IJBPAS, August, 2020, 9(8): 1841-1848

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

# VALIDATION OF HPLC METHOD FOR THE ESTIMATION OF CLONAZEPAM ODT AS PER USP

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**Received 8th Dec. 2019; Revised 9th Jan. 2020; Accepted 10th Feb. 2020; Available online 1st Aug. 2020**

https://doi.org/10.31032/IJBPAS/2020/9.8.5130

## ABSTRACT

According to USP method, a mixture of Potassium phosphate:acetonitrile (52:48) was used as mobile phase, the column having length of 4.6 mm × 25 cm and containing packing L7 which is maintained at 45 ± 1 ˚C temperature. The injection volume was 10 µl and the flow rate was 1.5 mL/min. In this method, the column used for experiment purpose, was packed with porous silica bonded to nitrile groups.C25 column contains stationary phase which is stable and it can tolerate pressure limit more than 6000 psi, for this reason mobile phase runs in uniform way and it provide uniform flow throughout the column. C25 column contains smaller particle size due to which plate increases resulting in higher efficiency. Octadecyl silane is used to increase the polarity of the stationary phase to give sharp and intense peak.

In this work we have performed the assay of the tablet according to united state Pharmacopoeia, according to USP, the assay of Clonazepam Orally Disintegrating tablet was performed by HPLC, and then performed validation according to ICH guidelines. The

method was validated for analytical parameters such as linearity, accuracy, precision, system suitability, robustness and range.

From the results it is concluded that as per USP method, HPLC is less time consuming and cost reducing, and the results obtained are more accurate, precise and reproducible as compared to UV.[1]

**Keywords: HPLC, Clonazepam, USP, ICH guidelines, Validation**

## 1. INTRODUCTION

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, in order to understand better the role of individual molecules. In liquid chromatography, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). The separation occurs because each component in the mixture interacts differently with the stationary phase. Molecules that interact strongly with the stationary phase will move slowly through the column, while the molecules that interact less strongly will move rapidly through the column. This differential rate of migration facilitates the separation of the molecules **[14, 15]**.

The main goal of this research activity is selected based on the increasing needs of the pharmaceutical/biopharmaceutical industry in developing suitable analytical methods. Among the various other available techniques, the scope of this work was focused on the modern chromatographic techniques such as HPLC which are accurate, precise, sophisticated and are having wide spectrum of application in pharmaceutical/biopharmaceutical industries.

The objective of current study is the validation of HPLC method for the estimation of Clonazepam Orally disintegrating tablet as per ICH guidelines **[2, 3, 5]**.

## 2. EXPERIMENTAL

### 2.1 Apparatus and Instruments

List mentioned in **Table 1**.

### 2.2 Reagents and Materials

List mentioned in **Table 2, 3**.

### 2.3 Assay of Clonazepam as Per USP:

**Mobile Phase:**

Prepare a filtered and degassed mixture of water acetonitrile and methanol (2:1:1), make adjustment if necessary.

**Standard Preparation:**

Dissolved accurately weighed quantity of Clonazepam RS in mobile phase and dilute, if necessary, to obtain a

solution having a known concentration of about 0.01 mg/mL of USP Clonazepam RS.

**Assay Preparation:**

Weigh and finely powder not fewer than 20 tablets. Transfer an accurately weighed portion of powder equivalent to about 2 mg of Clonazepam to a 200 ml volumetric flask, add 120 mL of mobile phase, and sonicate for 15 minutes, with intermittent shaking. Shake the flask on mechanical shaker, for about 30 minutes. Dilute with mobile phase to volume and mix. Pass a portion of this solution through a nylon membrane filter having a 0.45 µm or finer porosity and use the filtrate after discarding the first 4 mL of filtrate.(Note-Solution is stable for 48 hrs at room temperature.

**Chromatographic System:**

The liquid chromatograph is equipped with a 254nm Detector and a 4.6 mm×15 cm column that contains 5 µm packing L7.The flow rate is about 1.2 mL/min. The column temperature is maintained at 30°.Chromatograph the standard preparation, and record the peak response as directed for procedure, the column efficiency is not fewer than theoretical plates, the tailing factor is not more than 2.0;and the relative standard deviation for replicate injection is not more than 2.0%.

**Procedure:**

Separately inject equal volumes (about 60 µL) of the standard preparation and the assay preparation into the chromatograph, and measure the responses for the Clonazepam peak. Calculate the quantity in percentage of label claim of Clonazepam in the portion of tablets taken by the formula:

$$(C_s/Cu)(r_u/r_s)\ 100$$

In which C is the concentration, in mg per mL of USP Clonazepam RS in the standard solution; C is the nominal concentration based on label claim, in mg per mL of Clonazepam in the Assay preparation; ru is the peak response for Clonazepam obtained from the Assay preparation; and rs is the peak response for Clonazepam obtained from the Standard preparation **[1]**.

**Preparation of Stock Solution:**

Dissolve 100 mg of Clonazepam sample in 100ml of diluent. This solution was of 1000ppm.

**Preparation of solution:**

Pipette out 0.5 ml, 0.75 ml, 1 ml, 1.25 ml, 1.5 ml from above stock solution in different volumetric flask and volume made upto 10 ml by diluent to obtain 50%, 75%, 100%, 125% and 150% solution of Clonazepam **[14, 15]**.

**Table 1: Instruments Used**

| Sr. no. | Instruments | Specification |
|---|---|---|
| 1 | HPLC System | Shimadzu HPLC System |
| 2 | Pump | LC-20 AT Prominence Liquid Chromatography |
| 3 | Column | 4.6 mm X 25 cm column packed with porous silica bonded with nitrile groups. |
| 4 | Detector | SPD-M20A PDA Detector |
| 5 | Data Processor | LC solution |
| 6 | Degasser | DGU-20 A5 Prominence Degasser |

**Table 2: Name of API and Suppliers**

| Sr. no. | Name of API | Supplier |
|---|---|---|
| 2 | Clonazepam | Ray Chemicals Pvt Ltd, Yelahanka, Banglore (K.A) |

**Table 3: Marketed Formulation**

| Sr. no. | Brand Name | Strength | Name of company | Batch no. | Mfg. Date | Exp. Date |
|---|---|---|---|---|---|---|
| 1 | Clonotril-2 | Clonazepam- 2 mg | Torrent Pharmaceuticals Ltd. | 21883009 | JUNE 2013 | MAY 2016 |

## 3. RESULTS AND DISCUSSION

### Validation Results as Per USP Method:

According to USP method, a mixture of Potassium phosphate:acetonitrile (52:48) was used as mobile phase, the column having length of 4.6 mm × 25 cm and containing packing L7 which is maintained at 45 ± 1 ˚C temperature. The injection volume was 10 µl and the flow rate was 1.5 mL/min.

### A. Linearity:

Linear correlation was obtained between area versus concentration of Clonazepam in the range of 50-150 µg/ml. The slope, intercept and correlation coefficient values were found to be 32.55,-8.301 and 0.997 for Clonazepam. The result was shown in **Table 4**.

### B. Accuracy (Recovery Study):

Analysis results were obtained for accuracy study which is mentioned in **Table 5** for Clonazepam. This table representing accuracy with mean assay value 100.412%, SD 0.65 and %RSD 0.65% at 50%, accuracy with mean assay value 100.238%, SD 0.99 and %RSD 0.98 at 100%, accuracy with mean assay value 100.248%, SD 1.05 and % RSD 1.05 at 150%.

### C. Precision:

In this, there is Method and System precision study was performed. **Table 6** shows Method precision with mean area 3196.967, SD 32.50, %RSD 1.02% for Clonazepam. **Table 6** shows System precision with mean area 3298.151, SD 34.76, %RSD 1.05% for Clonazepam.

### D. Robustness:

This parameter was studied to determine how far an analytical method can withstand with different stress condition.

This study was performed by change in flow rate, change in mobile phase ratio.

**Table 7** shows robustness results (Flow rate) of Clonazepam in two different conditions. Flow rate (+0.2 mL/min) shows SD 12.61 and %RSD 0.38. Flow rate (-0.2 mL/min) shows SD 25.18 and %RSD 0.76%.

**Table 7** shows robustness results (mobile phase) of Clonazepam. Mobile Phase (90%) shows SD 26.25, %RSD 0.79%. Mobile Phase (110%) shows SD 11.911, %RSD 0.36%.

**E. System Suitability:**

System suitability testing is essential for the assurance of the quality performance of the chromatographic system.

**Table 9** shows the results of system suitability. From the results I concluded that systems were adequate for the analysis to be performed.

**F. Range:**

This study was performed by taking minimum concentration i.e. 50% and maximum concentration i.e. 150% six times.

**Table 10** shows results of range for Clonazepam with mean area 1644.103, SD 20.55, % RSD 1.25% at 50%, and mean area 4961.917, SD 21.71, %RSD 0.43% at 150%.

**Table 4: Linearity Data for Clonazepam**

| Sr. No. | Concentration | Peak Area |
|---|---|---|
| 1 | 50 | 1658.408 |
| 2 | 75 | 2378.976 |
| 3 | 100 | 3310.182 |
| 4 | 125 | 3944.429 |
| 5 | 150 | 4945.410 |

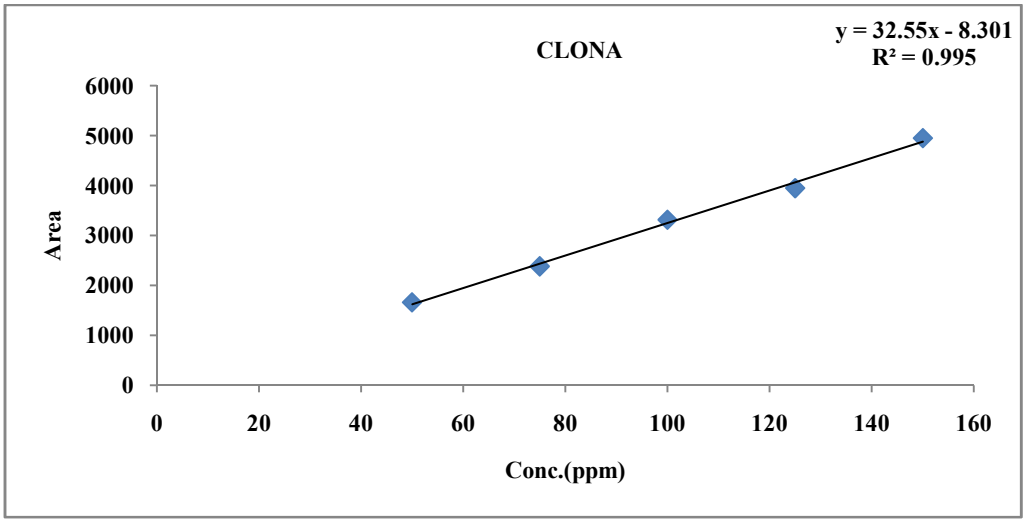


**Figure 1: Calibration curve of Clonazepam**

**Table 5: Result of % Accuracy Study of Clonazepam**

| % of spiked level of sample | Sample | Amount of Drug Added (µg/ml) | | Area Found | Amount of pure Drug found | % Recovery | Statistical Analysis of % Recovery |
|---|---|---|---|---|---|---|---|
| | | Pure | Formulation | | | | |
| 50 | 1 | 50 | 100 | 1655.215 | 50.155 | 100.311 | Mean:100.41 %RSD:0.65 |
| 50 | 2 | 50 | 100 | 1646.926 | 49.904 | 99.809 | |
| 50 | 3 | 50 | 100 | 1668.492 | 50.558 | 101.116 | |
| 100 | 1 | 100 | 100 | 3297.026 | 99.93 | 99.935 | Mean:100.23 %RSD:0.98 |
| 100 | 2 | 100 | 100 | 3280.513 | 99.43 | 99.434 | |
| 100 | 3 | 100 | 100 | 3343.546 | 101.346 | 101.346 | |
| 150 | 1 | 150 | 100 | 4965.281 | 150.517 | 100.344 | Mean:100.24 %RSD:1.05 |
| 150 | 2 | 150 | 100 | 4906.220 | 148.726 | 99.150 | |
| 150 | 3 | 150 | 100 | 5010.096 | 151.875 | 101.250 | |

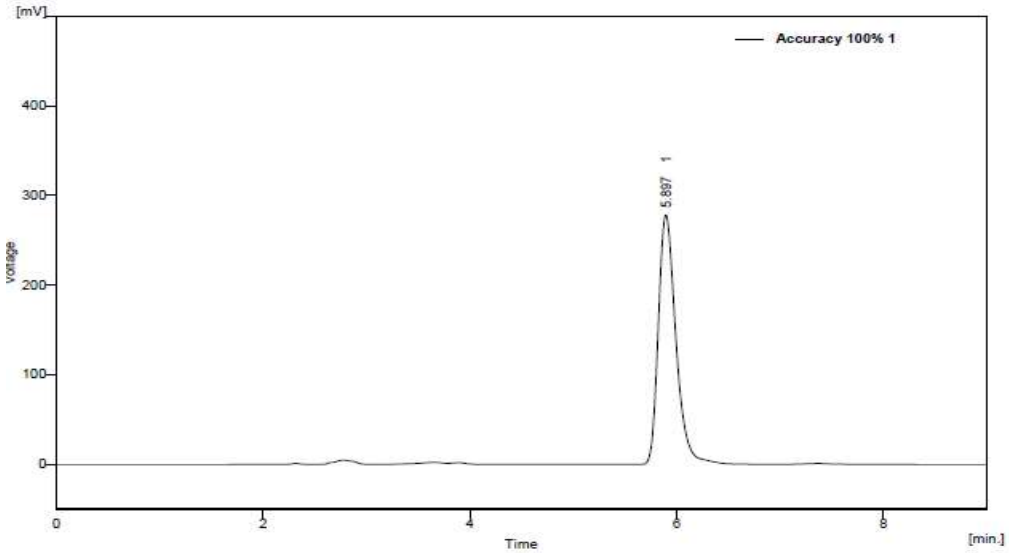


**Figure 2: Chromatogram of Clonazepam**

**Table 6: System Precision and Method Precision of Clonazepam**

| No. of Sample | System Precision | Method Precision |
|---|---|---|
| 1 | 3303.562 | 3199.060 |
| 2 | 3287.048 | 3183.068 |
| 3 | 3327.065 | 3224.710 |
| 4 | 3270.516 | 3173.471 |
| 5 | 3346.621 | 3243.964 |
| 6 | 3254.096 | 3157.530 |
| Mean | 3298.151 | 3196.967 |
| %RSD | 1.05% | 1.02% |

**Table 7: Flow Rate**

| Sample | Area | SD | %RSD |
|---|---|---|---|
| | Flow Rate (+0.2mL/min) | 12.612 | 0.38% |
| 1 | 3296.957 | | |
| 2 | 3280.415 | | |
| 3 | 3272.194 | | |
| | Flow Rate (-0.2 mL/min) | 25.183 | 0.76% |
| 1 | 3296.945 | | |
| 2 | 3280.454 | | |
| 3 | 3247.484 | | |

**Table 8: Mobile Phase**

| Sample | Area | SD | %RSD |
|---|---|---|---|
| | (90%organic) | 26.251 | 0.79% |
| 1 | 3280.454 | | |
| 2 | 3290.317 | | |
| 3 | 3330.045 | | |
| | (110%organic) | 11.911 | 0.36% |
| 1 | 3306.873 | | |
| 2 | 3290.366 | | |
| 3 | 3283.743 | | |

**Table 9: System Suitability Test Parameters**

| Drug | Retention Time | Theoretical Plate | Resolution | %RSD |
|---|---|---|---|---|
| CLONAZEPAM | 5.904 | 3202.279 | - | 0.65 |

**Table 10: Range for Clonazepam**

| Sr. No. | 50% | 150% |
|---|---|---|
| 1 | 1658.408 | 4945.41 |
| 2 | 1668.213 | 4975.326 |
| 3 | 1642.563 | 4928.652 |
| 4 | 1652.321 | 4969.021 |
| 5 | 1610.587 | 4989.201 |
| 6 | 1632.529 | 4963.892 |
| Mean | 1644.103 | 4961.917 |
| SD | 20.55778 | 21.71463 |
| %RSD | 1.25 | 0.43 |

**Table 11: Assay Result for Clonazepam**

| Formulation | Amount of Drug in Tablet (mg) | Amount of Drug taken (mg) | Amount of Drug found (mg) | %Amount of Drug found (mg) |
|---|---|---|---|---|
| Tablet | 2 | 2 | 1.85 | 92.51% |

## 4. ACKNOWLEDGEMENT

Authors are thankful to Management, Principals and Teaching & Non-Teaching Staff of Jamia & Ali-Allana college of Pharmacy, Akkalkuwa, Dist-Nandurbar, Swami Vivekanand Sanstha's Institute of Pharmacy, Mungase, Malegaon, Dist-Nashik, NBS Institute of Pharmacy, Ausa, Dist-Latur, Ismail Mehta College of Pharmacy, Ambad, Dist-Jalna, U.B.K.W.T' S D.pharm College, Kunjkheda Dist-Aurangabad for their kind Support and motivation during research work.

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