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Novel Development of RP-HPLC Method to Quantify Amoxicillin, Omeprazole and Rifabutin in Combination

Rama Kumar Kandula*, Raja Sundararajan
Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, INDIA.

ABSTRACT

Objectives: *Helicobacter pylori* infections are liable for most of the ulcers in the stomach and small intestinal. Talicia capsules was approved to be prescribed for the *Helicobacter pylori* infections. These capsules have combination of amoxicillin (ACN), omeprazole (OPE) and rifabutin (RFN). In this present study, for the first time, we developed a stability demonstrating RP-HPLC methodology to quantify ACN, OPE and RFN simultaneously. **Methods:** Assay of this combination was done with Thermo C_{18} stationary phase column using the mobile phase solvent system of 0.1M KH_2PO_4 buffer (3.5 pH): acetonitrile. Degradation tests were done on ACN, RFN and OPE solution by applying five different conditions, i.e. 0.1N HCl, 0.1N NaOH, 30% H_2O_2, 105°C and sun light. **Results:** Retention times of ACN, RFN and OPE were 2.539 min, 3.863 and 5.423 min, respectively. Method linearity scope was ranged from 125 – 375 µg/ml for ACN, 5 – 15 µg/ml for OPE and 6.25 – 18.75 µg/ml for RFN. The accuracy was computed in the range of 98.13–101.07% and the precision was between 0.282% and 0.569% relative standard deviation for three drugs. The method can effectively separate the degradation products from ACN, RFN and OPE. **Conclusion:** The results demonstrated that this method can be employed to quantify ACN, OPE and RFN simultaneously in presence of impurities produced during degradation investigation.

Key words: *Helicobacter pylori*, Amoxicillin, Rifabutin, Omeprazole, Stability indicating, Chromatography, Analysis.

Correspondence

Prof. S. Raja, M.Pharm., Ph.D.
GITAM Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam - 530 045, Andhra Pradesh, INDIA.
Phone no: +91-9885995823
Email: ramakumarkandula@gmail.com

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INTRODUCTION

Helicobacter pylori infections induced by the bacteria, *Helicobacter pylori*, are accountable for most of the stomach and small intestinal ulcers.[1,2] These bacteria will develop in the digestive tract and appear to target the lining of stomach. *Helicobacter pylori* afflicts the stomachs of about 60% of the adult population worldwide.[3] Talicia capsules (delayed release) is a fixed triple dose combination of amoxicillin (250 mg), omeprazole (10 mg) and rifabutin (12.5 mg). FDA approved Talicia capsules for the therapy of *Helicobacter pylori* infections in November 2019.[4,5]

Amoxicillin (ACN) is a semi-synthetic form of aminopenicillin and broad spectrum antibiotic.[6-8] ACN has bactericidal function and inhibits development of bacterial cell walls. It induces the bacterial cell wall to weaken and leads to the cell lysis. Rifabutin (RFN) is a semi-synthetic form of ansamycin and broad-spectrum antibiotic.[9-11] RFN has effective antimycobacterial features and impedes bacterial DNA-reliant RNA polymerase. This eventually led to the repression of RNA synthesis (transcription) followed by cell death in bacteria. Omeprazole (OPE) is a type of benzimidazole with proton-pump inhibitory activity.[12,13] OPE blocks H^+-K^+ ATPase enzyme present on the surfaces of parietal cells and prevents the transportation of hydrogen ions to the gastric lumen. Thus, OPE suppresses the release of the gastric acid.

Few analytical methods were described for quantifying ACN,[14-19] OPE[20-23] and RFN[24-27] alone in pharmaceutical formulations and samples of biological nature. No analytical method, for the ACN, OPE and RFN combined assay, has been published to date. Through this work, we for the first time, have established and validated a stability implying RP-HPLC method for simultaneous quantitation of ACN, OPE and RFN.

MATERIALS AND METHODS

Apparatus

ACN, OPE and RFN combined assay was performed in a Waters alliance model 2695 HPLC system fixed with column Thermo C_{18} (250 × 4.6 mm, particle dimension of 5 µm) and Waters model 2998 photodiode array detector. Waters software Empower2 program was used during ACN, OPE and RFN analyses to document and assess the chromatographic results.

Materials

Rainbow Pharma Training Lab (Telangana, India) provided reference standards of ACN, OPE and RFN. Chemicals like HCl, NaOH, H_2O_2, K_2HPO_4 and orthophosphoric acid were purchased from SD. Fine Chemicals Ltd., (Maharashtra, India). Acetonitrile was bought from Merck India Ltd., (Maharashtra, India). Pure water was bought from Milli Q purification apparatus.

Conditions for ACN, OPE and RFN Combined Assay

A Thermo C_{18} (250 × 4.6 mm, particle dimension of 5 µm) column set with 25°C temperature was used with an isocratic mobile phase having a flow at 1.0 ml/min rate. Mobile phase A was 0.1M potassium dihydrogen phosphate buffer. The buffer was fine-tuned to pH 3.5 units with 0.1% phosphoric acid. The mobile phase B was acetonitrile. Mobile phase A and B are mixed in 60:40 volume/volume ratio for analysis. Before using, mobile phase mixture was filtered through membrane filters of 0.45 pore size. 10 µl of sample was employed for the analysis. Photodiode array detector fine-tuned to 245 nm was employed for the ACN, OPE and RFN combined analyses.

ACN, OPE and RFN Combined Stock Solution

ACN, OPE and RFN combined stock solution was prepared at a concentration of 2500 µg/ml of ACN, 100 µg/ml of OPE and 125 µg/ml of RFN in mobile phase mixture solvent.

ACN, OPE and RFN Combined Working Solution

ACN, OPE and RFN combined working solution was prepared through diluting stock solution to a concentration of 250 µg/ml of ACN, 10 µg/ml of OPE and 12.5 µg/ml of RFN with mobile phase mixture solvent.

Placebo Mixture Solution

20 mg each of crospovidone, gelatin, hypromellose, hydroxypropyl cellulose, magnesium stearate, pregelatinized starch, mannitol-starch, silica, sodium lauryl sulphate, talc, sodium bicarbonate and triethyl citrate were weighed accurately into flask (100 ml). 60 ml of mobile phase mixture solvent was added. Sonicated the placebo mixture for 30 min. Filtered the placebo mixture solution through the membrane filters of 0.45 pore size and diluted to 100 ml using the mobile phase mixture solvent.

Validation

The proposed methodology was verified in keeping with "International Conference on Harmonization" strategies.[27]

Selectivity

The selectivity was assessed by comparing the chromatograms obtained after analysing the placebo solution, blank mobile phase mixture solution and combined working solution (250 µg/ml - ACN; 10 µg/ml - OPE; 12.5 µg/ml -RFN).

Linearity

Combined stock solution (2500 µg/ml - ACN; 100 µg/ml - OPE; 125 µg/ml - RFN) was diluted serially to obtain solutions in the concentration scope of 125 – 375 µg/ml for ACN, 5 – 15 µg/ml for OPE and 6.25 – 18.75 µg/ml for RFN. Each concentration solution was analysed by using the proposed method. Calibration curves of ACN, OPE and RFN were generated by determining peak area of each analyte and their respective concentrations. The regression line equations for ACN, OPE and RFN were established.

LOQ and LOD

Both the LOQ and the LOD were calculated using a signal-to - noise concept. LOQ was described as the minimal level of quantity of analyte leading to a peak height of ten times the baseline noise (i.e signal-to-noise ratio is ten). LOD was described as the minimal level of quantity of analyte leading to a peak height of three times the baseline noise (i.e signal-to-noise ratio is three).

Precision

Precision was obtained by the assessment of combined working solution (250 µg/ml - ACN; 10 µg/ml - OPE; 12.5 µg/ml -RFN) on the same day in six replicates. Determined the ACN, OPE and RFN mean peak area values and relative standard deviation values of ACN, OPE and RFN peak areas.

Accuracy

The accuracy was assessed using standard technique of addition. In this technique, previously analysed placebo solution was spiked with extra 50% (125 µg/ml - ACN; 5 µg/ml - OPE; 6.25 µg/ml - RFN), 100% (250 µg/ml - ACN; 10 µg/ml - OPE; 12.5 µg/ml -RFN) and 150% (375 µg/ml - ACN; 15 µg/ml - OPE; 18.75 µg/ml - RFN) contents of analytes. Applying the proposed RP-HPLC methodology analysed those mixtures again. The percent recovery for ACN, OPE and RFN at each level was appraised.

Robustness

Robustness was obtained by the assessment of combined working solution (250 µg/ml - ACN; 10 µg/ml - OPE; 12.5 µg/ml -RFN) with slightly modified conditions of assay. The conditions modified include: mobile phase composition (acetonitrile ratio 40 ± 5% volume), pH (3.5 ± 0.5 units), temperature (25 ± 2°C) and flow rate (1.0 ± 0.1 ml per min). The system suitability values for peaks of ACN, OPE and RFN were calculated in every modified condition of assay.

ACN, OPE and RFN Degradation Studies

0.1N HCl/0.1N NaOH Induced Hydrolysis

Accurately measured volume about 10 ml of combined stock solution (2500 µg/ml - ACN; 100 µg/ml - OPE; 125 µg/ml - RFN) was placed in a 100 ml flask. 10 ml of 0.1N HCl or 10 ml of 0.1N NaOH was added distinctly and left for 30 min ultrasonication at room temperature. The solution was neutralised with 0.1N NaOH or 0.1N HCl, respectively, after the specific time. Filtered the hydrolysed sample solution through membrane filters of 0.45 pore size and diluted to 100 ml using mobile phase mixture solvent. Applying the proposed RP-HPLC methodology, analysed the hydrolysed sample solutions. The percent recovery and percent hydrolysed values of ACN, OPE and RFN in each condition was appraised.

30% Peroxide Induced Oxidation

Accurately measured volume about 10 ml of combined stock solution (2500 µg/ml - ACN; 100 µg/ml - OPE; 125 µg/ml - RFN) was placed in a 100 ml flask containing 10 ml of 30% peroxide, mixed well and left for 30 min ultrasonication at room temperature. Filtered the oxidized sample solution through membrane filters of 0.45 pore size and diluted to 100 ml using mobile phase mixture solvent. Applying the proposed RP-HPLC methodology, analysed the oxidized sample solution. The percent recovery and percent hydrolysed values of ACN, OPE and RFN after oxidation was evaluated.

Dry heat/Sun Light Induced Degradation

Accurately measured volume about 10 ml of combined stock solution (2500 µg/ml - ACN; 100 µg/ml - OPE; 125 µg/ml - RFN) was placed in a 100 ml flask and kept in oven for 30 min at 105°C to study dry heat induced degradation and for 6 hr in sun light to study photo induced degradation. Filtered the degraded sample solutions through membrane filters of 0.45 pore size and diluted to 100 ml using mobile phase mixture solvent. Applying the proposed RP-HPLC methodology analysed the dry heat/sun light induced degraded sample solutions. The percent recovery and percent hydrolysed values of ACN, OPE and RFN after degradation was assessed.

Specificity

Specificity was obtained by the assessment of results from degradation studies. Specificity was evaluated by checking the retention times of the analyte peaks and degradation peaks in chromatograms obtained in conditions: 0.1N HCl/0.1N NaOH induced hydrolysis, 30% peroxide induced oxidation and dry heat/sun light induced degradation. Specificity was also evaluated through ACN, OPE and RFN peak purity analysis.

Statistical analysis

Statistical analysis during validation parameters study was performed by calculating standard deviation, relative standard deviation using Waters software Empower2 program.

RESULTS

Optimized Method Conditions

Complete resolution between ACN, OPE and RFN were obtained by employing Thermo C_{18} (250 × 4.6 mm, particle dimension of 5 µm) column set with 25°C temperature and with mobile phase system of 0.1M potassium dihydrogen phosphate buffer (fine-tuned to pH 3.5 units) - acetonitrile (60%:40% by volume). Flow rate was 1.0 ml per min with 10 µl of sample was injected for one analysis. Quantification of ACN, OPE and RFN simultaneously was done with photodiode array detector fine-tuned to 245 nm. Typical chromatogram of ACN, OPE and RFN using optimized method conditions was displayed in Figure 1.

Validation

The chromatograms of placebo solution and blank mobile phase mixture solution and combined working solution (250 µg/ml - ACN; 10 µg/ml - OPE; 12.5 µg/ml -RFN) are presented in Figure 2.

Linearity scope was 125 – 375 µg/ml for ACN, 5 – 15 µg/ml for OPE and 6.25 – 18.75 µg/ml for RFN. The obtained regression line equations along with regression coefficient were:

For ACN - y = 8822.3 x – 15359, (regression coefficient – 0.9992)

For OPE - y = 122654x - 6528.8 (regression coefficient – 0.9999)

For RFN - y = 117580x + 1996.8 (regression coefficient – 0.9998)

The LOD values for ACN, OPE and RFN were 1.148 µg/ml, 0.194 µg/ml and 0.114 µg/ml, respectively. The LOQ values for ACN, OPE and RFN were 3.826 µg/ml, 0.381 µg/ml and 0.648 µg/ml, respectively.

The mean peak area values were 2197763, 1168876 and 1528085 for ACN, OPE and RFN, respectively. The relative standard deviation values were 0.282 (ACN), 0.569 (OPE) and 0.291 (RFN).

The average recovery of ACN detected in the spiked placebo solution was 98.13%, 99.91% and 99.60% at 50%, 100% and 150% spiked levels, respectively (Table 1). The average recovery of RFN determined in the spiked placebo solution was 100.26% at 50% level spiked, 99.14% at 100% level spiked and 99.60% at 150% level spiked (Table 1). The average recovery of OPE determined at 50%, 100% and 150% spiked levels in placebo soliton were 101.07%, 99.33% and 100.42%, respectively (Table 1).

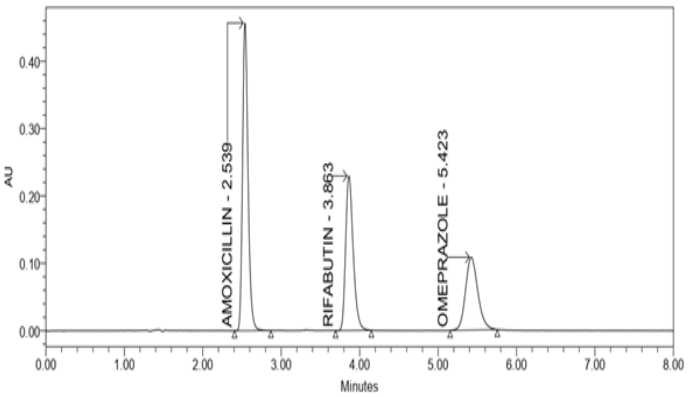

Figure 1: Typical chromatogram of ACN, OPE and RFN with optimized method conditions.

The system suitability values achieved with modified conditions of assay for parameters like plate count, resolution and tailing factor for the peaks of ACN, OPE and RFN were disclosed in Table 2. In all modified conditions of assay, good segregation between ACN, OPE and RFN was achieved.The percent recovery and percent hydrolysed values of ACN, OPE and RFN in conditions like 0.1N HCl/0.1N NaOH induced hydrolysis, 30% peroxide induced oxidation and dry heat/sun light induced degradation were summarized in Table 3. Chromatograms obtained in conditions 0.1N HCl/0.1N NaOH induced hydrolysis, 30% peroxide induced oxidation and dry heat/sun light induced degradation are shown in Figure 3. The retention times of analyte peaks

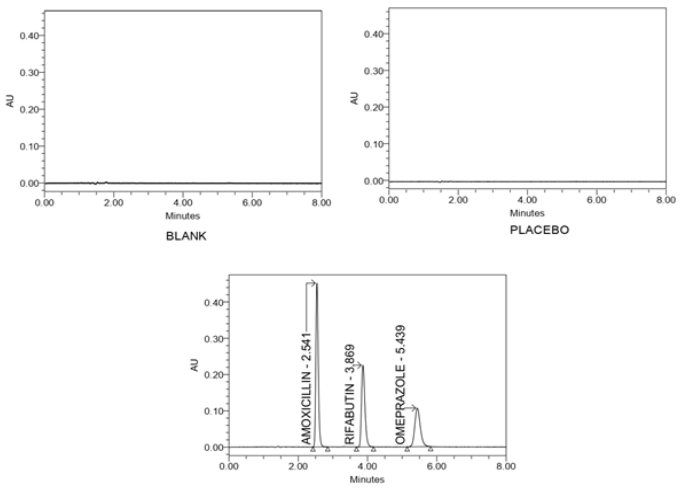

Figure 2: Chromatograms of selectivity investigation.

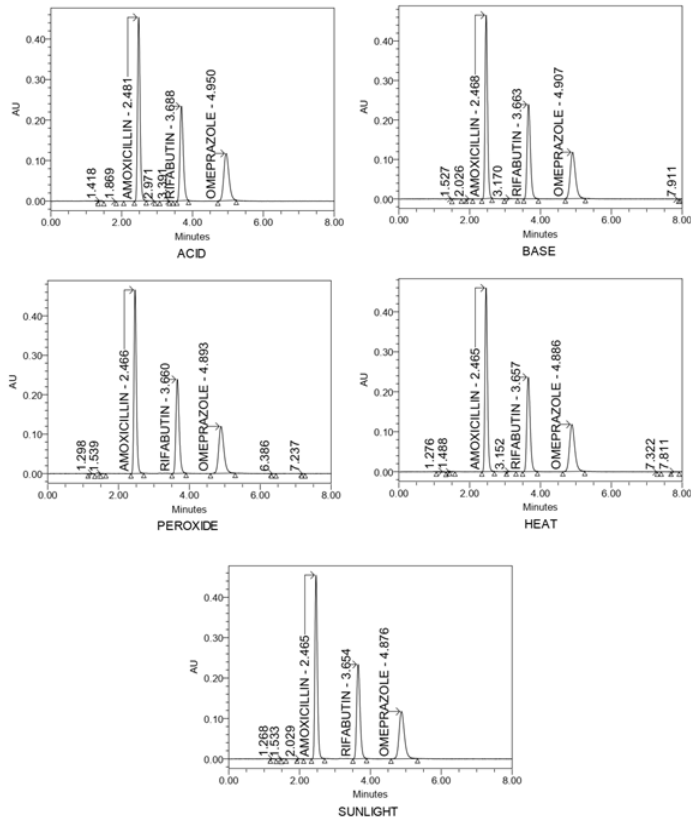

Figure 3: Representative chromatograms of ACN, OPE and RFN obtained after degradation conditions.

and degradation peaks are disclosed in Table 3. The purity angle and threshold values of ACN, OPE and RFN peaks were also disclosed in Table 3.

DISCUSSION

Quanmin and Zhanjun (2006, spectrophotometry),[14] Raju *et al.* (2016, RP-HPLC),[15] Fatma (2016, Electrochemical),[16] Marcel *et al.* (2018, HPLC),[17] Chen *et al.* (2019, HPLC-MS/MS)[18] and Ademar *et al.* (2020, Electrochemical)[19] reported methods to quantify ACN. Vital *et al.* (2009, LC-MS),[20] Preeta *et al.* (2010, HPTLC),[21] Shahrokhian *et al.* (2015, Voltametry),[22] and Alamen *et al.* (2018, spectrophotometry)[23] reported methods to quantify OPE. Jaiprakash *et al.* (20011, HPLC),[24] Hemanth *et al.* (2013, HPLC),[25] Singh and Srivastava (2018, HPLC),[26] and Sachin *et al.* (2020, HPTLC)[23] reported methods to quantify RFN. None of the methods reported quantified ACN, OPE and RFN simultaneously.[14-23] The methods of Fatma,[16] Chen *et al.*[18] Vital *et al.*[20] and Hemanth *et al.*[25] were not utilized for analysing drug in tablet dose type. A stability demonstrating HPLC methodology was developed for the first time to analyse ACN, OPE and RFN simultaneously. During development trails, critical parameters like mobile phase solvent system and stationary phase were investigated. During trails, the stationary phase investigated include YMC C_{18}, Aligent C_{18} and Thermo C_{18}. The mobile phase solvent systems investigated include 0.1 M NaH_2PO_4 buffer (3.5 pH): methanol, 0.1% phosphoric acid buffer (3.5 pH): methanol, 0.1M KH_2PO_4 buffer (3.5 pH): acetonitrile. Flow rate remains unchanged at 1.0 ml per min throughout the trails. After investigating the results of trail experiments, the mobile phase solvent system of 0.1M KH_2PO_4 buffer (3.5 pH): acetonitrile with Thermo C_{18} stationary phase column was observed as the best conditions. These conditions provided symmetrical ACN, OPE and RFN peaks and have the greatest separation efficiency and speed possible. Retention times of ACN (2.539 min), RFN (3.863) and OPE (5.423 min) recommended a fast methodology for the simultaneous evaluation of selected drug combination.

The flow rate of proposed method is less compared to Marcel *et al.*[17] method (flow rate 1.5 ml/min) and Hemanth *et al.*[25] method (flow

Table 2: Robustness calculations of method for ACN, OPE and RFN.

Condition modified	Modified value	Analyte	Plate count obtained	Resolution obtained	Tailing factor obtained
Acetonitrile ratio	35% by volume	ACN	5596	-	1.23
		RFE	6691	7.53	1.28
		OPE	4847	5.42	1.16
	45% by volume	ACN	6684	-	1.22
		RFE	7746	8.22	1.28
		OPE	5797	5.74	1.21
Temperature	23°C	ACN	5893	-	1.22
		RFE	6971	7.69	1.27
		OPE	5184	5.45	1.18
	27°C	ACN	6684	-	1.22
		RFE	7746	8.22	1.28
		OPE	5797	5.74	1.21
Flow rate	0.9 ml per min	ACN	5596	-	1.23
		RFE	6691	7.53	1.28
		OPE	4847	5.42	1.16
	1.1 ml per min	ACN	7004	-	1.23
		RFE	8234	8.46	1.29
		OPE	6309	5.92	1.23
pH value	3.4 unit	ACN	6362	-	1.22
		RFE	7423	8.51	1.30
		OPE	5481	6.55	1.19
	3.6 units	ACN	6271	-	1.23
		RFE	7437	8.47	1.30
		OPE	5598	6.51	1.18

Table 1: Accuracy calculations of method for ACN, OPE and RFN.

Added level	Added quantity (µg/ml)	Determined quantity (µg/ml)	Recovered percent (%)	Mean* Recovered percent (%)	SD and RSD
ACN recoveries					
50%	125	122.55	98.04	98.13	0.272 and 0.277
	125	123.05	98.44		
	125	122.4	97.92		
100%	250	250.45	100.18	99.91	0.442 and 0.442
	250	248.5	99.4		
	250	250.375	100.15		
150%	375	373.725	99.66	99.60	0.261 and 0.262
	375	374.325	99.82		
	375	372.4125	99.31		
RFN recoveries					
50%	6.25	6.26	100.19	100.26	0.163 and 0.162
	6.25	6.28	100.45		
	6.25	6.26	100.15		
100%	12.5	12.38	99.04	99.14	0.093 and 0.094
	12.5	12.40	99.17		
	12.5	12.40	99.22		
150%	18.75	18.72	99.86	99.62	0.317 and 0.319
	18.75	18.61	99.26		
	18.75	18.70	99.74		
OPE recoveries					
50%	5	5.06	101.1	101.07	0.098 and 0.097
	5	5.06	101.15		
	5	5.05	100.96		
100%	10	9.95	99.45	99.33	0.614 and 0.619
	10	9.87	98.66		
	10	9.99	99.87		
150%	15	14.95	99.64	100.42	0.773 and 0.769
	15	15.18	101.18		
	15	15.08	100.52		

* Mean obtained from three values; SD – standard deviation (n=3); RSD – relative standard deviation (n=3)

Table 3: Degradation and specificity calculations of method for ACN, OPE and RFN

Degraded with	Analyte	Recovered percent (%)	Degradation percent (%)	Purity angle	Purity threshold	Retention time (min) of	
						Analyte	Impurity formed
0.1N HCl	ACN	89.60	10.40	0.375	0.681	2.481	1.298, 1.539, 6.386, 7.237
	RFE	91.90	8.10	0.496	0.758	3.688	
	OPE	90.27	9.73	0.263	0.581	4.950	
0.1N NaOH	ACN	95.95	4.05	0.418	0.785	2.468	1.276, 1.488, 3.152, 7.322, 7.811
	RFE	93.91	6.09	0.395	0.661	3.663	
	OPE	93.87	6.13	0.289	0.492	4.907	
30% peroxide	ACN	93.18	6.82	0.402	0.783	2.466	1.298, 1.539, 6.386, 7.237
	RFE	96.47	3.53	0.413	0.760	3.660	
	OPE	92.75	7.25	0.388	0.592	4.893	
Dry heat	ACN	91.37	8.63	0.389	0.680	2.465	1.276, 1.488 3.152, 7.322, 7.811
	RFE	88.97	11.03	0.381	0.649	3.657	
	OPE	88.08	11.92	0.282	0.490	4.886	
Sun light	ACN	93.65	6.35	0.394	0.779	2.465	1.268, 1.533, 2.029,
	RFE	94.33	5.67	0.473	0.847	3.654	
	OPE	95.58	4.42	0.273	0.779	4.876	

rate 1.5 ml/min). Singh and Srivastava,[26] and Sachin *et al.*[23] methods use triple solvent type for mobile phase while proposed method uses binary solvent type. The runtime for single analysis is 8 min in proposed method while, it is 50 min in Quanmin and Zhanjun method,[14] more than 10 min in Raju *et al.*[15] Marcel *et al.*[17] and Hemanth *et al.*[25] methods, 9 min in Singh and Srivastava method,[26] and 20 min in Jaiprakash *et al.*[24] method. Unlike Alamen *et al.*[23] method, the proposed method did not need any derivatization of drug. No peaks were attained in chromatograms of placebo solution and blank mobile phase mixture solution at the retention times of ACN, OPE and RFN. This confirmed the absence of interreference from excipients at the studied concentrations and from mobile phase solvent mixture constituents. Thus, selectivity of the method to assay ACN, OPE and RFN simultaneously was confirmed.[28] The regression analysis data have disclosed a good linear association over the concentration scope of 25 – 375 µg/ml for ACN, 5 – 15 µg/ml for OPE and 6.25 – 18.75 µg/ml for RFN. Fair and reasonable linearity was achieved, as demonstrated by regression coefficients greater than 0.999 values in the concentration scope investigated.[28]

The proposed method has scored better regression coefficients than methods of Shahrokhian *et al.* (0.995),[22] Alamen *et al.*[23] (0.9990), Preeta *et al.* (0.9990),[21] Singh and Srivastava (0.9807),[26] and Sachin *et al.* (0.979).[27]

The values of LOQ and LOD fulfilled the sensitivity criteria for quantitative analysis of ACN, OPE and RFN simultaneously.[28] The proposed method has better LOD scores than methods of Quanmin and Zhanjun (2.0 µg/ml),[14] Ademar *et al.*[19] (3.0 µg/ml) and Alamen *et al.*[23] (0.364 µg/ml) and Sachin *et al.* (0.28 µg/ml).[27]

The relative standard deviation values of the peak areas of ACN, OPE and RFN corresponded to the precision of less than 2% relative standard deviation.[28] The proposed method was preciseness than methods of Shahrokhian *et al.*[22] (2.8%) and Hemanth *et al.*[25] (2.7% to 5.3%).

Accuracy determined at three concentrations levels ranged among 98.13 and 99.91% for ACN, 99.14 and 100.26% for RFN and 99.33 and 101.07% for OPE. The accuracy in established method was good compared to methods of Raju *et al.*[15] (< 98.0%) and Alamen *et al.*[23] (97.62-101.10%). The good recovery values demonstrated that the accuracy and also selectivity of the established method was acceptable in the quantitation of ACN, OPE and RFN when capsule excipients were present simultaneously.[28]

The findings of device suitability values demonstrated that all findings are within the acceptable boundaries, thus the process is robust. The tailing factor values for peaks of ACN, OPE and RFN are not more than 2.0%.[28] The plate count values for peaks of ACN, OPE and RFN are not less than 1000.[32] The resolution values were more than 2.[28]

Studies of degradation were conducted to test the stability of ACN, OPE and RFN under degradation conditions implemented. The order of stabilities of ACN, OPE and RFN were:

ACN: 0.1N NaOH >Sun light > Peroxide > Dry heat > 0.1 N HCl

RFN: Peroxide > Sun light > 0.1N NaOH > 0.1 N HCl > Dry heat

OPE: Sun light > 0.1N NaOH > Peroxide > 0.1 N HCl > Sun light

The stability of ACN, OPE and RFN under degradation conditions implemented were not presented in methods of Quanmin and Zhanjun,[14] Raju *et al.*[15] Fatma,[16] Marcel *et al.*[17] Chen *et al.*[18] Ademar *et al.*[19] Vital *et al.*[20] Shahrokhian *et al.*[22] Alamen *et al.*[23] Hemanth *et al.*[25] Singh and Srivastava,[26] and Sachin *et al.*[23] Specificity was assured by ample separation of ACN, OPE and RFN peaks from each other and from additional other peaks originated during 0.1N HCl/0.1N NaOH induced hydrolysis, 30% peroxide induced oxidation and dry heat/sun light induced degradation conditions.[29] In the specificity test, the angles of purity for ACN, OPE and RFN peaks were observed to be decreased than for the purity thresholds, in samples of 0.1N HCl/0.1N NaOH induced hydrolysis, 30% peroxide induced oxidation and dry heat/sun light induced degradation conditions. These results undoubtedly indicated that the ACN, OPE and RFN peaks were pure and this confirmed the specificity and stability indicating feature of the developed RP-HPLC methodology.[29]

CONCLUSION

In this present study, for the first time, we have developed an easy and speedy stability demonstrating RP-HPLC method to quantify ACN,

OPE and RFN simultaneously. Validation approaches disclosed adequate selectivity, sensitivity, precision, specificity, robust and accuracy for the developed method. These results suggested that this developed method can be employed as a reliable quantification method in the estimation of ACN, OPE and RFN simultaneously.

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CONFLICT OF INTEREST

The authors declare no Conflict of interest.

ABBREVIATIONS

RP - HPLC: Reverse phase High performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantitation; **RSD:** Relative standard deviation; **ICH:** International conference on Harmonization; **ACN:** Amoxicillin; **OPE:** Omeprazole; **RFN:** Rifabutin; **LC-MS:** Liquid chromatography with mass spectrometer; **HPTLC:** High performance thin layer chromatography.

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