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DEVELOPMENT & EVALUATION OF SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF ATOVAQUONE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT: A very simple and selective U. V. Method was developed and validated for the estimation of Atovaquone in pure form and the nanosuspension. The adequate drug solubility and maximum assay sensitivity was found in 8.00 Ph IPA Phosphate Buffer in 40:60 ratios v/v. The absorbances were measured at the $\lambda_{max.}$ of 494 nm in the wavelength range of 200-800 nm. The linear calibration range was found to be 20-140 µg/ml. The same method was applied and validated for the determination of Atovaquone in nanosuspensions. Also no interference was found from the excipients added to the nanosuspensions at the selected wavelength of 494 nm and at the analysis condition .It was found that the developed method was accurate, sensitive, precise, robust and reproducible. It can be applied directly for the estimation of drug containing the pharmaceutical formulations.

INTRODUCTION: Atovaquone, a hydroxy-1, 4 Napthoquinone (**Fig. 1**) is a structural analogue of protozoan ubiquinone, a mitochondrial protein involved in electron transport [1]. The drug has been approved in USA, Canada and in European countries for the treatment of *Pneumoncystis carinii* in Acquired Immunodeficiency Syndrome (AIDS) in patients that are intolerant to trimethoprim/Sulphamethoxazole. This drug was also found to be effective against toxoplasmosis another common opportunistic infection in patients with AIDS.

In this case, Atovaquone displays a patient in vitro activity against both the tachyzoite and cyst forms of *Toxoplasma gondii* [2]. Atovaquone has broad spectrum activity against *Plasmodium* spp. *Carinii*, *babesiosis* spp. and *Toxoplasma gondii*.

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Its mechanism of action has been most completely elucidated for Plasmodium spp. The drug is structurally similar to the inner mitochondrial protein ubiquinone (also called ubiquinone) which is an integral component of electron flow in aerobic respiration. Ubiquinone accepts electron from dehydogenase enzyme and passes them to electron transport cytochromes. The passage of electrons from ubiquinone to cytochrome bc1 Complex III requires binding of coenzyme Q Complex III at the Q_0 Cytochrome domain, it is this step which is inhibited by Atovaquone.

Several parasite enzymes are linked to the mitochrondrial electron transport system and are inhibited. Included among these enzymes is dihydro rotate dehydrogenase (DHOD) which is required in the biosynthesis of pyrimidines because plasmodia are unable to scavenge pyrimidines for DNA synthesis, inhibition of DHOD results in paralytic death. ATP generation is another physiologic process linked to decrease by the administration of Atovaquone. The effect of Atovaquone on Malarial parasites occurs at nanomolar concentrations [3].

Various method have been developed for the estimation of the Atovaquone by RP-HPLC using filtered and degassed mixture of Buffer and Acetonitrile in the ratio of 20:80 v/v.[4] 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 [5]. A U.V. method was developed for the determination of Atovaquone in Pharmaceutical Dosage form in Methanol at 251 nm [6]. The current study reveals a very simple precise and accurate method for the estimation of the Atovaquone in Pharmaceutical Dosage forms.

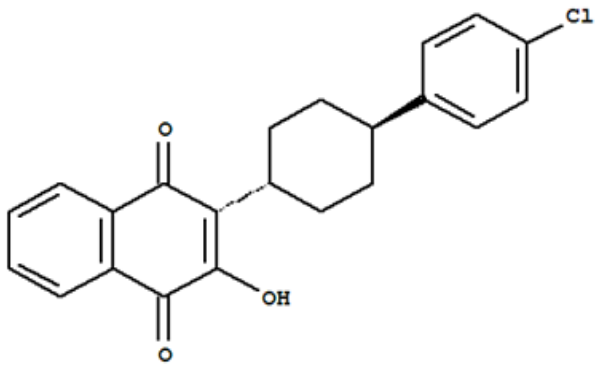


FIG. 1: STRUCTURAL FORMULA OF ATOVAQUONE

EXPERIMENTAL:

Chemical and Reagents: All the reagents used were of analytical grade IPA, Potassium Hydrogen Phosphate and Sodium Hydroxide. Atovaquone as a standard was obtained as a gift sample from Lupin Ltd Pune. Atovaquone nanosuspensions i. e. Meprone® was also obtained as a gift sample from Lupin Research Park Pune.

Instrumentation: Varian Cary C50 was used for the Spectrophotometric observations with spectral bandwidth of 1 nm with wavelength accuracy of ± 0.3 & 1.0 cm matched Quartz cells were used for the analytical method development.

Media Preparation: 8.00 Ph Phosphate Buffer was prepared as per the procedure of USP. Required quantity of NaOH (Sodium Hydroxide) and Monobasic Potassium Dihydrogen Phosphate were calculated and added to Millipore water. The IPA Phosphate Buffer was then prepared by the addition of IPA and Phosphate Buffer in ration of 40:60 v/v respectively. The final Ph of the buffer was found to be 8.50 Ph.

Standard Preparation: Standard stock solution was prepared by weighing about 10 mg of Atovaquone and transferred to 100 ml clean, dry standard volumetric flask. The volume was made up with the above prepared IPA Phosphate Buffer (40:60 v/v). Pipette out 2, 4, 6, 8, 1, 1.2 and 1.4 of the above stock solutions and was transfer to 10 ml volumetric flask to obtain 20, 40, 60, 80, 100, 120 and 140 ml concentration respectively. The linearity graph was plotted with concentration on X axis and absorbance on Y axis after scanning the solution.

Preparation of Sample solution: Mepron® Suspension (claiming 750 mg of Atovaquone in 5 ml) was taken.1.4 ml of the above suspension was added to 100 ml of volumetric flask and make up the volume with IPA Phosphate Buffer to obtain 100 µg/ml solution. The solution was filtered using 0.45µm pore filter and further measurements were done using U.V.

Selection of Wavelength: The 100µg/ml stock solution prepared above was scanned in the range of 200-800 nm. The absorbance maximum of 494nm (**Fig. 2**) was selected for analysis of Atovaquone.

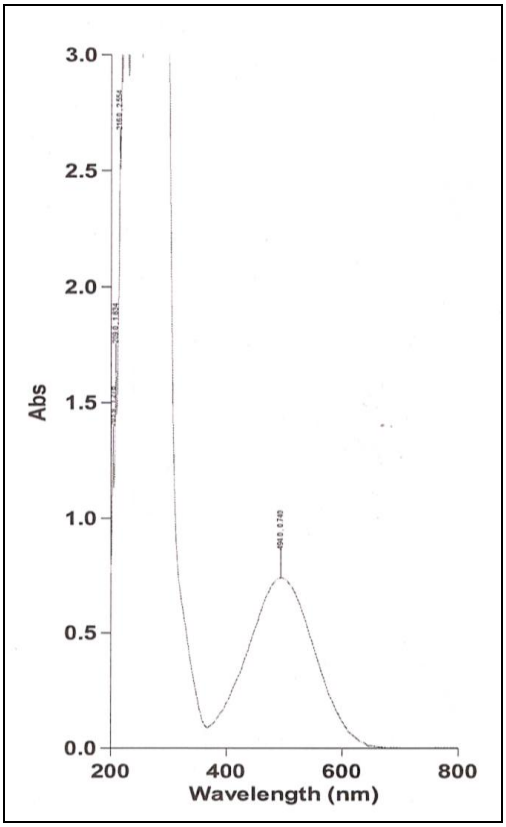
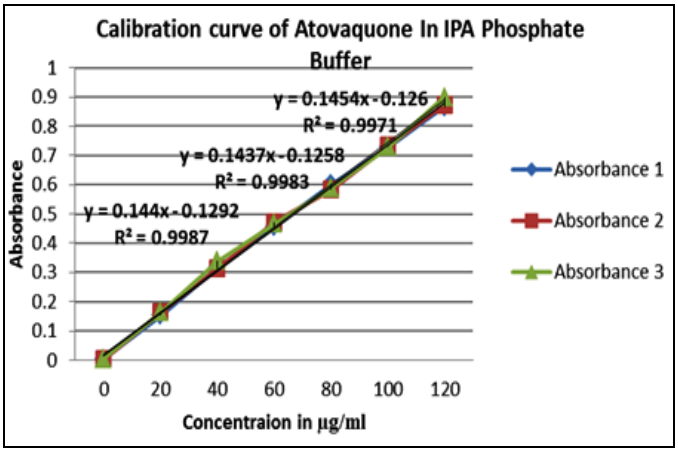


FIGURE 2: U.V SPECTRUM OF ATOVAQUONE IN IPA PHOSPHATE BUFFER (100µg/ml)

Calibration Curve: Absorbance's of above prepared dilutions was reported at 494 nm against IPA Phosphate Buffer as blank .The readings were taken in triplicates (**Table 1**).

Calibration Curve of Atovaquone in IPA Phosphate Buffer					
Sr. no.	**Concentration in µg/ml**	**Abs 1**	**Abs. 2**	**Abs. 3**	**SD**
1	20	0.0039	0.0028	0.0034	0.000551
2	40	0.1516	0.1633	0.1632	0.006726
3	60	0.3165	0.3135	0.3391	0.013995
4	80	0.4565	0.4727	0.4663	0.008159
5	100	0.6017	0.584	0.5882	0.009248
6	120	0.7299	0.7329	0.7296	0.001825
7	140	0.8672	0.8741	0.8999	0.017236

The graph was plotted by taking concentration on X axis and absorbance on Y axis. The coefficient of regression (r^2) of 0.998 (**Graph 1**) for Atovaquone drug was obtained.



GRAPH 1: CALIBRATION CURVE OF ATOVAQUONE IN IPA PHOSPHATE BUFFER

Method of Validation: Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result or a product meeting its predetermined specifications and quality characteristics [7]. The method was validated for different parameters like linearity, accuracy, precision, robustness, and ruggedness, limit of detection (LOD) and limit of quantification (LOQ) [8].

Linearity & Range: Linearity of Calibration Curve (Abs. vs Conc.) in Pure solution were checked over the concentration ranges of about (20-140 µg/ml) for the Atovaquone pure drug. The results were shown in **Table 1** and linearity graph were represented by **Graph 1**.

Accuracy: Accuracy of the method was checked by preparing the solution of different concentrations of 50%.100% and 150% where the concentration of Meprone® suspension was kept constant i.e. 20 mg & pure API was varied as 10mg, 20mg & 30mg respectively. The solutions were prepared in triplicates & the accuracy was indicated by % recovery (**Table 2**).

TABLE 2: ACCURACY OF THE METHOD WITH % RECOVERY

Sr. no.	Conc. of nano Suspension (µg/ml)	Pure drug (µg/ml)	% Drug added	Amount found	% recovery	Mean	SD	% RSD
1	20	10	50	10.14	101.4			
2	20	10	50	9.69	96.9	9.93	0.2273	0.0229
3	20	10	50	9.97	99.7			
4	20	20	100	20.06	100.3			
5	20	20	100	20.20	101	20.03	0.1762	0.0088
6	20	20	100	19.85	99.25			
7	20	30	150	29.96	99.867			
8	20	30	150	29.78	99.267	29.93	0.1375	0.0046
9	20	30	150	30.05	100.167			

Precision: Precision of method was determined by intraday and interday variation studies. 6 different solutions of same concentration i.e. 100µg/ml were prepared and analyzed 3 times a day i. e morning, afternoon and evening and the absorbances were noted. The results were indicated by % recovery. In the interday variation study, 6 different solutions of same concentrations (100 µg/ml) were prepared and analyzed three times for 3 consecutive days and the absorbances were noted. The results were calculated for % recovery (**Table 3**).

TABLE 3: PRECISION OF METHOD FOR INTERDAY AND INTRADAY VARIATIONS

Sr. no.	Concentrations µg/ml	Absorbances Interday			Avg % RSD	Absorbance Intraday			Avg % RSD
		Day 1	Day 2	Day 3		Morning	Afternoon	Evening	
1	100	0.6111	0.6019	0.5932	0.0091	0.6012	0.6011	0.6021	0.007
2	100	0.6040	0.6041	0.5944		0.6111	0.6012	0.6111	
3	100	0.6212	0.61102	0.5889		0.6121	0.6112	0.6112	
4	100	0.6110	0.6001	0.6012		0.6012	0.6031	0.6121	
5	100	0.6012	0.5991	0.5869		0.6012	0.6012	0.6112	
6	100	0.6121	0.6021	0.5966		0.6013	0.6021	0.6021	
%RSD		0.0117	0.00708	0.00874		0.00810	0.0059	0.00723	

Robustness: Robustness of the method was determined by carrying out the analysis at 5 different wavelengths (i.e. 494±1). The absorbances were noted and the results were indicated by %RSD (**Table 4**).

TABLE 4: ROBUSTNESS OF THE METHOD DEVELOPED

Sr. no	λ_{max}	Absorbance	Statistical analysis
1	495	0.601	Mean:0.6029 SD :0.001672 %RSD: .002774
2	495	0.6051	
3	495	0.6011	
4	493	0.6031	
5	493	0.605	
6	493	0.6021	

Ruggedness: The Ruggedness of the method was determined by carrying out the analysis by two different analysts and the respective absorbances were noted. The results were determined by %RSD (**Table 5**).

TABLE 5: RUGGEDNESS OF THE METHOD DEVELOPED

Sr. no.	λ_{max}	Absorbance	Statistical analysis
1	495	0.6011	Mean:0.6011 SD :0.0008 %RSD: 0.00012
2	495	0.6012	
3	495	0.6011	
4	493	0.6012	
5	493	0.6012	
6	493	0.6013	

Limit of Detection: The detection limit of individual analytical procedure is the lowest amount of analyte in the sample. The Limit of detection was determined by using the formula involving standard deviation of response and slope of calibration curve.

$$LOD=3.3*SD/S$$

Where.SD is the standard deviation of y intercept and S is slope of calibration curve.

LOD value for the method was found to be **0.0495**.

Limit of Quantification: The LOQ is the concentration that can be quantified reliably with the specified level of accuracy and precision. The LOQ was calculated using the formula involving standard deviation of response and slope of calibration curve.

$$LOQ=10*SD/S$$

Where, SD is the standard deviation of Y intercept and S is the slope of calibration Curve

The LOQ value for the method was found to be **0.06031**.

Interaction with placebo: The scan of placebo for the nanosuspensions was taken in 200 to 400 nm range. The scan shows that no interference of the excipients was found in the region of 494 nm at which Atovaquone shows the λmax value. This shows that the method is very accurate for the determination of the Atovaquone in Various pharmaceutical dosage forms (**Fig. 3, 4**).

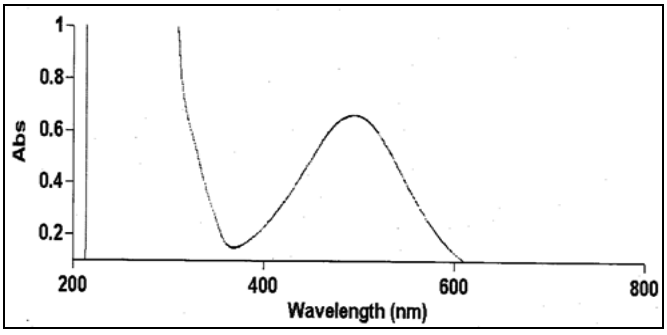


FIGURE 3: SCAN OF ATOVAQUONE NANOSUSPENSIONS IN IPA PHOSPHATE BUFFER AT 100 µg/ml CONCENTRATION (100µg/ml).

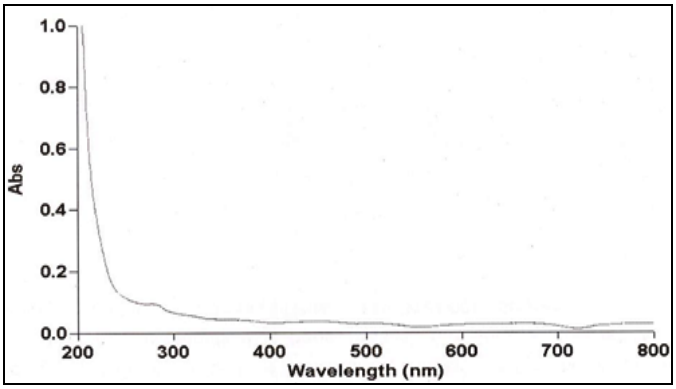


FIGURE 4: SCAN OF PLACEBO OF ATOVAQUONE NANOSUSPENSION IN IPA PHOSPHATE BUFFER (100µg/ml)

RESULTS AND DISCUSSION: The developed method was found to be precise as the % RSD values for intraday and interday were found to be less than **2 %.**Good recoveries **(96.9% to 101.4 %)** of the drug were obtained at each added concentration, which indicates that the method was accurate. The LOD and LOQ were found to be in sub microgram level **(i. e. 0.0495 & 0.06031)** which indicates the sensitivity of the method. The method was also found to be robust and rugged as indicated by %RSD values which are less than **2 %.**The assay results shows that the amount of drug was in good agreement with the labeled claim of the formulation as indicated by % recovery **(101.01 %).**

Summary of Validation: Parameters of proposed Spectrophotometric method were shown in **table 6**.

TABLE 6: PARAMETERS OF PROPOSED SPECTROPHOTOMETRIC METHOD

Parameters	Results
Linearity indicated by correlation coefficient	Range = 20-140 µg/ml R^2 = 09982
Precession indicated by % RSD	0.007 (%RSD Value))
Accuracy indicated by & Recovery	50% = 0.023 (%RSD Value) 100 % = 0.0088 (%RSD Value) 150% = 0.0045 (%RSD Value)
Limit of Detection (LOD)	0.0495 (sub microgram level)
Limit of Quantification(LOQ)	0.0631 (sub microgram level)
Linear regression equation	R^2 = 0.9982
Robustness indicated by % RSD	0.002774 (%RSD Value)
Ruggedness indicated by % RSD	0.00012 (%RSD Value)

CONCLUSION: An accurate and precise method for the determination of Atovaquone in different pharmaceutical dosage form was developed. The USP gives HPLC as a validated method for the determination of Atovaquone which is costly and time consuming. The above validated method can be used for the determination of the same in various dosage forms.

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