | 3256 |
|       |                                         | 27°C            | 0.82                      | 1.23           | 3968 |
| 3     | Wavelength of analysis ± 2nm            | 272 nm          | 0.59                      | 1.10           | 2965 |
|       |                                         | 270 nm          | 0.66                      | 1.14           | 2664 |
|       |                                         | 268 nm          | 0.80                      | 1.01           | 2723 |
| 4     | Organic composition of mobile phase by ± 2% | 48:52 | 0.65                      | 1.23           | 2527 |
|       |                                         | 50:50           | 0.78                      | 1.14           | 2692 |
|       |                                         | 52:48           | 0.85                      | 1.12           | 3052 |
Table 8. Report of robustness – levodopoa

| S.No. | Parameter                               | Condition | System suitability results | % RSD | USP tailing | USP plate count |
|-------|-----------------------------------------|-----------|---------------------------|-------|-------------|-----------------|
| 1     | Flow rate by ± 2%                       | 1.2 ml    | 1.05                      | 1.21  | 3638        |
|       |                                         | 1.0 ml    | 1.11                      | 1.23  | 3410        |
|       |                                         | 1.4 ml    | 1.20                      | 1.50  | 2308        |
| 2     | Column oven temperature by ± 2°C        | 23°C      | 0.96                      | 1.24  | 2603        |
|       |                                         | 25°C      | 0.85                      | 1.22  | 2850        |
|       |                                         | 27°C      | 0.86                      | 1.04  | 2652        |
| 3     | Wavelength of analysis ± 2nm            | 272 nm    | 0.99                      | 0.91  | 2921        |
|       |                                         | 270 nm    | 0.81                      | 0.96  | 3652        |
|       |                                         | 268 nm    | 0.79                      | 0.86  | 2121        |
| 4     | Organic composition of mobile phase by ± 2% | 48:52    | 0.69                      | 0.83  | 2542        |
|       |                                         | 50:50     | 0.58                      | 0.86  | 2721        |
|       |                                         | 52:48     | 0.72                      | 0.79  | 2533        |

Table 9. Report of robustness – carbidopoa

| S.No. | Parameter                               | Condition | System suitability results | % RSD | USP tailing | USP plate count |
|-------|-----------------------------------------|-----------|---------------------------|-------|-------------|-----------------|
| 1     | Flow rate by ± 2%                       | 1.2 ml    | 1.18                      | 0.55  | 2531        |
|       |                                         | 1.0 ml    | 1.05                      | 0.68  | 2456        |
|       |                                         | 1.4 ml    | 1.15                      | 0.70  | 3210        |
| 2     | Column oven temperature by ±2°C         | 23°C      | 1.22                      | 1.32  | 2900        |
|       |                                         | 25°C      | 1.14                      | 1.21  | 2533        |
|       |                                         | 27°C      | 1.17                      | 1.17  | 2411        |
| 3     | Wavelength of analysis ± 2nm            | 272 nm    | 0.56                      | 0.86  | 2865        |
|       |                                         | 270 nm    | 0.72                      | 0.84  | 2456        |
|       |                                         | 268 nm    | 0.65                      | 0.79  | 2741        |
| 4     | Organic composition of mobile phase by ± 2% | 48:52    | 0.79                      | 0.76  | 2648        |
|       |                                         | 50:50     | 0.73                      | 0.68  | 2315        |
|       |                                         | 52:48     | 0.75                      | 0.82  | 2145        |
7. CONCLUSION

There is no official compendial method was reported for the estimation of entacapone, levodopa and carbidopa. Therefore the proposed method which is new, simple, sensitive, precise and accurate economical analytical method can be used for the regular analysis and also can be applied to the commercial formulation. Depending on all the validated parameters it can be confirmed that this method is the best one that can be applied for the estimation of both active pharmaceutical industries and also commercial pharmaceutical labs. The high sensitivity (LOD), mobile phase utilized (eco-friendly) and run time (=5) can be determined as an important features for this proposal.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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