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Research Article

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The Study of Impact of Forced Degradation on the Assay of Methylene Blue by RP-HPLC

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Abstract The main objective of this study is to study the impact of forced degradation on the assay of Methylene Blue by RP-HPLC. A simple, specific, accurate and precise RP-HPLC method has been validated as per ICH guideline for determination of assay of Methylene Blue. Validation parameters like Linearity, Accuracy, Precision, System suitability, Specificity were tested. The Methylene Blue was dissolved in HPLC water at the concentration of 1 mg/mL and appropriate chromatographic condition was kept as per the monograph of Methylene Blue in USP. No interference was observed in the parameter specificity of any blank or other impurities. The Recovery of the Spiked Sample in parameter accuracy was within the acceptance limit of 97 to 103 % at three different concentrations 80 %, 100 % and 120 %. The Method Precision and Intermediate Precision of this method was achieved within the limit of RSD 2 %. The Linearity of the method was achieved through the correlation coefficient of 0.99983.Forced Degradation of Methylene Blue was performed through Acid, Base, Oxidation, and Thermal Degradation to get the % degradation data of methylene blue. The study was performed for 48 hr in which the % degradation was within the acceptance limit of 20 % except Thermal Degradation. This study can be applicable in the profiling of Methylene Blue and Sterile Preparation of Methylene Blue Injection. This study can be used for the Pharmaceutical Industries for the referral of forced degradation studies.

Keywords Methylene Blue, RP-HPLC, Validation, ICH, Forced Degradation

Introduction

Forced degradation studies were used to find reactions which may occur to damage a product. Usually conducted before final formulation, degradation uses external stresses to rapidly screen material stabilities. Longer term storage tests are usually used to measure similar properties when formulations were involved because of strict FDA regulations. The forced degradation study is a vital analytical aspect of drug development for molecules. Forced degradation is carried out to establish as specificity to developed a stability-indicating analytical method, using high-performance liquid chromatography (HPLC). As per International Conference on Harmonization (ICH) guidelines (Q1A), stability studies need to be performed to propose shelf life of new drug substances. Stability studies were part of various regulatory submissions to FDA [1-4].

Methylene Blue is a synthetic basic dye. Methylene blue stains to negatively charged cell components like nucleic acids; when administered in the lymphatic bed of a tumor during oncologic surgery [5].

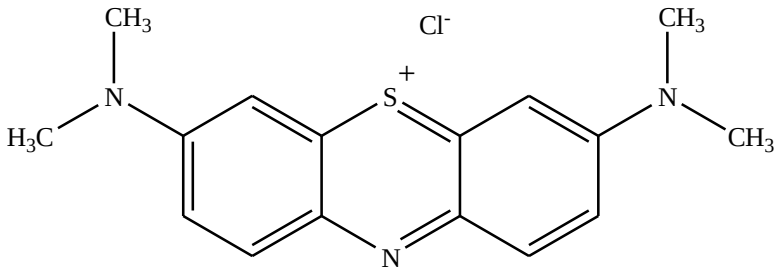

Figure 1: Chemical Structure of Methylene Blue

Methylene Blue is a synthetic basic dye and an oxidation-reduction agent. The intravenous form of methylene blue is approved by the FDA for the treatment of pediatric and adult patients with acquired methemoglobinemia. It is Soluble in Water and in Chloroform and sparingly soluble in alcohol. The Uses of Methylene Blue are in the treatment of Hepato-pulmonary Syndrome, Anti-Malarial, Methemoglobinemia, Anti-cancer, Neuro-toxicity and Alzheimer's Disease [5].

Methods

Materials

All chemicals and reagents used in method were of HPLC and analytical grade. Methylene Blue Working Standard was procured from Macsen Drugs, Udaipur. Methylene Blue API were kindly provided by Macsen Drugs, Udaipur.

Instrumentation and Chromatographic conditions

HPLC chromatographic was performed on Agilent Technologies Liquid Chromatographic system 1260 Infinity II, ultraviolet (UV) detector, and a fixed injector equipped 20 µl loop was used for the chromatographic separation. Chromatographic separation was carried out at a flow rate of 1ml/min using L11 (100 mm x 4.6 mm) Kromasil column. The wavelength was kept at 246nm. The mobile phase consists of Buffer (Troflluoroacetic acid (0.1 %)): Acetonitrile in gradient form mentioned below [6]:

Table 1: Mobile Phase Composition

Time (min.)	Solution A (%)	Solution B (%)
0	80	20
5	80	20
25	30	70
32	30	70
33	80	20
38	80	20

The diluent was prepared by Mobile Phase A: Mobile Phase B (70:30)

Preparation of Standard and Sample solution of Methylene Blue

The Standard and Sample solution was prepared at concentration of 1 mg/mL of Methylene Blue/Methylthioninium Chloride Working Standard in Diluent.

Forced Degradation Studies

Acid degradation- Acid decomposition studies were performed by refluxing 1ml of stock solution was transferred in to 10ml of volumetric flask. 2ml of 0.1N HCl solutions was added and mixed well and put for 6 hrs at 70ºC 250ml round bottom flask. After time period the content was cooled at RT. Then the volume was adjusted with diluents to get 20µg/ml for Methylene Blue [1].

Base degradation- Base decomposition studies were performed by refluxing 1ml of stock solution was transferred into 10ml of volumetric flask. 2 ml of 0.1N NaOH solutions was added and mixed well and put for 4 hrs at 70ºC

250ml round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluents to get 20µg/ml for Methylene Blue [3].

Oxidation degradation- Oxidative decomposition studies were performed by refluxing 1ml of stock solution was transferred in to 10ml of volumetric flask. 2ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs at 70ºC in 250ml round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluent to get 20µg/ml for Methylene Blue [2].

Thermal degradation- 1ml of stock solution was transferred in to 10ml of volumetric flask. This solution was put in oven 100ºC for 8 hrs. Then the volume was adjusted with diluent to get 20µg/ml for Methylene Blue [4].

Result and Discussion

HPLC method validation

The optimized chromatographic method was validated by evaluating specificity, linearity, precision, accuracy and robustness. The validation of the method was performed as per ICH Guidelines [7-8].

Specificity

The Chromatograms of Methylene Blue standards and Methylene Bluesample show no interference with the Chromatogram of Methylene BlueBlank, so the method is found to be Specific.

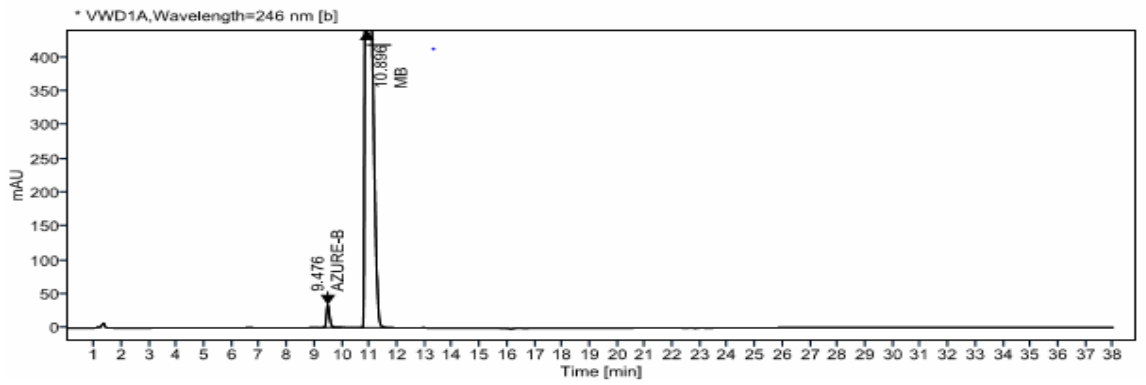

Figure 2: Chromatogram of Methylene Blue standard

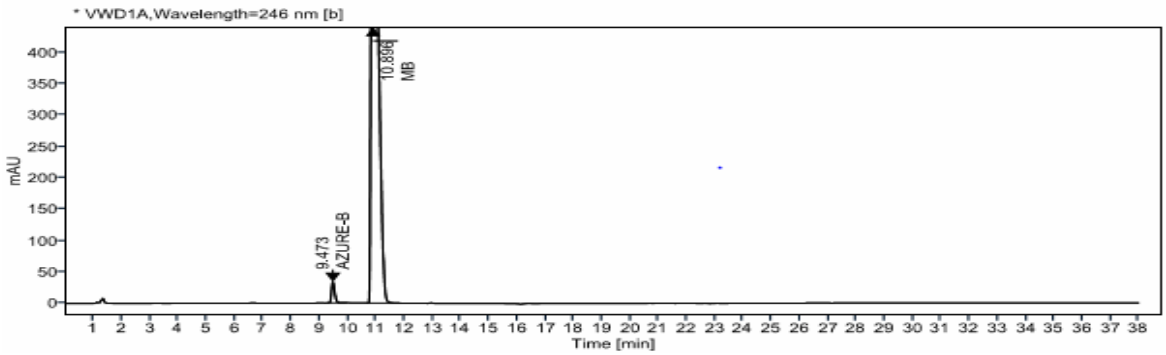

Figure 3: Chromatogram of Methylene Blue sample

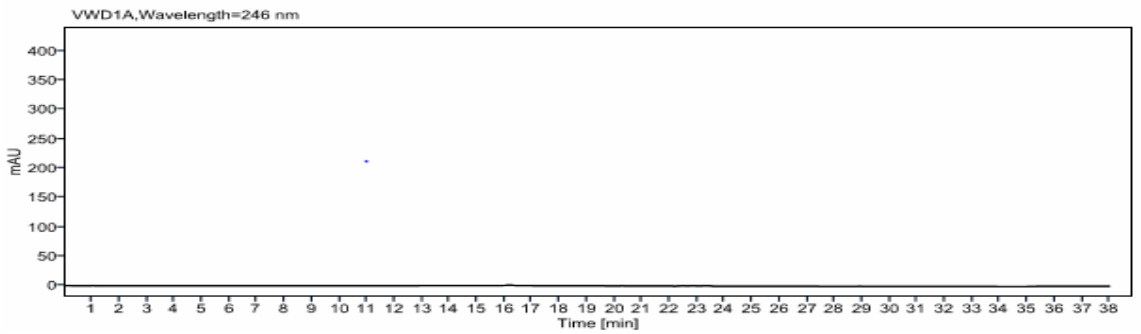

Figure 4: Chromatogram of Methylene Blue Blank

Linearity

The linearity for Methylene Blue were assessed by analysis of combined standard solution in range of 0.5-1.5mg/ml. The concentration of linearity was 0.5, 0.7, 1.0, 1.3 and 1.5 mg/ml for Methylene Blue. Correlation co-efficient of Methylene Bluewas found to be 0.99983respectively.

Table 2: Linearity data for Methylene Blue

Linearity Data Sheet				
Concentration (mg/mL)	**Concentration as % of analyte target**	**Peak Area (mean of the Injection)**	**Peak Area SD**	**Peak Area RSD (%)**
0.5	**50%**	7134.9971	10.020	**0.14**
0.7	**70%**	9967.3863	5.7586	**0.06**
1.0	**100%**	14413.1153	3.0699	**0.02**
1.3	**130%**	18981.5227	5.3396	**0.03**
1.5	**150%**	21704.1785	15.6662	**0.07**

Correlation Coefficient (r2) = 0.99983

Acceptance Criteria:Correlation Coefficient (r2) should be greater than 0.999

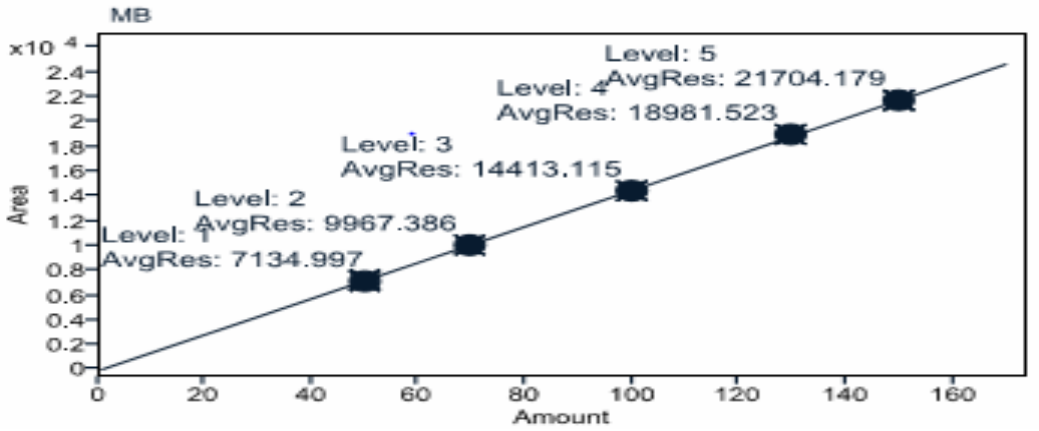

Figure 5: Calibration Curve of Methylene Blue

Precision

Method Precision

The data for repeatability of peak area measurement for Methylene Blue (1mg/ml) based on six measurements of same solution. The % RSD for Methylene Blue was found to be 0.4737respectively.

Table 3: Method Precision data for Methylene Blue

Sr. No.	Conc. (mg/ml)	Area	Mean ± S.D (n=6)	% R.S.D
1.	1	13954.0241 13965.3554 13968.0321 13991.3716 14038.7020 14128.2671	14007.6254	**0.4737**

Intermediate Precision

Standard solution of Methyleneblue was analyzed through variation in different instruments, days, analyst and column. The % R.S.D was achieved to be 0.19 % and 0.06 %.

Table 4: Intermediate precision of Methylene Blue

Sample Details	**Instrument I**	**Instrument II**
	Operator Name:	**Operator Name:**
	Operator I	Operator II
19-20/MB/002	Assay on Instrument-I	Assay on Instrument-II
Mean	**98.31**	**99.03**
SD	**0.1835**	**0.0568**
%RSD Instrument I and II	**0.19**	**0.06**
Acceptance Criteria:%Assay = RSD NMT 2.0%		

Accuracy

The Accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Recovery at each level, mean recovery and overall mean recovery should be 97.0% to 103.0%. Mean recovery and overall mean recovery should be between 98.0% and 102.0%. The recovery was found at 80 % was 98.44 %, 100 % was 98.04 and 120 % was 98.97 %.

Table 5: Recovery data for Methylene Blue

S. No.	Conc. Level (%)	Sample amount (mg/ml)	Amount recovered (mg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	0.8	0.8037	99.25	**98.44**
2		0.8	0.8022	98.57	
3		0.8	0.7996	97.52	
4	100 %	1	0.9924	98.28	**98.04**
5		1	0.9930	97.56	
6		1	0.9925	98.29	
7	120 %	1.2	1.2005	98.75	**98.975**
8		1.2	1.2224	100.05	
9		1.2	1.1198	98.125	

Forced Degradation Studies of Methylene Blue

Acid degradation- Acid decomposition studies were performed by refluxing 1ml of stock solution was transferred in to 10ml of volumetric flask. 2 mL of 0.1N HCl solutions was added and mixed well and put for 6hrs at 70ºC

250ml round bottom flask. After time period the content was cooled at RT. Then the volume was adjusted with diluents to get 1 mg/mL for Methylene Blue.

Table 6: Acid Degradation Data ofMethylene Blue

Time Frame	Assay of Sample (Methylene Blue)	%Degradation (Methylene Blue)
Initial (0 hr)	99.65 %	0 %
3 hrs	98.52 %	1.48 %
6 hrs	98.11 %	1.89 %
12 hrs	96.21 %	3.80 %
24 hrs	93.87 %	6.15 %
48 hrs	82.42 %	17.64 %

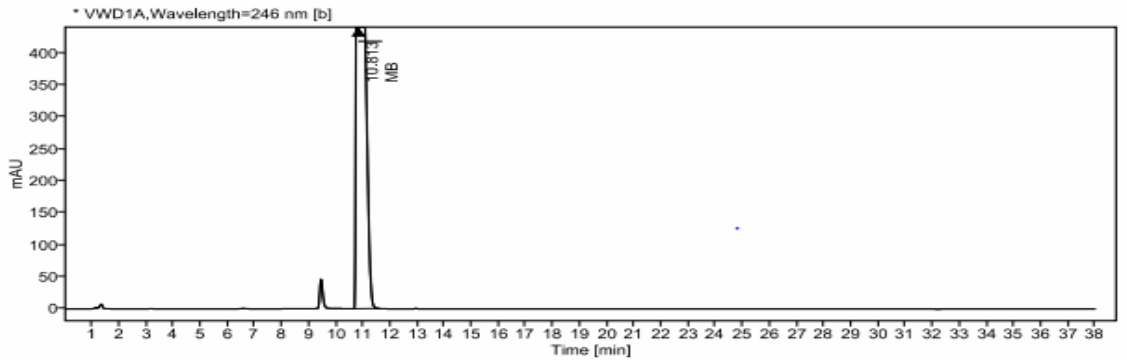

Figure 6: Chromatogram of Acid Degradation of Methylene Blue @ 24 hr

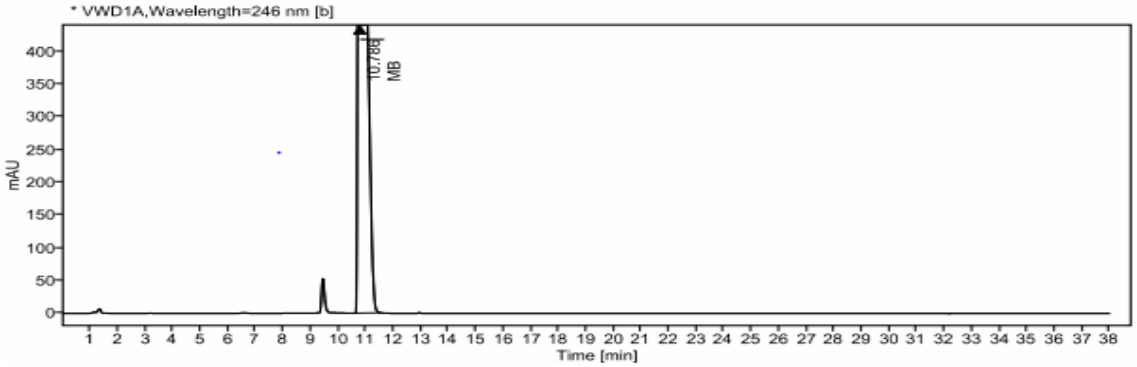

Figure 7: Chromatogram of Acid Degradation of Methylene Blue @ 48 hr

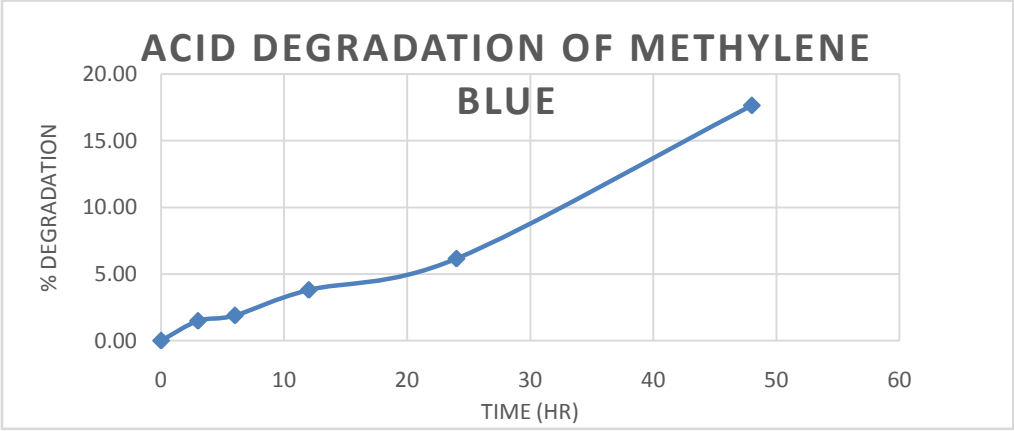

Figure 8: Schematic Curve of Acid Degradation of Methylene Blue

Base degradation-Base decomposition studies were performed by refluxing 1ml of stock solution was transferred into 10ml of volumetric flask. 2 ml of 0.1N NaOH solutions was added and mixed well and put for 4 hrs at 70ºC 250ml round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluents to get 1 mg/ml for Methylene Blue.

Table 7: Base Degradation Data of Methylene Blue

Time Frame	Assay of Sample	%Degradation
Initial (0 hr)	99.65 %	0 %
3 hrs	98.84 %	1.16 %
6 hrs	98.49 %	1.51 %
12 hrs	96.96 %	3.05 %
24 hrs	94.53 %	5.48 %
48 hrs	85.65 %	15.64 %

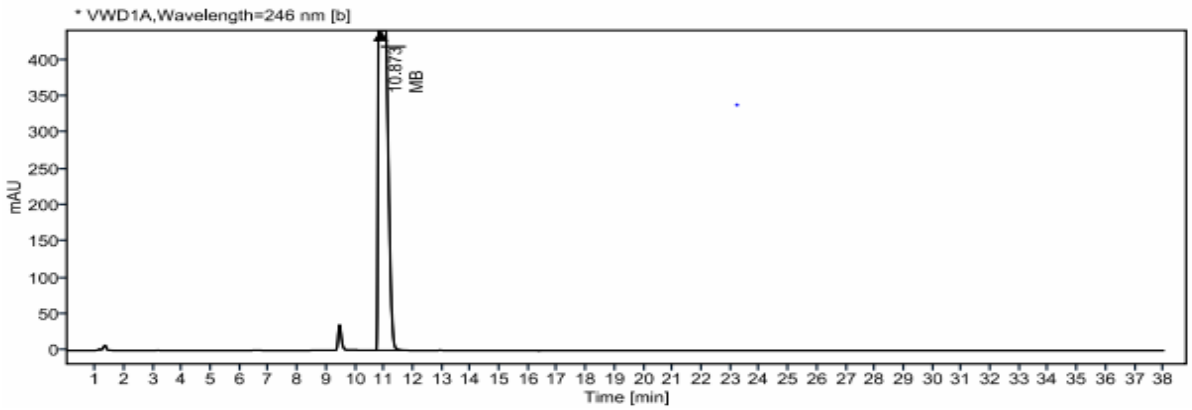

Figure 9: Chromatogram of Base Degradation of Methylene Blue @ 24 hr

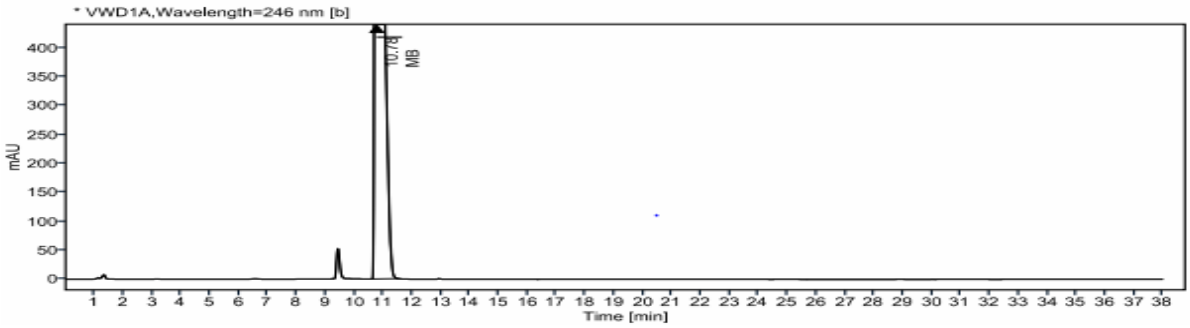

Figure 10: Chromatogram of Base Degradation of Methylene Blue @ 48 hr

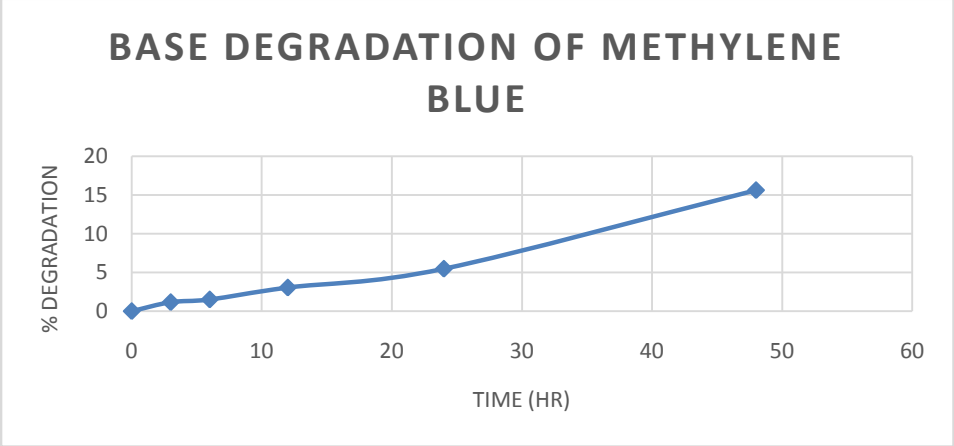

Figure 11: Schematic Curve of Base Degradation of Methylene Blue

Oxidation degradation- Oxidative decomposition studies were performed by refluxing 1ml of stock solution was transferred in to 10ml of volumetric flask. 2ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs at 70ºC 250ml round bottom flask. After time period the content was cooled to RT. Then the volume was adjusted with diluent to get 1 mg/ml for Methylene Blue.

Table 8: Oxidative Degradation Data of Methylene Blue

Time Frame	Assay of Sample	%Degradation
Initial (0 hr)	99.65 %	0 %
3 hrs	98.39 %	1.61 %
6 hrs	97.23 %	2.77 %
12 hrs	96.25 %	3.76 %
24 hrs	95.53 %	4.48 %
48 hrs	84.19 %	16.34 %

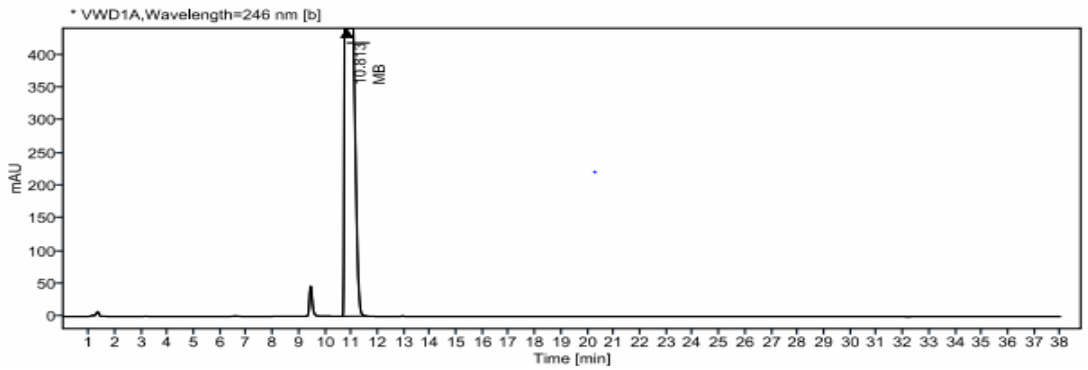

Figure 12: Chromatogram of Oxidation Degradation of Methylene Blue @ 24 hr

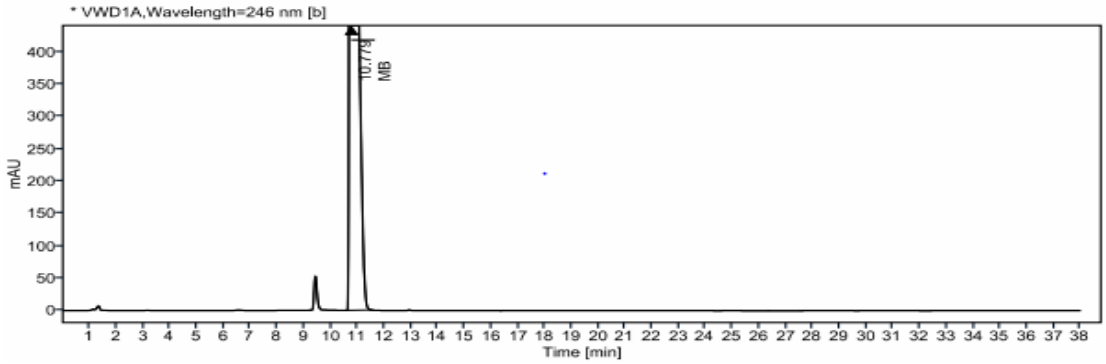

Figure 13: Chromatogram of Oxidation Degradation of Methylene Blue @ 48 hr

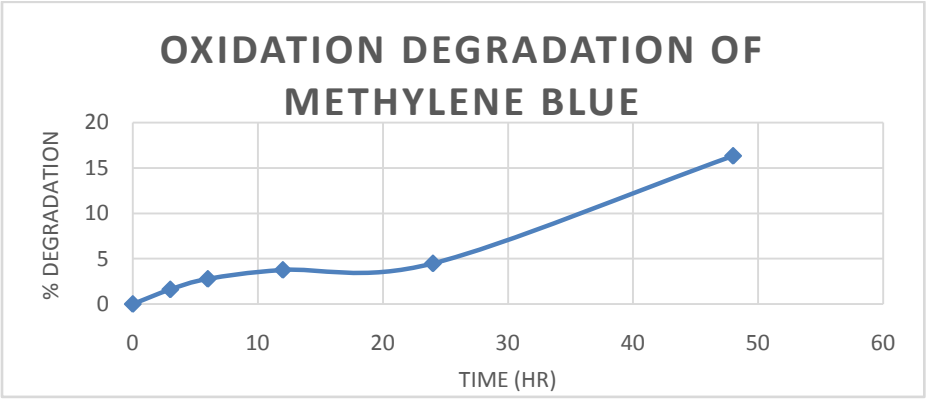

Figure 14: Schematic Curve of Oxidation Degradation of Methylene Blue

Thermal degradation- 1ml of stock solution was transferred in to 10ml of volumetric flask. This solution was put in oven 100ºC for 8 hrs. Then the volume was adjusted with diluent to get 1 mg/ml for Methylene Blue [6].

Table 9: Thermal Degradation Data of Methylene Blue

Time Frame	Assay of Sample	%Degradation
Initial (0 hr)	99.65 %	0 %
3 hrs	98.48 %	1.52 %
6 hrs	97.96 %	2.04 %
12 hrs	96.59 %	3.42 %
24 hrs	91.95 %	8.06 %
48 hrs	79.57 %	20.50 %

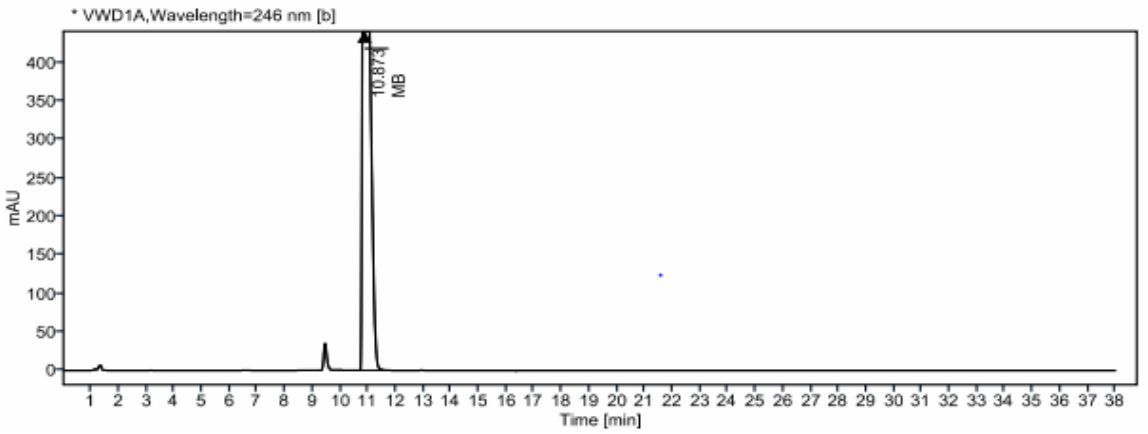

Figure 15: Chromatogram of Thermal Degradation of Methylene Blue @ 24 hr

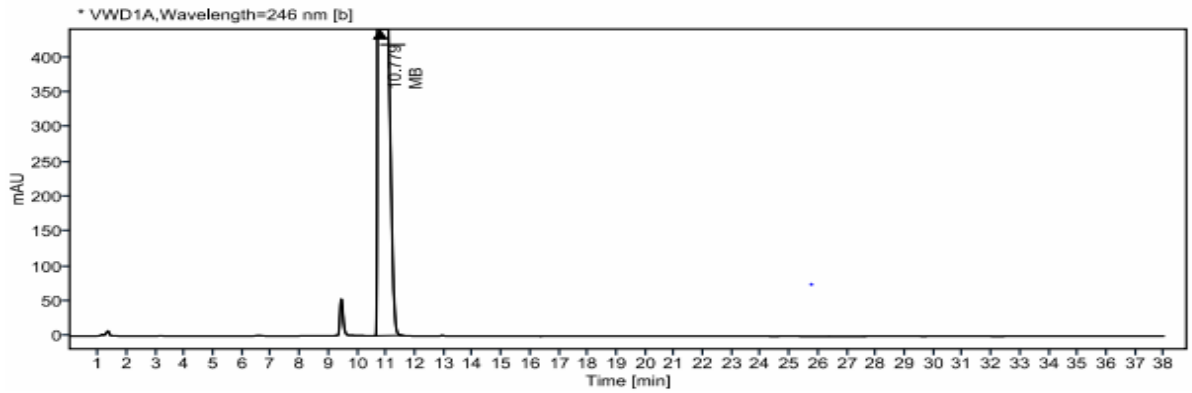

Figure 16: Chromatogram of Thermal Degradation of Methylene Blue @ 48 hr

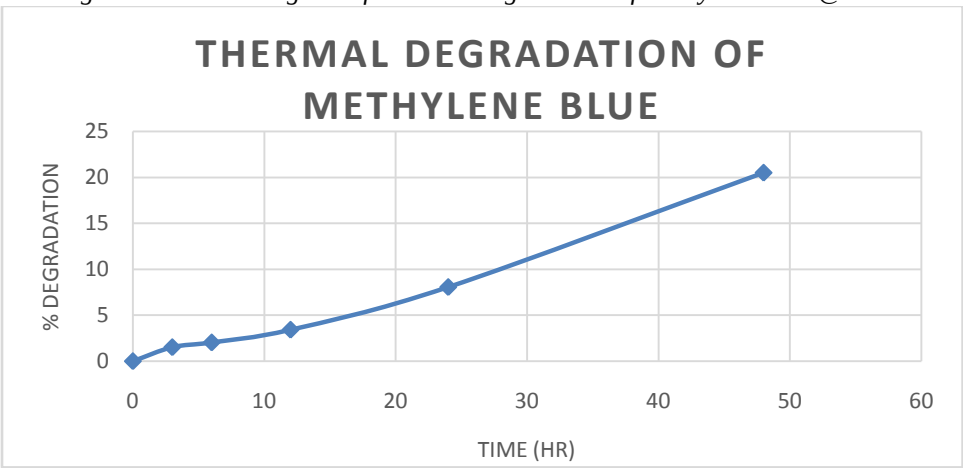

Figure 17: Schematic Curve of Thermal Degradation of Methylene Blue

Conclusion

The Method for determination of assay of Methylene Blue by RP-HPLC was validated as per ICH guidelines. Validation parameters like Linearity, Accuracy, Precision, System suitability, Specificity were tested.Forced Degradation of Methylene Blue was performed through Acid, Base, Oxidation, and Thermal Degradation to get the % degradation data of methylene blue. The study was performed for 48 hr in which the % degradation was within the acceptance limit of 20 % except Thermal Degradation. This study can be applicable in the profiling of Methylene Blue and Sterile Preparation of Methylene Blue Injection. This study can be used for the Pharmaceutical Industries for the referral of forced degradation studies.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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