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***Research Article** (Open Access)*

# DEVELOPMENT AND VALIDATION OF NEW RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF PROGUANIL AND ATOVAQUONE IN PHARMACEUTICAL DOSAGE FORM

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## 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 achieved by employing a mobile phase consists of Phosphate buffer: acetonitrile (50:50 v/v). The flow rate was 1.0ml/ minute and ultra violet detector at 287nm. 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 25-150 µg/ml for proguanil and 62.5 - 375 µg/ml for atovaquone.

***Keywords:*** *Proguanil, Atovaquone, Isocratic and RP-HPLC*



## INTRODUCTION
Proguanil (Wangboonskul *et al.*, 1993; Hussein *et al.*, l996) 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 bifunctionaldihydrofolatereductase-thymidylate synthase (DHFR-TS) of plasmodium parasite, thereby disrupting deoxythymidylate synthesis and ultimately blocking DNA and protein synthesis in the parasite.

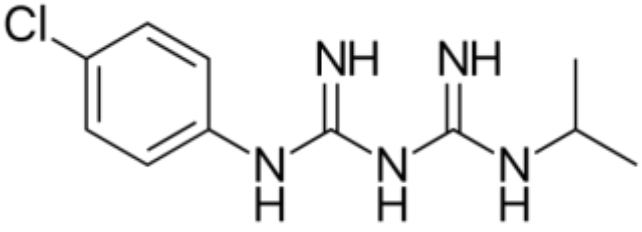


**Figure 1: Chemical structure of proguanil**

Atavaquone (Helsby *et al.*, 1993; Gemma *et al.*, 2013) [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.

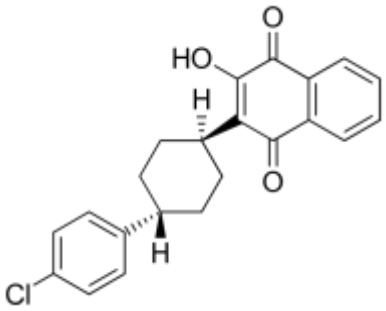


**Figure 2: Chemical structure of atovaquone**

(As per the available literature, HPLC methods have been reported for determination of proguanil (Satish *et al.*, 2009; and Nathalie *et al.*, 2006; Adewuyi *et al.*, 2010; Nandini and Seema 2013; Nandini and Seema 2013; Sompon and Pratt, 1995; Benjamin *et al.*, 2005) and atovaquone (Sanjay *et al.*, 2009; Allison *et al.*, 2017; Satish *et al.*, 2009; Viplava and HarithaPavani, 2012; Santosh *et al.*, 2018; Varsha *et al.*, 2013; Kalpesh *et al.*, 2010; Srujani *et al.*, 2015) in single pharmaceutical dosage forms and biological samples and few methods (Veer *et al.*, 2012; Lakshmana *et al.*, 2017; Lindegardh *et al.*, 2005; Naresh *et al.*, 2014; Nitin and Lalitha, 2014; Patil *et al.*, 2016; Naazneen and Sridevi 2017; Bhavyasri *et al.*, 2013; Julius *et al.*, 2009; Patil *et al.*, 2013; and Sahoo *et al.*, 2012) have been reported for the simultaneous determination of proguanil and atovaquone in 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) (Malarone tablets 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 acid were 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 50:50 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 acetonitrile 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/mL proguanil and 5 mg/mL atovaquone (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 proguanil and 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 DISSCUSION

### 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 (50:50 %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.

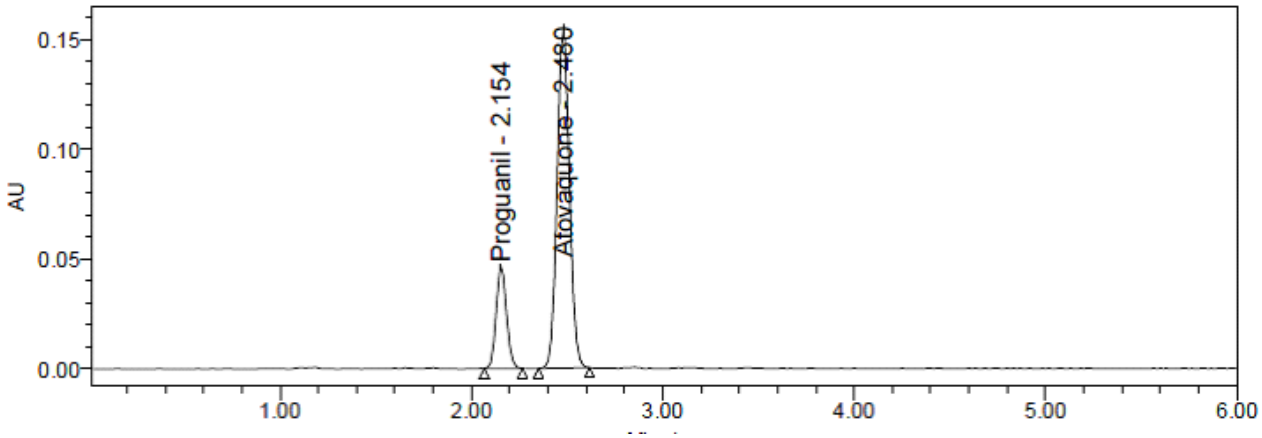


**Figure 3: Chromatogram of standard solution of proguanil and atovaquone**

**Table 1: Optimized chromatographic conditions for simultaneous estimation of proguanil and atovaquone.**

| | | |
|---|---|---|
| **Column** | **:** | Kromasil C18 (4.6 x 150 mm; 5 µm) |
| **Elution mode** | **:** | Isocratic |
| **Mobile phase** | **:** | Phosphate buffer: acetonitrile (50:50 v/v) |
| **Column Temp** | **:** | $30^0$ C |
| **Wavelength** | **:** | 287 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 25-150 µg/mL for proguanil and 62.5-375 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 of proguanil and atovaquone**

| **Proguanil** | | **Atovaquone** | |
|---|---|---|---|
| **Concentration (µg/ml)** | **Mean Peak area (n=3)** | **Concentration (µg/ml)** | **Mean Peak area (n=3)** |
| 25 | 57689 | 62.5 | 198563 |
| 50 | 114524 | 125 | 388425 |
| 75 | 172989 | 187.5 | 595305 |
| 100 | 235848 | 250 | 797212 |
| 125 | 288159 | 312.5 | 997584 |
| 150 | 346272 | 375 | 1189564 |

**Table 3: Analytical performance parameters of proguanil and atovaquone**

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

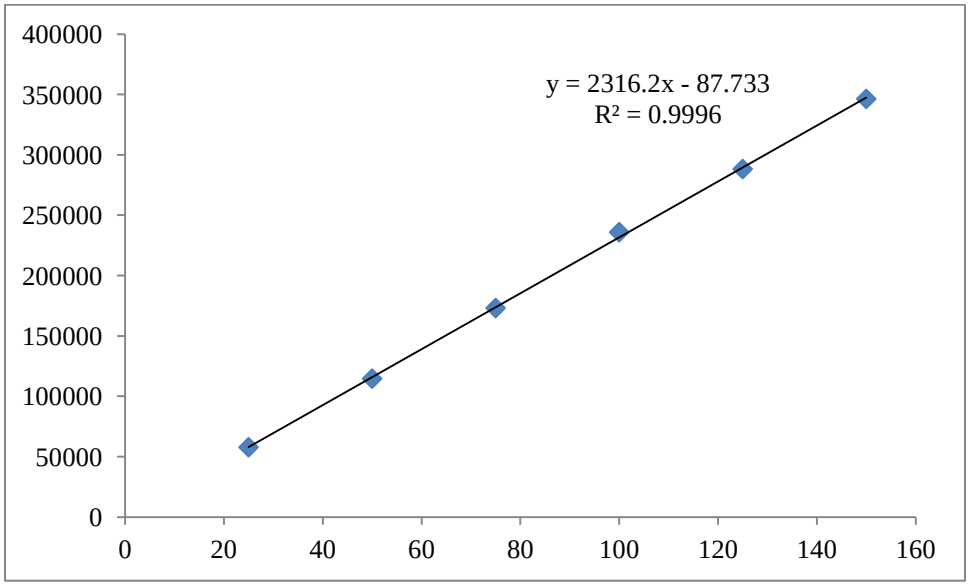


**Figure 4: Calibration curve of proguanil**

**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).

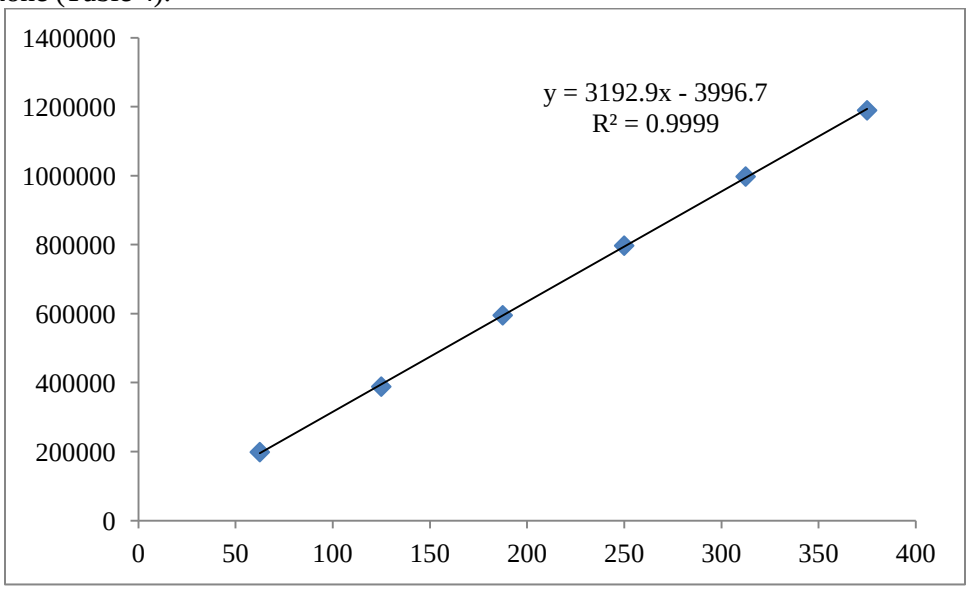


**Figure 5: Calibration curve of atovaquone**

**Table 4: Accuracy (recovery) data**

| **Preanalysed amount (µg/ml)** | | **Spiked Amount (µg/ml)** | | **% Recovered** | |
|---|---|---|---|---|---|
| **Proguanil** | **Atovaquone** | **Proguanil** | **Atovaquone** | **Proguanil** | **Atovaquone** |
| 50 | 125 | 25 | 62.5 | 99.77 | 99.79 |
| 50 | 125 | 25 | 62.5 | 99.75 | 99.84 |
| 50 | 125 | 25 | 62.5 | 99.76 | 99.67 |
| 50 | 125 | 50 | 125 | 99.86 | 99.52 |
| 50 | 125 | 50 | 125 | 99.84 | 99.66 |
| 50 | 125 | 50 | 125 | 99.79 | 99.75 |
| 50 | 125 | 75 | 187.5 | 99.06 | 99.71 |
| 50 | 125 | 75 | 187.5 | 99.11 | 99.65 |
| 50 | 125 | 75 | 187.5 | 99.15 | 99.72 |
| | | | **MEAN** | 99.56 | 99.70 |
| | | | **SD** | 0.346 | 0.092 |
| | | | **%RSD** | **0.35** | **0.09** |

**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 0.27 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 | 234847 | 7528 | 1.11 | 797232 | 6907 | 1.07 |
| 2 | 236511 | 7317 | 1.13 | 796975 | 7122 | 1.04 |
| 3 | 234925 | 7231 | 1.09 | 795987 | 6959 | 1.06 |
| 4 | 235391 | 7289 | 1.10 | 796947 | 7126 | 1.08 |
| 5 | 235768 | 7344 | 1.05 | 797045 | 7132 | 1.10 |
| 6 | 235001 | 7399 | 1.06 | 797144 | 7034 | 1.04 |
| MEAN | 235407.16 | | | 796888.33 | | |
| SD | 641.8 | | | 454.17 | | |
| % RSD | **0.27** | | | **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 0.38 and 0.09 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 | 235103.66 | 7388 | 1.08 |
| Day 2 | 235104.66 | 7412 | 1.10 |
| Overall average | 235102.66 | | |
| SD | 896.07 | | |
| % RSD | **0.38** | | |

**Table 6b: Results of Intermediate Precision of atovaquone**

| S. No. | Average area (n=6) | USP Plate Count | USP Tailing |
|---|---|---|---|
| Day 1 | 796794.0 | 7032 | 1.05 |
| Day 2 | 796792.0 | 7001 | 1.02 |
| Overall average | 796793.0 | | |
| SD | 682.0 | | |
| % RSD | **0.09** | | |

**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 | 234910 | 99.07 | ----- |
| Flow rate at 0.9 mL/min | 223557 | 99.15 | 0.08 |
| Flow rate at 1.1 mL/min | 235623 | 99.34 | 0.27 |
| Mobile phase: | | | |
| Buffer-Acetonitrile(55:45) | 234648 | 99.02 | 0.05 |
| Buffer-Acetonitrile(45:55) | 225885 | 98.97 | 0.1 |
| Column Temperature: | | | |
| at 25°C | 245678 | 98.79 | 0.28 |
| at 35°C | 235321 | 99.21 | 0.14 |

**Table 8: Robustness study for atovaquone**

| Condition | Mean area | % assay | % difference |
|---|---|---|---|
| optimised | 793832 | 99.81 | ---- |
| Flow rate at 0.9 mL/min | 797671 | 100.03 | 0.22 |
| Flow rate at 1.1 mL/min | 786588 | 99.97 | 0.16 |
| Mobile phase: | | | |
| Buffer-Acetonitrile(55:45) | 789671 | 100.07 | 0.26 |
| Buffer-Acetonitrile(45:55) | 793588 | 99.89 | 0.08 |
| Column Temperature: | | | |
| at 25°C | 797348 | 99.95 | 0.14 |
| at 35°C | 784598 | 99.77 | 0.04 |

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

LOD and LOQ values for proguanil were 1.10 and 3.33 µg/ml respectively and those for atovaquone were 0.88 and 2.65 µg/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 | 99.65 | 98.89 | 0.76 |
| atovaquone | 100.12 | 99.78 | 0.34 |

*n=6 for each parameter

## CONCLUSION

In the present study, a new simple, precise and accurate HPLC method was developed and validated for the simultaneous estimation of proguanil and atovaquone in combined tablet dosage form. In this method, a Kromasil C18 (4.6 x 150 mm; 5 µm) was selected as the stationary phase. A 50:50% v/v mixture of phosphate buffer and acetonitrile was used as the mobile phase at a flow rate of 1.0 mL/min. Under the optimized conditions, the retention times obtained for proguanil and atovaquone were 2.15 and 2.48 min

respectively. The method was validated as per the ICH guidelines. In this method, the number of theoretical plates is above 2000, tailing factor is less than 2 and RSD of peak area is less than 2 which indicates that the optimized method met the limits of system suitability parameters. The linearity ranges obtained for the drugs are sacubitril and valsartan shows in 25-150 µg/mL and 62.5-375 µg/mL respectively. The percent mean recovery values of proguanil and atovaquone were found to be 99.56 & 99.70 respectively and it showed that the proposed method is accurate. RSD values of repeatability and intermediate precision were ≤2 and hence the method is precise. The lowest values of LOD and LOQ as obtained by the proposed HPLC method indicate that the method is sensitive. The solution stability studies of method indicate that proguanil and atovaquone were stable up to 24 h. Chromatographic conditions were deliberately changed slightly to check the robustness of the method and the results showed the reliability of the method as no appreciable changes in the results were observed.
The proposed method has specific merits over the earlier reported methods. Both the drugs were eluted with a simple mobile phase with good resolution. The retention times of the drugs obtained in this method were short. The short run time shows the speed of analysis which enables more number of samples to be analyzed per unit time. The linearity range for both drugs obtained at lowest concentrations with least LOD & LOQ values were found by proposed method than the reported methods. The proposed RP-HPLC method is sensitive, robust, precise and accurate and can be used for routine quality control analysis for simultaneous determination of proguanil and atovaquone in their tablet dosage forms.

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