WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 13, 338-349. Research Article ISSN 2277– 7105

STABILITY INDICATING RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ATOVAQUONE AND PROGUANIL IN BULK AND TABLET DOSAGE FORM

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Article Received on
27 August 2017,

Revised on 17 Sept. 2017,
Accepted on 08 Oct. 2017

DOI: 10.20959/wjpr201713-9533

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ABSTRACT

The present work aimed to develop a stability indicating high Performance Liquid Chromatographic (HPLC) validated method for the cestimation of combined tablet formulation of Atovaquone and Proguanil Hydrochloride. Chromatographic separation was optimized by gradient HPLC on a C18 column [Inertsil Silica, 250 x 4.6 mm, 5µ] utilizing a mobile phase consisting a mixture of 10mM ammonium formate, pH 3.5 and 90:10 v/v acetonitrile - methanol in the ratio of 30:70 v/v at a flow rate of 0.9ml/min with UV detection at 254nm. The retention time of Atovaquone and Proguanil Hydrochloride was 7.3 min and 3.8 min respectively. The developed method was validated as per ICH guidelines. Linearity of the method was good over the concentration range 2.5µg/ml to 20µg/ml for proguanil and 6.25-50 µg/ml for atovaquone. Correlation coefficient was found to be 0.999&0.999 for proguanil & Atovaquone respectively. The % mean recovery of proguanil (98.38-101.09.%) & atovaquone (98.62-100.99%) at each level was within the limits of 98% and 102%.The results obtained for accuracy, precision, LOD, LOQ and Ruggedness were within the limits. Thus the validated economical method was applied for forced degradation study of Atovaquone and Proguanil Hydrochloride tablet.

KEYWORDS: Atovaquone and proguanil, stress study, HPLC method.

INTRODUCTION

Proguanil anti malarial drug chemically known as 1-(4-chlorophenyl)-2-(N'-propan-2-ylcarbamimidoyl) guanidine, a synthetic biguanide derivative of pyramiding. Atovaquone

Chemically known as trans-2-[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthalenedione, used for the Pneumocystis Carinii pneumonia in acquired immunodeficiency syndrome (AIDS).[1] After profound search from data and literature available, it was revealed that many methods have been reported including LC-MS,[2] ultraviolet spectrophotometry,[3] high performance liquid chromatography,[4-6] TLC method[3] for the analysis of Proguanil alone. Limited analytical methods were reported including UV,[7] HPLC,[8-9] capillary zone electrophoresis[10] and LC-MS[11] for the analysis of atovaquone alone. Few analytical techniques including mass spectrometric[12] and HPLC[13-14] methods have been reported for the simultaneous determination of Atovaquone and Proguanil hydrochloride from bulk drug and Pharmaceutical dosage forms. Whereas there is no stability indicating analytical methods were reported for simultaneous estimation of atovaquone and proguanil. Hence a simple, rapid, sensitive and accurate stability indicating HPLC method was developed for the simultaneous estimation of atovaquone and proguanil from bulk and pharmaceutical dosage form.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade methanol, acetonitrile, formic acid and analytical grade ammonium formate were purchased from Merck (Mumbai, India). Proguanil working standard was obtained as a gift sample from Alkem Laboratories, Ltd., (Mumbai, India) and Atovaquone working standard from Zhejiang Kangyo Pharmaceuticals Co., Ltd., Mumbai, India.

Instrumentation

Shimadzu gradient HPLC (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).

Preparation of standard solutions

Stock solutions (1mg/ml) of atovaquone and proguanil were prepared in methanol. Further dilutions were carried out using Methanol: Acetonitrile 50:50 v/v as diluent. Working standards of different concentrations ranging from 2.5-20µg/ml for poguanil and 6.25-50 µg/ml for atovaquone were prepared by diluting several aliquots of standard solution s of proguanil and atovaquone.

Preparation of sample solution

Twenty tablets each containing 100 mg of proguanil and 200 mg of atovaquone were weighed and powdered equivalent to dose, transferred to a 100 mL volumetric flask, and extracted with mixture of methanol and water (80:20). The mixture was sonicated for 20 min in an ultrasonic bath. The volume was adjusted to 100 mL with the same solvent mixture, filtered and from this solution 1.0 mL was pipetted and the volume was made up to 100mL with diluents to get the concentration 10 µg/mL of proguanil and 20 µg/mL of Atovaquone.

Chromatographic Conditions

Chromatographic Conditions The HPLC system consisted of Shimadzu gradient HPLC (JAPAN) with dual λ Absorbance UV detector. The wavelength of detection as set at 254nm. Separation was carried out in gradient mode on inertsil C18 column (4.6x250mmx5µm) and the retention time of atovaquone and proguanil was found to be 7.3 and 3.8 respectively (figure 1), using mobile phase consisting a mixture of 10mM ammonium formate, pH 3.5 and 90:10 v/v acetonitrile - methanol in the ratio of 30:70 v/v at a flow rate of 0.9ml/min. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultra sonicator prior to use. Chromatography was carried out at room temperature 25^{o}C and maintains the column temperature at 32 ^{o}C.

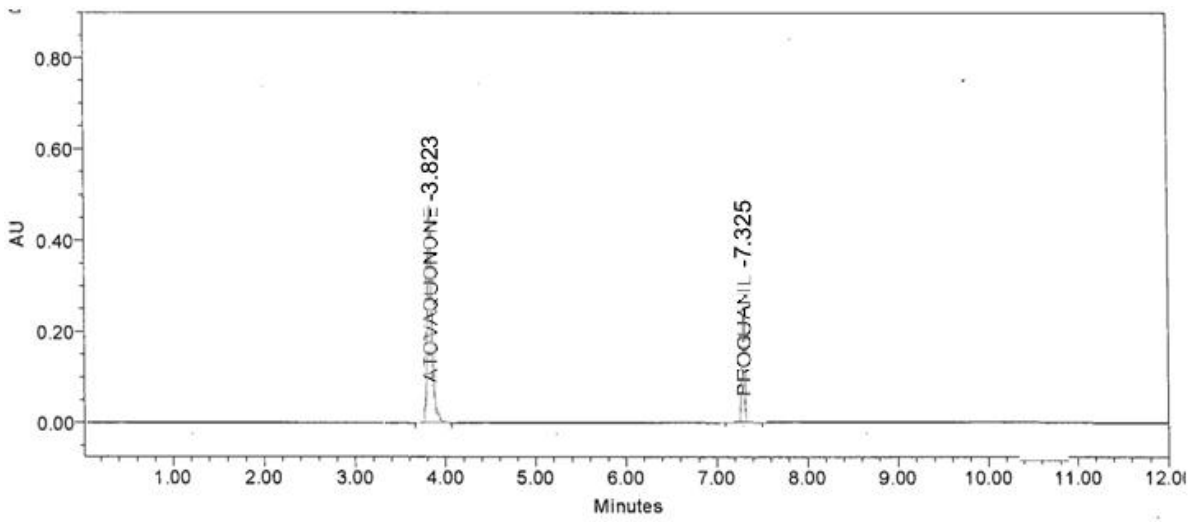

Figure 1: Chromatogram of Atovaquone and Proguanil

METHOD VALIDATION

Method validation was performed as per the ICH guidelines Q2 (R1) Validation of Analytical Procedure.[15] 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 2.5µg/ml to 20µg/ml (Table 1) for Proguanil and 6.25-50 µg/ml for Atovaquone. Correlation coefficient was found to be 0.999&0.999 for proguanil & atovaquone respectively (Fig 2&3).

Table 1: Linearity Data for Proguanil & Atovaquone.

Proguanil				
Conc (µg/ml)	**Area 1**	**Area 2**	**Area 3**	**Avg Area**
2.5	3225	3142	3210	3192
5	8013	7930	7998	7980
10	15994	15911	15979	15961
15	23989	23906	23974	23956
20	31966	31882	31951	31933
Intercept	-457.5	-541	-473	-490.5
slope	1628	1628	1628	1628
Intercept Standard Deviation				44.41565
LOD (µg/ml)				**0.090032**
LOQ(µg/ml)				**0.272823**
Atovaquone				
Conc (µg/ml)	**Area 1**	**Area 2**	**Area 3**	**Avg Area**
6.25	341930	341797	341865	341864
12.5	667964	667831	667899	667898
25	1323685	1323552	1323620	1323619
37.5	1951565	1951432	1951500	1951499
50	2579584	2579451	2579519	2579518
Intercept	30123	29990	30058	30057
Slope	51155	51155	51155	51155
Intercept Standard Deviation				66.50564
LOD (µg/ml)				**0.00429**
LOQ(µg/ml)				**0.013001**

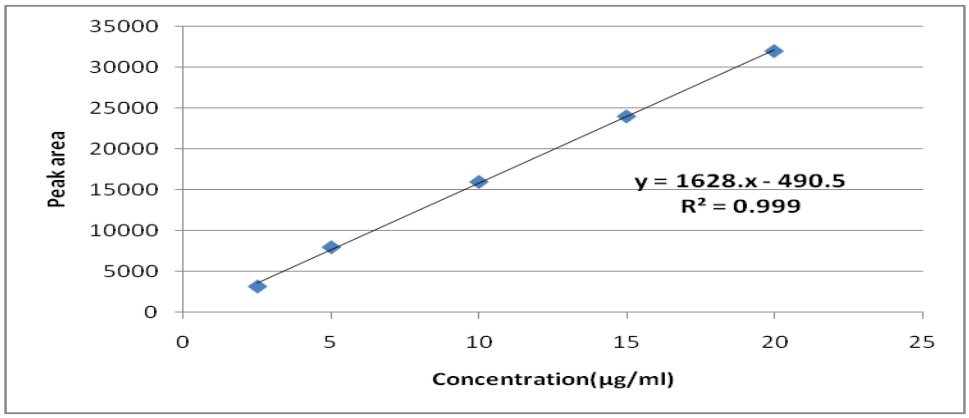

Figure 2: Calibration Curve of Proguanil.

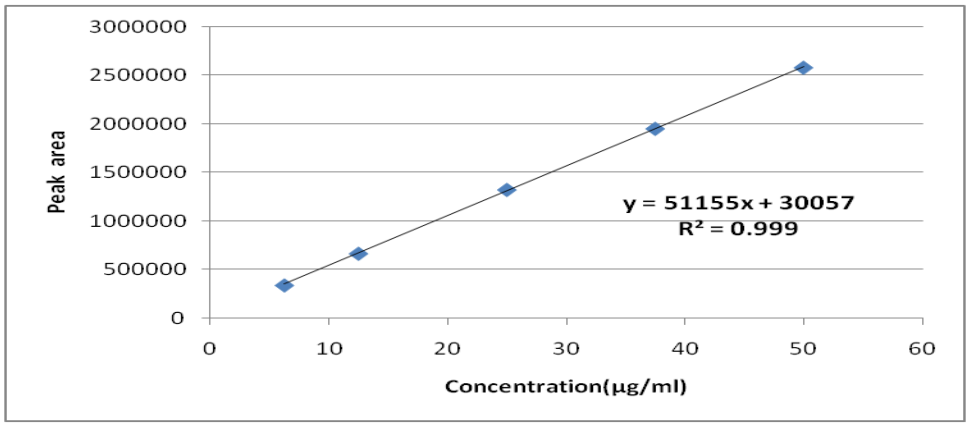

Figure 3: Calibration Curve of Atovaquone.

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

The LOD is calculated using the formula 3.3 times σ/s where "σ" is standard deviation of the intercept obtained for calibration curve and "s" is the slope of the calibration curve. Similarly LOQ is calculated using the formula 10 times σ/s. The calculated LOD and LOQ are shown in Table 1.

Precision

The intraday precision was demonstrated by injecting six test solutions of proguanil and atovaquone with 5µg/ml and 12.5µg/ml respectively as per the test procedure (Table 2) & recording the chromatograms of six test solutions. The % RSD of proguanil and atovaquone was found to be 1.0 and 0.72 respectively.

Table 2: Method Precision data of Proguanil and Atovaquone.

Proguanil (5µg/ml)		Atovaquone (12.5µg/ml)
S.No	**Area**	**Area**
1	8158	658954
2	7984	657814
3	8127	648757
4	8195	658314
5	8216	649931
6	8151	658769
Mean	8138	655423
SD	82.14	4739.95
%RSD	1.00	0.72

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 Proguanil and Atovaquone was found to be 0.46 and 0.63 respectively (Table 3).

Table 3: Precision Data of Proguanil & Atovaquone.

	Proguanil (5µg/ml)				Atovaquone (12.5µg/ml)			
S.No	**Day-1**	**Day-2**	**Day-3**	**Avg**	**Day-1**	**Day-2**	**Day-3**	**Avg**
1	8242	8234	8125	8200	657636	656977	656318	656977
2	8068	8160	8202	8143	646498	655841	645183	649174
3	8111	8103	8194	8136	657459	646811	646162	650144
4	8079	8170	8162	8137	666997	656339	655681	659672
5	8200	8191	8283	8225	658631	647981	647331	651315
6	8135	8127	8218	8160	657451	656793	646134	653459
Mean	8139	8164	8198	8167	657446	653457	649468	653457
SD	68.87	46.5	53.38	37.14	6519.51	4725.74	5108.78	4121.48
%RSD	0.84	0.57	0.65	**0.46**	0.99	0.72	0.79	**0.63**

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 % mean recovery of proguanil (98.62-100.99%) & atovaquone (98.38-101.09.%) at each level was within the limits of 98% and 102% (Table 4).

Table 4: Accuracy data of Proguanil and Atovaquone.

Accuracy of Proguanil						
S.N0.	**Conc.**	**Calculated Concn.**	**%Recovery**	**Mean Recovery**	**SD**	**%RSD**
1	5	4.95	98.93			
2	5	4.90	98.06	98.62	0.49	0.50
3	5	4.94	98.88			
1	10	10.0	99.99			
2	10	10.25	102.46	100.99	1.297	1.28
3	10	10.05	100.52			
1	15	15.20	101.33			
2	15	15.08	100.52	100.90	0.405	0.40
3	15	15.13	100.86			
Accuracy of Atovaquone						
S.N0.	**Conc.**	**Calculated concn.**	**%Recovery**	**Mean Recovery**	**SD**	**%RSD**
1	12.5	12.47	99.78			
2	12.5	12.18	97.43	98.38	1.24	1.26
3	12.5	12.24	97.93			

1	25	24.89	99.56			
2	25	25.20	100.80	100.50	0.84	0.84
3	25	25.29	101.16			
1	37.5	37.86	100.95			
2	37.5	37.96	101.22	101.09	0.14	0.13
3	37.5	37.91	101.10			

Ruggedness

The ruggedness of method was calculated with six injections of 10µg/ml proguanil and 25 µg/ml of atovaquone in two batches using two different columns. The % CV of ruggedness for proguanil was 1.56 with column-1 and 1.36 with column-2 and the % CV of ruggedness for atovaquone was 1.32 with column-1 and 0.93 with column-2 (Table-5), which is within acceptance limits.

Table 5: Results of Ruggedness.

	Proguanil 10µg/ml		**Atovaquone 25µg/ml**	
S.NO	**Column 1**	**Column 2**	**Column 1**	**Column 2**
1	10.02	10.08	25.04	25.08
2	9.76	10.02	25.02	24.88
3	9.74	9.89	24.34	24.62
4	9.84	9.82	24.72	24.96
5	9.79	9.71	24.55	24.59
6	9.55	9.94	24.29	24.51
Mean	9.78	9.91	24.66	24.78
± SD	0.15	0.13	0.33	0.23
% CV	1.56	1.36	1.32	0.93
% Accuracy	97.83	99.10	98.64	99.09

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. (Table 6&7).

Acid degradation studies

To 1ml of stock solution atovaquone and proguanil, 1ml of 2N HCl 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.

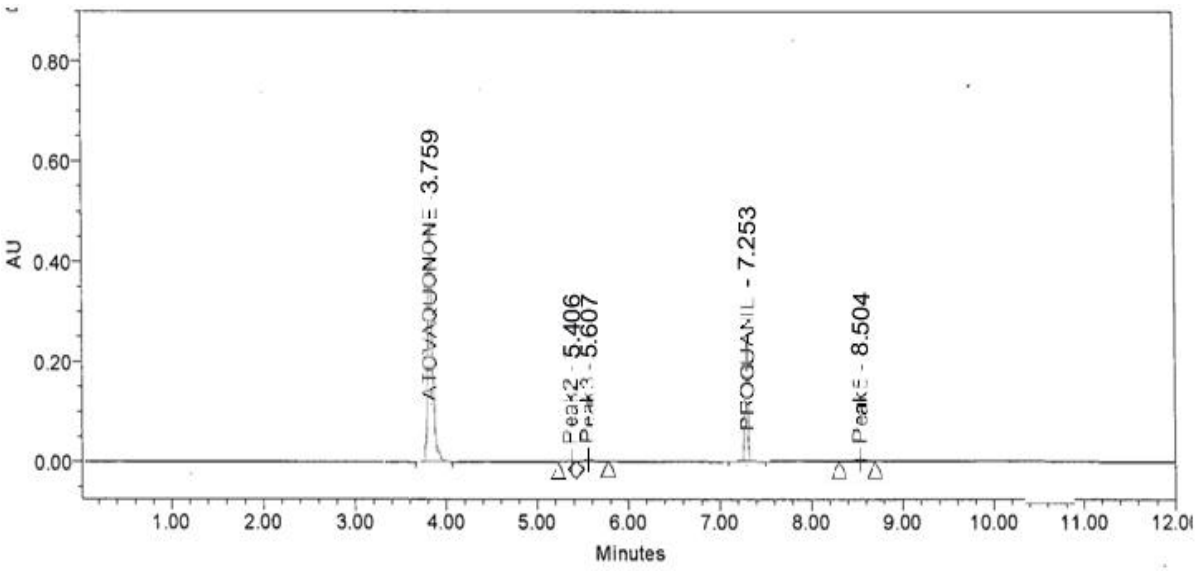

Figure 4: Chromatogram of Acid Hydrolysis.

Alkali Degradation Studies

To 1ml of stock solution of of standard drug and sample atovaquone and proguanil, 1ml of 2N NaOH 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.

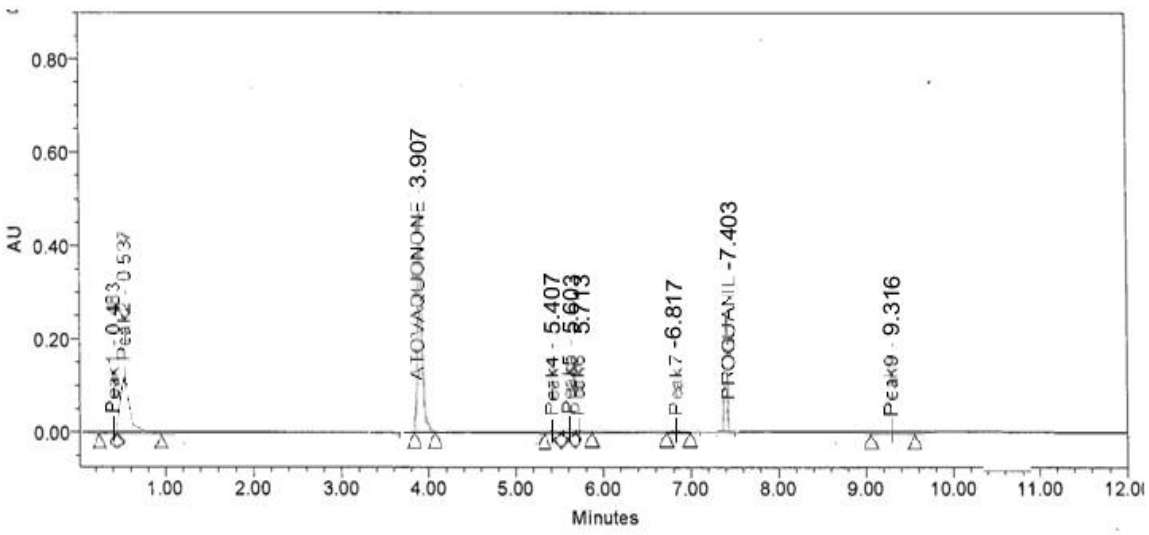

Figure 5: Chromatogram of Base Degradation.

Oxidation

To 1ml of stock solution of standard drug and sample of atovaquone and proguanil, 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.

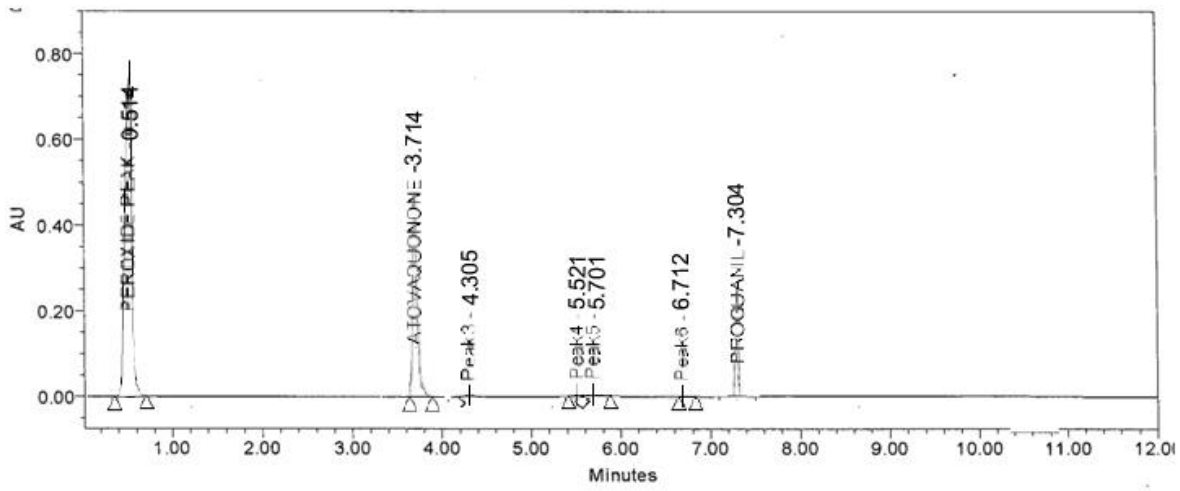

Figure 6: Chromatogram of Oxidative Degradation.

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).

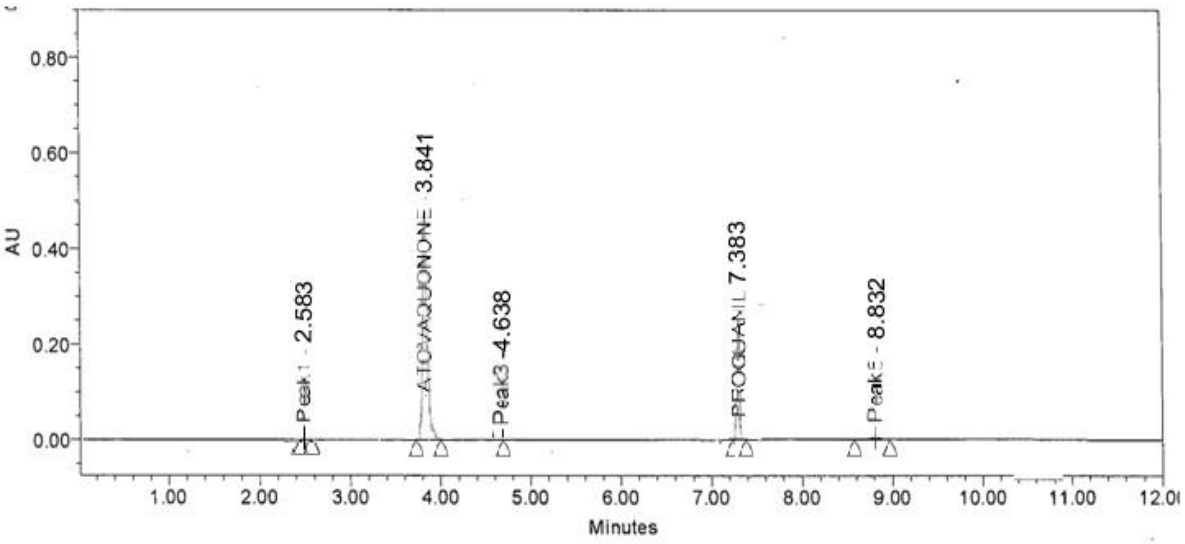

Figure 7: Chromatogram of Photo Degradation Study.

Thermal degradation studies

The 1ml of stock solution of standard drug and sample of atovaquone and proguanil was exposed to temperature 105^{o}C for 24hrs 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: 8).

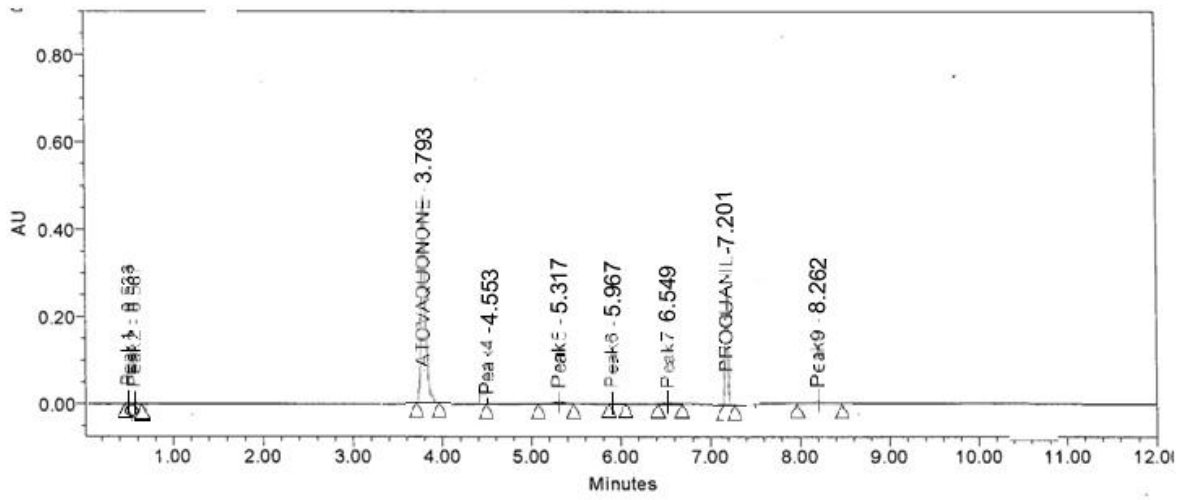

Figure 8: Chromatogram of Thermal Degradation Study.

Table 10: Results of stress degradation studies of Proguanil.

Sno	Stress conditions	Time	% Assay	% Degradation	Purity angle	Purity threshold
1	Acid Degradation	30 min	91.59	8.41	0.26	0.29
2	Base Degradation	30 min	86.13	13.87	0.19	0.22
3	Peroxide Degradation	30 min	97.10	2.9	0.24	0.26
4	UV Degradation	7 days	97.30	2.7	0.17	0.21
5	Thermal Degradation	24hrs	96.3	3.7	0.20	0.23

Table 11: Results of stress degradation studies of Atazanavir.

Sno	Stress conditions	Time	% Assay	% Degradation	Purity angle	Purity threshold
1	Acid Degradation	30 min	93.713	6.28	0.13	0.16
2	Base Degradation	30 min	94.985	5.02	0.16	0.21
3	Peroxide Degradation	30 min	92.798	7.20	0.20	0.23
4	UV Degradation	7 days	93.502	6.49	0.18	0.21
5	Thermal degradation	24hrs	94.8	5.2	0.14	0.19

Atovaquone and proguanil undergoes significant degradation in acidic, oxidation, alkaline, and UV. Comparatively More degradation was found with base for proguanil and with peroxide for atovaquone. As per ICH guidelines peak purity angle should be less than peak purity threshold. Hence, method of the analysis of atovaquone and proguanil in tablet dosage form shows that the degradation product doesn't interfere with the analytical determination. hence the proposed analytical method is also useful for the determination of atovaquone and proguanil stability in sample of pharmaceutical dosage form.

CONCLUSION

The developed stability indicating HPLC-UV method for simultaneous estimation of Proguanil and Atazanavir was novel, simple, precise, accurate, robust & cost-effective method. There are no HPLC method reported till now on selected combination drugs. Hence the developed method suitable for the routine analysis and quality control and percentage degradation of pharmaceutical preparations containing these drugs either individually or in combination.

ACKNOWLEDGEMENT

The author is acknowledged to Chairmen St Mary's College of Pharmacy, Secunderabad, Telangana, India, for the immense support & providing laboratory facilities for success full completion of work.

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