RP-HPLC Method development, Validation and Forced Degradation for Simultaneous estimation of Benserazide HCl and Levodopa in a Marketed Formulation

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Abstract: The objective of the study was to develop and validate a novel, stability indicating, simple, rapid, accurate, precise and isocratic reverse-phase high-performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of benserazide HCl and levodopa in a marketed formulation. Chromatographic separation was achieved by using C18 Cosmosil 4.6 × 250 mm column with a mixture of phosphate buffer pH 2 and acetonitrile in proportion of 95:5 as mobile phase at a flow rate of 1.0 ml/min and column temperature 25°C. The detection was carried out at 210 nm using UV detector. The retention time for benserazide and levodopa was found to be 3.1 minutes and 6.6 minutes respectively and recoveries from tablet were between 98 and 102 %.

Keywords: Benserazide HCl, Levodopa, Reverse-phase high-performance liquid chromatography, validation, forced degradation.

INTRODUCTION:

Parkinson's disease is a neurodegenerative disorder of the extrapyramidal nervous system. It affects the mobility and control of the skeletal muscular system. The level of the dopamine in parkinson's disease is very less or dopamine gets depleted. But administration of dopamine is ineffective in the treatment of Parkinson's disease. Hence levodopa (precursor of dopamine) is administered which on metabolism gives dopamine [1,2]. Levodopa (LEV) is chemically, (S)-2-amino-3-(3, 4-dihydroxyphenyl)propionic acid. As dopamine itself cannot cross the blood brain barrier, levodopa which is its precursor is used in treatment of parkinsons disease [3]. Benserazide is chemically named as 2-amino-3-hydroxy-N'-[2,3,4-trihydroxyphenyl]methyl)propane hydrazide. It is a peripherally acting aromatic L- aromatic amino acid decarboxylase (AADC) or DOPA decarboxylase inhibitor [4]. Benserazide is used in combination with levodopa because it inhibit dopamine production outside the brain and allows direct delivery of dopamine to the brain. By blocking the conversion of levodopa to dopamine, benserazide prevents the side effects such as palpitation, nausea and vomiting [5]. Chemical structure of both drugs are given in Fig. 1 and 2.

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Various HPLC methods have been reported for estimation of benserazide HCl and levodopa in combination with other drugs or alone such as Stability Indicating HPLC Method for Simultaneous Estimation of Entacapone, Levodopa and Carbidopa [1], RP-HPLC Method for Simultaneous Estimation of Levodopa and Carbidopa [3], HPLC Method for the Simultaneous Quantification of Levodopa, Carbidopa, Benserazide and Entacapone in Complex Matrices [6], HPLC method for the determination of levodopa [7], RP-HPLC-PDA Method for the Simultaneous Estimation of Levodopa, Carbidopa and Entacapone [8], HPLC method for simultaneous estimation of drug release of levodopa and carbidopa in entacapone, levodopa and carbidopa tablets [9].

From the literature survey it is evident that there has been only one RP-HPLC method reported for estimation of benserazide HCl and levodopa [4]. There is no forced degradation studies reported till now for benserazide HCl and levodopa combination, hence it was decided to develop and validate a novel method followed by forced degradation studies.

EXPERIMENTAL

Chemicals and reagents

Pharmaceutical grade working standard levodopa and benserazide were provided by Enaltech laboratories, Ambernath. The commercial tablet dosage form Madopar 250 was purchased from local market. All chemicals and reagents used were HPLC grade.

Instrument

The analysis was performed using shimadzu prominence-i 2030 model, data handling system (lab solution), The BIO-LAB (BL-135D) centrifuge model was used for centrifugation of sample. pH meter and the Kroma tech (KL-1.5) sonicator was used for sonication.

Chromatographic conditions

Chromatographic separation was achieved by using C18 Cosmosil 250×4.6mm column with mixture of phosphate buffer pH 2 and acetonitrile in ratio of (95:5) as the mobile phase at a flow rate of 1.0 ml/min and column temperature 25°C. The detection was carried out at 210 nm.

Selection of wavelength

Standard solution of benserazide HCl 5 ppm and levodopa 5 ppm were prepared and scanned by UV spectrophotometer separately, in the range of 200-400 nm and overlay UV spectra of benserazide HCl and levodopa was obtained as shown in Fig.3. 210 nm wavelength was selected as detection wavelength for separation of benserazide HCl and levodopa.

Preparation of phosphate buffer pH 2

2.73 gm of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 2000 ml of water and mixed well. The pH of above solution was adjusted to 2 with 10 % dilute orthophosphphoric acid, and the solution was filtered through 0.45 nylon filter.
Preparation of 10% orthophosphoric acid

10 ml of orthophosphoric acid was diluted to 100 ml of water to obtain 10% OPA.

Preparation of mobile phase

A mixture of 97 volumes of phosphate buffer and 3 volumes of HPLC grade acetonitrile was prepared and sonicated for 10 min to degas.

Preparation of diluent

Buffer pH 2 and acetonitrile was mixed in the ratio of 99:1.

Preparation of Benserazide HCl standard solution

25 gm of benserazide HCl was accurately weighed and transferred in 50 ml volumetric flask. 30 ml 0.1 N HCl was added and sonicated to dissolve and diluted up to the mark with diluents. Further 5 ml of stock solution was diluted to 50 ml with diluents to obtain final solution of 50 ppm.

Preparation of Levodopa standard solution

50 gm of levodopa was accurately weighed and transferred in 50 ml volumetric flask. 30 ml 0.1 N HCl was added and sonicated to dissolve and diluted up to the mark with diluent. Further 5 ml of stock solution was diluted to 50 ml with diluent to obtain final solution of 200 ppm.

Preparation of sample solution

5 Tablets were weighed and powdered finely and quantity corresponding to 50 mg of benserazide HCl and 200 mg of levodopa was taken and transferred to 100 ml volumetric flask and 30 ml of 0.1N HCl was added. The flask was sonicated for 30 min with intermittent shaking. Volume was adjusted upto the mark with diluent. The sample solution was centrifuged at 5000 rpm for 10 min and then filtered through whatman filter paper. Further 5 ml of sample solution was diluted to 50 ml with diluent.
Assay of marketed formulation

5 Tablets were weighed and powdered finely and quantity corresponding to 50 mg of benserazide HCl and 200 mg of levodopa was taken and transferred to 100 ml volumetric flask and 30 ml of 0.1N HCl was added. The flask was sonicated for 30 minutes with intermittent shaking. Volume was adjusted upto the mark with diluent. The sample solution was centrifuged at 5000 rpm for 10 minute and then filtered through Whatman filter paper. Further 5 ml of sample solution was diluted to 50 ml with diluent. The percent assay of marketed formulation was found to be 100.0% for benserazide HCl and 98.8% for levodopa as shown in table 9.

Method development

Chromatographic separation was achieved by using C18 Cosmosil (4.6 x 250m) with mixture of phosphate buffer pH 2 and acetonitrile in ratio of (95:5) as the mobile phase at a flow rate of 1.0 ml/min and column temperature 25°C. The detection was carried out at 210 nm. The developed optimized method resulted in the elution of benserazide at 3.1 ± 0.2 minute and levodopa at 6.3± 0.2 minute. The total runtime was 10 minutes. Chromatograms of standard and sample of benserazide and levodopa are shown in Fig.4 and Fig.5. The optimized chromatographic conditions are tabulated in Table 1.

![Optimized chromatogram of standard benserazide HCl and levodopa at 210nm.](image1)

![Optimized chromatogram of sample benserazide HCl and levodopa at 210nm.](image2)
Table 1: Optimized chromatographic conditions for benserazide and levodopa

| Parameters             | Optimized conditions                   |
|------------------------|----------------------------------------|
| Pump mode              | Isocratic                              |
| column                 | Cosmosil c 18 (250 × 4.6mm)             |
| Mobile phase           | Phosphate buffer pH 2 :acetonitrile (97:3) |
| Flow rate              | 1.0 ml/min                              |
| Column temperature     | 25°C                                   |
| Injection volume       | 10 µl                                   |
| Detection wavelength   | 210 nm                                  |
| Retention time         | 3.1 minutes and 6.6 minutes respectively |

Method validation

The developed method for benserazide HCl and levodopa was validated for parameter such as system suitability, precision, linearity, accuracy and robustness as per International Council for Harmonisation guidelines [10-13].

System suitability

The system suitability was done by injecting six replicates of standard solution and retention time, tailing factor, and number of theoretical plates were evaluated. The standard solution of benserazide HCl and levodopa were prepared as per optimize method and injected into a chromatographic system.

Precision

From multiple sampling of same homogeneous sample under the given condition, the closeness of agreement between a series of obtained measurements is known as precision. The system precision and method precision were performed by injecting six injection of benserazide HCl and levodopa standard and sample. The percentage relative standard deviation (% RSD) was calculated.

Accuracy

The accuracy was evaluated at three levels 50%, 100%, and 150% of working level concentration by the standard addition method. At each level, three replicates were injected into chromatographic system. Since the working concentration of benserazide HCl is 50 ppm and levodopa is 200 ppm. Percentage recovery and relative standard deviation were calculated.

Linearity

Linearity was evaluated in the range of 50-150 % of the working concentration level, As the working concentration of benserazide HCL is 50 ppm and levodopa is 200 ppm. The linearity curve was constructed by plotting peak area versus concentration and the regression coefficient was calculated.

Robustness

The method is said to be robust when there is no changes in the results occurs even when the deliberate changes in the method parameters are made. The developed method was evaluated for the robustness by making deliberate changes in optimized method such as change in flow rate (±0.2 ml/min), change in wavelength (±2 nm), change in temperature (±5°C) and the percentage relative standard deviation (% RSD) was calculated.

Forced degradation

Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions [14]. Forced degradation studies were carried out on marketed formulation of benserazide HCL and levodopa by treating it under stress condition to estimate the ability of developed method
to separate benserazide HCl and levodopa from degradant products. Forced degradation studies was carried out by treating sample under stress condition such as acid degradation, alkali degradation and peroxide degradation.

Table 2: System suitability parameter for Benserazide HCl and Levodopa

| Parameters                  | Benserazide HCl | Levodopa |
|-----------------------------|-----------------|----------|
| Retention time              | 3.1 min         | 6.6 min  |
| Tailing factor              | 1.0             | 1.05     |
| Number of theoretical plate | 3880            | 6500     |

Table 3: System precision results for Benserazide HCl and Levodopa

| Sr.no | Benserazide HCl Peak area | Levodopa Peak area |
|-------|---------------------------|-------------------|
| 1     | 2861179                   | 6262064           |
| 2     | 2864372                   | 6246001           |
| 3     | 2858284                   | 6237787           |
| 4     | 2855243                   | 6268842           |
| 5     | 2841637                   | 6231047           |
| 6     | 2830030                   | 6251282           |
| Average | 2851791          | 6249504           |
| SD    | 13245                     | 14318             |
| % RSD | 0.46                      | 0.23              |

Table 4: Method precision results for Benserazide HCl and Levodopa

| Sr.no | Benserazide HCl | Levodopa |
|-------|-----------------|----------|
| 1     | 100.6           | 97.8     |
| 2     | 99.3            | 98.3     |
| 3     | 98.8            | 98.9     |
| 4     | 99.8            | 98.4     |
| 5     | 99.6            | 98.8     |
| 6     | 100.0           | 98.4     |
| Average | 99.7            | 98.4     |
| SD    | 0.61            | 0.39     |
| % RSD | 0.61            | 0.47     |

RESULT AND DISCUSSION

System suitability

The system suitability was done by injecting six replicates of standard solution and retention time, tailing factor, and number of theoretical plates were evaluated. All the parameter results are within limit and are tabulated in Table 2.

Precision

The percentage relative standard deviation (%RSD) was calculated from chromatogram area and is <2%. From precision results, it was found that the method is precise. Results of precision are tabulated in Table 3 and 4.

Accuracy

The accuracy was evaluated at three levels 50%, 100%, and 150% of working level concentration. Since the working concentration of benserazide HCl is 50 ppm and levodopa is 200 ppm. Results of recovery of benserazide HCl and levodopa are tabulated in Table 5 and 6.
Linearity

The regression coefficient ($r^2$) was found to be 0.9983 for benserazide and 0.9993 for levodopa. From the linearity results, it was found that the developed method is linear as depicted in Fig. 6 and Fig. 7. Results are tabulated in Table 7 and 8.

Robustness

The % RSD was found to be within limits, and method was found to be robust. Results of robustness of benserazide HCl and levodopa are tabulated in Table 8.

Forced degradation

Forced degradation is a degradation of new drug substance and drug product at conditions more severe than accelerated conditions [14]. Forced degradation studies were carried out on marketed formulation of benserazide HCl and levodopa by treating it under stress condition to estimate the ability of developed method to separate benserazide HCl and levodopa from degradant products.

Acid degradation

In acidic condition (0.1 N HCl), both benserazide HCl and levodopa are not degraded, and no peak of any degradation was observed in chromatogram (Fig.8). The results of force degradation are tabulated in table 10.

Base degradation

In alkali degradation (0.1 N NaOH), both benserazide HCl and levodopa degraded and degradation was 6% and 0.9% for benserazide HCl and levodopa respectively, and no peak of degradant product was observed in chromatogram (Fig.9). The results of force degradation are tabulated in table 10.

Oxidative degradation

In oxidative degradation (30% H$_2$O$_2$), both benserazide HCl and levodopa were degraded and degradation was 11.4% and 4.1% for benserazide HCl and levodopa respectively, and peak of degradant product was observed in chromatogram at 4.5 minutes (Fig.10). The results of force degradation are tabulated in table 10.

Fig. 5: Optimized chromatogram of sample benserazide HCl and levodopa at 210nm.
Fig. 6: Linearity curve of standard Benserazide HCl

Fig. 7: Linearity curve of standard levodopa

Table 5: Accuracy results for Benserazide HCl

| Level  | % Recovery | Average | SD    | % RSD |
|--------|------------|---------|-------|-------|
| 50 %   | 99.63      | 99.52   | 0.1819| 0.1824|
|        | 99.62      |         |       |       |
|        | 99.31      |         |       |       |
| 100 %  | 99.65      | 99.81   | 0.1446| 0.1448|
|        | 99.91      |         |       |       |
|        | 99.89      |         |       |       |
| 150 %  | 99.65      | 99.56   | 0.1307| 0.1312|
|        | 99.62      |         |       |       |
|        | 99.42      |         |       |       |
### Table 6: Accuracy results for Levodopa

| Level | % Recovery | Average | SD  | % RSD |
|-------|------------|---------|-----|-------|
| 50 %  | 99.14      | 100.29  | 0.365 | 0.3   |
|       | 100.03     |         |      |       |
|       | 99.98      |         |      |       |
| 100 % | 100.14     | 99.81   | 0.500 | 0.5   |
|       | 100.03     |         |      |       |
|       | 100.71     |         |      |       |
| 150 % | 100.14     | 99.56   | 0.571 | 0.5   |
|       | 100.02     |         |      |       |
|       | 99.02      |         |      |       |

### Table 7: Linearity results for Benserazide HCl and Levodopa

| Concentration (PPM) | Area | Benserazide HCl | Levodopa |
|---------------------|------|-----------------|----------|
| 25                  | 1430589 | 3131032       |
| 35                  | 2145883 | 4696548       |
| 50                  | 2961179 | 6262064       |
| 65                  | 3566473 | 7827580       |
| 75                  | 4291774 | 9193096       |

### Table 8: Robustness results for Benserazide HCl and Levodopa

| Parameter                  | Benserazide HCl | Levodopa |
|----------------------------|-----------------|----------|
| Minus flow (0.8 ml / min)  | 101.5           | 100.3    |
| Plus flow (1.2 ml / min)   | 101.1           | 101.7    |
| Minus temperature (20°C)   | 99.0            | 100.0    |
| Plus temperature (30°C)    | 99.9            | 98.19    |
| Minus wavelength (208 nm)  | 101.2           | 101.6    |
| Plus wavelength (212 nm)   | 101.5           | 100.2    |

### Table 9: Assay of marketed formulation

| Tablet                        | Drug            | % Assay |
|-------------------------------|-----------------|---------|
| Madopar (benserazide HCl      | Benserazide HCl | 100.0   |
| 50 mg + levodopa 200mg        | Levodopa        | 98.8    |

### Table 10: Forced degradation studies for benserazide HCL and levodopa

| Condition                        | Benserazide HCl | Levodopa |
|----------------------------------|-----------------|----------|
|                                  | % Assay | Difference W.R.T control | % Assay | Difference W.R.T control |
| Control sample                   | 100.0   | NA                  | 98.8    | NA                      |
| Acid treated sample              | 98.2    | 1.8                | 98.0    | 0.8                     |
| Base treated sample              | 94.1    | 6                  | 97.9    | 0.9                     |
| Peroxide treated sample          | 88.8    | 11.4               | 94.7    | 4.1                     |
Fig. 8: Chromatogram of acid treated sample

Fig. 9: Chromatogram of base treated sample

Fig. 10: Chromatogram of peroxide treated sample
CONCLUSION

The stability-indicating method has been developed and validated for the simultaneous estimation of benserazide HCl and levodopa in bulk and marketed dosage form. The developed method was successfully applied for forced degradation. Results indicates that the method can be successfully used for separation of degraded products from sample. The developed method is novel, cost effective, precise and accurate for determination of benserazide HCl and levodopa and its forced degraded products.

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REFERENCES

1. Bhatnagar P, Vyas D, Sinha SK, Chakrabarti T. Stability Indicating HPLC Method for Simultaneous Estimation of Entacapone, Levodopa and Carbidopa in Pharmaceutical Formulation. J Chromatogr Sep Tech, 2015, 6, 304.
2. Robust and Efficient HPLC Method for the Simultaneous Quantification of Levodopa, Carbidopa, Benserazide and Entacapone in Complex Matrices. J Pharm Pharm Sci. 2017, 20, 258-269.
3. Kumar SS, Natraj K, Khan A, Kumar BK, Rao JV. Development and Validation of RP-HPLC Method for Simultaneous Estimation of Levodopa and Carbidopa in Pharmaceutical Dosage Forms. J. Pharm. Res, 2011, 4(11), 1-4.
4. B. Madhavi Latha et al. RP-HPLC PDA method for simultaneous estimation of benserazide and levodopa in pure and marketed formulation. J Cur Res 2018; 3(1):28-31.
5. Naushad Mu, Gupta VK, Wabaidur SM, Alothman ZA. Simultaneous Determination of Benserazide and Levodopa in Pharmaceutical Tablet, Human Serum and Urine Sample by Differential Pulse Voltammetry Using Modified Glassy Carbon Electrode. Int J Electrochem Sci, 2013, 8, 297 – 311.
6. Wollmer, E., & Klein, S. Development and Validation of a Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-A review. J Pharm Anal, 2014, 4, 159-65.
7. Elbarbry F, Nguyen V, Mirka A, Zwinkey H, Rosenbaum R. A new validated HPLC method for the determination of levodopa: Application to study the impact of ketogenic diet on the pharmacokinetics of levodopa in Parkinson's participants. Biomed. Chromatogr., 2019, 33, e4382.
8. S.Madhavi et al. Development and Validation of RP-HPLC-PDA Method for the Simultaneous Estimation of Levodopa, Carbidopa and Entacapone in Bulk and Pharmaceutical Dosage Forms. Indo Am J Pharm Res, 2014, 4(11), 5235-41.
9. Bhatnagar P, Vyas D, Sinha SK and Gajbhiye A. HPLC method for simultaneous estimation of drug release of levodopa and carbidopa in entacapone, levodopa and carbidopa tablets. Int J Pharm Sci Res, 2017, 8(3), 1091-01.
10. International Conference on Harmonization. Stability Testing of New Drug Substances and Products (Q1AR2). International Conference on Harmonization. Geneva, IFPMA, 2003.
11. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use. Validation of Analytical Procedures: Text and Methodology. ICH, 2005.
12. Food and Drug Administration. Reviewer Guidance Validation of Chromatographic Methods. Centre for Drug Evaluation and Research (CDER). Food and Drug Administration, 1994, 2.
13. Food and Drug Administration. ORA Validation and Verification Guidance for Human Drug Analytical Methods. Food and Drug Administration, 2003, 1.
14. Blessy M, Patel RD, Prajapati PN, Agrawal YK. Development of forced degradation and stability indicating studies of drugs-A review. J Pharm Anal, 2014, 4, 159-65.

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