

## A simple HPLC-UV method for quantification of Atovaquone in rabbit plasma

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**Abstract :** A new simple, sensitive and specific high performance liquid chromatography with UV detection (HPLC-UV) method was developed and validated for determination of Atovaquone in rabbit plasma. The method utilized 250  $\mu$ L of plasma and Atovaquone was extracted from the plasma by using liquid-liquid extraction method. Buparvaquone was used as the internal standard and the method was validated on a  $C_{18}$  column using acetonitrile-ammonium acetate (pH 3; 0.02 M) (85 : 15, v/v) with UV detection at 254 nm. The assay was conducted at 45  $^{\circ}$ C and a flow rate of 1 ml/min. The calibration curve was linear between 80–4000 ng/ml ( $r^2 = 0.9993$ ) with a limit of quantification (LOQ) of 80 ng/ml. The extraction recovery ranged from 84.5 to 97.9% and plasma samples containing Atovaquone were found to be stable in  $-20^{\circ}$ C for 4 weeks. Finally, the validated HPLC-UV method was successfully applied in an *in vivo* pharmacokinetic study involving New Zealand rabbits.

**Keywords :** HPLC-UV, rabbit plasma, Atovaquone, Buparvaquone, pharmacokinetic study.

### Introduction

Atovaquone or *trans*-2-[4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthalenedione (Fig. 1) is a structural analogue of protozoan ubiquinone. It has a broad-spectrum antiparasitic activity towards causing organisms of pneumocystis pneumonia<sup>1,2</sup>, toxoplasmosis<sup>3</sup> and malaria<sup>4</sup>. In *Plasmodium* species, Atovaquone inhibits the binding of coenzyme Q-complex III at  $Q_o$  cytochrome domain<sup>5</sup>. The inhibition will further cause collapse of the mitochondrial membrane potential. As several metabolic enzymes depend on this mitochondrial transport chain involving ubiquinone, Atovaquone will cause indirect inhibition of these enzymes. Consequently, the synthesis of nucleic acid and adenosine triphosphate will be blocked thus causing the parasite death.

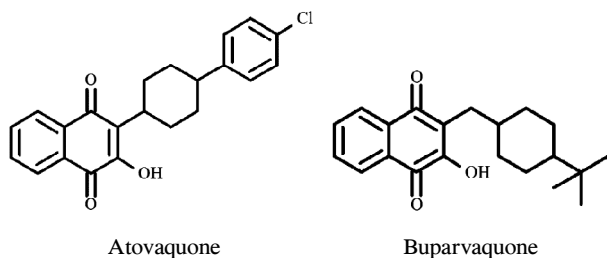


Fig. 1. Chemical structures of Atovaquone and Buparvaquone.

Several methods have been reported for quantification of Atovaquone in plasma using high-performance liquid chromatography with UV detector (HPLC-UV)<sup>6-8</sup>, ultra performance liquid chromatography<sup>9</sup> and gas chromatography with electron capture detector (GC-ECD)<sup>10</sup>. A steep gradient liquid chromatography method with UV detector was also described for the quantification of Atovaquone in the presence of Proguanil<sup>11</sup>. More recently, quantification of Atovaquone using advanced analytical methods were developed including liquid chromatography with tandem mass spectrometry (LC-MS-MS)<sup>12</sup> and liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS)<sup>13</sup>. Generally, lower limit of quantification (LOQ) was possible to achieve by using the mass spectrometry while HPLC-UV methods gave higher LOQ values. However, HPLC-UV is still among the most widely used analytical method due to the availability and cost efficiency. Therefore, we developed and validated a new simple, sensitive and specific HPLC-UV method to achieve lower LOQ by using smaller plasma volume.

Most of the studies used liquid-liquid extraction as the sample preparation method while some used protein precipitation<sup>8</sup> and solid phase extraction<sup>6,11</sup>. In this study,

liquid-liquid extraction procedure by Pingale *et al.*<sup>12</sup> was implemented and slightly modified in order to reduce the volume of plasma sample from 500 to 250  $\mu$ L. Most of the previous methods used Deschloroatovaquone (59C80) as the internal standard (IS). However, this study utilized Buparvaquone or 4-hydroxy-3-[(4-tertbutylcyclohexyl)methyl]naphthalene-1,2-dione (Fig. 1) as the IS. Finally, this method was applied for the quantification of Atovaquone in the *in vivo* pharmacokinetic study involving New Zealand rabbits to evaluate the comparative bioavailability of pure Atovaquone and Atovaquone-loaded solid lipid nanoparticles (Atovaquone-SLNs).

## Results and discussion

### Bioanalytical method validation :

The chromatographic conditions were adjusted in order to provide a good performance of the assay. The optimum wavelength for the detection of Atovaquone was found to be at 254 nm. As for the mobile phase, several compositions of acetonitrile to ammonium acetate were tested at ratios of 90 : 10, 85 : 15 and 80 : 20 (v/v). Increment in the percentage of acetonitrile had shown reduction in the retention time of both analytes. This can be explained by the highly hydrophobic nature of both compounds which demands high amount of organic modifier<sup>14</sup>, causing faster elution at higher acetonitrile composition. On the other hand, the increase in ammonium acetate composition had shown improvement in the resolution between the peaks of both analytes. Thus, a composition of acetonitrile : ammonium acetate (85 : 15, v/v) was chosen as it gave sufficient retention time to avoid interferences of plasma endogenous peak at lower retention time, peaks with high area and good shape, and ample resolution between Atovaquone and the IS.

pH of ammonium acetate was varied from pH 5 to pH 3. As the pH decreased, retention time of Atovaquone was reduced and the peak area was increased. Tailing factor was also improved indicating symmetry of both peaks. For a weak acid substance such as Atovaquone, pH above the pKa value will cause the acidic analyte to carry a negative charge and behave as an extremely polar molecule. Thus, increasing the pH value will cause a delay in the elution of analyte in hydrophobic mobile

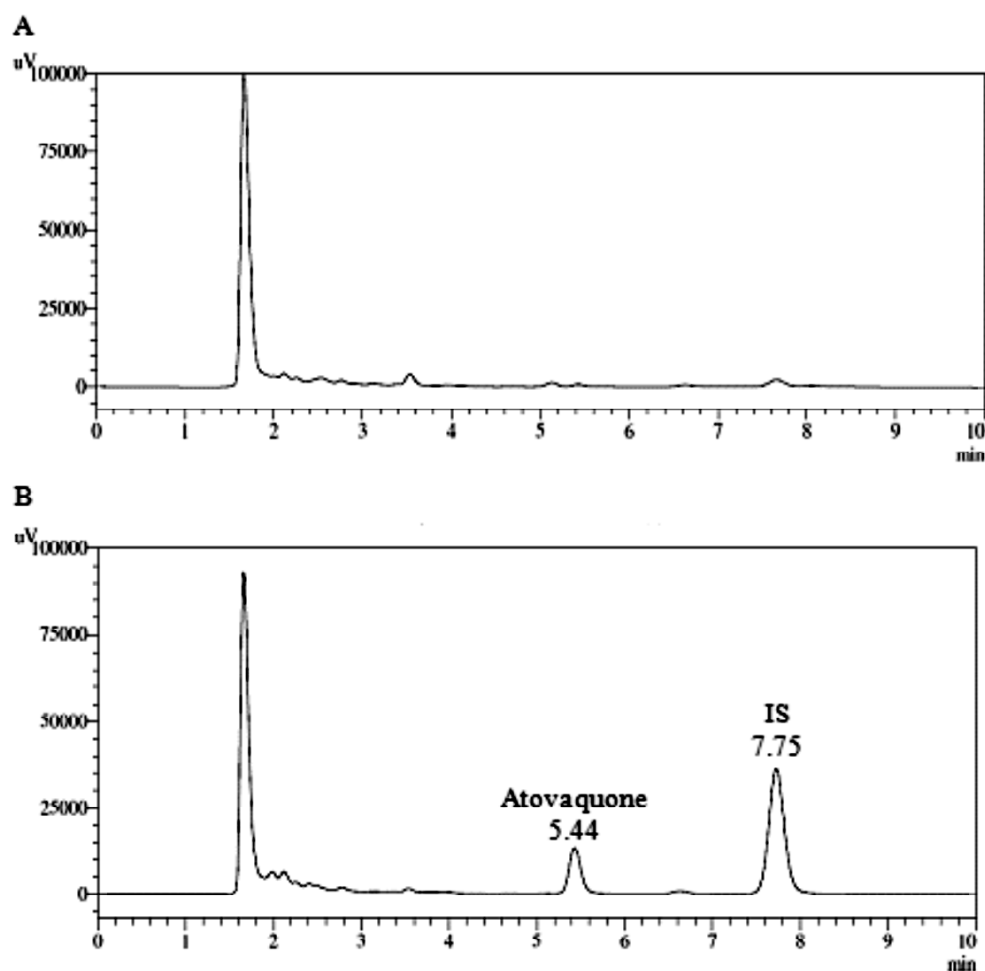
phase. Since pKa of Atovaquone is  $\approx 5.0^6$ , pH lower than pH 5 will cause faster elution of Atovaquone. Therefore, pH 3 was chosen as it gave sufficient retention time, highest peak area and good peak shape.

At first the temperature of the column was set at 25 °C. However, it did not give a stable baseline. Thus, a higher temperature of 45 °C was used as it gave stable baseline, and faster elution of Atovaquone with a better peak shape. Finally, a mobile phase containing a mixture of acetonitrile and ammonium acetate (pH 3; 0.02 M) (85 : 15, v/v) was chosen for the analysis at a flow rate of 1 ml/min and column temperature of 45 °C. The wavelength of detection was set at 254 nm.

Fig. 2 shows the representative chromatogram of blank rabbit plasma and plasma spiked with Atovaquone and IS. No endogenous interference was observed at the retention times of Atovaquone and IS in six blank rabbit plasma samples and a good resolution between both analytes was achieved. The retention time was 5.44 min for Atovaquone and 7.75 min for IS. IS is important especially in bioanalytical analysis involving extraction as the sample preparation procedure. It is added prior to the extraction to compensate for loss and error during the process. A suitable hydrophobic IS is crucial for this method as the chromatographic condition involved using high percentage of organic solvent in the mobile phase. Several compounds have been tested and Buparvaquone was chosen as the most suitable IS as it did not interfere with the matrix and was well resolved from Atovaquone. It is also stable and does not exist endogenously in plasma.

The linearity of the method was achieved over a concentration of 80–4000 ng/ml of Atovaquone on five consecutive days. Samples were quantified using peak area ratio of Atovaquone to IS. The mean linear regression equation from all five curves was determined as  $y = 0.0005 (\pm 0.0000)x + 0.0130 (\pm 0.0045)$  with a correlation coefficient of  $0.9993 (\pm 0.0009)$ .

The precision of a concentration point is described by percent relative standard deviation (%RSD) and the accuracy is conceived as percent relative error (%RE). The limit of detection (LOD) for the method is 40 ng/ml which was determined according to the USFDA guideline with



**Fig. 2.** Chromatogram of blank rabbit plasma (A) and rabbit plasma spiked with Atovaquone and IS (B).

a signal to noise ratio of 3 : 1<sup>15</sup>. The LOQ is 80 ng/ml with a precision of 5.8% and accuracy of 9.3%. The LOQ conformed to the USFDA requirement with signal to noise ratio of at least 5 : 1 and precision and accuracy value of within  $\pm 20\%$ <sup>15</sup>.

The mean extraction recoveries of low, medium and high concentration of standard solution are shown in Table 1. The mean recoveries were  $84.5 \pm 2.9\%$ ,  $96.0 \pm 3.3\%$ ,

and  $97.9 \pm 2.5\%$ , respectively. In most cases, recovery may not be 100% but it should be consistent, precise, and reproducible<sup>15</sup> as shown by the precision value ranging from 2.5 to 3.5%.

The results for intra-day and inter-day precision and accuracy for all quality control (QC) samples were shown in Table 2. The intra-day precision ranged from 2.4 to 5.0% and accuracy from -9.2 to 0.0% whereas the inter-day precision ranged from 3.0 to 3.8% and accuracy from -9.6 to -2.3%.

The precision and accuracy for all points were well within the requirement of USFDA which should be within  $\pm 15\%$ <sup>15</sup>. The result showed that the method was precise and accurate over the tested range. An improvement in the method sensitivity was successfully achieved when compared to previous methods using HPLC-UV<sup>6-8,16</sup>.

**Table 1.** Extraction recovery of Atovaquone in plasma  
Mean  $\pm$  SD,  $n = 5$

| Nominal concentration (ng/ml) | % Recovery     | Precision (%RSD) |
|-------------------------------|----------------|------------------|
| 250                           | $84.5 \pm 2.9$ | 3.5              |
| 1500                          | $96.0 \pm 3.3$ | 3.4              |
| 3500                          | $97.9 \pm 2.5$ | 2.5              |

**Table 2.** Experimental values of mean calculated concentration, %RSD and %RE for method validation

| Condition                     | Nominal concentration<br>(ng/ml) | Calculated concentration<br>(ng/ml) | Precision<br>(%RSD) | Accuracy<br>(%RE) |
|-------------------------------|----------------------------------|-------------------------------------|---------------------|-------------------|
| Intra-day <sup>a</sup>        | 250                              | 226.9 ± 11.3                        | 5.0                 | -9.2              |
|                               | 1500                             | 1500.3 ± 36.3                       | 2.4                 | 0.0               |
|                               | 3500                             | 3415.0 ± 160.1                      | 4.7                 | -2.4              |
| Inter-day <sup>b</sup>        | 250                              | 226.0 ± 8.7                         | 3.8                 | -9.6              |
|                               | 1500                             | 1460.9 ± 48.1                       | 3.3                 | -2.6              |
|                               | 3500                             | 3419.8 ± 103.0                      | 3.0                 | -2.3              |
| Bench top <sup>c</sup>        | 250                              | 219.4 ± 2.5                         | 1.1                 | -12.2             |
|                               | 1500                             | 1378.7 ± 29.0                       | 2.1                 | -8.1              |
|                               | 3500                             | 3434.2 ± 209.6                      | 6.1                 | -1.9              |
| Freeze and thaw <sup>d</sup>  | 250                              | 217.8 ± 3.0                         | 1.4                 | -12.9             |
|                               | 1500                             | 1390.9 ± 35.1                       | 2.5                 | -7.3              |
|                               | 3500                             | 3529.4 ± 85.5                       | 2.4                 | 0.8               |
| Post-preparative <sup>e</sup> | 250                              | 230.1 ± 10.1                        | 4.4                 | -8.0              |
|                               | 1500                             | 1506.1 ± 47.7                       | 3.2                 | 0.4               |
|                               | 3500                             | 3717.4 ± 81.5                       | 2.2                 | 6.2               |
| Short-term <sup>f</sup>       | 250                              | 234.5 ± 18.2                        | 7.8                 | -6.2              |
|                               | 1500                             | 1485.8 ± 134.5                      | 9.1                 | -0.9              |
|                               | 3500                             | 3552.6 ± 364.4                      | 10.3                | 1.5               |

<sup>a</sup>5 replicates for each concentration, <sup>b</sup>15 replicates (day 1,  $n = 5$ ; day 2,  $n = 5$ ; day 3,  $n = 5$ ) for each concentration, <sup>c</sup>after 6 h at room temperature ( $25 \pm 2$  °C),  $n = 3$ , <sup>d</sup>after 3 cycles of freeze and thaw at  $-20$  °C,  $n = 3$ , <sup>e</sup>after 24 h in autosampler,  $n = 3$  and <sup>f</sup>4 weeks at  $-20$  °C,  $n = 3$ .

Most of the studies reported an LOQ of more than 100 ng while Lindegårdh and Bergqvist<sup>6</sup> managed to achieve an LOQ of  $\sim 55$  ng (150 nM) but with higher plasma sample volume of 500  $\mu$ L and injection volume of 100  $\mu$ L. However, this method gave a low LOQ (80 ng) using smaller plasma volume of 250  $\mu$ L and injection volume of 20  $\mu$ L.

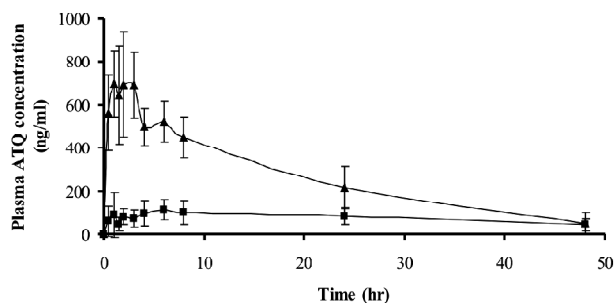
#### Stability studies :

The stability of the samples at all four conditions is shown in Table 2 where all QC samples were shown to be stable with precisions and accuracies of less than  $\pm 15\%$ , which comply with the USFDA guideline. The results show that Atovaquone was stable in blank plasma for 6 h at room temperature with precision values ranging from 1.1 to 6.1% and accuracy from -12.2 to -1.9%. The preparations were also shown to be stable in 24 h-post preparative procedures in the auto sampler with precision values ranging from 2.2 to 4.4% and accuracy from -8.0 to 0.4%. Repeated freeze and thaw conditions (three cycles) were shown not to affect sample stability with precision value ranging from 1.4 to 2.5% and accuracy from -12.9 to 0.8%. Beside that, the short term stability

results showed the precision values ranging from 7.8 to 10.3% and accuracy from -6.2 to -0.9%. This indicates that Atovaquone was stable within the matrix for 4 weeks when stored at  $-20$  °C.

#### Pharmacokinetic study :

Fig. 3 shows the mean plasma profile of Atovaquone-SLNs dispersion and pure Atovaquone dispersion after the oral administration of both formulations in the rab-



**Fig. 3.** Mean plasma concentration of Atovaquone over 48 h in rabbits after oral administration of Atovaquone-SLNs ( $\blacktriangle$ ) and pure Atovaquone ( $\blacksquare$ ),  $n = 5$ .

bits. The calculated mean area under the plasma concentration-time curve from time zero to the last measurable concentration ( $AUC_{0-t}$ ) of Atovaquone-SLNs was  $12870.0 \pm 2856.0$  ng/ml.h while the value for the pure drug suspension was  $3542.3 \pm 1380.2$  ng/ml.h, showing a significant 3.6-fold ( $p < 0.05$ ) increase in the absorption of Atovaquone in the former preparation.

The validated HPLC method was utilized in the pharmacokinetic study of Atovaquone in New Zealand rabbits. Higher  $AUC_{0-t}$  value of Atovaquone-SLNs was shown when compared to pure Atovaquone, indicating a significantly greater exposure of Atovaquone in the solid lipid nanoparticles formulation. The increase in absorption may be contributed by the size reduction of the drug in the solid lipid nanoparticles formulation where the nanosize can improve the solubility and hence increase the concentration gradient to assist the passive diffusion in the gastrointestinal tract<sup>17</sup>. Beside that, the small size of the solid lipid nanoparticles (20–500 nm) also offers an alternative uptake route via the lymphatic system into the systemic circulation and bypassing the hepatic metabolism<sup>18</sup>. Thus, an increase of the drug in blood circulation can be achieved.

## Experimental

### *Chemicals and reagents :*

Atovaquone was obtained from Hallochem Pharmaceutical Co. Ltd. (Chongqing, China) and Buparvaquone was purchased from Glaxo SmithKline (UK). Acetonitrile (HPLC grade) was purchased from J. T. Baker (Phillipsburg, USA) and ammonium acetate was bought from Bendosen Laboratory Chemicals (UK). Glacial acetic acid and ethyl acetate were bought from Quality Reagent Chemicals (New Zealand). Blank rabbit plasma was obtained from the marginal ear vein of New Zealand rabbits and stored at  $-20^{\circ}\text{C}$  before further use.

### *Instrumentation and chromatographic conditions :*

Analysis was performed using a Shimadzu liquid chromatographic system (Kyoto, Japan) with CBM-20A system controller, LC-20AD solvent delivery pump, SPD-20A UV-Vis detector, SIL-20A autosampler, and CTO-10ASvp oven system. Data acquisition and analysis were performed using Shimadzu LCsolution software (Kyoto, Japan).

Mobile phase containing acetonitrile-ammonium acetate (pH 3; 0.02 M; adjusted with glacial acetic acid) (85 : 15, v/v) was used in this study. Mobile phase was filtered under vacuum through 0.45  $\mu\text{m}$  Nylon membrane filter (Whatman International, Maidstone, UK) and degassed prior to use. The analysis was run at a flow rate of 1 ml/min using 254 nm as the wavelength for UV detection. The chromatographic separation of the analyte was achieved at  $45^{\circ}\text{C}$  using  $C_{18}$  column (Phenomenex,  $150 \times 4.6$  mm ID, 5  $\mu\text{m}$ ) fitted with a universal guard column (Thermoscientific,  $4 \times 4.6$  mm ID). The injection volume was 20  $\mu\text{L}$ .

### *Preparation of stock and working standard solutions :*

A primary standard stock solution of Atovaquone (400  $\mu\text{g/ml}$ ) was prepared in 1% v/v dimethylformamide in methanol. The stock solution was further diluted with mobile phase to obtain a set of working standard solutions in the range of 1.6–80  $\mu\text{g/ml}$ . The stock solution of Buparvaquone, as an IS was prepared at a concentration of 200  $\mu\text{g/ml}$  in methanol and further diluted with mobile phase to give a working concentration of 40  $\mu\text{g/ml}$ . These solutions were protected from light and stored at  $4^{\circ}\text{C}$  prior to use.

### *Preparation of calibration and quality control samples :*

237.5  $\mu\text{L}$  of blank rabbit plasma was spiked with 12.5  $\mu\text{L}$  of working standard solutions of Atovaquone to obtain a set of calibration standard solution with concentration of 80–4000 ng/ml. The quality control samples were prepared at 3 levels; low, medium and high, which were 250 ng/ml, 1500 ng/ml and 3500 ng/ml, respectively.

### *Sample extraction procedure :*

Liquid-liquid extraction was used as a sample preparation method in this study. 250  $\mu\text{L}$  of plasma was accurately measured into a glass tube followed by the addition of 12.5  $\mu\text{L}$  of IS working solution (40  $\mu\text{g/ml}$ ) before being vortexed for 10 s. 50  $\mu\text{L}$  of 5 mM ammonium acetate was added and the mixture was vortexed for 30 s. After that, 2 ml of ethyl acetate was added to the mixture and vortexed for 3 min followed by centrifugation at 3500 rpm for 2 min. The organic supernatant was transferred into a reaction vial and evaporated to dryness at  $40^{\circ}\text{C}$  under nitrogen gas. The residue was reconstituted with 100  $\mu\text{L}$  of mobile phase and subjected to centrifugation at 12000 rpm for 10 min to sediment any microparticles.

Then, 50  $\mu\text{L}$  of the supernatant was carefully transferred into an autosampler vial prior to the injection of 20  $\mu\text{L}$  aliquot into the HPLC for analysis.

*Bioanalytical method validation :*

The selectivity was carried out using six different sources of New Zealand rabbit plasma to define the ability of the analytical method to discriminate and quantify the analyte from other components. All plasma samples were processed and injected into HPLC to assess any interference at the retention times of the analyte and IS.

The linearity was evaluated using five calibration curves prepared on five consecutive days. The curve covers a concentration range of 80 to 4000 ng/ml of Atovaquone. All samples were prepared in five replicates and the calibration curves were plotted as a ratio of peak area of Atovaquone to peak area of IS against the nominal concentration using linear regression analysis. The LOD determination was carried out by injecting samples at subsequent low concentrations of Atovaquone. Accuracy and precision were determined for all calibration points and QC samples using 5 replicates per concentration excluding the blank samples. QC samples at low, medium and high Atovaquone concentration of 250, 1500, and 3500 ng/ml were used to determine the intra- and inter-day accuracy and precision. The extraction recovery was determined by comparing Atovaquone-to-IS peak area ratio of three plasma QC samples (250, 1500, and 3500 ng/ml) with Atovaquone-to-IS peak area ratio from unextracted aqueous standards with the same concentration of Atovaquone. Recovery from five replicates for each QC level was analyzed.

*Stability studies :*

The stability studies were conducted using three replicates of QC samples with concentration of 250, 1500 and 3500 ng/ml, respectively. Samples were subjected to situations likely to be encountered during the plasma samples handling and analysis. All QC samples were prepared using a freshly made stock solution of Atovaquone and compared with the mean of back-calculated values of all three concentrations from freshly prepared samples.

*Application to pharmacokinetic study in rabbits :*

A 2-phase, 2-sequence crossover design study with one month wash out period between the phases was con-

ducted and approved by the Animal Ethics Committee of Universiti Sains Malaysia (Approval No. USM/Animal Ethics Approval/2011/(68)(327); Date : 17th October 2011). The study involved five healthy male New Zealand rabbits (2.0–3.3 kg), which were housed in the animal care facility at  $25 \pm 2$  °C and relative humidity of 50–60% in a 12 h light/12 h dark cycle. The animals had free access to rabbit pellet and water throughout the experiment.

The rabbits were randomly divided into two groups containing 3 and 2 rabbits per group. They were fasted for 12 h prior to procedure with access of water *ad libitum*. Each rabbit in the first group received aqueous dispersion of Atovaquone-SLNs while the other group received aqueous dispersion of pure Atovaquone where both dispersions contain 3.57 mg/kg body weight of Atovaquone. Both formulations were administered orally to the rabbits and food was permitted 4 h after the dose administration. 0.5 ml of blood was collected using 1 ml syringe fitted with 27G  $\times$   $\frac{1}{2}$ " needle (0.40 mm  $\times$  13 mm) from the marginal ear vein at predetermined intervals of 0 (pre-dose), 0.5, 1, 1.5, 2, 3, 4, 6, 8, 24, and 48 h post administration. Samples were transferred into heparinised test tubes and centrifuged at 4000 rpm for 15 min. The plasma was collected and transferred into new centrifuge tubes without anticoagulant and stored at  $-20$  °C until further analysis using HPLC. After the wash-out period, the groups were switched and all procedures were repeated accordingly. The  $\text{AUC}_{0-t}$  was calculated using trapezoidal rule from the obtained data of both preparations.

*Statistical analysis :*

Statistical analysis was done on the  $\text{AUC}_{0-t}$  by applying t-test for independent group, using SPSS software (version 16.0, Chicago, USA), assuming equal variances within each group. The differences were statistically significant when  $p < 0.05$ . All values were expressed as their mean  $\pm$  SD.

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