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Stability-indicating High Performance Thin Layer Chromatographic Determination of Clozapine in Tablet Dosage Form

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Abstract
A simple, selective, precise and stability-indicating high-performance thin-layer chromatographic method of analysis of Clozapine in pharmaceutical dosage form was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system comprised of toluene - methanol - ethyl acetate - ammonia (8:2:1:0.1, v/v/v/v). This system was found to give compact spots for Clozapine (Rf value of 0.25). Clozapine was subjected to acid and alkali hydrolysis, oxidation, photochemical degradation and thermal degradation. Also, the degraded product was well separated from the pure drug. Densitometric analysis of Clozapine was carried out in the absorbance mode at 280 nm. The linear regression analysis data for the calibration plots showed good linear relationship with coefficient of regression value, r^2 = 0.9991 in the concentration range 10.8-108.0 ng $spot^{-1}$. The value of correlation coefficient, slope and intercept were 0.9995, 22.49 and 18.06, respectively. The method was validated for precision, recovery, ruggedness and robustness. The limits of detection and quantitation were 5.4 and 10.8 ng $spot^{-1}$, respectively. The drug undergoes degradation under acidic, basic, photochemical degradation and thermal degradation conditions. All the peaks of degraded product were resolved from the active pharmaceutical ingredient with significantly different R_f values. The samples degraded with hydrogen peroxide showed no additional peak. This indicates that the drug is susceptible to acid-base hydrolysis degradation, photochemical degradation and thermal degradation. Statistical analysis proves that the method is reproducible and selective for the estimation of said drug. As the method could effectively separate the drug from its degradation product, it can be employed as a stability-indicating one.
Keywords: Clozapine ; Chromatography ; Stability Indicating ; Degradation

1.Introduction:

Clozapine, chemically, 8 – chloro – 11 - (4 – methyl – 1 – piperazinyl) - 5 *H* – dibenzo (b,e) (1,4) diazepine [1,5] (Figure1) is an Antipsychotic drug. The drug is listed in Merck index [1]. Literature survey reveals that there are spectroscopic methods [2-4], HPLC method [5-9], for quantitation of Clozapine. Few HPLC - electrospray tandem mass spectrometry [10-12] is reported for evaluation of Clozapine. Few method for GC [13-15]and GCMS [16] is reported for determination of Clozapine. A solid phase extraction method [17] is reported for systematic toxicological analysis in biological fluid. An MS-MS libraries with triple quadrupole - tandem mass spectrometers [18] technique for drug identification and drug screening is reported. But there is no stability indicating HPTLC method for determination of Clozapine from its tablets, as its pharmaceutical dosage form.

The International Conference on Harmonization (ICH) guideline entitled 'Stability Testing of New Drug Substances and Products' requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [19]. Susceptibility to oxidation is one of the required tests. The hydrolytic and the photolytic stability are also required. An ideal stability-indicating method is one that quantifies the drug per se and also resolves its degradation products. A very viable alternative for stability-indicating analysis of Clozapine is high-performance thin-layer chromatography (HPTLC). The advantage of HPTLC is that several samples can be run simultaneously by using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis [20-22].

The aim of the present work was to develop an accurate, specific, reproducible, and stability indicating method for the determination of low levels of Clozapine in the presence of its degradation products

and related impurities as per ICH guideline [23].

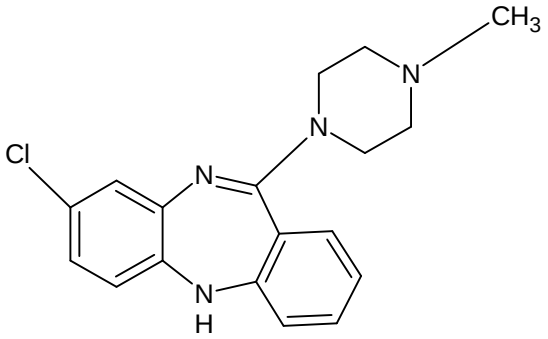

Figure 1: Chemical structure of Clozapine

2.**Experimental:**

2.1.*Materials*

Clozapine was supplied by Sun pharma India Ltd. and tablets (Label Claim: 25 mg tablet^{-1}, Product Name: Sizopin) were procured from the market. All chemical and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

2.2.*HPTLC Instrumentation*

The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm x 10 cm with 250 μm thickness; E.merck, Germany) using a Camag Linomat V (Switzerland). A constant application rate of 150 μL sec^{-1} was employed and space between two band was 5 mm.

The slit dimension was kept at 5 mm x 0.45 mm and 20 mm sec^{-1} scanning speed was employed. The mobile phase consisted of toluene - methanol - ethyl acetate - ammonia (8:2:1:0.1, v/v/ v/v). Methanol was used as diluent for standard and sample preparation. Linear ascending development was carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min. at room temperature. The length of the chromatogram run was 7 cm. Subsequent to the development; TLC plates were dried in a current of air with the help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 280 nm. The source of radiation utilized was deuterium lamp.

2.3.*Calibration Curves of Clozapine*

A stock solution of Clozapine (10.8 μg mL^{-1}) was prepared in diluent. Different volumes of stock solution 1, 3, 5, 7.5 and 10μL, were spotted on TLC plate to obtain concentration of 10.8, 32.0, 54.0, 81.0 and 108.0 ng per spot of Clozapine, respectively. The data of peak area versus drug concentration were treated by linear least-square regression analysis.

2.4*Method Validation*

2.4.1.*Precision*

Precision was measured in terms of repeatability of application and measurement. Repeatability of standard application was carried out using five replicates of the same spot (50 ng spot^{-1}for standard application). Repeatability of sample measurement was carried out in six different sample preparations from same homogenous blend of marketed sample (50 ng spot^{-1} for sample application). It showed very low % relative standard deviation (% RSD) of peak area of Clozapine.

2.4.2.*Ruggedness and Robustness*

Method ruggedness and robustness was determined by analysing same sample blend at normal operating conditions and also by changing some operating analytical conditions such as development distance, mobile phase composition, injection volume, chamber saturation time and analyst.

2.4.3.*Limit of detection and limit of quantitation*

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank diluent was spotted in replicates following the same method as explained in section 2.2. The signal to noise ratio was determined.

2.4.4.*Recovery Studies*

Recovery study was performed by spiking 30%, 50% and 70% of Clozapine working standard to a preanalysed sample. The preanalysed sample is to be weighed in such a way that final concentration is half

or 50% of the sample preparation before spiking. The percentage sum level of preanalysed sample and spiked amount of drug should be 80%, 100% and 120% of simulated dosage nominal or target concentration of sample preparation. The accuracy of the analytical method was established in duplicate across its range.

2.5.*Analysis of marketed formulation*

Weigh and finely powder not less than 20 tablets. Transfer blend equivalent to 25 mg of Clozapine to a 100 mL volumetric flask. Add about 60 mL of diluent and sonicate for 15 minutes and make up volume with diluent. Mix well and centrifuge the solution at 2500 rpm for 10 minutes. Spot the clear supernatant solution in the form of bands on the specified TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate. The possibility of excipient interference in the analysis was studied.

2.6.*Forced degradation of Clozapine*

2.6.1.Preparation of acid and based-induced degradation product

Tablet powder equivalent to 25 mg of Clozapine was transferred to 100mL volumetric flask. To it, 25 mL of diluent was added and sonicated for 10 minutes with intermittent shaking. To it, 1mL of 5N Hcl was added and 1mL of 5N NaOH was added separately. The sample was heated on a boiling water bath for 5 minutes. After cooling, to room temperature it was diluted to volume with diluent and mixed. This solution was centrifuged at 2500 rpm for 10 minutes. This acidic and basic forced degradation was performed in the dark in order to exclude the possible degradative effect of light. The resultant supernatant solution was applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.6.2.Preparation of hydrogen peroxide-induced degradation product

Tablet powder equivalent to 25 mg of Clozapine was transferred to 100mL volumetric flask. To it, 25 mL of diluent was added and sonicated for 10 minutes with intermittent shaking. To it, 1mL of 30.0% H_2O_2 was added. The sample was heated on a boiling water bath for 5 minutes. After cooling, to room temperature it was diluted to volume with diluent and mixed. This solution was centrifuged at 2500 rpm for 10 minutes. The resultant supernatant solution was applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.6.3.Photochemical degradation product

Tablet powder equivalent to 25 mg of Clozapine (previously kept in UV light for 24 hours), was transferred to 100mL volumetric flask. To it, 25 mL of diluent was added and sonicated for 10 minutes with intermittent shaking, diluted to volume with diluent and mixed. This solution was centrifuged at 2500 rpm for 10 min

The resultant supernatant solution was applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.6.4.*Thermal degradation product*

Tablet powder equivalent to 25 mg of Clozapine was transferred to 100mL volumetric flask. To it, 50 mL of diluent was added and sonicated for 10 minutes with intermittent shaking. This sample was heated on a boiling water bath for 5 minutes. After cooling to room temperature it was diluted to volume with diluent and mixed. This solution was centrifuged at 2500 rpm for 10 minutes. The resultant supernatant solution was applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.7.*Detection of the related impurities*

Weigh and finely powder not less than 20 tablets. Transfer blend equivalent to 25 mg of Clozapine to a 100 mL volumetric flask. Add about 60 mL of diluent and sonicate for 15 minutes and make up volume with diluent. Mix well and centrifuge the solution at 2500 rpm for 10 minutes. Spot the clear supernatant solution in the form of bands on the specified TLC plate and

the chromatograms were run as described in Section 2.2.

*3.***Result and discussion:**

3.1.Development of the optimum mobile phase

TLC procedure was optimized with a view to develop in stability- indicating assay method. Both the pure drug and the marketed products were spotted on TLC plates and run in different solvent system where bands closer to the solvent front and diffused bands were observed. Finally, the mobile phase of toluene - methanol - ethyl acetate - ammonia (8:2:1:0.1, v/v/v/v) gave good sharp and symmetrical peak with R_f value of 0.25 for Clozapine. Well defined spots were obtained when the chamber was saturated with the mobile phase for 5 min at room temperature.

3.2.*Calibration curves*

The linear regression data for the calibration curves indicate that the response is linear over the range 10.8 to 108.0 ng $spot^{-1}$ for Clozapine with coefficient of regression, r^2, value as 0.9991. The value of correlation coefficient, slope and intercept were 0.9995, 22.49 and 18.06, respectively.

3.3.*Validation of the method*

3.3.1.*Precision*

The %RSD for repeatability of standard application is 0.97%. Whereas the %RSD for repeatability of sample preparation is 1.13%. This shows that precision of the method is satisfactory as % relative standard deviation is not more than ±2.0% and mean recovery between 98.0 to 102.0%. Table 1 represents the precision of method.

3.3.2.Ruggedness and Robustness of the method

The parameters and results of normal operating condition (original) against changed conditions are indicated in Table 2. The low value of % RSD obtained after introducing the deliberate changes in parameters alters the results of Clozapine to -1.37% of method precision study, which is not a significant change. The ruggedness and robustness of the method was established, as the % deviation from mean assay value obtained from precision study was less than ±2.0%.

3.3.3.LOD and LOQ

The signal -to-noise ratios of 3 and 10 were considered as LOD and LOQ, respectively. The LOD and LOQ for Clozapine is 5.4ng/spot and 10.8ng/spot respectively.

3.3.4Recovery studies

$$\% \text{ Recovery} = \frac{\% \text{ Amount Recovered}}{\% \text{ Sum Level}} \times 100$$

The results of recovery are shown in Table 3. The results indicate that the individual recovery of Clozapine ranges from 97.99% to 101.08% with mean recovery of 99.66% and % relative standard deviation of 1.30%. The recovery of Clozapine by proposed method is satisfactory as % relative standard deviation is not more than ±2.0% and mean recovery between 98.0% to 102.0%.

3.4.Analysis of the marketed formulation

A single spot at R_f 0.25 was observed in the chromatogram of the drug sample extracted from tablets. There was no interference from the excipients commonly present in the tablets. The drug content was found to be 99.22% with a % RSD of 0.59%. It may therefore be inferred that degradation of Clozapine was not occurred in the marketed formulation that were analyzed by this method. The low % RSD value indicated the suitability of this method for routine analysis of Clozapine in pharmaceutical dosage form.

3.5.Stability- indicating property

The % assay and % degradation with stress conditions are shown in Table 4 with respective figures in Figure No.2. The no treatment sample (as control) has been evaluated relative to the standard concentration where as rest of the stressed condition samples (Sr.No.2 to 6) are evaluated relative to the control sample with respect to the % assay & % degradation. The percentage degradation results are calculated by area normalization method. The chromatogram of the acid degraded sample for Clozapine showed additional peak at R_f value of 0.28. The chromatogram of the alkali degraded sample for Clozapine showed additional

peak at R_f value of 0.19. The chromatogram of photochemical degraded sample for Clozapine showed additional peak at R_f value of 0.21. The chromatogram of thermal degraded samples for Clozapine showed additional peak at R_f value of 0.21.

The sample degraded with hydrogen peroxide showed no additional peak. In each forced degradation sample where

Table 1: Method precision of Clozapine

Sample Preparation	% Assay	% Deviation from Mean Assay Value
1	98.55	-0.67
2	99.66	0.45
3	99.44	0.22
4	97.29	-1.94
5	100.08	0.87
6	100.27	1.06
Mean	99.22	
± SD	1.12	
%RSD	1.13	

Table 2: Ruggedness and Robustness of Clozapine

Parameter	Normal (Original)	Changed conditions
Development Distance (from line of application)	70%	75%
Mobile Phase Composition (% v/v/v)	Toluene – Methanol – Ethyl Acetate – Ammonia (8:2:1:0.1)	Toluene – Methanol - Ethyl Acetate- Ammonia (7:3:1:0.1)
Injection Volume	1 µL	2 µL
Chamber Saturation time	20 Minutes	30 Minutes
Analyst	Zahid	Analyst II
% assay, Clozapine	99.22	97.86
% RSD from mean assay value obtained in method precision studies is -1.37% of Clozapine.		

Table 3: Recovery of Clozapine

Sample Preparation	% Simulated Dosage Nominal	% Sum Level	% Amount Recovered	% Recovery
Pre-analysed Sample				99.22
1	80	80.2	81.07	101.08
2	80	74.6	74.60	100.00
1	100	91.4	92.14	100.81
2	100	87.8	87.69	99.88
1	120	104.0	101.91	97.99
2	120	99.4	97.63	98.22
Mean				99.66
± Standard Deviation				1.29
% Relative Standard Deviation				1.30

Table 4: Stressed study data of Clozapine

Sr. No.	Condition	% Assay Clozapine	% degradation	
			Single maximum	Total
1.	No treatment (control sample)	99.22	nil	nil
2.	Acid	94.02	5.64	5.64
3.	Alkali	92.38	7.24	7.24
4.	H_2O_2	98.53	nil	nil
5.	UV	93.92	5.37	5.37
6.	Thermal	95.81	3.50	3.50

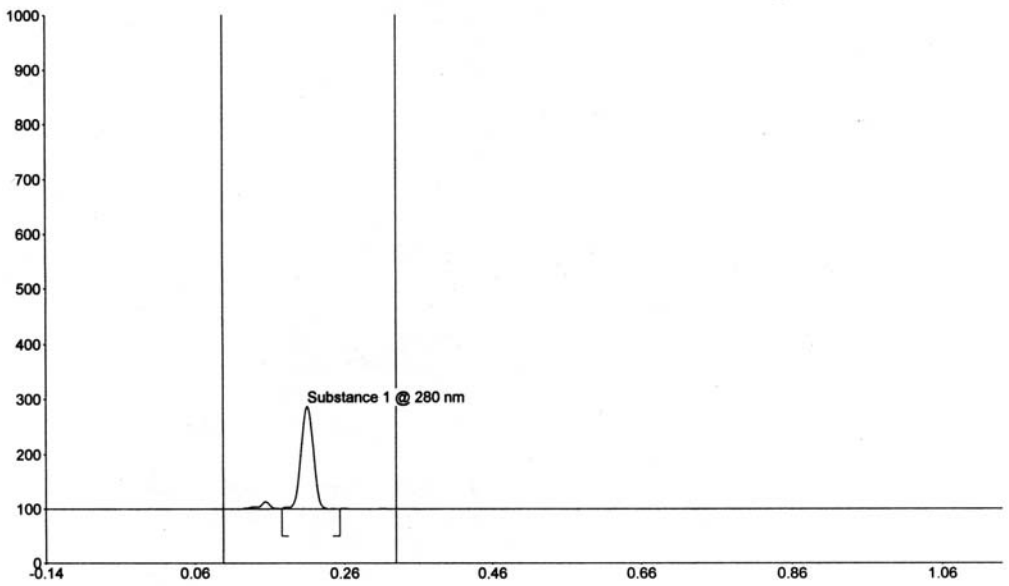

Figure 2.a Typical standard chromatogram of Clozapine

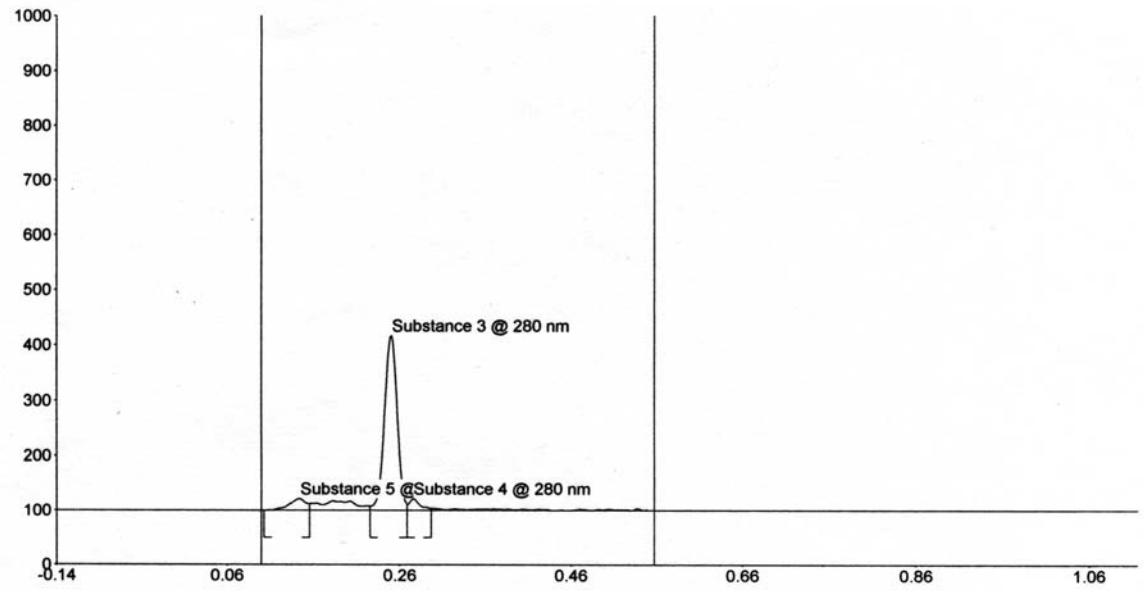

Figure No.2.b: Acid degradation of Clozapine

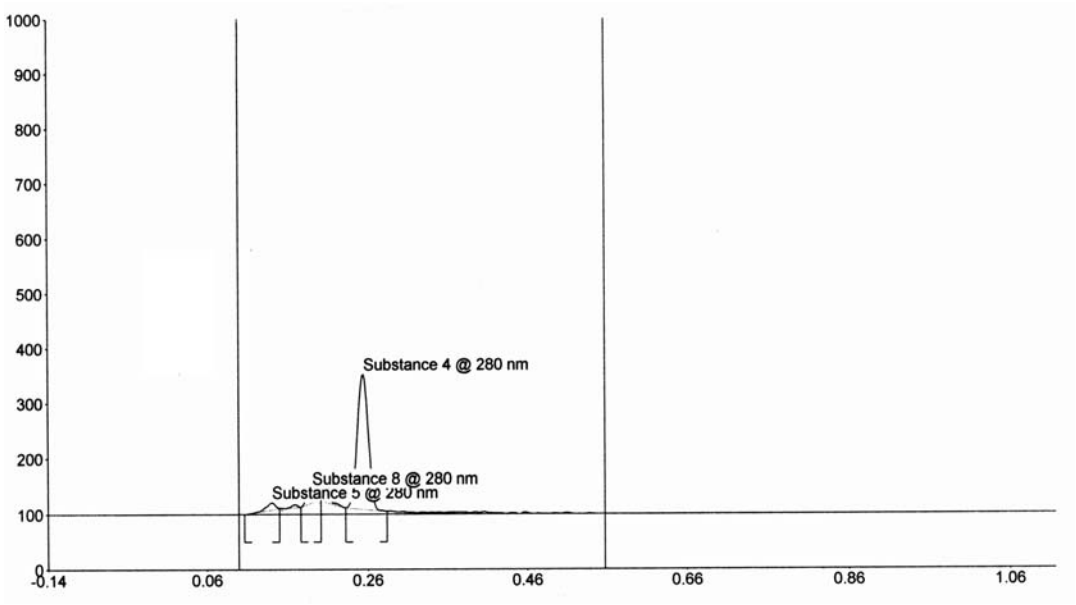

Figure No .2.c : Alkali degradation of Clozapine

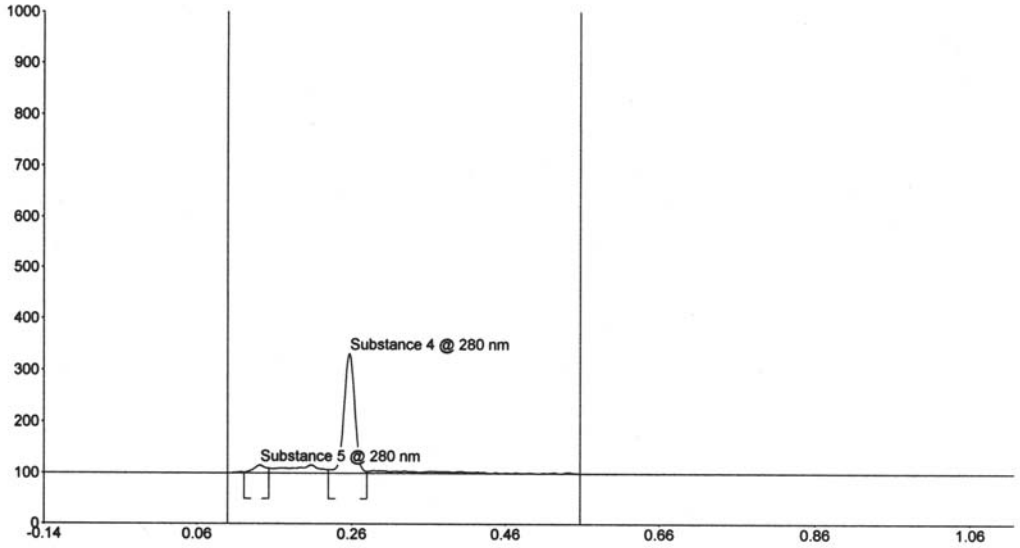

Figure No.2.d : Peroxide degradation of Clozapine

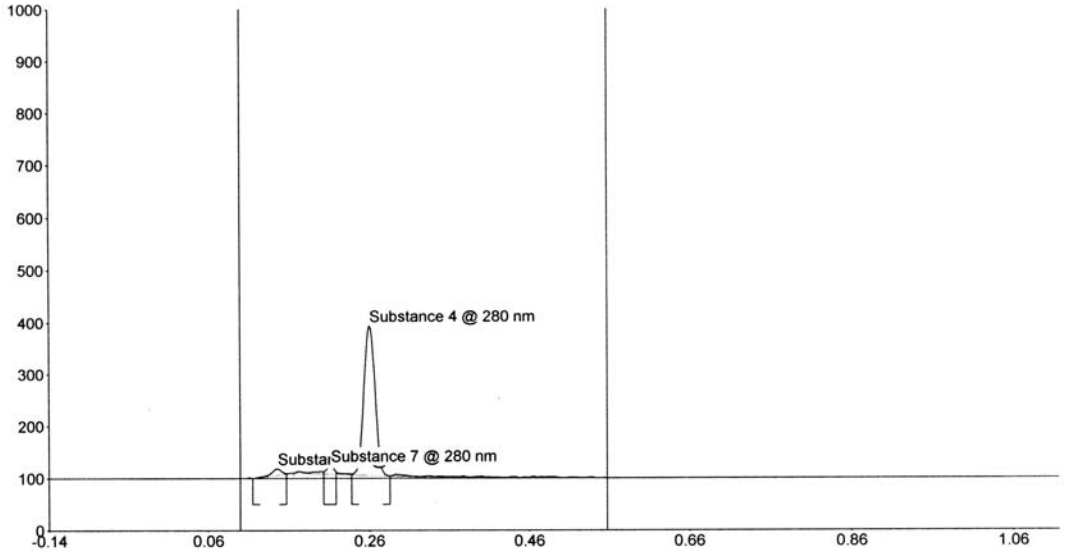

Figure No.2.e: UV degradation of Clozapine

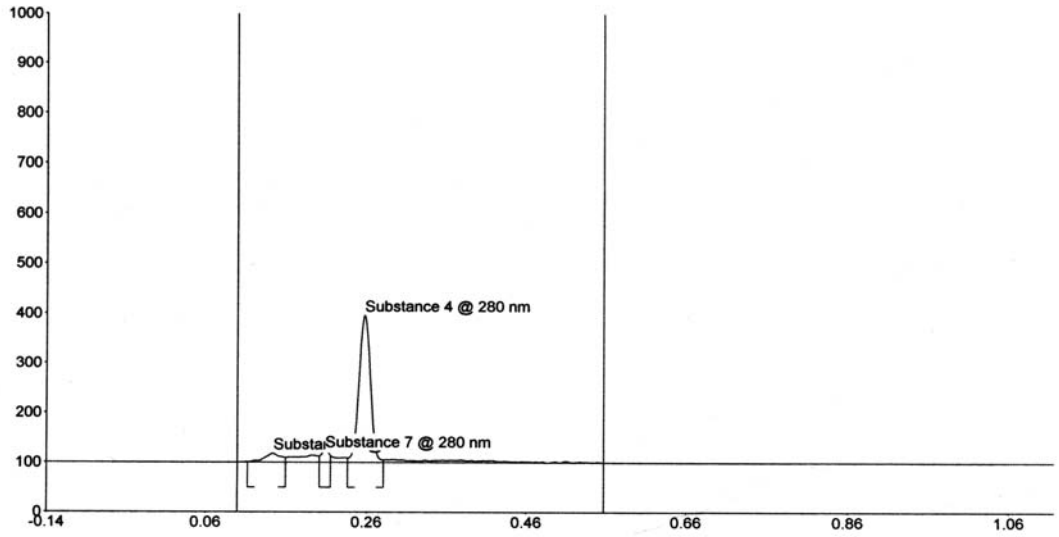

Figure No.2.f: Thermal degradation of Clozapine

additional peak were observed, the response of the drug was changing from the initial control sample. This indicates that the drug is susceptible to acid-base hydrolysis degradation, photochemical degradation and thermal degradation. The lower R_f values of the degraded components indicated that they were less polar whereas higher R_f values of the degraded components indicated they were more polar than the analyte itself.

3.6.*Detection of the related impurities*

The sample solution shows no additional spots other then principle spot. Hence no related impurities are present in the market sample.

*4.*Conclusion:

The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is reproducible and selective for the analysis of Clozapine in pharmaceutical dosage form. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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