International Journal of Pharmacy and Analytical Research (IJPAR)

IJPAR |Vol.13 | Issue 4 | Oct - Dec -2024

www.ijpar.com

ISSN: 2320-2831

DOI : https://doi.org/10.61096/ijpar.v13.iss4.2024.473-482

Research

Analytical method development and validation for estimation of clonazepam and brexpiprazole in bulk and tablet dosage form by rp-hplc

B. Sai Manisha, Dr. Gadipally Sai Kiran*, Dr. D. Venkata Ramana

[1]*Department Of Pharmaceutical Analysis, Holy Mary Institute Of Technology And Science (College Of Pharmacy), Kondapur Telangana, India.*

*Author for Correspondence: Dr. Gadipally Sai Kiran
Email: drsk9898@gmail.com

Published on: 5 Oct 2024

Published by:
DrSriram Publications

Abstract

This study presents the development and validation of a High-Performance Liquid Chromatographic (HPLC) method for the simultaneous estimation of Clonidine and Hydrochlorothiazide in both bulk and tablet dosage forms. Chromatographic separation was achieved using a Phenomenex Gemini C18 column with a mobile phase consisting of Methanol (80:20, v/v) at a flow rate of 1.0 ml/min. Detection of the analytes was performed at 230 nm, with retention times of 2.090 min for Clonidine and 3.289 min for Hydrochlorothiazide. Calibration curves exhibited linearity over the concentration ranges of 20 µg/ml for Clonidine and 25 µg/ml for Hydrochlorothiazide. The method was meticulously validated following ICH guidelines, encompassing assessments of specificity, accuracy, precision, linearity and range, ruggedness, robustness, and system suitability. The results demonstrated that the developed HPLC method is accurate, precise, and reproducible for the simultaneous quantification of Clonidine and Hydrochlorothiazide in pharmaceutical formulations. Importantly, the method exhibited high specificity, with no interference observed from tablet excipients or endogenous substances. Overall, the validated HPLC method offers a straightforward and rapid analytical approach suitable for routine quality control analysis in pharmaceutical laboratories. Its robust performance, confirmed through validation parameters including accuracy, precision (with RSD values meeting acceptable criteria), and linearity, supports its practical utility in ensuring the quality and consistency of Clonidine and Hydrochlorothiazide formulations during manufacturing and quality assurance processes.

Keywords: Clonidine and Hydrochlorothiazide, RP-HPLC, Accuracy, ICH Guidelines.

INTRODUCTION

Chromatography is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.[1]

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.[2]

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster.

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".
- Mobile phase: This phase is always composed of "liquid" or a "gaseous component."
- Separated molecules

The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (i.e. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, esther, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses [4].

Stationary phase in chromatography is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable and non-volatile samples [5].

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achieve a satisfactory separation within a suitable time interval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography [6].

- Column chromatography
- Ion-exchange chromatography
- Gel-permeation (molecular sieve) chromatography
- Affinity chromatography
- Paper chromatography
- Thin-layer chromatography
- Gas chromatography
- Dye-ligand chromatography
- Hydrophobic interaction chromatography
- Pseudo affinity chromatography
- High-pressure liquid chromatography (HPLC)

High-Pressure Liquid Chromatography (HPLC)

Using this chromatography technique it is possible to perform structural, and functional analysis, and purification of many molecules within a short time, This technique yields perfect results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules, In HPLC, mobile phase passes through columns under 10–400 atmospheric pressure, and with a high (0.1–5 cm//sec) flow rate. In this technique, use of small particles, and application of high pressure on the rate of solvent flow increases separation power, of HPLC and the analysis is completed within a short time. Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Duration of separation is controlled. Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Duration of separation is controlled with the aid of a computerized system, and material is accrued [25].

MATERIALS AND METHODS

Instruments used

HPLC from WATERS Alliance 2695 separation module, software: Empower 2, 996 PDA detector.

Chemicals used

Brexpiprazole and Clonazepam from Sura Pharma Labs, Water and Methanol for HPLC from LICHROSOLV (MERCK), Acetonitrile for HPLC from Merck.

Preparation of standard solution: (Brexpiprazole)

Accurately weigh and transfer 10 mg of Brexpiprazole, working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent.

Preparation of standard solution: (Clonazepam)

Accurately weigh and transfer 10 mg of Clonazepam working standard into a 10ml of clean dry volumetric flasks add about 7ml of diluent and sonicate to dissolve and removal of air completely and make volume up to the mark with the diluent. Further pipette 0.4 ml of Brexpiprazole, 0.5ml of Clonazepam from stock solutions in to a 10ml volumetric flask and dilute up to the mark with diluent.

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.

Preparation of mobile phase: Accurately measured 350 ml (350%) of HPLC Methanol and 650 ml of TEA (65%) were mixed and degassed in a digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filter.

Diluent Preparation

Accurately measured 350 ml (350%) of HPLC Methanol and 650 ml of TEA (65%) were mixed and degassed in a digital ultra sonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filter.

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard)

Mobile phase	:	Methanol: TEA Buffer pH-4.8 (35:65)
Column	:	Symmetry ODS C18 (4.6×150mm, 5.0 µm)
Flow rate	:	1 ml/min
Wavelength	:	276 nm
Column temp	:	Ambient
Injection Volume	:	10 µl
Run time	:	10 minutes

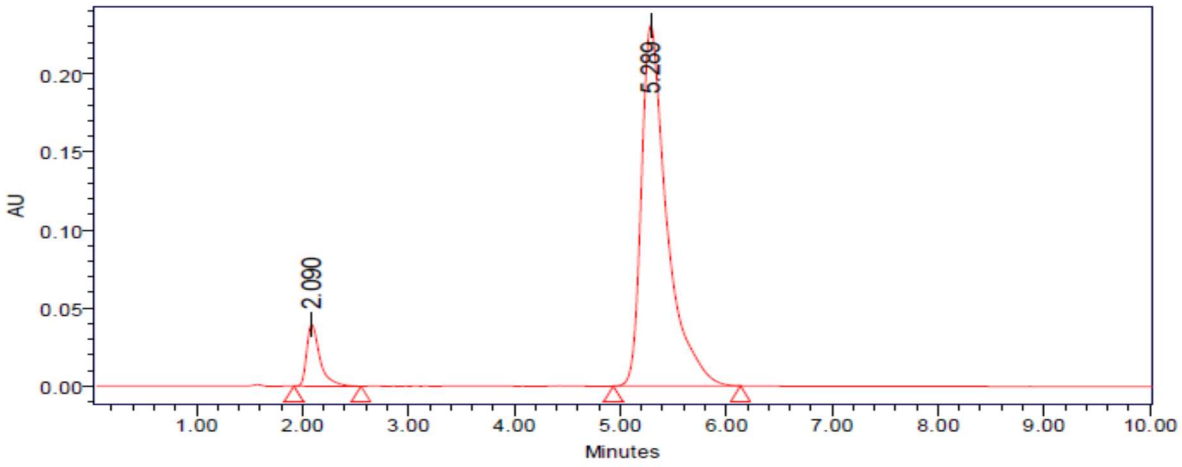

Fig 1: Optimized Chromatogram

Table 1: Peak Results for Optimized Chromatogram

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Brexpiprazole	2.090	327989	39785		1.72	5657
2	Clonazepam	5.289	3576856	232354	9.80	1.77	5869

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

Optimized Chromatogram (Sample)

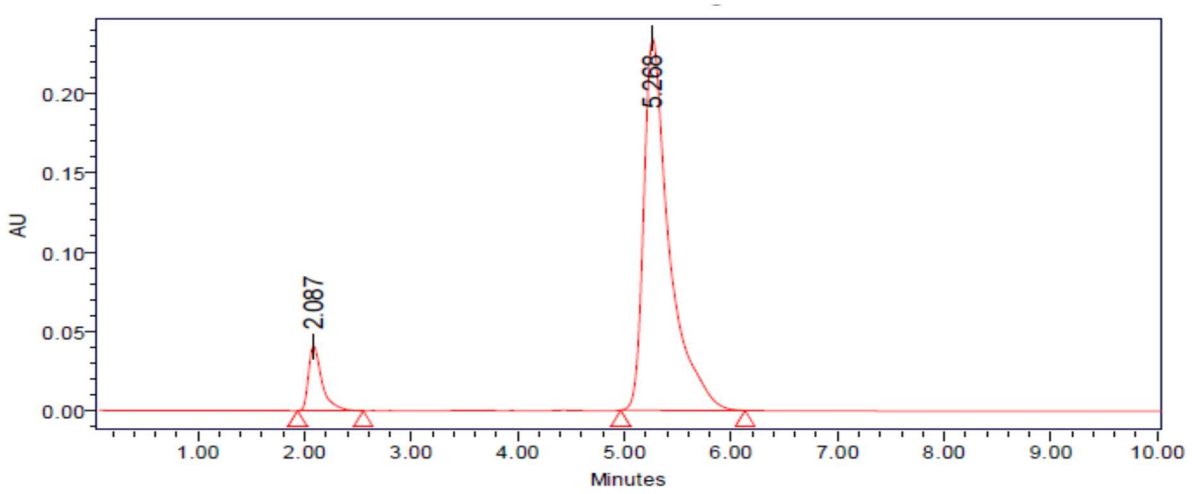

Fig 2: Optimized Chromatogram (Sample)

Table 2: Optimized Chromatogram (Sample)

S. No.	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Brexpiprazole	2.087	312548	41236		1.75	5568
2	Clonazepam	5.268	3498965	236584	9.83	1.94	5847

- *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 Brexpiprazole

S. No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Brexpiprazole	2.090	325896	39689	5653	1.42
2	Brexpiprazole	2.090	326989	39689	5695	1.42
3	Brexpiprazole	2.089	327985	39698	5598	1.44
4	Brexpiprazole	2.089	329477	40198	5569	1.43
5	Brexpiprazole	2.085	325858	40259	5612	1.47
Mean			**327241**			
Std. Dev			**1527.944**			
% RSD			**0.466917**			

- *%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 Clonazepam

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Clonazepam	5.289	3576859	232352	5785	1.46	9.80
2	Clonazepam	5.289	3585695	232365	5915	1.47	9.81
3	Clonazepam	5.338	3596885	232451	5895	1.48	9.81
4	Clonazepam	5.327	3565874	231653	5987	1.40	9.83
5	Clonazepam	5.262	3598654	233658	5861	1.43	9.82
Mean			3588946				
Std. Dev			3585486				
% RSD			11360.78				

- *%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

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Brexpiprazole	2.090	328966	39586		1.70	5563	1
2	Clonazepam	5.289	3574898	232356	9.80	1.77	5665	1
3	Brexpiprazole	2.089	327898	39568		1.66	5584	2
4	Clonazepam	5.338	3569854	232548	9.93	1.83	5646	2
5	Brexpiprazole	2.089	328657	40526		1.68	5584	3
6	Clonazepam	5.327	3565874	232547	9.91	1.86	5783	3

Assay (Sample)

Table 6: Peak Results for Assay Sample

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Brexpiprazole	2.088	336589	40365		1.69	5569	1
2	Clonazepam	5.276	3586985	232565	9.75	1.89	5658	1
3	Brexpiprazole	2.087	335684	41245		1.72	5548	2
4	Clonazepam	5.268	3587896	235685	9.82	1.91	5864	2
5	Brexpiprazole	2.085	335876	40898		1.75	5496	3
6	Clonazepam	5.262	3586848	234588	9.78	1.95	5754	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 Brexpiprazole and Clonazepam in pharmaceutical dosage form was found to be99.494%.

Linearity
Chromatographic data for linearity study:
Brexpiprazole

Concentration µg/ml	Average Peak Area
20	164436
30	255571
40	348687
50	439024
60	534830

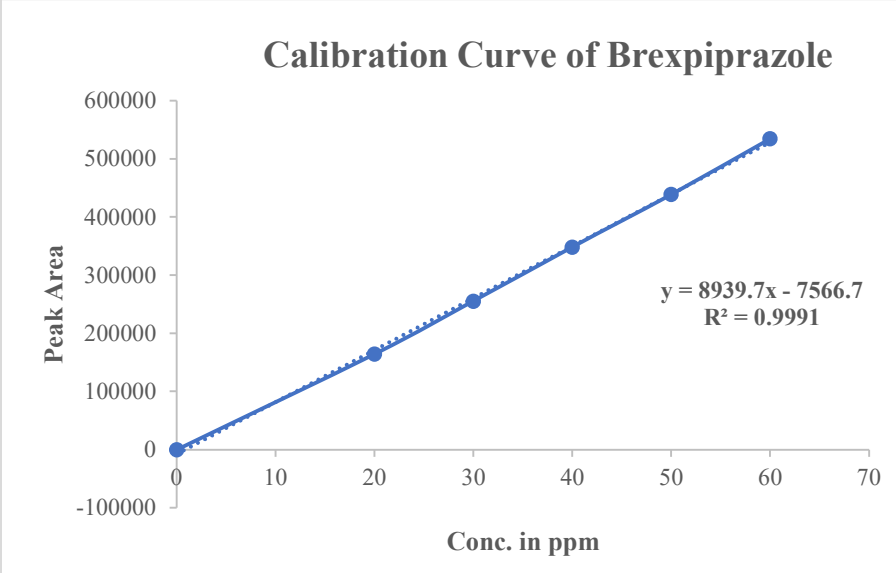

Fig 3: Calibration graph for Brexpiprazole

The response linearity is verified if the Correlation Coefficient is 0.99 or greater. Correlation Coefficient (r) is 0.99, and the intercept is 7566. These values meet the validation criteria.

Clonazepam

Concentration µg/ml	Average Peak Area
25	1782454
37.5	2728974
50	3688678
62.5	4658022
75	5592695

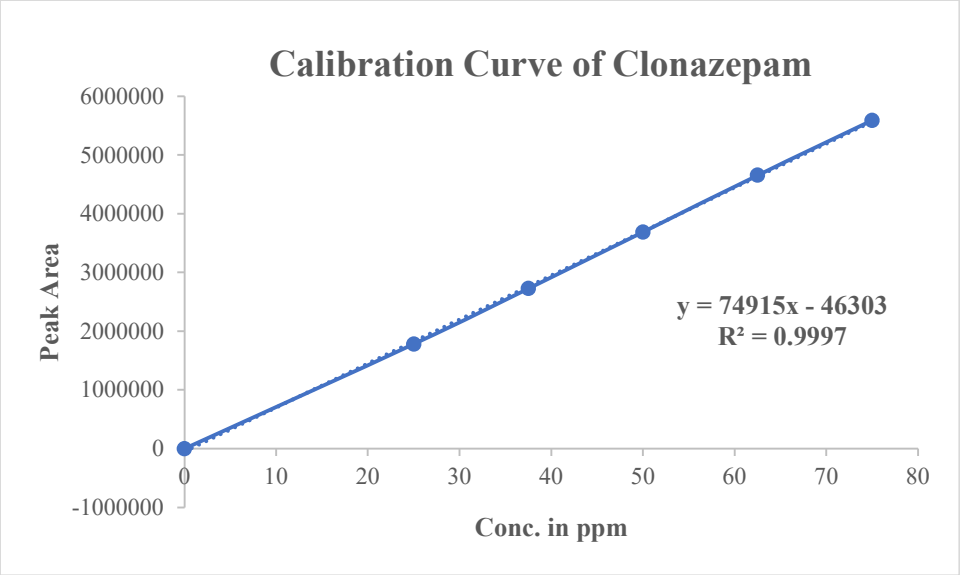

Fig 4: Calibration graph for Clonazepam

The response linearity is verified if the Correlation Coefficient is 0.99 or greater. Correlation Coefficient (r) is 0.99, and the intercept is 46303. These values meet the validation criteria.

Precision

Repeatability

Table 7: Results of Repeatability for Brexpiprazole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Brexpiprazole	2.086	327689	41697	5081.3	1.8
2	Brexpiprazole	2.083	327978	41402	5144.1	1.8
3	Brexpiprazole	2.083	327879	41540	5118.1	1.8
4	Brexpiprazole	2.081	327868	42256	5147.3	1.8
5	Brexpiprazole	2.081	327859	42143	5101.8	1.8
Mean			**327854.6**			
Std. Dev			**104.2176**			
% RSD			**0.031788**			

- *%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 Clonazepam

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Clonazepam	5.178	3576985	241253	5969.5	2.0	9.8
2	Clonazepam	5.199	3578989	2365824	5865.1	2.0	9.7
3	Clonazepam	5.235	3576859	239568	5936.4	2.0	9.9
4	Clonazepam	5.202	3578458	2386547	5964.4	2.0	9.8
5	Clonazepam	5.206	3579864	241425	5045.6	2.0	9.5
Mean			**3578231**				
Std. Dev			**1296.889**				
% RSD			**0.036244**				

- *%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
Day 1

Table 9: Results of Intermediate precision for Brexpiprazole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Brexpiprazole	2.083	328986	42365	5556.2	1.6
2	Brexpiprazole	2.083	328898	42685	5524.6	1.6
3	Brexpiprazole	2.089	327789	42544	5465.2	1.6
4	Brexpiprazole	2.083	328758	42685	5464.5	1.6
5	Brexpiprazole	2.082	328869	42256	5589.4	1.8
6	Brexpiprazole	2.080	329687	42365	5565.5	1.8
Mean			**328831.2**			
Std. Dev			**608.8985**			
% RSD			**0.185171**			

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

Table 10: Results of Intermediate precision for Clonazepam

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Clonazepam	5.229	3578659	243659	5252.1	2.2	10.2
2	Clonazepam	5.203	3578469	2436521	5256.4	2.1	10.0
3	Clonazepam	5.133	3574865	245664	5356.8	2.1	10.0
4	Clonazepam	5.229	3574824	243652	5265.6	2.2	10.2
5	Clonazepam	5.151	3579861	244254	5235.7	1.5	9.9
6	Clonazepam	5.112	3574898	236558	5986.2	1.6	9.9
Mean			**3576929**				
Std. Dev			**2112.55**				
% RSD			**0.05906**				

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

Day 2

Table 11: Results of Intermediate precision Day 2 for Brexpiprazole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Brexpiprazole	2.078	370979	42978	7083.0	1.9
2	Brexpiprazole	2.082	371041	42568	8583.2	1.8
3	Brexpiprazole	2.080	371386	42211	7533.2	1.8
4	Brexpiprazole	2.089	369246	42277	6537.8	1.6
5	Brexpiprazole	2.083	370840	42065	5489.3	1.6
6	Brexpiprazole	2.089	369246	42277	6537.8	1.6
Mean			**370456.3**			
Std. Dev			**954.6004**			
% RSD			**0.25**			

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

Table 12: Results of Intermediate precision for Clonazepam

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Clonazepam	5.077	3578985	246818	5208.0	1.5	10.1
2	Clonazepam	5.151	3578415	242854	5127.6	1.3	10.0
3	Clonazepam	5.112	3579864	242955	5269.7	1.5	10.2
4	Clonazepam	5.133	3579862	242955	5269.7	1.6	10.2

5	Clonazepam	5.203	3578948	242854	5127.6	1.5	10.0
6	Clonazepam	5.133	3586775	242955	5269.7	1.6	10.2
Mean			**3580475**				
Std. Dev			**3137.978**				
% RSD			**0.087641**				

- *%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 Brexpiprazole

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	186584.7	20	20.026	100.13	100.435%
100%	367968.7	40	40.32	100.80	
150%	545922	60	60.225	100.375	

Table 14: The Accuracy Results for Clonazepam

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1925532	25	25.084	100.336	100.284%
100%	3790965	50	49.985	99.970	
150%	5695646	75	75.410	100.546	

- *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.

Robustness

Table 15: Results for Robustness

Brexpiprazole

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	327989	2.090	5698	1.70
Less Flow rate of 0.9 mL/min	302986	2.736	5569	1.82
More Flow rate of 1.1 mL/min	316989	1.673	5598	1.91
Less organic phase	315989	2.736	5651	1.82
More organic phase	308986	1.673	5452	1.91

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

Clonazepam

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	3576856	5.289	5689	1.77
Less Flow rate of 0.9 mL/min	3458978	6.746	5658	1.88
More Flow rate of 1.1 mL/min	3589871	4.032	5245	1.91
Less organic phase	3579124	6.746	5154	1.88
More organic phase	3578698	4.032	5652	1.91

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

SUMMARY AND CONCLUSION

This study describes the development and validation of a High-Performance Liquid Chromatographic (HPLC) method for the simultaneous estimation of Clonidine and Hydrochlorothiazide in both bulk and tablet dosage forms. Chromatographic separation was achieved using a Phenomenex Gemini C18 column with a mobile phase composed of Methanol (80:20, v/v) at a flow rate of 1.0 ml/min. Detection of the drugs was performed at 230 nm, with retention times of 2.090 min for Clonidine and 3.289 min for Hydrochlorothiazide. Calibration curves

demonstrated linearity over the concentration ranges of 20 µg/ml for Clonidine and 25 µg/ml for Hydrochlorothiazide. The method was validated according to ICH guidelines, assessing specificity, accuracy, precision, linearity and range, ruggedness, robustness, and system suitability. The developed HPLC method offers an accurate, precise, and reproducible means for the simultaneous estimation of Clonidine and Hydrochlorothiazide in bulk substances and tablet dosage forms. Its simplicity and rapidity make it suitable for routine quality control analysis in pharmaceutical laboratories. The method showed no interference from excipients or endogenous substances present in tablet formulations, confirming its specificity. The validation results confirmed the method's accuracy, precision (with RSD values within acceptable limits), and linearity across the specified concentration ranges. Overall, this HPLC method is recommended for its practical applicability in ensuring the quality and consistency of Clonidine and Hydrochlorothiazide formulations in pharmaceutical manufacturing and quality assurance processes.

ACKNOWLEDGEMENT

The Authors are thankful to the Management and Principal, Department of Pharmacy, Holy Mary Institute of Technology and science (College of Pharmacy) in Kondapur, Telangana, for extending support to carry out the research work. Finally, the authors express their gratitude to the Sura Pharma Labs, Dilsukhnagar, Hyderabad, for providing research equipment and facilities.

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