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Effect of Ascorbic Acid on Pharmacokinetic Profile of Artemether in Male Rabbits (*Oryctolagus cuniculus)*

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ABSTRACT: Malaria is one of the major public health challenges in Nigeria. Treatment failure as a result of drug interaction is a major problem. A study of the effect of ascorbic acid on the antiplasmodial activity of artemether has been reported. This study was carried out to determine the effect of ascorbic acid on the pharmacokinetic profile of artemether. Fourteen male rabbits weighing between 2.0 – 2.5 kg were used in this study. Four of the rabbits were used for determination of the efficiency, recovery studies and calibration curve while ten rabbits were randomly selected into Group A and B consisting of five rabbits each were used for the pharmacokinetic studies. Group A was administered a single dose of 10 mg/kg artemether while Group B was co-administered a single dose of 10 mg/kg artemeter and10 mg/kg ascorbic acid intramuscularly. The dihydroartemisinin in the plasma was analysed using High Performance Liquid Chromatography. The results showed that ascorbic acid affect the pharmacokinetic parameters of artemether. The time to reach maximum concentration (T_{max}) increase from 30.00 min to 42.00 min while the maximum concentration (C_{max}) and Area-under the Concentration Curve (AUC) decrease from 1461.9 (ng/mL) and 6566.7 (ng hr/mL) to 1331.1 (ng/mL) and 6105.8 (ng hr/mL), respectively. Ascorbic acid altered the pharmacokinetic profile of artemether. In clinical practice patients should be adviced not to co-administer ascorbic acid with artemether.

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Malaria is reported in sub-saharan Africa, Asia and Latin America. In Nigeria it is among the five major public health challenges. According to a recent World Malaria Report, approximately about 619 000 malaria deaths occurred globally in 2021 compared to 625 000 in 2020 while the number of malaria deaths stood at 568 000 in 2019. The global incidence of malaria cases was 247 million in 2021, compared to 245 million in 2020 and 232 million in 2019. Thus malaria cases remain on the increase (WHO, 2021[a]). In addition, according to the 2021 World Malaria Report, the highest number of global malaria cases and deaths was in Nigeria (WHO, 2021[b]). Artemether is a derivative of artemisinin which is an antimalarial drug commonly used as artemisinin-based combination therapies (ACTs) for the treatment of malaria. It is one of the essential components in the treatment of multi-drug resistant falciparum malaria when combined with other long-acting antimalarial drugs such as Lumefantrine (Green *et al.*, 2001, FMoH, 2005, WHO, 2015). Artemether undergoes biotransformation to dihydroartemisinin (DHA), a metabolite that possesses antimalarial activity (Qi-Gui *et al.*, 1997). In 2016, artemisinin was reported to bind to a large number of targets which include calcium-dependent ATP, glutathione S-esterase, Deoxyribonucleic acid (DNA) and nicotinamide adenine dinucleotide (NADH). The reduction of the endoperoxide bridge produces desoxyartemisinin which is devoid of antimalarial activity (Wang *et al.*, 2015). The co-administration of antimalarial and formulations containing some vitamins and minerals possessing

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antioxidant activity is a common practice in Nigeria (Ocloo *et al.*, 2011, Amany *et al.*, 2015). Studies to assess the interactions between artemisinin derivatives and antioxidant vitamins like Vitamin A, C and E have been reported(Oreagba and Ashorobi, 2007, Awodele *et al.*, 2007, Ganiyu *et al.*, 2012). The assessment was based on the effects of antioxidants on the anti-plasmodia activities of artemisinin and its derivatives. Findings from their studies indicate that there was a decreased antimalarial activity when the artemisinin derivatives were co-administered with antioxidants. This decreased antimalarial activity was attributed to the free radicals (carbon-centered free radicals) generated as a result of haem-mediated decomposition of the endoperoxide bridge responsible for the death of the parasites which were scavenged by the co-administered antioxidants (Meshnick *et al.*,1996, Khrishna *et al.*, 2004). From available literature, there is paucity of reports on the effect of co-administration of ascorbic acid on the plasma concentration of artemether. Therefore, theobjective of this study is to evaluate the effect of ascorbic acid on the pharmacokinetic profiles of artemether in Male Rabbits (*Oryctolagus cuniculus)*

MATERIALS AND METHODS

Chemicals: Dihydroartemisinin (DHA) reference standard, acetonitrile (HPLC grade), sodium nitrite, acetic acid and ethyl acetate were purchased from Sigma-Aldrich. Anhydrous sodium sulfate (Merck) and sodium chloride (ABC Chemicals) all of analytical grade were also obtained. Other materials used include plasma tubes with sodium citrate solution, nitrogen gas (Vision air), deionised water, artemether injection 80 mg/mL (Arthec®) from Geneith Pharm. Limited (Manufacturing Date: 09-2020, Expiry Date: 09-2023, NAFDAC No: 04-9503, Batch No: 110200905), and ascorbic acid injection 500 mg/mL from Chupet Pharm. Co. Ltd (Manufacturing Date: 05-2020, Expiry Date: 04-2023, NAFDAC number A4-9139 and Batch No: 200526).

Apparatus: High Performance Liquid Chromatography system (HPLC) (Agilent Technologies 1200 series, Germany, with Chem station software Rev.B.04.03(16), Variable wavelength detector (VWD), Nylon membrane syringe filter with a diameter 25 mm and pores size 0.45 pm, analytical weighing balance, micro-pipettes and an evaporator were used in this study. Disposable latex hand gloves, razor blade, methylated spirit, cotton wool, polyethylene tubes, 1 mL syringe, 2 mL syringes, 23 g disposable needles and masking tape were sourced from Pharmacy premises in Benin City.

Methods: A total of fourteen (14) male rabbits weighing between 2.0 – 2.5 kg were used in this study. The rabbits were kept in the animal house of Pharmacology and Toxicology of the Faculty of Pharmacy, University of Benin, Benin City. They were fed on standard commercial rabbit pellets and allowed free access to water. They were acclimatized for two weeks before the commencement of the experiment and cared for in accordance with the Guideline for the care and use of laboratory animals (NRC, 2011). All the drugs and reagents used were not expired as at the time of the study. All the rabbits were tagged properly, fasted for 12 hours before treatment and retained in their respective cages during sampling procedure.

Ethical approval (EC/FP/021/09) for the use of laboratory animals for the study was obtained from Ethics Committee of the Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria.

Procedure for extraction of plasma sample: Dihydroartemisinin (DHA) extraction from plasma sample was carried out using the modified method developed by Stijn *et al* (Stijn *et al.*, 2008). Blood sample was aseptically withdrawn from the marginal ear vein of each rabbit and collected in plasma tubes containing sodium citrate solution. The tubes were centrifuged for 10 min at 2500 rpm. The plasma layer was aspirated and transferred into a sterile polypropylene tube. Immediately, 0.15 mL of 3 M sodium nitrite containing 1% acetic acid was added to the plasma (Keiser *et al.*, 2009). A volume of 0.5 mL of saturated sodium chloride solution was added and mixed and then 5 mL of 100% ethyl acetate was added. The sample was sonicated for 1 min and centrifuged at 3000 rpm for 5 min. The ethyl acetate phase was transferred to a clean glass tube. Anhydrous sodium sulphate (0.5 g) was added to the ethyl acetate layer. Thereafter, 4 mL of the ethyl acetate was concentrated to dryness under a stream of nitrogen at 40 ^{o}C in an evaporator and stored in the freezer at -20 ^{o}C until analysis.

Thereafter, the residue was dissolved in 100 µL acetonitrile / water (40:60 v/v) containing 0.15% formic acid. The re-dissolved residue was sonicated for 5 mins and centrifuged at 3500 rpm for 15 min and then filtered through nylon membrane syringe filter pore size 0.45 pm. The filtrate was transferred into an auto sampler vial. Then, the analysis was performed using high performance liquid chromatography (HPLC) with ultra-violet detector at 216 nm, using column, Zorbax Eclipse XBD CB RP 150 x 4.6mm, 5 µm at pressure of 50 bar and temperature of 30^{o}C. The injection volume and flow rate were 10 µL and 500

µL/min while acetonitrile/water 40:60 (v/v) and 0.15% formic acid was used as the mobile phase.

Development of calibration curve: Drug-free whole blood sample 6 mL was obtained from a rabbit. Thereafter, 1 mL was transferred to six labeled plasma tubes containing sodium citrate solution. Dihydroartemisin (DHA) reference standard 10 mg was dissolved in acetonitrile 10 mL in a volumetric flask to obtain 1000 µg/mL stock solution. The drug-free whole blood sample (1 mL) in sodium citrate solution tubes were spiked with the stock solutions of DHA to make a calibration curve in the range of 0 - 1000 ng/mL in sequence of 0, 100, 200, 400, 800 and 1000 ng/mL. Thereafter, the drug in the whole blood was extracted and analysed with HPLC. Reference standard (50 µg/mL of dihydroartemisin) was used to check the system suitability.

Determination of the efficiency of extraction of the drug in plasma: Three rabbits were used to determine the efficiency of extraction of dihydroartemisinin (DHA), an active metabolite of artemether using a method reported by Ocloo *et al.*, 2011.

The procedure was as follows:

Procedure for rabbit 1: Blood sample (2) mL was withdrawn from the marginal ear vein of the rabbit and transferred into sodium citrate tube without drug administration. Chromatogram was obtained for extract from the blood sample of the rabbit.

Procedure for rabbit 2: The rabbit was administered 10 mg/kg artemether alone intramuscularly into the cranial muscle of one of the hind limbs. Thereafter, 2 mL of blood sample was withdrawn from the marginal ear vein of the rabbit into a sodium citrate solution tube at 30 minutes after drug administration. Chromatogram was obtained for extract from the blood sample.

Procedure for rabbit 3: The procedure for rabbit 2 was repeated for rabbit 3. However, 10 mg/kg artemether and 10 mg/kg ascorbic acid were administered intramuscularly into the cranial muscle of one of the hind limb of rabbit 3. Thereafter, 2 mL blood sample was withdrawn from the marginal ear vein of the rabbit 3 into a sodium citrate solution tube at 30 minutes after drug administration. Chromatogram was obtained for extract from the blood sample. The chromatograms obtained for the extracts of blood samples withdrawn from the rabbits after drug administration were necessary to confirm that there was no potential interference with endogenous substances.

Recovery studies: This study was carried out for the purpose of assessing the recovery of dihydroartemisin (DHA) from blood sample of rabbit. Exactly 3 mL of blood sample from a rabbit was collected, and share in equal 1 mL to three sodium citrate solution tubes. Each of the tube was spiked with 20 µL of 50 µg/mL (1000 ng) of DHA reference standard solution. Recovery of DHA was carried out through extraction procedure. It was dissolved in 1 mL of the solvent phase and exactly 10 µL was injected into the HPLC column. The percentage recovering was determined by comparing the peak area obtained after extraction of the drug from the whole blood with that obtained after injecting the same concentration of the drug into the column of the HPLC.

*Determination of the effect of ascorbic acid on the pharmacokinetic parameters of artemether***:** Ten healthy rabbits were randomly selected into two groups (A and B) consisting of five (5) rabbits each. Rabbits in group A were administered a single dose of 10 mg/kg artemether intramuscularly, while rabbits in group B were co-administered a single dose of 10 mg/kg ascorbic acid and 10 mg/kg artemether, intramuscularly. Thereafter, blood sample (1 mL) was withdrawn from the marginal ear vein of each rabbit and transferred into sodium citrate tubes at 0, 0.25, 0.50, 1, 2, 4, 8 and 12 hours after drug administration for each groups. Extraction procedure for dihydroartemisinin (DHA) was carried out. The dried extract was stored at -20^{o}C. The DHA in the extract was analysed with HPLC. The results of pharmacokinetic parameters for group A and B which include maximum blood concentration (C_{max}), time to achieve maximum blood concentration (T_{max}), the elimination rate constant (K_{el}), elimination half-life ($t_{1/2}$), Area-under-the-concentration (AUC) curve, clearance rate (Cl) and volume of distribution (V_d) were obtained and statistically compared. The Pharmacokinetics analysis was done as described by Ocloo *et al.*, 2011.

Data Analysis: The results were expressed as mean ± standard deviation. Significance of the difference between the results for the pharmacokinetic parameters for control (Group A) and test group (Group B) were evaluated using the student *t*-test. P-value less than 0.05 ($p<0.05$) was taken as the significance level.

RESULTS AND DISCUSSION

The results for determination of the efficiency, recovery studies, calibration curve and effect of ascorbic acid on the pharmacokinetic parameters of artemether are presented.

Chromatogram for Dihydroartemisinin Reference Standard and Blood Extract of Rabbit without Drug Administration: The chromatograms obtained for 10,000 ng/mL dihydroartemisinin and blood extract before administration of drug(s) are shown in Figure 1A and 1B respectively. The complex interactions between the body and drug formulation are interpreted using pharmacokinetics. The maximum plasma concentration of a drug (C_{max}), the time to reach maximum plasma concentration (T_{max}) and the area under the concentration curve (AUC) are among the important pharmacokinetic parameters of antimalarial used in the treatment of malaria which is responsible for high mortality rate in sub-sahara Africa in which Nigeria accounts for a high percentage of deaths (54%) (WHO, 2021[a]). In order to effectively prevent the incidence of death caused by malaria, a good antimalarial drug must clear all the malaria parasites from the blood and liver quickly and rapidly relieve symptoms of the disease. This can only be achieved if the T_{max} is short enough; while the C_{max} and the AUC are adequate. The other pharmacokinetic parameters are also important in the determination of the right dose, duration for drug administration and drug toxicity (Qi-Gui *et al.*, 1997). A modified method of Stijn *et al.*, using High performance liquid chromatography – Ultra violet (HPLC-UV) technique was used in this study. A single dose of 10 mg/kg of all drugs were administered intramuscularly based on previously reported studies on pharmacokinetic studies on artemisinin derivatives (Qi-Gui *et al.*, 1997, Keiser *et al.*, 2009). The results from this study obtained from report on the chromatogram of blood extract without drug administration and 10,000 ng/mL of the reference standard dihydroartemisinin, revealed that the endogenous substances do not interfere with dihydroartemisinin which is the active metabolites (Figure 1A and 1B). The chromatogram obtained for 10,000 ng/mL of the reference standard dihydroartemisinin, shown a single peak at retention time 2.3 min (Figure 1A).

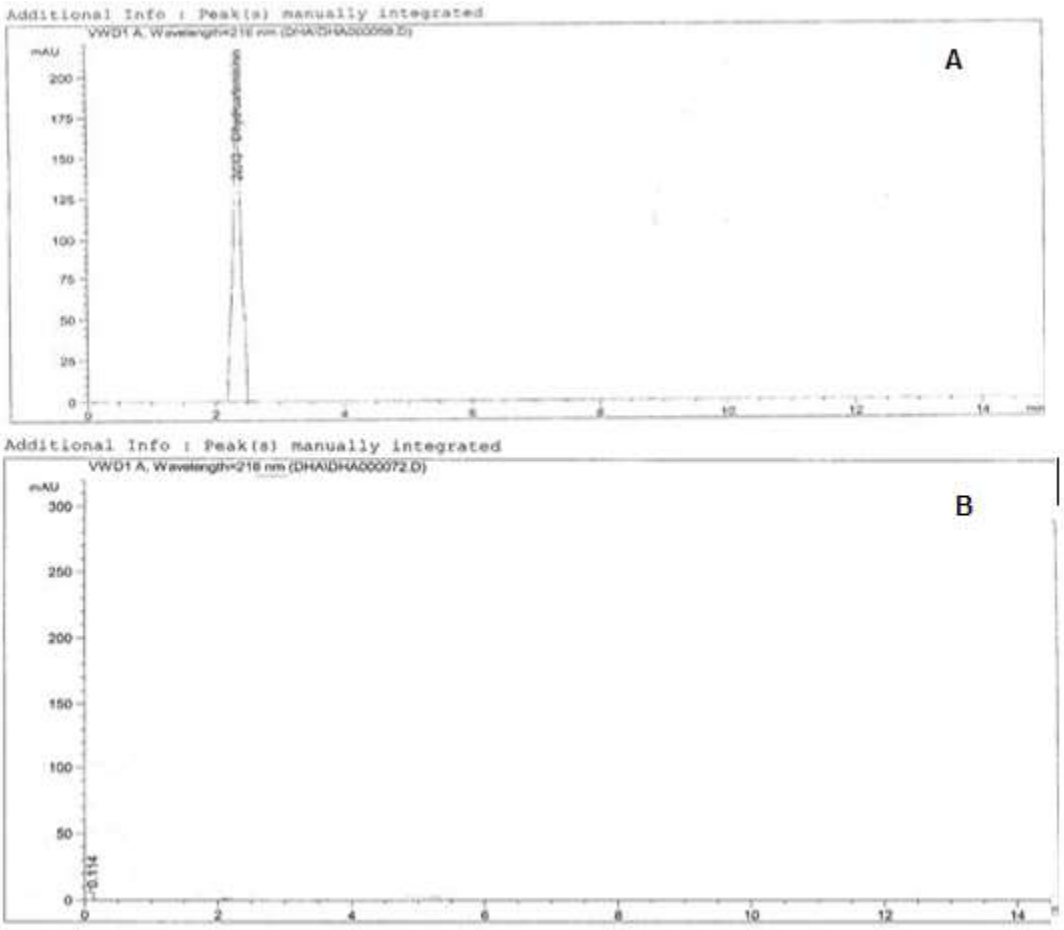

Fig 1: Chromatogram for Dihydroartemisinin reference sample (A); Chromatogram from blood extract without drug administration (B).

Effect of Ascorbic Acid on Pharmacokinetic Parameters of Artemether: The chromatograms obtained after 30 min for artemether alone and when artemether and ascorbic acid were co- administered

are shown in Figure 2A and 2B respectively. The Chromatogram obtained for rabbit administered single dose of artemether alone showed three peaks indicating three metabolites while that for rabbit concurrently administered artemether and ascorbic acid showed seven peaks indicating seven metabolites (Figure 2A and 2B). Artemether was reported to undergo bio transformation to the active metabolite dihydroartemisinin as well as the inactive metabolites desoxyartemisinin and 9,10-dihydroartemisinin when it was administered alone. Every drug has its own pharmacokinetic profile. However, the pharmacokinetic parameters can be affected by other compounds which could be from drugs, food, herbs, etc. (Zhao *et al.*, 1988).

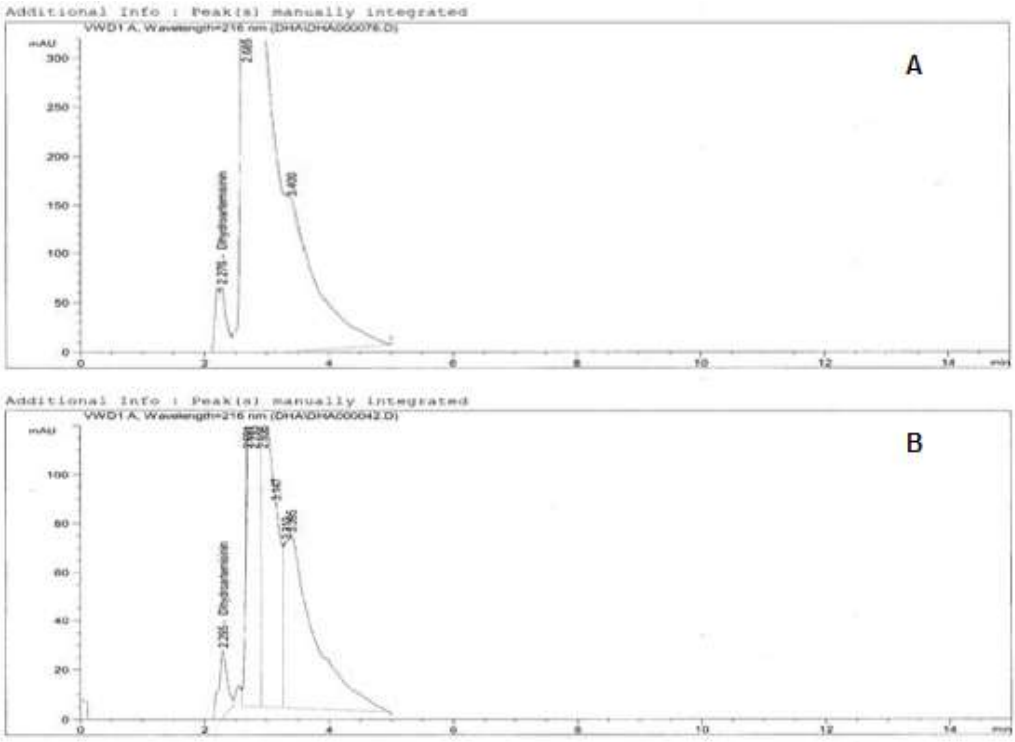

Fig 2: Chromatogram 30 minutes after artemether was administered to rabbit (A); Chromatogram 30 minutes after artemether was co-administered with ascorbic acid to rabbit (B)

*Calibration Curve for Determination of Plasma Concentration of Dihydroartemisinin***:** The results from the calibration graph obtained by spiking drug free whole blood with stock solution of Dihydroartemisinin in the concentration range of 0 – 1000 ng/mL gave a correlation coefficient of 0.983 as shown in Figure 3. The percentage recovery was 70.23 ± 2.89%.

Plasma Concentration versus Time Graph for Artemether and Artemether/Ascorbic Acid: The plasma concentration versus time graph for artemether alone and when artemether was co-administered with ascorbic acid is shown in Figure 4.

Effect of Ascorbic Acid on the Pharmacokinetic Parameters of Artemether: The results of pharmacokinetic parameters for the group administered artemether alone and co-administered artemether and ascorbic acid are as shown in Table 1.

This study on the concurrent administration of artemether and ascorbic acid showed that the usage of ascorbic acid altered all the pharmacokinetic parameters of dihydroartemisinin which is their active metabolite (Table 1). The parameters include maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), area under the curve (AUC), elimination rate constant (K_{el}), half-life ($t_{1/2}$), clearance (CL) and volume of distribution (Vd). The results from this study showed that the C_{max} for Artemether was 1427.6 ng/ while T_{max} was 30.00 min. In a report on comparison of the main pharmacokinetic parameters of Dihydroartemisinin, Artemether in rats after single intramuscular doses, the C_{max} for artemeter was 692 ng/mL while the T_{max} was 28.8 min (Qi-Gui *et al.*, 1997). The high C_{max} in this study, may be due to the to the addition of 3 M sodium nitrite containing 1% acetic acid which prevent the degradation of dihydroartemisinin in the blood sample collected for analysis (Keiser *et al.*, 2009).

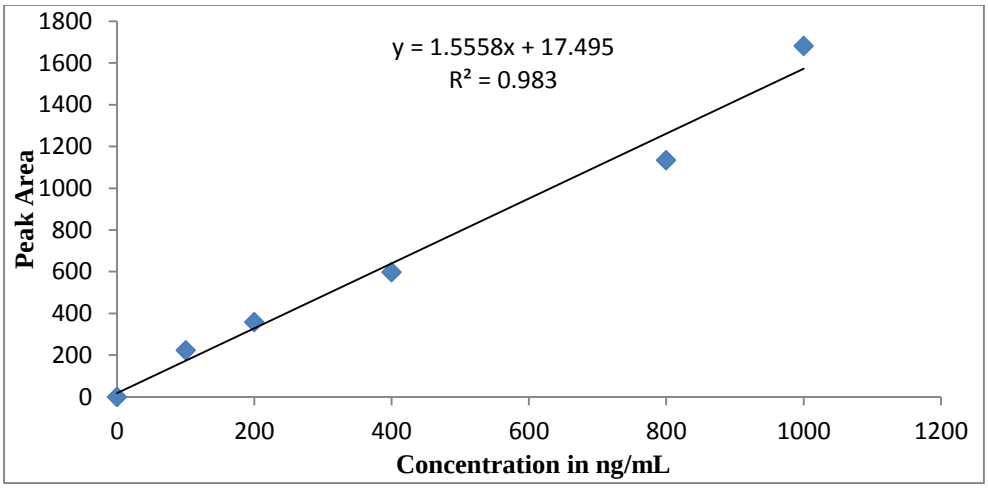

Fig 3: Calibration graph for dihydroartemisinin in plasma of rabbit

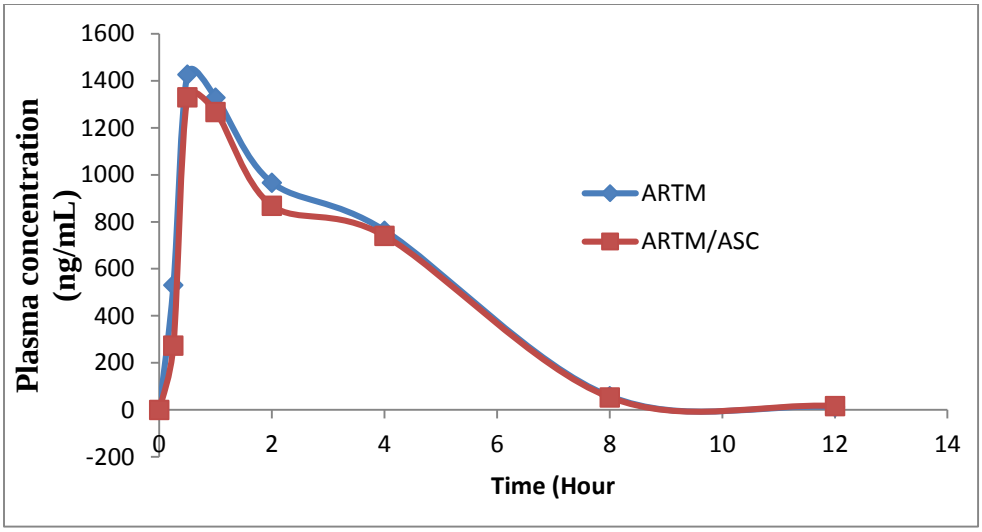

Fig 4: Plasma concentration versus time graphs for Artemether alone and when artemether and ascorbic acid were co-administered. *ARTM= Artemether alone; ARTM/ASC= Artemether and Ascorbic acid*

Table 1: Results of pharmacokinetic profiles of artemether alone and co-administered artemether and ascorbic acid in Rabbits

Serial number	Pharmacokinetics Parameters	Artemether alone (Group A)	Artemether and Ascorbic Acid (Group B)
1	C_{max} (ng/mL)	1427.6 ± 51.6	1331.1 ± 108.4*
2	T_{max} (min)	30.00	42.00
3	AUC (ng hr/mL)	6566.7 ± 47.83	6105.8 ± 26.37**
4	K_{el} ($hour^{-1}$)	0.08245 ± 0.02	0.0820 ± 0.01***
5	$t_{1/2}$ (hour)	8.4054 ± 0.15	8.4526 ± 0.12***
6	Cl ($L\ hour^{-1}Kg^{-1}$)	0.0152 ± 0.0013	0.0163 ± 0.0005**
7	V_d (LKg^{-1})	0.001256 ± 0.00016	0.0013440 ± 0.00012***

*Data are expressed as mean ± SD. Treatment group vs control group, significance *p<0.05, **p<0.01, ***p<0.001 (n = 5).*

Another report showed that the elimination half-life for rats was 16 hour after intramuscular injection of 80 mg Kg^{-1} artemether as against 8.40 hour in this study (Zhao *et al.*, 1988). The differences in formulations used in these studies may be responsible for the variations in the results. The Area under the Concentration time curve (AUC) after eight hours for the group administered artemether alone was 6566.7 (ng hour/mL) while the group co-administered artemether with ascorbic acid was 6105.8 (ng hour/mL) (Table 1). There was variation in the time to reach maximum concentration among the groups. The T_{max} was 30 mins for group A (artemether alone) while it was 42 min for the group B (co-administered artemether and ascorbic acid). The Co-administration of artemether and ascorbic acid increased the time to

reach maximum concentration (T_{max}) and elimination half-life ($t_{1/2}$) of artemether. The bioavailability of dihydroartemisinin was reduced in group B co-administered ascorbic acid (Table 1). The result obtained from comparative statistical analysis revealed that with the exception of the maximum concentration (C_{max}), all the parameters evaluated were not significantly different at $p < 0.05$. There was significant decrease in the maximum concentration (C_{max}) in the group administered artemether and ascorbic acid.

*Conclusion***:** The results of the study revealed that ascorbic acid altered all the pharmacokinetic parameters of artemether when co-administered. The difference in the maximum concentration (C_{max}) in the group administered artemether alone and those co-administered ascorbic acid was statistically significant. In clinical practice, patients should be advice not to administer ascorbic acid and artemether concurrently.

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