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Development and validation of a stability-indicating assay method for determination of metronidazole benzoate in bulk: Spectroscopic approach

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Abstract

A new, simple, rapid, accurate, sensitive and cost effective spectrophotometric stability indicating assay method was developed for the quantification of metronidazole benzoate in bulk and validated according to ICH guidelines using different parameters. In the present study an attempted has been made to develop an effective method which will surpass the disadvantages associate with other reported methods like tedious in use, less sensitive and costly etc. The assay method was based on the forced degradation study of metronidazole benzoate under different ICH recommended stress conditions like hydrolytic, oxidation, thermal, and photolysis. Drug shown maximum absorption at 322nm and a regression coefficient value (R^2) of 0.999 indicates linearity followed within the concentration range of 5µg/ml to 50µg/ml. During forced degradation study, metronidazole was significantly degraded in alkaline conditions whereas mild degradation occurs in acidic, neutral and oxidative stress. The drug was stable to dry heat and photolytic degradation conditions. Statistical validation result of the developed method indicates that it was a simple, precise, reproducible, selective, specific and accurate method for analysis of Metronidazole and could be successfully adopted to estimate the drug in bulk and formulations.

Keywords: Metronidazole; Stability indicating assay method; UV spectroscopy; Validation; Forced degradation study

1. Introduction

Metronidazole (MET) chemically known as 2-(2-methyl-5-nitro-1H-imidazole-1-yl) ethanol having molecular formula and molecular mass $C_6H_9N_3O_3$ and 171.15 g/mol respectively (Fig.1)[1]. Metronidazole has been used as an antibiotic for several decades, with added antiparasitic properties allowing it to treat a wide variety of infections caused by anaerobic bacteria and protozoa. It is frequently used in the treatment of parasitic infections like gastrointestinal infections, trichomoniasis, giardiasis, amoebiasis and vaginosis[2,3]. It belongs to nitroimidazole class of antibiotics works by binds deoxyribonucleic acid and electron-transport proteins of organisms, blocking nucleic acid synthesis. After administration, metronidazole enters cells by passive diffusion, following this, ferredoxin or flavodoxin reduce its nitro group to nitro radicals[4,5]. The redox potential of the electron transport portions of anaerobic or microaerophilic micro-organisms render metronidazole selective to these organisms, which cause nitro group reduction, leading to the production of toxic metabolites. These include N-(2-hydroxyethyl) oxamic acid and acetamide, which may damage DNA of organisms[6-8].

Recently there is an increased need for development of a stability-indicating assay method (SIAM), using the conditions mentioned in the ICH guidelines[8]. A stability method is a validated analytical technique used to measure the concentration of active constituents accurately in the presence of its degradation products, excipients, and other impurities. The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drug substances and products" requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance by the influence of temperature, humidity, light, oxidizing agent as well as susceptibility over a

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wide range of pH values[9,10]. Generally, chromatographic methods were established to monitor the changes in concentration of drug at different stressed conditions[11,12]. Spectrophotometric stability method may have advantages over chromatographic techniques in terms of simplicity, economic and time consumption. In degradation study, the intensity of stress parameters depends on physicochemical properties of drug substance and nature of drug or product[13,14]. In the Pharmaceutical industry time and expanses plays an important role hence simple, cost effective and more sensitive methods are highly acceptable for routine usage of study. The present study was aimed to develop and validate a UV- spectroscopic stability indicating assay method for metronidazole in distilled water at different stressed conditions[15]. The objective of the study is developing a suitable, efficient and economic analytical method to estimate drug in presence of its degradation product and impurities.

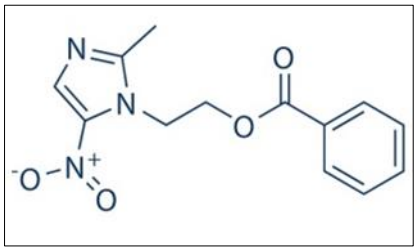

Figure 1 Chemical structure of Metronidazole Benzoate

2. Material and methods

2.1. Chemicals and Reagents

Pharmaceutical-grade API of metronidazole was procured as a gift sample from Abicee Pharmaceutical Pvt. Ltd, Bhubaneswar, Odisha, India and was used without further purification. Distilled water used as solvent during the study was prepared in the chemistry lab CPS, Puri and all other chemicals and reagents used were of analytical grade.

2.2. Instruments

For developing the stability-indicating assay method of MET, a PC controlled double beam UV- spectrophotometer (Systonic) having spectral bandwidth 3nm and of wavelength accuracy ±1nm with 1 cm quartz cell was used for spectral estimation and absorbance measurements of sample solutions. The work was carried out in an air-conditioned room maintained at a temperature 25 ± 2 °C. A precision mantel heater (Biotech, Mumbai) with temperature regulator equipped with a reflux condenser was used for degradation study in acid, alkali and neutral conditions. A dry-air oven was used to study the effect of dry heat. The photolytic study was carried out by exposing the drug to direct sunlight.

2.3. Preparation of standard stock solution and estimation of λ_{max}

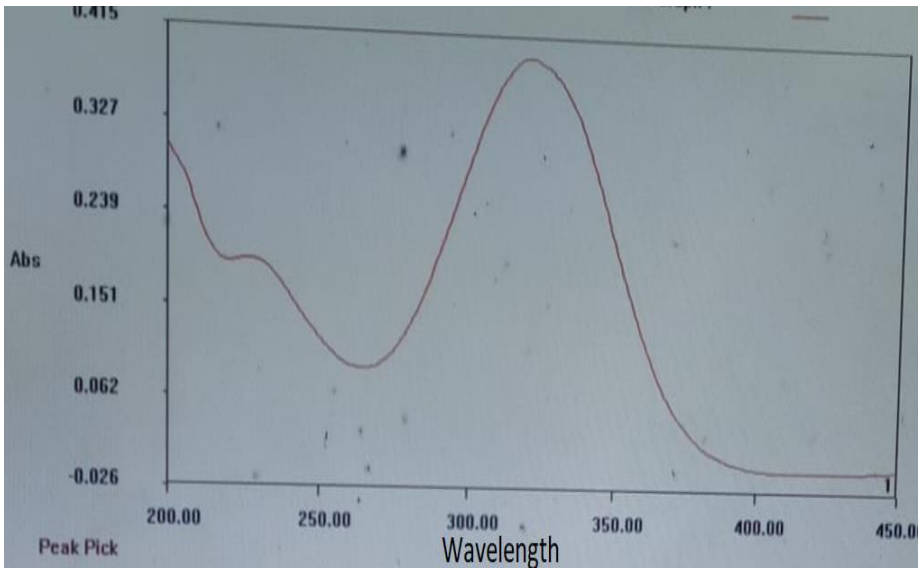

Figure 2 λmax of MET at 322nm

On the basis of solubility of MET, distilled water was selected as solvent to carry out the whole study. A standard stock solution of 1000 µg/ml of MET was prepared by dissolving 100 mg of pure drug in a 100ml volumetric flask by adjusting the final volume with distilled water. Working standard solution of 100 µg/ml of MET was further prepared by diluting 10ml of the above solution to 100ml with distilled water. Then further dilutions of 5- 50 µg/ml were made by diluting the required aliquot of 100 µg/ml solution with distilled water. The solution with a concentration of 30 µg/ml was scanned over the range of 200-450nm against blank solvent. The drug spectrum was showing maximum absorbance (λ_{max}) at 322nm which is considered as the analytical wavelength for further study (Fig. 02).

2.4. Validation of Method[16,17]

2.4.1. Linearity

Linearity study of the drug was performed for the dilutions of concentration 5-50 µg/ml which were scanned at 322nm and absorbance were recorded. The calibration curve was plotted between absorbance and concentration of standard solutions and the regression equation was determined. The experiment was carried out in five replicates.

2.4.2. Accuracy

The accuracy of the developed method was confirmed by performing recovery study. The recovery study was carried out by standard addition method in which pre-analyzed sample of 20 µg/ml were spiking with extra standard drug of MET in 80,100 and 120% and absorbance recorded at 322nm. The percent of recovery of the added drug was calculated. The study is carried out in triplicate.

2.4.3. Precision

The precision of the method was established by repeatability and intermediate precision. Three solutions of concentration 10, 20 and 30 µg/ml of MET were prepared to perform the precision study.

Repeatability

Repeatability (intra-day) was assessed by analysing the working solution of MET, analysed in triplicates for three different concentrations (10, 20 and 30 µg/ml) of three times a day at an interval of 1hr. The % relative standard deviation (RSD) was calculated for absorbance obtained for these solutions.

Intermediate precision

Intermediate precision or inter-day precision was established by analysing the working solution of three different concentrations (10, 20 and 30 µg/ml) of MET for three different days. The results was reported in terms of % RSD.

2.4.4. Detection and Quantitation limit

As per ICH guideline three approaches are to determine the detection limit (DL) and quantitation limit (QL), these includes visual evaluation, signal to noise ratio and the use of standard deviation of the response and the slope of the calibration curve. DL is the lowest amount of analyte in a sample, which can be detected and QL is the lowest amount of analyte which can be quantitatively determined by the developed method. The LOD and LOQ were separately determined based on the third approach and calculated using eq. 1 and eq.2, where, σ is the standard deviation of the intercept of the calibration plot and S is the slope of the calibration curve.

$$LOD = 3.3\ \sigma / S \quad \text{Eqn. 01}$$

$$LOQ = 10\ \sigma / S \quad \text{Eqn. 02}$$

2.5. Forced Degradation Studies[18-20]

As per the International Conference on Harmonization (ICH) guideline, stability testing of a drug substances and products requires that stress testing to be performed for revealing of the inherent stability characteristics[20]. In order to check the stability of MET in different ICH prescribed forced degradable conditions like hydrolytic (acid, base and neutral), thermal, photolytic and oxidative, drug at a concentration of 1 mg ml^{-1} was used for all degradation studies.

2.5.1. Acidic degradation study

For acidic degradation study, 10 ml of 1mg ml^{-1} standard stock solution of the MET was added to 10 ml of 1.0N HCl and refluxed for 06hrs at 60 ^{0}C. From this 0.4ml of aliquot was taken at regular interval of time in separate 10ml volumetric

flask and adjusted the volume with distilled water after neutralization with 1.0N NaOH to form a solution having concentration 20 µg/ml and absorbance was recorded at 322nm against blank solvent. Finally the absorbance of the sample were compared with the absorbance of standard solution and percentage of degradation was calculated.

2.5.2. Alkaline degradation Study

10 ml of 1mg ml^{-1} standard stock solution of the MET was added to equal volume of 1.0N NaOH and refluxed for 06hrs at 60 ^{0}C. From this 0.4ml of aliquot was taken at regular interval of time in a 10ml volumetric flask and adjusted the volume by distilled water after neutralization with 1.0N HCl to form a solution containing 20 µg/ml and absorbance was recorded at 322nm against blank solvent. The percentage of degradation was calculated by comparing the absorbance of the sample with the absorbance of standard solution.

2.5.3. Neutral Hydrolysis

In separate volumetric flask 20 ml of 1 mg ml^{-1} standard stock solution of the MET was refluxed for 06hrs at 60 ^{0}C. From this 0.2ml of aliquot was taken regularly in separate 10ml volumetric flask which was then adjusted the volume with distilled water to form a solution containing 20µg/ml and absorbance was recorded at 322nm. Absorbance of sample and standard solutions were compared to determine the percentage of decomposition.

2.5.4. Oxidation induced degradation

To perform oxidative degradation study 10 ml of 1mg ml^{-1} standard stock solution of the MET is mixed with 10 ml of 3% H_2O_2. The solution was kept at room temperature for a period for 24hrs. A volume of 0.4 ml of aliquot was taken after 24hrs in a separate 10ml volumetric flask and adjusted the volume with distilled water to form a solution of 20 µg/ml and at 322nm absorbance was recorded and percentage of degradation was calculated.

2.5.5. Thermal Degradation

A sample power of accurately weighed 100 mg of MET was exposed to 80 °C for 48hrs in a controlled-temperature hot air oven. Then required amount was dissolved distilled water and a solution of 20 µg/ml was prepared and the absorbance was recorded at 322nm and degradation percent was calculated.

2.5.6. Photo degradation

The pure drug sample accurately weighed 100 mg was exposed to sun light for 6hrs. Further suitably diluted to 20 µg/ml by dissolving required amount of drug in distilled water and absorbance was recorded. Finally percent of degradation was estimated by comparing absorbance of sample and standard solutions.

3. Results and discussion

3.1. UV Spectroscopic method development

In the present study, a stability-indicating assay method was developed and validated as per ICH guidelines for study of degradation behaviour of MET in bulk and marketed formulations using distilled water as solvent. The wavelength 322nm was found as λ_{max} and taken as the analytical wavelength for estimation of the drug. This developed method is found to be simple and economical as compared to the previously reported HPLC methods as it required less sample preparation and solvent consumption.

3.2. Validation of the developed method

3.2.1. Linearity and range

Linearity was evaluated by analysis of working standard solutions of MET for concentration 5-50 µg/ml. The absorbance of these solutions was recorded at 322 nm and a plot was made between the absorbance and concentration of standard solutions as shown in Figure 3. The results of the regression analysis are summarized in (Table 1). The result of the study showed that the drug obeyed Beer-Lambert's law in the above concentration range with a correlation coefficient of 0.999 which indicates that, the method is capable enough with good sensitivity.

The mean absorbance was found in between 0.036-0.957.

Table 1 Regression analysis of MET for developed method

S.NO	Parameters	Results
1	Absorption maxima λ_{max}	322 nm
2	Beer's-Lambert's range	5-50 µg/ml
3	Regression equation	y = 0.057x +0.0101
4	Correlation coefficient	0.999
5	Slope	0.057
6	Intercept	0.0101

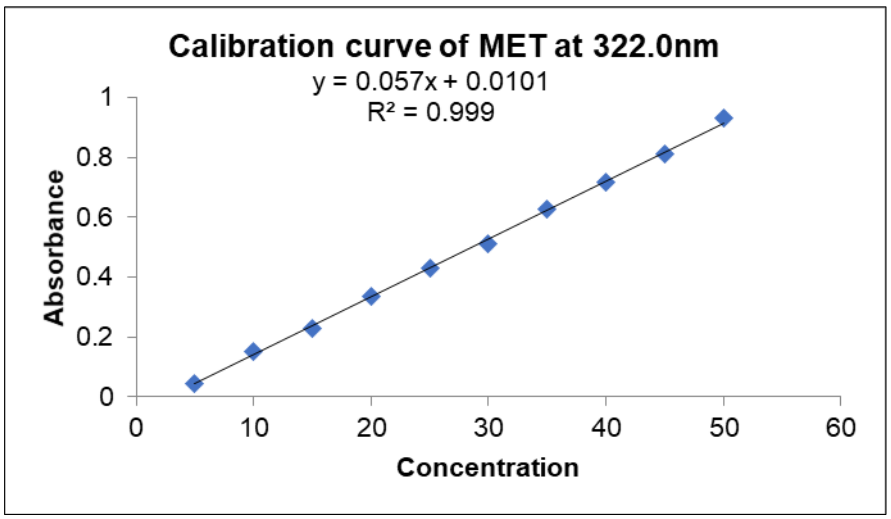

Figure 3 Linearity of MET at 322 nm

3.2.2. Accuracy Study

Accuracy study was performed to check the sensitivity of the developed method for the estimation of MET. The recovery study was investigated by analysing three concentrations of standard drug solution previously analysed using the standard addition technique. The standard addition technique was carried out by adding 80,100 and 120 % of the MET concentration in the sample. The % recoveries of the three concentrations were found to be 100.24 -100.88 %. The results of % recovery and % RSD are given in Table 2. The accuracy study results indicates that, the recoveries were well within the limits and the developed method is capable enough of showing good accuracy with reproducibility for analysis of MET.

Table 2 Result of Recovery Study

Initial Concentration (µg/ml)	Level of spiking %	Concentration of spiked sample (µg/ml)	Absorbance ± S.D	% Recovery (n=3)	% RSD (n=3)
20	80	16	0.694 ± 0.254	100.24	0.174
20	100	20	0.819 ± 0.145	100.57	0.112
20	120	24	0.947 ± 0.351	100.88	0.092

3.2.3. Precision Study

The results of the repeatability and intermediate precision study are given in Table 3. The developed method was found to be precise as the % RSD values for repeatability and intermediate precision studies were < 2.0 indicating the proposed method is repeatable when executed in different day also.

Table 3 Result of Precision Study

Repeatability Study			**Intermediate Precision study**		
Sample concentration (µg/ml)	**Mean Absorbance ± S.D**	**% RSD**	**Sample concentration (µg/ml)**	**Mean Absorbance ± S.D**	**% RSD**
10	0.126 ± 0.012	0.521	10	0.158 ± 0.063	0.089
20	0.281 ± 0.054	0.341	20	0.265 ± 0.154	0.184
30	0.577 ± 0.023	0.254	30	0.527± 0.897	0.279

3.2.4. Detection and Quantitation limits

The detection limit (DL) and quantitation limit (QL) were determined as per the ICH guidelines and were found to be 0.62 and 1.85 µg/ml for MET respectively (Table. 4).

Table 4 Result of LOD and LOQ

Ingredients	**LOD (µg/ml)**	**LOQ (µg/ml)**
MET	0.62	1.85

3.3. Result of forced Degradation Study

Table 5 Degradation studies of MET at different induced forced stress conditions at 322nm

S. No	**ICH prescribed different stressed conditions**	**Concentration (µg/ml)**	**Absorbance**	**Percentage of degradation**
1	Acidic condition	20	0.828	15.3
2	Alkali condition	20	0.642	58.6
3	Neutral condition	20	0.879	12.3
4	Oxidation	20	0.764	24.6
5	Thermal	20	0.952	5.8
6	Photolysis	20	0.924	5.6

The induced forced degradation study of MET was performed by exposing standard drug of MET to different stress conditions under ICH guidelines. Result of the study indicates that MET was undergoes significant degradation in alkali hydrolytic conditions, whereas mild degradation under acidic, neutral and oxidation conditions but stable to thermal and photolytic conditions. The results of forced degradation study summarized in (Table 5).

3.3.1. Hydrolytic studies

Acid induced degradation

For the acidic hydrolytic stress degradation study, MET dissolved in 1.0 N HCl and refluxed for 06hrs at 60 ^{0}C. After that a working solution of 20 µg/ml was prepared by further diluting with distilled water and absorbance was recorded at 322nm. The percentage of degradation was determined by comparing the absorbance of sample solution with standard solution. In strong acidic hydrolytic condition the drug was found to be moderately degraded by 25.3% (Fig.4).

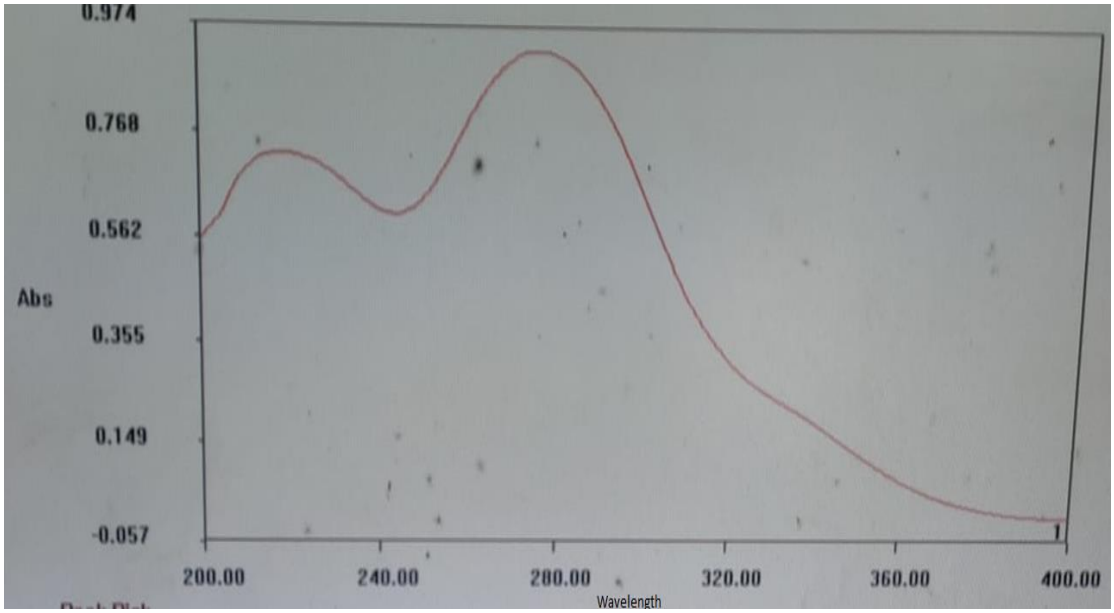

Figure 4 Acid induced degradation of MET

Alkali induced degradation

In stress degradation study under alkali conditions, MET exposed to 1.0 N NaOH solution and refluxed for 6hrs at 60 ^{0}C. After that the solution was neutralized by mixing with equivalent volume 1.0N HCl solution and working solution 20 µg/ml was prepared by dilute with distilled water and absorbance was determined at 322nm. The drug was found to be degraded to a significant percentage of 58.6 % (Fig. 5).

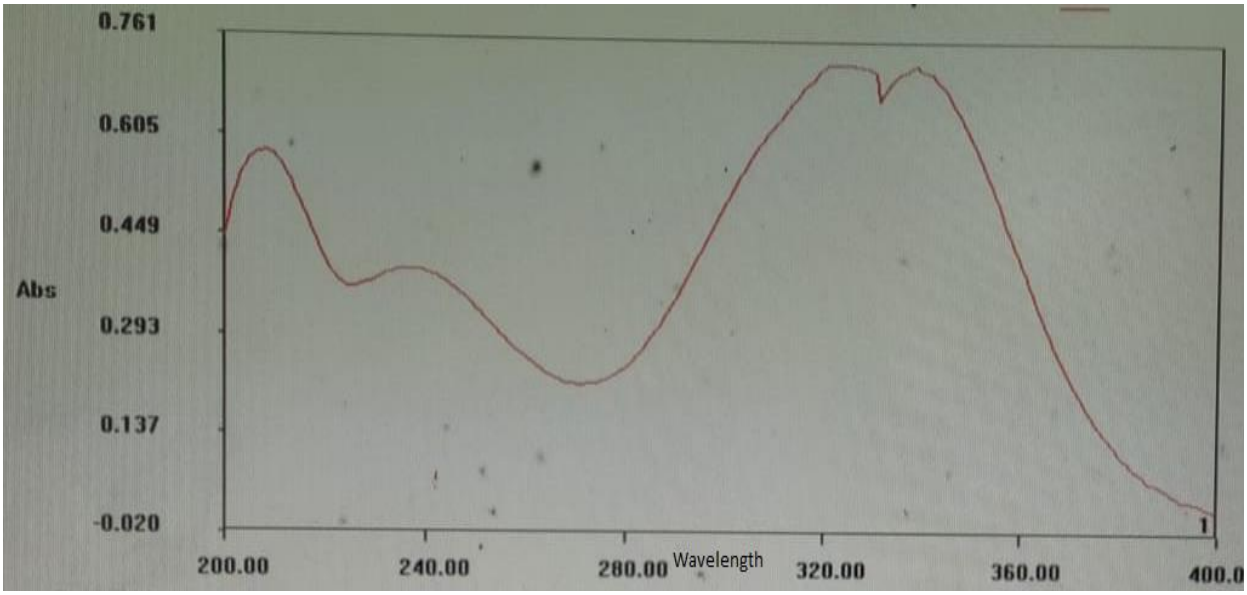

Figure 5 Alkali induced degradation of MET

Neutral induced degradation

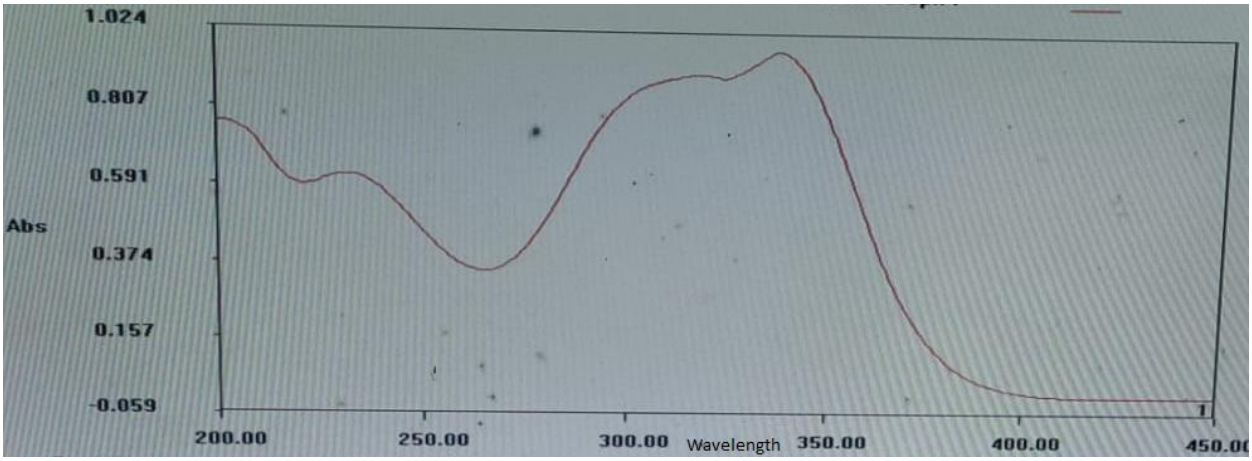

Figure 6 Neutral-induced degradation of MET

In neutral conditions the prepared drug solution was refluxed for 06hrs at 60 ^{0}C. The percentage of degradation was found to be 15.3% (Fig. 6).

3.3.2. Oxidation induced degradation

In oxidative stressed condition the drug solution is mixed to a 3% H_2O_2 solution and kept at room temperature for 24hrs. A 20 µg/ml solution was prepared from the above solution by dilute with distilled water and absorbance was taken at 322nm. The result of the study indicates that the drug was degraded by 24.6% in oxidative degradable conditions (Fig.7).

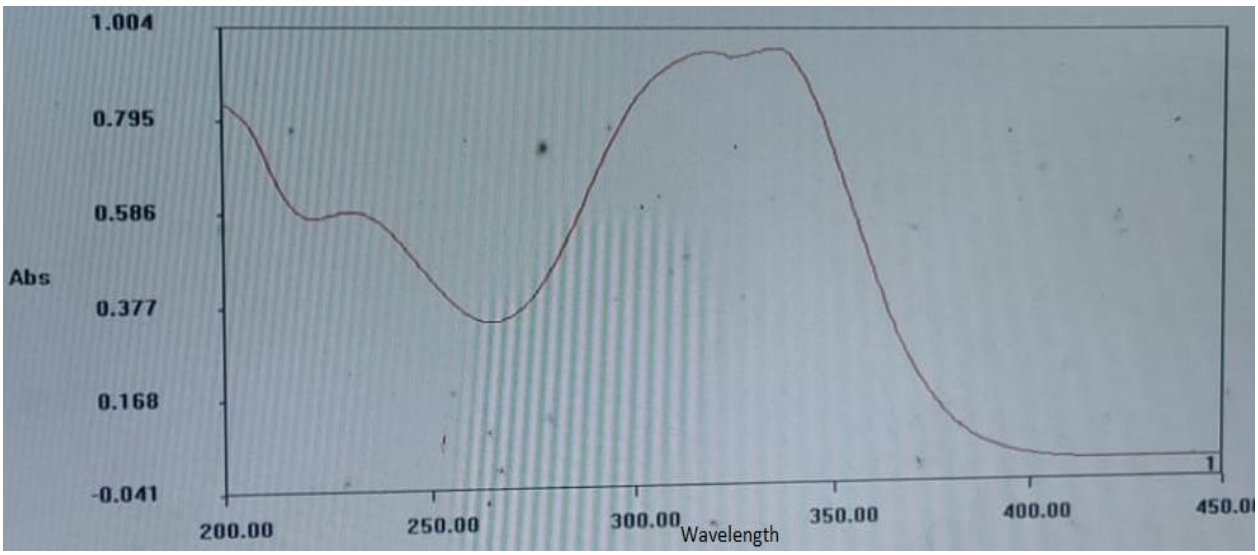

Figure 7 Oxidative degradation of MET in 3% H_2O_2

3.3.3. Thermal stress study

In stress degradation study under thermal condition pure sample powder of MET exposed at 80 ^{0}C for 48hrs. There was no notable degradation was found for thermal condition about 5.8% of standard drug was found to be degraded under this stress condition (Fig. 8).

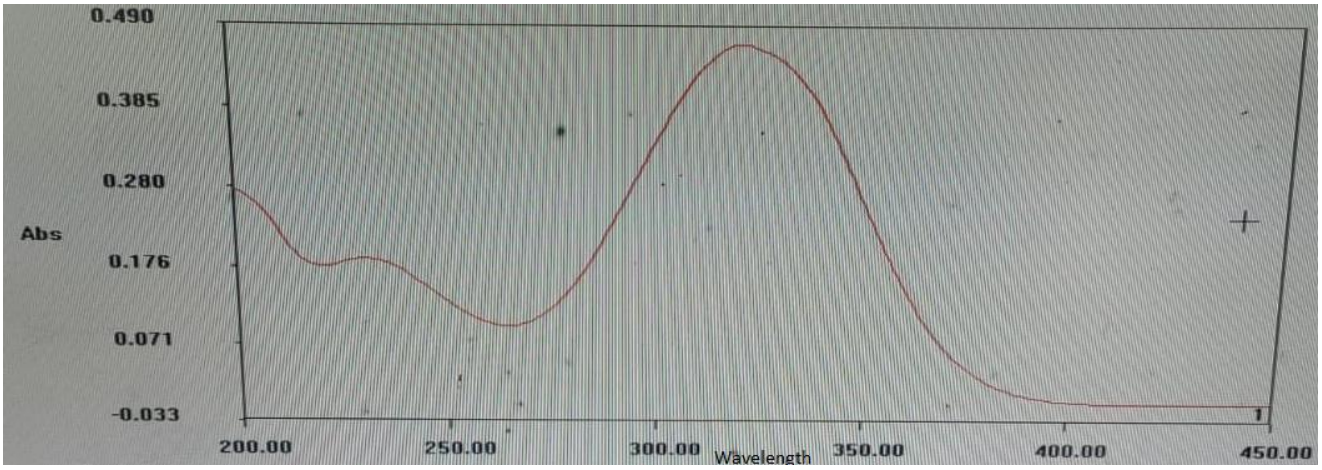

Figure 8 Thermal induced degradation of MET

3.3.4. Photolytic degradation

Photo degradation study of the drug was carried out in dry form where the drug was directly exposed to the sunlight for 6hrs on a hot sunny day. After that required amount was diluted with distilled water to make a working solution 20 µg/ml and absorbance recorded at 322nm. The percentage of degradation was estimate by comparing absorbance of sample solution with standard solution. The drug was found to be stable to the exposed degradation condition and about 5.6% degradation of drug was observed for thermal degradation study (Fig. 9).

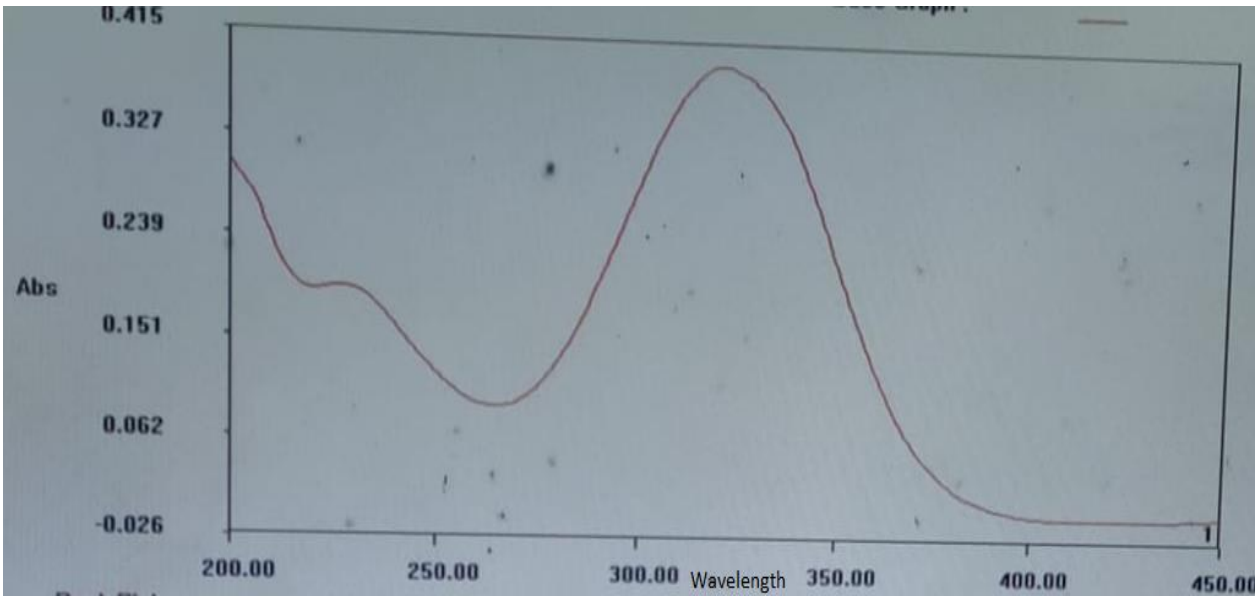

Figure 9 Photolytic degradation of MET

4. Conclusion

Changes in drug stability can risk patient safety by the formation of toxic degradation product(s).The stability of a drug product or a drug substance is a critical parameter which may affect purity, potency and safety. Therefore, it is essential to know the purity profile and degradation behaviour of a drug substance under various degradable conditions. The proposed method presents a simple, rapid, economic and useful stability indicating assay method for the determination of metronidazole in bulk and formulation. Majority of the reported methods employ organic solvents as diluents and suffers two disadvantages of narrow linear range and poor sensitivity. In this method distilled water is taken as diluent which makes this method inexpensive and wide linearity range with more sensitivity for drug estimation. This developed spectroscopic method was further validated as per ICH guideline. The values of % RSD for recovery and precision study are less than 2 indicates this method has accuracy, sensitivity and precision for estimation of metronidazole. LOD and LOQ were found to be within limits. Hence this method can be easily and conveniently adopted

for routine stability indicating assay of metronidazole in different dosage formulation before going to HPLC or HPTLC methods to save the time.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors have no conflict of interest.

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