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# DEVELOPMENT AND VALIDATION OF A REVERSE PHASE HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF CARBIDOPA AND LEVODOPA IN PHARMACEUTICAL DOSAGE FORMS

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**Abstract:**

*A new, simple, Accurate, precise, robust and rugged reverse phase-HPLC method was developed for the simultaneous estimation of the Levodopa and Carbidopa in pure and pharmaceutical dosage forms. Chromatogram was run through Hypersil C18 (250 mm×4.6 mm, 5µm) particle size. Mobile phase containing Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25%) was pumped through column at a flow rate of 1.0ml/min. Temperature was maintained at Ambient. Optimized wavelength selected was 226 nm. Retention time of Levodopa and Carbidopa were found to be 1.693min and 3.235min ± 0.02 respectively. The precision %RSD of the Levodopa and Carbidopa were and found to be 0.435 and 0.039 respectively. %Recovery was obtained as 100.06% and 100.083% for Levodopa and Carbidopa respectively. Regression equation of Levodopa is y = 48138x + 5396.0., and y = 71.91x + 42.07 of Carbidopa. The LOD and LOQ values were found to be for the Levodopa and Carbidopa are 1.27µg/ml, 1.16 µg/ml 3.81µg/ml, 3.48µg/ml and the proposed method was found to be simple, precise, accurate, rapid, economic and reproducible for the estimation of Levodopa and Carbidopa in pure form and pharmaceutical marketed formulation.*

***Keywords:*** *Levodopa and Carbidopa, HPLC, Method Development, Validation.*

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## INTRODUCTION:

**High Performance Liquid Chromatography**

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved .The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed,efficient ,accurate and highly resolved method of separation.

For the recent study metformin and Sitagliptin was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

- Speed many analysis can be accomplished in 20min (or) less.
- Greater sensitivity(various detectors can be employed).
- Improved resolution(wide variety of stationary phases).
- Re usable columns(expensive columns but can be used for many analysis).
- Ideal for the substances of low viscosity.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour).
- Precise and reproducible.
- Integrator itself does calculations.
- Suitable for preparative liquid chromatography on a much larger scale.

**HPLC Basic Instrumentation:**

**HPLC components**

The essential components of a complete HPLC system are solvent delivery system (Pump), detector, fixed volume injector loop or autosampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in above Figure.

**Column**

The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today.The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

**Pump**

The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

**Injector or Auto sampler**

Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependant on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction. As

HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

**Detector**

HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength.

**Solvent reservoir**
Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory.

**Data handling and analysis**
Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data.

## MATERIALS AND METHODS:

Carbidopa & Levodopa Procured from Sura labs, Water and Methanol for HPLC from LICHROSOLV (MERCK), Acetonitrile for HPLC from Merck, Triethylamine from Merck.

**HPLC METHOD DEVELOPMENT:**
**TRAILS**
**Preparation of standard solution:**
Accurately weigh and transfer 10 mg of carbidopa and levodopa working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.3ml of carbidopa and 1.98ml of levodopa from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

**Procedure:**
Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

**Mobile Phase Optimization**:
Initially the mobile phase tried was Methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile and water in proportion 75:25 v/v respectively.

**Optimization of Column:**
The method was performed with various C18columns like Symmetry, X terra and ODS column. Phenomenex Gemini C18 (4.6×250mm) 5µ was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

**OPTIMIZED CHROMATOGRAPHIC CONDITIONS**:
Instrument used : Waters Alliance 2695 HPLC with PDA Detector 996 model.
Temperature : 40ºC
Column : Phenomenex Gemini C18 (4.6×250mm) 5µ
Mobile phase : Acetonitrile and water (75:25% v/v)
Flow rate : 1ml/min
Wavelength : 240nm
Injection volume : 10µl
Run time : 6minutes

**VALIDATION**
**PREPARATION OF MOBILE PHASE:**
**Preparation of mobile phase:**
Accurately measured 750ml of Acetonitrile (75%) of and 250ml of HPLC Water (25%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

**Diluent Preparation:**
The Mobile phase was used as the diluent.

## RESULTS AND DISCUSSION:

Mobile phase : Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25)
Auto sample temperature : Ambient
Injection volume : 20µL
Column : Hypersil C18 (250 mm×4.6 mm, 5µm) particle size
Detector wavelength : 226 nm
Flow rate : 1.0ml/min
Run time : 6 minutes

**Procedure: a**
Inject 20µL of standard, sample into chromatographic system and measure the areas for the Levodopa and Carbidopa peeks and calculate the % assay by using the formula.

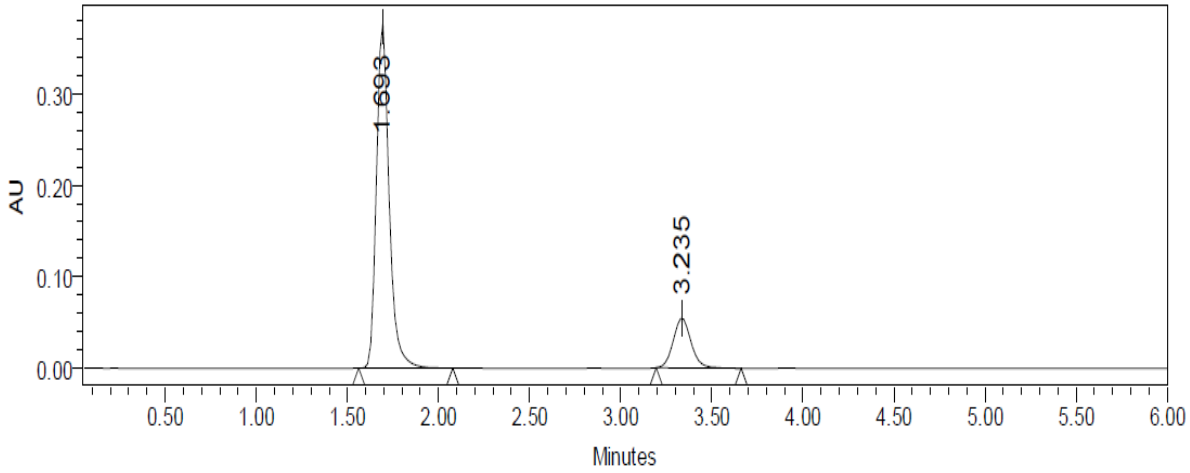


**Fig no: 12 Typical Chromatogram for optimized method**

**Table-: Results of optimized method Chromatogram**

| S. No. | Name | Retention Time | Area | USP Resolution | USP Tailing | USP Plate Count |
|---|---|---|---|---|---|---|
| 1 | Levodopa | 1.693 | 3658985 | | 1.58 | 5698 |
| 2 | Carbidopa | 3.235 | 6529 | 7.28 | 1.63 | 7529 |

**Observation:** Peeks are well separated all the parameters are within the limits.

**Table no: 10 Standard Results of Levodopa**

| S. no | Sample name | RT | Area | USP plate count | USP tailing |
|---|---|---|---|---|---|
| 1 | Injection 1 | 1.694 | 3658986 | 5698 | 1.58 |
| 2 | Injection 2 | 1.689 | 3659844 | 5655 | 1.59 |
| 3 | Injection 3 | 1.692 | 3659864 | 5682 | 1.58 |
| 4 | Injection 4 | 1.688 | 3654875 | 5674 | 1.58 |
| 5 | Injection 5 | 1.688 | 3654514 | 5628 | 1.59 |
| **Avg.** | | | 3657617 | | |
| **SD** | | | 2693.969 | | |
| **% RSD** | | | 0.073654 | | |

**Table no: 11 Standard Results of Carbidopa**

| S. no | Sample name | RT | Area | USP plate count | USP tailing |
|---|---|---|---|---|---|
| 1. | Injection 1 | 3.244 | 6598 | 7598 | 1.63 |
| 2. | Injection 2 | 3.238 | 6574 | 7549 | 1.64 |
| 3. | Injection 3 | 3.246 | 6523 | 7561 | 1.63 |
| 4. | Injection 4 | 3.265 | 6539 | 7592 | 1.63 |
| 5. | Injection 5 | 3.265 | 6578 | 7569 | 1.64 |
| **Avg.** | | | 6562.4 | | |
| **SD** | | | 30.59902 | | |
| **% RSD** | | | 0.466278 | | |

**ACCURACY:**

**Table no: Accuracy (%recovery) results of Levodopa**

| S. no | Accuracy Level | Sample name | µg/ml added | µg/ml found | % Recovery | % Mean |
|---|---|---|---|---|---|---|
| 1 | 50% | 1 | 40 | 39.949 | 99.872 | 99.979% |
| | | 2 | 40 | 40.098 | 100.245 | |
| | | 3 | 40 | 39.929 | 99.822 | |
| 2 | 100% | 1 | 80 | 80.071 | 100.088 | 100.124% |
| | | 2 | 80 | 80.180 | 100.225 | |
| | | 3 | 80 | 80.048 | 100.060 | |
| 3 | 150% | 1 | 120 | 120.080 | 100.066 | 100.091% |
| | | 2 | 120 | 120.092 | 100.076 | |
| | | 3 | 120 | 120.159 | 100.132 | |

**Table no: Accuracy (%recovery) results of Carbidopa**

| S. no | Accuracy Level | Sample name | µg/ml added | µg/ml found | % Recovery | % Mean |
|---|---|---|---|---|---|---|
| 1 | 50% | 1 | 45 | 45.124 | 100.275 | 100.131% |
| | | 2 | 45 | 44.999 | 99.997 | |
| | | 3 | 45 | 45.055 | 100.122 | |
| 2 | 100% | 1 | 90 | 90.028 | 100.031 | 100.108% |
| | | 2 | 90 | 90.056 | 100.062 | |
| | | 3 | 90 | 90.209 | 100.232 | |
| 3 | 150% | 1 | 135 | 134.987 | 99.990 | 100.010% |
| | | 2 | 135 | 135.112 | 100.082 | |
| | | 3 | 135 | 134.945 | 99.959 | |

**LINEARITY**

**Table no: Linearity data for Levodopa**

| S. no | Concentration (µg/ml) | Rt | Area |
|---|---|---|---|
| 1. | 40 | 1.689 | 1923835 |
| 2. | 60 | 1.691 | 2899874 |
| 3. | 80 | 1.692 | 3868985 |
| 4. | 100 | 1.689 | 4835984 |
| 5. | 120 | 1.688 | 5758747 |

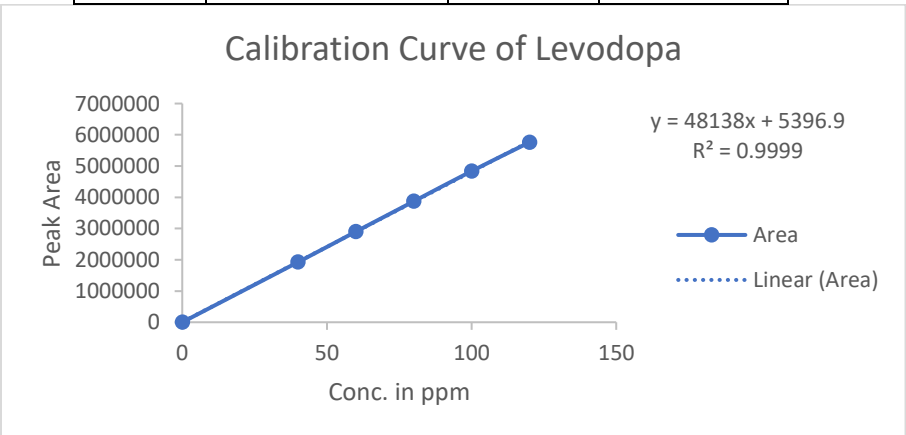


**Fig no: Linearity Curve of Levodopa**

**Table no: Linearity data for Carbidopa**

| S. no | Concentration (µg/ml) | Rt | Area |
|---|---|---|---|
| 1. | 50 | 3.203 | 3675 |
| 2. | 70 | 3.299 | 5108 |
| 3. | 90 | 3.294 | 6529 |
| 4. | 110 | 3.290 | 7954 |
| 5. | 130 | 3.288 | 9349 |

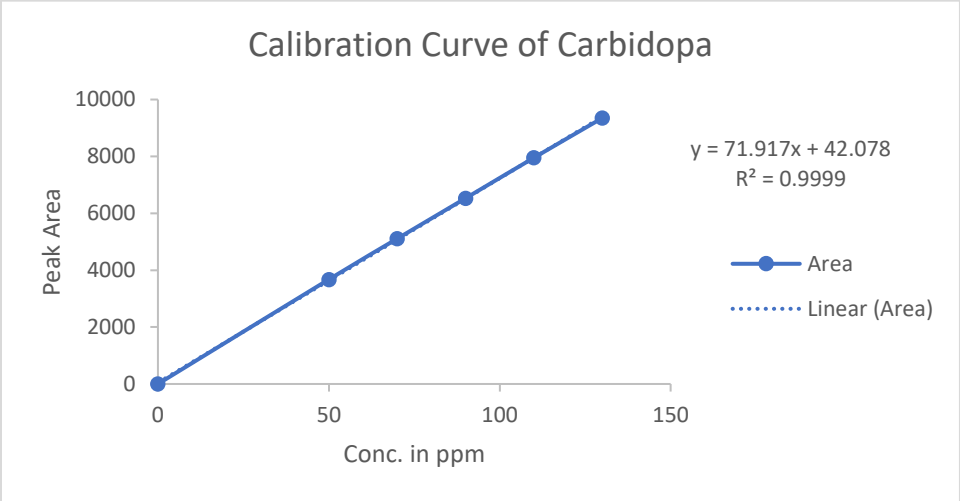


**Fig no: Linearity Curve of Carbidopa**

**Robustness**

**Table no: Robustness data for Levodopa**

| Parameter | Rt | Theoretical plates | Tailing factor |
|---|---|---|---|
| Decreased flow rate (0.8ml/min) | 1.868 | 5854 | 1.56 |
| Increased flow rate (1.2ml/min) | 1.544 | 5365 | 1.57 |
| Decreased temperature ($20^0$c) | 1.731 | 5418 | 1.53 |
| Increased temperature ($30^0$c) | 1.675 | 5496 | 1.54 |

**Table no: Robustness data for Carbidopa**

| Parameter | Rt | Theoretical plates | Tailing factor |
|---|---|---|---|
| Decreased flow rate (0.8ml/min) | 3.621 | 7598 | 1.62 |
| Increased flow rate(1.2ml/min) | 2.998 | 7612 | 1.61 |
| Decreased temperature ($20^0$c) | 6.242 | 7251 | 1.64 |
| Increased temperature ($30^0$c) | 2.302 | 7195 | 1.61 |

**PRECISION:**

**Table no: Precision studies for Levodopa and Carbidopa**

| S. no | Intraday precision for Levodopa | | | Intraday precision for Carbidopa | | |
|---|---|---|---|---|---|---|
| | Peak area | Mean peak area | %RSD | Peak area | Mean peak area | %RSD |
| 1 | 3658952 | | | 6598 | | |
| 2 | 3659854 | | | 6529 | | |
| 3 | 3659874 | 3665965 | 0.435 | 6537 | 6547 | 0.390 |
| 4 | 3658748 | | | 6538 | | |
| 5 | 3698547 | | | 6546 | | |
| 6 | 3659816 | | | 6534 | | |

**SPECIFICITY:**

**Table no: Specificity data for Levodopa and Carbidopa**

| S. no | Sample name | Levodopa | | Carbidopa | |
|---|---|---|---|---|---|
| | | Area | Rt | Area | Rt |
| 1 | Standard | 3658985 | 1.691 | 6529 | 3.299 |
| 2 | Sample | 3785984 | 1694 | 6695 | 3.234 |
| 3 | Blank | - | - | - | - |
| 4 | Placebo | - | - | - | - |

## CONCLUSION:

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative simultaneous estimation of Levodopa and Carbidopa in bulk drug and pharmaceutical dosage forms.

This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps.

Levodopa was found to be readily sol in dil. hydrochloric and formic acids; practically insoluble in ethanol, benzene, chloroform, ethyl acetate, soluble in water. Carbidopa was found to be slightly soluble in water, Soluble in 100% ethanol and in methanol, freely soluble in 3N HCl, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. Potassium dihydrogen phosphate (0.03M) (pH-2.8): Methanol (75:25) was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.

This method can be used for the routine simultaneous determination of Levodopa and Carbidopa in bulk drug and in Pharmaceutical dosage forms.

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