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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING UV SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN TRIHYDRATE AND METRONIDAZOLE IN BULK AND IN-HOUSE TABLET

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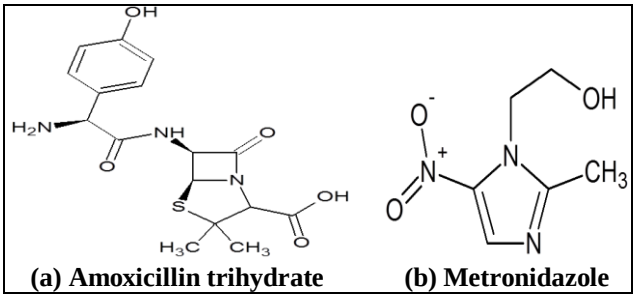
ABSTRACT

Stability indicating UV spectrophotometric methods for simultaneous estimation of amoxicillin trihydrate and metronidazole in bulk and in-house tablet has been developed by two methods. First method is simultaneous equation based on measurement of absorbance at 230 nm and 310 nm as two wavelengths selected for quantification of amoxicillin trihydrate and metronidazole. The second method is absorbance ratio based on the measurement of absorbance at iso-absorptive point at 237.3 nm and 310 nm as second wavelength selected as for quantification. Both methods obeyed Beer-Lambert's law in the concentration range of 15-90 µg/ml for amoxicillin trihydrate and 5-30 µg/ml for metronidazole with correlation coefficient (r^2) values 0.999 for amoxicillin trihydrate and 0.998 for metronidazole in method-I, whereas 0.998 for amoxicillin trihydrate and 0.998 for metronidazole in method-II. Both methods showed good accuracy and precision with % RSD less than 2. LOD and LOQ values were 0.349 µg/ml and 1.06 µg/ml for amoxicillin trihydrate and 0.215 µg/ml and 0.652 µg/ml for metronidazole in method-I, whereas 0.558 µg/ml and 1.689 µg/ml for amoxicillin trihydrate and 0.215 µg/ml and 0.652 µg/ml for metronidazole in method-II respectively. The % assay was found to be 99.85% and 99.86% in method-I, whereas 99.63% and 99.86% in method-II for amoxicillin trihydrate and metronidazole respectively. Stress degradation was studied in different acidic, basic, neutral, oxidative, photolytic and thermal condition.

KEYWORDS: Amoxicillin trihydrate, metronidazole, UV spectroscopy, simultaneous equation, absorbance ratio, stability indicating assay method, validation.

INTRODUCTION

Amoxicillin trihydrate is a broad spectrum semi-synthetic amino penicillin antibiotic. It is effective against a wide range of infections caused by wide range of Gram- negative bacteria and Gram- positive bacteria in both human and animals. Amoxicillin trihydrate is bactericidal against susceptible micro-organisms through the inhibition of biosynthesis of cell wall mucopeptide during bacterial multiplication. This results in inhibition of bacterial cell synthesis and ultimately death due to lysis of bacteria.[1] Chemically amoxicillin trihydrate (figure 1) is (2S,5R,6R)-6-[(R)-2-amino-2-(4-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0.] heptane-2-carboxylic acid trihydrate,[2] Metronidazole is a prodrug that acts as antibiotic, antiprotozoal, amoebicidal, bactericidal and trichomonicidal. Metronidazole is converted in anaerobic organisms by the redox enzyme pyruvate-ferredoxin oxidoreductase. The nitro group of metronidazole is chemically reduced by ferredoxin (or a ferredoxin-linked metabolic process) and the products are responsible for disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis.[3] Chemically metronidazole (figure 1) is 2-(-2-methyl-5-nitro-1-H-Imidazole-1-yl)-ethanol.[4] The combination is used in the treatment of infection of gum and dental cavities, brain infection, lung infection, bones infection, stomach infection, blood infection and other conditions.[5]



(a) Amoxicillin trihydrate **(b) Metronidazole**

Figure 1: Chemical structure of analyzed substances

Various spectrophotometric,[6-14] HPLC,[15-21] HPTLC[22-23] and stability indicating HPLC[24] methods are reported in the literature for the estimation of amoxicillin trihydrate and metronidazole individually and in combination with other drugs. According to literature survey no stability indicating UV spectrophotometric method has yet been reported for simultaneous estimation of amoxicillin trihydrate and metronidazole in combination by using methanol as solvent. The present work described two stability indicating UV spectrophotometric methods for the simultaneous estimation of amoxicillin trihydrate and metronidazole by simultaneous equation and absorbance ratio method.

MATERIAL AND METHOD

Pharmaceutically pure sample of amoxicillin trihydrate and metronidazole was obtained from Aarati drugs ltd. Tarapur as gift samples. All other chemicals used in the analysis were Analytical grade.

Instrumentation

A double beam UV-visible spectrophotometer (Shimadzu) model UV-1800 PC was used. The software employed was UV probe. The spectrum was recorded over range 200-400 nm against solvent in 1 cm quarts cells. Electronic analytical balance (Anamed) model AA-2200, Ultrasonicator (HMG India) was used.

Preparation of standard stock solution

Accurately weighed 10 mg of AMT and MET were transferred into 100 ml volumetric flasks separately and dissolved in methanol, then volume was made up to 100 ml with methanol to get a concentration of 100 µg/ml for both drugs.

Determination of absorption maxima and iso - absorptive point

Working standard solution was obtained by dilution of respective stock solution with methanol. Stock solutions (2 ml) were diluted to 10 ml to get a concentration of 20 µg/ml for AMT and MET. Both the solutions were scanned in the spectrum mode over the range of 200-400 nm against methanol as blank and the overlain spectra of the two were recorded. AMT showed an absorbance peak at 230 nm, whereas MET shows an absorbance peak at 310 nm. The overlain spectra also showed iso-absorptive point at 237.3 nm (Figure 2). Due to difference in absorbance maxima and having no interference with each other so both drugs can be simultaneously estimated by simultaneous equation method (method-I) and absorbance ratio method (method-II).

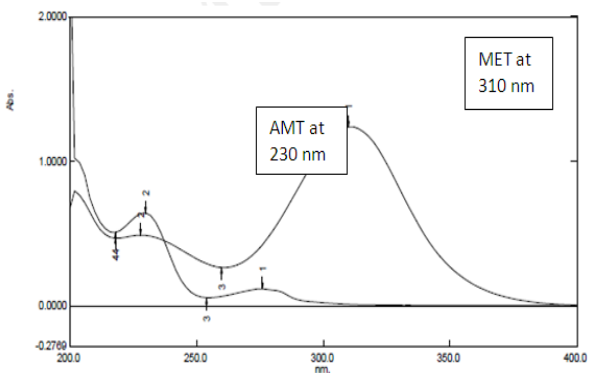


Figure 2: Overlain spectra of AMT and MET.

Method I (Simultaneous equation method)

Two wavelengths selected for the method were 230 nm and 310 nm that are absorption maxima of AMT and MET respectively in methanol. The stock solutions of both the drugs were further diluted separately with methanol to get a series of standard solutions of 15-90 µg/ml for AMT and 5-30 µg/ml for MET. The absorbances were measured at the selected wavelengths and absorptivities (A 1%, 1 cm) for both the drugs were determined as mean of five independent determinations. Concentrations in the sample were obtained by using following equations.

$$Cx = (A_2ay_1 - A_1ay_2)/ (ax_2ay_1 - ax_1ay_2) \text{ ……. (i)}$$
$$Cy = (A_1ax_2 - A_2ax_1)/ (ax_2ay_1 - ax_1ay_2) \text{ …… (ii)}$$

Where A_1 and A_2 are absorbance of mixture at 230 nm and 310 nm respectively, ax_1 and ax_2 are absorptivities of AMT at λ_1 and λ_2 respectively and ay_1 and ay_2 are absorptivities of MET at λ_1 and λ_2 respectively. Cx and Cy are concentration of AMT and MET respectively.

Method II (Absorbance ratio method)

From the overlain spectrum of AMT and MET, two wavelengths were selected one at 237.3 nm which is the iso-absorptive point for both the drugs and the other at 310 nm which is λmax of MET. The absorbances of the sample solutions are prepared in a similar manner as in the previous method, were measured and the absorbance ratio values for both the drugs at selected wavelengths were also calculated. The method employs Q-values and the concentrations of drugs in sample solution were determined by using the following formula,

$$Cx = Qm - Qy/ Qx - Qy \text{ X } A/ax_1 \text{ ……(iii)}$$
$$Cy = Qm - Qx/ Qy - Qx \text{ X } A/ay_1 \text{ ……(iv)}$$

Where,

Qm = Absorbance ratio of sample at 310 nm & 237.3 nm

Qx = Ratio of absorptivity of AMT at 310 nm & 237.3 nm

Qy = Ratio of absorptivity of MET at 310 nm & 237.3 nm

A = Absorbance of sample at iso-absorptive point

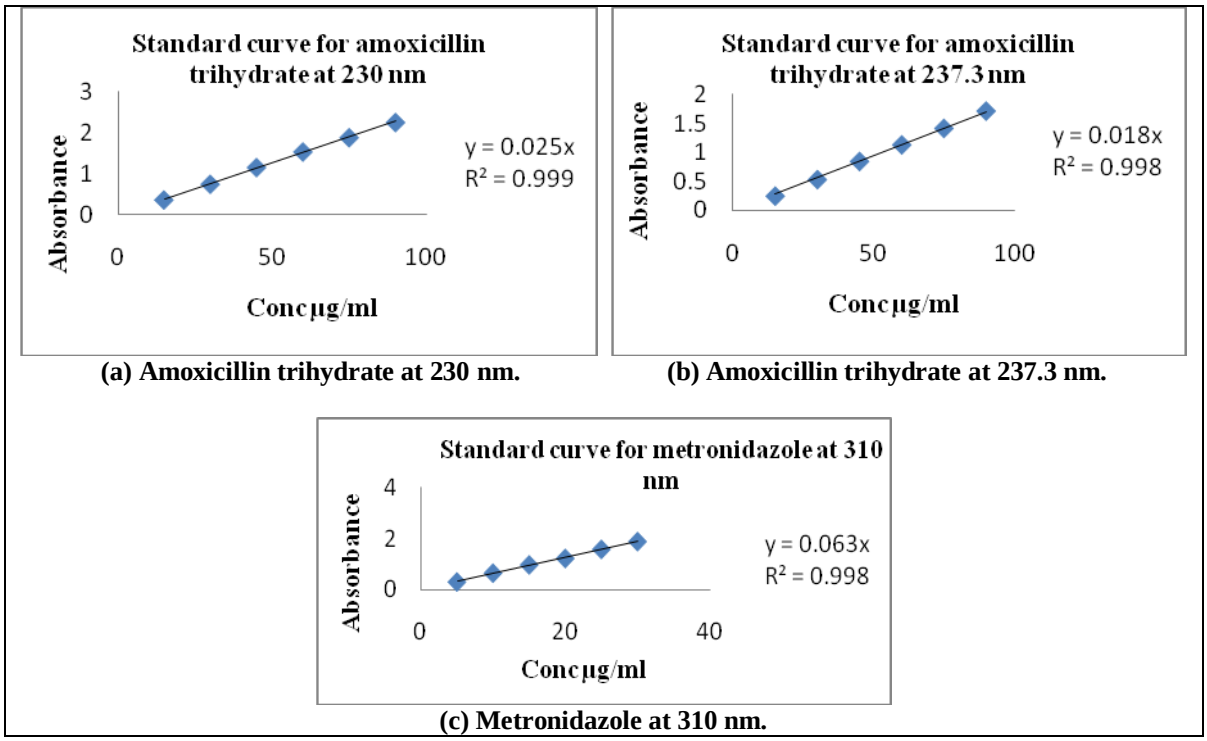
ax_1 & ay_1 = Absorptivity of AMT & MET at isoabsorptive point.

Validation of the method

Method was validated according to ICH guidelines for linearity, accuracy, precision, LOD and LOQ.

Linearity

Linearity was studied by preparing standard solution at different concentration levels. Standard stock solution of AMT and MET was further diluted to get concentration in the range of 15-90 µg/ml for AMT and 5-30 µg/ml for MET. The resultant absorbances of the solutions were measured at 230 nm, 237.3 nm and 310 nm against methanol as blank. The calibration curves were constructed by plotting absorbances versus concentrations of the drug and the regression equations were calculated (figure 3). These results shown there was an excellent correlation between absorbance and analyte concentration. Linearity parameters are tabulated in Table 1.



(a) Amoxicillin trihydrate at 230 nm. **(b) Amoxicillin trihydrate at 237.3 nm.**

(c) Metronidazole at 310 nm.

Figure 3: Calibration curves of linearity.

Table 1: Parameters of linearity study.

Parameters	Method - I		Method – II	
	AMT	MET	AMT	MET
Wavelength range (nm)	230 nm	310 nm	237.3 nm	310 nm
Linearity range (µg/ml)	15–90 µg/ml	5–30 µg/ml	15–90 µg/ml	5–30 µg/ml
Regression Coefficient (r2)	0.999	0.998	0.998	0.998
Slope (m)	0.025x	0.063x	0.018x	0.063x

Accuracy

The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100%, 120%. The recovery studies were carried out by adding known amount of standard drug to pre-analyzed sample. The resulting solutions were then re-analyzed by proposed methods. Whole analysis procedure was repeated to find out the recovery of the added drug sample. This recovery analysis was repeated at three replicate of three concentrations levels. The results of accuracy study are reported in table 2.

Table 2: Result of accuracy.

Recovery level (%)	Amount added (mg)		% Recovery*				% RSD*			
			Method - I		Method - II		Method – I		Method – II	
	AMT	MET	AMT	MET	AMT	MET	AMT	MET	AMT	MET
80	8	6.4	99.56	99.54	99.37	99.53	0.186	0.212	0.377	0.191
100	10	8	99.91	99.99	100.1	99.98	0.187	0.171	0.265	0.158
120	12	9.6	100.3	100.4	100.3	100.4	0.139	0.080	0.216	0.064

*Average of three determination.

Precision

Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). The precision was determined at different parameter like repeatability, intermediate precision (intra-day precision, inter-day). Repeatability was determined by analyzing AMT (30 µg/ml) and MET (24 µg/ml) for three times. Intraday precision was determined by analyzing same concentration of solution for three times within the day and Interday precision was determined daily for three days. Then % RSD was calculated and it was within limit (less than 2 %). The results of precision are reported in table 3.

Table 3: Result of precision.

Parameters	Conc. (µg/ml)		% RSD			
			Method-I		Method-II	
	AMT	MET	AMT	MET	AMT	MET
Repeatability	30	24	0.2648	0.1867	0.265	0.187
Intraday	30	24	0.444	0.4036	0.0928	0.1998
Interday	30	24	1.243	0.578	0.492	0.605

*Average of three determination.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. LOQ is the lowest concentration of analyte in the sample that can be quantitatively determined with precision and accuracy. Results of LOD and LOQ are given in table 4.

LOD and LOQ was calculated from linear curve using following formulae.

LOD = 3.3 σ/Slope, LOQ = 10 σ/Slope.

(Where σ = the standard deviation of the response and S = Slope of calibration curve).

Table 4: Result of LOD and LOQ.

Drug	Method - I		Method – II	
	LOD (µg/ml)	LOQ (µg/ml)	LOD (µg/ml)	LOQ µg/ml
Amoxicillin trihydrate	0.349	1.06	0.558	1.689
Metronidazole	0.215	0.652	0.215	0.652

Analysis of in-house tablet

Due to unavailability of tablet containing amoxicillin trihydrate and metronidazole in local Indian market, in-house tablet was prepared via direct compression technique using commonly used excipients containing 250 mg of amoxicillin trihydrate and 200 mg of metronidazole.

Twenty tablets were taken and their average weight was determined, crushed to fine powder; powder equal to 50 mg of AMT and 40 mg of MET was taken in 100 ml volumetric flask. Then 80 ml of methanol was added and the flask was sonicated for about 10 min to solubilize the drug present in tablet, after that drug solution was filtered through Whatman filter paper No. 41. The filtrate was collected and volume was made up to the mark with methanol. Further dilution was done in 10 ml volumetric flask to get final concentration of 30 µg/ml of AMT and 24 µg/ml of MET. Absorbance of sample solution was measured at 230 nm, 237.3 nm and 310 nm. Concentrations of both drugs were obtained from equation. Results of in-house tablet analysis are given in table 5.

Table 5: Analysis of in-house tablet.

Sr. No.	Drug	Conc. (µg/ml)	Amount found*	% label claim*	S.D.
Method 1	AMT	30 µg/ml	29.96 µg/ml	99.8	0.087
	MET	24 µg/ml	23.98 µg/ml	99.86	0.238
Method 2	AMT	30 µg/ml	29.92 µg/ml	99.63	0.22
	MET	24 µg/ml	23.97 µg/ml	99.86	0.244

* Average of three determinations.

Forced degradation studies

To further confirm the stability indicating nature of the analytical method, amoxicillin trihydrate and metronidazole were subjected to stress testing as per ICH recommended test conditions.

Acid Hydrolysis

Accurately weighed 10 mg of AMT and MET was transferred to 100 ml volumetric flask, to it 10 ml 0.1 N HCl was added. This flask was heated on water bath at 60°C for 3 hrs. Solution was cooled and neutralized with 0.1 N NaOH and volume was made up to mark with

methanol. This solution was further diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET and absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Base hydrolysis

Accurately weighed 10 mg of AMT and MET was transferred to 100 ml volumetric flask, to it 10 ml 0.1 N NaOH was added. This flask was heated on water bath at 60°C for 3 hrs. Solution was cooled and neutralized with 0.1 N HCl and volume was made up to mark with methanol. This solution was further diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET and absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Neutral hydrolysis

Accurately weighed 10 mg of AMT and MET was transferred to 100 ml volumetric flask, to it 50 ml water was added. This flask was refluxed for 3 hrs at 60°C. Solution was cooled and volume was made up to mark with methanol. This solution was further diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET. Absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Oxidative degradation

Accurately weighed 10 mg of AMT and MET was transferred to 100 ml volumetric flask, to it 10 ml 3% H_2O_2 was added. This flask was refluxed for 3 hrs at 60°C. Solution was cooled and volume was made upto mark with methanol. This solution was further diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET. Absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Photolytic degradation

Pure drugs were exposed to UV radiations for 12 hrs. The sample after exposure to light was diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET. Absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Thermal degradation

Thermal degradation was carried out by exposing pure drugs to dry heat at 80°C for 2 hrs. The sample after exposure to heat was diluted with methanol to get 30 µg/ml of AMT and 24 µg/ml of MET. Absorbance was measured at 230 nm, 237.3 nm and 310 nm. Finally absorbance of sample was compared with standard absorbance and percent degradation was calculated.

Summary of forced degradation study data is given in table 6 and 7.

Table 6: Forced degradation data (Simultaneous equation).

Sr. No.	Condition	% degradation		% assay	
		AMT	MET	AMT	MET
1	Acid hydrolysis	6.76	9.67	93.24	90.33
2	Base hydrolysis	7.43	7.1	92.57	92.90
3	Neutral hydrolysis	8.75	5.44	91.25	94.56
4	Oxidative hydrolysis	6.9	10.42	93.1	89.58
5	Photolytic degradation	4.51	3.32	95.49	96.68
6	Thermal degradation	8.49	12.54	91.51	87.46

Table 7: Forced degradation data (Absorbance ratio).

Sr. No.	Condition (absorbance ratio)	% degradation		% assay	
		AMT	MET	AMT	MET
1	Acid hydrolysis	11.03	9.67	88.97	90.33
2	Base hydrolysis	13.65	7.1	86.35	92.90
3	Neutral hydrolysis	10.47	5.44	89.53	94.56
4	Oxidative degradation	6.73	10.42	93.27	89.58
5	Photolytic degradation	2.24	3.32	97.76	96.68
6	Thermal degradation	8.22	12.54	91.78	87.46

RESULT AND DISCUSSION

As both the drugs are soluble in methanol estimation was carried out in methanol as solvent. The overlain spectra of AMT and MET exhibited λmax at 230 nm and 310 nm for AMT and MET respectively and iso-absorptive point were observed at 237.3 nm. The linear relationship was observed between absorbance and concentration over the range of 15-90 µg/ml for AMT and 5-30 µg/ml for MET with correlation coefficients (r^2) values 0.999 for AMT and 0.998 for MET in method-I, whereas 0.998 for AMT and 0.998 for MET in method-II. The accuracy of the method was confirmed by recovery studies at three different levels of addition (80%, 100%, and 120%). Both the methods of estimation showed good recoveries close to 100% and % coefficient of variation was less than 2%. The results of the recovery studies performed indicate the methods to be accurate. Precision was carried out as repeatability and intermediate precision. It was determined at three concentration levels with three replicates at each level. For all three concentration levels % RSD obtained was less than 2 % for both drugs. In

method-I, the LOD, LOQ values for AMT was found to be 0.349 µg/ml and 1.06 µg/ml and for MET 0.215 µg/ml and 0.652 µg/ml respectively; in method–II, 0.558 and 1.689 µg/ml for AMT and 0.215 µg/ml and 0.652 µg/ml for MET respectively. The % assay was found to be 99.85% and 99.86% in method-I, whereas 99.63% and 99.86% in method-II for AMT and MET respectively. The % degradation of AMT in acid, base, neutral, oxidative, photolytic and thermal condition was 6.76%, 7.43%, 8.75%, 6.9%, 4.51%, 8.49%, and of MET was 9.67%, 7.1%, 5.44%, 10.42%, 3.32%, 12.54% respectively in method-I; whereas the % degradation of AMT was 11.03%, 13.65%, 10.47%, 6.73%, 2.24%, 8.22%, and of MET was 9.67%, 7.1%, 5.44%, 10.42%, 3.32%, 12.54% respectively in method-II.

CONCLUSION

Both the developed analytical methods are simple, specific, accurate and stability indicating. Hence it can be used for routine quality control analysis as well as stability studies of pharmaceutical dosage forms. The degradation behavior of AMT and MET was determined by subjecting them in various stress conditions and no attempt was made to identify the degradation product.

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