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

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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.

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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 antimalarials [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 antimalarial 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](image)

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. Overlaid spectrum of Atovaquone and Mefloquine hydrochloride.](image)

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 determinations 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.](image-url)

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     |
| 100             | 2226409          | 2226410   | 100+100       | 4452729  | 4452733.5          | 100       |
| 120             | 3248615          | 3248658   | 120+100       | 5475298  | 5475294           | 99.99     |

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      |
| 50              | 3057399          | 3057398.5 | 50+50         | 6114726  | 6114728.5          | 99.9      |
| 60              | 3356069          | 3356070   | 60+50         | 6413571  | 6413569           | 99.9      |
Table 5(a). Intraday precision of Atovaquone and Mefloquine hydrochloride at 231 nm

| Drug                     | Atovaquone | Mefloquine hydrochloride |
|--------------------------|------------|--------------------------|
| **Concentration**        | 90 µg/ml   | 45 µg/ml | 50 µg/ml | 55 µg/ml |
| **Area**                 |            |          |          |          |
| 1                        | 1381901    | 3248701 | 2824642 | 3057666 |
| 2                        | 1381828    | 3248699 | 2824901 | 3057399 |
| 3                        | 1381892    | 3248723 | 2824864 | 3057962 |
| **Mean**                 | 1381874    | 3248708 | 2824802 | 3057676 |
| **SD**                   | 39.804     | 13.315  | 140.07  | 281.62 |
| **%RSD**                 | 0.003      | 0.0003  | 0.005   | 0.009  |

Table 5(b). Interday Precision of Atovaquone

| Day | Day 1 | Day 2 | Day 3 |
|-----|-------|-------|-------|
| Concentration 90 µg/ml | 100 µg/ml | 110 µg/ml | 100 µg/ml | 110 µg/ml | 100 µg/ml | 110 µg/ml |
| **Area** | 1381901 | 3248701 | 2824642 | 1381828 | 3248699 | 2824901 | 1381892 | 3248723 | 2824864 |
| 2226410 | 2226398 | 2226399 | 2226424 | 2226446 | 2226402 | 2226398 | 2226399 | 2226402 |
| 1381830 | 1381845 | 1381816 | 1381830 | 3248708 | 2824802 | 3057676 | 3355398 |
| **Mean** | 1381874 | 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(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 | 55 µg/ml |
| **Area** | 2824642 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 |
| 2226410 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 |
| 2226398 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 | 2824642 | 3057666 | 3354032 |
| **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.
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