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Simultaneous Determination of Atovaquone and Proguanil Hydrochloride in Tablet Dosage Form by High Performance Liquid Chromatography

Vijay S Veer[1], MA Badgujar[2]* and Kiran V Mangaonkar[1]

[1]Analytical Chemistry Research Laboratory, Mithibai College, Vile Parle (W), Mumbai-400056, India
[2]Department of Chemistry, Sheth J.N.Paliwala College Pali, Raigad, India

ABSTRACT

A simple, fast, precise and rapid isocratic reverse phase high performance liquid chromatographic method was developed for the simultaneous determination of atovaquone and proguanil hydrochloride from tablet dosage form. The chromatographic separation was performed on Supelcosil C8, DB 250 x 4.6 mm, 5 µm particle size. Mobile phase consisted of a mixture of ammonium formate buffer (10 mM $HCOONH_4$, pH adjusted to 3.5 using formic acid) and acetonitrile-methanol (90:10 v/v) in the ratio of 30:70 v/v at a flow rate of 1.0 mL/min. The wavelength was set at 254 nm. The proposed method was validated for linearity, accuracy, precision, LOD and LOQ. The calibration was linear over the range of 125-375 µg/mL for atovaquone and 50-150 µg/mL for proguanil hydrochloride. The retention times were found as 4.2 min for proguanil hydrochloride and 8.3 min for atovaquone. This method can be successfully employed for simultaneous quantitative analysis of atovaquone and proguanil hydrochloride in bulk drugs and formulations.

Keywords: atovaquone, proguanil hydrochloride, RP-HPLC, Validation

Corresponding author

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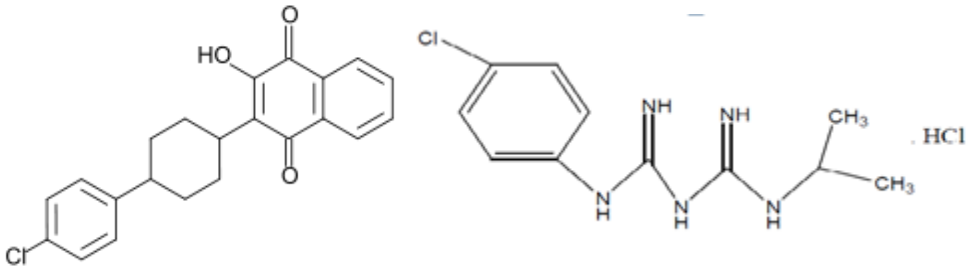
INTRODUCTION

Atovaquone, 2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthalenedione is a hydroxynaphthoquinone that has antimicrobial activity and is being used in antimalarial protocols. It is used for the treatment or prevention of *Pneumocystis carinii* pneumonia in patients who are intolerant to trimethoprim-sulfamethoxazole (TMP-SMX).

Proguanil,(E)-1-({amino[(4-chlorophenyl)amino]methylidene}amino)-N'-(propan-2-yl) methenimidamide is a prophylactic antimalarial drug, which works by stopping the malaria parasite, Plasmodium falciparum and Plasmodium vivax, from reproducing once it is in the red blood cells. It does this by inhibiting the enzyme, dihydrofolate reductase [1], which is involved in the reproduction of the parasite.

Bergqvist, Y. *et al*. developed a method for simultaneous estimation of Atovaquone and Proguanil hydrochloride in pharmaceutical preparations [2]. Hansson *et al*. developed a reverse phase HPLC method for the estimation of Atovaquone [3].

Literature survey reveals that various analytical techniques *viz*, HPLC [2-8] and mass spectrometric method [11]. Few HPLC [10] methods have been reported for the simultaneous determination of Atovaquone and Proguanil hydrochloride. Our aim was to develop proper method which estimates both the analytes in a shorter time and to develop low cost method. The present study describes an isocratic, reversed-phase HPLC method using ultraviolet detection for the determination of Atovaquone and Proguanil hydrochloride from tablet dosage form.



1(a) **1(b)**

Figure 1 Chemical structures of (1a) Atovaquone (1b) Proguanil hydrochloride

MATERIALS AND METHODS

Experimental

All chemicals were of analytical grade. Atovaquone and proguanil hydrochloride working standard were obtained from Glenmark Pharmaceutical Limited with the certificates of analysis. Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium formate (analytical grade) and formic acid were purchased from Merck (Mumbai, India).

Standard stock preparation

100 mg of Proguanil hydrochloride and 250 mg of Atovaquone were accurately weighed and transferred to a 100 cm^3 volumetric flask. It was dissolved in a 50-60 mL of mobile phase and 0.1 mL of ammonia solution, the solution was sonicated for 10 minutes at an ambient temperature with intermittent swirling and then diluted up to the mark with mobile phase. The concentration of the solution obtained was 1000 µg/mL for proguanil hydrochloride and 2500 µg/mL for atovaquone.

Preparation of Sample solution

Ten tablets were weighed and their average weight was calculated. These tablets were powdered and weight equivalent to one tablet containing 100 mg of proguanil hydrochloride and 250 mg of atovaquone was taken in a 100 mL dilution flask. Then about 50 mL of diluent and 0.1 mL of ammonia solution was added to it. The solution was sonicated for 20-25 minutes at an ambient temperature with intermittent swirling, cooled and diluted up to the mark with diluent, mixed well. Then solution from the flask was filtered through syringe filter. Further 10 mL of this solution was diluted to 100 mL with the diluent. This solution was used for further analysis.

Chromatographic conditions

The chromatography was performed using waters HPLC system having Waters 501 isocratic pump equipped with Waters™ 717 plus auto sampler and a Waters 486 tunable absorbance UV detector. The data was recorded using millenium^{32} chromatographic software. Separation was performed on a 250 mm × 4.6 mm i.d., 5 µ particle size, Supelcosil C8 column. Mobile phase consisted of a mixture of buffer and (acetonitrile: methanol, 90:10 v/v) in a ratio of 30:70 v/v, pH 3.5 adjusted with formic acid. Flow rate was kept at 1.0 mL/min. Wavelength was set at 254 nm.

Method validation

The method was validated as per ICH guidelines for specificity, linearity, quantification limit, precision, accuracy, recovery and stability.

Specificity was investigated by analyzing the blank diluents and samples of 100% level for any interference of the excipients at the retention times of ATQ and PG.
The accuracy of the method was determined by recovery experiments. A standard addition method was employed for this experiment. A known quantity of each drug substance (ATQ and PG) corresponding to 50%, 100% and 150% of the label claim of each drug was added. Each set of addition was repeated three times. The accuracy was expressed as a percentage of analytes recovered by the assay.

The precision of the method was demonstrated by interday and intraday variation studies, six repeated injections of standard and sample were made and percentage RSD was calculated. In the intraday variation studies six repeated injections of standard and sample solution was carried out by injecting on the same day at different intervals and percentage RSD was calculated. In the inter day variation studies six repeated injections of standard and sample solution were made for three consecutive days and percentage RSD was calculated.

The linearity of the method was demonstrated at six concentration levels of the mixed standards of ATQ and PG.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

In order to develop an isocratic reverse phase HPLC method for the determination of ATQ and PG in combined dosage form, the chromatographic conditions were optimized. For better separation and resolution the different buffers were tried. It has been found that ammonium formate buffer, pH 3.5 adjusted with formic acid gave better peak shape than other buffers. The different compositions of mobile phase were changed for getting better separation of analytes. Thus the mobile phase composed of the mixture of buffer (10 mM $HCOONH_4$, pH 3.5 adjusted with formic acid) and acetonitrile-methanol (90:10 v/v) in the ratio of (30: 70 v/v) was finalized. The better separation, peak symmetry and reproducibility were obtained with Supelcosil C8, 250 mm x 4.6 mm, 5 µm column. Both the analytes gave better response at 254 nm wavelength using UV detector. The flow rate kept was 1.0 mL/min. There was no peak tailing observed under these optimized chromatographic conditions. The retention times of PG and ATQ were found to be 4.2 min and 8.3 min respectively.

Validation

The proposed method was showed short elution time and good separation between ATQ and PG. The system suitability test was performed as per the USP and international conference of harmonization (ICH) guidelines [9] to confirm the suitability and the reproducibility of the method. Six consecutive injections of the standard solution were performed and evaluated for repeatability, tailing factor, theoretical plates and resolution. % RSD values were found to be 0.35 and 0.27 for ATQ and PG respectively. The tailing factor and theoretical plates were found to be perfectly within the limits.

The method was linear over the range 125-375 µg/mL for atovaquone and 50-150 µg/mL for proguanil hydrochloride. The calibration curve was constructed by plotting response factor against concentration of drugs. The slope and intercept value for calibration curve was Y= 69636x - 171995 (r^2= 0.9995) for PG and Y = 159366x - 419168 (r^2= 0.9994) for ATQ, the results shows that an excellent correlation between response factor and concentration of drugs.

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The limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The limit of quantification (LOQ) and limit of detection (LOD) was established at a signal-to-noise ratio.

The LOQ and LOD of ATQ and PG were experimentally determined. The LOD of ATQ and PG was found to be 0.25 µg/mL and 0.1 µg/mL respectively. The system precision study was performed to determine the repeatability of the method. Six solutions of standard were prepared at 100% level and assayed according to the procedure. The method precision study was performed to determine the reproducibility of the method. Six samples of tablets were prepared at 100% level and assayed according to the procedure. The accuracy of the method was determined by the standard addition method at three different levels. The sample solution of 100% level was considered as a zero level and 50, 100 and 150% of the standard drug of analytes were added respectively. Each determination was performed in triplicates. The accuracy was then calculated as the percentage of the standard drug recovered by the recovery study. Mean recoveries for ATQ and PG from the combination formulation are shown in Table 1. The results were well within the acceptance limit and hence the method is accurate. % RSD values were found to be 0.87, 0.67 and 0.20 for ATQ and 0.46, 0.14, 0.78 PG respectively. The tailing factor and theoretical plates were found to be perfectly within the limits.

Table 1. % Recovery of proguanil hydrochloride and atovaquone

Amount of proguanil hydrochloride, mg										
Sr. No.	% added	Amount added			Amount found			% Recovery	S.D.	% RSD
1	50	50.34	50.23	50.16	50.28	49.65	50.44	99.77	0.86	0.87
2	100	100.8	100.5	100.4	100.47	99.64	99.24	99.53	0.61	0.67
3	150	150.2	150.8	150.6	149.13	149.6	149.3	99.43	0.16	0.20
Amount of atovaquone, mg										
1	50	125.6	125.1	125.7	124.91	124.8	124.5	99.39	0.49	0.46
2	100	250.2	250.3	250.4	248.17	248.9	247.5	99.06	0.15	0.14
3	150	375.2	375.22	375.56	374.12	370.76	376.89	99.61	0.776	0.78

The stability of both the standard and the sample was determined by monitoring the peak area responses of the standard solution and the sample solution of ATQ and PG at 6, 12 and 24 hours at room temperature. The results showed that there were no significant differences. The results are shown in Table 2.

The specificity of the method was determined by exposing 100% sample solution of ATQ and PG. The chromatogram of the sample solution shows that there should not be any interference of the placebo at the retention times of the analytes. It is shown in Figure 2.

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Figure 2 Chromatogram of atovaquone and proguanil hydrochloride in 100% sample solution

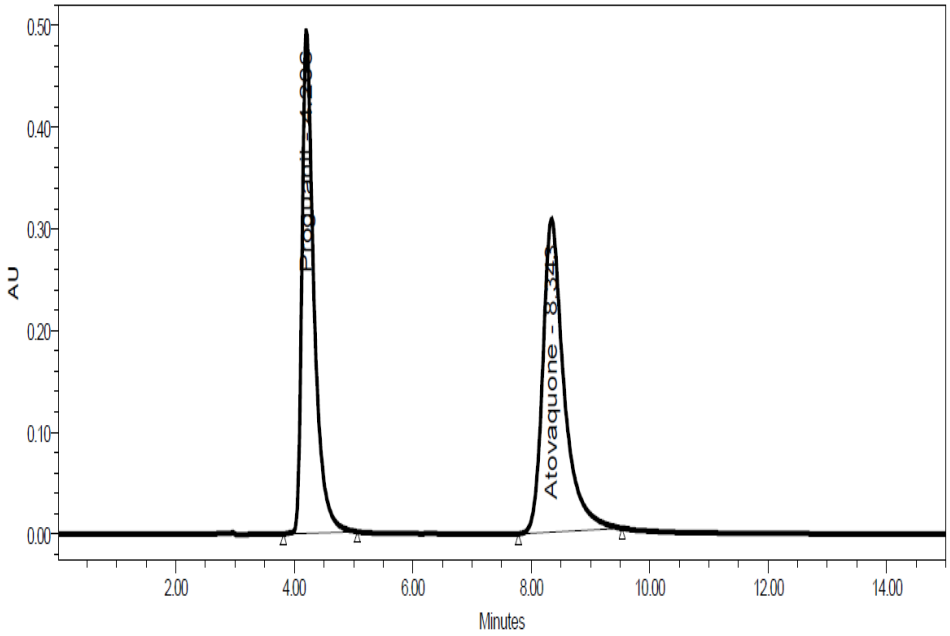

Table 2 Solution stability of atovaquone and proguanil hydrochloride sample solution

Conditions	Level in %	Peak area	Assay in mg/tab	% Label claim	% Relative Deviation
Atovaquone					
Initial- 0 h	100	15289881	247.71	99.08	-
Initial- 6 h	100	15252641	248.3	99.32	0.24
Initial- 12 h	100	15271261	247.39	98.96	- 0.37
Initial- 24 h	100	15261951	247.55	99.02	0.06
	Mean	15268933	247.74	99.10	
	S.D.	15899.64	0.40	0.16	
	% RSD	0.10	0.16	0.16	
Proguanil hydrochloride					
Initial- 0 h	100	6673825	99.27	99.27	-
Initial- 6 h	100	6692165	100.26	100.26	0.99
Initial- 12 h	100	6684470	99.22	99.22	-1.04
Initial- 24 h	100	6688318	99.04	99.04	-0.18
	Mean	6684694	99.45	99.45	
	S.D.	7898.14	0.55	0.55	
	% RSD	0.12	0.55	0.55	

Method applications

The validated HPLC method was applied to the simultaneous determination of ATQ and PG in tablet dosage form. The samples were analysed and the assay results are as per the label claim shown in Table 3.

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Table 3 Results of assay experiments

Drug	Label claim, mg	Amount found, mg (n= 6)	S.D.	% RSD	% Assay
Atovaquone	250	247.95	1.2	0.28	99.18
Proguanil hydrochloride	100	99.43	0.78	0.62	99.43

CONCLUSION

The isocratic RP- HPLC method has proved to be simple, specific, precise and accurate and is suitable for simultaneous quantification of atovaquone and proguanil hydrochloride. The proposed method gives a good resolution among the analytes. The method is very simple, rapid and no complicated sample preparation is needed. High percent of recovery shows the method is free from interference of excipients present in the formulations and the method is accurate.

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