**Original Article**

# BIOEQUIVALENCE STUDIES OF MARKETED CLOZAPINE TABLETS BY USING OPTIMIZED VALIDATED LIQUID CHROMATOGRAPHIC METHOD

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## ABSTRACT

**Objective:** A simple, selective, precise and accurate method was developed for the estimation of Clozapine by RP-HPLC technique.

**Methods:** Chromatographic conditions used are stationary phase, Phenomenex BDS (150 mm x 4.6 mm, 5µ), Mobile phase was methanol and water in (80:20) ratio and flow rate was maintained at 1.0 ml/min, column temperature was set at 25 °C, detection wavelength was 240 nm, and diluent was mobile phase. These conditions were finalized for the optimized method.

**Results:** Linearity study was carried out between 10-60 µg/ml, the $R^2$ value was found to be as 0.995. Precision was found to be as follows for system precision 1.052, method precision 1.662, and intraday precision 1.02 and for interday precision 0.93. The % Recovery was found to be 98.60%. LOD and LOQ were found to be 2.7 µg/ml and 8.4 µg/ml respectively. By using the above method assay of the marketed formulation was carried out and the % purity was found to be 99.28 %. Stability studies of Clozapine were done, in all conditions degradation was found to be within the acceptable range.

**Conclusion:** The current validated method was finally applied in bioequivalence studies of four different brands of Clozapine by using dissolution apparatus and percentage drug release was found to be 99.48%, it was within the acceptable limit (NLT 85 %) as per USP.

**Keywords:** Clozapine, RP-HPLC, Degradation, Bioequivalence

## INTRODUCTION

Clozapine is a tricyclic benzodiazepine, classified as an atypical antipsychotic agent. It binds several types of central nervous system receptors and displays a unique pharmacological profile. Clozapine is a serotonin antagonist, with strong binding to 5-HT 2A/2C receptor subtype. It also displays a strong affinity to several dopaminergic receptors but shows only weak antagonism at the dopamine D2 receptor, a receptor commonly thought to modulate neuroleptic activity. Agranulocytosis is a major adverse effect associated with the administration of this agent [1]. (fig. 1)

Extensive literature survey revealed that a few HPLC [2, 3] and bio-analytical [4-6] methods have been reported for quantitative estimation of clozapine in pharmaceutical formulations and biological fluids, which include Spectrophotometric, and bioanalytical methods. Current research work was mainly to develop a simple, rapid, precise, accurate and reproducible isocratic RP-HPLC method for the determination of clozapine and the developed method is validated with respect to specificity, linearity, precision, accuracy, range, LOD, LOQ,robustness, ruggedness, stability and bioequivalence studies. The method validated in accordance with International Conference on Harmonization (ICH) guidelines.

## MATERIALS AND METHODS

### Standard drugs

Clozapine was procured from the Arvind Remedies Pvt Ltd, Chennai.

### Chemicals and reagents

HPLC Grade Methanol (Merck), HPLC Grade Water (Merck and Rankem).

### Instruments

HPLC (SHIMADZU-SPD), UV (Agilent Technologies, Cary 60 Uv-Vis), Detector (UV detector), Column (Phenomenex ODS $C_{18}$, (150×4.6 mm, 5µ), Software (Spinchrome), Ultra-Sonicator (Tharmo Lab-Cleaner).

### Preparation of mobile phase

Accurately measured 800 ml of Methanol(HPLC grade) mixed with 200 ml of Water (HPLC grade) was degassed in an ultrasonicator for 10 min and then filtered through 0.45 µ filter under vacuum filtration. The mobile phase was used as diluent.

### Standard preparation

Accurately weighed 50 mg of Clozapine was transferred into 50 ml V. F and final volume was made with diluent to get 1000 µg/ml. 1 ml of above solution was pipetted into 10 ml V. F and volume was made with diluent to get 100 µg/ml. From the above solution, 1 ml was transferred into 10 ml V. F and final volume was diluted to obtain 10 µg/ml and injected into HPLC (fig. 4)

### Selection of wavelength (λmax)

Standard solution of 10 µg/ml Clozapine was prepared and scanned in the UV-Visible spectrophotometer in the range of 200 to 400 nm by using methanol and water (80:20 v/v) as blank to determine λmax (fig. 2)

### Quantitative determination of clozapine

### Preparation of sample solution

20 tablets of Clozapine were weighed and finely powdered. An accurately weighed quantity of powder equivalent to 50 mg of clozapine was taken in a 50 ml V. F and 25 ml of diluent was added. The flask was sonicated for 10 min and final volume was made with diluent to get 1000 µg/ml. From this further dilution was made to get a final concentration of 10µg/ml. The percentage purity of drug present in the sample was calculated (fig. 5).

### Method development

### Chromatographic conditions

The column used was a$C_{18}$ column (150 × 4.6 mm, i.d., 5µ). The mobile phase consisted of a mixture of 80 volumes of methanol and

20 volumes of water. The mobile phase was filtered through 0.45 μ filter and degassed in ultrasonicator prior to use. Chromatography was performed at a temperature of 25 °C by pumping the mobile phase at a flow rate of 1.0 ml/min. The analyte was monitored at a wavelength of 240 nm using UV-detector. The retention time of both standard and sample Clozapine was found to be 4.790. The optimized conditions are shown in table.1.

## Method validation

The developed method was validated as per ICH guidelines for the following parameters [7].

### Specificity

Specificity is the ability of the analytical method to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the solution. The specificity of the method was determined by spiking the placebo at a specific level to standard drug solution (10 μg/ml). The blank, placebo and mixture of placebo and Clozapine solutions were injected to observe any interference with the drug peak.

### Linearity

Linearity is the ability of the method to produce results that are directly proportional to the concentration of the analyte in samples with given range. The linearity of Clozapine was performed in the range of 10-60 μg/ml. A calibration curve was plotted and regression coefficient was calculated (fig. 3)

### Accuracy

Accuracy is the closeness of results obtained by a method to the true value. The accuracy of the method was evaluated by standard addition method. Recovery of the method was determined by spiking a known concentration the pure drug (80, 100, 120 %) at three different concentration levels to sample.

### Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. It is expressed as standard deviation or relative standard deviation.

### System precision

The standard solution of Clozapine 10 μg/ml was injected and measured the area for all injections in HPLC. The % RSD for the area of replicate injections was found to be within the specified limits.

### Method precision

The sample solution of Clozapine 10 μg/ml was injected and measured the area for all injections in HPLC. The %RSD for the area of replicate injections was found to be within the specified limits.

### Intraday precision

Intraday precision studies were performed by injecting a standard solution of 10 μg/ml at several intervals i.e., 1, 3 and 5 h within a day.

### Interday precision

The Interday precision was carried out for three days with 10 μg/ml Clozapine standard solutions.

### Limit of detection and limit of quantification

Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value and quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ of Clozapine were separately determined based on the standard deviation of the response of the respective calibration curve.

$LOD = 3.3 \times \sigma/s$

$LOQ = 10 \times \sigma/s$

Where $\sigma$=Standard deviation

S= Slope

### Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness of test method was demonstrated by carrying out Flow rate variation±0.1 ml, i.e. (0.9 ml and 1.1 ml/min) and Wavelength variation±2 nm i.e. (238 nm and 240 nm).

### Ruggedness

The ruggedness of analytical method is a degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators, and materials. Ruggedness studies were performed by analyzing the two sets of 10 μg/ml standard solutions of Clozapine prepared by two analysts under the same experimental conditions.

### System suitability

System suitability is defined as "the checking of a system, before or during analysis of unknowns, to ensure system performance."A Standard solution of Clozapine 10 μg/ml was prepared and injected six times into HPLC. Then the system suitability parameters were evaluated from standard Chromatograms obtained by calculating the retention time, tailing factor, theoretical plates and peak areas from six replicate injections.

### Stability studies

These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products without interference. Standard solution of Clozapine was exposed to 0.1N HCL, 0.1N NaOH, 3%$H_2O_2$, heat, UV-Light, and water. The degraded samples were analyzed using HPLC to determine if there are interferences with the standard. Thus, the stability-indicating property was evaluated [8-10].

### Bioequivalence studies

It is a relative term which denotes that the drug substance in two or more identical dosage forms, reaches the systemic circulation at the same relative rate and to the same relative extent i.e. their plasma concentration-time profiles will be identical without significant difference.

### Preparation of acetate buffer (pH 4.0)

Accurately weighed 2.2 gm of sodium hydroxide pellets in 1000 ml of V. F and about 900 ml of distilled water was added and mixed. Then it was made up to the mark with distilled water. Then pH was adjusted to 4.0 with a glacial acetic acid solution.

### Preparation of clozapine standard drug solution

50 mg of Clozapine standard was accurately weighed and transferred into a clean and dry 50 ml V. F, dissolved in sufficient volume of acetate buffer and sonicated for 5 min. The final volume made up to 50 ml with acetate buffer (1000 μg/ml). From the above stock solution 1 ml was pipetted out and transferred into 10 ml V. F and the remaining volume was made with mobile phase to get 100 μg/ml. From the above solution, 1 ml was taken into 10 ml volumetric flask and the final volume was made up to the mark with mobile phase (10μg/ml) and injected into HPLC.

### Dissolution procedure

900 ml of pH 4.0 acetate buffer was added in each of the eight vessels of dissolution apparatus 2 (paddle). After reaching a temperature of 37 °C, four brands of clozapine tablets were added (two vessels for each brand). The machine was operated at the rotation of paddle speed at 100 rpm. After 45 min, samples were withdrawn from the vessels and filtered through Whatman filter paper. From the filtrate, 4 ml was transferred into 10 ml volumetric flask and remaining volume was made with diluent. The final diluted sample solutions were filtered by using 0.45 μm membrane filter

and injected into HPLC. The amount of clozapine present in each formulation and its percentage of drug release was calculated by comparing with the standard.

## RESULTS AND DISCUSSION

### Quantitative determination of clozapine

The percentage purity of specified Clozapine formulation was found to be 99.28 % w/v. The results are shown in table 2.

### Specificity

The Specificity Studies are carried out by varying specific conditions i.e. Placebo study. Chromatogram of placebo doesn't show interference at the retention time of Clozapine. Therefore this method is specific for determination of Clozapine.

### Linearity

From the Linearity data, it was observed that the method was showing linear in the concentration range of 10-60 μg/ml for Clozapine. The correlation coefficient was found to be 0.995.

### Accuracy

The recovery studies were performed. They found to be in the range of 98.38%-99.23%, which shows that the method was accurate. The results are shown in table 3.

### Precision

The precision of the method was evaluated by carrying system precision, method precision, Intra-day and inter-day precision studies. The percentage relative standard deviation (% RSD) of each study was calculated and was found to be less than 2% hence the method was found to be precise. The results are shown in table 4.

### LOD and LOQ

Limit of detection (LOD) and limit of quantitation (LOQ) of the method was found to be 2.7 μg/ml and 8.4 μg/ml respectively. Limit of detection (LOD) and limit of quantitation (LOQ) of the method was found to be 2.7 μg/ml and 8.4 μg/ml respectively.

### Robustness

No significant change in the chromatographic parameters was observed when changing the optimized condition like a change in the flow rate and wavelength. The results are shown in table 5

### Ruggedness

Comparison of the results obtained by two analyst's shows that the method is rugged for an analyst to analyst variability. The results are shown in table 6.

### System suitability

All the values of system suitability were found to be within in the acceptable limits. It concluded that the method and systems are adequate for the analysis to be performed. The result of system suitability studies are shown in table 7.

### Stability studies

The standard solution was exposed to water, acid (0.1N HCl), base (0.1N NaOH), an oxidizing agent (3% $H_2O_2$), Heat, UV-Light, and water. Degradation of drug substances are between 5 to 20% has been accepted for validation of chromatographic assays. From the results, degradation of Clozapine was shown more in alkaline condition. The results are shown in table 8.

### Bioequivalence studies

The percentage release of Clozapine formulations by dissolution was found to within the acceptable limits i.e., NLT 85% in 45 min as per USP. The results are shown in table 9.
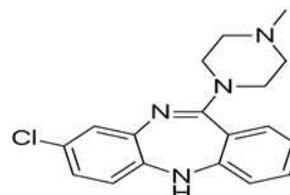


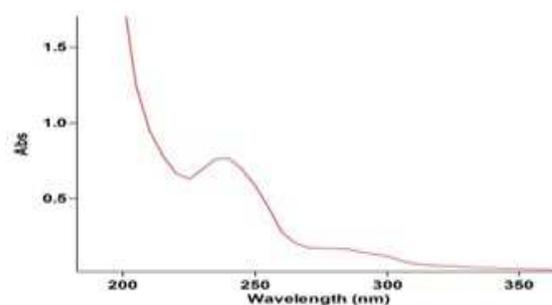Fig. 1: Structure of clozapine
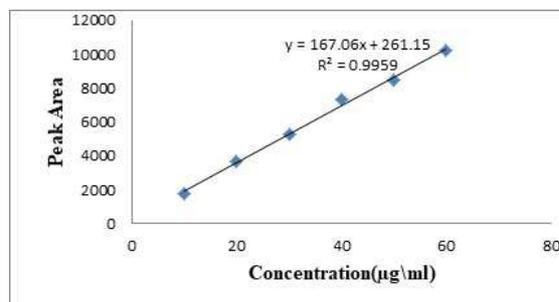


Fig. 2: UV spectrum of clozapine



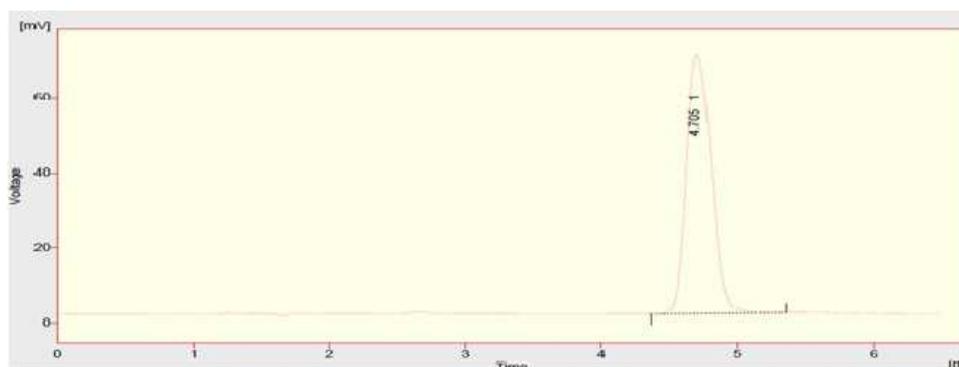Fig. 3: Linearity plot for clozapine



Fig. 4: HPLC chromatogram for standard

**Fig. 5: HPLC chromatogram of a sample**
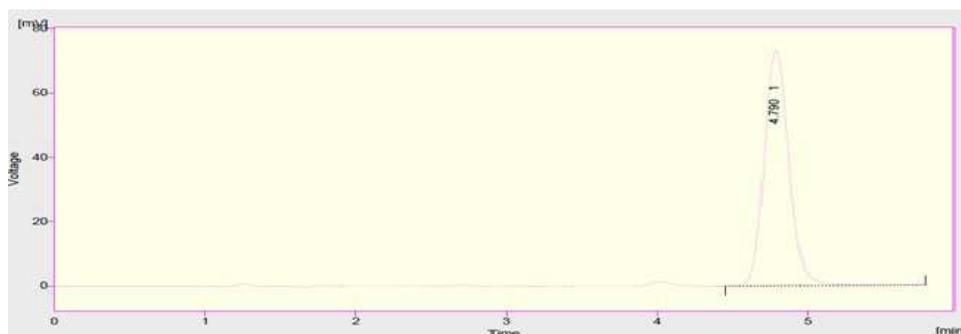
**Table 1: Optimized conditions for clozapine**

| Parameter | Method |
|---|---|
| Column | Phenomenex ODS C$_{18}$(150 x 4.6 mm, 5 μ) |
| Mobile Phase | Methanol and Water (80:20) |
| Flow rate (ml/min) | 1.0 ml/min |
| Column temperature ( °C) | 25 °C |
| Injection volume (μl) | 20 μl |
| Detection wavelength (nm) | 240 nm |
| Drug RT (min) | 4.790 |

**Table 2: Quantitative determination of clozapine**

| S. No. | Formulation | Concentration (μg/ml) | Retention time | Label claim (mg) | Amount found (mg) | Percentage purity |
|---|---|---|---|---|---|---|
| 1 | Clopine (Clozapine) | 10 | 4.790 | 25 | 24.83 | 99.28 |

**Table 3: Recovery studies of clozapine**

| S. No. | Recovery level | Sample concen. (μg/ml) | Amount spiked (ml) | % recovery | Average | SD | % RSD |
|---|---|---|---|---|---|---|---|
| 1 | 80% | 10 | 0.8 | 98.17 | | | |
| 2 | 80% | 10 | 0.8 | 99.39 | 98.38 | 0.918 | 0.933 |
| 3 | 80% | 10 | 0.8 | 97.59 | | | |
| 4 | 100% | 10 | 1 | 98.10 | | | |
| 5 | 100% | 10 | 1 | 94.73 | 98.21 | 1.458 | 1.485 |
| 6 | 100% | 10 | 1 | 96.82 | | | |
| 7 | 120% | 10 | 1.2 | 98.16 | | | |
| 8 | 120% | 10 | 1.2 | 100.28 | 99.23 | 1.060 | 1.068 |
| 9 | 120% | 10 | 1.2 | 99.25 | | | |

**Table 4: Precision data foe clozapine**

| S. No. | Precision | % RSD | Acceptance Limit |
|---|---|---|---|
| 1 | System Precision | 1.052 | NMT 2% |
| 2 | Method Precision | 1.662 | NMT 2% |
| 3 | Intraday Precision | 1.023 | NMT 2% |
| 4 | Interday Precision | 0.930 | NMT 2% |

**Table 5: Robustness data for clozapine**

| S. No. | Parameter | Modifications | Retention time | Peak area | Tailing factor | % RSD |
|---|---|---|---|---|---|---|
| 1 | Flow rate (±0.1 ml/min) | 0.9 ml/min | 4.907 | 2319.648 | 1.161 | 1.619 |
| | | 1.0 ml/min | 4.701 | 2071.125 | 1.130 | 1.191 |
| | | 1.1 ml/min | 4.026 | 1912.358 | 1.164 | 1.421 |
| 2 | Wavelength (nm) | 238.00 | 4.412 | 2058.057 | 1.093 | 1.300 |
| | (±2 nm) | 240.00 | 4.701 | 2071.125 | 1.130 | 1.191 |
| | | 242.00 | 4.812 | 1591.185 | 1.415 | 1.052 |

**Table 6: Ruggedness data for clozapine**

| S. No. | Analysts | Retention time | Peak area | % RSD |
|---|---|---|---|---|
| | | 4.700 | 2061.892 | |
| 1 | Analyst-1 | 4.713 | 2082.236 | 1.711 |
| | | 4.703 | 2013.835 | |
| | | 4.758 | 2083.148 | |
| 2 | Analyst–2 | 4.757 | 2058.260 | 1.795 |
| | | 4.760 | 2132.052 | |

**Table 7: System suitability data for clozapine**

| S. No. | Parameters | Values obtained | Acceptance criteria |
|---|---|---|---|
| 1 | Plate count | 4567 | ≥2000 |
| 2 | Tailing factor | 1.205 | ≤2.0 |
| 3 | Retention time | 4.790 | |

**Table 8: Summary of the stability studies**

| S. No. | Stress degradation | Stress condition | Time | Peak area | % degradation |
|---|---|---|---|---|---|
| 1 | Acidic | 0.1N HCL | 30 min | 1635.768 | 8.5 |
| 2 | Alkaline | 0.1N NaoH | 30 min | 3239.679 | 16.1 |
| 3 | Oxidative | 30% $H_2O_2$ | 30 min | 1620.186 | 5.2 |
| 4 | Thermal | 105 °C | 7 h | 1904.289 | 6.5 |
| 5 | Photolytic | UV Chamber | 7days | 1141.810 | 9.5 |
| 6 | Neutral | $H_2O$ | 30 min | 2486.083 | 6.7 |

**Table 9: Bioequivalence studies data**

| S. No. | Brands | Retention time | Label claim (mg/ml) | Amount found (mg/ml) | % drug release |
|---|---|---|---|---|---|
| 1 | Sizopin | 1.55 | 25 | 25.036 | 100.142 |
| 2 | Skizoril | 1.55 | 25 | 24.623 | 99.154 |
| 3 | Clozaril | 1.55 | 25 | 24.880 | 99.113 |
| 4 | Clomach | 1.55 | 25 | 24.778 | 99.537 |

## CONCLUSION

A simple, precise and accurate method was developed for the estimation of Clozapine by RP-HPLC technique. Bioequivalence studies and forced degradation studies were also performed using the optimized method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH Q2B guidelines and shown to be accurate, precise and specific. *In vitro* bioequivalence studies were performed based on the USP Pharmacopeia. The analytical method validation and bioequivalence studies of Clozapine by RP-HPLC method was found to be satisfactory and could be used for the quality control analysis and pharmacokinetic studies of Clozapine.

## ABBREVIATION

RP-HPLC: Reverse phase-High performance liquid chromatography, ICH: International Conference on Harmonization, LOD: Limit of detection, LOQ: Limit of quantification, USP: The United States of Pharmacopeia, RSD: Relative standard deviation, CONCEN: Concentration,V. F: Volumetric flask.

## ACKNOWLEDGEMENT

## CONFLICT OF INTERESTS

The authors declare no conflicts of interest

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