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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN TRIHYDRATE, METRONIDAZOLE AND FAMOTIDINE

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ABSTRACT

The objective of the current study was to develop and validate a simple, accurate, precise and rapid reversed-phase HPLC method for simultaneous estimation of Amoxicillin trihydrate (AMOX), Metronidazole (METRO) and Famotidine (FAMO). The chromatographic separation of AMOX, METRO and FAMO were achieved on RP-HPLC having Luna C-18-ODS bonded column of length 250mm using UV detection at 267 nm. The optimized mobile phase consisted of a mixture of 0.03M disodium hydrogen phosphate buffer–acetonitrile (93:7, v/v) adjusted to pH 6.5 at a flow rate of 1.5 ml/min. The retention times were 2.560, 3.657 and 6.983 min for AMOX, FAMO and METRO respectively. The proposed method provided linear responses within the concentration ranges of 0-50µg/ml for Amoxicillin trihydrate & Metronidazole and 0-30µg/ml for Famotidine. The LOD values were 0.0252, 0.0098 and 0.0288 µg/ml and LOQ values were 0.0765, 0.0298 and 0.0875µg/ml for Amoxicillin trihydrate & Metronidazole and Famotidine, respectively. High recovery and low % coefficient of variation (COV) revealed the reliability of the method for quantitative study of the three drugs in combined dosage form.

K**eywords:** Amoxicillin trihydrate, Metronidazole, Famotidine, Simultaneous estimation, Reversed-phase HPLC method.

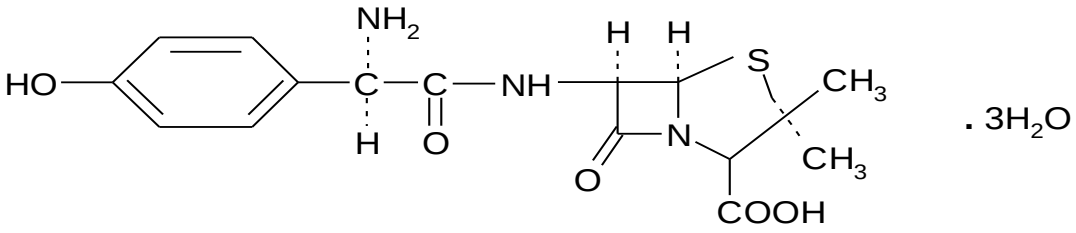
INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped, Gram-negative bacterium that chronically infects the gastric mucosa of >50% of the human population, causing chronic inflammation of the stomach and development of gastroduodenal diseases, such as gastritis, peptic ulcer and gastric cancer [1]. The most widely recommended treatment in international guidelines for the eradication of *H. pylori* is combination of two antibiotics with an acid-suppressing agent for at least 14 days [2].

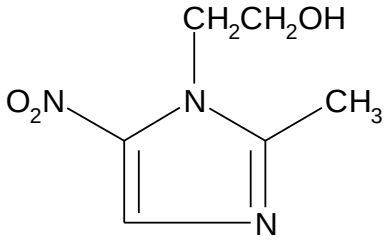
Amoxicillin trihydrate (AMOX), 6(R)-6-{α-D- (4- hydroxyl phenyl) glycyl amino} penicillanic acid trihydrate is an orally absorbed, semi-synthetic broad-spectrum antimicrobial drug [3-4]. Metronidazole (METRO), 1-(β-hydroxy-ethyl)-2-methyl-5-nitroimidazole is used as antiprotozoal and antibacterial agent [5-6]. Famotidine (FAMO), N'–(aminosulfonyl)-3–[[[2-[(di-aminomethylene) amino] 4-triazolyl] methyl] thio] propanimidamide is a potent, competitive and reversible inhibitor of histamine action at the H2 receptor [7-8]. The combination of Amoxicillin trihydrate, Metronidazole and Famotidine, is now

widely used in a standard eradication treatment of gastric and duodenal ulcers, which are associated with *H. pylori* infection. These triple therapies are provided to be effective in clinical application [9-11]. Combination of three drugs has shown more effective against the peptic ulcer caused by *H. pylori*. Till date there is no HPLC method for the simultaneous estimation of these three drugs combination. Extensive literature survey suggested that a formulation containing these three drugs in combination has not been reported so far and hence the method of analysis is also not available [12-15].

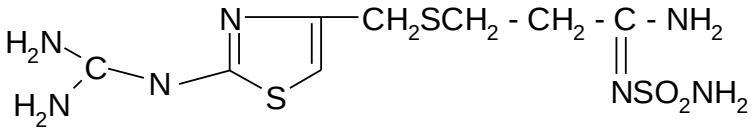
The present study reveals a simple, specific, precise and rapid RP-HPLC method for simultaneous estimation of Amoxicillin trihydrate, Metronidazole and Famotidine in combined dosage form. The developed method is further validated a per ICH guidelines Q2A and Q2B [16-18].



Amoxicillin trihydrate



Metronidazole



Famotidine

Figure 1. Chemical structures of Amoxicillin trihydrate (AMOX), Metronidazole (METRO) and Famotidine (FAMO).

MATERIAL AND METHODS

Reagents and chemicals

The gift samples of drugs i.e. Amoxicillin trihydrate, Metronidazole and Famotidine were provided by Lapiz Pharma, Sagar, Khandelwal laboratory, Mumbai and Lupin Research Park, Pune, respectively. Disodium hydrogen phosphate, sodium hydroxide, acetonitrile and all other chemicals were purchased from Himedia labs, USA. All the chemicals of HPLC and analytical grades were used without any further purification. All the excipients used for the development of placebo formulations were obtained from commercial sources and were used as such. Double distilled water was used during entire HPLC procedure.

Equipment/instrumentation

Shimadzu LC 10 AT VP series HPLC was used having photodiode array detector. Luna C-18-ODS bonded column of length 250 mm and an inter diameter 4.6 mm was selected for the analysis. The particle size of the stationary phase was 5 µm. The mobile phase was a degassed and filtered (0.45 µm, Milipore) mixture of 0.03M disodium hydrogen phosphate buffer–acetonitrile (93:7, v/v) adjusted to pH 6.5 at a flow rate of 1.5 ml/min.

Preparation of Standard Stock Solutions and Sample Preparations

The standard stock solutions of Amoxicillin trihydrate, Metronidazole and Famotidine were prepared by dissolving 50mg of each drug in 100ml of mobile phase separately. From the above solution 10mL of solution was taken and diluted to 50ml with the same to get a solution containing 100 µg/ml of each drug. From the stock solutions, eight working standard solutions for three drugs having concentration 5, 10, 15, 20, 25, 30, 35, 40, 45, 50µg/ml were prepared in mobile phase and their area was noted by injecting 20µL into the system. After that calibration curves were plotted between concentration against their respective area for AMOX, Metronidazole and Famotidine separately. From the calibration curve it was found that AMOX and Metronidazole have linearity range between 0-50µg/ml whereas Famotidine has range between 0-30µg/ml.

Preparation of Mixed Standard Solutions

Five mixed standards solutions with concentration of Amoxicillin trihydrate, Metronidazole and Famotidine in µg/ml of 5:25:30, 10:20:25, 15:15:20, 20:10:15, 25:5:10 were prepared in mobile phase by diluting appropriate volumes of the standard stock solutions. The solutions were loaded in the injector fitted with a 20 µl fixed volume loop and area was recorded.

Method Validation

Linearity

For checking linearity standard stock solutions of Amoxicillin trihydrate, Metronidazole and Famotidine were prepared by dissolving 50mg of each drug in 100ml of mobile phase separately. From the above solution 10ml of solution was taken and diluted to 50ml with the same to get a solution containing 100 µg/ml of each drug. From the stock solutions, ten working standard solutions for three drugs having concentration 5, 10, 15, 20, 25, 30, 35, 40, 45, 50µg/ml were prepared in mobile phase and their area was noted by injecting 20µL into the system. After that calibration curves were plotted between concentration against their respective area for Amoxicillin trihydrate, Metronidazole and Famotidine, separately.

Accuracy and precision

Accuracy is performed to check similarity of results obtained by analytical value to the true value. The precision is defined as degree of this similarity. To check the accuracy of the proposed methods, recovery studies were carried out at 80, 100, and 120% of the standard concentration as per ICH guidelines [19, 20]. The recovery study was performed three times at each level.

Intermediate Precision- (Inter-day and Intra-day precision)

Intermediate Precision of the method was inter-day and intra-day analysis i.e. the analysis of formulation was repeated six times in the same day and on three successive days [21].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of Amoxicillin trihydrate, Metronidazole and Famotidine determined by calibration standard method. LOD and LOQ were calculated using the following equations; LOD = 3.3 (σ/S) and LOQ = 10 (σ /S), where σ is standard deviation (SD) of the y-intercept of calibration curve and S is slope of regression equation [22].

For LOD and LOQ, 1µg/ml of solution of three drugs was prepared from standard stock solution containing 100µg/ml by diluting appropriate volume with mobile phase. Five standard solutions for Amoxicillin trihydrate having concentration 0.4, 0.8, 1.0, 1.2, 1.4 µg/ml and for Metronidazole and Famotidine having concentration 0.8, 1.0, 1.2, 1.4, 1.6 µg/ml were prepared in mobile phase from 1 µg/ml of solution area was noted.

Results and discussion

Linearity

From the calibration curve (Figure no. 1, 2 and 3) it was found that Amoxicillin trihydrate and Metronidazole have linearity range between 0-50µg/ml whereas Famotidine has range between 0-30µg/ml. For each drug, appropriate dilutions of standard stock solutions were assayed as per the developed methods. The linear regression equation for three drugs was;

For AMOX $Y= 12628x – 1465.4$ (r^2 =0.9981)

For METRO $Y= 18837x + 4117.8$ (r^2 =0.9987)

For FAMO $Y = 22925x – 7449.8$ (r^2 = 0.9975)

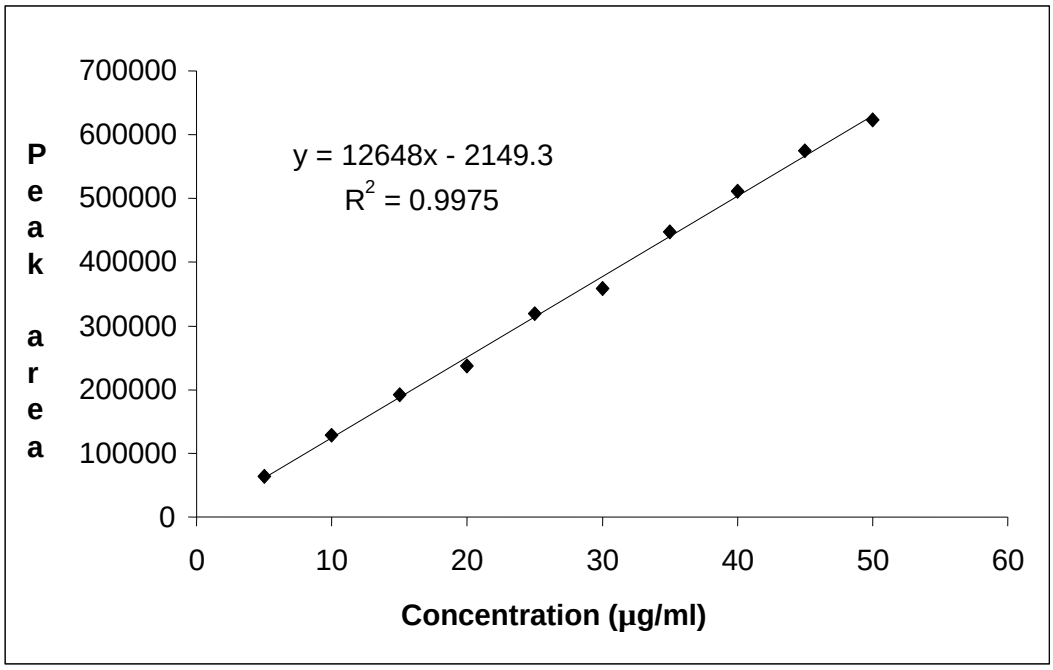


Figure 1: Calibration Curve of Amoxicillin Trihydrate

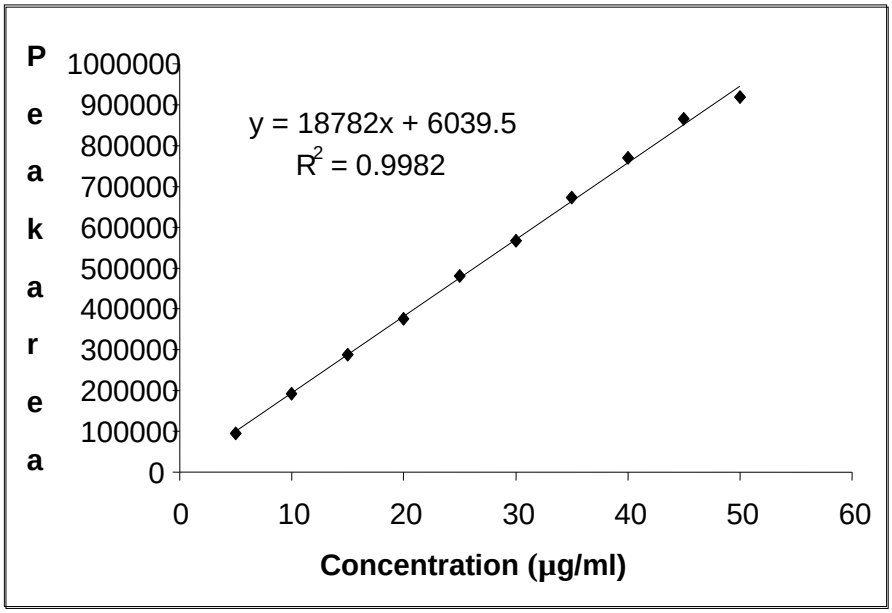


Figure 2: Calibration Curve of Metronidazole

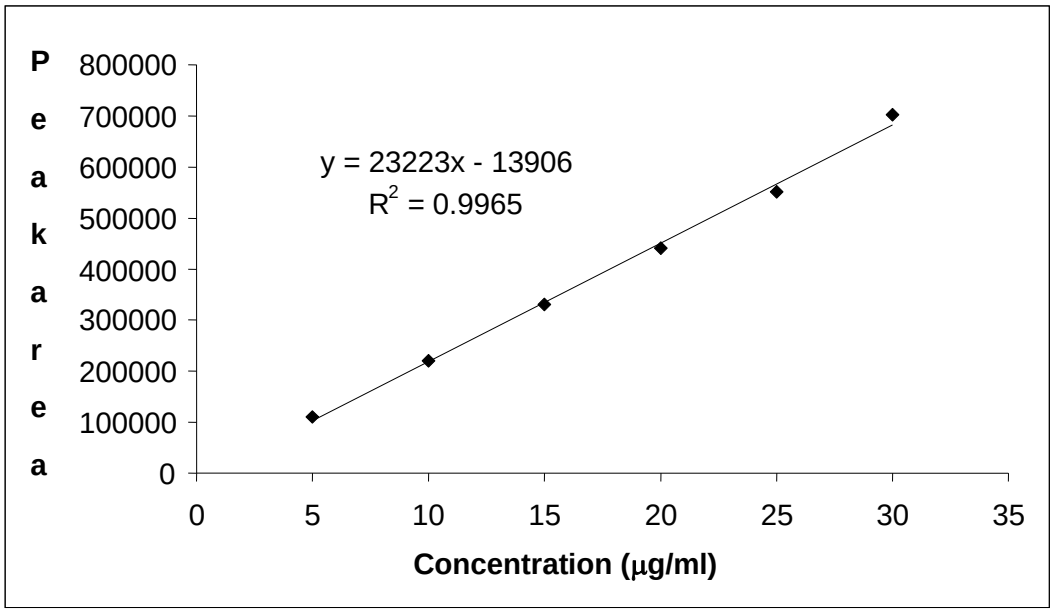


Figure 3: Calibration Curve of Famotidine

Specificity

The Figure no. 4 shows two-dimensional chromatogram for three drugs indicating no interference between all three drugs at 267 nm. Good separation is seen as the retention times were 2.560, 3.657 and 6.983 min for Amoxicillin trihydrate, Metronidazole and Famotidine, respectively. Although there is less difference between Amoxicillin trihydrate and Famotidine but still peaks are clear distinguished which was further supported by validation data. The total samples were run for 10 min to allow late eluting peak. The 10 min run is sufficient for any sample analysis to allow analysis of large no. of sample in less time [23].

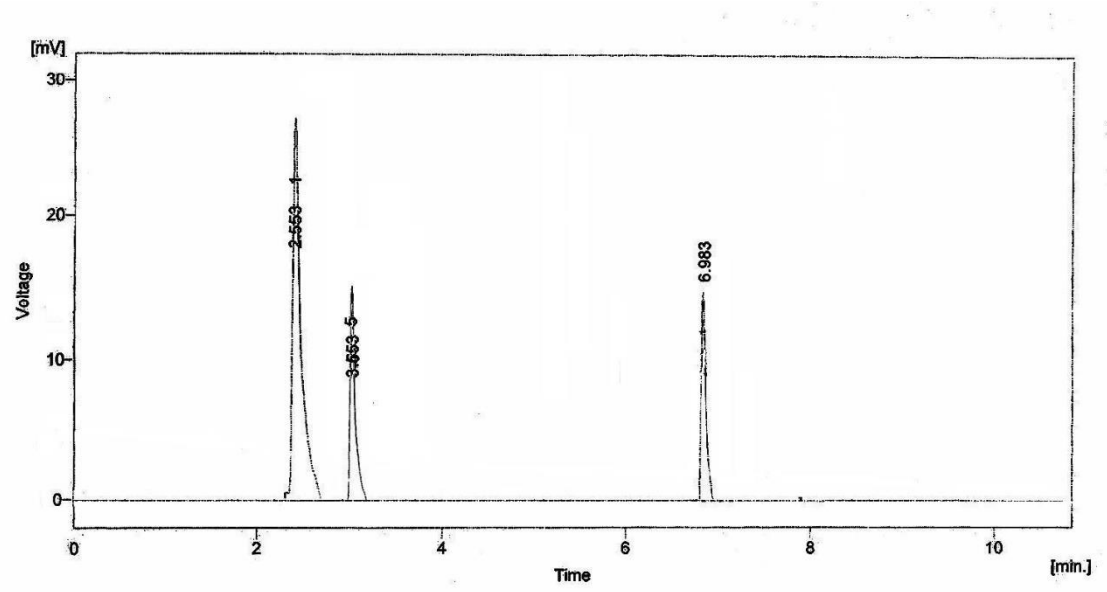


Figure 4: Chromatogram of AMOX, METRO and FAMO in Sample Solution and its Retention Time at 267nm.

Accuracy and precision

The data obtained (Table no. 1) shows that % recovery of all drugs lies between 99.50-100.50 %, which proves that developed method is accurate and lies well within recommended tolerance of 80- 115 %. It is considered that method is validated when its accuracy is within ± 15% and precise when COV is below 15% [24].

Table 1 Analysis of the results of recovery experiments

	Amount taken (µg/ml)			Amount added at (µg/ml)				% Recovery		
	AMOX	**METRO**	**FAMO**	**%**	**AMOX**	**METRO**	**FAMO**	**AMOX**	**METRO**	**FAMO**
1	20	10	4	80%	16	8	3.2	99.50 ± 0.17	99.80 ± 0.10	100.00 ±0.22
2	20	10	4		16	8	3.2	100.10 ± 0.11	100.40 ± 0.24	100.20 ± 0.16
3	20	10	4		16	8	3.2	100.00 ± 0.25	99.60 ± 0.13	100.50 ± 0.10
1	20	10	4	100%	20	10	4	99.50 ± 0.18	99.70 ± 0.19	100.00 ± 0.28
2	20	10	4		20	10	4	100.20 ± 0.12	99.90 ± 0.25	100.50 ± 0.31
3	20	10	4		20	10	4	100.30 ± 0.28	99.70 ± 0.16	99.50 ± 0.19
1	20	10	4	120%	24	12	4.8	99.00 ± 0.22	100.30 ± 0.20	99.75 ± 0.26
2	20	10	4		24	12	4.8	100.00 ± 0.16	100.00 ± 0.07	100.30 ± 0.12
3	20	10	4		24	12	4.8	99.50 ± 0.35	99.50 ± 0.28	99.80 ± 0.10

Intermediate Precision- (Inter-day and Intra-day precision)

Precision was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) and was expressed as the relative standard deviation (RSD) of a series of measurements. The repeatability was evaluated by assaying six samples at the same concentration (12.0 µg/mL) during the same day. The intermediate precision was evaluated by repeating the studies on three different days and comparing the obtained results.

The data of intra-day and inter-day precision and accuracy for the method are listed in Table 2.

Intermediate precision study expresses the intra-day and inter-day precision was determined by assay of the sample solution on the same day at different time intervals and on different days, respectively. The data obtained (table no. 2) shows that for all the methods, % coefficient of variation (COV) was not more than 2.0% which indicates well intermediate precision.

Table 2 Results of intra-day and inter-day precision

Intraday precision				Inter day precision			
% Retention				% Retention			
Time	**AMOX**	**METRO**	**FAMO**	**Day**	**AMOX**	**METRO**	**FAMO**
After 1hr	99.50±0.17	100.50±0.17	100.25±0.17	First day	99.10±0.25	100.10±0.10	100.20±0.22
After 2hr	99.63±0.17	100.00±0.17	100.40±0.17	Second day	99.20±0.22	99.90±0.12	99.95±0.26
After 3hr	99.10±0.17	100.25±0.17	100.50±0.17	Third day	99.35±0.12	99.70±0.13	100.20±0.22
After 4hr	99.20±0.17	100.10±0.17	100.35±0.17				
After 5hr	99.50±0.17	100.20±0.17	100.40±				
After 6hr	99.30±0.17	100.30±0.17	100.50±0.17				
Mean	99.37	100.22	100.40	Mean	99.21	99.90	100.08
SD	0.2040	0.1724	0.0948	SD	0.2000	0.1258	0.2158
%COV	**0.2052**	**0.1720**	**0.0944**	**% COV**	**0.2015**	**0.1259**	**0.2156**

AMOX: Amoxicillin trihydrate, METRO: Metronidazole, FAMO: Famotidine, S.D.: Standard deviation, COV: Coefficient of variation, Values represent mean ± SD (n = 3)

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The Data for LOD and LOQ for different drugs are shown in table no 3, 4, and 5. The LOD values were 0.0252, 0.0098 and 0.0288 µg/ml and LOQ values were 0.0765, 0.0298 and 0.0875µg/ml for Amoxicillin trihydrate, Metronidazole and Famotidine, respectively. High recovery and low % COV revealed the reliability of the method for quantitative study of three drugs in combined dosage form.

LOD values of calibration curves indicates the lowest concentration of analyte(s) in a sample that can be detected under a stated experimental conditions and LOQ values of calibration curves indicates the lowest concentration of analyte(s) in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions [25].

Table 3 HPLC analysis of AUC of AMOX

Std. conc. (µg/ml)	Replicate					
	1	**2**	**3**	**4**	**5**	**Mean**
0.6	5378±117	5278±122	5478±119	5270±105	5288±109	5278
0.8	10553±123	10753±102	10653±127	10650±111	10656±0124	10653
1.0	13416±115	13216±132	13316±106	13300±118	13332±135	13316
1.2	15779±103	15979±109	15850±114	15908±126	15879±124	15879
1.4	18625±119	18425±108	18500±122	18550±131	18525±128	18525
Analytical parameters	LOD	0.0252µg/ml		LOQ	0.0765µg/ml	
	S**D**	120.968		Slope	15799.8	

Values represent mean ± SD (n = 3)

Table 4 HPLC analysis of AUC of METRO

Standard concentration (µg/ml)	Replicate					
	1	**2**	**3**	**4**	**5**	**Mean**
0.8	12433±337	12633±265	12533±279	12733±310	12333±283	12533
1.0	15660±268	15760±301	15560±254	15460±279	15860±281	15660
1.2	20692±290	20892±264	20792±307	20592±291	20992±279	20792
1.4	24357±304	24157±294	24257±306	24457±293	24057±300	24257
1.6	27822±313	27622±299	27922±280	27522±291	27722±297	27722
Analytical parameters	LOD	0.0098µg/ml		LOQ	0.0298µg/ml	
	SD	296.985		Slope	19488	

Values represent mean ± SD (n = 3)

Table 5 HPLC analysis of AUC of FAMO

Standard concentration (µg/ml)	Replicate					
	1	**2**	**3**	**4**	**5**	**Mean**
0.8	14392 ± 168	14192 ± 180	14292 ± 153	14200 ± 193	14492 ± 172	14292
1.0	17812 ± 175	17212 ± 192	17512 ± 186	17712 ± 155	17312 ± 182	17512
1.2	21000 ± 181	21028 ± 179	21514 ± 192	20514 ± 188	21014 ± 173	21014
1.4	25604 ± 184	25404 ± 183	25504 ± 172	25304 ± 191	25704 ± 177	25504
1.6	30247 ± 191	30047 ± 170	30147 ± 182	30157 ± 179	30137 ± 183	30147
Analytical parameters	LOD	0.0288µg/ml		LOQ	0.0875µg/ml	
	SD	**173.534**		Slope	**19829.4**	

Values represent mean ± SD (n = 3)

Conclusion

The proposed RP-HPLC method is rapid, sensitive, and reproducible, allows accurate, precise and reliable measurement of Amoxicillin trihydrate, Metronidazole and Famotidine simultaneously in combined dosage form. The RSD for all parameters was found to be less than 2%, which indicates the validity of method. Thus, the developed method can be used for routine quantitative simultaneous estimation of AMOX, Metronidazole and Famotidine in combined dosage form.

Acknowledgement:

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