Assessment of Clinical Pharmacokinetic Drug-Drug Interaction of Antimalarial Drugs α/β -Arteether and Sulfadoxine-Pyrimethamine

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ABSTRACT Antimalarial drug combination therapy is now being widely used for the treatment of uncomplicated malaria. The objective of the present study was to investigate the effects of coadministration of intramuscular α/β -arteether (α/β -AE) and oral sulfadoxine-pyrimethamine (SP) on the pharmacokinetic properties of each drug as a drug-drug interaction study to support the development of a fixed-dose combination therapy. A single-dose, open-label, crossover clinical trial was conducted in healthy adult Indian male volunteers (18 to 45 years, $n = 13$) who received a single dose of AE or SP or a combination dose of AE and SP. Blood samples were collected up to 21 days postadministration, and concentrations of α -AE, β -AE, sulfadoxine, and pyrimethamine were determined by using a validated liquid chromatography-tandem mass spectrometry method. Pharmacokinetic parameters were calculated and statistically analyzed to calculate the geometric mean ratio and confidence interval. Following single-dose coadministration of intramuscular AE and oral SP, the pharmacokinetic properties of α/β -AE were not significantly affected, and α/β -AE had no significant effect on the pharmacokinetic properties of SP in these selected groups of healthy volunteers. However, more investigations are needed to explore this further. (This study has been registered in the clinical trial registry of India under approval no. CTRI/2011/11/002155.)

KEYWORDS pharmacokinetics, malaria, combination therapy, α/β -arteether, sulfadoxine, pyrimethamine

Malaria is one of the dreadful infectious diseases in the world, affecting 95 countries, and approximately 214 million malaria cases were reported in 2015 across the globe (1). There are five species of *Plasmodium* that infect humans, namely *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae*, and *P. knowlesi*. *P. falciparum* causes potentially severe and life-threatening malaria, characterized by cerebral malaria, pulmonary edema, acute renal failure, and anemia (2). As older monotherapy-based regimens have become less effective due to resistance development, a consensus has emerged for the use of combination therapy for malaria. Resistance to most of the antimalarial monotherapies is well documented worldwide (3, 4).

The World Health Organization (WHO) currently recommends the use of combination drug therapy as the first-line treatment (especially artemisinin-based combination therapy [ACT]) for treating all cases of uncomplicated malaria infection (5, 6). The use of a combination antimalarial with different mechanisms of action can ensure very high cure rates and prevent the onset of drug resistance or delay its emergence (1). The

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commonly used ACT treatments are artemether-lumefantrine, artesunate-amodiaquine, dihydroartemisinin-piperaquine, artesunate-mefloquine, and artesunate plus sulfadoxine-pyrimethamine (SP) (1, 7–9). Artemisinin derivatives rapidly eliminate the parasites by up to 4 logs in 48 h of the asexual cycle and have a high rate of parasite clearance (10–13). To avoid recrudescence, a therapeutic concentration should be maintained for three life cycles by multiple-dose administration (12, 14). For this, a long-acting malarial drug and extended-release antimalarials are required.

α/β -Arteether (α/β -AE) is an ethyl ether derivative of dihydroartemisinin developed by CSIR—Central Drug Research Institute (CSIR-CDRI), India. It is a mixture of α - and β -diastereomers at a ratio of 30:70, respectively. It is clinically used for uncomplicated falciparum malaria with a high cure rate. α/β -Arteether interacts with heme in the parasite by virtue of its endoperoxide bridge. Iron-mediated cleavage of the bridge releases a highly reactive free-radical species that binds to membrane proteins, leading to lipid peroxidation, damage to endoplasmic reticulum, and inhibition of protein synthesis, which results in lysis of the malarial parasite (15). It has an elimination half-life ($t_{1/2}$) of ~ 24 h and is more stable than the other artemisinin compounds (12, 14, 16). Current efforts have been directed to tackle the major concern of multidrug resistance. Because of the high relapse rates with short-course AE monotherapy, AE is combined with long-acting antimalarial agents, such as SP, for the treatment of uncomplicated falciparum malaria. Sulfadoxine and pyrimethamine are slow-acting blood schizontocides and are more effective in action against *P. falciparum* than *P. vivax*. Sulfadoxine and pyrimethamine inhibit dihydrofolate reductase of plasmodia, which is essential in the biosynthesis of tetrahydrofolate. Sulfadoxine and pyrimethamine have long half-lives of ~ 169 h and ~ 111 h, respectively (10, 17). α/β -Arteether is a substrate of CYP3A4/5, whereas sulfadoxine-pyrimethamine does not interact with CYP3A4/5 (18, 19). The combination of α/β -arteether and sulfadoxine-pyrimethamine is expected to be a safe treatment with less drug-drug interaction. However, clinical pharmacokinetics of the drug-drug interaction of AE in combination with SP has not been reported.

The synergistic combination of AE and SP for the treatment of severe/multidrug-resistant cerebral malaria has also been reported (R. Tripathi, S. Puri, J. Srivastava, S. Singh, O. Asthana, and A. Dwivedi, U.S. patent application 11/022,929). The drugs given in a combination may have an undesirable pharmacokinetic interaction. The clinical pharmacokinetics, safety, and efficacy of AE (12, 20–22) and of SP (10, 17, 19, 23, 24) given alone have been reported in healthy volunteers. However, the pharmacokinetics of the drug-drug interaction of AE in combination with SP has not been reported. The objective of this study was to evaluate the pharmacokinetic interaction between AE and SP after the administration of intramuscular (i.m.) AE alone and in combination with oral SP in healthy adult volunteers. The rationale behind this combination was to mingle the benefits of AE (fast onset of action) with those of SP (long duration of action and high cure rate) and assess its interaction potential for its safe use.

RESULTS

Bioanalytical assay. The described liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was fully validated and proved to be sensitive, selective, and accurate for quantification of AE, sulfadoxine, and pyrimethamine in human plasma samples. No peaks interfering with quantification were observed throughout the validation process. The intraday and interday accuracy and precision values met the acceptance criteria according to U.S. Food and Drug Administration guidelines (Table 1). The analytes and internal standard (IS) were stable in an autosampler at 4°C for 24 h, at room temperature for 24 h, after three freeze-thaw cycles, and during storage at -80°C for 60 days (data not shown). The assay method exhibited a lack of significant ion suppression/enhancement when assessed for matrix effect.

Baseline characteristics. The baseline characteristics of the study population are given in Table 2. A total of 15 volunteers were assessed according to inclusion/

TABLE 1 Assessment of accuracy and precision of the LC-MS/MS method in human plasma

Drug	Concn ^a	Accuracy (% bias)		Precision (% RSD) ^b		Recovery (%) (mean $\pm$ SD)
		Interday	Intraday	Interday	Intraday	
α -Arteether	0.78	3.56	12.29	3.56	3.99	82.56 $\pm$ 3.34
	3.13	6.78	8.45	5.90	2.11	84.16 $\pm$ 4.78
	50	-3.09	3.23	0.89	5.80	86.45 $\pm$ 2.45
	200	-5.56	-4.45	7.76	5.18	83.56 $\pm$ 3.34
β -Arteether	0.78	-1.23	-5.34	9.73	2.63	78.19 $\pm$ 4.23
	3.12	1.80	1.55	8.24	2.34	81.23 $\pm$ 2.14
	50	-1.34	-2.67	11.17	8.29	85.59 $\pm$ 3.89
	200	-0.90	-2.67	3.83	5.16	82.79 $\pm$ 3.46
Pyrimethamine	3.12	6.61	8.47	7.32	3.68	81.23 $\pm$ 4.87
	12.5	-3.45	-1.98	9.30	4.78	76.45 $\pm$ 4.92
	200	6.39	4.41	7.63	5.14	83.45 $\pm$ 3.45
	800	3.78	-2.67	5.69	-2.30	81.90 $\pm$ 5.30
Sulfadoxine	3.12	4.21	5.47	8.23	10.68	72.23 $\pm$ 3.56
	5.0	3.19	2.45	5.15	12.14	74.56 $\pm$ 4.56
	70	-3.45	-2.41	9.40	3.56	75.32 $\pm$ 5.67
	140	-5.39	6.89	4.67	7.40	78.45 $\pm$ 4.56

^aConcentrations are given in ng/ml except for those for sulfadoxine, which are in μ g/ml.^bRSD, relative standard deviation.

exclusion criteria, 15 healthy Indian males were randomized, and 13 volunteers completed the study, while two volunteers were lost to follow-up.

Treatment tolerability and safety. There were no clinically relevant changes in laboratory parameters and vital signs. At the end of the treatment period, the overall tolerability assessment by the investigator was considered “good” in all volunteers. No adverse event was found during the study.

Pharmacokinetics of α/β -arteether. The mean plasma concentration-time profiles of α - and β -AE are shown in Fig. 1a and b, respectively. The absorption of AE from the i.m. injection site was rapid initially, with both isomers being detected from the first sampling point at 30 min onwards. Lag time was not observed in any of the cases. The β -isomer, which constituted 70% of the i.m. dose, showed high systemic exposure, with approximately double and 2- to 3-fold higher $t_{1/2}$ s and volume of distribution (V) than those of the α -isomer. The main pharmacokinetic parameters of α - and β -AE are shown

TABLE 2 Baseline characteristic features of the study population after receiving a dose of AE alone and in combination with SP

Characteristic ^a	Value ^b (mean $\pm$ SD) for subjects receiving:		
	AE	AE + SP	Range
Age (yr)	28.11 $\pm$ 6.63		19–42
Ht (cm)	162.27 $\pm$ 8.71		149–178
Wt (kg)	67.7 $\pm$ 7.4		60–85
Systolic blood pressure (mm Hg)	124.2 $\pm$ 6.9	122.5 $\pm$ 8.4	110–130
Diastolic blood pressure (mm Hg)	77.7 $\pm$ 4.2	73.4 $\pm$ 5.9	70–84
Pulse (beats/min)	76.8 $\pm$ 4.2	77.05 $\pm$ 3.6	70–82
Hemoglobin (g/liter)	13.16 $\pm$ 2.06	13.18 $\pm$ 2.05	10–15.5
Bilirubin (IU/dl)	0.95 $\pm$ 0.35	0.99 $\pm$ 0.33	0.65–1.3
SGPT (U/liter)	24.5 $\pm$ 10.8	22.7 $\pm$ 7.4	16.2–49.3
SGOT (U/liter)	30.8 $\pm$ 15.7	31.73 $\pm$ 16.37	17.2–36.4
Alkaline phosphatase (U/liter)	130.95 $\pm$ 23.99	134.76 $\pm$ 25.71	90–165
Total cholesterol (mg/dl)	134.12 $\pm$ 20.36	139.20 $\pm$ 17.13	90.6–170.8
Fasting glucose (mg/dl)	83.73 $\pm$ 7.97	81.01 $\pm$ 8.82	72–96

^aSGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase.^bValues are means $\pm$ SD ($n = 13$) unless stated otherwise. Age, height, and weight are the same in both treatment columns.

FIG 1 Overlay of mean ($\pm$ SEM) plasma concentration-time profiles of α -arteether (a) and β -arteether (b) after a single dose of α/β -arteether given alone (AE) and in combination with sulfadoxine-pyrimethamine (AE + SP).

in Table 3. Pharmacokinetic parameters for α - and β -AE after administration of AE alone or in combination with SP were not significantly different ($P > 0.05$). There was no significant change in observed time to reach $C_{\max}$ ($T_{\max}$) (where $C_{\max}$ is the maximum observed plasma concentration at time $[T_{\max}]$ after administration) and $t_{1/2}$ of α - and β -isomers between treatment alone and treatment in combination. The main pharmacokinetic parameters for the two isomers were compared by the Wilcoxon signed-rank test. The pharmacokinetic parameters of AE showed interindividual variability in plasma levels. This finding may be caused by several factors, the most important of which is related to drug metabolism or physiological parameters. Geometric mean ratios (90% confidence interval [CI]) of the $C_{\max}$, last area under the concentration-time curve (AUC_{last}), and predicted AUC after the last dose from zero time to infinity ($AUC_{0-\infty}$) for α/β -arteether are shown in Table 4. The 90% CIs were within the standard bioequivalence boundaries of 80 to 125%. These results suggest that the drug exposure of α - and β -AE treatment in combination with SP was not significantly higher than that of AE alone, indicating that no dose adjustment of AE is required when coadministered.

Pharmacokinetics of sulfadoxine-pyrimethamine. The mean plasma concentration-time profiles of AE and SP are shown in Fig. 2a and b, respectively. Plasma concentrations of sulfadoxine and pyrimethamine were monitored up to 504 h. The main pharmacokinetic parameters of sulfadoxine and pyrimethamine are shown in Table 5. No significant difference was detected in the main pharmacokinetic parameters for sulfadoxine and pyrimethamine alone and in combination with AE, as compared by the

TABLE 3 Noncompartmental pharmacokinetic estimates of α/β -arteether in healthy male subjects after receiving a dose of AE alone and in combination with SP

Drug	PK variable	Median (range) ($n = 13$) for subjects receiving:		<i>P</i> value
		AE	AE + SP	
α -Arteether	$C_{\max}$ (ng/ml)	68.0 (57.1–90.0)	76.0 (49.4–94.0)	0.45
	$T_{\max}$ (h)	4.0 (4.0–6.0)	4.0 (2.0–6.0)	0.23
	$AUC_{0-\infty}$ (h · ng/ml)	1,584.56 (428.56–1,310.72)	1,648.16 (930.26–2,206.33)	0.94
	$t_{1/2}$ (h)	18.25 (13.31–22.2)	18.5 (12.23–27.44)	0.63
	V/F (liters)	10.62 (7.50–18.72)	10.40 (6.86–14.95)	0.63
	CL/F (liters/h)	0.40 (0.322–0.70)	0.38 (0.28–0.68)	0.94
	MRT (h)	23.43 (18.95–32.19)	27.39 (19.02–38.88)	0.68
β -Arteether	$C_{\max}$ (ng/ml)	52.6 (45.70–62.12)	51.8 (40.0–69.50)	0.73
	$T_{\max}$ (h)	8.0 (6.0–8.0)	6.0 (4.0–8.0)	0.12
	$AUC_{0-\infty}$ (h · ng/ml)	1,723.74 (1,175.91–2,275.42)	1,856.75 (1,435.53–2,295.73)	0.58
	$t_{1/2}$ (h)	28.23 (14.05–38.02)	22.82 (16.82–34.09)	0.37
	V/F (liters)	33.90 (18.84–41.07)	30.72 (23.11–39.33)	0.27
	CL/F (liters/h)	0.87 (0.63–1.25)	0.79 (0.64–1.04)	0.58
	MRT (h)	40.48 (25.91–52.76)	37.37 (28.26–54.44)	0.63

Data are medians (ranges), and *P* values were calculated using the paired Wilcoxon signed-rank test at 95% CI; a *P* of >0.05 indicates statistical nonsignificance. $C_{\max}$, maximum observed plasma concentration after administration; $T_{\max}$, observed time to reach $C_{\max}$; CL/F, elimination clearance; V/F, apparent volume of distribution; $t_{1/2}$, terminal elimination half-life; $AUC_{0-\infty}$, predicted area under the plasma concentration-time curve after the last dose from zero time to infinity; MRT, mean residence time.

TABLE 4 Bioequivalence analysis of α/β -arteether, sulfadoxine, and pyrimethamine after administration of α/β -arteether alone and in combination with SP

Parameter ^a	Estimated value ^b for:			
	α -Arteether (n = 13)	β -Arteether (n = 13)	Sulfadoxine (n = 13)	Pyrimethamine (n = 13)
$C_{\max}$ (ng/ml)	102 (93–110)	97 (89–105)	104 (98–111)	102 (96–108)
AUC_{last} (h · ng/ml)	101 (87–118)	108 (100–117)	115 (103–124)	96 (92–99)
$AUC_{0-\infty}$ (h · ng/ml)	101 (85–119)	105 (94–117)	112 (100–121)	95 (91–99)

^a $C_{\max}$, maximum observed plasma concentration; $AUC_{0-\infty}$, predicted area under the plasma concentration-time curve after the last dose from zero time to infinity.

^bData are presented as geometric mean ratios (90% CI).

Wilcoxon signed-rank test ($P \geq 0.05$). Geometric mean ratios (90% CI) of $C_{\max}$, AUC_{last} , and $AUC_{0-\infty}$ for AE and SP are shown in Table 4. The 90% CIs were within the standard bioequivalence boundaries of 80 to 125%. The absence of a substantial pharmacokinetic interaction in our study population supports the safety of AE plus SP in uncomplicated falciparum malaria treatment. The effect of the longer elimination half-life ($t_{1/2}$) of SP may possibly increase the posttreatment prophylactic effect of SP plus AE.

DISCUSSION

Artemisinin-based combination therapies (ACTs) are now recommended as the first-line treatment for falciparum malaria throughout the world, where malaria is endemic, and are now specifically recommended for the treatment of uncomplicated falciparum malaria (25). This is a pragmatic compromise, as ACTs may become the only available effective antimalarials and the evidence to date indicates that artemisinin derivatives are safe and effective (26).

The synergistic preclinical and clinical efficacy of the combination of intramuscular AE and oral SP was reported previously (27; Tripathi et al., U.S. patent application 11/022,929). This is, to the best of our knowledge, the first controlled clinical pharmacokinetic drug-drug interaction study of a fixed-dose combination of AE and SP in healthy male volunteers. The main purpose of this investigation was to evaluate the potential for pharmacokinetic drug-drug interactions between AE and SP. The route of administration and dose were selected based on a published clinical efficacy pilot study (27). The clinical trial was designed to investigate the influence of SP on the pharmacokinetics of AE and vice versa. The duration of the trial was chosen based on the pharmacokinetic profiles of the drugs, balanced with a desire to limit overall exposure in healthy participants. The findings of the present study demonstrated that after treatment with AE alone, the $AUC_{0-\infty}$ and $C_{\max}$ were comparable (Table 3) to those when α/β -AE was given in combination with SP; however, this finding was independent of the treatment sequence, and the point estimates and 90% CIs for the ratios of AUC and $C_{\max}$ (combined treatment versus alone) were entirely contained within the acceptance range of 80 to 125%. The results showed that the pharmacokinetics of α/β -AE were not influenced by the coadministration of SP and vice versa. Furthermore,

FIG 2 Overlay of mean ($\pm$ SEM) plasma concentration-time profiles of sulfadoxine ($\mu\text{g}/\text{mL}$) (a) and pyrimethamine (ng/mL) (b) after a single dose of sulfadoxine-pyrimethamine given alone (SP) and in combination with α/β -arteether (AE + SP).

TABLE 5 Noncompartmental pharmacokinetic estimates of sulfadoxine and pyrimethamine in healthy male subjects after receiving a dose of SP alone or α/β -arteether in combination with SP

Drug	PK variable	Median (range) ($n = 13$) for subjects receiving:		<i>P</i> value
		SP	AE + SP	
Sulfadoxine	$C_{\max}$ ($\mu\text{g/ml}$)	106.646 (84.40–117.00)	111.0 (98.67–130.0)	0.21
	$T_{\max}$ (h)	9.692 (4–12)	12 (4–12)	0.39
	$\text{AUC}_{0-\infty}$ ($\text{h} \cdot \text{ng/ml}$)	24,847.425 (15,795.41–44,474.16)	26,417.57 (17,828.68–44,385.00)	0.14
	$t_{1/2}$ (h)	202.341 (149.77–282.03)	173.58 (126.76–205.17)	0.45
	V/F (liters)	17.73 (11.86–29.07)	15.34 (12.21–19.05)	0.12
	CL/F (liters/h)	0.065 (0.03–0.1)	0.06 (0.03–0.08)	0.056
	MRT (h)	276.907 (172.67–385.59)	240.64 (169.84–297.62)	0.375
Pyrimethamine	$C_{\max}$ (ng/ml)	598 (490–722)	588 (497–761)	0.37
	$T_{\max}$ (h)	6.0 (4.0–8.0)	8.0 (4.0–8.0)	0.29
	$\text{AUC}_{0-\infty}$ ($\text{h} \cdot \text{ng/ml}$)	68,335.88 (56,634.76–114,618.46)	68,294.4 (54,386.45–118,666.3)	0.11
	$t_{1/2}$ (h)	117.78 (96.80–135.64)	112.67 (88.40–125.55)	0.339
	V/F (liters)	169.54 (114.19–227.44)	172.88 (96.17–199.73)	0.89
	CL/F (liters/h)	1.09 (0.75–134)	1.1 (0.64–1.37)	0.125
	MRT (h)	152.84 (118.39–177.33)	150.23 (104.34–180.86)	0.49

Data are presented as Median (range) and *P* values calculated using the paired Wilcoxon signed-rank test at at 95% CI; $p > 0.05$ statically non-significant.

there were no significant differences in the $t_{1/2}$ s of α/β -AE and of sulfadoxine and pyrimethamine ($P > 0.05$) when given alone or in combination. The pharmacokinetic parameters determined in these clinical trials were in accordance with data reported in the literature for AE (12, 20–22) and SP (10, 17, 19, 23, 24).

The lack of an effect of SP on the pharmacokinetic parameters of AE might be the result of the difference in metabolic pathways of AE and SP. While most drug interactions are thought to be mediated by the CYP system, this is unlikely to be the source for a potential drug-drug interaction. In this regard, AE is known to be only 5% converted into its active metabolite dihydroartemisinin by CYP3A4 (12, 28) and further conjugated by the UDP-glucuronosyltransferase system (29). Conversely, sulfadoxine is excreted primarily unchanged in urine, whereas pyrimethamine is metabolized in the liver by unknown enzymes before renal excretion (29). The lack of significant hepatic CYP enzymes for metabolism of AE and SP may be the possible mechanism for the nonsignificant drug-drug interaction of AE and SP.

Recent clinical trials showed that artesunate in combination with SP produced a 100% curative effect and no pharmacokinetic interactions were reported, but artesunate has poor stability and a lower 50% lethal dose (LD_{50}) than AE (30). The synergistic combination of AE and SP for the treatment of severe/multidrug-resistant cerebral malaria has also been reported (Tripathi et al., U.S. patent application 11/022,929). However, its pharmacokinetics have not been reported. Therefore, the combination of AE and SP may result in a better clinical curative effect without any pharmacokinetic drug-drug interaction.

This trial represents the first report of combination therapy of AE with SP in a clinical trial setting; it is not known to what extent these results may be generalizable to patients with malaria who may be receiving α/β -AE and may be taking additional concomitant malaria medication. However, no effect of SP on the plasma levels of α/β -AE was observed in these subsequent clinical trials. In the present trials, no clinically relevant changes in clinical laboratory values or cardiovascular or electrocardiogram (ECG) parameters were observed during the administration of α/β -AE and SP either alone or in combination. No serious adverse events were observed during the trial. Two participants withdrew from the trial due to personal reasons.

Conclusion. In conclusion, the administration of intramuscular α/β -AE and oral SP alone or in combination was well tolerated, and with single-dose coadministration of AE and SP, the pharmacokinetic properties of α/β -arteether were not significantly affected. In addition, α/β -AE had no significant effect on the pharmacokinetic properties of SP in these selected groups of healthy volunteers. In the absence of any

Study	Period-I	Period -II	Period -III
Healthy volunteer screened	Single dose of α/β -arteether (150mg, i.m.) Blood sampling up to 72 hrs (3 days) Followed by Washout period 3 Weeks	Combination of α/β -arteether (150mg, i.m.) + Sulfadoxine/pyrimethamine (3 tablet containing, 500mg Sulfadoxine and 25 mg pyrimethamine each tablet) Blood sampling up to 504 hrs (7 days) Followed by Washout period 8 weeks	Single dose of Sulfadoxine/pyrimethamine (3 tablet containing, 500mg Sulfadoxine and 25 mg pyrimethamine each tablet) Blood sampling up to 504 hrs (7 days)

FIG 3 Study design to evaluate the effect of sulfadoxine-pyrimethamine on the pharmacokinetics of α/β -arteether.

significant drug-drug interaction, this study supports the new combination therapy of AE and SP in the treatment of malaria.

MATERIALS AND METHODS

Drug formulation and chemicals. The drugs used in the study, Laridox (IPCA Laboratories Ltd., Mumbai, India), containing 500 and 25 mg per tablet of sulfadoxine and pyrimethamine (SP), respectively, and Rapither AB for α/β -arteether (AE) (IPCA Laboratories Ltd., Mumbai, India), containing 75 mg/ml of α - and β -arteether at a 30:70 ratio in arachis oil, were bought from the Local Pharmacy Stores, Lucknow, India. Pure standards of α -arteether and β -arteether (≥ 98.0) were generous gifts from the Medicinal Chemistry Division, CSIR-CDRI, and IPCA Laboratories Ltd., Mumbai, India, respectively. Sulfadoxine ($\geq 95.0\%$), pyrimethamine ($\geq 98.8\%$), artemisinin ($\geq 98.0\%$), and Sigmacote were purchased from Sigma-Aldrich, St. Louis, MO, USA. Intraperitoneal heparin sodium injection (Beparine, 5,000 IU/ml; Biological E Limited, Hyderabad, India) was obtained by local purchase. All other chemicals, reagents, and solvents used for the assay were of analytical grade or high-performance liquid chromatography (HPLC) grade.

Ethical approval. This clinical study was conducted in accordance with the principles established by the World Health Assembly on Ethics in Human Experimentation, the revised version of the Helsinki Declaration, and good clinical practices. The protocol was approved by the Institutional Ethics Committee (approval no. XLIX ECM A-/P6). This study was registered in the clinical trial registry of India under approval no. CTRI/2011/11/002155. Each volunteer was informed of the objectives, nature, and possible risks of the trial. Written informed consent was obtained from every volunteer participating in the study. The volunteers were informed that they were free to withdraw consent at any time.

Pharmacokinetics study design. The pharmacokinetics study design is shown in Fig. 3. Healthy male volunteers aged 18 to 45 years were enrolled in the study. This is the first pharmacokinetics study of AE-SP clinical drug-drug interaction, and, hence, for safety point of view male volunteers were included. However, subsequent clinical pharmacokinetics/efficacy studies will include female subjects. Written informed consent was obtained from the participants before the commencement of the study, and the participants were judged to be healthy based on physical examination, medical history, and standard laboratory tests. Subjects were excluded from the study if they were smokers, were taking any form of medication, had a recent history of chronic medication, had been treated with any known enzyme-inducing or -inhibiting drugs within 30 days of the commencement of study, or had consumed alcohol during the 7-day period before the study.

Volunteers ($n = 15$) were enrolled into three treatment periods, AE, AE plus SP, and SP, by crossover design. In period I, volunteers received a single intramuscular injection of 150 mg of AE (i.m. injection) and were sampled up to 72 h (3 days). In period II, after 3 weeks of a washout period, volunteers received a combination of AE and SP (i.m. injection of 150 mg of AE and three tablets containing 500 mg of sulfadoxine and 25 mg of pyrimethamine in each tablet) and were sampled up to 504 h (21 days). In period III, after 8 weeks of a washout period, the same volunteers received SP (three tablets containing 500 mg of sulfadoxine and 25 mg of pyrimethamine in each tablet) and were sampled up to 504 h (21 days). The washout period was calculated based on the half-life reported in the literature (12, 17), and the washout period was kept for more than 8 elimination half-lives of the drug, which was more than the 5-half-life period as recommended by FDA guidelines (31). All the subjects were monitored for any adverse reactions and to make sure that the medicine was not vomited. The sampling schedule was predose (0 h) and 0.5, 1, 2, 4, 6, 8, 12, 24, 48 (day 2), 72 (day 3), 168 (day 7), 336 (day 14), and 504 (day 21) h, and a disposable syringe in clean, heparinized test tubes was used. Approximately 1.5 ml of blood was transferred immediately to silanized heparin tubes (22 IU of heparin/ml of blood), while the remaining ~ 0.5 ml of blood was transferred into a separate glass tube and sent for biochemical analysis. The plasma samples were separated by centrifuging the blood samples at $2,000 \times g$ for 10 min at 4°C .

TABLE 6 Summary of MS/MS parameters^a

Sr. no.	Drug	RT	MRM transition <i>m/z</i> (Q1/Q3)	DP	CE	CXP
1	α -Arteether	4.46	330.0/276.0	35	13	10
2	β -Arteether	6.02	330.0/267.0	35	13	10
3	Sulfadoxine	3.03	311.0/156.0	95	24	10
4	Pyrimethamine	2.08	249.0/198.0	95	50	10
5	Artemisinin	3.21	300.0/209.0	35	16	10

^aThe parameters include the precursor ion, fragment ions, expected retention time (RT), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) for analytes.

and stored in sealed glass tubes at -80°C , until analysis. The volunteers were admitted and housed in the clinical facility for the first 24 h and discharged after the collection of blood samples at 24 h postdose. For the rest of the sampling time points, volunteers were asked to report to the Clinical Pharmacology Unit, Department of Medicine, CSM Medical University, Lucknow, India, per the protocol schedule. Vital checks and adverse drug reaction (ADR) monitoring were done during the day at a predetermined time schedule.

Treatment tolerability and safety assessment. ADRs were monitored throughout the study. Assessment of the frequency of ADRs, vital signs (blood pressure, pulse rate), physical examinations, clinical laboratory tests (hematology, coagulation, electrolytes, enzymes, substrates, urine pH, etc.), and an overall tolerability assessment by the investigator (good, satisfactory, not satisfactory, or bad) were performed on the basis of safety evaluation. Physical and clinical examinations of subjects were done at admission, prior to dosing, and at 2, 6, 12, and 24 h postdosing in each period and each ambulatory visit. Subjects underwent laboratory investigations at 0 h (prior to dosing), at 24 h postdosing, and at day 21.

LC-MS/MS conditions. An API QTrap 4000 mass spectrometer from Applied Biosystems (Foster City, CA) equipped with LC-10ADVP pumps, a SIL-10 ADVP autosampler, a CTO-10AS column oven, an SCL-10 AVP system controller, and a DGU-20A 3 degassing unit, all from Shimadzu Co. (Columbia, MD), was used. Analytes were separated on a Waters SymmetryShield C_{18} column (150 by 4.6 mm, 5 μm) preceded with a guard column at 30°C . The analytes were eluted at a flow rate of 0.7 ml/min, with the mobile phase consisting of 10 mM ammonium acetate buffer (pH 4.0) and methanol (8:92, vol/vol). Artemisinin was used as the internal standard (IS). The mass spectrometer was operated in the electrospray ionization (ESI) positive ion mode, and the analytes were quantified using the multiple-reaction monitoring (MRM) mode. Optimized precursor (ammonium adducts, $\text{M} + \text{NH}_4^{+}$ or $\text{M} + \text{H}^{+}$)-to-product ion transitions were used for quantification of analytes given in Table 6. The ion spray voltage was set at 5 kV, and the source temperature was set at 500°C . The MS was operated with unit resolution for both Q1 and Q3 detection. The Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA) was used for instrument control and data acquisition.

Stock, calibration standard, and QC sample preparation. Stock solutions (1 mg/ml) of α -arteether, β -arteether, sulfadoxine, pyrimethamine, and artemisinin were prepared in dimethyl sulfoxide (DMSO). The calibration standard stocks of all analytes were prepared by stepwise dilution of the stock solution in methanol. The method was linear in the ranges of 0.78 to 200 ng/ml for α -AE and β -AE, 3.12 to 800 ng/ml for pyrimethamine, and 3.12 to 140 $\mu\text{g}/\text{ml}$ for sulfadoxine ($r^2 > 0.999$). Quality control (QC) samples at four different concentrations, namely lower limit of quantification (LLOQ), low quality control (LQC), middle quality control (MQC), and high quality control (HQC), were prepared separately in five replicates (Table 1), independent of the calibration standards. Calibration curve (CC) and QC samples were prepared by spiking appropriate working stocks in blank plasma. All the stocks were found stable for complete validation.

Sample preparation. α - and β -arteether and pyrimethamine were extracted from human plasma samples by a double liquid-liquid extraction method. For the assay, 100 μl of plasma was spiked with 10 μl of IS (artemisinin, 5 $\mu\text{g}/\text{ml}$), followed by liquid-liquid extraction with *n*-hexane and ethyl acetate (50:50, two times at 3 ml). The separated organic layer was evaporated to dryness at reduced pressure, and the dry residue was reconstituted in 100 μl of methanol. The clear supernatants were transferred into autosampler vials for LC-MS/MS analysis.

Sulfadoxine was extracted from human plasma samples by a protein precipitation method. Briefly, 5 μl of the working stock solution was added to 45 μl of plasma and vortex mixed for 5 s, and plasma proteins were precipitated with 500 μl of acetonitrile containing IS (artemisinin). The resulting mixture was centrifuged at $13,000 \times g$ for 15 min at room temperature to precipitate plasma proteins. The clear supernatants were transferred into autosampler vials for sulfadoxine analysis.

Pharmacokinetic and statistical analysis. The pharmacokinetic parameters were evaluated by a noncompartmental analysis approach using Phoenix WinNonlin version 6.3 (Pharsight Corporation, California, USA). Total drug exposure up to the last measured concentration ($\text{AUC}_{0-\text{last}}$) was calculated using the linear trapezoidal method for ascending concentrations and the logarithmic trapezoidal method for descending concentrations. The $\text{AUC}_{0-\text{last}}$ was defined as the area under the concentration time curve from the time of dose until the last concentration above the lower limit of quantitation. The plasma drug concentration was estimated by log-linear regression of three to six concentrations in the terminal elimination phase. Maximum concentration (C_{max}) and time to reach maximum concentration (T_{max}) were taken directly from the observed data. Apparent volume of distribution (*V*) and clearance (CL) were computed individually according to standard procedures.

Pharmacokinetic estimates for a single dose of each drug administered alone and for that in combination with the other drug were compared using the paired Wilcoxon signed-rank test using Prism 5 (GraphPad Software, Inc., CA, USA). The intraindividual differences between the two treatment conditions (single drug treatment and combined treatment), at corresponding 90% confidence intervals (CIs), were estimated using Phoenix WinNonlin version 6.3. Formal ranges for bioequivalence were applied (90% CI, 80% to 125% for $AUC_{0-\infty}$ and C_{max}) (32).

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