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Research Article

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF AMOXICILLIN, OMEPRAZOLE AND RIFABUTIN IN SYNTHETIC MIXTURE

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ABSTRACT

Helicobacter pylori (*H. pylori*) infection is one of the most prevalent infectious diseases worldwide, which exists in almost 50% of the world's population. *H. pylori* infection plays an important role in gastric adenocarcinoma and the development of chronic gastritis, gastric ulcer, duodenal ulcer and gastric mucosa-associated lymphoid tissue lymphoma. A simple, sensitive and rapid isocratic RP-HPLC method has been developed for simultaneous estimation of amoxicillin (AMX), omeprazole (OMP) and rifabutin (RFB) in synthetic mixture. The analysis was performed on a Thermo C_{18} analytical column (250 mm × 4.6mm i.d., 5.0µm) with a mobile phase consisting of 20mM KH_2PO_4: acetonitrile (pH 4.0 with OPA) in the ratio of 20:80v/v. The UV detector was operated at 230mm and the effluents were pumped with a flow rate of 1.0 ml/min. The run time under the optimum chromatographic a condition is less than 7 min. Linearity, accuracy and precision were found to be acceptable over the concentration range of 5-25µg/ml for AMX and 1-5µg/ml for OMP and RFB. The sensitivity of the method allows the determination of the studied drugs with a limit of quantification of 1.05, 0.020 and 0.015µg/ml for AMX, OMP and RFB respectively. The proposed method was fully validated according to ICH guidelines. The high sensitivity and the simplicity of the proposed method allow the successful determination of such a ternary mixture with a percentage recovery of 99.09%±0.260, for AMX, 99.49%±0.163 for OMP and 98.10%±0.921 for RFB. The results showed that AMX, OMP and RFB together could be separated and determined simultaneously with low LOD and LOQ values using the proposed HPLC method. The method proved to be valuable for the therapeutic drug monitoring after oral administration of ternary mixture.

Keywords: *Helicobacter pylori,* RP-HPLC, Amoxicillin, Omeprazole, Rifabutin, Method validation.

1. INTRODUCTION

H. pylori, a gram-negative, microaerophilic bacterium, has been associated with gastritis, duodenal ulcer, gastric ulcer and the epidemic form of gastric ulcers [1, 2]. The association between chronic *H. pylori* infection and development of gastric cancer is well established [3, 4]. However, eradication of *H. pylori* infection has proven to be difficult [5, 6]. The current management of *H. pylori* infections relied on antibiotic therapy, consisting of a combination with two different antibiotics together with a proton-pump inhibitor as antisecretory agent with or without colloidal bismuth, which in most cases achieve a high eradication rate [7, 8]. Triple therapies used PPI, primarily omeprazole (OMP), combined with amoxicillin (AMX) and rifabutin (RFB), which are given four capsules every 8 hours with food for 14 days regimen [9]. Omeprazole (OMP, Fig. 1A) is a substituted benzamidazole (5-methoxy-2-[[(4- methoxy-3, 5-dimethyl-2-pyridinyl) methyl] sulfinyl]-1H-benzimi-dazole). OMP belongs to the class of drug known as proton pump inhibitor and is the prototype of this group [10]. OMP inhibits (Hþ=Kþ)-Atpase in the gastric parietal cells [11] and is used in the treatment of symptomatic acid reflux disease, Zollinger-Ellison syndrome [12-14] and for the eradication of *H. pylori* combined with antibacterials in dual or triple therapy like metronidazole, amoxicillin, tinidazole, clarithro-mycin and doxycycline [15]. A ternary mixture of OMP, AMX and RFB is co-formulated for treatment of *H. pylori*. OMP is extensively metabolized in the liver [16] by cytochrome P450 isoenzymes CYP2C19 and CYP3A4, to 5-Hydroxy-OMP (5-OH-OMP) and OMP-Sulfone(OMP-S), respectively [17]. Different analytical methods for determination of OMP were developed. The United States Pharmacopeia recommends HPLC as a method

for determination of OMP [18] while the British Pharmacopeia [19] recommends a potentiometric method. A review has been reported which summarized analytical methodologies for determination of OMP in formulations and biological fluids till 2007 [20] is presented. Additionally, new methods have been published including: spectrophotometry [21-26], polaro-graphy [27], TLC [28], HPLC [29-38] and capillary electrophoresis [39]. Amoxicillin (AMX Fig. 1B) is a β-lactam antibiotic drug which belongs to the group of penicillin group drugs [40]. It is a moderate-spectrum β-lactam antibiotic used to treat infections caused by penicillin-sensitive Gram-positive bacteria as well as some Gram-negative bacteria [41]. AMX is named chemically as (2S, 5R, 6R) [[(2R)-2-amino-2 (4 hydoxyphenyl) acetyl] amino]-3, 3-dimethyl-7-oxo-4-thia1-azabicyclo [3.2.0] heptanes-2-carboxyic acid [42, 43]. Various spectrophotometric, [44-47] HPLC, [48-53] HPTLC [54] and spectrofluorimetric [55] methods are reported in the literature for the estimation of AMX individually and in combination with other drugs. Rifabutin (RFB Fig. 1C) is a synthetic derivative of rifamycin S isolated from *Amycolatopsis rifamycinica* that acts by inhibiting the DNA dependent RNA-polymerase of bacteria; it has been shown to have significant mycobactericidal (hence anti-tuberculosis) activity. RFB is a less potent microsomal enzyme inducer than rifampin, therefore it is the preferred rifamycin class antibiotic for treatment of TB in HIV-infected patients. RFB is readily absorbed from the gastrointestinal tract with a Cmax of about 375ng/ml reached 3.3 h after a single 300-mg oral dose, under fasting conditions. RFB is actively degraded to its 25-O-desacetyl derivative in vitro with an activity almost equivalent to that of its parent compound [56, 57]. Few HPLC, [57-61] and capillary electrophoresis [62] methods are reported in the literature for the estimation of RFB individually and in combination with other drugs. Till now no method was reported for the simultaneous determination of OMP, AMX and RFB by RP-HPLC in their raw materials and synthetic mixture. In this paper, we report an isocratic reversed-phase HPLC method to assay OMP, AMX and RFB using a Thermo C_{18} analytical column (250 mm × 4.6mm i.d., 5.0μm) and UV detection at 230 nm. Thus results in a method with high recoveries and good linearity, accuracy, and precision.

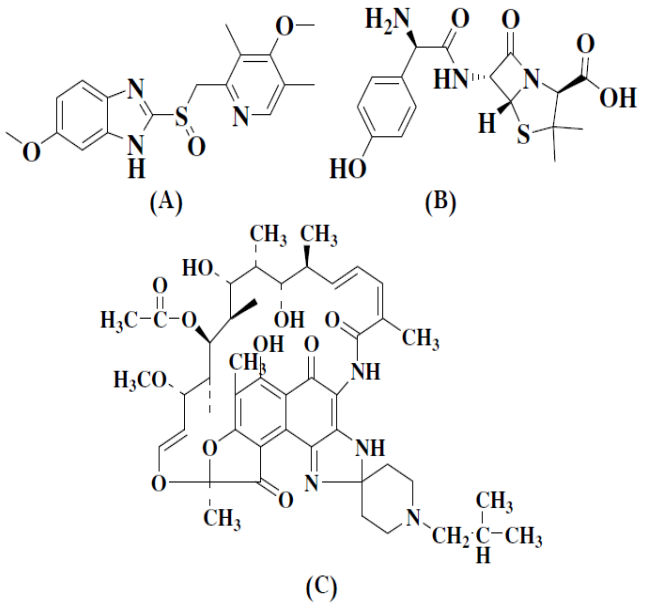


Fig. 1 Chemical structure of (A) Omeprazole (B) Amoxicillin (C) Rifabutin

2. MATERIAL AND METHODS

2.1. Instrument

Liquid chromatographic system from Waters model no 784 comprising of manual injector, water 515 binary pump for constant flow and constant pressure delivery and UV-Visible detector connected to software Data Ace for controlling the instrumentation as well as processing the generated data. A Labindia 3000+ UV/VIS spectrophotometer with 1 cm matched quartz cells was used for the estimation.

2.2. Reagents and chemicals

Amoxicillin was purchased from Zoetic Formulations Ltd. (Chennai, India) Rifabutin and omeprazole were procured as a gift sample from Simpex Pharma Pvt. Ltd (India) and Aristro Pharma (India), respectively. Potassium di hydrogen phosphates (AR grade), Disodium hydrogen phosphate (AR grade), glacial acetic acid and acetonitrile, methanol (HPLC Grade) was purchased from E. Merck Ltd. Worli, Mumbai, India. All other chemicals used were of analytical grade. Reverse osmosis water was used throughout the study.

2.3. Diluents

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials Acetonitrile was used as diluent.

2.4. Selection of mobile phase

Initially to estimate AMX, OMP and RFB in fix dosage form simultaneously, number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was 20mM KH_2PO_4: Acetonitrile (pH 4.0 with OPA) in the ratio of 20:80v/v, run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by Sonication. Flow rate employed for analysis was 1 ml/min.

2.5. Chromatographic conditions

The isocratic mobile phase consisted of 20mM KH_2PO_4: Acetonitrile (pH 4.0 with OPA) in the ratio of 20:80v/v, flowing through the column at a constant flow rate of 1.0 ml/min. A Thermo C_{18} column (250 mm × 4.6mm i.d., 5.0µm) was used as the stationary phase. By considering the chromatographic parameter, sensitivity and selectivity of method for three drugs, 230 nm was selected as the detection wavelength for UV-PDA detector. The HPLC system was operated at room temperature 25°C.

2.6. Preparation of standard stock solution

Accurately weighed 10 mg of AMX, OMP and RFB was transferred into 10 ml volumetric flasks separately and dissolved in 5 ml of acetonitrile and sonicate for 10 min., then volume was made up to 10 ml with acetonitrile. Concentration of AMX, OMP and RFB in acetonitrile was 1000µg/ml. (stock- A)

2.7. Preparation of sub stock solution

1 ml of solution was taken from stock-A of AMX, OMP and RFB and transferred into 10 ml volumetric flask separately and diluted up to 10 ml with diluent (Acetonitrile) to give concentration of 100 µg/ml (Stock-B).

2.8. Preparation of different solution

0.5ml, 1.0ml, 1.5ml, 2.0ml and 2.5ml of stock-B was taken separately in 10 ml volumetric flask and volume was made up to 10ml with (Acetonitrile). This gives the solutions of 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml for AMX. In same manner 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml, 5µg/ml of OMP and RFB also prepared.

2.9. System suitability parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1ml/min. After complete saturation of column, three replicates of working standard of AMX 15µg/ml and 3µg/ml for OMP and RFB was injected separately. Peak report and column performance report were recorded at 230 nm for all chromatogram.

2.10. Linearity and calibration graph

To establish the linearity of analytical method, a series of dilution ranging from 5-25 µg/ml for AMX, 1-5µg/ml for OMP and 1-5µg/ml for RFB were prepared. All the solution were filtered through 0.2µm membrane filter and injected, chromatograms were recorded at 230 nm and it was repeat for three times. A calibration graph was plotted between the mean peak area and respective concentration and regression equation was derived.

2.11. Analysis of synthetic mixture

Synthetic mixture were weighed and ground to a fine powder; amount equal to 25mg of AMX (1.0mg OMP and 1.25 mg RFB) was taken in 10 ml volumetric flask. Then 5ml of acetonitrile was added and the flask was sonicated for about 10 min to solubilizing the drug present in powder mixture and the volume was made up to the mark with acetonitrile. After sonication filtration was done through 0.45µ membrane filter. Filtrate was collected and further diluted with acetonitrile to get the final concentrations of all drugs in the working range. The mean area of final dilutions was observed, the concentrations were obtained from calibration curve method. The procedure was repeated for five times.

3. VALIDATION OF METHOD

As per ICH guideline the method was validated and following parameters were evaluated [63, 64].

3.1. Linearity

Linearity of AMX, OMP and RFB was established by response ratios of drug. The response ratios (response factor) were calculated by dividing the AUC with respective concentration. The curve was plotted between response ratios and concentration which shows the good linearity of drugs in the concentration ranging from 5-25µg/ml for AMX and 1-5µg/ml for OMP and RFB respectively.

3.2. Specificity
Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

3.3. Precision
Precision was determined by repeatability, Intermediate precision and reproducibility of all three drugs.

3.4. Repeatability
The repeatability was performed for five replicate at five concentrations in linearity range for AMX, OMP and RFB that indicates the precision under the same operating condition over short interval time.

3.5. Intermediate precision
3.5.1. Day to day precision
Intermediate precision was also performed within laboratory variation on different days for all three drugs simultaneously in five replicate at five concentrations.

3.5.2. Analyst- to- analyst precision
Analyst to analyst variation was performed by different analyst in five replicate at five concentrations.

3.5.3. Reproducibility
The reproducibility was performed by chemical to chemical (use of Rankem chemicals in place of Merck chemicals) variation in five replicate at five concentrations.

3.6. Accuracy (% recovery)
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 solution of AMX, OMP and RFB to preanalysed synthetic mixture solutions. The resulting solutions were then re-analysed by proposed methods. Whole analysis procedure was repeated to find out the recovery of the added drug sample. This recovery analysis was repeated at 3 replicate of 5 concentrations levels.

3.7. Robustness
As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of mobile phase was change from, 20mM KH_2PO_4: acetonitrile (20:80 % v/v), to (25:75 % v/v).

3.8. LOD and LOQ
The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

4. RESULTS AND DISCUSSION
4.1. Method development
The goal of this work was to develop and validate a simple, rapid and sensitive assay method for the quantitative determination of AMX, OMP and RFB from synthetic mixture dosage form. Initially to estimate AMX, OMP and RFB simultaneously number of mobile phases in different ratios was tried. Taking into consideration the system suitability parameter (Table 1) like RT, tailing factor, number of theoretical plates and HETP, the mobile phase was found to be most suitable for analysis was 20mM KH_2PO_4: Acetonitrile (pH 4.0 with OPA) in the ratio of 20:80v/v, run as isocratic system. The mobile phase was filtered through 0.45 m filter paper and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min. Separation variable (Table 2) was set and mobile phase was allowed to saturate the column at 1.0 ml/min. After complete saturation of column, six replicates of reference standard, 15µg/ml of AMX and 3µg/ml of OMP and RFB were injected separately.

Table 1: Results of system suitability parameters

Parameters	% Mean± SD*		
	AMX	OMP	RFB
No. of Theoretical Plates	3050±14.450	3258±14.778	3361.50±12.927
Tailing Factor	1.163±0.056	1.333±0.015	1.183±0.057
Retention time	2.128±0.015	4.618±0.005	5.894±0.002

Table 2: Separation variable of RP-HPLC method

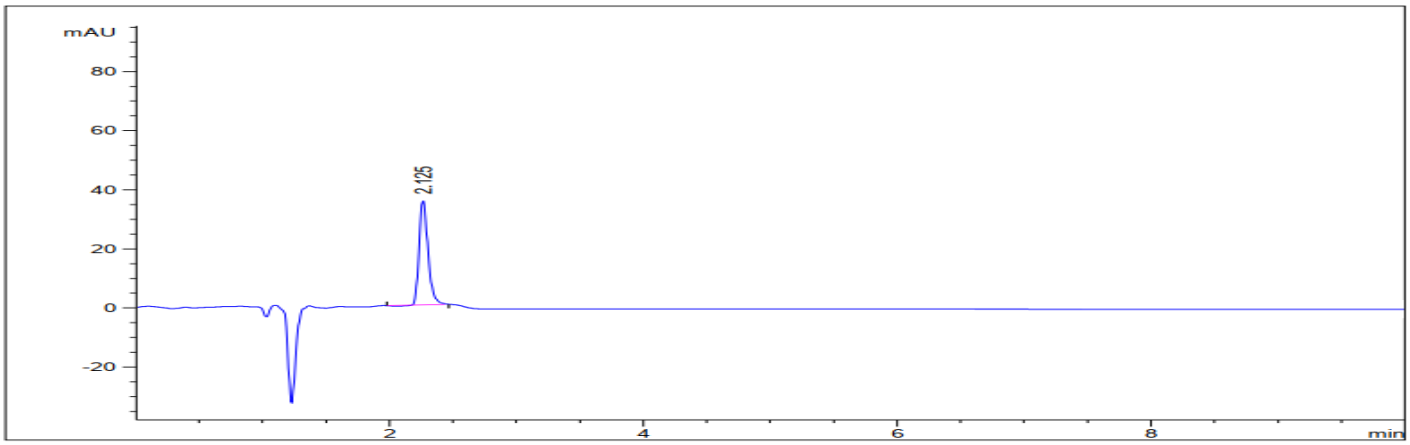
Variable	Condition
Column	
Dimension.	250mm x 4.60mm
Particle Size	5µ
Bonded Phase	Octadecylsilane (C_{18})
Mobile Phase	
20mM KH_2PO_4	20
Acetonitrile	80
Diluent	Acetonitrile
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20 µl
Detection wavelength	230mm
Retention time	
Amoxicillin	2.123 ± 0.3min
Omeprazole	4.613 ± 0.3min
Rifabutin	5.891 ± 0.3min

Peak report and column performance report were recorded. The chromatogram was recorded at 230 nm Figure 2. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph Figure 3-5. The result of their optical characteristics and linearity data of all three drugs has been reported in the Table 3.

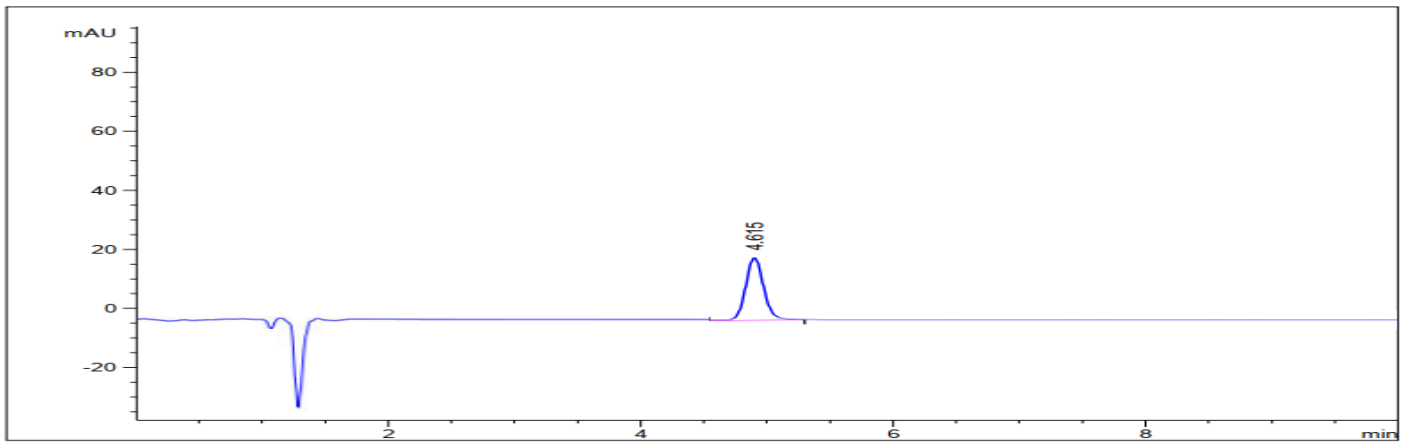
Table 3: Optical characteristics and linearity data of AMX, OMP and RFB

S. No.	Parameters	RP-HPLC Method		
		AMX	OMP	RFB
1	**Working λ**	230	230	230
2	**Concentration (µg/ml)**	5-25	1-5	1-5
3	**Correlation Coefficient (r^2)***	0.999	0.999	0.999
4	**Slope (m)***	65.54	108.4	120.6
5	**Intercept (c)***	20.88	0.071	0.083

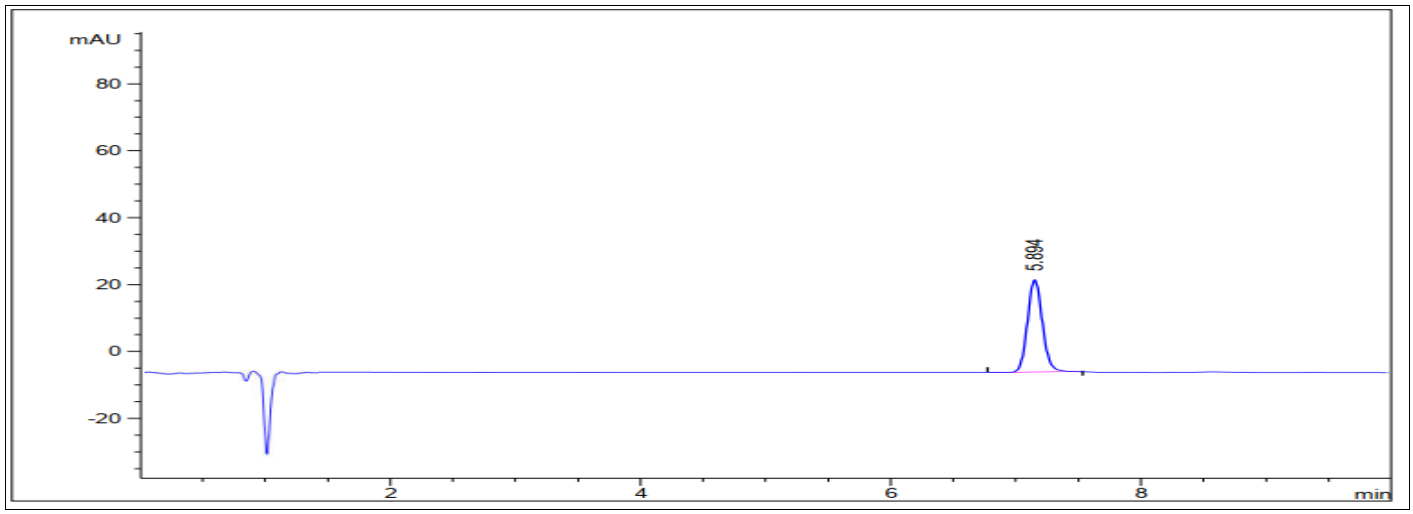
*Average of five determinations



(A)



(B)



(C)

Fig. 2: HPLC chromatogram of (A) AMX 15µg/ml, (B) OMP 3µg/ml (C) RFB 3µg/ml

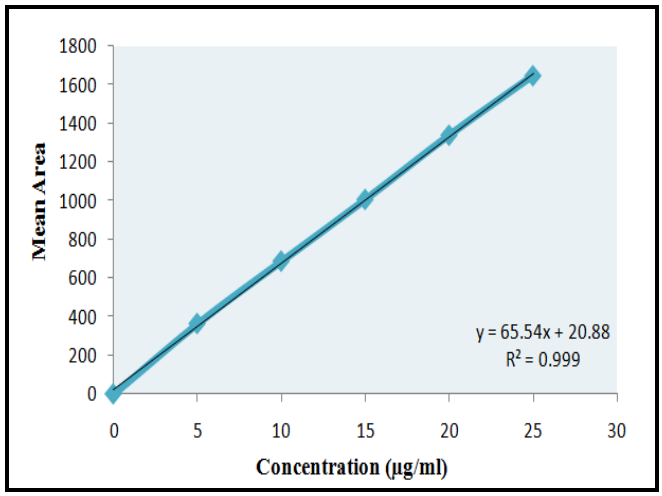


Fig. 3: Calibration curve of AMX

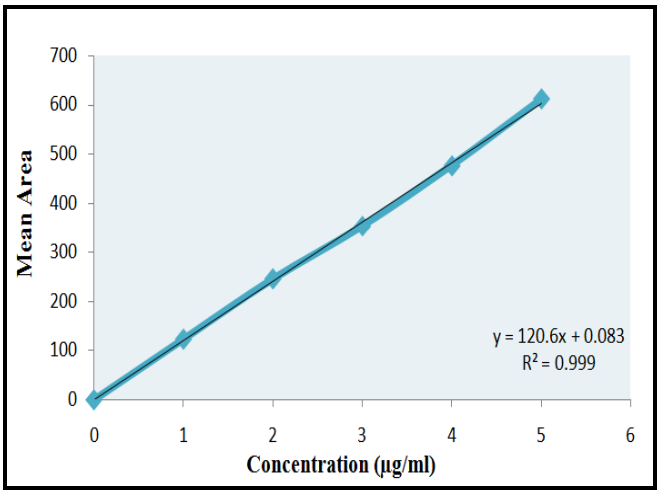


Fig. 5: Calibration curve of RFB

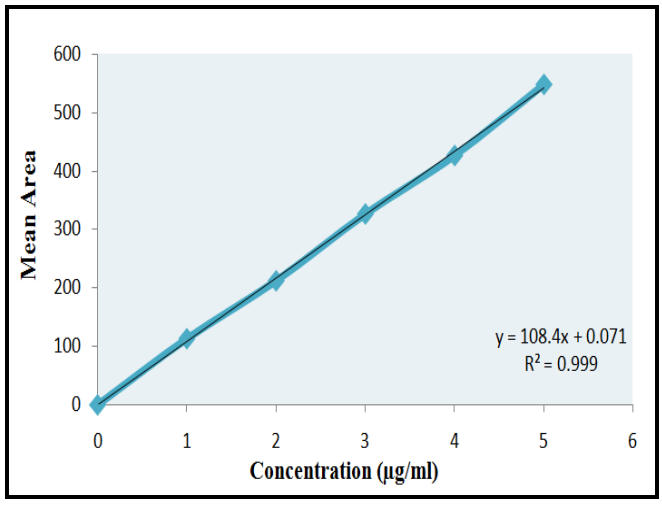


Fig.4: Calibration curve of OMP

4.2. Method validation

4.2.1. Linearity

The proposed method was found to be linear in the range of 2-25µg/ml for AMX and 1-5µg/ml for OMP and RFB respectively with correlation coefficient 0.999, 0.999 and 0.999 for AMX, OMP and RFB respectively. Linearity of AMX, OMP and RFB were established by response ratios of drug. Response ratio of three drugs was calculated by dividing the absorbance or peak area with respective concentration (Table 4).

4.2.2. Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as matrix

components. The result of specificity is shown in Figure 6 and Figure 7 as compare to blank, there was no interference seen in chromatogram.

4.2.3. Precision

Precision of the methods was studied at three levels as at repeatability, intermediate precision (Day to Day and analyst to analyst) and reproducibility (Table 5).

4.2.4. Robustness

The robustness of developed method was checked by changing in the deliberate variation in solvent. Result of robustness is shown in Table 5.

4.2.5. Accuracy

The validity and reliability of proposed methods were assessed by recovery studies. The recovery of added standards (80 %, 100 % and 120 %) was found at five replicate and five concentrations level. The values of % mean just close to 100, SD and % RSD were less than 2 which indicate the accuracy of method. Result of recovery study is shown in Table 6.

4.2.6. LOD and LOQ

Detection limit and Quantitation limit of described method were observed as 1.05 µg/ml and 2.98µg/ml for AMX, 0.020µg/ml and 0.065µg/ml for OMP, 0.015µg/ml and 0.045µg/ml for RFB, based on the SD of response and slope, which meet the requirement of new method.

4.2.7. Assay of synthetic mixture

The results of the analysis of synthetic mixture were reported. The assay value of AMX, OMP and RFB were close to 100, SD and % RSD are less than 2 which indicate that the no interference of excipients in the estimation of AMX, OMP and RFB was observed. The statistical evaluation of tablet analysis by methods has been reported in Table 7.

Table 4: Response ratios of AMX, OMP and RFB

Concentration (µg/ml)				RP-HPLC Method					
				AMX		OMP		RFB	
	AMX	OMP	RFB	AUC	RR	AUC	RR	AUC	RR
1	5	1	1	364.320	72.86	113.294	113.29	123.278	123.27
2	10	2	2	686.759	68.67	212.319	106.15	245.853	122.92
3	15	3	3	1005.956	67.06	326.615	108.87	353.541	117.84
4	20	4	4	1338.203	66.91	426.684	106.67	476.824	119.20
5	25	5	5	1645.859	65.83	548.249	109.64	610.999	122.19

Table 5: Results of precision and robustness

Parameter	% MEAN±SD*		
	AMX	OMP	RFB
Repeatability	98.737±0.116	97.043±0.081	96.563±0.093
Intermediate precision			
Day to day precision	99.371±0.059	97.716±0.039	96.889±0.069
Analyst to Analyst	99.365±0.055	96.835±0.082	97.250±0.057
Reproducibility	98.736±0.135	96.535±0.066	97.012±0.048
Robustness	98.803±0.045	98.780±0.023	98.060±0.384

*****Value of five replicate and five concentrations

Table 6: Results of recovery study

% Level	% MEAN±SD*		
	AMX	OMP	RFB
80%	99.05±0.880	98.47±1.067	98.68±0.311
100%	98.63±0.578	97.81±1.283	97.27±0.996
120%	98.90±1.262	99.06±0.808	98.91±0.956

******Value of five replicate and five concentrations*

Table 7: Analysis on synthetic mixture

Synthetic mixture	Talicia		
Label claim	AMX(250mg)	OMP (10mg)	RFB (12.5mg)
Assay (% of label claim*) Mean ± S. D.	99.09±0.260	99.49±0.163	98.10±0.921

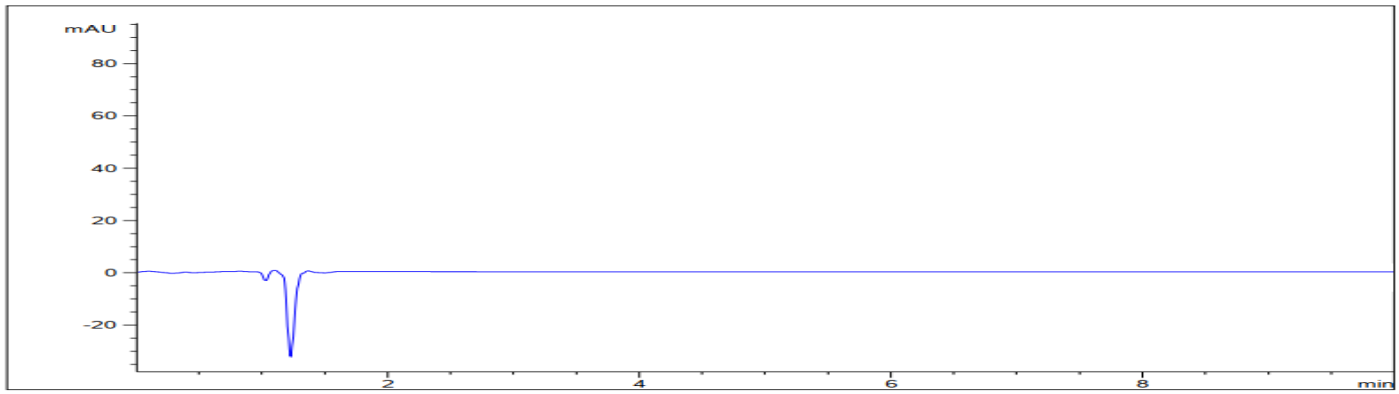


Fig. 6: Chromatogram of blank

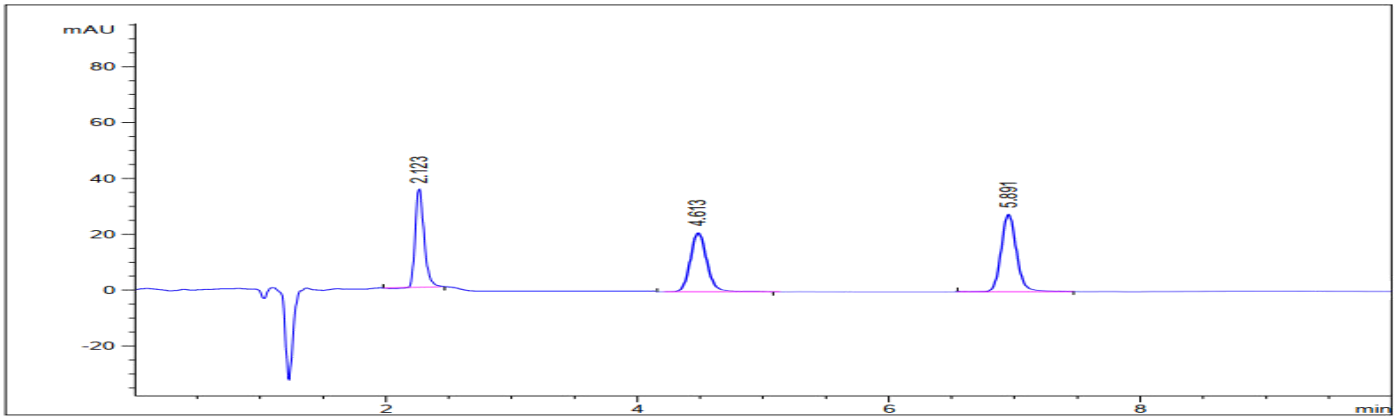


Fig.7: Chromatogram of AMX, OMP and RFB at 230nm

5. CONCLUSION

A rapid HPLC method has been developed for the simultaneous determination of AMX, OMP and RFB in their raw materials and synthetic mixture. These drug agents have been quantified with UV-Vis detector of the HPLC instrument at 230 nm wavelength. With reverse-phase Thermo C_{18} analytical column and 20mM KH_2PO_4 (pH4.0): acetonitrile (20:80v/v) mobile phase, AMX, OMP and RFB could be separated, calibrated and determined in their mixture solutions. The linear calibration curves of them were obtained in the ranges of 5-25µg/ml and 1-5µg/ml for AMX, OMP and RFB, with excellent calibration correlations (R^2: 0.999, 0.999 and 0.999) and with low LOD (1.05, 0.020, 0.015µg/ml), respectively. The percentage recoveries of the amoxicillin, lansoprazole, and levofoxacin in commercial pharmaceuticals were 99.09%, 99.49%, and 98.10%, respectively. The results showed that amoxicillin, omeprazole and rifabutin could be separated and determined rapidly and simultaneously without any separation using proposed HPLC method.

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