Development and Validation of Rapid RP-HPLC Method for the Determination of Chlordiazepoxide in Bulk form and Pharmaceutical Dosage form

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ABSTRACT : *A new, economical, simple, rapid, precise, accurate and reproducible RP-HPLC method for determination of Chlordiazepoxide in bulk form and marketed pharmaceutical formulation. Separation of Chlordiazepoxide was successfully achieved on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm column in an isocratic mode of separation utilizing Phosphate Buffer: Methanol in the ratio of 46:54% v/v (pH-3.2) at a flow rate of 1.0mL/min and the detection was carried out at 206nm. The response was found to be linear in the drug concentration range of 0-140mcg/mL for Chlordiazepoxide. The correlation coefficient was found to be 0.9993 for Chlordiazepoxide. The LOD and LOQ for Chlordiazepoxide were found to be 0.08µg/mL and 0.24µg/mL respectively. The proposed method was found to be good percentage recovery for Chlordiazepoxide, which indicates that the proposed method is highly accurate. The method was validated according to ICH guidelines for linearity, sensitivity, accuracy, precision, specificity and robustness. The specificity of the method shows good correlation between retention times of standard solution with the sample solution. Therefore, the proposed method specifically determines the analyte in the sample without interference from excipients of pharmaceutical dosage forms.*

Keywords: Chlordiazepoxide, RP-HPLC, Accuracy, Precision, ICH Guidelines.

I.INTRODUCTION

Chlordiazepoxide[1] is a benzodiazepine used to treat the withdrawal symptoms of acute alcoholism, to treat preoperative anxiety, and to treat anxiety over a short term period. Chlordiazepoxide is a long-acting benzodiazepine with anxiolytic, sedative and hypnotic activity. Chlordiazepoxide exerts its effect by binding to the benzodiazepine site at the gamma-aminobutyric acid (GABA) receptor-chloride ionophore complex in the central nervous system (CNS). This leads to an increase in the opening of chloride channels, membrane hyperpolarization and increases the inhibitory effect of GABA on the CNS. Chlordiazepoxide[2] is an orally available benzodiazepine used for therapy of anxiety and alcohol withdrawal syndromes. As with other benzodiazepines, Chlordiazepoxide therapy is not associated with serum aminotransferase or alkaline phosphatase elevations, and clinically apparent liver injury from Chlordiazepoxide has been reported, but is very rare. Chlordiazepoxide has antianxiety, sedative, appetite-stimulating and weak analgesic actions. The drug seems to block EEG arousal from stimulation in the brain stem reticular formation. The drug has been studied extensively in many species of animals and these studies are suggestive of action on the limbic system of the brain, which recent evidence indicates is involved in emotional responses. Hostile monkeys were made tame by oral drug doses which did not cause sedation. Chlordiazepoxide revealed a "taming" action with the elimination of fear and aggression. The taming effect of Chlordiazepoxide was further demonstrated in rats made vicious by lesions in the septal area of the brain. The drug dosage which effectively blocked the vicious reaction was well below the dose which caused sedation in these animals. The IUPAC Name of Chlordiazepoxide[3] is 7-chloro-4-hydroxy-N-methyl-5-phenyl-3H-1, 4-benzodiazepin-2-imine. The Chemical Structure of Chlordiazepoxide is as follows

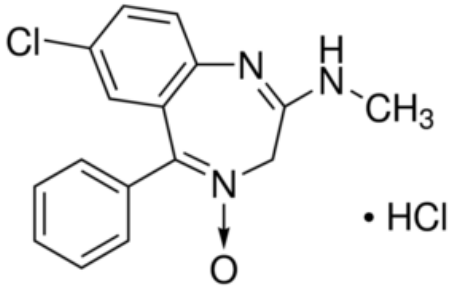

Fig.1. Chemical Structure of Chlordiazepoxide

II. EXPERIMENTAL

Table-1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table-2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Selection of Wavelength:

The standard & sample stock solutions[4,5] were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Chlordiazepoxide, so that the same wave number can be utilized in HPLC UV detector[6] for estimating the Chlordiazepoxide. The scanned UV spectrum is attached in the following page,

Sample & Standard Preparation for the UV-Spectrophotometer Analysis:

25 mg of Chlordiazepoxide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Preparation of 0.01M Potassium dihydrogen orthophosphate Solution:

About 1.36086grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade[7] water. The pH was adjusted to 3.20 with diluted orthophosphoric acid.

Preparation of mobile phase:

460ml of Phosphate buffer (0.05M) pH 3.20 and 540ml of HPLC Grade Methanol were mixed well and degassed

in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration[8].

III. Method Validation

3.1. Accuracy:

Recovery Study: To decide the exactness[9] of the proposed strategy, recuperation contemplates were completed by including diverse sums (80%, 100%, and 120%) of unadulterated medication of CHLORDIAZEPOXIDE were taken and added to the pre-examined plan of fixation 100µg/ml. From that rate recuperation esteems were figured. The outcomes were appeared in table-4.

3.2. Precision:

3.2.1. Repeatability: The accuracy[10] of every technique was found out independently from the pinnacle regions and maintenance times gotten by real assurance of six recreates of a fixed amount of drug Chlordiazepoxide (API). The percent relative standard deviation[11] was calculated for Chlordiazepoxide is presented in the table-5.

3,2.2. Intermediate Precision:

2.2.1. Intra-assay & inter-assay: The intra & inter day[12,13] variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Chlordiazepoxide revealed that the proposed method is precise.

3.3. Linearity & Range:

The calibration curve[14] showed good linearity[15] in the range[16] of 0 – 140 µg/ml (Table-4, for Chlordiazepoxide (API) with correlation coefficient (r^2) of 0.999 (Fig-7). A typical calibration curve has the regression equation[17] of y = 48313x + 71968 for Chlordiazepoxide.

3.4. Method Robustness:

Impact of little changes in chromatographic conditions[18], for example, change in Flow rate (± 0.1ml/min), Wavelength[19] of location (±2nm) and organic phase content in mobile phase[20] (±5%) concentrated to decide the Robustness[21] of the technique are additionally for (Table-8, % RSD < 2%) the created RP-HPLC strategy[22] for the examination of Chlordiazepoxide (API).

3.5. Limit of Detection:

The detection limit[23] 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.

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where,

σ = Standard deviation of the response

S = Slope of the calibration curve

3.6. Limit of Quantitation:

The quantitation limit[24] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$\mathbf{LOQ = 10\times\sigma/S}$$

Where,

σ = Standard deviation of the response

S = Slope of the calibration curve

3.7. System Suitability Parameter

Framework appropriateness testing is a necessary piece of numerous explanatory methodologies. The tests depend on the idea that the gear, hardware, logical tasks and tests to be examined comprise a necessary framework that can be assessed thusly. Following framework reasonableness test parameters[25,26] were built up. The information is appeared in Table-9.

3.8. Estimation of Chlordiazepoxide in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms were taken and the I.P. technique was taken after to decide the normal weight. Above measured tablets were at long last powdered and triturated well. An amount of powder comparable to 25 mg of medications were exchanged to 25 ml volumetric jar, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with mobile phase[27]. The arrangement was separated through a film channel (0.45 µm) and sonicated to degas. The arrangement arranged was infused in five repeats into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard[28] arrangement was likewise infused into the HPLC framework[29] and the pinnacle zones were recorded. The Assay information is appeared in Table-10.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of medication acquired with test readiness
AS = Peak Area of medication acquired with standard readiness
WS = Weight of working standard taken in mg
WT = Weight of test taken in mg
DS = Dilution of Standard arrangement
DT = Dilution of test arrangement
P = Percentage virtue of working standard

IV. RESULTS AND DISCUSSION

Selection of Wavelength:

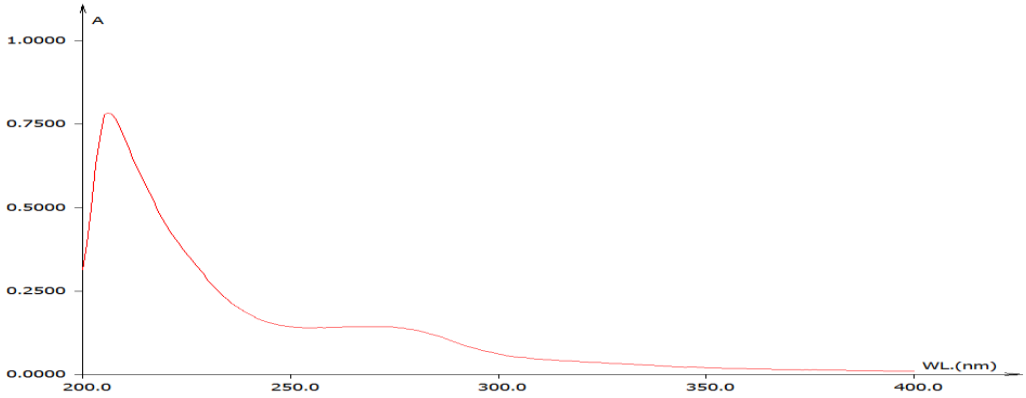

Fig.2. UV spectrum for Chlordiazepoxide

Summary of Optimized Chromatographic Conditions:

Table-3: Summary of optimised Chromatographic conditions

Mobile phase	Phosphate Buffer : Methanol = 46:54 (pH-3.2)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	206 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	3.622 minutes

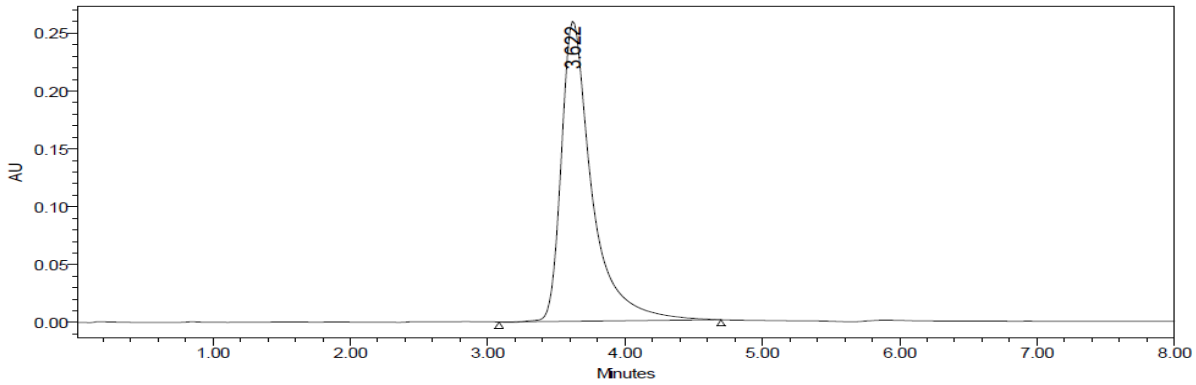

Fig.3. Chromatogram of Chlordiazepoxide in Optimized Condition

Method Validation:

4.1. Accuracy:

Table-4: Accuracy Readings

Conc. In ppm	Conc. Found	Peak Area		% Recovery
80	80.461	3959294		100.576
80	80.095	3941634		100.118
80	80.194	3946409		100.242
			Avg.	100.312
			S.D	0.236888
			%RSD	0.236151
Conc. In ppm	**Conc. Found**	**Peak Area**		% Recovery
100	100.932	4948323		100.932
100	99.879	4897463		99.879
100	100.030	4904741		100.030
			Avg.	100.2803
			S.D	0.569388
			%RSD	0.567796
Conc. In ppm	**Conc. Found**	**Peak Area**		% Recovery
120	120.019	5870480		100.015
120	119.907	5865040		99.922
120	119.794	5859590		99.828
			Avg.	99.92167
			S.D	0.0935
			%RSD	0.093574

4.2. Precision:

4.2.1. Repeatability:

Table-5: Repeatability Readings

HPLC Injection Replicates of Chlordiazepoxide	Retention Time (Minutes)	Peak Area
Replicate – 1	3.639	3948323
Replicate – 2	3.622	3935751
Replicate – 3	3.575	3979135
Replicate – 4	3.525	3971013
Replicate – 5	3.526	3919463
Replicate – 6	3.523	3974741
Average		**3954738**
Standard Deviation		**24108.89**
% RSD		**0.609621**

4.2.2. Intermediate Precision:

4.2.2.1. Intra-assay & inter-assay:

Table-6: Results of Intra-Assay & Inter-Assay

Conc. of Chlordiazepoxide (API) (µg/ml)	Observed Conc. of Chlordiazepoxide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	79.35	0.88	80.36	0.56
100	100.57	0.65	99.86	0.36
120	119.87	0.93	120.18	0.87

4.3. Linearity & Range:

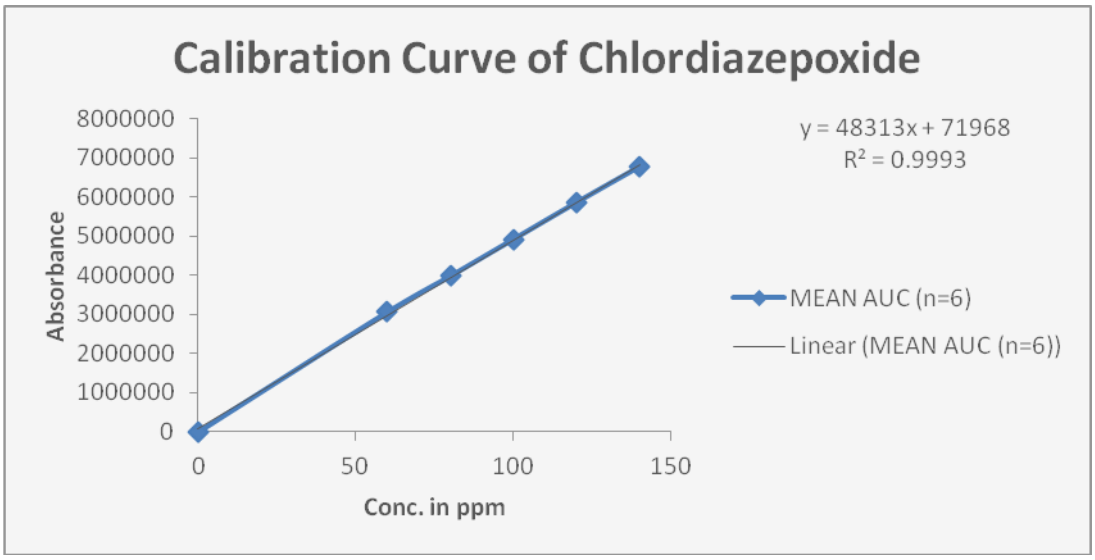

Fig.4. Calibration Curve of Chlordiazepoxide (API)

Table-7: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
60ppm	3059294
80ppm	3979280
100ppm	4919463
120ppm	5859590
140ppm	6770480

4.4. Method Robustness:

Table-8: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.61

Flow (0.9 ml/min)	0.75
More Organic	0.69
Less Organic	0.81
Wavelength of Detection (208 nm)	0.89
Wavelength of detection (204 nm)	0.99

4.5. LOD & LOQ:

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.

4.6. System Suitability Parameter:

Table-9: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.34
2	Asymmetry	T ≤ 2	Chlordiazepoxide=0.16
3	Theoretical plate	N > 2000	Chlordiazepoxide=3065
4	Tailing Factor	T<2	Chlordiazepoxide=1.55

4.7. Estimation of Chlordiazepoxide in Pharmaceutical Dosage Form

Table-10: Recovery Data for estimation Chlordiazepoxide in Librium

Brand name of Chlordiazepoxide	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Librium (10mg) (Abbott)	10mg	9.752 (± 0.546)	99.82 (± 0.287)

Result & Discussion: The amount of drug in Librium Tablet was found to be 9.752 (± 0.546) mg/tab for Chlordiazepoxide & % assay was 99.82 %.

V. SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Chlordiazepoxide, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm column was preferred because using this column peak shape, resolution and absorbance were good.

Recognition wavelength was chosen in the wake of checking the standard arrangement of medication more than 200 to 400nm. From the U.V range of Chlordiazepoxide it is apparent that a large portion of the HPLC works can be refined in the wavelength scope of 206 nm helpfully. Further, a stream rate of 1 ml/min and an infusion volume of 10µl were observed to be the best examination.

The outcome demonstrates the created strategy is amazingly, one more appropriate technique for test and steadiness related contamination thinks about which can help in the examination of Chlordiazepoxide in various definitions.

Finally a sensitive and particular RP-HPLC technique has been created and approved for the examination of Chlordiazepoxide in API form and marketed pharmaceutical dosage form.

Promote the proposed RP-HPLC technique has magnificent affectability, exactness and reproducibility.

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