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RP-HPLC ESTIMATION OF CLOFAZIMINE IN SOFT GELATINE CAPSULE: DEVELOPMENT, VALIDATION AND STABILITY INDICATIONS

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ABSTRACT
Stability indicating reverse phase high performance liquid chromatography method was developed for the estimation of Clofazimine in bulk drug and soft gelatin capsules. The chromatographic separation was achieved on ODS C18 column using mobile phase comprising 10 mM citrate buffer: acetonitrile, in which pH of buffer is adjusted with 1N HCL solution in ratio of (70:30 v/v), flow rate was 1.0 ml/min and eluents were detected by UV detector at 283 nm. Retention time of Clofazimine was found to be 5.205 min. Linearity range was found over the concentration range 25-75 µg/ml. Clofazimine was subjected to various stress conditions i.e. Acid, Base, Oxidative, Thermal and Photolytic degradation. The degradation peak of formulation of Clofazimine were well resolved from the pure peak. The proposed method were validated according to the ICH guidelines and it can be suitable for quality control analysis of Clofazimine in soft gelatin capsule.

KEYWORDS: Clofazimine, Reverse Phase High Performance Liquid Chromatography Method, soft gelatin capsule, degradation, Validation.

INTRODUCTION
Clofazimine is an Anti-inflammatory agent which acts by the inhibition of mycobacterial DNA leading to disruption of the cell cycle and that eventually kills the bacterium. It may also bind to bacterial potassium transporters, thereby inhibiting their proliferation function1-5. Chemically Clofazimine is N, 5-bis (4-chlorophenyl)-3-[(propan-2-yl)imino]-3,5-dihydrophenazin-2 amine. Chemical formula of Clofazimine is C27H22C12N46 (Figure 1). Clofazimine is official in Indian Pharmacopoeia (IP) 7, British Pharmacopoeia (BP)8 and United States Pharmacopoeia (USP)9.

Literature survey was carried out and it revealed that UV visible spectroscopy in formulation10, HPTLC and HPLC in human and in animal11-15 has been reported. It was also revealed that there was no method reported on the stability indicating HPLC in the formulation of Clofazimine.

MATERIALS AND METHODS
Materials and Reagents
Clofazimine API was obtained from Sangrose Laboratory of Kerala and soft gelatin capsules were procured from local pharmacy. Water for HPLC, Acetonitrile and Methanol (HPLC grade) were purchased from Merck specialist Pvt. Ltd, Mumbai. Hydrochloric acid, Sodium hydroxide, Potassium dihydrogen phosphate & Di potassium hydrogen phosphate, Trisodium citrate dehydrate, Formic acid, and Hydrogen peroxide were obtained from Dr. Reddy's Laboratories.

Instrumentation chromatographic conditions
Different kinds of equipment like Digital weighing balance (Shimadzu ATX 224, Japan), HPLC system (Shimadzu Prominence with pump LC-20AD, Japan), Injector (Rheodyne, 20 µl), Sonicator (Toshco instrument), pH meter (Janki impex Pvt. Ltd), vacuum filter pump, millipore filtration kit, mobile phase reservoir, humidity chamber (Pheonix Lab), hot air Oven (Janki Instruments).

Chromatographic conditions
Stationary Phase: C18 (Shim pack XR ODS II, 250mm×4.6mm, 5µm).

Mobile Phase: 10 mM citrate buffer pH 6.0: Acetonitrile (70:30% v/v).

Column temperature: Room temperature.

Injector volume: 20µm.

Detector: UV- detector SPD-20A.

Detection Wavelength: 283 nm.

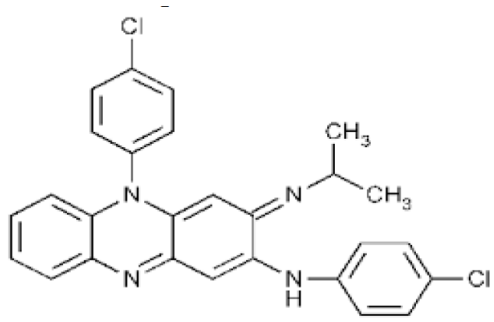

Figure 1: Chemical structure of Clofazimine.

Table 1: Optimized parameters for method development of Clofazimine.

Parameters	Optimized condition
Elution	Isocratic
Mobile Phase Composition	10mM citrate buffer pH 6.0: Acetonitrile(70:30)
Column	C_{18}(Shim pack XR ODS II. 250mm×4.6mm, (5µm)
Flow Rate	1.0 ml/min
Wavelength Detection	283 nm
Injection Volume	20 µg/ml
Run Time	10 min
Retention Time	5.205 min
Sample Concentration	25 µg/ml
Peak Characteristics	Good and symmetric

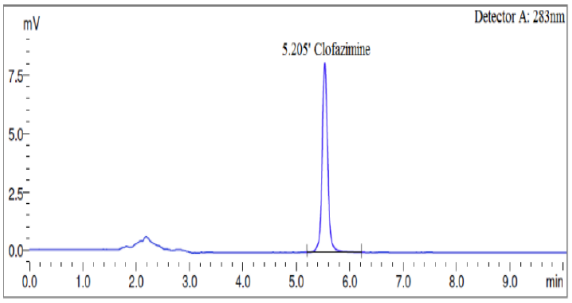

Figure 1: Optimized chromatogram of clofazimine.

Table 2: System Suitability Testing (n=6).

Parameters	Data Obtained
Mean Area	7016429
Retention Time	5.207 min
Theoretical Plates Per Column	15311
Tailing Factor	1
Resolution	-

Table 3: Linearity Data (n=5) of Clofazimine.

Concentration (µg/ml)	Mean Area	%R.S.D
50	3571217	0.47
50	5007201	0.57
50	7157208	0.30
50	8588390	0.19
50	10782742	0.38

Table 4: Repeatability of Clofazimine (n=6).

Concentration (µg/ml)	Mean Area	S.D	% R.S.D
50	6966181		
50	6997904		
50	6984645		
50	6990782	22054.16	0.31
50	7029610		
50	7012056		

Table 5: Data for Intermediate Precision.

Concentration (µg/ml)	Interday Precision (n=3)	Intraday Precision (n=3)
	Peak area ± % RSD	Peak area ± % RSD
25	3560538 ± 1.06	3549159 ± 0.38
50	7073124 ± 1.19	7161707 ± 0.00
75	10682618 ± 0.97	10784321 ± 0.00

Table 6: Data of LOD and LOQ.

Parameters	Result
LOD	0.85
LOQ	2.6

Diluents: Methanol.

Preparation of Solutions

Preparation of standard stock solution

10 mg of Clofazimine was accurately weighed and transferred in 100ml of volumetric flask, 70ml of methanol was added and it was sonicated for about 15 minutes to dissolve the API completely. The solution was made up to the mark using methanol and it was mixed well (concentration 100 ug/ml).

Calibration standards Clofazimine:

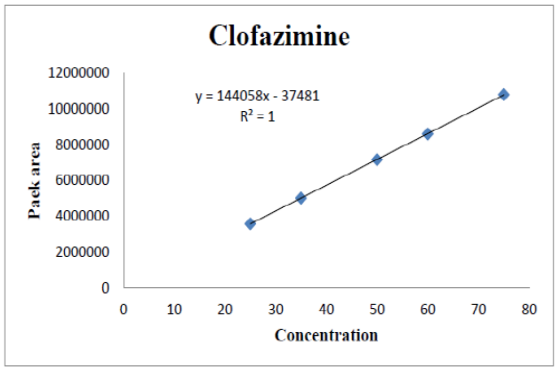

Figure 3: Calibration curve of Clofazimine(25-75 µg/ml).

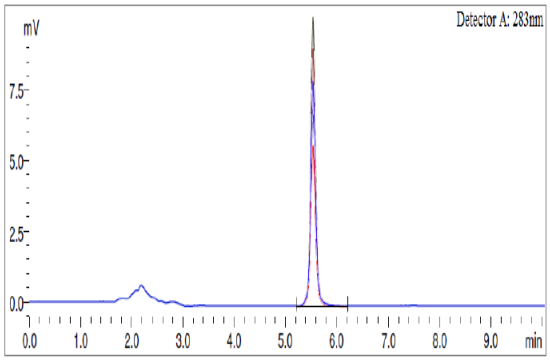

Figure 4: Overlain chromatogram of Clofazimine (25-75 µg/ml).

Table 7: Data for Recovery Study of Clofazimine (n=3)

Level %	Amount Taken (µg/ml)	Amount Spiked (µg/ml)	Mean Area	Amount Recovered	% Recovery	% RSD
80	25	20	2802449	19.71	98.55	
	25	20	2809410	19.76	98.80	0.19
	25	20	2798850	19.68	98.44	
100	25	25	3538826	24.82	99.28	
	25	25	3535711	24.80	99.20	0.13
	25	25	3529189	24.75	99.03	
120	25	30	4225229	29.59	98.63	
	25	30	4238211	29.68	98.93	0.21
	25	30	4220986	29.56	98.53	

From standard stock solution Clofazimine (100 ug/ml) final Concentration was made in ranges 25-75 µg/ml.

Method validation

The developed RP-HPLC method validated according to ICH guidelines (ICH Q2 (R1)) 16, 17. 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 25-75 µg/ml.

Table 8: Data for Robustness Method (n=3).

Sr. No	Parameters	Variation	Mean Area	% RSD
	Flow Rate	0.9	7700523	0.4
1	(±0.1 ml/min)	1	7016429	0.2
		1.1	6373286	0.6
2		68:32	7045673	0.4
	Mobile Phase Composition	70:30	7016429	0.2
	(±2%v/v)	72:28	7047412	0.7
3		5.8	7025202	0.2
	Mobile Phase pH	6	7016429	0.2
	(±0.2)	6.2	7023564	0.2

Table 9: Stability study of Clofazimine

Time	Amount of drug estimated, mg	% label claim	%RSD
30 min	49.75	99.50	0.32
1hr	49.65	99.30	0.56
2 hr	49.52	99.04	0.45
4 hr	49.48	98.96	0.11
8hr	49.36	98.72	0.25
24 hr	49.25	98.50	0.37

Table 10: Assay of marketed Formulation.

	Label Claim (mg/ml)	Amount Found (mg/ml)	% Assay ± R.S.D
Clofazimine	50	49.74	99.48 ± 0.08

Accuracy

Recovery studies were carried out by spiking three different known amounts of pure drug (at 80%, 100% and 120% level) to the pre-analyzed sample solution (standard addition method). The known amount of standard solution of Clofazimine were added to the pre analysed sample solution 25 µg/ml of Clofazimine.

Precision

The precision of the method was verified by repeatability and intermediate precision studies. Repeatability was determined by injecting six replicates of the working standard solution 50µg/ml. The intermediate precision of the method was checked by repeating studies on three different days. The intraday and interday precision of the proposed methods were performed by injecting three different concentrations of the standard in triplicates on the same day and on different days. Concentrations of standard solutions of Clofazimine selected were (25, 50, 75µg/ml).

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,

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 and pH of mobile phase on peak area were studied. The solution containing 50 µg/ml of Clofazimine was injected (in triplicate) into sample injector of HPLC three times under the varied conditions.

Stability
Stability in sample solutionsSample solution of Clofazimine (100 µg/ml) was prepared and was kept at room temperature (30 ± 20°C) protected from day light. The sample solution was assayed after 30 min, 1 h, 2 h, 4h, 8 h and 24 h and hence results of the remaining analysis times were compared with it.

System suitability
To ascertain resolution and reproducibility of proposed chromatographic system for estimation of Clofazimine 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 100µg/ml Clofazimine. The diluted standard solutions were filtered through 0.2 µ membrane filter.

Preparation of sample solution
Transfer 5 soft gelatin capsules into 100ml volumetric flask, add about 20-25 ml of water, sonicate for 45 minutes and then shake for 30 minutes by mechanical means, check visually if the capsules are dispersed or not and then make up the volume up to the mark with methanol and mix well. Filter the solution through 0.45µ PVDF filter. Further transfer 1ml of filtrate to 50ml of volumetric flask and make up the volume up to the mark with methanol and inject.

For Force degradation studies
In order to evaluate the stability indicating property of the developed HPLC methods stress studies were carried out under ICH recommended conditions18. Intentional degradation was tried by exposing the tablet sample to the following stress conditions: acid (0.1N HCL at room temperature for 12 hours), base (0.1N NaOH at room temperature for 30 minutes), peroxide degradation (3% H2O2 at 80ºC at room temperature for 12 hours), thermal degradation (80ºC) and sunlight degradation (24 hours). Ability of the proposed methods to measure the analyte response in presence of its degradation products was studied.

RESULTS AND DISCUSSION

Optimization of separation conditions

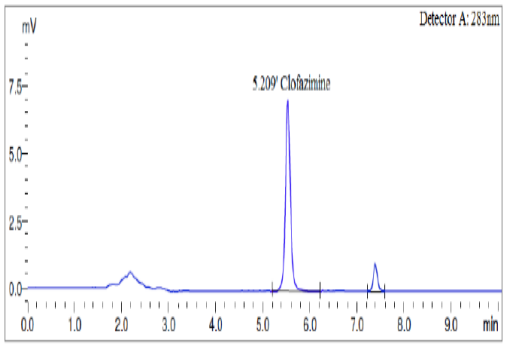

Figure 5: Chromatogram of acid degradation.

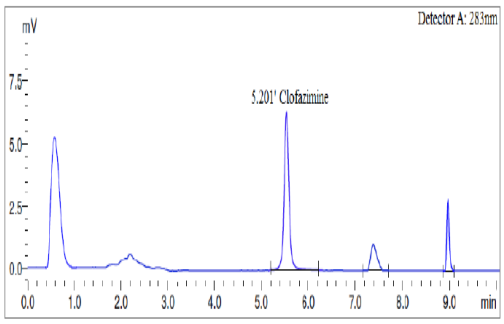

Figure 6: Chromatogram of base degradation.

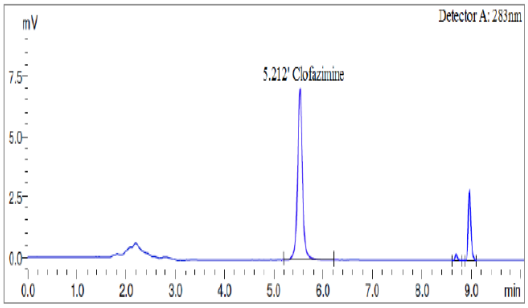

Figure 7: Chromatogram of peroxide degradation.

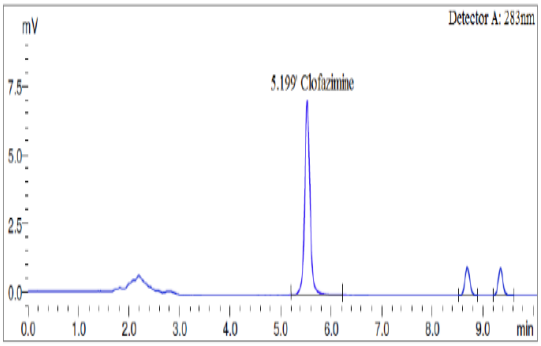

Figure 8: Chromatogram of thermal degradation.

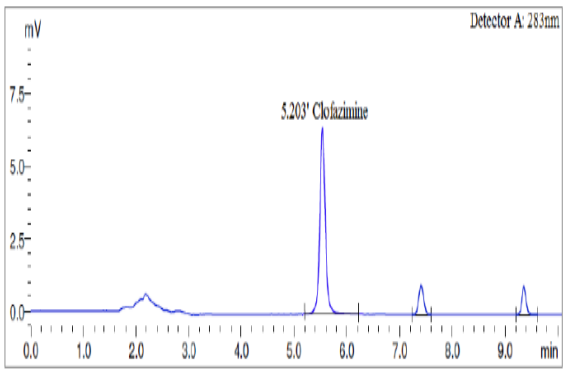

Figure 9: Chromatogram of sun light degradation.

Table 11: Results for Force Degradation Study.

Parameters	Retention time (min)	Peak area	Resolution	% Assay	% Degradation
Acid	5.209	5918436	-	84.40%	15.40%
	7.399	79321	3.8		
Base	5.212	5960510	-	84.99%	14.81%
	8.705	14859	6.5		
	8.989	54896	1.9		
Peroxide	5.201	5813251	-	82.90%	16.90%
	7.398	83254	3.8		
	8.972	51236	3.3		
Thermal	5.199	5904411	-	84.19%	15.61%
	8.712	45632	6.4		
	9.375	44985	2.2		
Sunlight	5.203	5862007	-	83.60%	16.20%
	7.395	65023	3.8		
	9.378	42569	3.9		

By carrying out different trial for the mobile phase selection, 10mM citrate buffer pH 6.0: Acetonitrile (70:30) was optimized since it gave a good and symmetric peak. (Table 1 and figure 1)

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 Clofazimine was recorded for each injection shown in Table 2.

Validation of the method

Linearity

A linear relationship was observed between peak area and concentration in the range of 25-75 µg/ml Clofazimine. The correlation coefficients for the calibration curve were found to be 1.000 for Clofazimine. Mean peak areas for Clofazimine at selected wavelength are shown in Table 3 and Figure 3 and 4.

Precision

Repeatability and reproducibility of the proposed method was determined by intra-day and inter-day precision studies. The capsule 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 thepercent label claim determined by developed method as shown in Table 4 and 5.

Limit of Detection (LOD) and Quantification (LOQ):

The limit of detection was 0.85µg/ml and limit of quantification was 2.6µg/ml for Clofazimine respectively as shown in Table 6.

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 7.

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, mobile phase ratio and pH of mobile phase on peak area were studied. The solution containing 50 µg/ml of Clofazimine was injected (in triplicate) into sample injector of HPLC 3 times under the varied conditions. Robustness data is given in Table 8.

Stability

The stability evaluation in sample solutions (constituted with methanol) was performed up to 24 h. The sample solutions were analysed periodically at 1, 2, 4, 8 and 24 h. The results are shown in Table 9. The peak areas of eachdrug were not considerably different from each other. Moreover, RSD value of each sample was <1%.

Assay of soft gelatin capsule

Commercially available soft gelatin capsule of Clofazimine was analysed for %recovery. The analysis of marketed formulation of Clofazimine was carried out and results are shown in the following table 10.

Force degradation studies

In the force degradation studies, Clofazimine was found to degrade under acidic, basic, peroxide, thermal and sunlight stress conditions employed. The results for forced degradation studies are included in Table 11.

Typical chromatography obtained for Clofazimine under different stress conditions are shown in Figures 5-9. 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 RP-HPLC method for determination of Clofazimine was proved to be simple, rapid, precise, accurate and robust in their pharmaceutical dosage and validated as per ICH guidelines. Moreover, Clofazimine was found stable in the sample solutions placed at room temperature up to 24h. Accordingly, the proposed analytical procedure with detection time of 10 min can

be used for reliable determination of Clofazimine in bulk and capsule. The Force degradation studies method was found to be simple, sensitive, selective, and suitable for determination of Clofazimine in presence of its degradation products. From the force degradation studies it was concluded that the formulation was in stable in acidic, basic, oxidative, thermal and photolytic conditions. Clofazimine Statistical analysis proved that the method is repeatable, reproducible, accurate and specific for the analysis of Clofazimine. The developed RP-HPLC method which confirms the stability indicates power of the developed method.

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