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Development and Validation of HPLC Method for Estimation of Clozapine inBulk drugs and Formulations.

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Abstract: A new, simple, and rapid high-performance liquid chromatographic method was developed and validated for quantitative determination of Clozapine. The sample was analyzed by Agilent, HPLC instrument. Using Nucleosil 100-5 C_{18} (250 mm X 4.6 mm, 5 μm) and HiQSiL C_{18} Column (250 xmm, 5μm) column as stationary phase and potassium dihydrogen orthophosphate: acetonitrile (65:35 v/v) as mobile phase at 1.0 ml/min flow rate. UV detector was used for the detection at 259 nm wavelength. The retention time for Clozapine was found about 4.5 minute. The linearity for the drug was obtained for the concentration of 5, 10, 15, 20, 25 and 30μg/ml. The method was successfully applied to pharmaceutical formulation because no significant interferences from tablet excipient were found. The method retained its accuracy and precision when certain variations in method parameters wereapplied.

Key Words: CLZ (Clozapine), HPLC, ACN (Acetonitrile), Methanol, Bulk drug, Formulations

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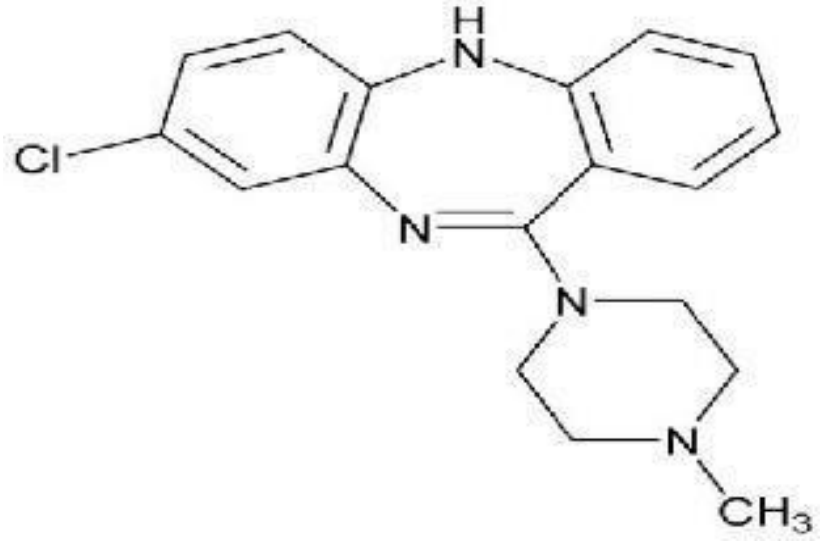
I. INTRODUCTION

Clozapine, an atypical antipsychotic drug, is a tricyclic dibenzodiazepine derivative, 8-chloro- 11-(4-methyl-1- piperazinyl)-5H-dibenzo [b, e] [1.4} dizaepine. It is slightly soluble in water, soluble in acetone, and highly soluble in chloroform. Its solubilityin water is 188.9 mg/L (25 °C) Chemical Formula: $C_{18}H_{19}ClN_4$ Mol. Weight: 326.823 g/mol[1].

Various methods have been reported for the determination of CLZ in pharmaceuticalpreparationsincluding spectrophotometric methods, gas-liquid chromatography (GC), FTIR, planar chromatography, and high performance liquid chromatography (HPLC). Most of the methods reported are highly sophisticated, costly, and time consuming and require special sample preparation. So simple method for the determination of clozapine using high performance liquid chromatography (HPLC) was developed. The method was validated successfully for the determination of clozapine and found simple andrapid.

structure of clozapine



II. EXPERIMENTAL-[2,3,4,5]

2.1 Method development of Clozapine-

A. Selection of mobile phase and chromatographicconditions:

Chromatographic separation studies were carried out on the working standard solution of Clozapine (10 µg/ml). Initially, trials were carried out using Acetonitrile in various proportions along with buffer of varying pH, to obtain the desired system suitability parameters. Finally 10 mM potassium dihydrogen orthophosphate (pH adjusted to 3.0 using *o*-phosphoric acid): acetonitrile in ratio 65: 35, v/v was chosen as the mobile phase which shown good resolution and acceptable peak parameters at a flow rate of 1 ml/min.

B. Preparation of mobilephase:

Mobile phase was prepared by mixing 10 mM potassium dihydrogen orthophosphate (pH adjusted to 3.0 using *o*-phosphoric acid) and acetonitrile in ratio 65: 35, v/v. It was then filtered through 0.45 µm membrane filter paper using filtration assembly and then sonicated on ultrasonic water bath for 10min.

C. Preparation of Standard stock solution:

Standard stock solution of Clozapine was prepared by dissolving 10 mg of drug in 10 ml of Methanol to get concentration of 1000 µg/ml. From the standard stock solution, 1 ml was further diluted to 10 ml with mobile phase to get 100 µg/ml solution of Clozapine.

D. Selection of DetectionWavelength:

From the standard stock solution further dilutions were done using mobile phase and scanned over the range of 200 - 400 nm and the spectrum was obtained. It was observed that Clozapine showed considerable absorbance at λmax 259 nm (Fig. 2.1)

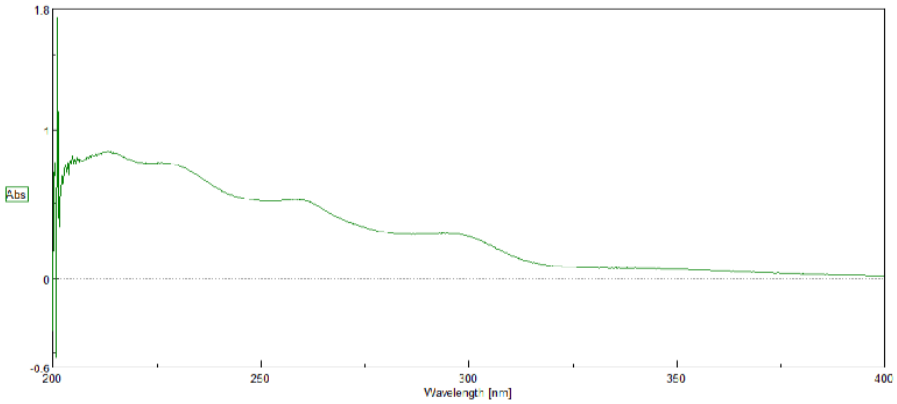


Fig. 2.1: UV Spectra of Clozapine (10 µg/ml)

E. Preparation of sample solution(Assay):

Twenty tablets [Sizopin 25 mg, Sun Pharma Laboratories Ltd.] were weighed and powdered. Tablet powder equivalent to 10 mg of Clozapine was weighed and transferred to 10 mlvolumetric flask and was diluted with Methanol. It was sonicated for 10 mins and filtered soas to get solution having concentration 1000 µg/ml. 0.1 ml of this solution was further dilutedwith mobile phase to get the final concentration of 10 µg/ml Clozapine. Six determinationswere carried out from homogenous sample to determine %assay.

F. Chromatogram and system suitability parameters ofdrug:

The column was saturated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of Clozapine (10 µg/ml) was injected on system. The retention times of repeated injections was found to be,

Sr. No.	RT
1	4.533
2	4.533
3	4.547
4	4.493
5	4.493
6	4.56
AVG	4.527
STDEV	0.028
% RSD	0.615

Table 2.1 Retention time

Clozapine = 4.527 ± 0.028 min

Chromatogram of mobile phase blank and Clozapine are shown in Fig. 2.2,2.3 and 2.4 respectively. System suitability parameters of Clozapine are summarised below in Table 2.2

Name	**RT (Min)**	**Concentration (µg/ml)**	**Area (µV.Sec)**	**Plates**	**Asymmetry**
Clozapine	4.527 ± 0.028	10	1262547.58	10584.28	1.08

Table 2.2: System suitability parameter

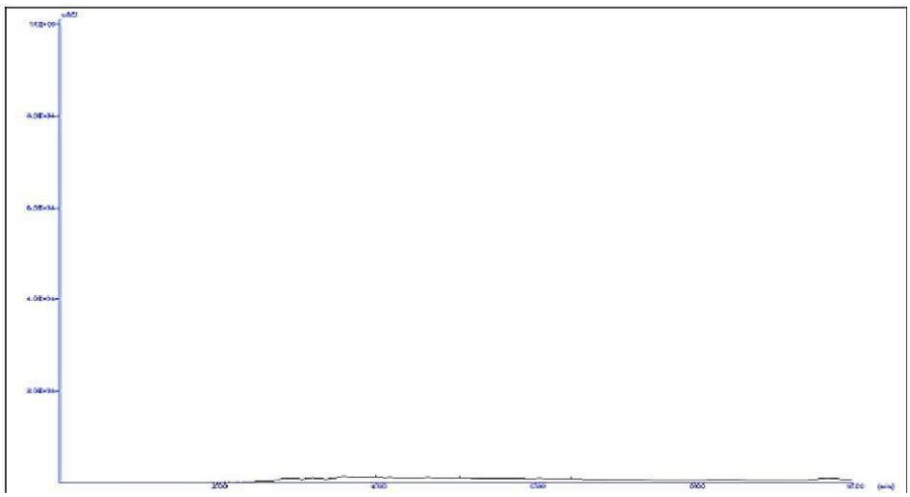


Fig. 2.2: Mobile Phase blank

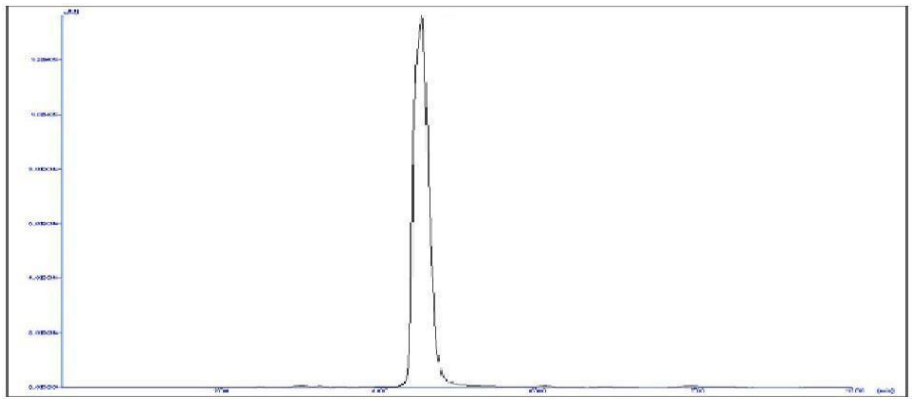


Fig. 2.3: Chromatogram of Standard Clozapine (10 µg/ml)

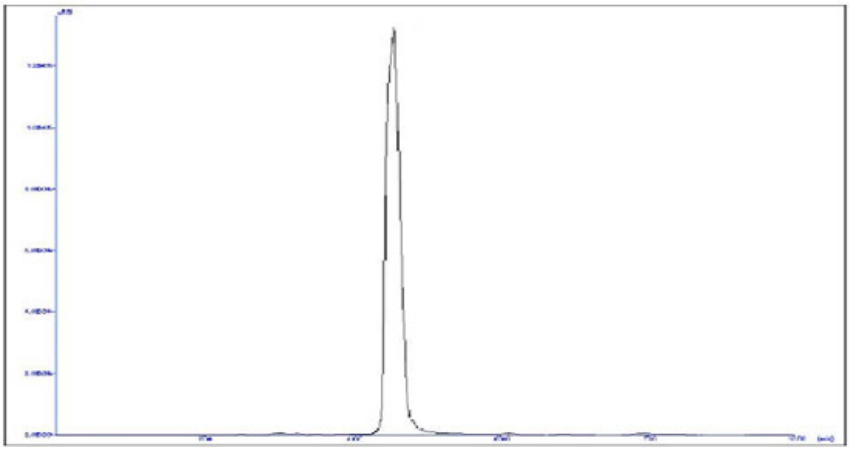


Fig. 2.4 Chromatograph of Marketed Formulation (10 µg/ml)

Summary of Chromatographic parameters selected:

Sr. No.	Parameter	Conditions used for Analysis
1.	Mobile phase	0.01 M potassium dihydrogen orthophosphate (pH 3.0 adjusted with o-phosphoric acid) : Acetonitrile (65: 35,v/v)
2.	Flow rate	1 ml/min
3.	Detection Wavelength	259 nm
4.	Sample injector	20 µl loop
5.	Column	HiQSil C8 (250 x 4.6 mm, 5µm)
6.	Column temperature	Ambient

Table 2.3 Summary of Chromatographic parameter

2.2. Validation of Analytical Method

A. Linearity

From the standard stock solution (100 µg/ml) of Clozapine further dilutions were made with mobile phase to obtain range of solution containing six different concentrations. Six replicates per concentration were injected. The linearity (relationship between peak area and concentration) was determined over the concentration range of 5-30 µg/ml ofClozapine.The results obtained are shown in Table 2.4. The peak area was plotted against the corresponding concentrations to obtain the calibration curve as shown in Fig. 2.4.

	Concentrations of Clozapine					
	5 µg/ml	**10 µg/ml**	**15 µg/ml**	**20 µg/ml**	**25 µg/ml**	**30 µg/ml**
Replicates	**Peak Area**					
1	808535.9	1233526	1666912	2189545	2613275	3248799
2	822200.5	1248641	1666236	2257805	2600322	3250365
3	803450.2	1271196	1725427	2271831	2615872	3259195
4	812598.9	1239725	1675288	2200548	2626407	3265125
5	823848.2	1261063	1669575	2262130	2605533	3256879
6	804254.5	1275481	1727154	2274105	2618490	3262457
Mean	812481.4	1254939	1688432	2242661	2613316	3257137
Std. Dev.	8814.632	17049.33	29502.98	37530.73	9317.592	6508.199
% RSD	1.085	1.359	1.747	1.673	0.356	0.199

Table 2.4: Linearity study of Clozapin

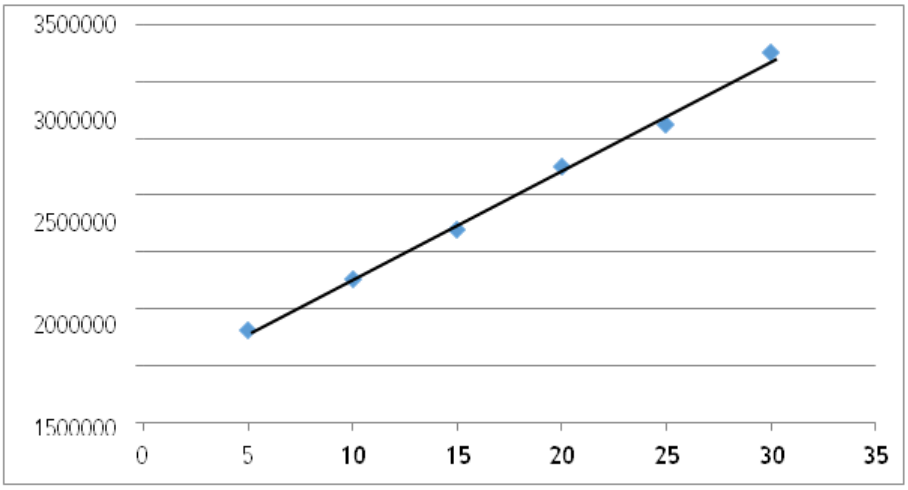


Fig. 2.4: Calibration curve for Clozapine

B. Range

Clozapine = 5-30 µg/ml

C. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD is calculated from the formula: -

$$LOD = 3.3\ \sigma / S$$

Where,

σ = standard deviation of response for the lowest conc. In the range

S = slope of the calibration curve.

LOD of Clozapine = 0.508 µg/ml

The quantitation limit is expressedas:LOQ= $10\ \sigma / S$

Where,

σ = standard deviation of response for the lowest conc. in the range S = slope of the calibration curve.

LOQ of Clozapine = 1.539 µg/ml

D. Precision

The precision of the method was demonstrated by Intra-day and Inter-day variation studies. In the Intraday studies, 3 replicates of 3 different concentrations (10, 20, 30 µg/ml) of Clozapine were analyzed in a day and percentage RSD was calculated. For the inter day variation studies, 3 replicates of different concentrations were analyzed on 3 consecutive days and percentage RSD were calculated. The results obtained for Intraday and Inter day variations are shown in Table 2.5 and 2.6.

Concentration (µg/ml)	Area (µV. Sec)	% Recovery ± SD	Mean % Recovery* ± SD	% RSD*
10	1259411			
10	1272122			
10	1243944	100.27 ± 1.47		
20	2220172			
20	2187273			
20	2210929	99.34 ± 0.88		
30	3154712			
30	3166741		99.88 ± 1.16	1.16
30	3226213	100.02 ± 1.33		

*Average of three determinations

Table 2.5: Intra-day precision study Clozapine

Concentration ((µg/ml)	**Area (µV. Sec)**	**% Recovery ± SD**	**Mean % Recovery* ± SD**	**% RSD***
10	1262791	100.56 ± 1.12	101.71 ± 1.13	1.11
10	1271254			
10	1249894			
20	2266244	101.93 ± 0.46		
20	2251744			
20	2250384			
30	3259411	102.65 ± 0.49		
30	3272122			
30	3243944			

*Average of three determinations

Table 2.6: Inter-day precision of Clozapine

E. Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 995, indicating the no interference of any other peak of degradation product, impurity or matrix.

Drug	**Purity tail**	**Purity front**
Clozapine	995.858	996.142

Table 2.7: Peak purity of Clozapine

F. Assay

Sizopin 25 mg tablet formulation analysis was carried out. Procedure was repeated for six times. Sample solution was injected and area was recorded. Concentration and % recovery was determined from linear equation. (Table 2.8)

Sr. No.	**Area**	**Concentration (µg/ml)**	**% Recovery**	**% Recovery Mean ± SD**	**% RSD**
01	1254712	9.988	99.876	99.679 ± 1.244	1.248
02	1266741	10.113	101.125		
03	1256213	10.003	100.032		
04	1232469	9.757	97.566		
05	1246542	9.903	99.028		
06	1260240	10.045	100.450		

Table 2.8. Results of Tablet formulation analysis (Assay)

G. Accuracy

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 50, 100 and 150 %. Basic concentration of sample chosen was10 µg/ml of Clozapine from tablet solution. These solutions were injected in stabilized chromatographic conditions in triplicate to obtain the chromatograms. The drug concentrations of Clozapine were calculated by using linearity equation of Clozapine. The results obtained are shown in Table2.9.

Level	Conc. (µg/ml)		Area	% Recovery	Mean	% RSD
	Sample	Std				
50 %	10	5	1728432.00	99.38	98.695	0.656
			1709826.05	98.09		
			1717410.57	98.62		
100 %	10	10	2225016.159	100.32	100.204	0.376
			2214746.265	99.78		
			2228770.869	100.51		
150 %	10	15	2701260.737	100.03	100.561	0.478
			2723936.015	100.98		
			2716599.139	100.67		

Table 2.9: Recovery study of Clozapine

H. Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, detection wavelength, flow rate were altered and the effects on the area were noted. The results obtained are shown in Table 2.10

% RSD Found For Robustness Study(peak area)								
MP COMPOSITION			**DETECTION WAVELENGTH (± 2 nm)**			**FLOW RATE (± 0.05 ml/min)**		
62:38	**65:35**	**68:32**	**257**	**259**	**261**	**0.95**	**1.0**	**1.05**
1.515	0.551	1.429	0.437	0.441	0.390	0.146	0.551	0.517

Table 2.10 Robustness study

Summary of validation study

The summary of validation parameters are summarised in Table2.11

Sr. No.	Validation Parameter	Results
		Clozapine
1.	Linearity	y = 96301 x + 292897 R^2 = 0.995
2.	Range	5-30 µg/ml
3.	Assay (Mean ± % RSD)	99.679 ± 1.248
4.	Precision	(% RSD)
	A) Intraday precision	1.16
	B) Interday precision	1.11
5.	Accuracy	% Recovery (Mean ± % RSD)
	50 %	98.695 ± 0.656
	100 %	100.204 ± 0.376
	150 %	100.561 ± 0.478
6.	LOD	0.508 µg/ml
7.	LOQ	1.539 µg.ml
8.	Specificity	Specific
9.	Robustness	Robust

Table 2.11: Summary of validationstudy

III. CONCLUSION

A new HPLC method has been developed for the identification and quantification of CLZ. Low cost, faster speed, and satisfactory precision and accuracy are the main features of this method. Method was successfully validated as per ICH guidelines and statistical analysis proves that method is sensitive, specific, and repeatable. It can be conveniently employed for routine quality control analysis of CLZ as bulk drug in

marketed tablets.

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2. Conflict

The authors have no any conflict of interest.

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