International Journal of Pharma Research and Health Sciences, 2022; 10(1): 3351-3358.
DOI:10.21276/ijprhs.2022.01.01
Patra and Sahithi

CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

Original article

A Novel RP-HPLC Method Development and Validation of Anti-Malarial Drugs Atovaquone and Proguanil Hydrochloride in Bulk Drug and Marketed Pharmaceutical Dosage Form

Pratap Kumar Patra*, R Sahithi

Department of Pharmaceutical Analysis, Sree Dattha Institute of Pharmacy, Sheriguda (V), Ibrahimpatnam (M), Ranga Reddy, 501510, India.

ARTICLE INFO:
Received: 12 Feb 2022
Accepted: 27 Feb 2022
Published: 28 Feb 2022

Corresponding author *
Pratap Kumar Patra,
Department of Pharmaceutical Analysis, Sree Dattha Institute of Pharmacy, Sheriguda (V), Ibrahimpatnam (M), Ranga Reddy, 501510, India.

ABSTRACT:
A Novel Analytical simple, reproducible and efficient RP-HPLC method was developed for simultaneous estimation of Atovaquone and Proguanil Hydrochloride in pure form and marketed combined pharmaceutical dosage forms. A column having Develosil ODS HG-5 RP C18, 15cmx4.6mm, i.d. Columnin isocratic mode with mobile phase containing Methanol: Acetonitrile in the ratio of 85:15% v/vwas used. The flow rate was 1.0 ml/min and effluent was monitored at 258nm. The retention times and linearity range for Atovaquone and Proguanil Hydrochloride was found to be (2.217, 5861min) and (0-14, 0-28), respectively. The method has been validated for linearity, accuracy and precision, robustness and limit of detection and limit of quantitation. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.08µg/ml and 0.24µg/ml for Atovaquone and 0.1µg/ml 0.3µg/ml for Proguanil Hydrochloride respectively. The proposed method was found to be accurate, precise and selective for simultaneous estimation of Atovaquone and Proguanil Hydrochloridein pure form and marketed combined pharmaceutical dosage forms.
Keywords: Atovaquone and Proguanil Hydrochloride, RP-HPLC, Validation, Accuracy, Precision.

1. INTRODUCTION

Atovaquone [1] is an antimicrobial indicated for the prevention and treatment of Pneumocystis jirovecii pneumonia (PCP) and for the prevention and treatment of Plasmodium falciparum malaria. Atovaquone is a naphthoquinone used for the prevention and treatment of Pneumocystis jevorici (formerly carinii) pneumonia and, in combination with Proguanil, prevention and treatment of P. falciparum malaria. Atovaquone therapy is associated with low rates of serum enzyme elevations and has been linked to only rare cases of clinically apparent liver injury. Atovaquone [2] is a synthetic hydroxynaphthoquinone with antiprotozoal activity. Atovaquone blocks the mitochondrial electron transport at complex III of the respiratory chain of protozoa, thereby inhibiting pyrimidine synthesis, preventing DNA synthesis and leading to protozoal death. Atovaquone is a naphthoquinone compound having a 4-(4-chlorophenyl) cyclohexyl group at the 2-position and a hydroxy substituent at the 3-position. It has a role as an antimalarial, an antifungal agent, an EC 1.3.5.2 [dihydroorotate dehydrogenase (Quinone)] inhibitor, an EC 1.6.5.3 [NADH: ubiquinone reductase (H(+)-translocating)] inhibitor and an EC 1.10.2.2 (quinol--cytochrome-c reductase) inhibitor. It is a member of monochlorobenzenes and a hydroxy-1, 4-naphthoquinone. The IUPAC Name of Atovaquone [3] is 3-[4-(4-chlorophenyl) cyclohexyl]-4-hydroxynaphthalene-1,2-dione. The Chemical Structure of Atovaquone is as follows

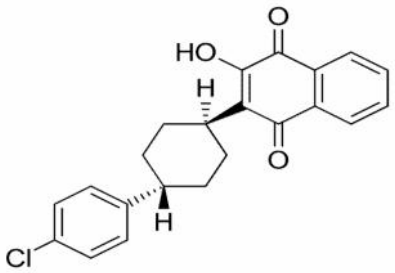

Fig 1: Chemical Structure of Atovaquone

Proguanil [4] is a biguanide derivative which is active against several protozoal species and is used in combination with Atovaquone and Chloroquine for the prevention and therapy of malaria. Proguanil has not been evaluated extensively as a single agent, but the combinations of Proguanil [5] with Atovaquone or Chloroquine have been used to treat malaria and have been linked to serum enzyme elevations during therapy and rare instances of clinically apparent acute liver injury.Proguanil is a biguanide compound which has isopropyl and p-chlorophenyl substituents on the terminal N atoms. A prophylactic antimalarial drug, it works by inhibiting the enzyme dihydrofolate reductase, which is involved in the reproduction of the malaria parasites Plasmodium falciparum and P. vivax within the red blood cells. It has a role as an antimalarial, an antiprotozoal drug and an EC 1.5.1.3 (dihydrofolate reductase) inhibitor. It is a member of biguanides and a member of monochlorobenzenes.The IUPAC Name of Proguanil [6, 7] hydrochloride is (1E)-1-[amino-(4-chloro anilino) methylidene]-2-propan-2-ylguanidine; hydrochloride. The Chemical Structure of Proguanil hydrochloride [7] is as follows

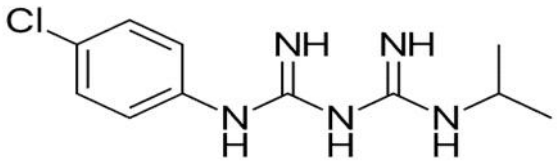

Fig 2: Chemical Structure of Proguanil hydrochloride

2. MATERIALS AND METHODS

Table 1: List of instrument used

S. No.	Instruments/Equipment/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator(Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5mm, 15mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table 2: List of chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Method development:

Atovaquone Standard Solution Preparation

Weigh accurately 10 mg of standard Atovaquone and it transferred into a clean & dry 100 ml of volumetric flask. Add 10ml mobile phase and further do sonication in order to dissolve. Finally make up to the volume up to mark with the mobile phase. The final resulted solution contained about 100 µg/ml of Atovaquone.

Proguanil Hydrochloride Standard Solution Preparation

Weigh accurately about 10 mg of standard Proguanil Hydrochloride and transferred into a clean and dry 100 ml volumetric flask. Add 10ml mobile phase and further do sonication in order to dissolve. Finally make up the volume with the same mobile phase [8] i.e. same solvent system. The volume was made up to the mark with same solvent. The final solution contained about 100µg/ml of Proguanil Hydrochloride.

Initialization of the HPLC instrument

First switched on the HPLC instrument. The selected column was washed with the HPLC grade water for 45 minutes. Then selected column was saturated with the mobile phase for 45 minutes. Then keep the mobile phase for stabilization. The mobile phase was run to obtain the peaks. After completion of stabilization [9]. After 20 minutes the standard drug solution was injected in HPLC.

Method validation:

Accuracy

The accuracy [10] of the method was determined by calculating % recovery. A known amount of Atovaquone and Proguanil HCL was added to a placebo and the amounts were estimated by measuring the peak area. These studies were carried out in triplicate over the specified concentration range and the amount of Atovaquone and Proguanil HCL was estimated by measuring the peak area ratios. The percentage recovery [11] and standard deviation of percentage recovery were calculated.

Precision

The precision of the method was determined in terms of Intra-day [12] and inter-day precision [13]. For intra-day precision studies, a standard solution of 10 ppm was injected at various time intervals and percent related standard deviation [14] (%RSD) was estimated. The inter-day precision was studied by injecting the same concentration of standard solution on consecutive days and the % RSD [15] of the signal was calculated. The repeatability, intermediate precision and reproducibility of the developed method were determined.

Specificity

Specificity [16] is the ability to assess unequivocally the analyte in the presence of components etc. The blank (diluent), placebo, standard (100 ppm), sample (100 ppm) were prepared and injected to prove that the method developed was specific to Atovaquone and Proguanil HCL.

Linearity and range

The linearity [17, 18] of the method was determined at six concentration levels ranging from 12-28 ppm of Atovaquone and Proguanil HCL. A regression line [19] was plotted of peak area v/s concentration. The correlation coefficient [20] and equation of the regression line were calculated. The interval of lowest assessed concentration to the highest is the linearity range [21] of the procedure.

LOD and LOQ

The detection limit [22] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit [23] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision [24] and accuracy [25].

Where, = the standard deviation of the response, Slope = slope of the calibration curve [26].

Robustness

Robustness [27] of the developed method was studied by changing the flow rate and column [28] temperature. The effect of flow rate was studied by keeping all chromatographic conditions same except the flow rate, i.e. 0.9ml/min and in the next run 1.1ml/min respectively. Similarly, the effect of temperature was studied by keeping all chromatographic conditions same except the temperature, i.e. 35 °C and in the next run with 25 °C respectively.

System suitability

The system suitabilityparameters [29, 30] like retention time, the number of USP theoretical plates, USP tailing, and peak area and peak height were evaluated.

Stability Studies

The APIs of Atovaquone and Proguanil HCL was subjected to different stability conditions [31, 32] in various ways to observe the rate and extent of degradation [33] occur in the course of storage after administration to body. This is one type of accelerated stability studies [34] that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing [35].

3. RESULTS AND DISCUSSION

Method development:

Selection of wavelength

Selectivity of HPLC method that uses UV detector [36-38] depends on proper selection of Wavelength. A wavelength which gives good response for the drug to be detected is to be selected. From the UV spectra 284 nm was selected as the wavelength for study. The λ max of this method can be determined as 284 nm.

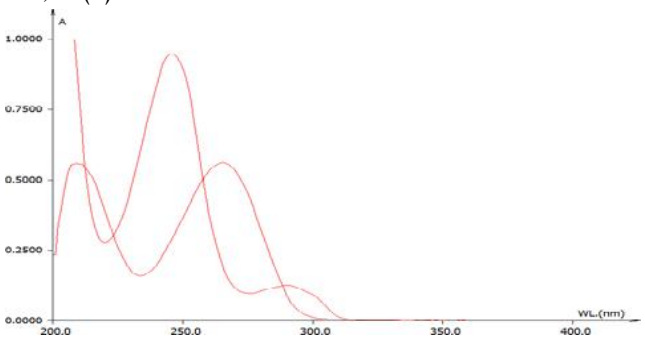

Fig 3: Isobestic point Atovaquone and Proguanil Hydrochloride (258nm)

Optimized chromatographic method:

Table 3: Summary of Optimized Chromatographic Conditions

Mobile phase	Methanol: Acetonitrile 85:15% v/v
Column	Develosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d.
Column Temperature	Ambient
Detection Wavelength	258 nm
Flow rate	1.0 ml/ min.
Run time	15 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

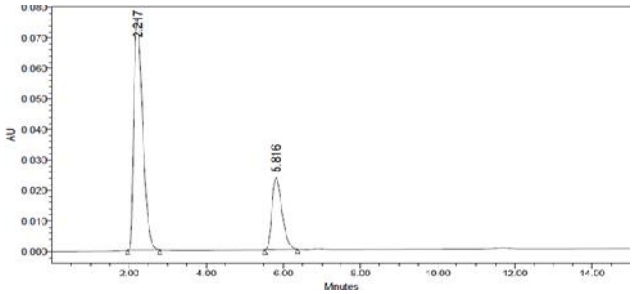

Fig 4: Optimized Chromatographic Condition of Atovaquone and Proguanil Hydrochloride

Method validation

Linearity and range: To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml and 12-28µg/ml for Atovaquone and Proguanil HCL respectively. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Plotting of Calibration Graphs: The result ant area so linearity peaks are plotted against Concentration.

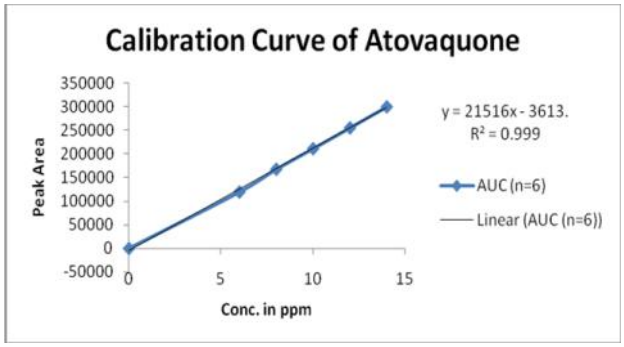

Fig 5: Standard curve for Atovaquone

Table 4: Linearity results for Atovaquone

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
6	119571
8	167873
10	211264
12	255428
14	299987

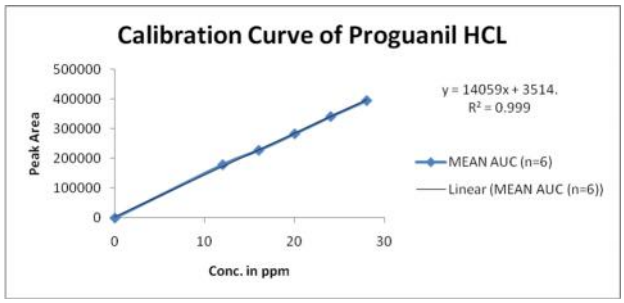

Fig 6: Standard curve for Proguanil HCL

Table 5: Linearity Results for Proguanil HCl

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	179371
16	227893
20	283264
24	341428
28	394987

Accuracy:

Recovery study: To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Atovaquone and Proguanil HCLwere taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 21516x + 3613.2. The results were shown in table-6.

Table 6: Accuracy Readings for Atovaquone

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	7.997368	115949	99.9671	Mean= 100.7003%
S_2 : 80 %	8	8.106622	117485	101.3328	S.D. = 0.6884036
S_3 : 80 %	8	8.064087	116887	100.8011	% R.S.D.= 0.683616%
S_4 : 100 %	10	9.904901	142767	99.04901	Mean= 100.36157%
S_5 : 100 %	10	10.02966	144521	100.2966	S.D. = 1.346221
S_6 : 100 %	10	10.17391	146549	101.7391	R.S.D.= 1.3413706%
S_7 : 120 %	12	12.01807	172476	100.1506	Mean= 100.183756%
S_8 : 120 %	12	11.88079	170546	99.00657	S.D. = 1.19411
S_9 : 120 %	12	12.16729	174574	101.3941	% R.S.D. = 1.19191%

Observation: The mean recoveries were found to be 100.7003, 100.361 and 100.183% for Atovaquone and Proguanil HCL. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Recovery Study: Proguanil HCL

Recovery study: To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Atovaquone and Proguanil HCLwere taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 14059x + 3514.9. The results were shown in table-7.

Table 7: Accuracy results for Proguanil HCL

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	16.08685	229679	100.5428	Mean= 100.54488%
S_2 : 80 %	16	15.93079	227485	99.56745	S.D. =0.97847%
S_3 : 80 %	16	16.2439	231887	101.5244	R.S.D.=0.9731%
S_4 : 100 %	20	20.07632	285767	100.3816	Mean= 99.97095%
S_5 : 100 %	20	19.98769	284521	99.93847	S.D. = 0.395406
S_6 : 100 %	20	19.91856	283549	99.59279	R.S.D.= 0.39552%
S_7 : 120 %	24	23.75432	337476	98.97634	Mean= 100.27718%
S_8 : 120 %	24	24.11494	342546	100.4789	S.D. = 1.21262
S_9 : 120 %	24	24.33032	345574	101.3763	% R.S.D. = 1.20927%

Observation: The mean recoveries were found to be 100.544, 99.970 and 100.277% for Atovaquone and Proguanil HCL. The limit for mean % recovery is 98-102% and as both the values are within the limit, hence it can be said that the proposed method was accurate.

Precision: The precision of each method was ascertained separately from the peak areas obtained by actual determination of six replicates of a fixed amount of drug

Atovaquone and Proguanil HCL. The percent relative standard deviations were calculated for Atovaquone and Proguanil HCL is presented in the Table-8.

Repeatability

Table 8: **Data showing repeatability analysis for Atovaquone & Proguanil HCL**

HPLC Injection Replicates	AUC for Atovaquone	AUC for Proguanil HCL
Replicate – 1	113568	241022
Replicate – 2	113241	240137
Replicate – 3	115408	242911
Replicate – 4	117412	245245
Replicate – 5	112541	241941
Replicate – 6	112546	240444
Average	**114119.3333**	**241356.6667**
Standard Deviation	**1925.83838**	**1416.95812**
% RSD	**1.68756**	**0.58708**

Observation: The repeatability study which was conducted on the solution having the concentration of about 20µg/ml for Atovaquone and Proguanil HCL(n =6) showed a RSD of 0.58708% for Atovaquone and Proguanil HCL. It was concluded that the analytical technique showed good repeatability.

Intermediate precision:

Intra-assay & inter-assay: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Atovaquone and Proguanil HCLrevealed that the proposed method is precise.

Table 9: Results of intra-assay & inter-assay

Conc. of Atovaquone (API) (µg/ml)	Observed Conc. of Atovaquone (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.09	0.97	8.03	0.96
10	10.05	0.45	10.04	0.47
12	11.98	0.37	11.90	0.12

Table 10: Data for Proguanil HCL intra-assay & inter-assay analysis

Conc. of Proguanil HCL (API) (µg/ml)	Observed conc. of Proguanil HCL (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.97	0.27	8.09	0.59
10	10.14	1.29	9.95	0.64
12	12.08	0.61	11.94	0.26

Result and discussion:

The Intraday and interday related studies shows that the % RSD was found to be within limit i.e. (≤2%). So it is indicated that the developed is within the limits. Hence finally we concluded that the developed method was found to be precise.

Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3 (SD/S).

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & discussion

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24 µg/ml respectively for Atovaquone.

The LOD was found to be 0.1 µg/ml and LOQ was found to be 0.3 µg/ml for Proguanil HCL which represents that sensitivity of the method is high.

Method robustness:

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^0C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Atovaquone (API).

Table-11: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	1.05
Flow (0.9 ml/min)	0.67
Temperature (27^0C)	0.58
Temperature (23^0C)	0.61
Wavelength of Detection (280 nm)	0.38
Wavelength of detection (270 nm)	0.17

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^0C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Proguanil HCL (API).

Table 12: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.09
Flow (0.9 ml/min)	0.07
Temperature (27^0C)	0.28
Temperature (23^0C)	0.74
Wavelength of Detection (235 nm)	0.86
Wavelength of detection (240 nm)	0.67

System suitability parameter: It is an integral part of so many analytical procedures. The parameters are based on the idea that the equipment, electronics, analytical operations and the samples to be analyzed constitute as an integral system which can be examined. Finally system suitability

test parameters are established. The obtained data is shown in the following table 13.

Table 13: Data of system suitability parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs < 2	3.65
2	Asymmetry	T ± 2	Atovaquone = 0.35 Proguanil HCL = 0.23
3	Theoretical plate	N < 2000	Atovaquone = 3771 Proguanil HCL =2437

Estimation of Atovaquone and Proguanil HCL in pharmaceutical dosage form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table-14.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution

DT = Sample solution dilution

P = Working standard percentage purity

The assay was performed as explained in the previous chapter (Above). The results which are obtained are following:

Table 14: Recovery data for estimation Atovaquone and Proguanil HCL in Lemel

Brand name of Atovaquone and Proguanil HCL	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method(n=6)	Assay % (± SD)
Lemel Tablets (Livealth Biopharma Pvt. Ltd.)	250/100	249.787 (±0.598)/99.867 (±0.796)/	99.875 (±0.598)/99.698 (± 0.467)

Result & Discussion: The % purity of Atovaquone & Proguanil HCL for Tablets was found to be 99.875% and 99.698%respectively.

Stability studies:

Results of degradation studies: The results of the stress studies indicated the specificity of the method that has been developed. Atovaquone and Proguanil HCL were stable only in photolytic stress conditions and little bit in thermal stress conditions. The results of forced degradation studies are given in the following Table-15.

Table 15: Results of forced degradation studies of Atovaquone and Proguanil HCl

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

4. SUMMARY AND CONCLUSION

Isocratic elution is easy, needs only one pump & flat standard splitting up for easy and also reproducible results. So, it was preferred for the present research over gradient elution.In case of RP-HPLC various columns are offered, however below Develosil ODS HG-5 RP C18, 5µm, l5cmx4.6mm i.d. column was preferred since using this column top shape, resolution as well as absorbance were great.

Mobile stage & diluent for preparation of different examples were completed after researching the solubility of API in various solvents of our disposal (methanol, Acetonitrile, water, 0.1 N NaOH, 0.1 N HCl).Discovery wavelength was picked after checking the basic remedy of drug over 200 to 400nm. From the U.V spectrum of Atovaquone and also Proguanil HCL it is evident that the majority of the HPLC work can be achieved in the wavelength variety of 200-300 nm easily. Even more, a circulation rate of 1 ml/min & an injection volume of 10µl were found to be the best evaluation.

5. REFERENCES

1. Drugbank. https://go.drugbank.com/drugs/DB01117 (Accessed on 12th Jan 2022).
2. Atovaquone. https://pubchem.ncbi.nlm.nih.gov/compound/Atovaquone (Accessed on 10th Jan 2022).

3. Atovaquone. https://en.wikipedia.org/wiki/Atovaquone (Accessed on 29th Dec 2021).
4. Drug bank. https://go.drugbank.com/drugs/DB01131 (Accessed on 10th Jan 2022).
5. Drug bank. https://go.drugbank.com/salts/DBSALT001187 (Accessed on 22th Oct 2021).
6. Proguanil. https://en.wikipedia.org/wiki/Proguanil (Accessed on 27th Oct 2021).
7. Atovaquone https://pubchem.ncbi.nlm.nih.gov/compound/Atovaquone (Accessed on 09th Sep 2021).
8. British Pharmacopoeia.(International Ed.) 1993; Vol. 1:429, 483. Published on the Recommendation of the Medicines Commissions Pursuant to Medicines Act 1968, 1993.
9. United States Pharmacopoeia 29 NF 24, Published on the Recommendation of the Medicines Commissions Pursuant to Medicines, page no. 587.
10. Skoog DA, Holler FJ, Nieman TA. Principles of Instrumental Analysis, 5th edition, Harcourt Publishes Int Company, Skoog, Holler and Nieman, Chapter 28, p.726-66.
11. Beckett AH, Stenlake JB. Practical Pharmaceutical Chemistry. 4th ed. CBS Publishers and Distributors; 2002. p. 157-174.
12. Pharmacopoeia I, Volume II. Published by the controller of Publication. Vol. I, New Delhi. 2007;655.
13. Caude M. HPLC Columns Theory, Technology and Practice. Analusis. 1997;9:M74.
14. Elena Kartz. Handbook of HPLC, Vol.78, Marcel Dekker Inc.
15. Galen W. Ewing. Instrumental Methods of Chemical Analysis, 5th Edition, McGraw Hill Book Company 1988.
16. "HPLC in Pharmaceutical Industry", Fong and Long, Marcel Dekker Series
17. Chatwal Anand. Instrumental Method of Chemical Analysis", Himalaya Publishing House, p.no.615-623.
18. Kealey and Haines PJ. Analytical Chemistry, 1st edition, Bios Publisher, 2002: P1-7.
19. Skoog, West, Holler, Crouch, "Fundamentals of analytical chemistry", eighth edition, 2009 (Indian edition), Cengage learning India Pvt ltd, New Delhi, Page no. 271-80.
20. Kasture AV, Mahadik KR, Wadodkar SG, More HN. A textbook of pharmaceutical analysis, Instrumental methods", Nirali Prakashan, vol.2, 9th edition, page no. 5-7, 28-30.
21. Settle FA, In: Handbook of Instrumental Techniques for Analytical Chemistry. 1st Ed, Singapore, Pearson Education Inc.2004.
22. Willard HH and Dean AJ. Instrumental Methods of Analysis. CBS Publishers and distributors, 7th Ed, 1986, 513-515.
23. Connors AK. In: A Text Book of Pharmaceutical Analysis. A Wiley Interscience Publication, 3rd Ed, 2005; 373-400.
24. Ahuja S. In: High Pressure Liquid Chromatography of Comprehensive Analytical Chemistry. Elsevier Publications. 2006.
25. Biosciences A. Reversed Phase Chromatography. Principles and Methods. 2013:6-8.
26. Synder LR, Kirkland JJ and Glajch JL. In: Practical HPLC Method Development, 2nd Ed, John Wiley and Sons Inc. Canada. 1997.
27. Mohammad T et al., HPLC Method Development and Validation for Pharmaceutical Analysis- A Review. International Pharmaceutica Sciencia. 2012; 2: 14.
28. Snyder LR, Kirkland JJ and Glajch JL. In: Practical HPLC Method Development. 2nd ed, 2001.
29. Vibha G et al., Development and validation of HPLC method - a review. International Research Journal of Pharmaceutical and Applied Sciences. 2012; 2: 22-3.
30. Bliesner DM. In: Validating Chromatographic Methods. John Wiley & sons Inc. 2006, 88-92.
31. Validation of Analytical Procedures: Methodology. ICH-Guidelines Q2B, Geneva. 1996, 11. (CPMP/ICH/281/95).
32. Development and validation of HPLC method - A Review, Vibha Gupta et al, International Research Journal of Pharmaceutical and Applied Sciences, 2012; 2(4):17-25.
33. Bhardwaj SK. A Review: HPLC Method Development and Validation,. International Journal of Analytical and Bioanalytical Chemistry, accepted 20 November 2015.
34. Method Development: A Guide to Basics Quantitative & Qualitative HPLC, LC, GC chromacademy.
35. Lalit V Sonawane. Bioanalytical Method Validation and Its Pharmaceutical Application- A Review Pharmaceutica Analytical Acta 2014, 5:3Center for Drug Evaluation and Research (CDER) Reviewer Guidance.
36. EMEA I. Topic Q 2 (R1), Validation of Analytical Procedures: Text and Methodology. InInternational Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 2006.
37. Pattanaik P, Subrahmanyam KV, Roy H. UPLC Method Development and Validation for Terconazole in Active Ingredient. Int J Pharma Res Health Sci 2015; 3(2): 22-26.
38. Sharanya M, Siddiqua H, Chaitanya Padavala HK, and Kauser R, Development and Validation of RP-Hplc Method for the Simultaneous Estimation of Atovaquone and Proguanil Hydrochloride in Pharmaceutical Dosage Form. World Journal of Pharmacy and Pharmaceutical Sciences 2019; 9: 2331-445.

ACKNOWLEDGEMENT: None.

CONFLICT OF INTEREST: The authors declare no conflict of interest, financial or otherwise.

SOURCE OF FUNDING: None.

AVAILABILITY OF DATA AND MATERIALS: Not applicable.

CONSENT FOR PUBLICATION: Not applicable.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE: Not applicable