A Validated Stability – Indicating HPLC Method estimation of Clonazepam In the bulk drug and Pharmaceutical Dosage Form

Pallavi Mangesh Patil[1]*, Sagar Baliram Wankhede[2] and Praveen Digambar Chaudhari[1]

[1]P.E.Society's Modern College of Pharmacy Yamunanagar, Nigdi, Pune-411044 Maharashtra, India, 411044
[2]Padm. Dr.D.Y. Patil Institute of Pharmaceutical Sciences & Research, Pimpri, Pune-411018, Maharashtra, India

Abstract

A rapid, accurate, linear, and sensitive RP-HPLC method has been developed and validated for estimation of Clonazepam in the bulk and Pharmaceutical Dosage Form. The chromatographic separation was performed on C18 Column (250 mm × 4.6 mm, 5 µm particle size) using a mobile phase Acetonitrile: Methanol (60:40 v/v) at flow rate of 1.0 ml/min and 30°C column temperature with the detection wavelength at 254 nm. The Clonazepam (RT 6.11 min).The linearity was performed in the concentration range of 5 to 25 µg/ml for Clonazepam R^2 0.9993, Clonazepam. The percentage purity of Clonazepam was found to be 99-101%. Precision (Inert-day) of the system was found to be 0.48% Clonazepam and all the method was found to be specific and found to be within the limits of the acceptance criteria. The limit of detection was 0.092 µg/ml and limit of quantification was 0.97 µg/ml. Clonazepam was found to be specific. The Proposed method has been validated for which were within the acceptance limit according to ICH guidelines. Forced degradation conditions of hydrolysis (neutral, acidic and alkaline), oxidation, photolysis and thermal stress, as suggested in the ICH guideline Q1A (R2). The drug showed instability in alkaline and oxide, while it remained stable in acid conditions.

Keywords: Clonazepam; Validation; Force degradation studies

Introduction

Clonazepam is a light yellow crystalline powder which is practically odourless. Literature survey states that Paroxetine hydrochloride and Clonazepam is official in IP [1], BP [2] with HPLC methods for the estimation individually. Clonazepam (CZ) in Figure 1 chemically, 5-(2-chlorophenyl)-7-nitro-2, 3-dihydro-1H-1, 4-benzodia zepin-2-one (Figure1) belongs to the drug class benzodiazepines. Mechanism of action involves Allosteric interactions between central benzodiazepine receptors and gamma-minobutyric acid (GABA) receptors potentiate the effects of GABA. It's chemical formula and molecular weight is $C_{15}H_{10}ClN_3O_3$ and 315.7 [3-8], HPLC [3], LCMS based methods have been reported. A UV and Spectrophotometric methods was reported for the estimation of Paroxetine and Clonazepam in combined formulations [9-10]. But no stability-indicating assay method has been reported for the simultaneous estimation of Clonazepam in the presence of their degradants using the ICH [11-13] approach of stress testing. Therefore, the present work was aimed to develop a simple, rapid, precise, and accurate isocratic reversed-phase stability indicating HPLC method for estimation of Clonazepam in the bulk drug and tablet dosage forms. The developed method was validated as per ICH Guidelines.

Materials and Methods

All the reagents as Acetonitrile and Methanol (HPLC grade), were purchased from Merck Chemicals, India. Reference standard Clonazepam was procured from Reddy Hyderabad laboratories, India as gift samples.

Instrumentation and chromatographic conditions

Different kinds of equipment like Analytical weighing balance, HPLC system (SHIMADZU-SPD 20A), Injector (Rheodyne, 20 µl), Sonicator, pH meter, vacuum filter pump, Millipore filtration kit, mobile phase reservoir. Grace C18 ACME 9000, C18 reverse phase column of 250 × 4.0 mm i.e., 5 µm dimensions Detector: UV, D2 lamp, 254 nm Column Temperature: Controlled room temperature (25°C) Injection: 20 µL sample loop was used for Clonazepam with the help of mobile phase consisted of a mixture.

Preparation of standard concentrations

Accurately weighed quantity (100 mg) of CZ respectively were transferred to 100.0 ml volumetric flask, dissolved and diluted up to the mark with mobile phase. From this solution, 5.0 ml was transferred to 50.0 ml volumetric flask and diluted to the mark with mobile phase (concentration 100 µg/ml) CZ respectively. The solution was mixed and filtered through 0.2 µ membrane filter.

Calibration standards CZ

From standard stock solution CZ (100 ug/ml) final Concentration was made in ranges 5-25 µg/ml.

Method validation

The validated HPLC method proposed according to ICH guidelines (ICH 1994, 1996). The following parameters were used for validation of the developed method.

Linearity: Linear relationship between peak area and concentration of the drugs were evaluated, making six measurements at concentration levels in the range of 5-25 µg/ml.

Accuracy: Recovery studies were carried out by spiking three different known amounts of pure drug (at 80%, 100% and 120% of label claim) to the pre-analyzed powder (standard addition method). Hence, an accurately weighed quantity of pre-analyzed tablet 10 mg CZ to the pre-analyzed.

*Corresponding author: Pallavi Mangesh Patil, P.E.Society's Modern College of Pharmacy, Yamunanagar, Nigdi, Pune-411044, India, Tel: +91 09823720695; E-mail: pallavipatil_2007@yahoo.com, psadanshio@yahoo.co.in
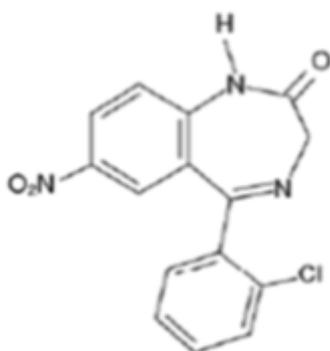
Figure 1: Chemical Structure of Clonazepam.

Precision: The precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysing the table sample six times at 100% of test concentration on the same day. The intermediate precision of the method was checked by repeating studies on three different days.

Limit of detection and quantitation: In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ) linear were separately determined based on the standard deviation (σ) of the response and the slope (S) of the calibration curve and using the formula LOD=3.3 σ /S and LOQ=10 σ /S, the LOD and LOQ for CZ was estimated.

Robustness: To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of change in flow rate and mobile phase ratio on retention time and tailing factor were studied. The solution containing 100 µg/ml of CZ was injected (in triplicate) into sample injector of HPLC three times under the varied conditions.

Stability

Stability in sample solutions

Sample solution of CZ (100 µg/ml) was prepared and was kept at room temperature (30 ± 20°C) protected from day light. The sample solution was assayed after 20 min, 1 h, 2 h, 4h, 8 h and 24 h and hence results of the remaining analysis times were compared with it. The percent label claim and the RSD values are shown in Table 1. The result of solution stability is stable for 24 hours.

System suitability

To ascertain resolution and reproducibility of proposed chromatographic system for estimation of CZ in Pharmaceutical dosage form, system suitability parameters like tailing factor (T), resolution (R) and column efficiency (number of theoretical plates, N) were studied. From stock solution D, appropriately diluted with mobile phase to obtain 100 µg/ml CZ. The diluted standard solutions were filtered through 0.2 µ membrane filter.

Force degradation studies

In order to evaluate the stability indicating property of the developed HPLC method stress studies were carried out under ICH recommended conditions. Intentional degradation was tried by exposing the tablet sample to following stress conditions: acid (0.1 N HCl at 60°C), base (0.1 N NaOH at 60°C), oxidation (3% H_2O_2 at 60°C), and heat (60°C), and UV light (254 nm). Ability of the proposed method to measure the analyte response in presence of its degradation products was studied.

Results and Discussion

Optimization of separation conditions

Different mobile phases containing Acetate buffer, Phosphate buffer, Methanol and Acetonitrile in different ratio, and various pH were tried and finally Acetonitrile: Methanol in the ratio (60:40 v/v) was selected as an appropriate mobile phase that resulted in good resolution and acceptable system suitability parameters for CZ was delivered at a flow rate of 1 ml/min with detection wavelength 254 nm for CZ, The injection volume was 20 µl. Analysis was performed at a temperature of 30°C, which shown in Figure 3.

Sample solution

The corresponding chromatograms were recorded and area of each peak for CZ was measured at 254.0 nm. Amount of CZ in sample (mg) was calculated by comparing the mean peak area of standard and sample solution.

Results of analysis of sample laboratory mixture are shown in Tables 2 and 3.

System suitability

For system precision six replicate injections of standard solution were given, tailing factor (T), and column efficiency (number of theoretical plates, N) were studied. For CZ was recorded for each injection shown in Table 7.

Validation of the method

Linearity of response: A linear relationship was observed between peak area and concentration in the range of 5-25 µg/ml CZ respectively. The correlation coefficients for the calibration curve were found to be 0.9993, for CZ. Mean peak areas for CZ at selected wavelength are shown in Table 2 and Figure 2.

Precision of the assay: Repeatability and reproducibility of the proposed method was determined by intra-day and inter-day precision studies. The Tablet was assayed three times on the same day (intra-day) and on three consecutive days (inter-day). The results of precision studies were expressed in terms of relative standard deviation (RSD) less than 2 of the percent label claim determined by developed method shown in Table 4.

Limit of Detection (LOD) and Quantification (LOQ): The limits of detection was 0.92 µg/ml for CZ and limit of quantification were 0.97 µg/ml for CZ respectively shown in Table 5.

Accuracy: The results of accuracy study are expressed in terms of percent recovery. The percent recovery at three levels (80 %, 100 % and 120 %) was found to be in the range of 98-102 % Results of recovery studies are shown in Table 2 and 3.

Clonazepam (CZ)	
Concentration (µg/ml)	Mean Peak Area
0	0
5	344,337
10	4 52,756
15	4 226,664
20	8 518,328
25	8 79,1890

Table 1: Standard Calibration Table for Clonazepam.

Level of recover	Weight of tablet taken (mg)	Amount of drug added (mg) CZ	Amount of drug recovered (mg) CZ	% Recovery CZ
80%	10	8	18.01	100.01
	10	8	18.21	100.27
	10	8	18.00	100.02
100%	10	10	19.11	99.91
	10	10	19.13	99.95
	10	10	19.17	99.97
120%	10	12	22.02	100.02
	10	12	22.10	100.10
	10	12	21.97	99.95

Table 2: Results of Accuracy Studies.

Mean	100.18	99.94	100.04
S.D.	± 0.5812	± 0.4178	± 0.219
C.V.	± 0.5810	± 0.4170	± 0.2222

Table 3: Results of Statistical Validation for Accuracy Studies.

Intra-day Precision			
Drug	% Mean*	S. D.	C. V.
CZ	99.98	± 0.4189	0.4812
Inter-day Precision			
CZ	99.91	± 0.3714	0.3516

Table 4: Results of Precision.

Parameter	CZ
Limit of Detection (µg/ml)	0.92
Limit of Quantification (µg/ml)	0.97

Table 5: LOD and LOQ of CZ.

Robustness: To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of change in flow rate and mobile phase ratio on retention time and tailing factor were studied. The solution containing 25 µg/ml of CZ was injected (in triplicate) into sample injector of HPLC 3 times under the varied conditions. Robustness data is given in Table 6.

Stability: The stability evaluation in sample solutions (constituted with methanol) was performed up to 24 h. The sample solutions placed in the autosampler at room temperature were analysed periodically at 1, 2, 4, 8 and 24 h. The results are shown in Table 7. The peak areas of each drug were not considerably different from each other. Moreover, RSD value of each sample was <1%.

The corresponding chromatogram was recorded and area of each peak for CZ at 254.0 nm. Amount of CZ in sample (mg) was calculated by comparing the mean peak area of standard and sample solution.

Assay of tablet: The resulting mixture of Tablet dosage form was centrifuged at 3500 rpm for 20 min. After centrifugation 20 µl of this mixture was injected into the chromatograph. The resulting solution was mixed and filtered through Whatman filter paper and to get approximate concentration and to obtain final concentration of 100 µg/ml CZ. The diluted solution was filtered through 0.20 µm filter.

The corresponding chromatograms were recorded and area of each peak for CZ was measured at 254.0 nm. Amount of CZ in sample (mg) was calculated by comparing the mean peak area of standard and sample solution are shown in Table 8.

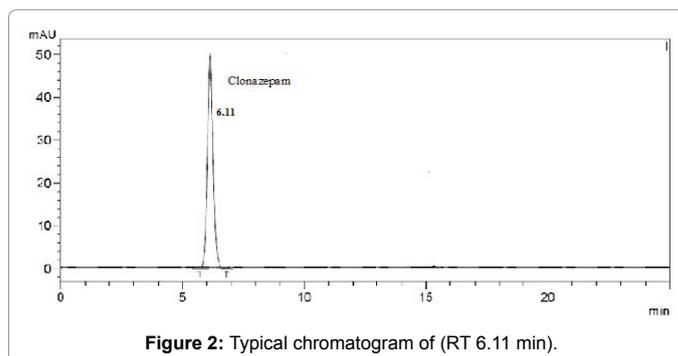**Force degradation studies:** In the forced degradation studies CZ

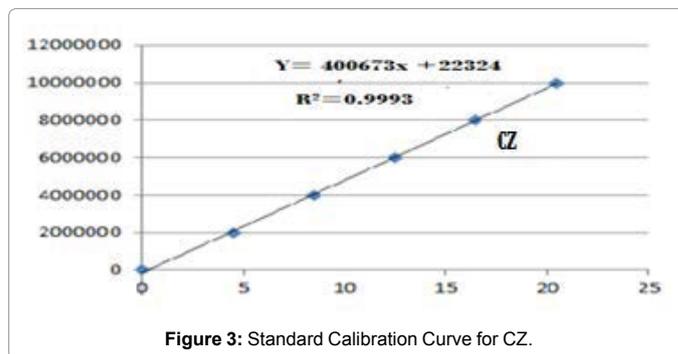**Figure 2:** Typical chromatogram of (RT 6.11 min).

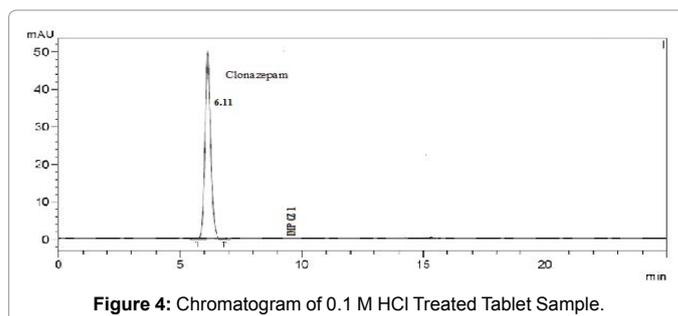**Figure 3:** Standard Calibration Curve for CZ.

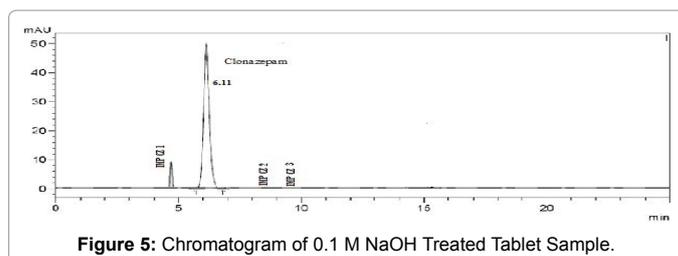**Figure 4:** Chromatogram of 0.1 M HCl Treated Tablet Sample.
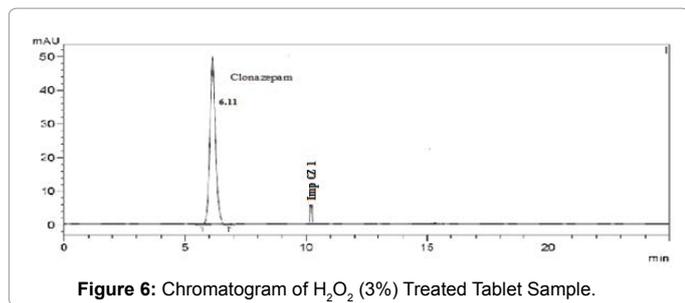

Figure 5: Chromatogram of 0.1 M NaOH Treated Tablet Sample.

Figure 6: Chromatogram of H_2O_2 (3%) Treated Tablet Sample.
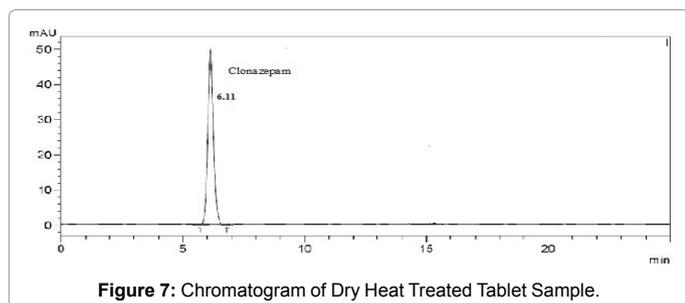

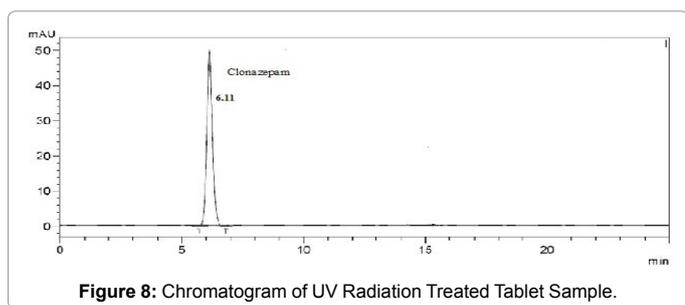**Figure 7:** Chromatogram of Dry Heat Treated Tablet Sample.

Figure 8: Chromatogram of UV Radiation Treated Tablet Sample.

Factor		Chromatographic Changes	
	Level	Retention time	Tailing factor
Flow Rate(mL/min)		CZ	CZ
0.9	-0.1	6.12	1.15
1.0	0	6.11	1.16
1.1	+0.1	6.14	1.18
	Mean	6.13	1.16
	S.D.	± 0.2118	± 0.2412
Mobile Phase (± 1ml changing Acetonitrile: Methanol)	Level	CZ	CZ
59:39	-1.0	6.10	1.17
60:40	0	6.11	1.15
61:41	+1.0	6.12	1.16
	Mean	6.11	1.16
	S.D.	± 0.2189	± 0.1446

Table 6: Results of Robustness Studies

were found to degrade under acidic (0.1 M HCL) and oxidative (3% H_2O_2) stress conditions employed. However it was found to be stable to the alkaline (0.1M NaOH) and unstable in photo (240 nm) degradation, thermal (60°C) and neutral conditions employed. The results for forced degradation studies are included in Tables 9 and 10. Typical chromatography obtained for CZ under different stress conditions are shown in Figures 4-8. The developed HPLC method could effectively resolve the drugs from their degradation products which confirm the stability indicating power of the developed method.

Conclusion

The present HPLC method for determination of CZ was proved to be simple, rapid, precise, accurate and robust in their pharmaceutical dosage and validated as per ICH guidelines. Moreover, CZ was found stable in the sample solutions placed at room temperature up to 24 h. accordingly the proposed analytical procedure with detection time of 10 min can be used for reliable determination of CZ in bulk and tablet.

The Force degradation studies method was found to be simple, sensitive, selective, and suitable for determination of CZ in presence of its degradation products CZ is instable in alkaline and acidic and stable in thermal and photo light. Statistical analysis proved that the method is repeatable, reproducible, accurate and specific for the analysis of CZ. The developed HPLC method which confirms the stability indicates power of the developed method.

As the method in cost effective and less time consuming, thus, it can represent another good alternative for the already existing HPLC methods.

1.	Tailing factor (T)	1.16
2.	No. of theoretical plates (N)	7330
3.	System precision (% RSD for peak area)	0.549
4.	Method precision (% RSD for peak area)	0.358

Table 7: System Suitability Parameters.

Sr.No	Weight of tablet taken CZ (mg)	Amount of drug estimated CZ (mg)	% Label Claim
1	100	99.98	99.97
2	100	99.97	99.96
3	100	100.02	100.01
4	100	100.13	100.12
5	100	99.86	99.85
6	100	99.72	99.71
		Mean	99.96
		S.D.	± 0.5242
		C.V.	± 0.5458

*denotes average of three determinations

Table 8: Results of Analysis of Tablet.

Sr. No.	Stress Condition	Percent assay of active substance (CZ)	RT Value of degraded product
1.	Acid (0.1 M HCl)	98.60	10.1
2.	Alkali (0.1 M NaOH)	97.53	4.8,8.3,9.5
3.	Oxide (3 % H_2O_2)	98.76	10.2
4.	Heat (60°C)	99.82	
5.	UV (240 nm)	99.98	--

Table 9: Results of Degradation Study.

Time	Amount of drug estimated CZ	% Label Claim*	S.D. (±)	% C.O.V.
20 min	99.98	99.88	0.376	0.452
1 h	99.78	99.67	0.311	0.389
2 h	99.04	99.89	0.378	0.392
4 h	100.00	10.00	0.408	0.455
12 h	100.10	10.07	0.455	0.478
24 h	99.98	99.91	0.377	0.394

Table 10: Results of stability.

References

1. The Indian Pharmacopeia (2014) Government of India, Delhi 2:1435-2439.

2. British pharmacopeia (2014) Stationary office, London: Medicines and Health care products regulatory agency: 1-2: 587-588.

3. Meghana D, Lahari K, KS Kumari, Prakash K (2012) Development and Validation of RP-HPLC Method for Simultaneous Estimation of Clonazepam and Propranolol Hydrochloride in Bulk and Pharmaceutical Dosage Forms. Inventi Rapid: Pharm Analysis & Quality Assurance 3: 12-16.

4. Chusena N, D Rao Garikapati, Usha P (2012) Development And Validation of An RP-HPLC Method for the Simultaneous Determination of Escitalopram Oxalate And Clonazepam in Bulk and its Pharmaceutical Formulations, International Current Pharmaceutical Journal 1:8: 193-198.

5. Sai T, Sakinala A, Shilpa C, Bala Vaidya S, J Rao (2012) Development and Validation of A RP-HPLC Method for Simultaneous Estimation of Propranolol HCL and Clonazepam in Bulk and Pharmaceutical Dosage Forms , International Research Journal of Pharmacy 3 :13-19

6. Chakole R, Charde M , N.Bhavsar, Marathe RP (2012) Simultaneous Estimation of Escitalopram and Clonazepam by RP-HPLC in Pharmaceutical Formulations. International Journal of Phytopharmacy 2: 25-29.

7. Moinuddin R Syed, Seema hashmi, Jitendra B Naik (2010) UV Spectrophotometric Method Development and Validation for Determination of Paroxetine Hydrochloride in Pharmaceutical Dosage Form, International Journal of Pharmacy And Pharmaceutical Sciences 2: 43-45.

8. MC Sharma, Smita Sharma (2010) Validated Simultaneous Spectrophotometric Estimation of Paroxetine HCL Bulk and Tablet Dosage Form Using Ferric Chloride, J. Optoel and Biomed. Mater 2: 185-89.

9. Janvika M, Hardik A Bhalodiya, Parula B Patel (2012) UV-Spectroscopic Method for Simultaneous Estimation of clonazepam and paroxetine Hydrochloride Hemihydrate in Combined Pharmaceutical Formulation, Inventi 21:333.

10. Sheeja VK, Swapna AS, Sosamma CE, Kumar P (2014) Method Development and Validation for the Simultaneous Estimation of Clonazepam and Paroxetine in Combined Dosage form using Colorimetry, Asian journal of Research in chemistry 7: 48.

11. Text on Stability Testing of new Drug Substances and Products Q1A (R2) (2003) In ICH, Harmonised Tripartite Guideline.

12. Text on validation of analytical Procedures Q2 (R1) (2005) In: ICH, Harmonised Tripartite Guideline.