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Optimization of a new artemisinin-based combination therapy of artesunate-piperaquine fixed-dose combination tablet with enhanced *in vivo* antimalarial effects

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ABSTRACT

This work aimed to develop a new fixed-dose combination tablet (FDCT) containing artesunate (ART) 50 mg and piperaquine 320 mg for enhanced malaria parasites treatment. Firstly, the physico-chemical properties of ART were improved effectively by forming solid dispersions (SDs) with β-cyclodextrin. Secondly, the FDCT was prepared by direct compression method using ART SDs and piperaquine phosphate (PQP). The formulation was experimented with full factorial design and optimized by Modde 5.0 software. FDCT were assessed for physical properties, drug content assay, dissolution, stability, acute toxicity, and *in vivo* antimalarial efficacy. ART and PQP assays were performed by using the validated high-performance liquid chromatography and UV spectrophotometry methods. Dissolution tests were conducted as per US Pharmacopoeia version 43 (USP43). The in vivo antimalarial activity of ART-PQP FDCT was assessed on mice infected with chloroquine-resistant *Plasmodium berghei*. The obtained FDCT met all desirable physical properties with an average weight of 670 mg, weight variation range of 690.5–702.9 mg per tablet, hardness range of 64-68N, friability of 0.5%–0.6%, and disintegration of 7–8 minutes. The contents of ART and PQP per tablet were 51.3–52.6 mg and 322.1–330.8 mg, respectively. The dissolution of ART and PQP within 30 minutes were more than 90% and 80%, respectively. Acute toxicity was identified with LD_{50} value in mice of 1,550.5 mg/kg. The cure rate of FDCT on mice of 93.3% was distinctly higher than that of single ART or PQP. The new ART-PQP FDCT showed enhanced antimalaria activity over monotherapies against chloroquine-resistant *Plasmodium berghei*.

INTRODUCTION

According to the World Health Organization (WHO), malaria is still a significant health burden in many tropical areas, recently causing thousands of deaths worldwide (World Health Organization,2020). Malaria infection is preventable and curable by using antimalarial medicines. To date, artemisinin and its derivatives have still been considered the most effective and safest antimalarial drugs. However, multidrug resistance has hindered the effect of monotherapy for malaria in many areas worldwide. Alternative regimens including artemisinin-based combination therapies (ACTs) are, therefore, urgently in need (World Health Organization,2020). ACTs are well known for their efficacy enhancement and low drug resistance effect. Several WHO-recommended ACTs were artemether + lumefantrine, artesunate (ART) + mefloquine, ART + amodiaquine, and ART + sulfadoxine-pyrimethamine (World Health Organization,2020). Although the first two combinations give the highest cure rates, they are too expensive for low-income countries (Baird,2005). Artemether

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+ lumefantrine also needed to be used with fat-enriched food to ensure adequate bioavailability and it required 6-dose regimes to ensure efficacy (Davis *et al*., 2005b). Mefloquine could cause side effects relating mainly to neurotoxicity (Martins *et al*., 2021). However, the efficacy of the two latter, ART + amodiaquine and ART + sulfadoxine-pyrimethamine, has dropped significantly in many areas during the past years. Dihydroartemisinin (DHA) + piperaquine (Artekin® and Duo-Cotecxin®) is used for the treatment of all forms of malaria, including those caused by multidrug-resistant strains, but its efficacy has been diminished in parts of Cambodia where there are rising levels of drug resistance (Lyu *et al*., 2021). ART + pyronaridine (Pyramax®) is the most recent ACT therapy to be approved for the treatment of both of the two main strains of malaria: *Plasmodium falciparum and Plasmodium vivax* (Lyu *et al*., 2021).

Typical ACTs should consist of a short half-life ($t_{1/2}$) of artemisinin derivative and another drug with a longer $t_{1/2}$ (>4 days) (Tran *et al*., 2004). Amongst those available artemisinin derivatives, ART has the most desirable pharmaceutical characteristics for use in ACTs (Davis *et al*., 2005b). ART is a white crystalline powder that can be prepared for oral, rectal, intramuscular, and intravenous routes (Barradell and fitton, 1995). ART is slightly soluble in water which results in a short half-life and poor bioavailability of only about 30% after oral administration (Shapiro and Goldberg, 2006). Several approaches to improve its solubility and dissolution rate include the formation of solid dispersions (SDs), synthesis prodrugs, or fabrication of drug complexation. SDs were first proposed to increase the solubility and oral absorption of poorly water-soluble drugs in 1961. While a wide range of excipients have been introduced as carriers for SDs, cyclodextrin and its analogs are SDs of choice because of their low toxicity, high aqueous solubility, high drug loading capacity, and well physiological tolerance. Poor water-soluble drugs are molecularly dispersed into the hydrophobic matrix of cyclodextrins leading to enhanced solubility. For instance, the solubility of artemisinin and its derivatives could be significantly increased through complexation with HP-β-Cyclodextrin (β-CD) at a molar ratio of 1:1 (Shaundarya *et al*., 2014).

Piperaquine is an aminoquinoline antimalarial drug with good safety, tolerability, efficacy, and pharmacokinetic profile. It has a low cost and is an ideal partner drug for use as part of an ACT (Davis *et al*., 2005a, 2005b). Piperaquine is effective against *P. vivax* and *P. falciparum*, including strains of *P. falciparum* being resistant to Chloroquine. It can be used as monotherapy (ex. in China) or ACTs (ex. CV8® in Vietnam, Artekin® and Duo-Cotecxin® in several South-East Asian countries) (Lyu *et al*., 2021; Myint *et al*., 2007; Tran *et al*., 2004). However, parasite resistance to piperaquine can develop when being used extensively as monotherapy. Therefore, combined therapies with piperaquine may be much more potential. Indeed, preparations containing DHA and piperaquine (Artekin® and Duo-Cotecxin®) have shown high effectiveness and good toleration at a reasonable price for patients in the developing countries.

Fixed-dose combination (FDC) products are common in the treatment of various diseases including malaria. They offer efficacy and safety improvement, product exclusivity, patient compliance, and medicinal cost reduction (Kim and Weon, 2021). There has been no known report about ART-piperaquine phosphate (PQP) fixed-dose combination tablets (FDCT) as an alternative convenient choice for malaria treatment available in the literature. The only published combination is piperaquine and DHA—an ART metabolite, which is indicated for uncomplicated falciparum malaria in Asia, Africa. ART has many advantages over other derivatives of artemisinin and is the most potential candidate used in ACTs. Thereby, we, inspired by the above ACTs, developed in this work a novel ART-PQP FDCT (containing ART 50 mg and PQP 320 mg) with a high dissolution rate, high stability, and enhanced *in vivo* antimalarial effect against chloroquine-resistant *P. berghei*.

MATERIALS AND METHODS

Materials

ART (in-house specification) was supplied by the Department of Pharmaceutical Industry, Hanoi University of Pharmacy (Vietnam). PQP (Chinese Pharmacopoeia specification) was purchased from Chongqing Kangle (China). β-CD was of British Pharmacopoeia (BP) grade and was procured from Zhiyuan Biotech (China). Corn starch from Sunar Pazarlama Ve Dis Ticaret Ltd. and talc from Imerys Talc Luzenac France (France) were of BP grade. Stearic acid was obtained from ChemCeed LLC (United States of America) and was of USP grade. ART standard (purity 98.60%) and PQP working standard (purity 92.26%) were supplied by the National Institutes of Drug Quality Control (Vietnam). High-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) was purchased from Merck (Germany). Water was purified by distillation. All other chemicals and solvents were of the pharmaceutical grade obtained commercially and were used as such without further purification.

Animals

The chloroquine-resistant *P. berghei* strain (K70 resistant strain) was obtained from the National Institutes of Malariology and Entomology (Vietnam).

Swiss albino mice Balb-C strain (18–25 g weight) were obtained from the National Institute of Hygiene and Epidemiology (Vietnam) for antimalaria tests. They were kept in a clean room at a temperature of 25°C ± 2°C with a 12-hour light/dark cycle. The relative humidity (RH) was 55% ± 15% with an air ventilation frequency of 15–20 times/hour. All mice were freely fed with water and a commercial diet. The protocol of animal study (No. 022C/21) was approved by the Animal Care and Use Committee of Vietnam Military Medical University, Vietnam.

Methods

Preformulation study

The compatibility of ART and PQP was evaluated by using Differential Scanning Calorimetric (DSC) analysis and an accelerated stability study. For these tests, three following samples were meticulously prepared: ART raw material (S1), PQP raw material (S2), and ART-PQP physical mixture (5:32 mass ratio, equivalent to the ratio of the two drugs in FDCT) (S3).

For DSC analysis, the DSC diagrams of S1, S2, and S3 were recorded using a DSC-TGA 131 Cetaram, France, and were analyzed. Samples, 2–3 mg each, were heated in a hermetic sealed aluminum pan at a constant heating rate of 10°C per minute in

about 30–35 minutes range under an airflow of 20 ml/minute. An empty aluminum pan was used as the reference.

For the accelerated stability study, S1, S2, and S3 samples were filled in open vials and placed in a stability chamber (Gallenkamp SANYO, Japan) at conditions of 60°C ± 2°C/85% ± 5% RH for 3 weeks. At predetermined time intervals of 7, 14, and 21 days, the content of each drug in samples was determined by HPLC. Also, any changes in the appearance of drugs were observed.

Preparation and characterization of ART-β-CD SD system

ART-*β*-CD SDs were prepared by spray-drying technique. Briefly, ART was dissolved in 100 ml of 96% ethanol, and β-CD was dissolved in 100 ml of preheated water at 80°C and then cooled down to 40°C. These two solutions were then combined, sonicated for 15 minutes (Ultrasonic LC60H Sonicator, Germany), and spray-dried on a mini spray-dryer (B-191 Buchi, Switzerland) with the following operational parameters: inlet temperature (80°C), outlet temperature (50°C 60°C), inlet airflow (700 l/hour), exhaust gas pump (maximum, approximate 35 m^3/hour), nozzle pressure (0.34 MPa), and pump speed (10 ml/minute). The SDs were characterized by loss on drying (YMC IB-30 balance, Japan), flow velocity (Mettler Toledo FP62, Germany), density (ERWEKA SVW, Germany), morphology (Joel 5410 JSM Scanning Electron Microscopy, Japan), size and size distribution (Dimension Tools, CorelDraw 12.0 software), drug content, dissolution (ERWEKA DT 60 dissolution tester, Germany), X-ray diffraction pattern (D8-Advance Brucker, Germany), DSC (DSC-TGA 131 Cetaram, France), stability (Gallenkamp microchamber SANYO, Japan), and antimalarial efficacy.

Preparation and characterization of ART-PQP tablets

Tablets preparation

ART-PQP FDCT was fabricated by direct compression method using the following basic formulation for each tablet [ART-*β*-CD SDs (1:4, w/w) 230 mg Eq. to ART 50 mg]; PQP tetrahydrate 350 mg (Eq. to PQP anhydrous 320 mg); talc 15 mg; stearic acid 15 mg; and corn starch q.s). Each trial was performed with a batch of 60 tablets. The drugs and other excipients (talc, stearic acid, and corn starch) were equally mixed in a plastic bag and then subjected to compression into flat-faced cylindrical tablets of 670 mg of weight (unless otherwise specified) and 12 mm of diameter and more than 30 N of hardness. The compression process was performed on either a manual tableting machine (Pye Unicam, Thailand) or single punch tableting machine (KP2, Germany). With the Pye Unicam manual tableting machine, every premixed powder amount corresponding to each tablet weight was carefully weighed, placed in dies, and compressed under a predetermined compression force. With the KP2 single punch tableting machine, the well-premixed powder was placed in the hopper and compressed. Tablet weight and hardness were adjusted using the weight and hardness regulator. Preliminary experiments were conducted on the Pye Unicam machine to examine the effects of compression force and the number of other excipients on the physicochemical properties of ART-PQP FDCT. Then, three batches of 300 tablets of the optimal formulation obtained from the optimization step conducted on the Pye Unicam machine were made to validate the consistency of the developed process and to evaluate the stability of the ART-PQP FDCT, using the KP2 machine.

Optimization of the formulation of FDCT

A full factorial design with MODDE 5.0 software (Umetrics AB, Malmö, Sweden) was employed to optimize the formulation of ART-PQP FDCT (Nguyen *et al*., 2018). In this study, the amount of corn starch (X1) and compression force (X2) were the two independent variables. The dependent variables were characteristics of tablets including hardness (Y1), friability (Y2), and disintegration time (Y3). The amount of talc and stearic acid was fixed at the same amount of 15 mg/tablet. Using a full factorial design with two independent variables, each at three different levels gave $3^2 = 9$ formula as shown in Table 1.

Tablets physical analysis

The physical analysis of the prepared ART-PQP FDCT included hardness (hardness tester TBH 20, ERWEKA, Germany), friability (Friabilator TA 10, ERWEKA, Germany), disintegration time (disintegrating apparatus ZT 31, ERWEKA, Germany), weight variation (Mettler Toledo AB204-S), and drug content uniformity.

Drug content

Drug content assay was performed by using HPLC or spectrophotometry method. HPLC instrument (Shimadzu, Japan) was equipped with a degas unit DGU-14A, a high-pressure pump LC-10ADVP, a column-contained chamber CTO-10AVP,

Table 1. Formulations of ART-PQP FDCT obtained on Pye Unicam machine and their physicochemical characteristics (means ± SD, $n = 3$).

No	Starch (mg/tablet)	Compression force (ton)	Hardness (*N*)	Friability (%)	Disintegration time (minutes)
1	30	0.5	72 ± 8	1.1 ± 0.2	18 ± 2
2	45	0.5	53 ± 7	1.7 ± 0.2	11 ± 2
3	60	0.5	35 ± 3	2.3 ± 0.3	3 ± 1
4	30	1.0	106 ± 8	0.2 ± 0.1	33 ± 3
5	45	1.0	81 ± 4	0.4 ± 0.1	19 ± 2
6	60	1.0	68 ± 4	0.5 ± 0.1	7 ± 1
7	30	1.5	165 ± 10	0.1 ± 0.1	66 ± 4
8	45	1.5	133 ± 5	0.2 ± 0.1	29 ± 3
9	60	1.5	112 ± 9	0.3 ± 0.1	15 ± 2

a controller unit SCL-10AVP, photodiode array detector SPD-M10AVP and a software Class VP 6.14, analysis column (Discovery C18, 150 × 4.6 mm, 5 µm), and guard column (Discovery C18, 20 × 4 mm, 5 µm). Twenty tablets were weighed, crushed, and mixed thoroughly; then, a sufficient amount of samples equal to 100 mg ART or 50 mg anhydrous PQP were taken and the assay procedures for ART and PQP were conducted separately. Before analysis, the assay methods were developed and validated according to ICH guidelines, and the methods satisfied all validation criteria for selectivity, linearity, precision, and accuracy (ICH Harmonised Traprtite Guideline, 1994).

ART samples were put in a 25 ml volumetric flask, dissolved, and diluted with ACN to mark. HPLC conditions applied for ART analysis were as follows: ACN: phosphate buffer pH 3.0 (50:50, v/v) as the diluent; 30°C temperature; 1.0 ml/minute flow rate; 20 µl injection volume; and UV detection at 216 nm. Method validation results were linear range 0.5–5.0 mg/ml, regression equation of y = 345,119.5× − 197.4 (R^2 = 0.9994), precision [repeatability, mean = 50.28 mg/tablet, standard deviation (SD) = 0.649, RSD = 1.29%, n = 6], and accuracy (high recovery, 98.29%–101.46%).

PQP samples were dissolved and diluted with 0.1 M aqueous hydrochloric acid solution in a 100 ml volumetric flask. This sample was again diluted 50 times with the same solvent before UV absorbance analysis using a spectrophotometer (Varian Cary 100 UV-VIS Spectrophotometer, USA) at a wavelength of 347 nm, and the blank sample was 0.1 M aqueous hydrochloric acid solution (Pharmacopoeia Commission, 2015). Method validation results were linear range 4–20 µg/ml, regression equation of y = 0.0328× + 0.0065 (R^2 = 0.9999), precision (high repeatability, mean = 322.2 mg/tablet, SD = 2.16, RSD = 0.67%, n = 6), and accuracy (high recovery, 98.86%–101.50%). The UV absorbance method was also compared with the HPLC method developed by our lab as follows: the PQP samples were dissolved and diluted with 1.0% aqueous acetic acid solution in a 50 ml volumetric flask. This sample was again diluted 10 times before HPLC analysis with phosphate buffer pH 2.7:ACN (94:6, v/v) as the mobile phase (phosphate buffer containing 0.01 M potassium dihydrophosphate and 1% triethylamine, pH was adjusted by using phosphoric acid), 30°C temperature, 1.0 ml/minute flow rate, 20 µl injection volume, and 347 nm wavelength. Method validation results were linear range 80–120 µg/ml, regression equation of y = 23,130× − 271.74 (R^2 = 0.9996), precision (high repeatability with mean = 319.3 mg/tablet, SD = 2.37, RSD = 0.74%, n = 6), and accuracy (high recovery, 99.02%–102.06%).

In vitro dissolution testing

Dissolution studies to determine drug release patterns from ART SDs or ART PQP FDCT were performed by using the USP apparatus type II (paddle apparatus) set at 100 rpm stirring speed and 900 ml distilled water as a dissolution medium at a temperature of 37°C ± 0.5°C.

For ART SDs samples, an amount of 50 mg ART raw material or SDs equivalent to 50 mg ART was used in the dissolution rate test. At predetermined time intervals of 5, 10, 15, 30, 45, and 60 minutes, 5 ml of dissolution medium was withdrawn, filtered through a 0.45 µm diameter membrane, and subjected to HPLC analysis as the above chromatography program with injection volume set at 100 µl. Parallelly, 5 ml fresh medium was replenished to maintain the "sink" condition.

For ART-PQP FDCT, at the end of the dissolution time, samples were collected and filtered through a 0.45 µm diameter membrane. The filtered solutions for the determination of ART concentration were analyzed by HPLC according to the conditions mentioned in the above *Drug Content* section with the exception that the injection volume was set at 100 µl. For PQP dissolution determination, 1 ml filtered solution was diluted with aqueous hydrochloric acid solution 0.1 M in a 25 ml volumetric flask and then we measured UV absorbance at 347 nm and compared it with PQP ($C_{29}H_{32}Cl_2N_6.4H_3PO_4$) standard solutions prepared in 0.1 M hydrochloric acid solution.

Stability studies

Stability studies were conducted with ART-PQP FDCT from the optimal formulation batches of 300 tablets. The ART-PQP FDCT was placed in well-capped glass vials following ICH guidelines at 30°C ± 2°C/75% ± 5% RH in a stability chamber (Gallenkamp microchamber SANYO, Japan) for 24 months. The physicochemical and aesthetic properties of the tablets were evaluated for parameters including appearance, hardness, moisture, drug content, and *in vitro* dissolution profile.

Determination of oral acute toxicity [lethal dose (LD_{50})]

The conventional test was carried out for determining the acute toxicity, using Swiss albino mice (60 mice, 18–22 g body weight, n = 10 per group), following the Organization for Economic Cooperation and Development guidelines for the testing of chemicals for safety evaluation. Doses ranged from 1,000 to 2,200 mg/kg. Drugs were dispersed homogeneously in a 1% aqueous solution of Arabic gum and were administered orally by gavage. The volume of administration was 0.4 ml/20 g body weight. The general states and mortality rate of experimental mice in each group were monitored for 72 hours and infra LD_0 and absolute LD_{100} were determined. The mean LD_{50} value was calculated using EPA Probit Analysis Program (Version 1.5).

Antimalarial efficacy on *P. berghei* - infected mice

The *in vivo* antimalarial activity was assessed in mice infected with chloroquine-resistant *P. berghei*. Five-week-old (23 ± 2 g) male and female mice were used according to the 28-day routine protocol of the National Institute of Malariology, Parasitology, and Entomology (Vietnam) based on Peter's 4-day suppressive test (Peters *et al*., 1975). The animals were randomly divided into four groups: Control, ART, PQP, and ART-PQP FDCT group (n = 30 mice per group). On the first day (day 0), mice were infected by the intravenous route with 10^7 *P. berghei*–infected erythrocytes from mice in 0.2 ml of saline. The animals were treated 1 day after infection, by oral administration with a total dose over 3 days of each group as follows: ART group at 217 mg/kg ART as SDs form, PQP group at 512 mg/kg PQP raw material, and ART-PQP FDCT group at 592 mg/kg (equivalent to 80 mg of ART and 512 mg of PQP). The drugs were given once a day, as a homogeneous suspension in a 1% aqueous solution of Arabic gum. The control group received only 0.2 ml of 1% Arabic gum solution.

Thin blood films were made from 2 to 3 drops of tail vein blood of all animals to determine parasitemia through observation under an optical microscope (1,000×). The percentage of infected erythrocytes was determined in blood films fixed with methanol and stained with Giemsa. Parasites were counted per 10,000 RBCs per slide, minimum of 20 fields (500 RBCs for each field) were counted in one slide, and average parasitemia was measured (Osmo *et al*., 1993). Parasitemia levels and survival rate were monitored and calculated up to 28 days after infection. The percent of parasitemia and survival rate were calculated using the following formulas:

Mean percent parasitemia = Infected RBCs/total number of RBCs examined × 100

Survival rate = Survived animals/total number of animals tested × 100.

Statistical analysis

All data are expressed as mean ± SD from triple replicates except otherwise specified. The significance of differences was assessed by analysis of variance and considered statistically significant when $p < 0.05$ in all cases.

RESULTS

Preformulation studies

The DSC thermograms of ART, PQP, and their physical mixture are presented in Figure 1. The DSC diagram of ART showed a sharp endothermic peak at 163.0°C which was the melting point of pure drug and followed by an exothermic peak at 171.9°C corresponding to its thermal decomposition. The DSC thermogram of PQP had two endothermic peaks, which were 149.5°C and 256.2°C corresponding to the water evaporation and the melting point, respectively (Li *et al*., 2018). The DSC of the physical mixture presented all typical peaks of ART and PQP and no new peak was detected at the temperature range below 200°C.

During the accelerated stability test, the drug admixtures showed free-flowing characteristics, and no apparent changes were detected, indicating that both drug substances were physically compatible with each other. Table S1 shows the drug content loss during storage time. It can be seen that after 3-week storage, the decline in ART content of S3 and S1 samples showed no significant difference ($p_{3,1} = 0.270 > 0.05$), which means the presence of PQP does not affect the stability of ART. Also, there was no statistically significant difference in PQP content loss of S3 and S2 samples ($p_{3,2} = 0.642 > 0.05$).

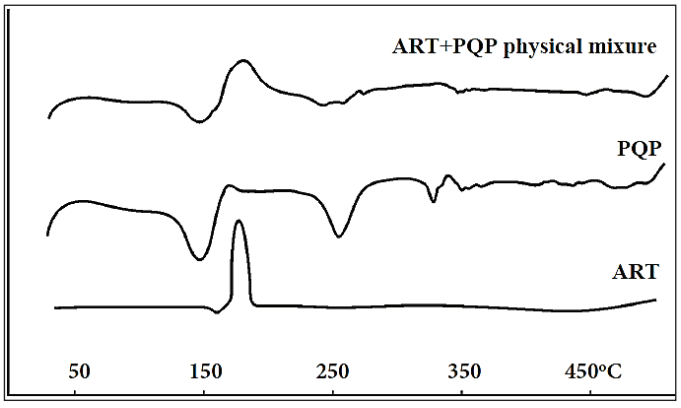

Figure 1. DSC of ART, PQP, and their physical mixture (5:32, w/w) after storage in 60°C ± 2°C/85% ± 5% RH for 3 weeks in preformulation studies.

Preparation and characterization of ART - β-CD SDs

After preliminary experiments (data not shown), three batches of ART SDs were prepared to get white dry fine powder containing lots of spherical particles with the following characteristics: loss on drying of 1.1% ± 0.2%, drug content of 22.1% ± 0.6% w/w, flow velocity of 9.2 ± 1.3 g/sec, bulk density of 0.33 ± 0.05 g/ml, mean diameter of 2.4 ± 1.8 µm, and the dissolution rate of 98.6% ± 1.3% (after 15 minutes) (Fig. 2a–c). The spray-drying process offered a high recovery of 77.6% ± 5%. The solubility of ART in water was significantly improved from only 0.41 ± 0.03 mg/ml in the case of ART raw material to 2.62 ± 0.04 mg/ml in SDs (about 6.4-fold enhancement) (Fig. 2f). Besides, the dissolution rate also changed greatly, while 75.3% of ART raw material dissolved after 60 minutes, almost all of ART in SDs dissolved after only 15 minutes (Fig. 2g).

DSC analysis indicated that ART raw material had a sharp endothermic peak at 163.0°C which was its melting point followed by an exothermic peak at 171.9°C corresponding to its thermal decomposition (Fig. 2e). β-CD showed a wide endothermic peak at 60°C–150°C corresponding to its dehydration. In ART-β-CD PM, there were still all characteristic peaks of both ART and β-CD while in SDs, the peak at the melting point of ART disappeared. This confirmed possible interactions due to the complexation between ART and carrier. Moreover, characteristic peaks at the decomposition point of ART in SDs reduced sharply indicating a more stable state of ART in SDs in comparison with that of raw material. The intensity of peaks at the decomposition points of ART in SDs decreased in the following order: ART/β-CD = 1/2 > ART/β-CD = 1/4 > ART/β-CD = 1/6 meaning that the protective effects of β-CD increased proportionally to its ratio in SDs. The ART SDs also showed good stability after 6 months of storage at accelerated conditions (40°C ± 2°C/75% ± 5% RH) (Fig. 3). More importantly, when compared to ART raw material, ART SDs showed enhanced antimalarial effects against chloroquine-resistant *P. berghei* in the mice model. Using a dosing regimen with total doses of 297 mg/kg ART in 3 days, one dose per day, and observed in 28 days, the ART SDs group reduced parasites in the blood more rapidly than that of ART raw material on day 1, day 2, and day 3 ($p < 0.01$, $n = 10$, data not shown) while the control group (used Arabic gum 1%) exhibited an increase in the number of parasites in blood from day 0 to day 7. In a word, ART SDs exhibited spherical particles with smooth surfaces, high stability and dissolution rate, enhanced antimalarial effects, good flowability, and *compressibility* for direct compression into FDCT.

Preparation and optimization of ART-PQP FDCT

There are numerous factors in formulation and process affecting the qualities of tablets such as the type and quantity of excipients, preparation method, mixing conditions, type of tableting machine, compression force, and speed. Among

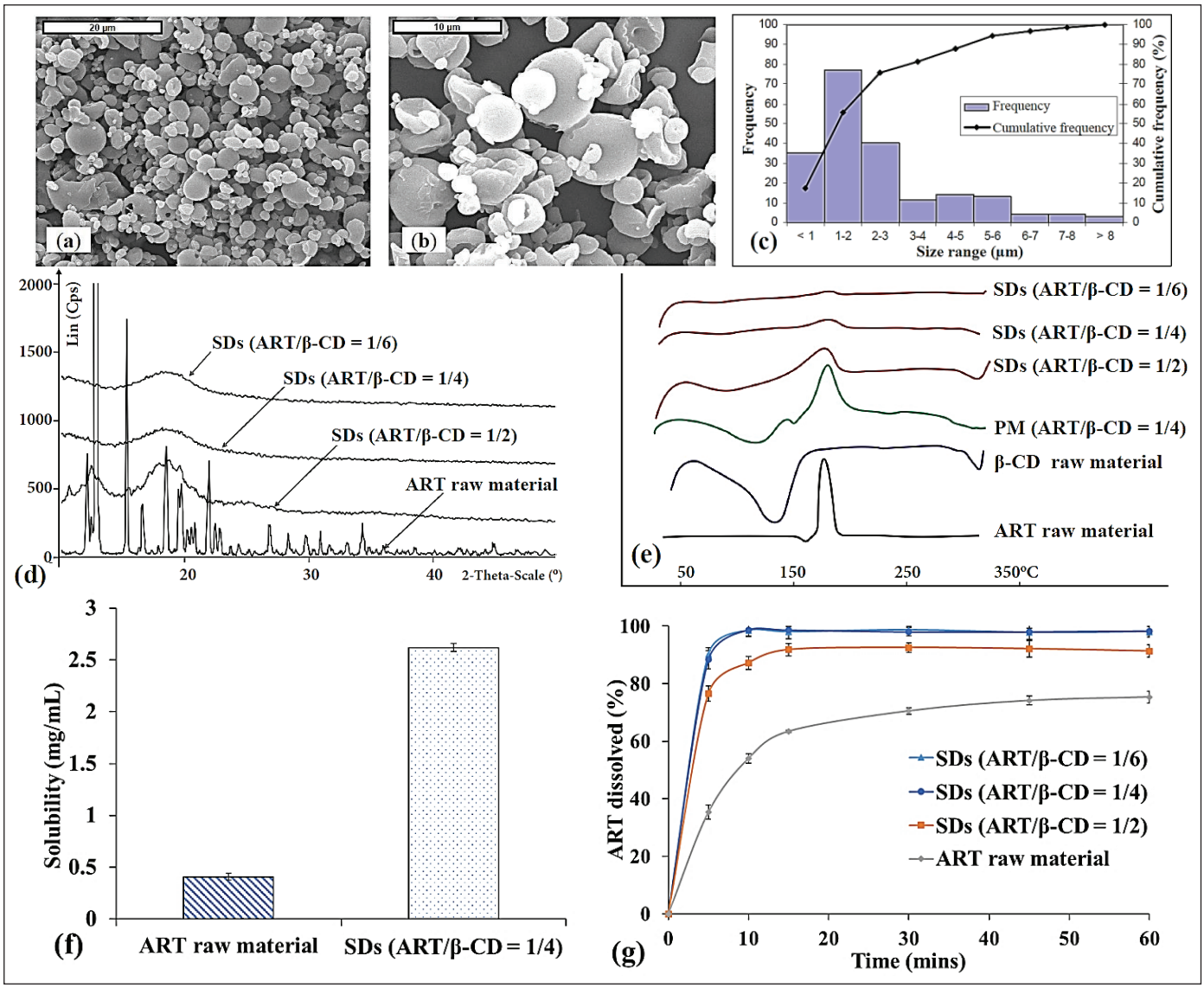

Figure 2. Characteristics of ART SDs. Morphology analysis of SDs (ART/β-CD = 1/4, w/w) with SEM images with different magnifications (a) 2,000-fold, bar scale = 20 µm and (b) 3,500-fold, bar scale = 10 µm. (c) Size distribution of SDs (ART/β-CD = 1/4, w/w). (d) X-ray diffraction patterns of ART raw material and SDs with different ratios of ART/β-CD. (e) DSC analysis of ART, β-CD raw material, physical mixture (PM) at ART/β-CD = 1/4, and SDs with different ratios of ART/β-CD. (f) Solubility of ART from raw material and SDs (ART/β-CD = 1/4, w/w) in water at 25°C. (g) Dissolution of ART from ART raw material and SDs with different ratios of ART/β-CD. The results are presented as means ± SDs, n = 3.

those, compression force is a crucial factor in determining many important physicochemical attributes of tablets including hardness, friability, disintegration time, dissolution rate, and further bioavailability and efficacy. Pye Unicam is a manual tableting machine that has the capability of determining and adjusting compression forces. Thus, it was used to investigate the influences of compression forces as well as the number of other excipients on the physicochemical properties of ART-PQP FDCT in this work. From our preliminary experiments conducted on the Pye Unicam machine, the formulations containing ART SDs and PQP only were already capable of being directly compressed to generate robust and stable tablets. The amount of lubricants (talc: 15 mg/unit, stearic acid: 15 mg/unit) was suitable for compressing process, tablets were easily pushed out of the mortar, and no sticky phenomenon was observed. Using compression force of 0.5 tons, the hardness of the tablets was satisfactory (about 150 N), but the disintegration time was still quite long (> 40 minutes), so we tried a widely used disintegrant, corn starch, with the aim of reducing the disintegration time. Table 1 showed ART-PQP FDCT formulations along with their physical characteristics including hardness, friability, and disintegration time from nine formulations according to full factorial design. In our work, ART-PQP FDCTs were obtained with hardness, friability, and disintegration time ranging from 35 to 165 N, 0.1% to 2.3%, and 3 to 66 minutes, respectively. To assess the experimental repeatability, Cochrane verification was used and the C_t value for each response was calculated and presented in Table S2. It could be seen that all C_t values of dependent variables were smaller than C(n-1; m; p) = C(2; 9; 0.95) = 0.478 (refer to Cochrane's table), meaning that the highest error was not more than the general error of experiment. Therefore, it could be concluded that the experiments were repeatable. The effects of independent variables on the responses

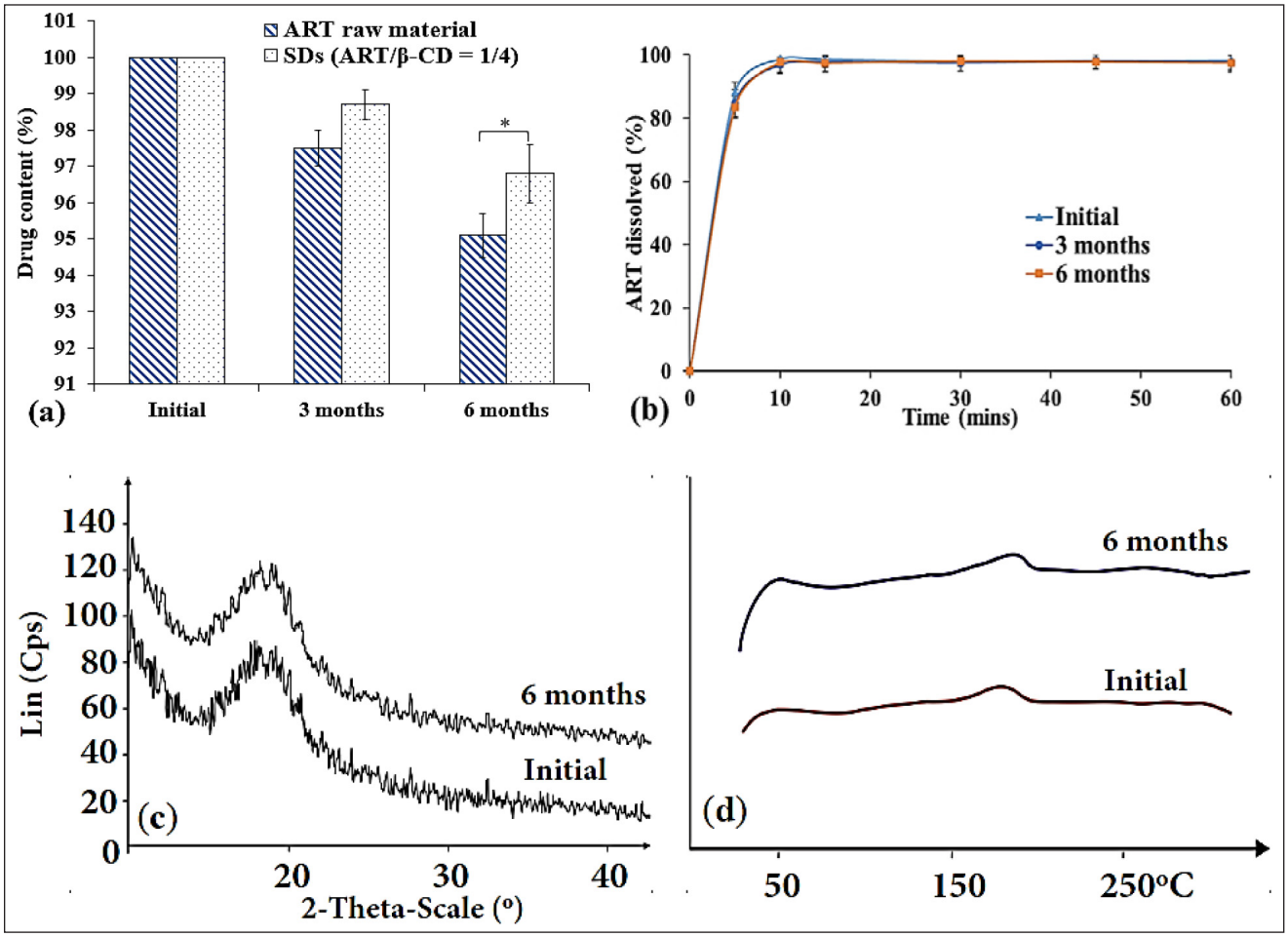

Figure 3. Stability of ART SDs (ART/β-CD = 1/4, w/w) after storage at 40°C±2°C/75% ± 5% RH. (a) Drug content of ART at initial and after 3 and 6 months ($n = 3$), * $p < 0.05$. (b) Dissolution of ART from SDs at initial, after 3 and 6 months ($n = 6$). (c) X-ray diffraction patterns of SDs at initial and after 6 months. (d) DSC analysis of SDs at initial and after 6 months. The results are presented as means ± SDs.

were calculated using MODDE 5.0 software and the results were shown in Table S3 and Figure 4.

Based on the data from the experimental results, the range of optimal conditions for dependent variables was designated as Y1: not less than 55N; Y2: not more than 0.8%; and Y3: not more than 10 minutes. Running MODDE 5.0 optimization software program, the input variables' values were determined: X1 = 60 mg/tablet, X2 = 1.1 tons. Because the optimal formulation determined by the software was very close to formulation No. 6, so the final formula was ART-*β*-CD SDs (1:4, w/w) 230 mg (equivalent to ART 50 mg), PQP tetrahydrate 350 mg (equivalent to PQP anhydrous 320 mg), talc 15 mg, stearic acid 15 mg, and corn starch 60 mg. The average weight of the FDCT was 670 mg. Triple batches of 300 tablets of the selected optimal formulation were prepared by the same direct compression method using the KP2 eccentric press tableting machine. The machine was adjusted to get tablets with an average weight of 670 mg and a hardness range of 60–80 N (equivalent to that value obtained from the Pye Unicam machine upon using a one-ton compression force). Critical properties of the tablets (hardness, friability, disintegration time, weight variation, drug content variation, and dissolution) were evaluated and shown in Table 2. Besides good appearance, the results of hardness (> 55 N), friability (< 0.8%), and disintegration (< 10 minutes) of all ART-PQP FDCT samples were within limits. The max bias of weight variation, ART content, and PQP content was 4.2%, 11.4%, and 7.3%, respectively. And more than 90% of ART and more than 80% of PQP dissolved from FDCT after 30 minutes in dissolution tests.

Stability studies

Specifications of ART-PQP FDCT including appearance, hardness, disintegration time, and loss on drying after 24 months in ambient storage conditions were similar to that at the beginning of the storage time (data not shown). Drug contents and dissolution results from the stability study in ambient conditions were summarized in Table 3. Those figures indicated that during storage, there was a slight decrease in the percentage of drugs solubilized and the content of the drugs as well. But all these observed changes remained within acceptable limits of in-house specification which were not less than 70% of ART and PQP dissolved in 30 minutes, and the contents ART and PQP were within 90%–110% and 93%–107% of the label claims, respectively. Therefore, it could be concluded that ART-PQP FDCT were stable for 24 months in ambient conditions (30°C ± 2°C/75% ± 5% RH). The shelf life of ART-PQP FDCT was estimated at 35 months according to STAB software (Lee and Lee, 2021).

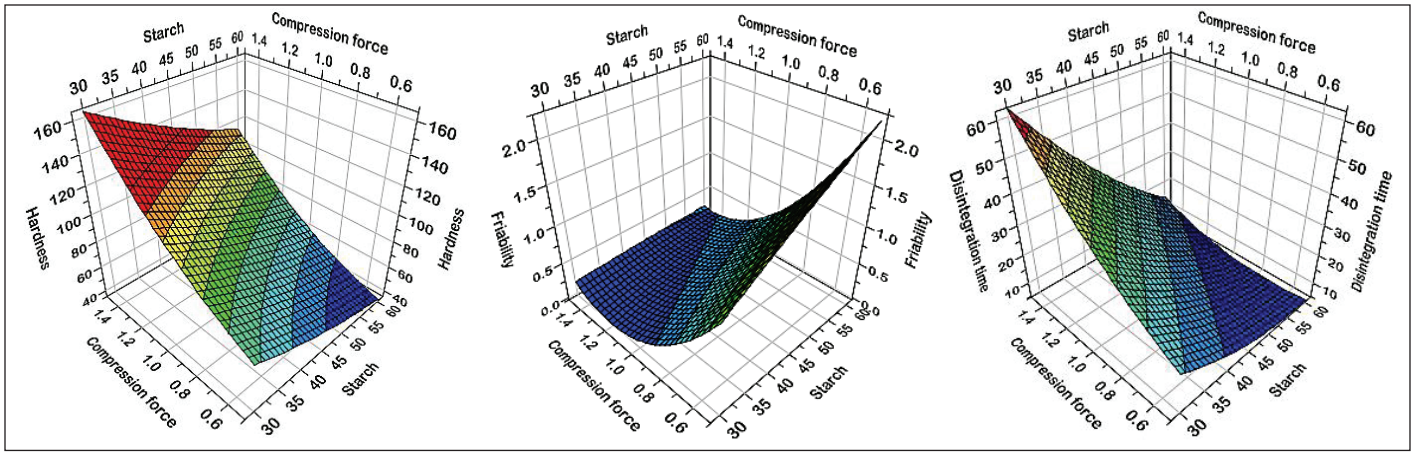

Figure 4. Response surface graphs of Y1 (hardness, N, left), Y2 (friability, %, middle), and Y3 (disintegration time, minutes, right) with input variables of X1 (starch, mg/tablet) and X2 (compression force, ton).

Oral acute toxicity

Data in Table S4 showed that the infra LD_0 and the absolute LD_{100} were 1,000 and 2,200 mg/kg, respectively. LD_{50} value in mice by oral administration of ART-PQP combination was 1,550.5 mg/kg (95% confidence interval, 1,393.9–1,734.8 mg/kg) as estimated by EPA Probit Analysis Program (Version 1.5), which was higher than that of other antimalarial drugs. The sign of oral acute toxicity observed as follows: mice were lethargic and unresponsive to stimuli, refused foods, and convulsed before dying. Also, this LD_{50} value of ART-PQP FDCT was 26 times higher than the total intended treatment dose in humans (4 doses of 2 tablets in 3 days, equivalent to 29.6 mg/kg on the first day and 14.8 mg/kg on the next 2 days), presenting a broad putative therapeutic index of this new combination.

In vivo antimalarial efficacy

In vivo antimalarial efficacy of ART, PQP, and FDCT was summarized in Figure 5. In the untreated control group, parasitemia increased rapidly within 14 days to 2,031 parasites/10,000 RBCs. Animal death was first observed on day 14 after infection and 29/30 animals (96.7%) died by day 28; only 1/30 mice survived but with very high parasitemia (3,264 parasites/10,000 RBCs).

In the ART group, with a total dose of 297 mg/kg, after an initial drop in parasitemia compared to the control mice during the first 3 days of treatment, the parasitemia increased rapidly in all mice after treatment ended. ART caused a significant delay in the development of parasitemia but it had no curative action. By day 28, only 3 out of 30 mice treated with ART were alive and with high parasitemia levels (3,121 parasites/10,000 RBCs). This result was in agreement with other studies (Barradell and fitton, 1995). Incomplete clearance of parasitemia will result in high recrudescence rates. This is the major issue of ART and other artemisinin derivatives, which could be contributable to their short half-life.

In the PQP group, with a total dose of 512 mg/kg, a disappearance of parasitemia was observed on day 4 in all mice. The onset of recrudescence was observed at day 10 in 20/30 mice (66.7%), but in low parasitemia levels (0.01% to 1.3%). After that, 14/20 mice of recrudescence had a clearance of parasitemia again. Ten out of 30 mice were cured and these remained free of parasites up to 28 days after infection (33.3%). Only one out of 30 died during 28 days with a high parasitemia state.

In the group treated with ART-PQP FDCT, with a total dose of 592 mg/kg (80 mg ART + 512 mg PQP), a disappearance of parasitemia was observed at day 3 in all mice, more rapid than that in the group treated with PQP alone. The radical cure rate is 93.3% (28/30 mice). The onset of recrudescence was observed at day 14 in 2/30 mice (6.7%). But all these two mice had a clearance of parasitemia on day 24 and all mice were surviving on day 28 (100%).

DISCUSSION

First, we conducted the preformulation studies to examine the possible interactions between ART and PQP because the compatibility of components combined in one formula is a prerequisite factor for the formulation development of FDC preparation (Bashira *et al*., 2019; Kim and Weon, 2021). ART is well known as a sensitive substance with extreme conditions such as high temperature, humidity, pH, oxidants, and heavy metal ions. Meanwhile, PQP has weak acidic properties [0.25% aqueous solution has a pH range of 3 to 4 (Pharmacopoeia Commission, 2015)] which may theoretically affect the stability of ART. The compatibility investigations were carried out and analyzed by using DSC and drug content as indicators after a 3-week period of preservation at highly accelerated conditions (60°C ± 2°C/85% ± 5% RH). DSC is a modern thermal analysis technique that could detect interactions that occurred between drugs and drug excipients. The advantages of DSC are rapid and accurate and requires little amount of sample for analysis. The results of our study indicated that there were no physicochemical interactions between ART and PQP, and the two drugs could be readily coincorporated into one combination. Indeed, the ART-PQP FDCT showed enhanced antimalarial efficacy than that of monotherapy and it was stable for 24 months under ambient storage conditions (see the following parts).

Second, we tried to enhance the physicochemical characteristics of ART before preparing ART-PQP FDCT because ART, especially in an acid form, has poor aqueous solubility, thus, resulting in low bioavailability after oral administration (Newton *et al*., 2000). Moreover, due to its ester group and peroxide bridge,

Table 2. Physicochemical of ART-PQP FDCT from three consecutive batches of the optimal formulation using KP2 machine.

Batch		No. 1	No. 2	No. 3	Max bias* (%)
Hardness (*N*, *n* = 10)		64 ± 6	68 ± 4	65 ± 4	-
Friability (%, *n* = 3)		0.6 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	-
Disintegration time (minutes, *n* = 6)		7 ± 2	8 ± 2	7 ± 1	-
Weight uniformity (mg, *n* = 20)		691.5 ± 15.9	702.9 ± 14.5	690.5 ± 18.0	4.2
Drug content uniformity (mg/tablet, *n* = 10)	ART	51.7 ± 3.1	52.6 ± 3.9	51.3 ± 3.3	11.4
	PQP	324.9 ± 13.5	330.8 ± 18.4	322.1 ± 12.8	7.3
Dissolution after 30 minutes (%, *n* = 6)	ART	92.4 ± 2.2	92.6 ± 2.4	91.2 ± 4.2	-
	PQP	85.7 ± 2.9	86.8 ± 4.4	83.6 ± 2.6	-

*Max Bias (%) indicates the deviation (%) (weight or drug content) of the tablet having the highest deviation compared to average weight or drug content.

Table 3. Contents and dissolution (after 30 minutes) of ART and PQP from optimal formulation during storage at 30°C ± 2°C/75% ± 5% RH. The results were presented as means (*n* = 3) for drug content and as means ± SDs (*n* = 6) for drug dissolution.

Time (months)	Drug content (%, *n* = 3)						Drug dissolution (%, *n* = 6)					
	Batch No. 1		Batch No. 2		Batch No. 3		Batch No. 1		Batch No. 2		Batch No. 3	
	ART	PQP	ART	PQP	ART	PQP	ART	PQP	ART	PQP	ART	PQP
Initial	100	100	100	100	100	100	92.4 ± 2.2	85.7 ± 2.9	91.2 ± 4.2	84.6 ± 2.6	94.6 ± 2.4	88.6 ± 4.4
3	99.8	99.3	98.7	99.5	99.1	99.6	90.2 ± 3.6	87.1 ± 2.5	92.7 ± 2.7	84.2 ± 2.7	94.7 ± 4.3	87.3 ± 3.5
6	98.8	99.7	97.9	99.1	98	98.8	89.6 ± 3.8	84.5 ± 3.4	89.4 ± 2.5	83.9 ± 3.4	92.2 ± 3.5	86.5 ± 3.4
9	98.4	98.4	96.9	98.3	97.4	98.7	90.5 ± 4.5	84.3 ± 2.6	88.2 ± 3.4	82.4 ± 2.7	90.7 ± 3.2	86.0 ± 2.8
12	97	98.3	98.3	97.8	97.2	98.3	90.8 ± 2.6	83.5 ± 2.5	88.4 ± 3.2	81.6 ± 3.5	89.4 ± 4.3	84.6 ± 3.7
18	95.8	97.3	96.2	96.8	95.8	97.5	87.3 ± 3.7	82.7 ± 3.4	85.6 ± 2.5	81.3 ± 3.6	87.5 ± 4.5	83.7 ± 3.2
24	93.6	96.1	94.5	96.2	94.6	96.6	84.5 ± 4.4	80.4 ± 4.1	82.2 ± 3.6	79.8 ± 4.2	86.2 ± 5.4	84.3 ± 3.9

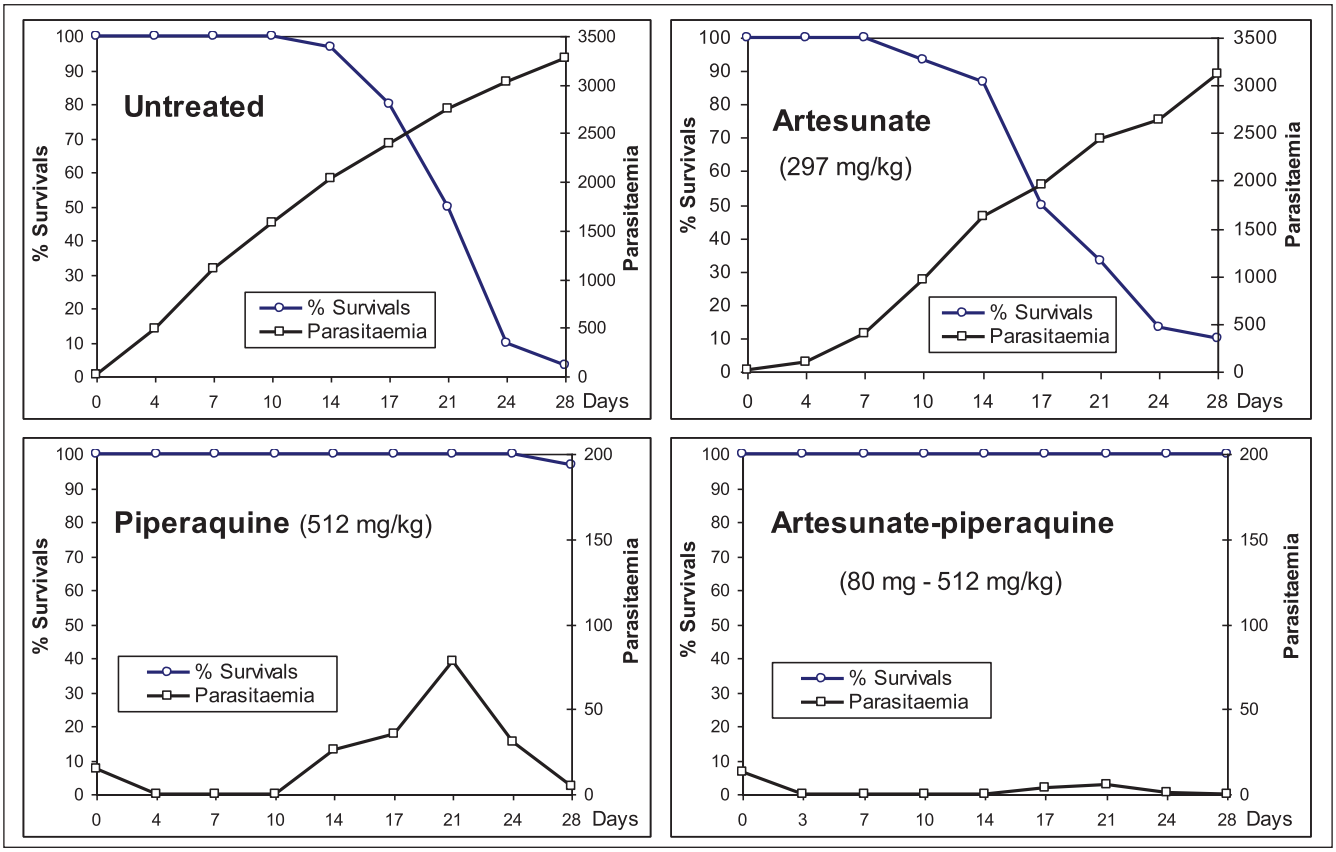

Figure 5. *In vivo* antimalarial efficacy of ART, PQP, and ART-PQP FDCT on mice infected with *P. berghei* (K70, a Chloroquine-resistant strain.

ART can be easily decomposed. Several attempts have been made to improve its aqueous solubility and stability for further application in various dosage forms. Hartell demonstrated that ART and β-CD had the capability of forming stable complex in an aqueous medium and thus had significant solubility enhancement (Hartell *et al*., 2004). Others used particle size reduction or hydrophilic binder excipient approaches to better the dissolution rate of ART in solid dosage forms (Bashira *et al*., 2019; Ogbonn *et al*., 2017). Among those strategies, SDs are one of the most classical but simple and effective methods to tackle solubility-related problems of low water-soluble drugs (Ashok *et al*., 2003; Vasconcelos *et al*., 2007). The important point that needs to be taken into account is choosing a suitable carrier and preparation method due to ART's poor stability with heat and humidity. Accordingly, an effort using a spray-drying technique with a hydrophilic β-CD carrier to form a complex with ART was performed in this work. The advantages of the spray-drying technique with β-CD carrier were the very short exposure of ART to heat and humidity and the protective effects of β-CD on ART during the preparation process, leading to forming SDs with high drug content as afore-designed. Indeed, there were no decomposed peaks of ART appearing on the HPLC chromatograms of SDs (data not shown). The solubility enhancement ability of β-CD and its derivatives toward poorly water-soluble drugs had been confirmed and demonstrated by some authors (Ashok *et al*., 2003; Shaundarya *et al*., 2014). The main mechanisms were that β-CD, which are bucket-shaped oligosaccharides, formed inclusion complexes with ART, resulting in higher solubility and faster dissolution rate. Besides, hydrophilic hydroxyl groups anchored on the outer surface of β-CD molecules significantly increased the water permeation of ART. Moreover, the micro size of SDs particles (~2.4 μm) and the amorphous state of ART in SDs (confirmed by X-ray diffraction analysis, Fig. 2d) were other crucial factors for dissolution enhancement. DSC analysis confirmed possible interactions due to the complexation between ART and carrier and the protective effects of β-CD in SDs (Fig. 2e). In regard to biological properties, ART SDs also showed enhanced antimalarial effects against chloroquine-resistant *P. berghei* in mice models when compared to that of ART raw material (data not shown). Thus, due to those above significant improvements, ART SDs were suitable for direct compression with PQP to form FDCT.

Third, we optimized the preparation of ART-PQP FDCT by using the Design of Experiment (DoE) concept. DoE is a combination of mathematical and statistical approaches for the optimization of the processes; it provides an alternative to assess the impacts more efficiently than the one-factor-at-a-time experiments (Ho *et al*., 2015; Nguyen *et al*., 2018), in which the application of a factorial design in pharmaceutical formulation development has played a crucial role in the relationship between independent factors and responses. Considering that ART is a heat and humidity labile drug, PQP is a light-sensitive substance and both PQP raw material and ART SDs had good compressibility and flowability, and a direct compression method was adopted to avoid unfavorable impacts of heat and moisture on the drug's stability. To further ensure the stability of ART, another important point that needs to be considered was that excipients used in formulation were preferably free or less of contaminated metal ions, less humidity adsorption, and no alkaline agents. Therefore, talc and stearic acid were chosen as lubricants and corn starch was used as a disintegration agent. Taking into account that the lubricants are hydrophobic, their contents were carefully calculated and controlled at minimum levels of 15 mg/tablet for each. These amounts were sufficient for flowability assistance and were antisticky of punches and die effects during the compression process. The effects of starch content and compression force were investigated by using the Pye Unicam machine. Nine formulations were generated by full factorial design (Table 1). The repeatability of experiments was confirmed by Cochrane verification. The results of the effects of the input variables on the responses obtained from MODDE 5.0 software showed that the hardness and disintegration time decreased with increasing the starch content and reducing the compression force while an increase in starch content and reduction of compression force led to an increase in tablets' friability (Table S3). Furthermore, Figure 4 shows the correlation between input variables and disintegration time (Y3) of ART-PQP FDCT. When the amount of starch was low (30–45 mg/tablet), the disintegration time did not meet the requirements (more than 15 minutes). Besides, when a low level of starch was used, for example, an amount equal to 30 mg/tablet made Y3 change significantly when changing the compression force; Y3 rose from 18 to 66 minutes when the compression force increased from 0.5 to 1.5 tons, respectively. However, an increasing amount of starch to 60 mg/tablet made the disintegration not only faster but also less varied when the compression force changed. Y3 saw a minor rise, from 3 to 15 minutes, when increasing the compression force from 0.5 to 1.5 tons, respectively. With starch content at 60 mg per tablet and compression force at 1.0 tons, the friability was below 0.8%, which complied with the requirements of USP. There is, sometimes, a large concern associated with the direct compression method that tablets obtained by this means are likely to have lower weight and drug content uniformity compared to the compaction-mediated tablet compressing method due to its inherent disadvantages such as poor flow properties of powders and drug-excipients separation during the compression process. However, besides good appearance, the results of hardness, friability, and disintegration of all ART-PQP FDCT samples were within acceptable limits and were also not much different from that obtained on the Pye Unicam manual tableting machine. Weight variation and drug content variation met the requirements of USP which required none tablet to weight out of ±5% of the mean weight and none tablet has drug content out of ±15% of average drug content. Regarding industrial production, tablets with a weight of 670 mg and a diameter of 12 mm are widely acceptable. In addition, ART-PQP FDCT in this work showed good and consistent dissolution rates which were more than 90% of ART and more than 80% of PQP dissolved after 30 minutes. The FDCT were also stable for 24 months in ambient conditions (30°C ± 2°C/75% ± 5% RH). Its shelf life was estimated at 35 months according to STAB software (Lee and Lee, 2021). Thus, ART-PQP FDCT formulated using an eccentric press tableting machine with commonly used excipients and a simple preparation method met all general quality requirements of tablet dosage form and showed a high dissolution rate.

Concerning that severely possible toxicological effects could be one of the limiting steps for a new drug or combination to be considered when used therapeutically in reality, we investigated the possible toxic effects of oral acute treatment with FDCT in mice. ART-PQP FDCT is less toxic (LD_{50} = 1,550.5 mg/kg) in

comparison with its components and other antimalarial drugs which are of ART (LD_{50} = 1,000–1,300 mg/kg) (WHO Prequalifacation Team, 2016), PQP (LD_{50} = 1,098 mg/kg), Artekin® (LD_{50} = 961.7 mg/kg), chloroquine (LD_{50} = 437 mg/kg) (Li *et al*., 2018), Mefloquine (LD_{50} = 880 mg/kg) (Drug Bank: mefloquine), and amodiaquine (LD_{50} = 550 mg/kg) (Drug Bank: amodiaquine). We also evaluated *in vivo* antimalarial performance of the prepared tablets. The cure rate of the combination assessed on mice, with a total dose of 592 mg/kg, was 93.3%, markedly higher than that of single ART or PQP. And it showed a more rapid action and much more activity than that of ART or PQP monotherapies. This is the first time ART-PQP FDCT was assessed on preclinical safety and antimalarial efficacy and appeared to be a promising combination in malaria treatment.

CONCLUSION

This study was successful in developing a new FDCT of ART 50 mg and PQP 320 mg, using a direct compression method with β-CD (used under SDs form with ART), corn starch, talc, and stearic acid as excipients. The FDCT met all the general quality requirements including hardness (64–68 N), disintegration (7–8 minutes), weight variation (690.5–702.9 mg/tablet), drugs content uniformity (51.3–52.6 mg ART and 322.1–330.8 mg PQP per tablet), dissolution (more than 90% and 80% for ART and PQP after 30 minutes, respectively), and stability (24 months at ambient conditions). Acute toxicity tests showed LD_{50} in mice of FDCT was 1,550.5 mg/kg by oral administration. The cure rate of the combination assessed on mice, with a total dose of 592 mg/kg, was 93.3%, markedly higher than that of single ART or PQP. The enhanced *in vivo* activity over monotherapy against the chloroquine-resistant *P. berghei* line indicated the therapeutic potential of the ART-PQP combination. However, deeper preclinical and clinical data will be necessary to confirm the efficacy and safety of this new FDC of ACTs.

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AUTHOR CONTRIBUTIONS

Van Han Nguyen did project administration, conceptualization, methodology, supervision, writing—review and editing. Trong Bien Tran contributed to conceptualization, methodology, investigation, writing, and editing. Ngoc Bao Tran carried out data curation, review, and editing. Viet Hoang Nguyen carried out the investigation. Quang Anh Luong participated in the investigation and visualization. Thi Tien Nong contributed to the methodology and investigation.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

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ETHICAL APPROVAL

The protocol of animal study (no. 022C/21) was approved by the Animal Care and Use Committee of Vietnam Military Medical University, Vietnam.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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SUPPLEMENTARY MATERIALS

Table S1. The percentage of drug degradation after storage in 60°C ± 2°C/85% ± 5% RH for 3 weeks in preformulation studies (%, means ± SD, n = 6).

Time (days)	S1 (ART)	S2 (PQP)	S3	
			ART	PQP
7	1.28 ± 0.09	0.72 ± 0.07	2.20 ± 0.12	0.95 ± 0.08
14	2.97 ± 0.13	1.42 ± 0.11	2.83 ± 0.14	1.14 ± 0.13
21	3.54 ± 0.15	2.13 ± 0.16	4.21 ± 0.17	1.92 ± 0.09
p-value			$p_{3.1}$= 0.270	$p_{3.2}$= 0.642

Table S2. Cochrane verification for dependent variables.

Variables	Hardness	Friability	Disintegration time
$C_t = \frac{\max S_i^2}{\sum_1^n S_i^2}$	0.162	0.353	0.369

Table S3. The effects of dependent variables processed by MODDE 5.0 software.

Independent variables	Hardness	Friability	Disintegration time
Starch	−42.7	0.6	−30.7
Compression force	83.3	−1.5	26.0

Table S4. Results of oral acute toxicity of ART-PQP FDCT.

Group	Dose (mg/kg)	Number of mice died
1	1,000	0/10
2	1,200	2/10
3	1,400	5/10
4	1,600	5/10
5	1,900	6/10
6	2,200	10/10