

Original Article

COMPARATIVE EVALUATION OF FIRST ORDER, ABSORBANCE RATIO AND BIVARIATE SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF ATOVAQUONE AND PROGUANIL IN PHARMACEUTICAL FORMULATION MALARONE®

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Received: 07 Jun 2015 Revised and Accepted: 11 Jul 2015

ABSTRACT

**Objective:** Three simple, rapid, accurate and precise spectrophotometric methods have been developed for the simultaneous estimation of atovaquone and proguanil hydrochloride in pharmaceutical preparations.

**Methods:** The determination of drugs was carried out using the first order derivative, absorbance-ratio and bivariate spectrophotometric methods. The methods were validated for their linearity, accuracy and precision, recovery and ruggedness according to the ICH guidelines.

**Results:** The linearity was established in the concentration range of 1.0-10 µg/ml for atovaquone and 0.5-8.0 µg/ml for proguanil hydrochloride by all three methods. The limit of detection (LOD) and the limit of quantitation (LOQ) of the methods varied from 0.252 to 0.270 µg/ml and 0.764 to 0.825 µg/ml for atovaquone and 0.119 to 0.156 µg/ml and 0.361 to 472 µg/ml for proguanil hydrochloride respectively. The intra-and inter-batch accuracy (% recovery) and precision (% RSD) ranged from 99.16 to 101.05 % and 0.603 to 1.048 for atovaquone and 99.74 to 101.12 % and 0.593 to 1.001 for proguanil respectively.

**Conclusion:** The proposed methods were applied to a pharmaceutical formulation with acceptable accuracy and precision without any interference from commonly used excipients and additives. The results show that all three methods are comparable, cost effective and rapid and thus can be readily used in quality control labs for routine analysis of these drugs.

**Keywords:** Atovaquone, Proguanil hydrochloride, Derivative spectrophotometry, Absorbance-ratio method, Bivariate Method, Malarone®

INTRODUCTION

Atovaquone [*trans*-2-[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthalene dione, ATQ] is chemically similar to ubiquinone, which plays an essential role in mediating electron transfer between mitochondrial respiratory enzyme complexes. It is a selective inhibitor of parasite mitochondrial electron transport, and is also reported that ATQ disturbs the mitochondrial membrane potential of *Plasmodium falciparum* [1]. It was developed by the Wellcome Laboratories in the 1980s as an antimalarial drug, a rare venture for a large pharmaceutical company [2].

However, a 30% failure rate with ATQ was unacceptable, leading to a search for a synergistic drug. Combination with proguanil, an antimalarial of long-standing use, resulted in close to complete cure rate with no evidence for recrudescence [3]. Proguanil [1-(p-chlorophenyl)-5-isopropylbiguanide, PRG] is a pro drug which is converted by the hepatic cytochrome P-450 isoenzyme to its active metabolite, cycloguanil and the inactive metabolite, 1-(4-chlorophenyl) biguanide [4]. PRG was found to enhance significantly the ability of atovaquone to collapse mitochondrial trans-membrane potential. In large clinical studies, the fixed dose combination of ATQ and PRG has been shown to give improved outcomes over single dose formulations of either ATQ or PRG [5]. The synergistic fixed dose combination of ATQ and PRG has been approved by the US Food and Drug Administration (FDA) and European Medicines Agency for the treatment of acute, uncomplicated *Plasmodium Falciparum* malaria. In addition it has been shown that this combination is of grave importance in areas where the drugs chloroquine, halofantrine, mefloquine and amodiaquine may have unacceptable failure rates, presumably due to drug resistance [5, 6].

There have been very few reports on the estimation of ATQ either from fixed dose formulation or biological specimens and are mainly based on chromatographic techniques [7]. A differential pulse polarographic method is described for the determination of ATQ in

its pharmaceutical formulations [8]. ATQ has been also estimated from serum samples using a capillary zone electrophoresis method [9]. Nevertheless, quite less efforts have been devoted to study PRG in pharmaceutical formulations [10, 11]. PRG along with other drugs/metabolites has been quantified from biological specimens using micellar electro kinetic chromatography [12], HPLC [13, 14] and LC-MS [15]. Several non-chromatographic methods for PRG are also available which include spectro fluorimetry [16], titrimetry [17, 18], and microbially [19]; however, some of them lack both specificity and sensitivity and thus are of limited use for the simultaneous analysis with ATQ. Adewuyi *et al.* [20] have reported the use of sodium benzoxazole-2-sulphonate as a derivatizing reagent for the determination of PRG using HPLC. Recently, a renewable silver amalgam film electrode has been developed [21] for the estimation of PRG.

There are some reports on chromatographic methods for simultaneous determination of ATQ and PRG; however they require different approaches to conventional liquid chromatography [22, 23]. So far there have been no reports on the use of spectrophotometry for the simultaneous estimation of ATQ and PRG. The primary objective of the present study was to develop simple UV-Visible spectrophotometry based methods to quantify these drugs from their pharmaceutical preparation for routine analysis in quality control labs.

MATERIALS AND METHODS

Chemicals and materials

Reference standards of atovaquone (ATQ, 98.74 %) and proguanil hydrochloride (PRG, 99.16 %) were purchased from Clear synth Laboratories Pvt. Ltd., Mumbai, India. Spectroscopic grade methanol was obtained from E. Merck, Mumbai, India. Twenty tablets of the proposed binary drug combination i.e. Malarone® (GlaxoSmithKline USA, 250 mg ATQ and 100 mg PRG) were commercially purchased from a local medical store.

### Instrumentation and analysis conditions

A Jasco V-570 double beam UV-Visible spectro photometer (Easton, MD) with matched 10 mm quartz cells was used for spectral measurements. The wavelength accuracy was within  $\pm 0.5$  nm, and a bandwidth of 1 nm was kept for all the methods. Data processing was done with Spectra manager software version 1.53.01.

A scan speed of 400 nm/min was used to record the spectra. Weighing of samples was done on a Sartorius GD503 (Bradford, MA, USA) analytical balance having a readability of 0.0001 g. For studying the ruggedness of the methods, Shimadzu UV-1700 UV-Visible Spectrophotometer (Tokyo, Japan) was also used.

### Stock solutions

Separate stock solutions of ATQ and PRG equivalent to 200  $\mu\text{g/ml}$  were prepared by dissolving carefully weighed amount i.e. 20 mg of each reference standard in 100 mL methanol.

### Spectral characteristics of analytes

The zero-order absorption spectrum for 5.0  $\mu\text{g/ml}$  of ATQ and PRG were recorded against methanol as a blank over 200–400 nm wavelength range.

### Construction of calibration curves

Calibration standards were prepared in the concentration range of 1.0–10.0  $\mu\text{g/ml}$  for ATQ and 0.5–8.0  $\mu\text{g/ml}$  for PRG from standard stock solutions (200  $\mu\text{g/ml}$ ) in two separate series of 10 mL volumetric flasks in methanol. The spectra of these standard solutions were recorded from 200 to 400 nm wavelength range.

### Derivative spectrophotometric method

In our present work, the zero order absorption spectra were recorded for 5.0  $\mu\text{g/ml}$  of ATQ and PRG, using methanol as a blank over 200–400 nm wavelength range. These zero order spectra of ATQ and PRG were treated to obtain corresponding first derivative spectra with an inter-point distance of 5 nm and scaling factor of 10. Using memory channels the zero and first order derivative spectra of both the drugs were overlaid for wave length selection and data treatment.

The absorbance spectra of pure ATQ and PRG were recorded in a wavelength range of 200–400 nm within the range of 1.0–10.0  $\mu\text{g/ml}$  for ATQ and 0.5–8.0  $\mu\text{g/ml}$  for PRG and the first derivative spectra were obtained using Spectra manager version 1.53.01 software. Using these first derivative spectra, the regression equations for ATQ and PRG were calculated at 216.0 nm and 277.0 nm respectively. These wavelengths correspond to the zero crossing points in the derivative spectra of PRG and ATQ respectively. The quantification of drugs was performed using these regression equations and the derivative response of the pharmaceutical sample solution.

### Absorbance ratio method

In order to construct two regression lines, two series of solutions were prepared and the absorbance values were measured at 221.0 ( $\lambda_{\text{max}}$  of ATQ), 258.0 ( $\lambda_{\text{max}}$  of PRG) and 277.0 nm (iso absorptive point,  $\lambda_{\text{iso}}$ ).

(a) Series 1: (for ATQ): Five aliquots equivalent to 10, 20, 30, 40 and 50  $\mu\text{g}$  of ATQ respectively were transferred to a series of 10-mL measuring flasks, containing an aliquot equivalent to 25  $\mu\text{g}$  of PRG. Each flask was diluted to volume with methanol. The absorbance of each solution was measured at 221.0 nm and 277.0 nm. The relative absorbance ( $Q_{\text{ATQ}} = A_{\text{ATQ}}/A_{\text{iso}}$ ) and the corresponding relative concentration ( $C_{\text{ATQ}}/\{C_{\text{ATQ}} + C_{\text{PRG}}\}$ ) was plotted and the regression equation for ATQ was computed.

(b) Series 2: (for PRG): Five aliquots equivalent to 10, 15, 25, 40 and 60  $\mu\text{g}$  of PRG respectively was transferred to a series of 10-mL measuring flasks, containing an aliquot equivalent to 30  $\mu\text{g}$  of ATQ. Each flask was diluted to volume with methanol. The absorbance of each solution was measured at 258.0 nm and 277.0 nm. The relative absorbance ( $Q_{\text{PRG}} = A_{\text{PRG}}/A_{\text{iso}}$ ) and the corresponding relative concentration ( $C_{\text{PRG}}/\{C_{\text{ATQ}} + C_{\text{PRG}}\}$ ) was plotted and the regression equation for PRG was computed.

### Bivariate method

Standard solutions covering the range of 1.0–10  $\mu\text{g/ml}$  and 0.5–8.0  $\mu\text{g/ml}$  were prepared for ATQ and PRG respectively and the spectra was acquired from 200 to 300 nm and stored on computer and the regression equations were computed at 221.0 and 265.0 nm. Thereafter the solutions prepared from synthetic laboratory mixtures and pharmaceutical formulations were scanned for absorbance values and the regression parameters were used to resolve equations for  $C_{\text{ATQ}}$  and  $C_{\text{PRG}}$ .

### Method validation parameters

All the proposed spectrophotometric methods were validated as per ICH guidelines for linearity, accuracy, precision, specificity, recovery and ruggedness [24].

### Linearity

The linearity of each method was ascertained by analyzing five calibration standards for absorbance-ratio method and seven calibration standards for other methods within the range of 1.0–10.0  $\mu\text{g/ml}$  for ATQ and 0.5–8.0  $\mu\text{g/ml}$  for PRG. The analytical response of each method was plotted against the concentration of each drug, and the slope, intercept and correlation coefficient were calculated. For an acceptable analytical method  $r^2$  must be  $\geq 0.99$ .

### Limit of detection and quantitation

The sensitivity of each method was evaluated in terms of limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were calculated according to the ICH guidelines [24], using the standard deviation of the response. For this purpose, 1.0  $\mu\text{g/ml}$  solutions of ATQ and PRG, was analyzed in three replicates; the standard deviation was calculated and the LOD and LOQ were evaluated using equations 1 and 2 below,

$$\text{LOD} = \frac{3\sigma}{S} \quad (1)$$

$$\text{LOQ} = \frac{10\sigma}{S} \quad (2)$$

### Accuracy and precision

Intra and inter-batch accuracy and precision were evaluated by carrying out measurements of three replicate samples at 80 %, 100 % and 120 % levels to that of the anticipated amount of the drugs in the sample solution on the same day and on three consecutive days respectively. The concentration values for known standards were calculated as % recovery and the precision of the method was reported in terms of percent relative standard deviation (% RSD). The methods were considered reproducible if the results were within the recommended range [24].

### Selectivity and ruggedness

The selectivity of the methods was established by analysis of the laboratory prepared standard mixtures. The interference due to commonly used excipients was also evaluated using the standard addition method (at 80 %, 100 % and 120 % levels to that of the amount in the sample). Known aliquots from the standard solutions of both the drugs were spiked into a pre-analyzed standard sample solution. For both the solutions, the amounts recovered were calculated and expressed in terms of mean recovery based on ICH guidelines for the assessment of the selectivity [24]. The ruggedness of the method was evaluated by analyzing standard samples of both the drugs and their combinations on two different spectrophotometers and also by using methanol obtained from two different sources. The methods were considered rugged for the proposed application if the % RSD were within the acceptable range.

### Analysis of marketed formulations

To ascertain the applicability of the developed methods, they were used for the quantitation of these drugs in their marketed combined dosage form. Twenty tablets of MALARONE® were weighed and ground to fine powder. A quantity of the powder equivalent to 12.5 mg ATQ and 5.0 mg PRG was accurately weighed and transferred to a 100 mL volumetric flask. To this 60 mL methanol was added,

sonicated for 15 min and briefly vortexed for the few seconds. The solution was filtered through Whatman filter paper 41 and the volume made up to 100 mL using methanol to give a final solution containing 125.0 µg/ml ATQ and 50.0 µg/ml PRG respectively. The working solutions were prepared by appropriate dilution of these stock solutions with methanol.

## RESULTS AND DISCUSSION

### Method development

#### Derivative spectrophotometric method [25, 26]

Adequate separation of overlapping peaks can be achieved by correct selection of derivative order. The zero order spectra for both ATQ and PRG showed intense overlapping as evident in fig. 1.

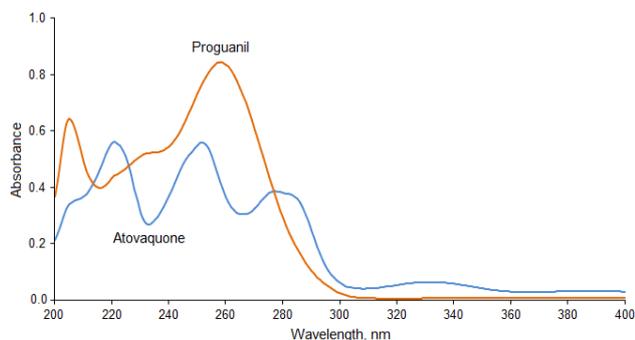


Fig. 1: Zero order absorbance spectra for atovaquone (5.0 µg/ml) and proguanil (5.0 µg/ml) in methanol

Optimal derivative order is a function of signal height and the distance between maxima in basic spectrum. Several manipulations were made to enable the mixture resolution using first, second and third derivative of the absorption spectra with an inter-point distance of 5 nm and scaling factor of 10. Using memory channels, the zero, first, second and third order derivative spectra of both the drugs (5.0 µg/ml of each) were overlapped for wavelength selection and data treatment. First order derivative spectra (<sup>1</sup>D) afforded the highest accuracy and detection limits compared to second and third order spectra and hence was selected for the analysis. In contrast to zero-order spectra, first derivative spectra showed enhanced resolution in terms of zero crossing point. The first derivative spectra were obtained using  $\Delta\lambda=5$  nm and a scaling factor of 10 for the calibration standards of both the drugs (fig. 2).

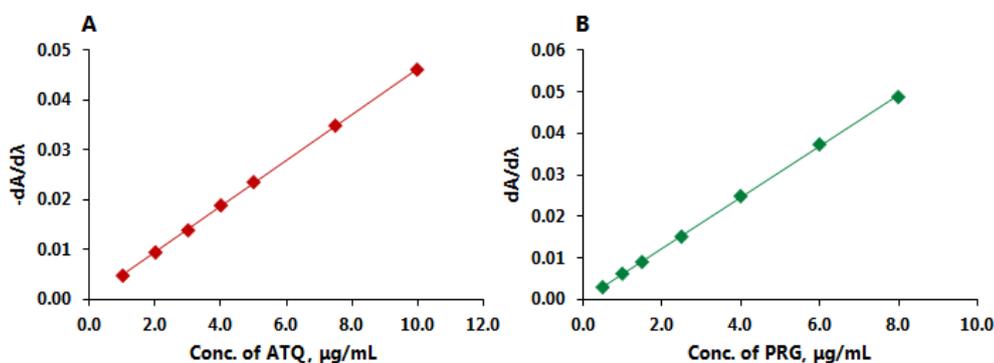


Fig. 3: Calibration curves of (A) Atovaquone (1.0-10.0 µg/ml) and (B) Proguanil (0.5-8.0 µg/ml) using the proposed first derivative method

**Absorbance ratio method [27]:** This method is based on the selection of optimum wavelengths *i.e.*  $\lambda_1$ ,  $\lambda_2$  and  $\lambda_{iso}$ . The mathematical treatment for the method can be expressed through equations 5 and 6 as shown below,

$$C_1 = \frac{Q_1 - b_1}{a_1} \times \frac{A_{iso}}{a_{iso}} \times 10^3 \quad (5)$$

$$C_2 = \frac{Q_2 - b_2}{a_2} \times \frac{A_{iso}}{a_{iso}} \times 10^3 \quad (6)$$

where,  $Q_1$  is the absorbance ratio ( $A_1/A_{iso}$ ) for the first component (ATQ);  $Q_2$  is the absorbance ratio ( $A_2/A_{iso}$ ) for the second component (PRG);  $C_1$  and  $C_2$  are the concentrations of ATQ and PRG respectively;  $A_{iso}$  is the absorbance at isoabsorptive point;  $a_{iso}$  is the

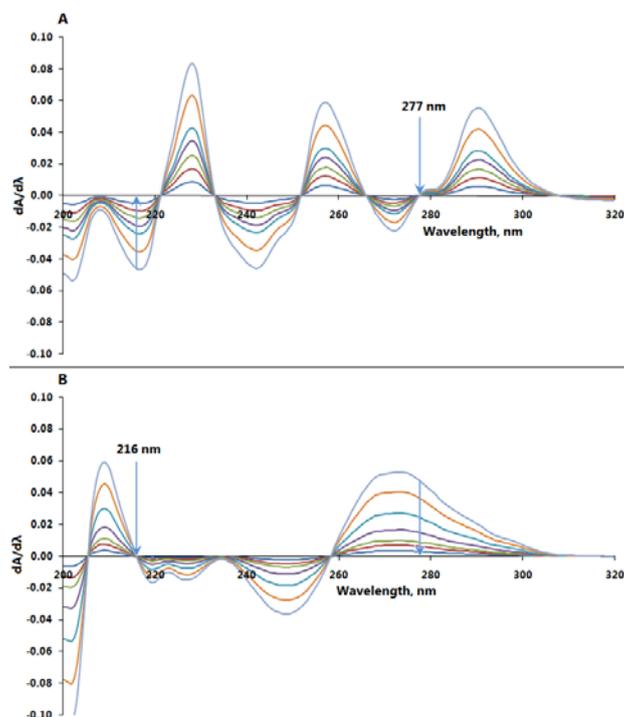


Fig. 2: First order absorbance spectra for (A) Atovaquone (1.0-10.0 µg/ml) and (B) Proguanil (0.5-8.0 µg/ml)

ATQ was determined at 216.0 nm without significant interference due to PRG. Since ATQ showed negligible contribution at 277.0 nm, PRG was accurately determined at this wavelength.

Two calibration curves were established for both the drugs using the analytical response at respective zero crossing points and a straight line curve was observed in the concentration range of 1.0-10.0 µg/ml for ATQ and 0.5-8.0 µg/ml for PRG as shown in fig. 3. The regression equations and correlation coefficients for both the drugs were calculated using equations 3 and 4,

$$-dA/d\lambda = 0.0046 C_{ATQ} + 0.00036 \quad (r^2 = 0.9998) \text{ at } 216 \text{ nm} \quad (3)$$

$$dA/d\lambda = 0.00615 C_{PRG} + 0.00008 \quad (r^2 = 0.9996) \text{ at } 277 \text{ nm} \quad (4)$$

absorptivity at iso absorptive point which equals to  $(A_{iso}/\{C_1+C_2\})$ ;  $a_1$  is the slope of regression equation ( $Q_1$  vs.  $C_1/\{C_1+C_2\}$ );  $a_2$  is the slope of the regression equation ( $Q_2$  vs.  $C_2/\{C_1+C_2\}$ );  $b_1$  and  $b_2$  are intercept values of these regression equations and  $A_1$  and  $A_2$  denotes the absorbance values of the mixture measured at  $\lambda_1$  and  $\lambda_2$  respectively.

The zero order absorption spectra of ATQ (5  $\mu\text{g/ml}$ ) and PRG (5  $\mu\text{g/ml}$ ) showed an iso absorptive point at 277.0 nm (fig. 1). The synthetic mixtures of the components were prepared as described earlier and the absorbance values were measured at 221.0 nm ( $\lambda_{\text{max}}$  of ATQ), 258.0 nm ( $\lambda_{\text{max}}$  of PRG), and 277.0 nm ( $\lambda_{\text{iso}}$ ). A linear relation between the absorbance ratio value of the binary mixture

and the relative concentration of such a mixture was established as shown in fig.4. There was good linearity between the two variables as evident from the correlation coefficients values in the following equations 7 and 8,

$$Q_1 = 0.274 \left( \frac{C_{\text{ATQ}}}{C_{\text{ATQ}}+C_{\text{PRG}}} \right) + 1.178 \quad (r^2 = 0.9997) \quad (7)$$

$$Q_2 = 1.156 \left( \frac{C_{\text{PRG}}}{C_{\text{ATQ}}+C_{\text{PRG}}} \right) + 1.094 \quad (r^2 = 0.9995) \quad (8)$$

From the above regression equations 7 and 8, the values of slopes and intercepts were obtained and used to resolve equations 5 and 6 in order to determine the concentration of ATQ and PRG.

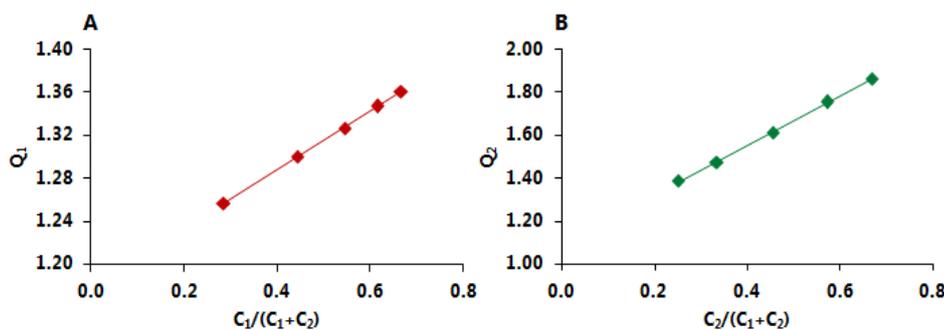


Fig. 4: Plot of (A)  $Q_1$  vs.  $C_1/(C_1+C_2)$  and (B)  $Q_2$  vs.  $C_2/(C_1+C_2)$  for Atovaquone and Proguanil using absorbance-ratio method

**Bivariate method:** This is different from Vierordt's method [28] in that it determines the linear regression parameters for the different set of wavelengths other than respective absorbance maxima [29]. To assure the highest sensitivity and selectivity a suitable pair of wavelengths was optimized by using Kaiser's method of matrix determinant [30]. The slope values of the regression line were determined for both the components (drugs) at the pre-selected wavelengths and a series of sensitivity matrices  $K$  was created for every pair of pre-selected wavelengths as shown below,

$$K = \begin{bmatrix} m_{\text{ATQ}1} & m_{\text{PRG}1} \\ m_{\text{ATQ}2} & m_{\text{PRG}2} \end{bmatrix} \quad (9)$$

Where,  $m_{\text{ATQ}1}$ ,  $m_{\text{ATQ}2}$  and  $m_{\text{PRG}1}$ ,  $m_{\text{PRG}2}$  are the slopes for components ATQ and PRG at two selected wavelengths ( $\lambda_1$  and  $\lambda_2$ ), which are

considered as the sensitivity parameters. Absolute matrix determinant values were calculated for each set of wavelength and the set for which the highest determinant values were obtained, was selected for the analysis.

Firstly, nine wavelengths were considered for the selection of a set of wavelengths using the method of Kaiser [30]. By analyzing calibration standards at selected wavelengths, the slope values of the linear regression ( $m_{Ai}$  and  $m_{Bi}$ ) were estimated for both the analytes. Using the obtained data, the sensitivity matrices were formed for each set of wavelength and the respective absolute matrix determinant values were acquired. From the results obtained as shown in table 1, 221.0 nm and 265.0 nm were used for sensitive bivariate determination of ATQ and PRG.

Table 1: Application of the method of Kaiser for the selection of wavelength set <sup>a</sup>

$\lambda_1/\lambda_2$ nm	210	221	230	240	252	258	265	280	290
210	0.0	46.1	12.7	6.2	2.0	38.8	48.7	32.6	33.4
221		0.0	58.6	55.9	73.5	112.5	113.0	2.0	19.9
230			0.0	7.6	17.7	18.2	30.6	40.9	36.9
240				0.0	7.3	32.0	43.9	39.3	37.8
252					0.0	56.8	72.6	51.9	52.3
258						0.0	23.8	77.9	64.7
265							0.0	77.8	60.8
280								0.0	12.6
290									0.0

<sup>a</sup> The absolute values of determinants of sensitivity is  $K \times 10^{-5}$

Thereafter, absorbance values were obtained for standard solutions of ATQ and PRG within their linearity ranges, and the resultant linear regression calibration formula used for the bivariate algorithm are presented as,

$$y = 0.109137 \cdot C_{\text{ATQ}} + 0.008484, \text{ at } 221 \text{ nm} \quad (10)$$

$$y = 0.059443 \cdot C_{\text{ATQ}} + 0.004621, \text{ at } 265 \text{ nm} \quad (11)$$

$$y = 0.088781 \cdot C_{\text{PRG}} + 0.001208, \text{ at } 221 \text{ nm} \quad (12)$$

$$y = 0.151912 \cdot C_{\text{PRG}} + 0.002067, \text{ at } 265 \text{ nm} \quad (13)$$

The above equations 10-13 was solved for ATQ and PRG, and were utilized to determine the amount of the drugs present in the mixture.

#### Method validation results

**Linearity:** For each spectrophotometric method, three calibration curves were prepared by analyzing five/seven non-zero concentrations to define the linearity of the methods. The calibration curves were linear in the range of 1.0-10.0  $\mu\text{g/ml}$  and 0.5-8.0  $\mu\text{g/ml}$  for ATQ and PRG, respectively. The detailed summary of linearity

along with the regression equation, correlation coefficient, LOD and LOQ for the selected drug combination is presented in table 2. The high values of correlation coefficient for both the drugs demonstrate good linearity of the proposed methods and their fitness for analysis. *Limits of detection and quantification*: Limit of detection (LOD) and

limit of quantification (LOQ) were calculated according to the ICH guidelines [24]. Standard deviation (SD) was calculated by triplicate analysis of pure standard solutions containing 1.0 µg/ml of ATQ and PRG respectively. The sensitivity of the proposed method can be confirmed by the low LOD and LOQ values as shown in table 2.

**Table 2: Regression parameters obtained for the proposed spectrophotometric methods**

Drug	Linearity range (µg/ml)	Slope	Intercept	Correlation coefficient (r <sup>2</sup> )	LOD (µg/ml)	LOQ (µg/ml)
Derivative spectrophotometric method						
ATQ	1.0-10.0	0.0046	0.00004	0.9998	0.270	0.825
PRG	0.5-8.0	0.0062	0.00008	0.9996	0.156	0.472
Absorbance ratio method						
ATQ	1.0-10.0	0.2744	1.1777	0.9997	0.262	0.795
PRG	0.5-8.0	1.1556	1.0936	0.9995	0.119	0.361
Bivariate method						
ATQ	1.0-10.0	0.1091	0.0085	0.9998	0.252	0.764
PRG	0.5-8.0	0.1519	0.0021	0.9996	0.134	0.407

### Accuracy and precision

Intra-and inter-batch accuracy and precision were calculated by analyzing standard samples at three concentration levels by using the same procedure as described previously. The concentrations were calculated using the corresponding regression equations. A concise summary of the obtained accuracy and precision values are given in table 3, which indicates that the % RSD were within the acceptable range for both the drugs. The precision values expressed as % RSD were less than 2 %, while the accuracy ranged from 99.16 to 101.12 % for both the drugs.

### Selectivity and ruggedness

Selectivity of the developed method was confirmed by analysis of laboratory prepared mixtures in three replicates. Standard mixtures were prepared at three different concentration levels and the percentage drug content was calculated. Here, the % mean

recoveries were considered to confirm the accuracy of a method and the detailed results obtained by applying the standard addition technique are shown in table 4.

All three spectrophotometric methods were found to be selective for the proposed application using methanol as the solvent. In the ruggedness study with different instrumentation and solvent source, no significant changes in the response of the methods were found for both the drugs. The % RSD for all the developed spectrophotometric methods was constantly less than 2 %.

### Stability of solutions

ATQ and PRG stock and calibration solutions remained unaffected for a minimum period of 2 weeks when stored at 4 °C. There was practically no change in concentration as evident from spectrophotometric measurements and no degradation occurred under refrigerated conditions during the storage period.

**Table 3: Accuracy and precision results of atovaquone and proguanil in bulk form using the proposed methods**

Amount added (µg/ml)	Intra-batch <sup>a</sup>			Inter-batch <sup>b</sup>		
	% Recovery (% RSD)			% Recovery (% RSD)		
	<sup>1</sup> DSM <sup>c</sup>	ARM <sup>d</sup>	BSM <sup>e</sup>	<sup>1</sup> DSM <sup>c</sup>	ARM <sup>d</sup>	BSM <sup>e</sup>
Atovaquone						
4.0	101.05 (0.955)	100.36 (0.639)	99.79 (0.726)	100.42 (0.603)	99.58 (0.941)	100.37 (0.787)
5.0	100.22 (0.975)	100.37 (0.839)	100.24 (0.756)	99.44 (1.048)	99.79 (1.016)	99.95 (0.853)
6.0	100.56 (0.764)	100.27 (0.952)	99.97 (0.622)	99.16 (0.741)	100.17 (0.851)	100.33 (0.807)
Proguanil						
1.6	99.86 (0.603)	100.48 (0.906)	100.39 (0.562)	100.27 (0.766)	100.13 (0.794)	100.16 (0.646)
2.0	100.48 (0.754)	100.54 (0.798)	99.74 (0.727)	101.12 (1.001)	100.00 (0.987)	100.47 (0.911)
2.4	99.76 (0.820)	100.37 (0.593)	99.94 (0.665)	99.78 (0.613)	99.78 (0.692)	99.99 (0.717)

<sup>a</sup>Mean values obtained in triplicate on the same day; <sup>b</sup>Mean values obtained in triplicate on three consecutive days; <sup>c</sup>First derivative spectrophotometric method; <sup>d</sup>Absorbance ratio method; <sup>e</sup>Bivariate spectrophotometric method

**Table 4: Recovery of atovaquone and proguanil by the standard addition technique**

Drug	Amount taken (µg/ml)	Amount added (µg/ml)	% Mean recovery (±SD), n = 3		
			<sup>1</sup> DSM <sup>a</sup>	ARM <sup>b</sup>	BSM <sup>c</sup>
ATQ	2.5	4.0	100.37±0.84	100.97±0.77	100.45±0.58
		5.0	100.29±0.70	100.21±1.06	99.28±0.72
		6.0	99.71±0.65	99.80±0.71	99.01±0.82
PRG	1.0	1.6	99.55±0.97	99.50±0.75	99.83±0.61
		2.0	100.75±0.59	98.84±0.95	100.25±6.0
		2.4	99.54±0.80	99.39±1.17	99.65±0.69

<sup>a</sup>First derivative spectrophotometric method; <sup>b</sup>Absorbance ratio method; <sup>c</sup>Bivariate spectrophotometric method

Table 5: Analysis of marketed formulation MALARONE® by the proposed methods

Drug	Amount claimed (mg)	% Mean recovery ( $\pm$ SD), n = 3		
		First derivative	Absorbance ratio	Bivariate
Atovaquone	250	100.75 $\pm$ 1.02	99.68 $\pm$ 0.91	100.19 $\pm$ 0.52
Proguanil	100	100.29 $\pm$ 0.84	99.26 $\pm$ 0.93	99.42 $\pm$ 0.79

#### Analysis of the marketed formulation MALARONE® by the proposed methods

The proposed spectrophotometric methods were successfully applied for the determination of selected drugs in the marketed formulation. Recoveries were calculated using regression analysis and also by the standard addition method. The results obtained were precise and in good agreement with the labeled claim as apparent from the satisfactory values of recovery and % RSD shown in table 5. Further, the recovery studies with all the methods indicate that there was no interference from the excipients commonly present in the tablet formulation.

#### Comparison of the method performance with those of reported method

The developed methods offer significant advantages over few reported analytical methods [8, 16-19] i.e. high sensitivity, adequate accuracy and precision. Though the sensitivity is less compared to advanced methodologies based on electrophoresis [9], chromatography [7, 10, 11-13, 15, 20] and voltammetry techniques [8, 21], these methods are more preferable owing to their simplicity and ease of analysis. In addition, the major advantage is that they do not require any type of expensive instrumentation. They can be operated using a simple double beam UV-Visible spectrophotometer which can be easily found in any chemical or quality control laboratory.

The only available methods for simultaneous quantitation of ATQ and PRG require tedious and time consuming modifications to existing HPLC equipment [22, 23]. On the other hand, the present methods do not need any such modifications and the only essential requirement is the computational programs for data processing. Though the results of HPLC methods are to some extent better than the present methods in terms of sensitivity and selectivity, but the long analytical run time of about 20 min makes it less preferable choice for routine quality control laboratories. Moreover due to the large difference in their lipophilicity it is quite difficult to achieve a rapid simultaneous elution and quantitation [22, 23].

However, use of spectrophotometry for such task seems fairly easy as the difference in their lipophilic nature does not affect their spectral characteristics, and hence could be computationally resolved from their combination spectra. In the present study, this has been achieved by using the first derivative, absorbance ratio and bivariate methods. Additionally, the method developed by Bergqvist and Hopstadius [22] mainly focused on method development, and less on the validation parameter. On the contrary, the present methods were validated following the ICH guidelines [24] and then applied for the analysis of drugs from their pharmaceutical preparations. Furthermore, the validation results were found comparable to the results of Lindegårdh *et al.* [23].

#### CONCLUSION

In the present study three spectrophotometry based methods namely derivative spectrophotometry, absorbance ratio method, and bivariate method was investigated for the simultaneous analysis of atovaquone and proguanil. By careful selection of the optimum wavelength, it was possible to determine their concentration in binary mixtures. The developed methods were suitably validated following the standard guidelines, and were successfully applied for the quantitation of these drugs in their pharmaceutical formulation. All three methods were comparable in terms of accuracy (recoveries) and precision for both the analytes. Finally, based on the overall performance of these methods they can be readily applied in quality control laboratories which do not have sophisticated instruments like HPLC.

#### ACKNOWLEDGEMENT

One of the authors, Nejal M. Bhatt wishes to thank UGC, New Delhi for BSR fellowship and the Department of Chemistry, Gujarat University for providing necessary facilities to carry out this work.

#### CONFLICT OF INTERESTS

The authors declare no conflict of interest

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