



A new rp-hplc method for the simultaneous estimation of levodopa, benserazide in its pure and pharmaceutical dosage form as per ich guidelines

Sheik Sufiya*¹, B. Sravanasree¹, D. Appalaraju¹

¹Department of Pharmaceutical Analysis, Pydah College of Pharmacy Patavala, Andhra University, Kakinada, Andhra Pradesh, India.

*Corresponding Author: Sheik Sufiya

Published on: 14.08.2023

ABSTRACT

A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of Levodopa, Benserazide in bulk and pharmaceutical formulations. Separation of Levodopa, Benserazide was successfully achieved on a Phenomenex Luna C18 (4.6×250mm, 5µm) particle size or equivalent in an isocratic mode utilizing Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v) at a flow rate of 1.0mL/min and elutes was monitored at 245nm, with a retention time of 2.102 and 3.537 minutes for Levodopa, Benserazide respectively. The method was validated and the response was found to be linear in the drug concentration range of 6µg/mL to 14µg/mL for Ertugliflozin and 18µg/mL to 42µg/mL for Metformin. The values of the slope and the correlation coefficient were found to be 77824 and 0.999 for Ertugliflozin and 10515 and 0.999 for Metformin respectively. The LOD and LOQ for Ertugliflozin were found to be 0.6µg/mL and 1.8µg/mL respectively. The LOD and LOQ for Metformin were found to be 0.8 µg/mL and 2.4µg/mL respectively. This method was found to be good percentage recovery for Levodopa, Benserazide were found to be 100.351 and 100.93 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analytes in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, Specificity and Robustness.

Keywords: Levodopa, Benserazide, RP-HPLC, Accuracy, Precision, ICH Guidelines.

INTRODUCTION

Chromatography²

Analytic method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantify or purifying compounds of interest. This technical brief will focus on development and validation activities as applied to drug products.

Method development

Effective method development ensures that laboratory resources are optimized, while methods meet the objectives

required at each stage of drug development. Method validation, required by regulatory agencies at certain stages of the drug approval process, is defined as the “process of demonstrating that analytical procedures are suitable for their intended use” [1-2]. Understanding of the physical and chemical characteristics of drug allows one to select the most appropriate high performance liquid chromatography method development from the available vast literature. Information concerning the sample, for example, molecular mass, structure and functionality, pKa values and UV spectra, solubility of compound should be compiled. The requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the

concentration, extraction (liquid or solid phase), derivatization for detection etc. should be checked. For pure compound, the sample solubility should be identified whether it is organic solvent soluble or water soluble, as this helps to select the best mobile phase and column to be used in HPLC method development.

Method development in HPLC can be laborious and time consuming. Chromatographers may spend many hours trying to optimize a separation on a column to accomplish the goals. Even among reversed phase columns, there is astonishing diversity, owing to differences in both base silica and bonded phase characteristics. Many of these show unique selectivity. What is needed is a more informed decision making process for column selection that may be used before the chromatographer enters the laboratory. The method of column selection presented here involves a minimal investment in time initially, with the potential of saving many hours in the laboratory.

Analytic methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods that support safety and characterization studies or evaluations of drug performance are also to be evaluated. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate the potential degradation of the API in the presence of formulation excipients [3, 4].

The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age) analysis conditions (% organic, pH, flow rate, temperature, wavelength, and column age), and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization [5]. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually [6-8]. The degraded drug samples obtained are subjected to preliminary chromatographic separation to study the number and types of degradation products formed under various conditions [9]. Scouting experiments are run and then conditions are chosen for further optimization [10]. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development [11]. Selectivity can be manipulated by combination of different factors like solvent composition, type of stationary phase, mobile phase, buffers and pH. Changing solvents and stationary phases are the most comfortable approaches to achieve the separation. The proper range of pH is an important tool for separation of ionizable compounds. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in

this range would result in a dramatic shift in retention time. However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention time that can be exploited in method development but also the method can be made more robust which is a desirable outcome with validation in minutes.

MATERIALS AND METHOD

Levodopa-Sura labs, Benserazide-Sura labs, Water and Methanol for HPLC-LICHROSOLV (MERCK), Acetonitrile for HPLC -Merck

HPLC method development

Trails

Preparation of standard solution

Accurately weigh and transfer 10 mg of Levodopa and Benserazide 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 0.1ml of the above Levodopa and 0.3ml of the Benserazide 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 Water: Acetonitrile and Methanol: Phosphate Buffer: ACN with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Phosphate Buffer in proportion 45:55 v/v respectively.

Optimization of Column

The method was performed with various columns like C18 column, Symmetry and Zodiac column. Phenomenex Luna C18 (4.6×250mm, 5µm) particle size was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Optimized chromatographic conditions

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.

Temperature : 35°C

Column : Phenomenex Luna C18 (4.6×250mm, 5µm) particle size

Buffer : Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 4.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration and ultra sonication.

pH : 4.6

Mobile phase : Acetonitrile: Phosphate Buffer(45:55 v/v)

Flow rate : 1ml/min

Wavelength : 245 nm

Injection volume : 10 µl

Run time : 7 min

Method validation

Preparation of mobile phase

Preparation of mobile phase

Accurately measured 450 ml (45%) of Methanol, 550 ml of Phosphate buffer (55%) were mixed and degassed in digital ultra sonicator for 15 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 : Acetonitrile: Phosphate Buffer (pH-4.6) (45:55 v/v)
 Column : Phenomenex Luna C18 (4.6×250mm, 5μm) particle size
 Flow rate : 1 ml/min
 Wavelength : 245 nm
 Column temp : 35°C
 Injection Volume : 10 μl
 Run time : 7 minutes

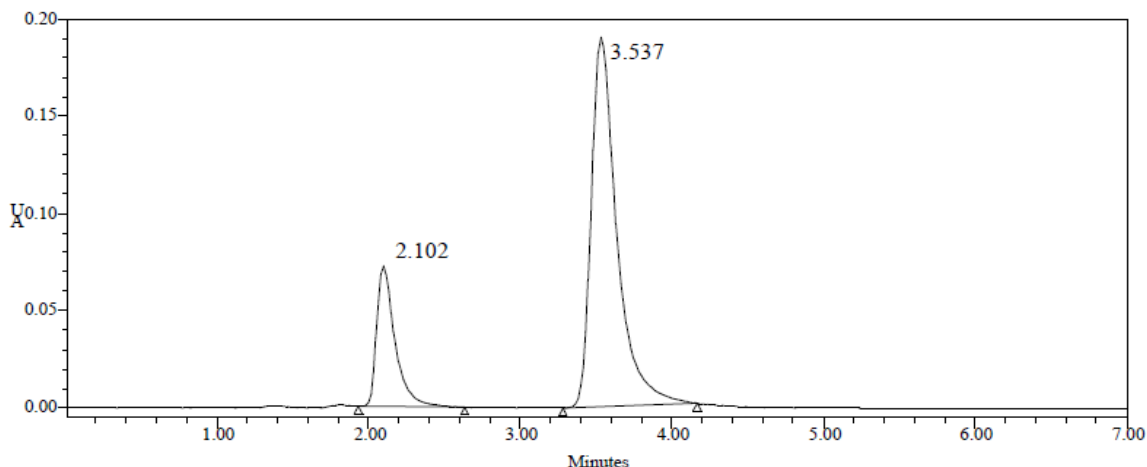


Fig 1: Optimized Chromatogram

Table 1: Peak results for Optimized Chromatogram

S. No	Peak name	R _t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Benserazide	2.102	765789	69584		0.97	5587.0
2	Levodopa	3.537	2532158	190049	2.97	1.26	5398.0

From the above chromatogram it was observed that the Levodopa and Benserazide peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

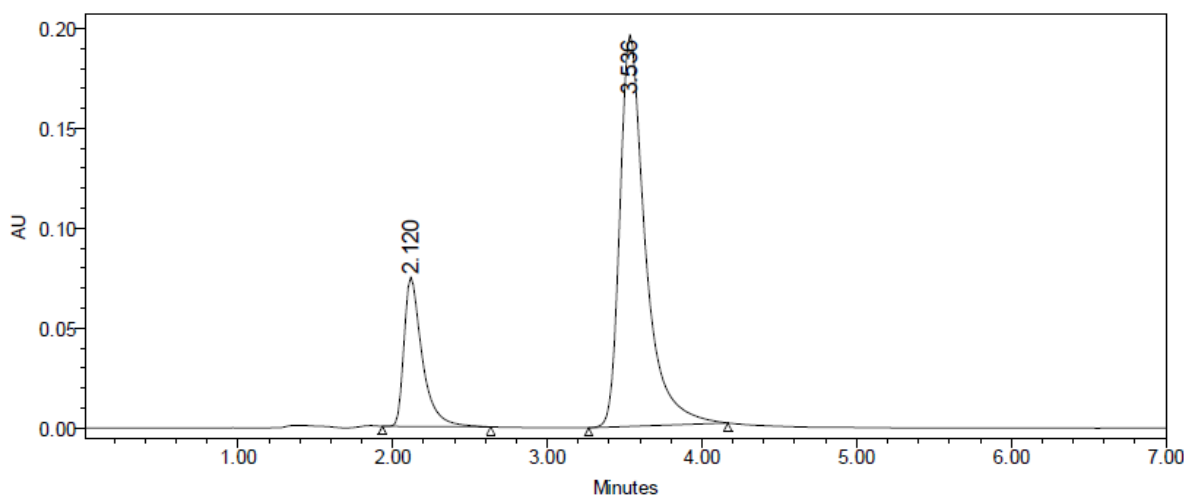


Fig 2: Optimized Chromatogram (Sample)

Table 2: Optimized Chromatogram (Sample)

S. No	Peak name	R _t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Benserazide	2.120	775684	13124		0.99	6365.0
2	Levodopa	3.536	2658478	937405	5.06	1.23	7458.0

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

System Suitability

Table 3: Results of system suitability for Benserazide

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Benserazide	2.117	765843	69587	5589	1.9
2	Benserazide	2.118	766594	69854	5576	1.6
3	Benserazide	2.116	765487	70211	5658	1.6
4	Benserazide	2.109	765928	69213	5642	1.7
5	Benserazide	2.102	765426	69558	5685	1.6
Mean			765855.6			
Std. Dev			466.6522			
% RSD			0.060932			

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table 4: Results of system suitability for Levodopa

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levodopa	3.547	2534658	190058	5365	1.2	2.07
2	Levodopa	3.539	2536854	190052	5348	1.4	2.05
3	Levodopa	3.547	2535879	190078	5389	1.5	2.0
4	Levodopa	3.565	2533564	190035	5347	1.6	2.01
5	Levodopa	3.537	2534214	190085	5364	1.6	2.01
Mean			2535034				
Std. Dev			1183.309				
% RSD			0.046678				

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Assay (Standard)

Table 5: Peak results for assay standard

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Benserazide	2.102	759868	71255		1.7	5689	1
2	Levodopa	3.537	2458754	215654	2.04	1.6	5362	1
3	Benserazide	2.105	759458	72541		1.7	5748	2
4	Levodopa	3.552	2465885	226565	2.00	1.6	5452	2
5	Benserazide	2.112	759245	72584		1.7	5584	3
6	Levodopa	3.560	2489578	221542	2.04	1.6	5456	3

Table 6: Peak results for Assay sample

Sno	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Benserazide	2.120	756985	68958		0.98	7253	1
2	Levodopa	3.536	2569856	198564	2.06	1.23	8836	1
3	Benserazide	2.120	758745	69857		1.05	6530	2

4	Levodopa	3.537	2598654	195682	2.04	0.99	7270	2
5	Benserazide	2.102	756848	69588		1.7	7586	3
6	Levodopa	3.537	2587454	192541	2.04	1.6	8371	3

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Levodopa and Benserazide in pharmaceutical dosage form was found to be 99.8%.

Linearity

Chromatographic data for linearity study: levodopa

Concentration µg/ml	Average Peak Area
10	349877
20	688574
30	999895
40	1326522
50	1673877

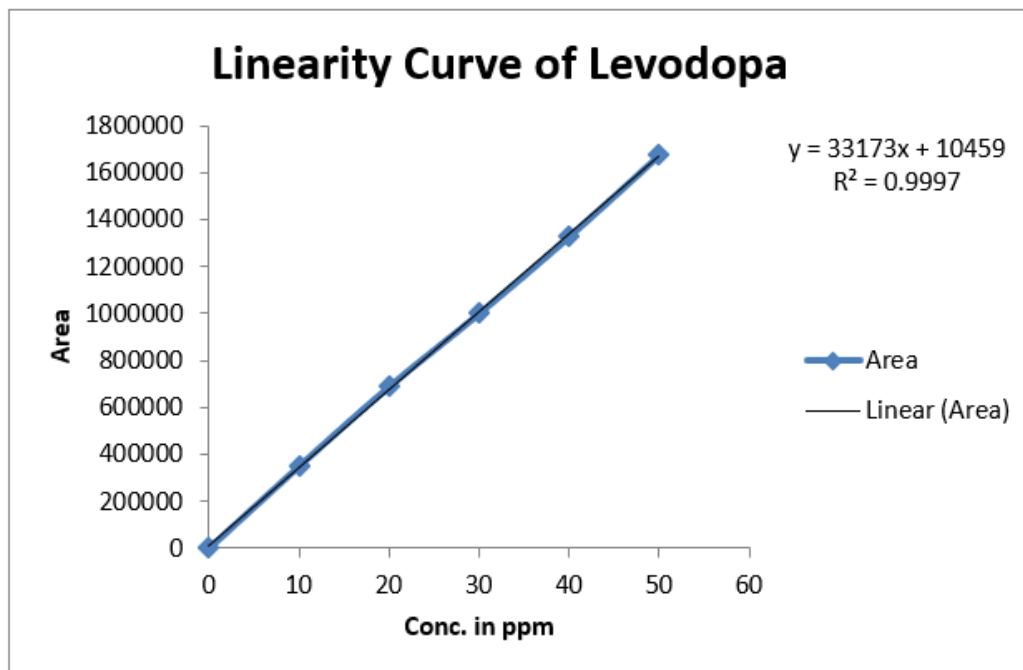


Fig 3: Calibration Graph for Levodopa

BENSERAZIDE

Concentration µg/ml	Average Peak Area
10	1896545
20	3685798
30	5389557
40	7096443
50	8878478

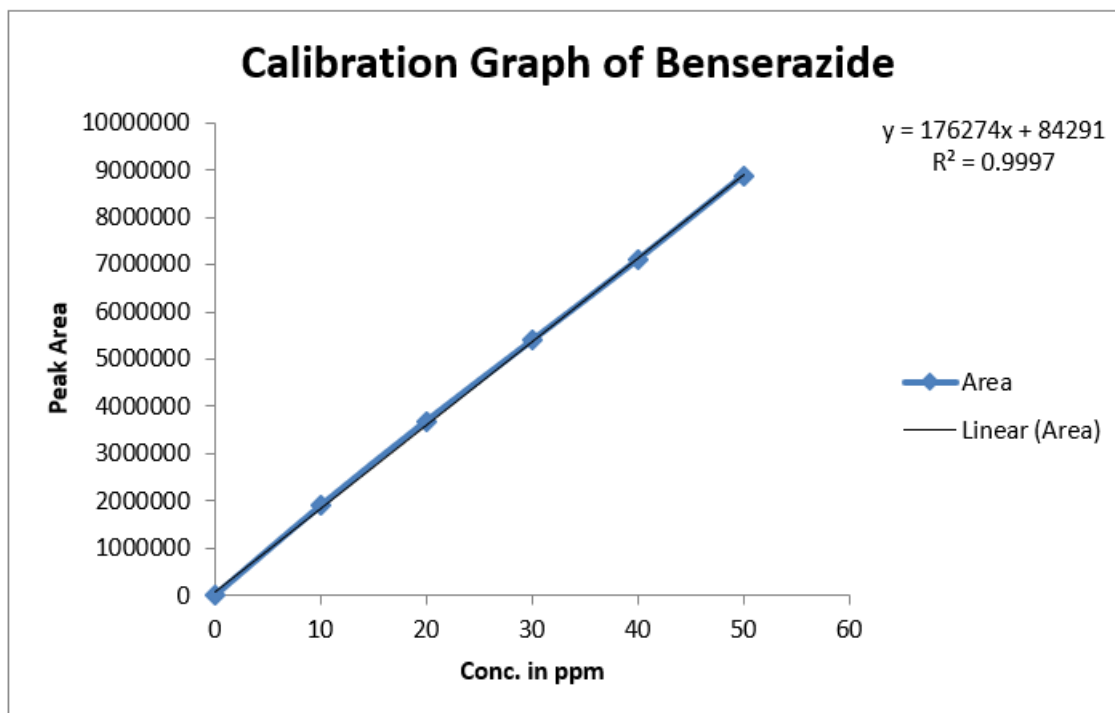


Fig 4: Calibration Graph for Benserazide

REPEATABILITY

Table 7: Results of Repeatability for Benserazide

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Benserazide	2.108	766854	702564	5685	1.6
2	Benserazide	2.105	765884	698789	5584	1.4
3	Benserazide	2.113	765842	701235	5521	1.6
4	Benserazide	2.109	768985	700124	5525	1.9
5	Benserazide	2.109	765845	698986	5578	1.7
Mean			766682			
Std. Dev			1357.973			
% RSD			0.177123			

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table 8: Results of method precision for Levodopa

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Levodopa	3.552	2569865	2231111	5365	1.6
2	Levodopa	3.550	2578474	2674210	5425	1.6
3	Levodopa	3.564	2568985	2231261	5368	1.5
4	Levodopa	3.564	2586845	2421301	5359	1.5
5	Levodopa	3.565	2545898	2324710	5498	1.6
Mean			2570013			
Std. Dev			15309.45			
% RSD			0.595695			

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision

Table 9: Results of Intermediate precision for Benserazide

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Benserazide	2.108	758955	68986	5785	1.6
2	Benserazide	2.105	759869	68957	5698	1.4
3	Benserazide	2.113	758985	68545	5689	1.6
4	Benserazide	2.109	756894	68952	5781	1.9
5	Benserazide	2.109	759854	68595	5785	1.7
6	Benserazide	2.102	756985	68952	5693	1.6
Mean			758590.3			
Std. Dev			1339.793			
% RSD			0.176616			

- %RSD of Six different sample solutions should not more than 2.

Table 10: Results of Intermediate precision for Levodopa

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levodopa	3.552	2659852	190025	5485	1.5	2.04
2	Levodopa	3.550	2648574	190048	5421	1.6	2.03
3	Levodopa	3.564	2659865	190054	5468	1.6	2.01
4	Levodopa	3.564	2658547	190078	5487	1.6	2.05
5	Levodopa	3.565	2648981	190016	5492	1.6	2.02
6	Levodopa	3.537	2654652	190057	5463	1.6	2.03
Mean			2655079				
Std. Dev			5242.086				
% RSD			0.197436				

- %RSD of Six different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is rugged.

Table 11: Results of Intermediate precision Day 2 for Benserazide

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Benserazide	2.102	766895	69858	5586	1.5
2	Benserazide	2.105	765988	69854	5636	1.6
3	Benserazide	2.112	766532	69824	5432	1.6
4	Benserazide	2.113	766214	69875	5468	1.6
5	Benserazide	2.109	765897	69854	5546	1.9
6	Benserazide	2.109	765245	69848	5507	1.7
Mean			766128.5			
Std. Dev			567.7234			
% RSD			0.074103			

- %RSD of Six different sample solutions should not more than 2.

Table 12: Results of Intermediate precision for Levodopa

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levodopa	3.537	2653254	190110	5428	1.6	7.98
2	Levodopa	3.552	2648985	190058	5452	1.6	6.4
3	Levodopa	3.560	2658213	190142	5498	1.6	8.9
4	Levodopa	3.564	2653652	190031	5442	1.5	8.3
5	Levodopa	3.564	2648978	190058	5489	1.5	7.5
6	Levodopa	3.565	2658985	190047	5463	1.6	5.3
Mean			2653678				
Std. Dev			4313.355				
% RSD			0.162543				

- %RSD of Six different sample solutions should not more than 2

- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY

Table 13: The accuracy results for Benserazide

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	392891.7	5	5.027	100.540%	100.351%
100%	781996	10	10.026	100.260%	
150%	1171988	15	15.038	100.253%	

Table 14: The accuracy results for Levodopa

% Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	204962	15	15.156	101.040%	100.93%
100%	365018	30	30.378	101.260%	
150%	521064.3	45	45.218	100.484%	

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Levodopa

Table 15: Results for Robustness

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	765789	2.102	5587	1.7
Less Flow rate of 0.9 mL/min	758698	2.330	5458	1.7
More Flow rate of 1.1 mL/min	7689584	1.950	5696	1.7
Less organic phase	758412	2.290	5586	1.4
More organic phase	769852	1.998	5355	1.5

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

Benserazide

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	2532158	3.537	5398	1.6
Less Flow rate of 0.9 mL/min	2458692	3.885	5329	1.7
More Flow rate of 1.1 mL/min	2658642	3.263	5256	1.7
Less organic phase	2452148	4.435	5214	1.2
More organic phase	2653894	3.009	5524	1.0

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION

A new method was established for simultaneous estimation of Levodopa and Benserazide by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Levodopa and Benserazide by using Phenomenex Luna C18 (4.6×250mm, 5µm) particle size, flow rate was 1ml/min, mobile phase ratio was (45:55 v/v) Acetonitrile: Phosphate Buffer (pH-4.6 was adjusted with orthophosphoric acid), detection wave length was 245nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.102mins and 3.537mins. The % purity of Levodopa and Benserazide was found to be 99.8%. The system

suitability parameters for Levodopa and Benserazides such as theoretical plates and tailing factor were found to be within limits. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study in Levodopa and Benserazide was found in concentration range of 6µg-14µg and 18µg-42µg and correlation coefficient (r^2) was found to be 0.999 and 0.999, % recovery was found to be 100.351% and 100.93%, %RSD for repeatability was 0.177 and 0.595. The precision study was precise, robust, and repeatable. LOD value was 0.6 and 0.8, and LOQ value was 1.8 and 2.4 respectively.

Hence the suggested RP-HPLC method can be used for routine analysis of Levodopa and Benserazide in API and Pharmaceutical dosage form.

ACKNOWLEDGEMENT

The Authors are thankful to the Management and Principal, Department of Pharmacy, Pydah College of Pharmacy ,

Kakinada, Andhra Pradesh for extending support to carry out the research work. Finally, the authors express their gratitude to the Sura Labs, Dilsukhnagar, Hyderabad, for providing research equipment and facilities.

REFERENCES

1. Dr. Kealey, Haines PJ. Analytical chemistry. 1st ed. Bios Publisher; 2002. P. 1-7.
2. Braithwait A, Smith FJ. Chromatographic methods. 5th ed. Kluwer Academic Publishers; 1996. P. 1-2.
3. Weston A, Phyllisr. Brown, HPLC principle and practice. 1st ed. Academic press; 1997. P. 24-37.
4. Kazakevich Y, Lobrutto R. HPLC for pharmaceutical scientists. 1st ed. Wiley Interscience A JohnWiley & Sons, Inc Publishing House; 2007. P. 15-23.
5. Chromatography [online]. Wikipedia. Available from: <http://en.wikipedia.org/wiki/Chromatography>.
6. Meyer VR. Practical high-performance liquid chromatography. 4th ed. England: John Wiley & Sons Ltd; 2004. P. 7-8.
7. Sahajwalla CG a new drug development. Vol. 141. New York: Marcel Dekker, Inc; 2004. P. 421-6.
8. Shewiy DH, Kaale E, Risha PG, Dejaegher B, Verbeke JS, Heyden YV. J Pharm Biomed Anal. 2012;66:11-23.
9. Rockville MD, Chapter 621. Chromatography system suitability, United States pharmacopeial convention (USP), USP. In: General Tests. Vol. 31; 2009.
10. FDA guidance for industry-analytical procedures and method validation, chemistry, manufacturing, and controls documentation. Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER); 2000.
11. Korany MA, Mahgoub H, Fahmy OT, Maher HM. Application of artificial neural networks for response surface modelling in HPLC method development. J Adv Res. 2012;3(1):53-63. doi: 10.1016/j.jare.2011.04.001.
12. Swartz ME, Jone MD, Fowler P, Andrew MA. Automated HPLC method development and transfer. LC GC N Am. 2002;75:49-50.
13. Snyder LR, Kirkland JJ, Glajach JL. X. Practical HPLC methods development. New York: John Wiley, 295; 1997. p. 643-712.
14. Swartz M, Murphy MB. New Frontiers in chromatography. Am Lab. 2005;37:22-4.
15. Dolan JW. Peak tailing and resolution. LC GC N Am. 2002;20:430-6.
16. Chan CC, Leo YC, Lam H. Analytical method validation and Instrument Performance Validation. Vol Wiley Interscience. 2004.