



**DEVELOPMENT AND VALIDATION OF NOVEL METHOD FOR
 SIMULTANEOUS ESTIMATION OF ATOVAQUONE AND
 MEFLOQUINE HYDROCHLORIDE IN BULK DRUG USING
 RP-HPLC**

Sanyam Sharma, Amar Deep Ankalgi, Rahul Sharma, Arti Devi, Shammy Jindal, Kanya Goyal*

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ABSTRACT

Atovaquone and Mefloquine hydrochloride are well known anti-malarial drugs. Literature survey reveals that there was no method available for the selected drug combination. In this way, here an endeavour has been made to develop simple, precise, fast method for simultaneous estimation of atovaquone and mefloquine hydrochloride in bulk drug by using RP-HPLC method. The method was carried out by using gradient HPLC on C18 column using Shimadzu prominence LC 20 AD and mobile phase comprised of Methanol:ACN:Water in the ratio of 85:7.5:7.5 (pH 2.9 was adjusted with OPA). The method was performed with 10µl injection volume. The UV detection was done at 231nm. The retention times of atovaquone and mefloquine hydrochloride were 7.6 and 2.6 min respectively. The proposed method was validated according to ICH guidelines. The validation parameters were linearity, accuracy, precision (inter-day, intra-day and repeatability) and robustness etc. Linearity was in the range of 80-120µg/ml for atovaquone and 40-60µg/ml for mefloquine hydrochloride. The percent recoveries of both drugs were 99.99-100% and 92.05-99.09%. This method is suitable for the routine analysis of atovaquone and mefloquine hydrochloride in bulk drugs either individually or in mixture

*Laureate Institute of Pharmacy, V.P.O. Kathog, Teh. Jawalamukhi, Distt. Kangra-176031, Himachal Pradesh, India

***For Correspondence:** kanya.goyal7@gmail.com

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INTRODUCTION

Atovaquone and Mefloquine hydrochloride (Figure 1) are broad spectrum anti-parasitic drugs used in the treatment of malaria [1-4]. Atovaquone is a hydroxy naphthaquinone or an analogue of ubiquinone which is highly lipophilic in nature and used for treatment and prevention of chloroquine-resistant *P. falciparum* in combination with proguanil [5-6]. Atovaquone plays a wide important role in disease management of malaria because of drug resistance, intolerable side effects of other existing anti-malarials [7-8]. Atovaquone is a competitive inhibitor of ubiquinol. It inhibits mitochondrial electron transport chain at the bc1 complex that leads to loss of mitochondrial function. During intra-erythrocytic phase of infection, a key job of the parasite in mitochondria is to give orotate for pyrimidine through the action of dihydro-orotate dehydrogenase (DHODH). Inhibition of bc1 complex by atovaquone impacts on the concentration of metabolites in the pyrimidine biosynthesis pathway [9-16].

Mefloquine hydrochloride is a phospholipid-interacting anti-malarial drug. It is very effective against *Plasmodium falciparum* with very few side effects [5-6][17-19]. Basically Mefloquine is a quinoline-methanol derivative with antimalarial, anti-inflammatory and potential chemosensitization and radiosensitization activities [20-21].

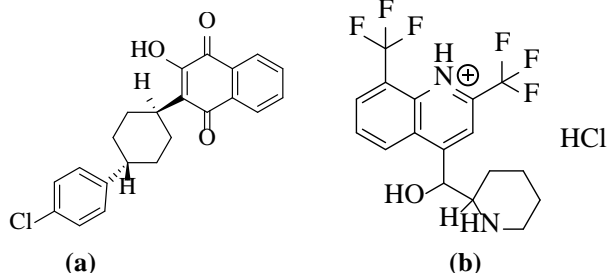


Figure 1. Structure of (a) Atovaquone (b) Mefloquine hydrochloride

MATERIALS AND METHOD

The drugs atovaquone and mefloquine hydrochloride were purchased from Sanjay Biologicals Amritsar, Punjab India. All the solvents were of HPLC grade and purchased through S.D. fine-chem. Ltd., India.

Instrumentation and chromatographic conditions

The lambda max and iso-absorptive point were determined by using double beam UV-spectrophotometer, Lab India with UV win software. Method development and validation studies were carried out by using Shimadzu prominence LC 20 AD. Lab

solution software used for instrument control. Column used for LC separation was Shimadzu octadecylsilane Hypersil (ODS) C18 having length of 150 × 4.6 mm, with particle size of 5 μ was used for the chromatographic separation of drugs. Injection volume was 10 μl and mobile phase composed of Methanol:ACN:Water in the ratio 85:7.5:7.5 (pH 2.9 was adjusted with OPA). The wavelength of detection was at 231 nm and run time was 15 minutes.

Selection of wavelength

The sensitivity of HPLC technique that utilizes UV detection relies upon appropriate selection of detection wavelength. For the selection of wavelength 50 μg/ml concentration of Atovaquone and 50 μg/ml of Mefloquine Hydrochloride used and overlain spectrum is taken. Atovaquone and Mefloquine hydrochloride was prepared. The solvent ratio was 50:50 v/v of water and methanol. Iso-absorptive point was obtained at 231 nm. The overlain spectrum of drugs was showed in figure 2.

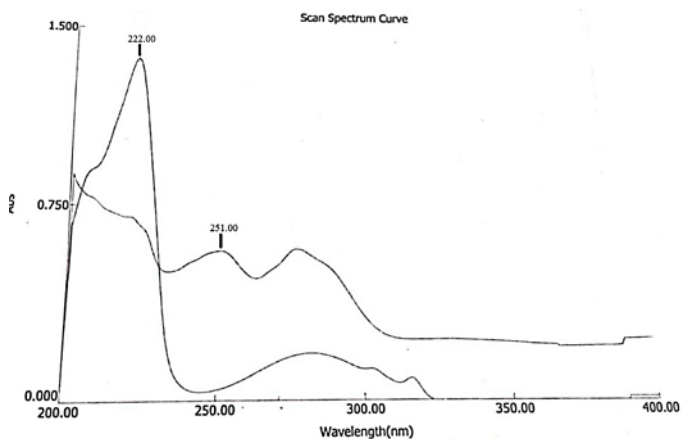


Figure 2. Overlain spectrum of Atovaquone and Mefloquine hydrochloride.

Analytical Method Validation

The method was validated as per ICH guidelines. The parameters studied were linearity, accuracy, precision (intraday and interday precision and repeatability) and robustness [22].

Preparation of standard solution

Atovaquone standard solution

Standard Atovaquone (50 mg) was weighed and transferred to 100 ml volumetric flask and dissolved with solvent (Methanol:ACN:Water pH 2.9 was adjusted with OPA). The contents were mixed and volume was made with solvent to obtain a solution containing 1000 μg/ml concentrations. From

the standard Atovaquone stock solution, volume of 0.8, 0.9, 1, 1.1, 1.2 ml was pipetted out from 1000 µg/ml and transferred to volumetric flasks of 10 ml capacity. Then volume was made up to the mark with conc. of 80, 90, 100, 110, 120 µg/ml.

Mefloquine hydrochloride standard stock solution

Standard Mefloquine hydrochloride 50 mg was weighed and transferred to 100 ml volumetric flask and dissolved in solvent. The contents were mixed and volume was made with solvent to obtain a solution containing 1000 µg/ml concentrations. From the standard Mefloquine hydrochloride stock solution the volume of 0.4, 0.45, 0.5, 0.55, 0.6 ml was pipetted out from 1000 µg/ml and transferred to volumetric flasks of 10 ml capacity. Then volume was made up to the mark with conc. of 40, 45, 50, 55, 60 µg/ml.

Linearity

The linearity range for Atovaquone was 80-120 µg/ml and for Mefloquine hydrochloride was 40-60 µg/ml. The R^2 value was calculated for both drugs. The linearity was plotted for peak area versus concentration. The injection was given at time interval of 15 min with run time of 15 min.

Accuracy

The accuracy was done by performing recovery studies at 80, 100 and 120% of test concentration of both the drugs. The samples were prepared in triplicate of Atovaquone (80, 100, 120 µg/ml) and Mefloquine hydrochloride (40, 50, 60 µg/ml) and the accuracy was calculated by recovery studies.

Precision

In analytical method, precision is the degree of closeness of agreement between a series of measurements acquired from the different testing of a similar sample. Precision includes repeatability, inter and intraday precision and reproducibility. The repeatability was performed by six determinants of test concentration of each drug. The interday readings were taken as for intraday one. The SD and %RSD was calculated and evaluated.

Robustness

Robustness is a measure of its capacity to stay unaffected by little, but deliberate variations in method parameter. HPLC robustness was done by changing flow rate and wavelength respectively.

RESULTS AND DISCUSSION

Analytical Method Validation

The validation parameters were summarized in table 8. The UV spectrum was obtained for both drugs at 200-400 nm scan as spectrum was mentioned in figure 2. The retention time for atovaquone was 7.6 and 2.6 and each chromatogram was mentioned in figure 3. The linearity of both drugs was found within the limits ($R^2 \geq 0.992$) and results were in figure 4 and 5 and table 1 and 2.

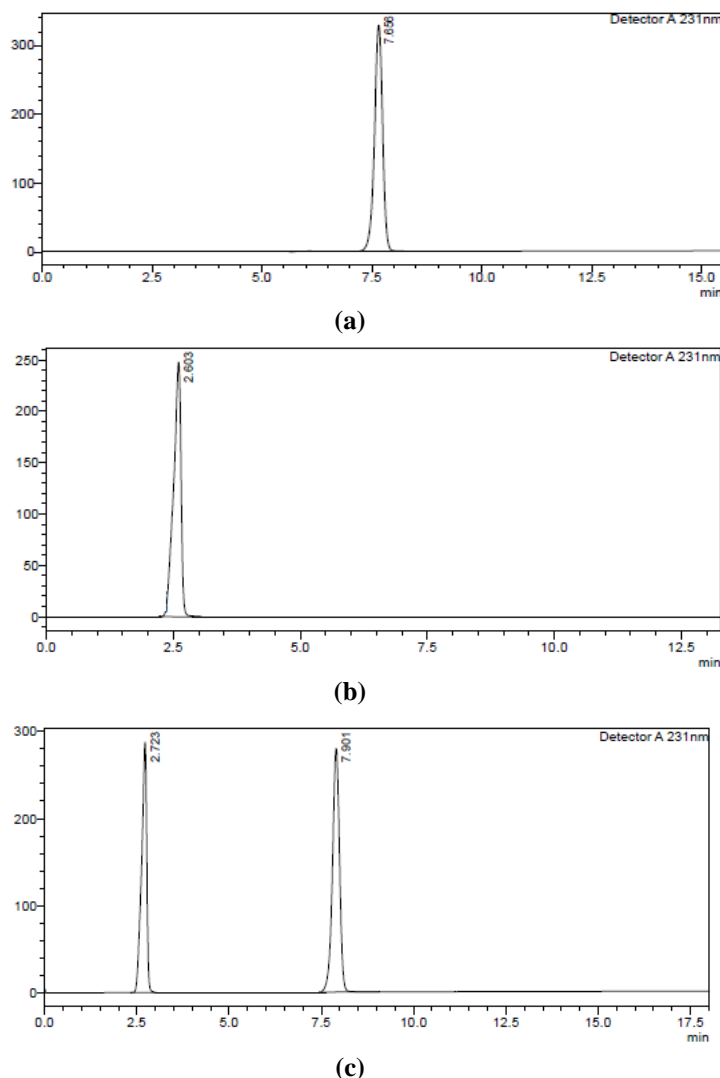
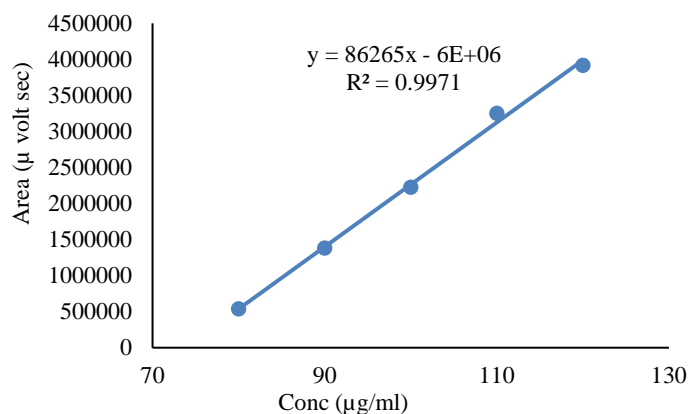


Figure 3. Chromatogram of (a) Atovaquone (b) Mefloquine hydrochloride (c) mixture of both drugs.

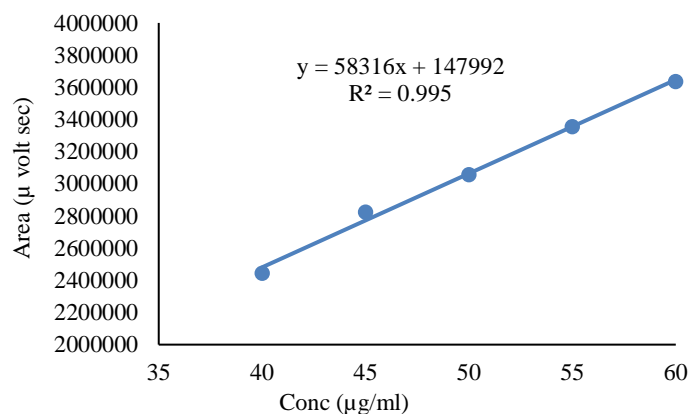
The accuracy was done by performing recovery studies at 80, 100 and 120% of test concentration of both the drugs. The samples were prepared in triplicate and the accuracy was calculated by recovery studies. The results of accuracy were mentioned in table 3 and 4 respectively.

Table 1. Results of linearity curve of Atovaquone at wavelength 231 nm.

Sr. No	Conc (µg/ml)	Area (µ volt sec.)
1	80	536965
2	90	1381901
3	100	2226410
4	110	3248701
5	120	3916799

**Figure 4. Linearity curve of Atovaquone at 231 nm.****Table 2. Results of linearity curve of Mefloquine hydrochloride at wavelength 231 nm.**

Sr. No	Conc. (µg/ml)	Area (µ volt sec.)
1	40	2444173
2	45	2824901
3	50	3057399
4	55	3356069
5	60	3636497

**Figure 5. Linearity curve of Mefloquine hydrochloride at 231 nm.****Table 3. % Drug Recovery of Atovaquone at wavelength 231 nm**

Recovery Sample			Fortified Sample			% Recovery
Conc. (µg/ml)	Peak Area	Mean Peak Area	Conc. (µg/ml)	Peak Area	Mean Peak Area	
80	1381901	1381895.5	80+100	3608243	3608244	99.98
	1381890			3608245		
100	2226409	2226410	100+100	4452729	4452733.5	100
	2226411			4452738		
120	3248615	3248658	120+100	5475298	5475294	99.99
	3248701			5475290		

Table 4. % Drug Recovery of Mefloquine Hydrochloride at wavelength 231 nm

Recovery Sample			Fortified Sample			% Recovery
Conc. (µg/ml)	Peak Area	Mean peak area	Conc. (µg/ml)	Peak area	Mean peak area	
40	2824901	2824902	40+50	5648704	5648705	92.5
	2824903			5648706		
50	3057399	3057398.5	50+50	6114726	6114728.5	99.9
	3057398			6114719		
60	3356069	3356070	60+50	6413571	6413570	99.9
	3356071			6413569		

Table 5(a). Intraday precision of Atovaquone and Mefloquine hydrochloride at 231 nm

Drug	Atovaquone			Mefloquine hydrochloride		
	Concentration	90 µg/ml	100 µg/ml	110 µg/ml	45 µg/ml	50 µg/ml
Area	1381901	2226410	3248701	2824642	3057666	3354032
	1381828	2226398	3248699	2824901	3057399	3356070
	1381892	2226399	3248723	2824864	3057962	3356092
Mean	1381874	2226402	3248708	2824802	3057676	3355398
SD	39.804	6.658	13.315	140.07	281.62	1183.042
%RSD	0.003	0.0003	0.0004	0.005	0.009	0.035

Table 5(b). Interday Precision of Atovaquone

Day	Day 1			Day 2			Day 3		
	Concentration	90 µg/ml	100 µg/ml	110 µg/ml	90 µg/ml	100 µg/ml	110 µg/ml	90 µg/ml	100 µg/ml
Area	1381901	2226410	3248701	1381828	2226410	3248699	1381825	2226438	3248682
	1381828	2226398	3248699	1381845	2226424	3248672	1381849	2226424	3248655
	1381892	2226399	3248723	1381816	2226446	3248685	1381816	2226452	3248639
Mean	1381874	2226402	3248708	1381830	2226427	3248685	1381830	2226438	3248659
SD	39.804	6.658	13.315	14.57166	18.14754	13.5	17.058	14	21.733
%RSD	0.003	0.0003	0.0004	0.001	0.008	0.004	0.001	0.0006	0.0006

Table 5(c). Interday Precision of Mefloquine hydrochloride

Day	Day 1			Day 2			Day 3		
	Concentration	45 µg/ml	50 µg/ml	55 µg/ml	45 µg/ml	50 µg/ml	55 µg/ml	45 µg/ml	50 µg/ml
Area	2824642	3057666	3354032	2824642	3057666	3354232	2824698	3057623	3354222
	2824901	3057399	3356070	2824939	3057381	3356025	2824939	3057390	3356025
	2824864	3057962	3356092	2824811	3057999	3356186	2824847	3057999	3356012
Mean	2824802	3057676	3355398	2824797	3057682	3355481	2824828	3057671	3355420
SD	140.07	281.62	1183.042	148.97	309.31	1084.6	121.61	307.2854	1037.23
%RSD	0.005	0.009	0.035	0.005	0.01	0.03	0.004	0.01	0.03

Table 6(a). Repeatability of atovaquone at 231 nm

Sr. No	Area (µ volt sec.)
1	2226438
2	2226424
3	2226452
4	2226422
5	2226419
6	2226474
Mean	2226438
SD	21.47
%RSD	0.0009

Table 6(b) Repeatability of mefloquine hydrochloride at 231 nm

Sr. No	Area (µ volt sec.)
1	3057623
2	3057390
3	3057999
4	3057647
5	3057332
6	3057927
Mean	3057653
SD	271.27
%RSD	0.008

Interday and intraday precision of concentration for Atovaquone 90, 100, 110 µg/ml and Mefloquine hydrochloride 45, 50, 55 µg/ml was prepared and data was obtained. 3 replicates were prepared for 3 days. The results were shown in table 5(a), 5(b), 5(c) for Atovaquone and Mefloquine hydrochloride.

For repeatability minimum of 6 determinants were prepared of 100 µg/ml for Atovaquone and 50 µg/ml for Mefloquine hydrochloride and the chromatograms were obtained. The results were shown in table 6(a) for Atovaquone and table 6(b) for Mefloquine hydrochloride.

Robustness results were shown in table 7(a) for change in flow rate and table 7(b) for change in mobile phase.

Change in flow rate of mobile phase

Table 7(a). Robustness of Atovaquone and Mefloquine hydrochloride at wavelength 231 nm by changing the flow rate.

Flow rate (ml/min.)	Difference	R _t of Atovaquone (min.)	R _t of Mefloquine hydrochloride (min.)
0.9	-0.1	7.429	2.502
1	0	7.520	2.656
1.1	+0.1	7.565	2.606

Change in Wavelength

Table 7(b). Robustness of Atovaquone and Mefloquine hydrochloride at the wavelength 231±2 nm.

Wavelength	Difference	R _t of Atovaquone (min.)	R _t of Mefloquine hydrochloride (min.)
229	-2	7.549	2.606
231	0	7.546	2.605
233	+2	7.548	2.602

CONCLUSION

The above developed RP-HPLC method for simultaneous estimation of Atovaquone and Mefloquine hydrochloride was simple, economic, precise, robust and accurate method. There were no HPLC method revealed till now on chosen combination of drugs. Subsequently, the developed method is great for the regular analysis and quality control of bulk drugs either individually or in combination.

Table 8. Summary of validation parameters of RP-HPLC at 231 nm wavelength.

Parameter	Atovaquone	Mefloquine hydrochloride
Linear range (µg/ml)	80-120	40-60
Regression coefficient (R ²)	0.997	0.995
%Recovery	99.99	98.42
Repeatability (n=6)	%RSD NMT 2	%RSD NMT 2
Precision		
Interday precision	%RSD	%RSD
Intraday precision	NMT 2	NMT 2

FINANCIAL ASSISTANCE

Nil

CONFLICT OF INTEREST

The authors declare no conflict of interest

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