Journal of Applied Pharmaceutical Science Vol. 6 (03), pp. 071-076, March, 2016
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2016.60312
ISSN 2231-3354

Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Pyrimethamine and Sulphadoxine in Bulk and Tablet Dosage Form

Veeragoni Anil Kumar[1*], Vasudeva Murthy Sindgi[2], Shoba Rani Satla[3], Manish Kumar Thimmaraju[4]

[1]Department of Pharmaceutical Analysis, Pathfinder institute of Pharmacy Education and Research, Warangal-Telangana. [2]Jayamukhi College of Pharmacy, Narsampet, Warangal, Telangana. [3]Center for Pharmaceutical Sciences, Institute of Science and Technology, JNTUH, Hyderabad, Telangana. [4]Central Analytical Laboratory, Balaji Institute of Pharmaceutical Sciences, Narsampet, Warangal-Telangana.

ARTICLE INFO

Article history:
Received on: 24/12/2015
Revised on: 15/01/2016
Accepted on: 08/02/2016
Available online: 30/03/2016

Key words:
Pyrimethamine, sulphadoxine, ICH guidelines and stress degradation studies.

ABSTRACT

A stability indicating simple, selective, accurate high Performance Liquid Chromatographic (HPLC) method was developed and validated for the combined tablet formulation of pyrimethamine & sulphadoxine. Chromatographic separation was optimized by gradient HPLC on a C18 column [Inertsil Silica, 250 x 4.6 mm, 5µ] utilizing a mobile phase of potassium dihydrogen phosphate and acetonitrile taken in the ratio 70:30 at a flow rate of 1.0 ml/min with UV detection at 221nm. The retention time of pyrimethamine and sulphadoxine was 2.77 and 6.57 min respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation, robustness and stress degradation studies. Validation of the method was done in accordance with ICH guidelines for the assay of active ingredients. Thus validated method can be recommended for the routine laboratory analysis.

INTRODUCTION

Pyrimethamine is an anti-malarial drug which inhibits the dihydrofolatereductase of plasmodia and there by blocks the biosynthesis of purines and pyrimidines, which are essential for DNA synthesis and cell multiplication. Sulfadoxine is a sulfa drug, often used in combination with pyrimethamine to treat malaria. Review of literature for pyrimethamine & sulphadoxine gave information regarding the various studies conducted and analytical methods established for the drugs alone, in combination and in combination with other drugs in pharmaceutical dosage forms and in biological fluids (Minzi *et al.*, 2013, Sinnaeve *et al.*, 2005). There are few methods reported in the literature for analysis of pyrimethamine & sulphadoxine alone or in combination with other drugs in the pure form, pharmaceuticals formulations and biological fluids by UV-spectrophotometer (Onah and Odeiani, 2002, Meena and Sandhya, 2013), HPTLC (Meena and Sandhya, 2013), Capillary zone electrophoresis (Amin *et al.*, 2012), RP-HPLC (Green *et al.*, 2002, Bergqvist *et al.*, 1991; Astier *et al.*, 1997; Bergqvist *et al.*, 1985; SaeedArayne *et al.*, 2010), LC-MS (Sinnaeve *et al.*, 2005).

There was no stability indicating HPLC methods established for the simultaneous estimation of pyrimethamine & sulphadoxine in formulation. The aim of the present work is to develop a stability indicating analytical method for the combined tablet formulation of pyrimethamine & sulphadoxine. Validation of the method was done in accordance with ICH guidelines for the assay of active ingredients. Thus validated method can be recommended for the routine laboratory analysis.

MATERIALS AND METHODS

Pyrimethamine (PYR) and Sulphadoxine (SUL) were procured as gift samples from Taj pharmaceuticals, Mumbai. REZIZ (Pyrimethamine -25mg and Sulphadoxine - 500mg) tablets manufactured by Shreya life sciences pvt. Ltd. India was procured from a local pharmacy. Acetonitrile (HPLC grade), ortho phosphoric acid, Potassium dihydrogen ortho phosphate**,** Methanol (HPLC grade), Tri ethyl amine and TDW (Triple Distilled Water).

* *Corresponding Author*
Veeragoni Anil Kumar, Department of Pharmaceutical Analysis Pathfinder Institute of Pharmacy Education And Research, Warangal-Telangana. Email: v_aneel@yahoo.co.in

Instrumentation

Shimadzu gradient HPLC MODEL NO (JAPAN), HPLC column Inertsil (250 x 4.6mm, 5µm), Mobile phase filtration unit (Pall Life sciences, Mumbai, India), LAB-INDIA U.V with UV Win software, Sonicator, P^H meter (LAB-INDIA), digital balance (Denver).

METHOD DEVELOPMENT

Preparation of standard solutions

Accurately weighed and transferred 10 mg of Pyrimethamine and 10 mg of Sulphadoxine working Standards into two separate 100 ml clean dry volumetric flasks, add 30ml of diluent , sonicated for 5 minutes and make up to the final volume with diluent.

Chromatographic Conditions

The HPLC system consisted of Shimadzu gradient HPLC (JAPAN) with dual λ Absorbance UV detector. The wavelength of detection as set at 221nm. Separation was carried out in gradient mode on inertsil C18 column (4.6x250mmx5µm) and the retention time of pyrimethamine and sulphadoxime was found to be 2.952 and 6.832 respectively (figure 1), using 70:30 v/v dihydrogen orthophosphate : acetonitrile as mobile phase at a flow rate of 1 ml/min. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultrasonicator prior to use. Chromatography was carried out at room temperature 25^0c and maintains the column temperature at 32^0c.

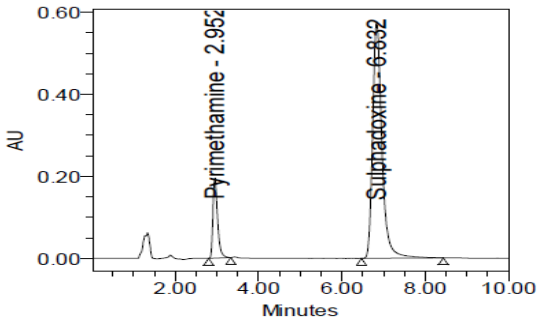

Fig. 1: Chromatogram of pyrimethamine and sulphadoxime.

Preparation of Standard Solutions

Stock solutions of pyrimethamine (0.5mg/ml) and sulphadoxime (1mg/ml) were prepared in methanol. Further dilutions were carried out in 60% acetonitrile and calibration standards were prepared freshly with pyrimethamine and sulphodoxime stock solutions to give the concentrations of 5, 10, 15, 20, 25 and 30 µg/ml.

Sample Preparation (Assay)

5 tablets were weighed and calculate the average weight of each tablet then the weight equivalent transferred into a 100 mL volumetric flask, 60mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 0.2ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with diluent.

RESULTS & DISCUSSIONS

Method Validation

Method validation was performed as per the ICH guidelines Q2 (R1) Validation of Analytical Procedure. The developed method was validated for the following parameters.

Linearity

Linear concentrations of both drugs were prepared and the best fit line was calculated. Wide range calibration was determined by solutions containing 5µg/ml to 30µg/ml (table 1). Correlation coefficient was found to be 0.999 & 0.997 for Pyrimethamine & Sulphadoxine respectively (shown in fig 2&3).

Table 1: Linearity results of pyrimethamine & sulphadoxine.

Sno	Pyrimethamine		Sulphadoxine	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
1	5	329102	5	2279923
2	10	876684	10	5805764
3	15	1225460	15	8254245
4	20	1753561	20	11870562
5	25	2109537	25	14481530
6	30	2614420	30	18076428

Precision

The intraday precision was demonstrated by injecting six test solutions at 25 µg/ml concentration as per the test procedure (shown in table no 2&3) & recording the chromatograms of six test solutions. The % RSD of pyrimethamine and sulphodixime was found to be 0.207 and 0.324 respectively.

Table 2: Method Precision of Pyrimethamine.

Sno	Pyrimethamine (25µg/ml)		
	Retention time(Rt)	Peak area	% Assay
1	2.90	2109429	100.01
2	2.930	2109837	99.99
3	2.839	2109941	99.98
4	2.914	2107535	100.09
5	2.845	2108530	100.05
6	2.872	2119528	99.53
Mean		2110800	99.94
SD		4371.18	0.206
RSD		0.207	0.206

Table 3: Method Precision of Sulphadoxine

Sno	Sulphadoxine (25µg/ml)		
	Retention time(Rt)	Peak area	% Assay
1	6.828	14482536	99.99
2	6.823	14481829	100.00
3	6.729	14599259	99.19
4	6.799	14499743	99.87
5	6.712	14481539	100.00
6	6.722	14481645	100.00
Mean		14504425	99.84
SD		47005.99	0.3220
RSD		0.324	0.3225

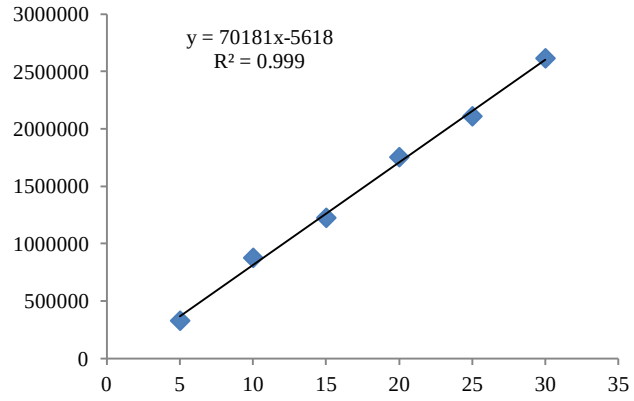

Fig. 2: Linearity of pyrimethamine.

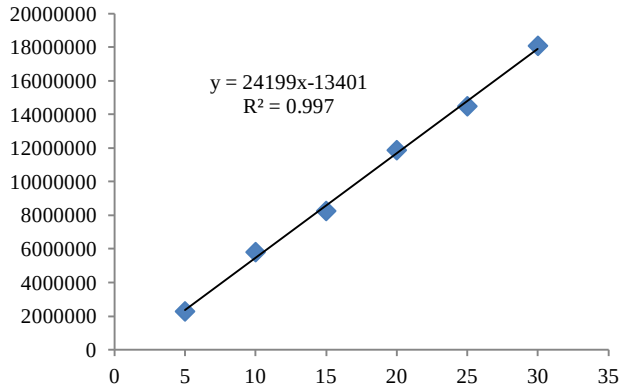

Fig. 3: Linearity of sulphadoxine.

Intermediate Precision

Intermediate precision of the analytical method was determined by performing method precision on in three successive days by different analysts under same experimental condition. Assay of all six replicate sample preparations was determined and the mean % RSD of pyrimethamine and sulphodixime was found to be 0.259 and 0.353 respectively (shown in table no 4).

Table 4: Intermediate Precision of pyrimethamine & sulphadoxine.

S.No	Parameter	%RSD			Mean RSD
		Day-1	Day-2	Day-3	
Pyrimethamine (25µg/ml)	Peak Area	0.199	0.264	0.318	0.260
	% Assay	0.198	0.263	0.317	0.259
sulphadoxine (25µg/ml)	Peak Area	0.317	0.288	0.462	0.356
	% Assay	0.315	0.287	0.4584	0.353

Accuracy

Accuracy of the method was established by performing recovery studies according to the ICH guidelines. Spiked samples were prepared by spiking pre-analyzed sample solutions with pure drug at three different concentration levels each in triplicate. Mean percentage recovery values at three different concentrations of the two drugs was calculated. The % recovery of Pyrimethamine (98.02-100.45%) & Sulphadoxine (99.79-100.20%) at each level was within the limits of 98% and 102% (shown in table no 5&6). Hence, accuracy was established for the present work and the method was said to be accurate.

Table 5: % recovery of Pyrimethamine.

S.no	Conc(µg/ml)	Conc(µg/ml) found	% recovery	Mean accuracy	%RSD
1	15	14.8	101.35		
2	15	15.1	99.34	100.45	1.024
3	15	14.9	100.67		
4	20	20.3	98.52		
5	20	19.7	101.52	98.02	1.530
6	20	20.1	99.50		
7	25	25.2	99.21		
8	25	24.9	100.40	100.14	0.832
9	25	24.8	100.81		

Table 6: % Recovery of Sulphadoxine.

Sno	Conc (µg/ml)	Conc(µg/ml) found	% recovery	Mean accuracy	%RSD
1	15	14.91	100.60		
2	15	15.11	99.27		
3	15	14.89	100.74	100.20	0.811
4	20	20.12	99.40		
5	20	19.89	100.55		
6	20	20.12	99.40	99.79	0.664
7	25	25.09	99.64		
8	25	25.1	99.60		
9	25	24.91	100.36	99.87	0.428

Limit of Detection & Quantification

In the present study, the LOD and LOQ were calculated according to the standard deviation of the response and the slope of the calibration curve i.e., 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.The lowest possible concentration of Pyrimethamine that can be detected and quantified by the present method was found to be 0.264 µg/ml and 0.800 µg/ml respectively and that of Sulphadoxine was found to be 0.53 µg /ml and 1.62µg/ml respectively.

Robustness

Robustness of the proposed method was determined by varying various parameters, the %RSD reported was found to be less than 2 %.As the system suitability parameters for the standard and test chromatograms of Pyrimethamine & Sulphadoxine were within limits for variation in flow rate (±0.1ml) and mobile phase composition, the allowable variation in flow rate, organic solvent ratio in mobile phase composition and column temperature should be 1±0.1ml/min, 65±2ml and 30±5^{0}c respectively (shown in table no 7 & 8).

STRESS DEGRADATION STUDIES

Stress degradation studies were performed as per the ICH guidelinesQ1A (R2) Stability Testing of New Drug Substances and Products, using the proposed validated analytical method and the results were shown in table no 9 & 10.

Table 7: Robustness of Sulphadoxine.

Parameter	Variation in flow		Variation in Mobile phase		Variation in column temp	
Stds	flow rate (0.9ml/mim)	flow rate (1.1ml/mim)	Buffer:Acetonitrile (75:25)	Buffer:Acetonitrile (80:20)	$30\text{-}5^0$	$30\text{+}5^0$
1	9709676	8894030	8990708	9020771	8962774	8921543
2	9721151	8862782	8982909	9025663	8965300	8925743
Mean	9715414	8878406	8986809	9023217	8965537	8922143
SD	8114.7	22095.7	5515	2044.6	3163.4	2262.4
%RSD	0.1	0.2	0.1	0.1	0.1	0.1
Retention time	7.09	2.715	6.03	7.07	6.47	6.37
Tailing factor	1.33	1.33	1.32	1.3	1.32	1.43
Theoretical plates	5217	5082	5089	5306	5253	5346

Table 8: Robustness of Pyrimethamine.

Parameter	Variation in flow		Variation in Mobile phase		variation in column temp	
Stds	flow rate (0.9ml/mim)	flow rate (1.1ml/mim)	Buffer:Acetonitrile (75:25)	Buffer:Acetonitrile (80:20)	$30\text{-}5^0$	$30\text{+}5^0$
1	1588766	1485375	1435845	1539898	1454227	1434907
2	1603920	1484112	1425867	1549198	1480862	1440355
Mean	1596343	1484743	1430856	1544548	1467545	1437631
SD	10715	892.8	7055.5	6576.4	18833.7	3852.6
%RSD	0.7	0.1	0.4	0.4	1.3	0.3
Retention time	2.95	2.715	2.43	3.076	2.71	2.66
Tailing factor	1.47	1.44	1.42	1.39	1.44	1.43
Theoretical plates	3679	3665	3797	3824	3746	3981

Table 9: Results of stress degradation studies of Sulphadoxine.

Sno	Stress conditions	Time	% Assay	% Degradation
1	Acid Degradation	30 min	88.082	11.918
2	Base Degradation	30 min	87.281	12.719
3	Peroxide Degradation	30 min	93.214	6.786
4	UV Degradation	7 days	92.887	7.113

Table 10: Results of stress degradation studies of Pyrimethamine.

Sno	Stress conditions	Time	% Assay	% Degradation
1	Acid Degradation	30 min	92.713	7.287
2	Base Degradation	30 min	85.985	14.015
3	Peroxide Degradation	30 min	82.798	17.202
4	UV Degradation	7 days	93.502	6.498

Acid degradation studies

To 1ml of stock solution pyrimethamine and sulphadoxine, 1ml of 2N HCl was added and refluxed for 30min at 60^0c. From the above solution10µl was injected into the system and the chromatograms were recorded to detect the stability of sample (figure 4).

Alkali Degradation Studies

To 1ml of stock solution of of standard drug and sample pyrimethamine and sulphadoxine, 1ml of 2N NaOH was added and refluxed for 30min at 60 °C. From the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample (figure 5).

Oxidation

To 1ml of stock solution of standard drug and sample of pyrimethamine and sulphadoxine, 1ml of 20% $H_2O_2$was added and refluxed for 30min at 60^0c. From the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample (figure 6).

Photo Stability Studies

The photochemical stability of the drug was also studied by exposing the 25 µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, from the above solution10 µl was injected into the system and the chromatograms were recorded to detect the stability of sample (figure 7).

Pyrimethamine and Sulphadoxine undergoes degradation in acidic, oxidation, alkaline, and UV. More degradation was found for oxidation. As per ICH guidelines peak purity angle should be less than peak purity threshold. Hence, method of the analysis of PYR and SUL in tablet dosage form shows that the degradation product doesn't interfere with the analytical determination. The stress degradation studies showed that the drug formulation containing pyrimethamine and sulphadoxine undergoes degradation in acidic, oxidation, alkaline, and UV (7.29% ,15.01% ,16.13% , 7.88% , 5.51% and 12.92% , 10.72% , 7.13% , 13.32% , 6.01%). hence the proposed analytical method is also useful for the determination of pyrimethamine and sulphadoxine stability in sample of pharmaceutical dosage form.

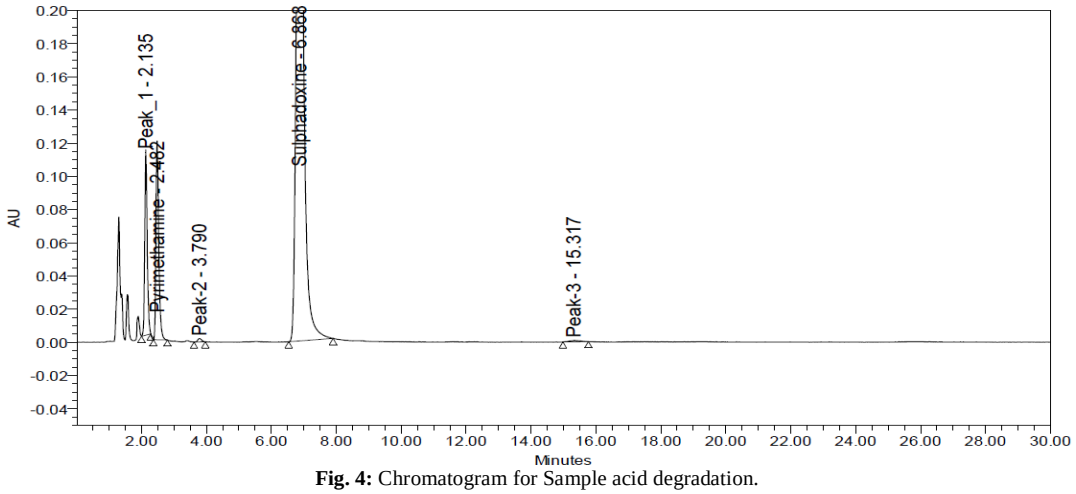

Fig. 4: Chromatogram for Sample acid degradation.

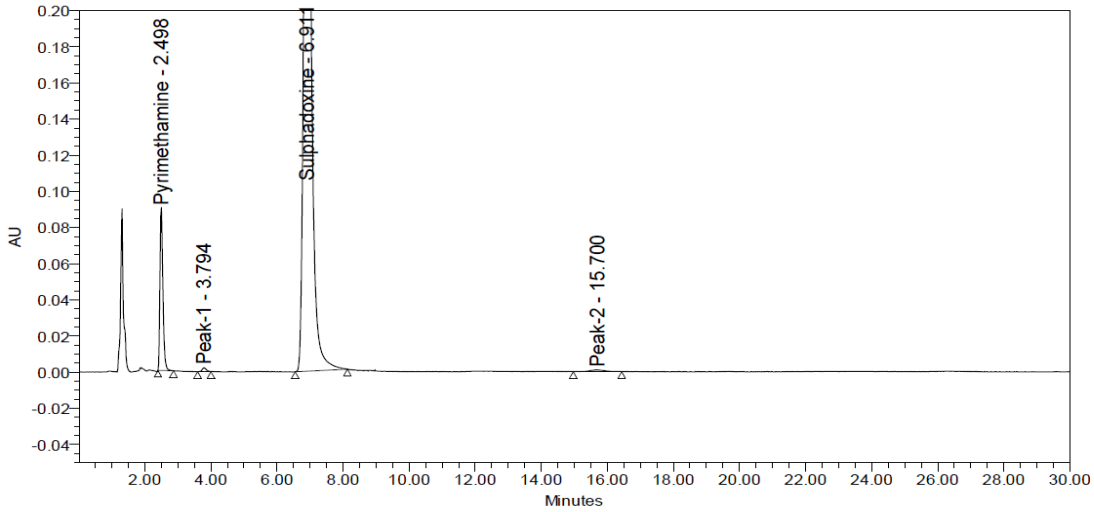

Fig. 5: Chromatogram for Sample Alkali degradation.

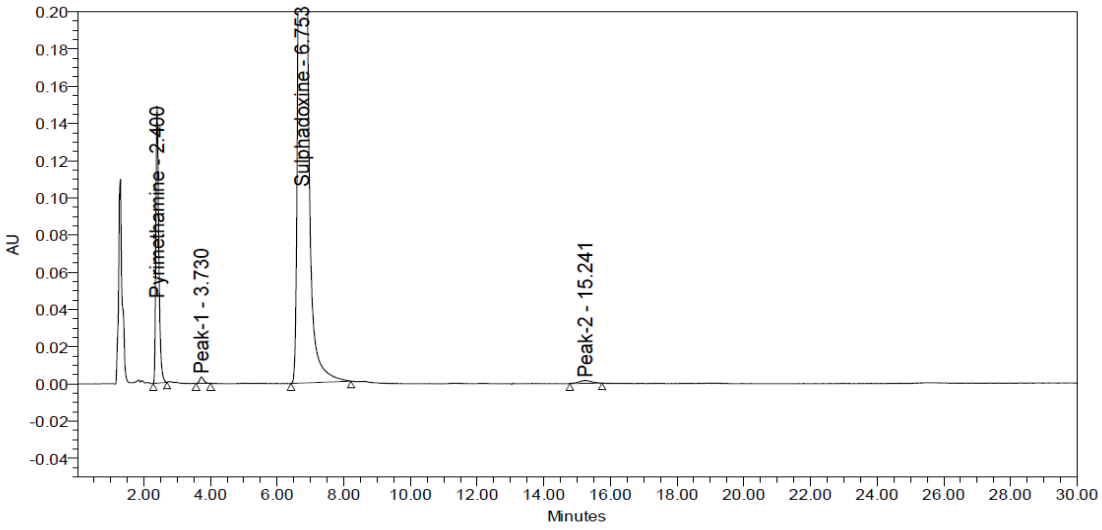

Fig. 6: Chromatogram for Sample Peroxide degradation.

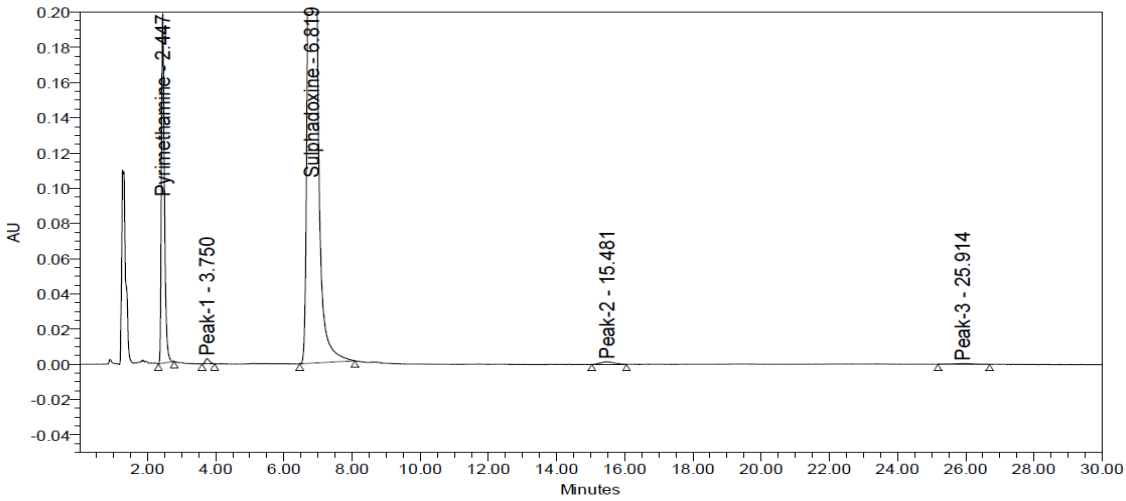

Fig. 7: Chromatogram for U.V degradation studies.

CONCLUSION

The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of pyrimethamine & sulphadoxine in bulk and tablet dosage form and was found to be suitable for the routine analysis and quality control and percentage degradation of pharmaceutical preparations containing these drugs either individually or in combination.

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How to cite this article:

Anil Kumar V, Sindgi VM, Satla SR, Thimmaraju MK. Stability Indicating Rp-Hplc Method for the Simultaneous Estimation of Pyrimethamine and Sulphadoxine in Bulk and Tablet Dosage Form. J App Pharm Sci, 2016; 6 (03): 071-076.