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RESEARCH ARTICLE

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Simultaneous determination of Amoxicillin trihydrate, Pantoprazole sodium and Clarithromycin in bulk powder and tablet formulation by an isocratic RP-HPLC method

Abstract:

Sensitive, rapid and precise an isocratic RP-HPLC method was developed and validated for simultaneous determination of Amoxicillin trihydrate (AMO), Pantoprazole Sodium (PAN) and Clarithromycin (CLA) in bulk powder and pharmaceutical formulation. A mixture of Methanol: Buffer (Ammonium acetate) (70:30 v/v, pH 4) was used as a mobile phase. The stationary phase used was HiQsil C18HS (4.6 × 250 mm, 5µm) analytical column. The flow rate was 1 ml/min and the detection was set at 240.2 nm. The method was linear in the range of 5-25 µg/ml for AMO, PAN and CLA respectively. The selectivity of the proposed method was checked using laboratory prepared mixtures. The validated method was successfully applied to the analysis of AMO, PAN and CLA in mixture and in their pharmaceutical dosage form without interference from other additives.

Key words: Isocratic, RP-HPLC, analytical column, flow rate.

1. INTRODUCTION:

Amoxicillin trihydrate (AMO) is chemically known as (2S,5R,6R)-6-{[(2R)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-carboxylic acid trihydrate (Figure 1). Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as penicillin. For this cause, it is frequently combined with clavulanic acid, a β-lactamase inhibitor. This drug combination is commonly called co-amoxiclav. Mixing the drugs increases effectiveness by reducing susceptibility to β-lactamase resistance.(Xiaofeng&Zhenghua, 2006).

Amoxicillin is used in the handling of a number of infections, including acute otitis media, streptococcal pharyngitis, pneumonia, skin infections, urinary tract infections, Salmonella infections, Lyme disease, and chlamydia infections (Chakravarthy et al., 2010). Amoxicillin trihydrate is official in the B.P., (Madhura et al., 2011) where it was found out by chromatography system. A study of the literature revealed that Amoxicillin has been estimated in pharmaceuticals by UV-visible spectrophotometric (Kamal et al., 2008; Hesham et al., 2002), spectrofluorimetry (El Walily et al., 1999) and chromatographic methods (Jani , 2014; Dhoka& Joshi, 2010; Shanmugasundaram et al., 2009; Angela & Kumar, 2011; Solanki&Badri, 2013; Rajput &Bhamre, 2014; Jadhav&Salunkhe, 2013; Numan&Majed, 2009; Patel &Varshney, 2014).
Pantoprazole Sodium (PAN), 5-(difluoromethoxy)-2-[[(3,4-dimethoxy-2-pyridinyl)methyl]sulphinyl]-1H-benzimidazole (Figure 2), is a selective and long acting proton-pump inhibitor used for treatment of acid-associated gastrointestinal disorders. The proton-pump inhibitor Pantoprazole inhibit gastric acid by blocking the H+ /K+ -adenosine triphosphatase enzyme system (the proton pump) of the gastric parietal cell. It is employed for short-term treatment of erosion and ulceration of the esophagus (Edwin, 2001).

Different analytical methods are described in the literature for the assay of Pantoprazole Sodium in dosage forms and in biological fluids, including spectrophotometry (Moustafa, 2000), HPTLC (Seema et al., 2006) and HPLC (Khanage et al., 2013; Siddartha&Sudheer, 2013; Jagatiya, 2012; Shitole&Gurjar, 2015; Mohideen, et al., 2011).

Clarithromycin (CLA) is a semi-synthetic macrolide antibiotic derived from Erythromycin with parallel actions and uses. Because of its good antimicrobial activity against a broad range of Gram-positive and Gram-negative organisms, Clarithromycin is used to treat the respiratory tract infections and skin and soft tissue diseases (Sweetman, 2011; Rodvold, 1999). Clarithromycin inhibits bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit. Chemically, Clarithromycin is described as (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R) -6-{[(2S,3R,4S,6R) -4-(dimethylamino) -3-hydroxy-6-methyloxan-2- yl] Oxy} -14-eth yl-12,13-dihydroxy-4-{[(2R,4R,5S,6S) -5-hydroxy-4- methoxy -4,6-dimethyloxan-2-yl] Oxy} -7-methoxy-3,5,7,9,11,13-hexa methyl-1-oxacyclotetradecane-2,10-Dione (Figure 3).

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Clarithromycin is as a Cytochrome P450 3A4 inhibitor, Cytochrome P450 3A Inhibitor, and P-Glycoprotein Inhibitor (Chey& Wong, 2007, Soichiro& Bruce, 2010). A study of the literature revealed that Clarithromycin has been estimated in pharmaceuticals by UV-visible spectrophotometric (Bakhtiar et al., 2013), TLC (Salem &Safa, 2013) and HPLC (Birch & Chan, 2001; Salem, 2014; Seyed, 2013).

Amoxicillin trihydrate, Pantoprazole sodium and Clarithromycin are present in tablets (PANTOCID HP Compound) are applied for treatment of respiratory, genitourinary, skin and soft tissue infection. As per literature triple therapy can be used with Pantoprazole, Clarithromycin and Amoxicillin for eradication in patients with Helicobacter pylori positive duodenal ulcers (Ghazzawi*et al*., 2004). Up to our cognition, there is no isoocratic RP-HPLC method was identified for the simultaneous determination of the three studied drugs in their laboratory prepared mixtures and in the pharmaceutical dosage form. The present work aimed to develop and validate an isocratic RP-HPLC method for simultaneous determination of AMO, PAN and CLA in laboratory prepared mixtures and pharmaceutical dosage form.

2. EXPERIMENTAL PART:

2.1 Instruments

Liquid chromatography was performed on JASCO Isocratic HPLC system model LC-NET II/ADC (JASCO Corporation, Japan) The system built with highly sensitive PDA detector and HiQsil C18HS (4.6 × 250 mm, 5µm) column with a 20 µL manual sample injector. The HPLC system was equipped with Chrom-NAV software for data processing.

2.2 Chemicals and reagents

The standard drug Amoxicillin Trihydrate was obtained from Wockhardt Pharmaceutical Ltd., Aurangabad, India. Pantoprazole Sodium was obtained fromVasudhaPharma, Chemical Pvt. Ltd., Ahmedabad, India. Clarithromycin was procured from Ajanta pharma Ltd. Paithan, Dist-Aurangabad, India. HPLC grade water was acquired from LobaChemie Mumbai, India. HPLC grade Acetonitrile, Acetic acid, Iso-propyl Alcohol and Methanol were purchased from Merck Ltd., India. EP grade buffering agent Orthophosphoric Acid, Ammonium acetate and triethylamine were purchased from Fisher scientific, Mumbai, India.

2.3 ANALYTICAL METHOD:

2.3.1 Chromatographic system and conditions

The compounds AMO, PAN and CLA were eluted off the column with a mobile phase containing Methanol: Buffer (Ammonium acetate) (70:30 v/v) and the Buffer was adjusted to pH 4 by Glacial acetic acid for RP-HPLC system. The flow rate was 1.0 mL/min and effluent was monitored at analytical wavelength 240.2 nm. The retention time of AMO, PAN and CLA were 1.941 min, 3.331 min and 2.518 min, respectively and the total run was 10 min as specified in Table 1.Prior to analysis mobile phase was filtered through a 0.45 µm nylon filter and then ultrasonicated for 30 min. The method was validated in accordance with the International Conference onHarmonization guidelines for validation of analytical procedures (ICH, 2000; ICH, 2005).

2.3.2 Preparation of buffer solution

The buffer preparation was done by dissolving 1.925 gm of Ammonium acetate in 100 Ml HPLC grade water, pH adjusted to 4 by using glacial acidic acid.

2.3.3 Preparation of mobile phase

Initially buffer was prepared by using the 1.925 gm of Ammonium acetate in 100 mL HPLC grade water, pH adjusted to 4 by using Glacial acidic acid, then 20 min ultra-sonication of this buffer solution was done and Methanol: Buffer (70:30 v/v), the prepared mobile phase was degassed by ultra-sonication for about 20 min, lastly the mobile phase after degassing was filtered through 0.45µm membrane nylon filter.

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2.3.4 Preparation of standard stock solutions
AMO 10 mg, PAN 10 mg and CLA 10 mg were accurately weighed on electronic balance and dissolved in 50 mL of mobile phase separately with shaking. Then the resulting solutions were sonicated and the volume was made up to 100 mL by addition of mobile phase to get the conc. 100 µg/mL. From the standard stock solution of drugs, appropriate dilutions were made with the mobile phase and the sample was filtered through 0.2 µm membrane nylon filter.

2.3.5 Loading of mobile phase
Filtered and degassed mobile phase was loaded in the 500 mL reservoir. Priming was done in each freshly prepared mobile phase.

2.3.6 Baseline stabilization
The detector was turned on for an hour before the actual run in order to obtain the stable UV light. The mobile phase run was started at the desired flow rate and the run was continued until the stable baseline was obtained.

2.3.7 Loading of samples
Well prepared and filtered samples of AMO, PAN and CLA were loaded into the Rheodyne injector port using a 2 mL glass syringe and then the sample was injected.

2.3.8 Construction of calibration curves
Working solutions were prepared immediately before use to cover the concentration ranges from (5-25 µg/mL) for AMO, PAN and CLA injected into the column and the chromatogram was performed. A graph was plotted as concentration of each drug against response (peak area) and it was found to be linear for all the drugs.

2.4 Assay of pharmaceutical formulations
Twenty tablets of each formulation containing 750 mg AMO, 40 mg of PAN and 500 mg CLA were weighed and powdered. The tablets were crushed to fine powder and the amount of powder equivalent to 75 mg AMO, 4 mg PAN and 50 mg CLA was weighed accurately, and then transferred to 100 mL dried volumetric flask. Sufficient quantity of mobile stage was added to break up the content and resulting solution was stirred for 20 minute. The volume was made up to 100 mL with the mobile phase and then filtered through membrane filter and degassed in sonicator. From this solution appropriate dilutions of AMO, PAN and CLA were made to make the final concentrations. After that sample was injected into the HPLC system to get chromatogram. The chromatogram obtained is presented in Figure 9 and the area obtained in each chromatogram of three replicates was correlated with regression equation and the quantity found was calculated, which was within the limit and results obtained are recorded in Table 2.

3. RESULTS AND DISCUSSION:

3.1 Optimization of chromatographic conditions
Chromatographic parameters comprising wavelength detection, mobile phase composition and proportions, pH and flow rate were prudently studied in order to identify the most appropriate chromatographic condition for analysis. The choice was based on the number of theoretical plates and best resolution in a reasonable time.

3.2 Selection of analytical wavelength
By appropriate dilution of each standard stock solution in the mobile phase, various concentrations of AMO, PAN and CLA were prepared separately. Each solution was scanned in between the range of 200-400 nm using UV-Visible double beam spectrophotometer (V-630 JASCO Corporation, Japan),Spectroscopic analysis of the drugs showed that AMO, PAN and CLA have maximum absorbance at 230 nm, 254 nm and 260 nm, respectively, then their overlain spectrum was taken (Figure 4). The isoabsorptive point was observed at 240.2 nm in the overlain spectrum. The wavelength selected for the HPLC analysis was 240.2 nm to which these three drugs showed maximum absorbance and good resolution of peaks.

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3.3 Linearity and range

Linearity study for the proposed method was established by least square linear regression analysis. Linearity was assessed by a plot of concentration versus peak area. The calibration graphs were found to be linear in the range of 5-25 µg/mL, 5-25 µg/mL and 5-25 µg/mL, respectively for AMO, PAN and CLA with correlation coefficient values 0.998, 0.997 and 0.998 respectively as indicated in Table 3, 4 and 5.

3.4 Accuracy (Recovery study)

The accuracy was performed by standard addition method. Three replicate injections, each of three different test concentrations at the level of 50, 100 and 150% were studied. The accuracy and reproducibility is apparent from the data as results are close to 100% and the value of standard deviation and % R.S.D were found to be < 2%, which shows the method is highly précised and accurate. The recovery study is indicated in Table 6, 7 and 8.

3.5 Precision

Precision/repeatability study was carried out using analysis of the drug by intra-day and inter-day variability. Results indicated that the % RSD found less than 2. The precision study for AMO, PAN and CLA was carried out by inter-day, which is discussed in Table 9, 10 and 11 and intra-day study has shown in Table 12, 13 and 14.

3.6 Limit of Detection (LOD)

The value for LOD was calculated from the following formula

$$LOD=3.3\sigma/S$$

Where, σ= Standard deviation of the response,

S= Slope of the calibration curve.

The Limit of detection (LOD) for AMO, PAN and CLA was found to be 0.02667 µg/mL, 0.0153 µg/mLand 0.0886 µg/mL respectively.

3.7 Limit of Quantitation (LOQ)

The value for LOQ was calculated from the following formula

$$LOQ=10\sigma/S$$

Where, σ= Standard deviation of the response,

S= Slope of the calibration curve.

The Limit of Quantitation (LOQ) for AMO, PAN and CLA was found to be 0.08084 µg/mL, 0.0464 µg/mL and 0.08272 µg/mL respectively.

3.8 Selectivity

After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity and accuracy. The optimized parameters were found to be suitable as well as there was no observation of any peak of the excipients or impurity other than the peak of AMO, PAN and CLA during experimental work, hence the proposed method was selected for development. Comparison of the chromatograms obtained from the mobile phase (blank), AMO, PAN, CLA standards and the tablet formulation revealed no significant interference, using same chromatographic conditions for all samples. Figure 5-10 are referring to the selective method for the analyte concerned.

3.9 Ruggedness

Different parameters like different laboratory condition, different source of reagents/solutions and different analyst, as a result, there was no any significant change in the optimized parameters were observed as indicated in Table 15.

3.10 Robustness

The method must be robust enough to withstand slight changes and allow routine analysis of samples. Robustness of the method were determined by carrying out the analysis under conditions during which change in flow rate, change in the organic composition of the mobile phase, change in pH, and change in analytical wavelength were studied. Variation of organic composition in the mobile phase, pH, wavelength and flow rate were seemed to have no significant impact on resolution, peak area, tailing factor, retention time and theoretical plate. The observations of robustness study are shown in Table 16-19.

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3.11 Solution stability

Stability in solution was evaluated by the standard solution and the test preparation. The solution was stored at ambient temperature without protection from light and tested after 12, 24, 36, and 48 hrs. The responses for the aged solution were evaluated by comparison with freshly prepared solutions. The stability study of the stored standard solution and test preparation was performed and solutions were found to be stable for up to 48 hrs. The assay values obtained after 48 hr were statistically identical with the initial value without measurable loss as shown in Table 20.

4. CONCLUSIONS:

An isocratic RP-HPLC method has been developed for the simultaneous estimation of mixture a of AMO, PAN and CLA. The developed method was validated in accordance with ICH guidelines and it was found to be simple, precise, accurate and sensitive. Excipients present in the tablets show no interference in the determination. The proposed method can be used in quality control laboratories for routine analysis of AMO, PAN and CLA in their pure mixtures and pharmaceutical preparations.

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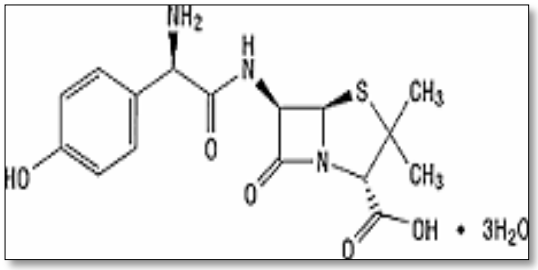

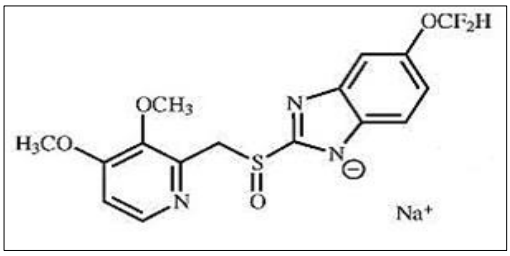

Figure 1.Chemical Structure of AMOFigure 2. Chemical Structure of PAN

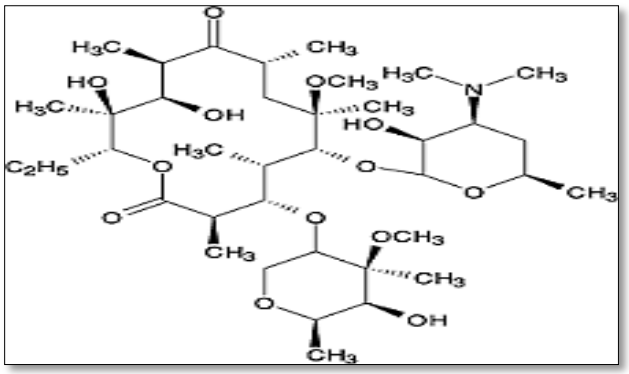

Figure 3. Chemical Structure of CLA

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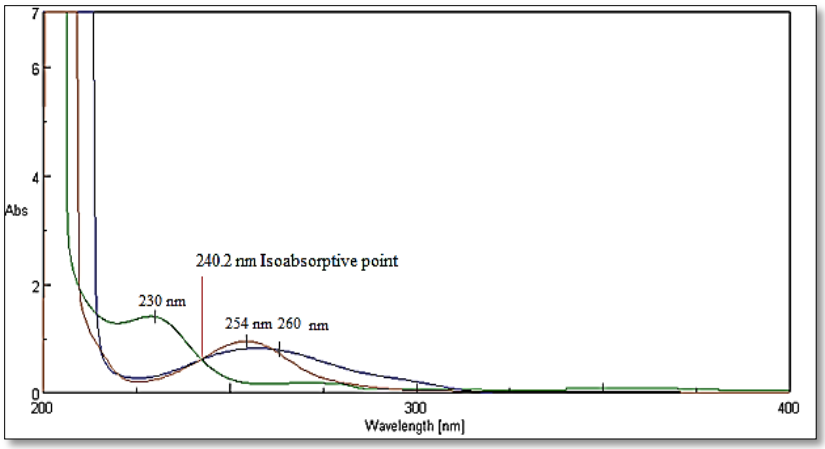

Figure 4.Overlain Spectra of AMO, PAN and CLA (240.2 nm) in optimised mobile phase

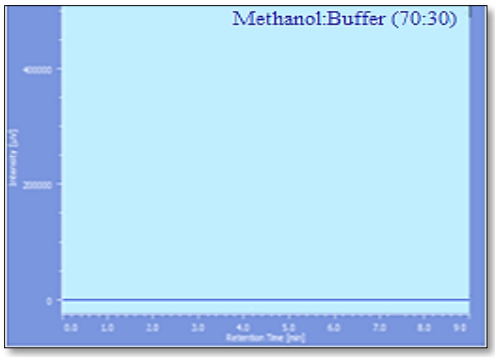

Figure 5.Chromatogram of selected M.P

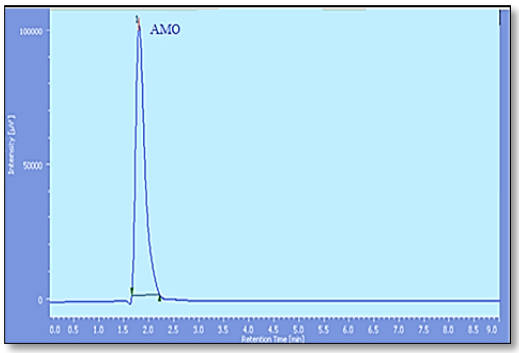

Figure 6. Chromatogram of AMO 5 µg/mL

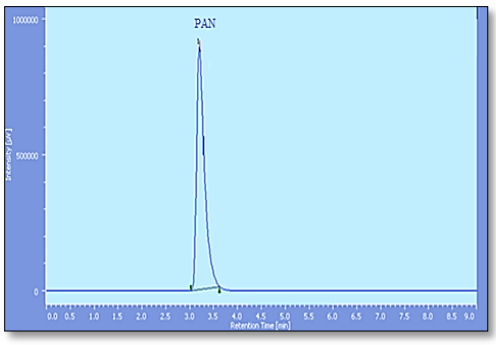

Figure 7.Chromatogram of PAN 5 µg/mL

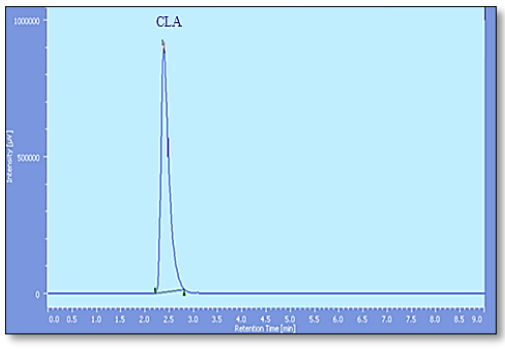

Figure 8. Chromatogram of CLA 5µg/mL

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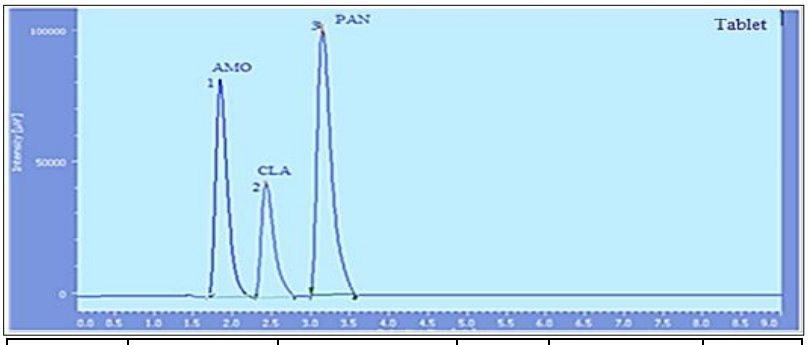
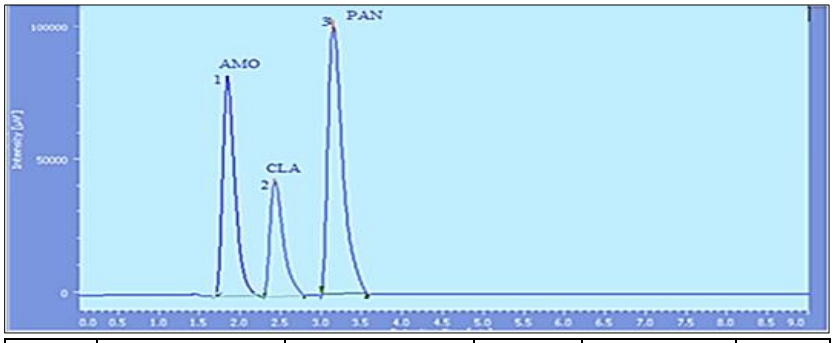


Drug	Retention Time(min)	Peak Area(µV/sec)	% area	Symmetric factor	NTP
AMO	1.941	109408	100	1.42	3053
PAN	3.331	105279	100	1.36	2535
CLA	2.518	107566	100	1.58	3359

Figure 9.Chromatogram of Tablet formulation



Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Symmetric factor	NTP
AMO	1.923	104046	100	1.409	3043
PAN	3.319	104663	100	1.350	2517
CLA	2.532	107765	100	1.521	3361

Figure 10. Chromatogram of working standard

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Table 1. Optimal chromatographic conditions of RP-HPLC analysis

Parameters	Chromatographic conditions
HPLC System	Jasco HPLC system, Japan
Pump	PU-2080 plus HPLC pump
Detector	UV-2075 plus as UV-VIS PDA detector
Column	HiQsil C18 HS (4.6 mm×250 mm) column
Column temperature	Ambient
Mobile phase	Methanol: Buffer (Ammonium acetate) (70:30 v/v), pH 4
Detection of Wavelength	240.2 nm
Flow rate	1 mL/min
Sample volume	20 µL
Run time	10 min
Retention time	Amoxicillin trihydrate : 1.941 min Pantoprazole sodium : 3.331 min Clarithromycin : 2.518 min

Table 2. Analysis of Tablet Formulation

Brand Name of Tablet Formulation	Drug	Label Claim	Peak area (µV/sec)	% of label claim determined	Mean %	SD*	RSD*
PANTOCID HP (Compound) By-Sun Pharma Ltd.	AMO	750	109256	99.89%	99.73%	0.2192	0.2197
	PAN	40	105196	99.12%	99.17%	0.2547	0.2568
	CLA	500	107245	99.75%	99.65%	0.1446	0.1451
PANTOP HP By-Aristo Pharma, Ltd.	AMO	750	109364	99.58%	99.48%	0.2433	0.2451
	PAN	40	104555	99.22%	99.76%	0.1918	0.1962
	CLA	500	109421	99.55%	98.69%	0.2316	0.2389

* indicates avreage of three determination

Table 3. Linearity data for AMO

Standard conc →	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates ↓	**Peak area**				
1	109213	221210	341939	432446	541892
2	109281	221392	342333	432314	542013
3	109301	221991	342415	432610	541916
4	109340	221492	342319	432214	542011
5	109410	222113	342412	432319	542115
Mean	109309	221639	342283	432380	541989
±SD	65.15	395.21	197.60	115.38	88.94
RSD	0.05960	0.1783	0.0577	0.0266	0.01640
Correlation coefficient (R^2)	0.998				

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Table 4. Linearity data for PAN

Standard conc. →	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates ↓	Peak area				
1	104550	231644	361268	457984	548588
2	104566	231656	361366	457983	548434
3	104538	231632	361310	457881	548434
4	104432	231522	361322	457921	548510
5	104511	231620	361323	457822	548521
Mean	104519	231614	3613171	457918	548494
±SD	47.25	47.92	79.25	141.18	202.41
RSD	0.0451	0.0206	0.0219	0.0308	0.0036
Correlation coefficient (R^2)	0.997				

Table 5. Linearity data for CLA

Standard conc. →	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates ↓	Peak area				
1	107683	211644	333912	422432	531892
2	107551	211323	333401	422533	531911
3	107682	212213	333821	422725	531635
4	107462	211492	333951	422419	532116
5	107552	212013	333816	422735	531965
Mean	107586	211737	333902	422568	531903
±SD	95.37	371.62	81.537	153.66	174.08
RSD	0.0886	0.1755	0.0244	0.0363	0.0327
Correlation coefficient (R^2)	0.998				

Table 6. Recovery study of AMO

Recovery level %	Amoxicillin trihydrate						
	Amt. Taken (µg/mL)	Amt. added (µg/mL)	Total amount (µg/mL)	Amt. recovered (µg/mL)	% recovery	Average recovery % ± SD	RSD
50%	5.0	2.5	7.5	7.428	99.04	98.92 ±0.7071	0.714
	5.0	2.5	7.5	7.440	98.66		
	5.0	2.5	7.5	7.433	99.06		
100%	5.0	5.0	10	9.803	98.03	98.28 ±0.5186	0.527
	5.0	5.0	10	9.901	99.01		
	5.0	5.0	10	9.782	99.82		
150%	5.0	7.5	12.5	12.413	99.58	99.01 ±0.0469	0.047
	5.0	7.5	12.5	12.401	99.02		
	5.0	7.5	12.5	12.339	98.43		

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Table 7. Recovery study of PAN

Recovery level %	Pantoprazole sodium						
	Amt. taken (µg/mL)	Amt. added (µg/mL)	Total amount (µg/mL)	Amt. recovered (µg/mL)	% recovery	Average recovery% ± SD	RSD
50%	5.0	2.5	7.5	7.476	99.68	99.05 ±0.7273	0.341
	5.0	2.5	7.5	7.482	99.16		
	5.0	2.5	7.5	7.479	99.06		
100%	5.0	5.0	10	9.989	99.89	99.60 ±0.4558	0.018
	5.0	5.0	10	9.982	99.83		
	5.0	5.0	10	9.983	99.15		
150%	5.0	7.5	12.5	12.345	98.70	98.382 ±0.0917	0.042
	5.0	7.5	12.5	12.473	99.76		
	5.0	7.5	12.5	12.251	98.00		

Table 8. Recovery study of CLA

Recovery level %	Clarithromycin						
	Amt. taken (µg/mL)	Amt. added (µg/mL)	Total amount (µg/mL)	Amt. recovered (µg/mL)	% recovery	Average recovery% ± SD	RSD
50%	5.0	2.5	7.5	7.469	99.13	99.57 ±0.3894	0.391
	5.0	2.5	7.5	7.478	99.86		
	5.0	2.5	7.5	7.480	99.73		
100%	5.0	5.0	10	9.893	98.93	98.92 ±0.3351	0.389
	5.0	5.0	10	9.925	99.25		
	5.0	5.0	10	9.858	98.58		
150%	5.0	7.5	12.5	12.422	99.36	99.43 ±0.0750	0.075
	5.0	7.5	12.5	12.435	99.44		
	5.0	7.5	12.5	12.439	99.51		

Table 9. Inter-day variability of AMO

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD*	RSD*
	Day 1	Day 2	Day 3			
5	109213	109330	109225	109256	97.22	0.0163
15	341910	342235	342307	342150	138.12	0.0185
25	541892	541982	541822	541898	315.61	0.0145

* indicates avreage of three determination

Table 10. Inter-day variability of PAN

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD*	RSD*
	Day 1	Day 2	Day 3			
5	104550	104552	104549	104550	526.86	0.0368
15	361261	361460	361663	361461	211.00	0.0581
25	548588	548316	548141	548315	235.24	0.0493

* indicates avreage of three determination

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Table 11. Inter-day variability of CLA

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD*	RSD*
	Day 1	Day 2	Day 3			
5	109412	109314	109438	109288	65.39	0.0498
15	332392	332593	332513	332499	101.19	0.0028
25	531792	531992	532093	531959	443.48	0.0130

* indicates avreage of three determination

Table 12. Intra-day variability of AMO

Conc. (µg/mL)	Peak area (µV/ sec)			Mean area (µV/sec)	± SD*	RSD*
	Trial 1	Trial 2	Trial 3			
5	109280	109416	109422	109372	126.62	0.0168
15	341933	341287	341810	341676	58.021	0.0360
25	541882	541922	541983	541929	97.505	0.0311

* indicates avreage of three determination

Table 13. Intra-day variability of PAN

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/Sec)	± SD*	RSD*
	Trial 1	Trial 2	Trial 3			
5	104650	104552	104754	104652	201.01	0.0975
15	361335	361429	361633	361465	201.53	0.0590
25	548041	548440	548342	548274	222.92	0.0322

* indicates avreage of three determination

Table 14. Intra-day variability of CLA

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD*	RSD*
	Trial 1	Trial 2	Trial 3			
5	109225	109296	109321	109280	68.80	0.0464
15	332417	332619	332393	332476	148.96	0.0250
25	531321	531420	531523	531421	122.00	0.0170

* indicates avreage of three determination

Table 15. Ruggedness data for AMO, PAN and CLA

Parameter	% Assay			SD*			RSD*		
	AMO	PAN	CLA	AMO	PAN	CLA	AMO	PAN	CLA
Analyst -1st	99.48	98.86	99.53	0.0339	0.5782	0.02657	0.0340	0.584	0.0266
Analyst-2nd	98.57	98.79	99.45	0.0452	0.0904	0.05169	0.0458	0.0915	0.05197
Lab-1st	98.74	98.27	99.35	0.0603	0.0701	0.0421	0.0616	0.0713	0.0423
Lab-2nd	99.15	96.86	99.29	0.0413	0.0628	0.0510	0.0415	0.0648	0.0513
Reagent-1st	99.43	99.59	99.62	0.0405	0.0521	0.0431	0.0407	0.0523	0.0405
Reagent-2nd	99.57	99.64	99.75	0.0402	0.0903	0.0680	0.0403	0.0682	0.0681

* indicates avreage of three determination

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Table 16. Robustness study of system suitability parameter: Change in flow rate (mL/min)

System suitability parameter*	Drug	Change in flow rate (mL/min)			RSD*		
		0.98	1.0	1.02	0.98	1.0	1.02
Peak area*	AMO	104046	104870	104799	0.0063	0.0016	0.0014
	PAN	104663	104412	104698	0.0143	0.0289	0.0125
	CLA	107765	106921	107516	0.0012	0.0025	0.0058
Theoretical plates*	AMO	3043	3136	3192	0.6014	0.3668	0.9172
	PAN	2517	2522	2561	0.5186	0.3437	0.3477
	CLA	3361	3379	3361	0.1653	0.2188	0.2857
Tailing factor*	AMO	1.409	1.412	1.486	0.4622	0.6679	0.3267
	PAN	1.350	1.336	1.311	0.7414	0.4969	0.3078
	CLA	1.521	1.565	1.526	0.8570	0.6908	0.5891
Retention Time*(Min)	AMO	1.923	1.926	1.927	0.4941	0.1454	0.1754
	PAN	3.319	3.326	3.337	0.3060	0.2034	0.2017
	CLA	2.532	2.517	2.522	0.2155	0.1213	0.1245

* indicates avreage of three determination

Table 17. Robustness study of system suitability parameter: Change in O.C. of M.P. Ratio

System suitability parameter*	Drug	Change in O.C. of M.P. Ratio			RSD*		
		75:25	70:30	65:35	75:25	70:30	65:35
Peak area*	AMO	104126	104870	104740	0.0158	0.0061	0.0151
	PAN	104321	104314	104654	0.0745	0.0623	0.0360
	CLA	107869	106965	107515	0.0108	0.0050	0.0034
Theoretical plates*	AMO	3044	3137	3180	0.4066	0.3823	0.3432
	PAN	2519	2521	2551	0.2278	0.4308	0.3259
	CLA	3370	3380	3362	0.1518	0.2773	0.2159
Tailing factor*	AMO	1.405	1.416	1.476	0.2178	0.6078	0.1821
	PAN	1.349	1.362	1.316	0.5458	0.1832	0.3788
	CLA	1.550	1.566	1.535	0.2378	0.6671	0.6409
Retention Time* (Min)	AMO	1.943	1.927	1.920	0.1145	0.1245	0.1645
	PAN	3.336	3.322	3.388	0.2359	0.2219	0.3026
	CLA	2.526	2.517	2.551	0.1544	0.0728	0.9405

* indicates avreage of three determination

Table 18. Robustness study of system suitability parameter: Change in pH

System suitability parameter*	Drug	Change in pH			RSD*		
		3.8	4.0	4.2	3.8	4.0	4.2
Peak area*	AMO	1046598	104879	104423	0.0160	0.0132	0.0187
	PAN	1045571	104423	105565	0.0577	0.0936	0.0573
	CLA	1077344	106794	107415	0.0069	0.0061	0.0572
Theoretical plates*	AMO	3043	3171	3170	0.2329	0.4267	0.3455
	PAN	2518	2527	2554	0.0780	0.2298	0.1459
	CLA	3360	3376	3356	0.1768	0.1193	0.1186
Tailing factor*	AMO	1.406	1.458	1.468	0.5298	0.5884	0.2763
	PAN	1.359	1.326	1.315	0.9410	0.2824	0.4622
	CLA	1.560	1.568	1.540	0.725	0.5766	1.0428
Retention Time*(Min)	AMO	1.953	1.972	1.930	0.1459	0.1506	0.0720
	PAN	3.338	3.323	3.348	0.2655	0.2256	0.3409
	CLA	2.537	2.571	2.560	0.1165	0.1821	0.1232

* indicates avreage of three determination

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Table 19. Robustness study of system suitability parameter: Change in Wavelength (nm)

System suitability parameter*	Drug	Wavelength (nm)			RSD*		
		240	242	244	240	242	244
Peak area*	AMO	1047894	1048382	1044527	0.0042	0.0075	0.0189
	PAN	1045681	1045249	1054754	0.0803	0.0811	0.0678
	CLA	1076827	1078497	1075264	0.0167	0.0336	0.0349
Theoretical plates*	AMO	3045	3180	3150	0.3432	0.1854	0.3216
	PAN	2522	2530	2564	0.2524	0.1277	0.1826
	CLA	3370	3378	3358	0.1784	0.0830	0.2482
Tailing factor*	AMO	1.415	1.422	1.436	0.5253	0.3867	0.2385
	PAN	1.361	1.378	1.398	0.1946	0.7695	0.4487
	CLA	1.540	1.535	1.549	0.1142	0.6385	0.6721
Retention Time*(Min)	AMO	1.968	1.958	1.944	0.1721	0.1701	0.2318
	PAN	3.358	3.392	3.352	0.3025	0.3016	0.1759
	CLA	2.536	2.574	2.580	0.1669	0.1143	0.1430

* indicates avreage of three determination

Table 20.Solution stability of AMO, PAN and CLA

Drug	% AssayInitial	After 12 hrs.	After 24 hrs.	After 36 hrs.	After 48 hrs.
AMO	99.28%	99.12%	99.35%	99.64%	98.52%
PAN	99.68%	99.24%	99.77%	99.34%	99.05%
CLA	99.57%	99.07%	99.54%	99.48%	99.19%

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