International Journal of Health care and Biological Sciences

Content available at www.saap.org.in Online ISSN: 2582-7499

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VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF PROGUANIL AND ATOVAQUONE IN PHARMACEUTICAL DOSAGE FORM

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Article History

Received on: 11-08-2023
Revised on: 25-09-2023
Accepted on: 15-10-2023

Abstract

The aim of the study was to develop and validate a rapid, sensitive and accurate method for simultaneous estimation of proguanil and atovaquone in Pharmaceutical dosage forms by liquid chromatography.The chromatographic separation was achieved on Kromasil C18 (4.6 x 150 mm; 5 µm) at ambient temperature.The separation was achievedby employing a mobile phase consists of Phosphate buffer: acetonitrile (40:60 v/v). The flow rate was 1.0ml/ minute and ultra violet detector at 280 nm. The retention time for proguanil and atovaquone found to be 2.15 min and 2.48 min respectively. The proposed method was validated for selectivity, precision, linearity and accuracy. All the results obtained from various validation parameters were within the acceptable range. The method was found to be linear from concentrations of 20-120 µg/ml for proguanil and 50 - 300µg/ml for atovaquone.

Keywords: Proguanil, Atovaquone, RP-HPLC.

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DOI: https://doi.org/10.46795/ijhcbs.v4i4.549

Introduction

Proguanil [1-2] is chemically (1E)-1-[amino-(4-chloroanilino) methylidene]-2-propan-2-ylguanidine [Fig:1] which is an anti-malarial drug used in combination with atovaquone or chloroquine to treat malaria and have been linked to serum enzyme elevations during therapy. It is a biguanide derivative that is converted to an active metabolite called cycloguanil. It exerts its antimalarial action by inhibiting parasitic dihydrofolatereductase enzyme. Upon hydrolysis, proguanil is converted to its active cyclic triazine metabolite, cycloguanil, by a cytochrome P450 dependent reaction. Cycloguanil selectively inhibits the bi functional dihydro folate reductase-thymidylate synthase (DHFR-TS) of plasmodium parasite, thereby disrupting deoxythymidylate synthesis and ultimately blocking DNA and protein synthesis in the parasite.

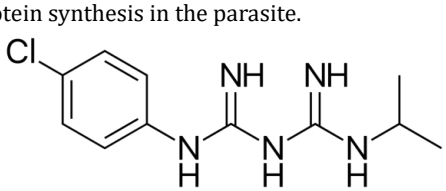

Figure:1- Chemical structure of proguanil

Atavaquone [3-4] [Fig:2]is chemically trans-2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthalenedione.Atovaquone selectively inhibits the malarial cytochrome bc_1 complex in the parasitic electron transport chain, collapsing the mitochondrial membrane potential.

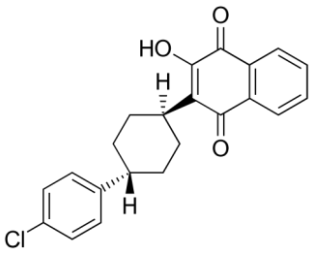

Figure: 2- Chemical structure of atovaquone

As per the available literature, HPLC methods have been reported for determination of proguanil[5-9] and atovaquone[10-15] in single pharmaceutical dosage forms and biological samples and few methods [16-21] have been reported for the simultaneous determination of proguanil and atovaquonein combined dosage forms.

Materials and methods

Chemicals and Solvents

The reference samples of proguanil and atovaquone(API) were obtained from M/s. Mylan labs, Hyderabad, India. The branded formulation (tablets) (Malaronetablets containing proguanil and atovaquone) manufactured by M/s. GSKIndia healthcare limited, Gurgaon were procured from the local market. HPLC grade acetonitrile, potassium

dihydrogen phosphate, ortho phosphoric acidwere obtained from M/s. Rankem Chemicals Ltd, Mumbai, India. Milli-Q water dispensed through a 0.22 µ filter of the Milli-Q water purification system (Millipore, Merck KGaA, Darmstadt, Germany) was used throughout the study.

Preparation of phosphate buffer solution

About 1.42 gm disodium hydrogen phosphate was weighed, transferred into a 1000 mL flask and 400ml of Milli-Q water was added, then mixed well. Then volume was made up to 1000 mL, sonicated for five minutes and cooled to room temperature. The pH of above buffer solution was adjusted to 3.0±0.05 with orthophosphoric acid solution and then filtered through a 0.45 µ membrane filter.

Preparation of the mobile phase

A 40:60 v/v mixture of the above phosphate buffer and acetonitrile was prepared and used as the mobile phase in the study.

The diluent

A 50:50 v/v mixture of methanol and water was prepared and used as the diluent for the preparation of drug dilutions.

Preparation of mixed standard solution of proguanil and atovaquone

About 100 mg of proguanil and 250 mg of atovaquone were accurately weighed and transferred into a 50 mL clean dry volumetric flask containing 30 mL of the diluent. The solution was sonicated for 10 min and then volume was made up to the mark with a further quantity of the diluent to get a concentration of 2 mg/mLproguanil and 5 mg/mLatovaquone (Stock solution). A mixed working standard solution was further prepared by diluting the above stock solution to obtain a concentration of200 µg/mL of proguanil and 500 µg/mL of atovaquone**.**

Preparation of the tablet solution

Twenty tablets of the commercial sample of "Malarone" were weighed and finely powdered. An accurately weighed portion of powdered sample equivalent to 100 mg of proguaniland 250 mg of atovaquone was transferred into a 50 mL volumetric flask containing 30 mL of the diluent. The contents of the flask were sonicated for about 10 min for complete solubility of the drugs and the volume made up with a further quantity of the diluent. Then, this mixture was filtered through a 0.45 µ membrane filter. Further, 1 mL of the above filtrate was pipetted into a 10 mL volumetric flask and the volume was made up with the diluent.

Results and Discussion

Method Development

After several initial trails with mixtures of methanol, water, Acetonitrile and buffer in various combinations and proportions, a trail with a mobile phase mixture of phosphate buffer: acetonitrile (40:60 %v/v). at flow rate was 1.0 mL/ minute brought sharp peaks. The optimized chromatographic conditions used were shown in Table 1. The chromatogram was shown in Fig 3.The retention times obtained under the optimized conditions were 2.15 min for proguanil and 2.48 min for atovaquone.

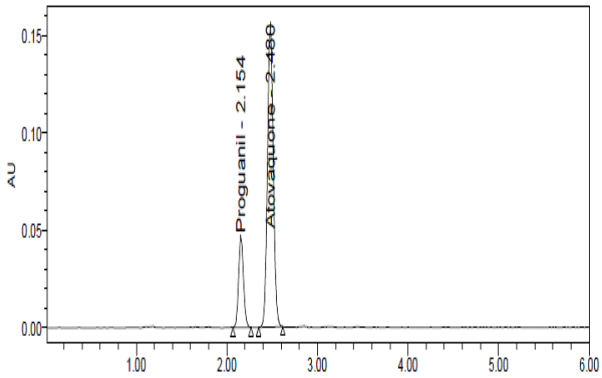

Figure 3: Chromatogram of standard

Table 1: Optimized chromatographic conditions

Column	:	Kromasil C18 (4.6 x 150 mm; 5 µm)
Elution mode	:	Isocratic
Mobile phase	:	Phosphate buffer: acetonitrile (40:60 v/v)
Column Temp	:	30^0 C
Wavelength	:	280 nm
Injection Volume	:	10 µL
Flow rate	:	1 mL/min
Run time	:	6 min

Linearity

Linearity was studied by analyzing five standard solutions covering the range of 20-120µg/mLfor proguanil and 60-300 µg/mL for atovaquone. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method. (Tables 2&3) (Figures 4 & 5).

Table 2: Linearity data

Proguanil		**Atovaquone**	
Concentration (µg/ml)	**Mean Peak area (n=3)**	**Concentration (µg/ml)**	**Mean Peak area (n=3)**
20	57689	50	198563
40	114524	100	388425
60	172989	150	595305
80	235848	200	797212
100	288159	250	997584
120	346272	300	1189564

Table 3: Analytical performance parameters

Parameters	**Proguanil**	**Atovaquone**
Slope (m)	2316.2	3192.9
Intercept (c)	87.73	3996.7
Correlation coefficient (R2)	0.999	0.999

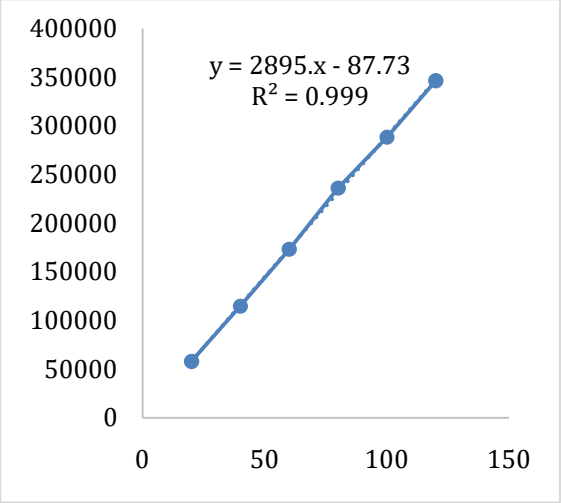

Figure 4: Calibration curve of proguanil

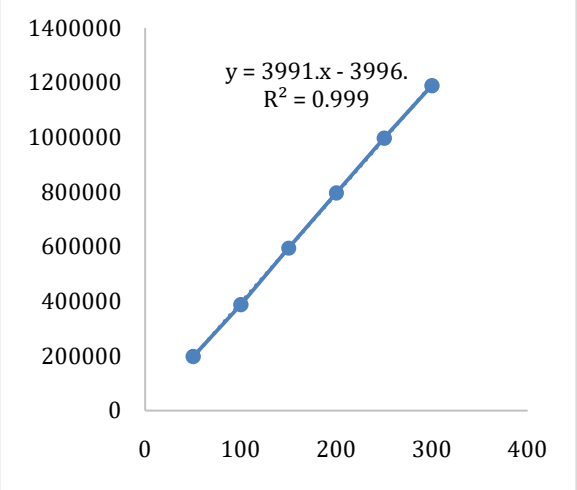

Figure 5: Calibration curve of atovaquone

Accuracy

The accuracy of the method was determined by calculating the recoveries of proguanil and atovaquone by analyzing solutions containing approximately 50%, 100% and 150% of the working strength of proguanil and atovaquone(Table 4).

Table 4: Accuracy (recovery) data

Preanalysed amount (µg/ml)		Spiked Amount (µg/ml)		% Recovered	
Proguanil	**Atovaquone**	**Proguanil**	**Atovaquone**	**Proguanil**	**Atovaquone**
60	150	30	75	99.67	99.67
60	150	30	75	99.57	99.52
60	150	30	75	99.76	99.61
60	150	60	150	99.68	99.25
60	150	60	150	99.45	99.69
60	150	60	150	99.82	99.85
60	150	90	225	99.03	99.17
60	150	90	225	99.11	99.36
60	150	90	225	99.14	99.58
			MEAN	99.47	99.52
			SD	0.285	0.209
			%RSD	**0.29**	**0.21**

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was determined as repeatability and intermediate precision, in accordance with ICH guidelines. The repeatability and intermediate precision were determined by analyzing the samples of proguanil and atovaquone. The repeatability and intermediate precision data were assessed by the use of standard solutions of proguanil and atovaquone and are summarized in Table 7 and 8 respectively.

Repeatability

Six replicate injections of proguanil and atovaquone were analyzed on the same day for assessing repeatability. The % RSD for proguanil and atovaquone were found to be 0.28 and 0.06 respectively. These values were found to be within acceptable limit of ≤2 and hence, the method is reproducible. The corresponding results are shown in the Table 5.

Table 5: Results of repeatability of proguanil and atovaquone

S. No.	Proguanil			Atovaquone		
	Area	**USP Plate Count**	**USP Tailing**	**Area**	**USP Plate Count**	**USP Tailing**
1	234748	7535	1.12	797582	6921	1.02
2	236622	7321	1.11	797015	7161	1.03
3	234852	7245	1.08	796025	6895	1.05
4	235658	7198	1.06	796852	7025	1.10
5	235698	7268	1.06	797158	7254	1.11
6	234888	7401	1.05	797098	7151	1.05
MEAN	235411			796955		
SD	662.8			471.5		
% RSD	**0.28**			**0.06**		

Intermediate Precision:

Six replicate injections of the same dilution were analyzed on two different days by different analyst for verifying the variation in the precision. The % RSD of the results for proguanil and atovaquone were found to be 0.03 and 0.44 respectively, which are within acceptable limit of ≤2. Hence, the method is reproducible on different days. This indicates that the method is precise. The results are shown in the Table 6a and 6b.

Table 6a: Results of Intermediate Precision of proguanil

S. No.	Average area (n=6)	USP Plate Count	USP Tailing
Day 1	235251.38	7454	1.07
Day 2	235111.25	7398	1.08
Overall average	235181.31		
SD	70.06		
% RSD	**0.03**		

Table 6b: Results of Intermediate Precision of atovaquone

S. No.	Average area (n=6)	USP Plate Count	USP Tailing
Day 1	789856	7123	1.03
Day 2	796852	7025	1.08
Overall average	793354		
SD	3498		
% RSD	**0.44**		

Robustness

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method (Table 7&8).

Table 7: Robustness study for proguanil

Condition	Mean area	% assay	% difference
optimised	234855	99.14	-----
Flow rate at 0.9 mL/min Flow rate at 1.1 mL/min	223958 234582	99.13 99.52	0.01 0.38
Mobile phase: Buffer-Acetonitrile(45:55) Buffer-Acetonitrile(35:65)	234875 225248	99.11 99.25	0.03 0.11
Column Temperature: at 25°C at 35°C	245558 235245	99.35 99.15	0.24 0.01

Table 8: Robustness study for atovaquone

Condition	Mean area	% assay	% difference
optimised	794217	99.32	----
Flow rate at 0.9 mL/min Flow rate at 1.1 mL/min	797582 786859	99.65 99.58	0.33 0.26
Mobile phase: Buffer-Acetonitrile(45:55) Buffer-Acetonitrile(35:65)	788859 796582	99.28 99.58	0.04 0.26
Column Temperature: at 25°C at 35°C	798547 786985	99.42 99.36	0.10 0.04

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

LOD and LOQ values for proguanil were 0.98 and 3.23 µg/ml respectively and those for atovaquone were 0.61 and 2.01µg/ml respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive.

Stability of the formulation solution:

The sample solution injected after 24 h by keeping at room temperature (30°C) did not show any appreciable change. The deviation in the assay was not more than 2 and the results are shown in Table 9.

Table 9: Stability data of proguanil and atovaquone

Drug	%Assay at 0 h*	%Assay at 24 h*	Deviation
proguanil	99.23	99.61	0.38
atovaquone	99.85	99.42	0.43

*n=6 for each parameter

Conclusion

In this current investigation, we developed and validated a novel HPLC method characterized by its simplicity, precision, and accuracy for concurrently determining proguanil and atovaquone in a combined tablet formulation. The stationary phase employed was Kromasil C18 (4.6 x 150 mm; 5 µm), while the mobile phase consisted of a 40:60% v/v blend of phosphate buffer and acetonitrile, flowing at 1.0 mL/min. Under the optimized conditions, proguanil and atovaquone exhibited retention times of 2.15 and 2.48 min, respectively. Method validation was conducted following ICH guidelines, revealing a number of theoretical plates exceeding 2000, a tailing factor below 2 and an RSD of peak area below 2, meeting the system suitability parameters.

The linear ranges for the drugs, sacubitril, and valsartan, were determined to be 20-120 µg/mL and 60-300 µg/mL, respectively. Mean recovery values for proguanil and atovaquonewere 99.47% and 99.52%, respectively, affirming the method's accuracy. Repeatability and intermediate precision demonstrated RSD values ≤2, establishing the precision of the method. The method's sensitivity was confirmed by the lowest LOD and LOQ values. Stability studies indicated that proguanil and atovaquone remained stable for up to 24 hours. Robustness of the method was tested by intentionally altering chromatographic conditions, demonstrating the method's reliability with no significant changes in results. Notably, our proposed method offers advantages over previously reported methods, such as the use of a simple mobile phase for elution, achieving good resolution for both drugs. The method's short run time enhances analysis speed, allowing for the analysis of a higher number of samples per unit time. Furthermore, the proposed method exhibited a broader linear range at lower concentrations with lower LOD and LOQ values compared to reported methods. Overall, this RP-HPLC method stands out as a sensitive, robust, precise, and accurate approach suitable for routine quality control analysis in the simultaneous determination of proguanil and atovaquone in tablet formulations.

Conflict of Interest

Authors are declared that no conflict of interest

Funding
No Funding

Ethical Considerations and Inform Consent
Not Applicable

Author Contribution
Authors are contributed equally.

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