Enhancement of solubility of Artemisinin and Curcumin by co-solvency approach for application in parenteral drug delivery system

Vaishali Thakkar1*, Rachana Dhankecha1, Mukesh Gohel1, Purvi Shah1, Tosha Pandya1, Tejal Gandhi1

A b s t r a c t

The aim of present study was to enhance solubility of poorly soluble antimalarial drugs, Artemisinin and Curcumin by adopting Co-solvency approach and to develop parenteral aqueous injectable solution. Solubility enhancement of both drugs was achieved using co-solvency approach. The parenteral injection was prepared by using a ternary-solvent system which comprised of benzyl alcohol, PEG 400 and tween 80 (as surfactant). Solubility of Artemisinin and Curcumin was found to be higher in benzyl alcohol and PEG 400. Co-solvent system comprising of benzyl alcohol, PEG 400 and tween 80 in volume fraction of 0.3, 0.9 and 0.2 respectively showed the minimum required solubility of Artemisinin (90 mg per ml) and Curcumin (180 mg per ml). The parenteral injectable formulation was characterized for pH, clarity, viscosity, osmolarity and sterility and the stated parameters were found in acceptable range. In-vitro erythrocyte toxicity study showed that intravenous administration of optimized formulation will be safe. In-vitro antimalarial assay indicated that efficacy of artemisinin and curcumin parenteral formulation was greater than quinine and combination of Artemether and Lumefantrine. Stability study of the optimized batch showed no change in physical and chemical characteristics. Potential implications: Based on study, one can conclude that Artemisinin and Curcumin can be successfully formulated as parenteral injectable formulation by co-solvency approach for the effective treatment of malarial infection.

Keywords: Solubility, Co-solvency, Artemisinin, Curcumin, Parenteral injection, In-vitro evaluation

Introduction

Malaria is caused by a parasite called plasmodium, which is transmitted via the bites of infected mosquitoes. In the human body, parasites multiply in the liver, and then infect red blood cells. The symptoms of malaria include fever, headache, and vomiting, and they usually appear between 10 and 15 days after the mosquito bite. If malaria is not treated, it can quickly become life-threatening by disrupting the blood supply to the vital organs [1]. From the five species that infect humans, P. falciparum is the one responsible for majority of the mortality and morbidity associated with malaria [2]. Malaria is curable if effective treatment is started early. Delay in treatment may lead to serious consequences including death. Burden of malaria in India is 180 million, with as many as 90 million cases of P. falciparum malaria per year. Malaria occurs during the blood-stage of the parasite's life-cycle where the parasite is known to replicate exclusively within erythrocytes. Infected individuals can also suffer relapses years after P. vivax and P. ovale infections due to parasites surviving in hepatocytes [3]. Two classes of drugs are currently available for the treatment of severe malaria: the cinchona alkaloids– Quinine and the Artemisinin derivatives.

The major problem with the currently available ant malarial drugs is that some malaria parasites have continuously developed resistance to these drugs [4]. Artemisinin (ART) derivatives have given efficacious result against malarial parasites. However, the requirement of a 7 day ART monotherapy has led to incomplete patient compliance and possible resistance development. As per the recommendation of World Health Organization (WHO), ART-based 3-day combination therapy may enhance the efficacy of this treatment and delay development of resistance. However, there is a need to develop newer ART-based combination therapy [5]. Combination therapy of Artemisinin and Curcumin adds a new dimension to malaria therapy in terms of its potential to prevent parasite recrudescence and relapse in falciparum and vivax malaria as well as to protect against cerebral malaria [6]. Curcumin, a hydrophobic polyphenol extracted from the roots of Curcuma longa L. is a staple of traditional Indian medicine including Ayurveda [7]. Recently a synergistic effect of Artemisinin and Curcumin against the malarial plasmodium, both in vitro and in vivo, has been proved. Curcumin is well tolerated even at very high doses and is a economical drug, hence its combination with Artemisinin may offer several advantages over conventional Artemisinin combination therapy, including a decreased Artemisinin dosage and reduced cost of therapy [8]. However, the poor water
solubility, incomplete absorption and poor bioavailability due to extensive first-pass metabolism of both compounds challenge their oral pharmaceutical formulations [9]. To resolve the issue of poor bioavailability and incomplete absorption, it is necessary to develop parenteral formulation with enhanced solubility of Artemisinin and Curcumin. Co-solvency, pH adjustment, surfactant addition, and complication are the most commonly used pharmaceutical approaches for solubilizing drug candidates with low aqueous solubility. Among them, use of co-solvent is one of the most popular approaches for improving the solubility of poorly aqueous soluble drugs in pharmaceutical liquid formulations. Addition of a co-solvent to a formulation improves the solubility of the drug by reducing strong water-water interactions and thereby reducing the ability of water to squeeze out non-polar solutes [10]. In this study ethanol, glycerin, PEG-400 and propylene glycol were used as co-solvents to enhance solubility of Artemisinin and Curcumin [11]. The objective of the present study has to prepare and evaluate the injectable dosage form of the two drugs.

Materials and methods

Materials

Artemisinin was obtained from Arihantam Life Care, Vapi, India. Curcumin was obtained from Pharmanza (India) Pvt. Ltd, Khambhat, India. Ethanol, benzyl alcohol and propylene glycol were obtained from Atul Chemicals, Anand, India. Glycerine and PEG-400 were obtained from Astron Chemicals, Ahmadabad, India. Tween 80 was obtained from S.D. Fine Chemical Ltd. Boisar, India.

Solubility of Artemisinin and Curcumin in individual solvents and binary system

Screening of solvents was done on the basis of solubility of Artemisinin and Curcumin in various solvents such as ethanol, benzyl alcohol, glycerin, PEG-400 and propylene glycol. Solubility was determined by equilibrium solubility method [12]. Excess amount of Artemisinin and Curcumin were individually added to screw-capped glass vials containing 5ml of the solvent. The vials were shaken mechanically for 24 hours on a mechanical shaker (RIS-24BL, REMI) at 37 ± 2°C. After 24 hours, the mixture was transferred into Eppendorf tubes and centrifuged at 6000 rpm for 20 min. The supernatants were collected from each Eppendorf tube and analyzed for drug content, after appropriate dilutions by UV-Visible spectrophotometer (Shimadzu, 1650 PC, Kyoto, Japan) at 292nm and 470nm for Artemisinin and Curcumin respectively. The study was performed in triplicate. Table 1 and Table 2 shows the solubility data of the drugs.

Construction of ternary phase diagram

Ternary phase diagram was constructed on the basis of measurement of refractive index of ternary solvent system [13]. Ternary solvent systems containing different composition of solvents were prepared and their refractive index was measured. Measured refractive index of solvent system was compared with refractive index of water. Ternary phase diagram was constructed by using Prosim software [14]. Figure 1 shows the ternary phase diagram.

Preparation of parenteral injection

On the basis of solubility data obtained from ternary solvent system, formulation of parenteral injection of Artemisinin and Curcumin was prepared using optimized solvent system. This formulation also contained permissible excipients such as the osmogen (4%) and antioxidant (0.1%).

Drug-excipient compatibility studies

UV spectral studies

UV spectral study of Artemisinin and Curcumin was performed. UV spectra of both the drugs in different solvents was measured for change in wavelength maxima [15]. The change in wavelength was considered as drug and excipient interaction. Figure 2 and Figure 3 shows the results.

Fourier Transform Infrared Spectroscopy (FTIR) study

The infra-red spectra of Artemisinin, Curcumin and co-solvent system having benzyl alcohol, PEG 400 and Tween 80 were recorded on Fourier Transform Infrared Spectrophotometer (Perkin Elmer – spectrum Bx, USA) in order to detect the existence of interaction between drug and solvents. The procedure consisted of uniformly dispersing a sample(10%w/w) in potassium bromide and finally compress it into pellets. The pellet was placed in light path and spectrum was recorded at a resolution of2 cm⁻¹ over a frequency range of 4000 to 400 cm⁻¹. The background spectrum of KBr was used as blank for determination.

Differential scanning calorimetric study

Differential scanning calorimeter (DSC) was used (Perkin Elmer DSC-7, Norway, USA) to study the thermal behavior of Artemisinin, Curcumin and co-solvent system comprising of benzyl alcohol, PEG 400 and Tween 80. The instrument comprised of calorimeter (DSC-60), flow controller (FCL-60), thermal analyzer (TA-60) and operating software (TA-60). The samples (2-4 ml) were heated in hermetically sealed flat-bottomed aluminum pans under nitrogen flow (20ml/min) at a scanning rate of 10°C/min from
25°C to 340°C. Empty aluminum pans were used as the reference standard.

**Characterization of aqueous injection containing Curcumin and Artemisinin**

**pH, clarity, viscosity, surface tension and specific gravity**

The pH of prepared aqueous injection was measured employing a pH meter (Lab India Pico+ Japan). The parenteral formulation was inspected against black and white background to assess presence of particulate matters. Viscosity, surface tension and specific gravity of the aqueous injection were measured using an Ostwald viscometer, Stalagmometer and relative density bottle method respectively. The measurements were performed in triplicate and the mean parameters of the aqueous injection were calculated.

**Drug content estimation**

Ratio derivative first order method was adopted for simultaneous estimation of Artemisinin and Curcumin in an aqueous injection. The data of zero order spectra for both drugs were divided by optimized divisor concentration. The ratio spectra of each solution were obtained and converted into first derivative. The converted ratio first derivative spectra were obtained at 293.17 nm and 457 nm for detection of Artemisinin and Curcumin respectively.

**Osmolarity**

The osmolarity of prepared co-solvent systems was determined with a vapor pressure osmometer (Supratech Laboratory, Ahmedabad).

**Leaching/extraction from the test container**

It was carried out by filling prepared aqueous injection in different container like glass vial, amber colour glass ampoule and flint glass ampoule. The containers were incubated for 15 days at ambient storage condition and the effect on pH, viscosity and drug content of formulation was measured [16].

**In-vitro erythrocyte toxicity study**

The *in-vitro* erythrocyte toxicity study was conducted for estimating the damage to the membrane which may be caused by formulation components [17]. Blood sample was collected in the vial containing EDTA (anticoagulant). Red blood cells (RBCs) were isolated by centrifugation (5,000 rpm for 5 min) and the RBCs were washed three times with isotonic phosphate buffer pH 7.4 before diluting with buffer to prepare erythrocyte stock dispersion (three parts of centrifuged erythrocytes plus eleven parts buffer). The buffer consisted of Na₂PO₄ 10H₂O (7.95 g), KH₂PO₄ (0.76 g), NaCl (7.2 g), and distilled water (q.s to 1000 ml). The washing step was repeated in order to remove debris and serum protein. A 100 microlitres aliquot stock dispersion was added per milliliter of test samples. The resulting solution was incubated at 37°C for a period of 1 h. After incubation under shaking, debris and intact erythrocytes were removed by centrifugation. One hundred microlitres of supernatant was added to 2 ml of an ethanol/HCl mixture [(39 parts ethanol (99% v/v) + 1 part of HCl (37% w/v)]. This mixture dissolved all components and avoided the precipitation of hemoglobin [18]. The absorbance of the mixture was determined at 398 nm by spectrometer monitoring against a blank sample. Control sample of 100% lyses (in TritonX100) was employed in the experiment [19]. The percentage of haemolysis caused by the test sample was calculated by following equation: Haemolysis caused by sample (%) = (Absorbance of test sample/ Absorbance at 100% lyses)*100

**In-vitro anti-malarial assay**

The test concentration which inhibits the complete maturation of ring stage parasites into schizonts was recorded as the minimum inhibitory concentration (MIC) [20]. It was carried out at Microcare Laboratory, Seurat. The injection of Artemisinin and Curcumin was taken as test sample and MIC was recorded and results were compared with Quinine (reference drug) and marketed combination of Artemether and Lumefantrine.

The *in-vitro* ant malarial assay was carried out in 96 well microstate plates according to the micro assay protocol of Reichmann et al. 1978 [21]. The cultures of *P. falciparum* strain were maintained in medium RPMI-1640 supplemented with 25mM HEPES, 1%D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum. The synchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitaemia of0.8to1.5% at 3% haematocrit in a total volume of 200µl of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining to assess the percent parasitaemia (rings) and uniform molly maintained with 50% RBCs (O+) [22]. A stock solution of 5mg/ml of each of the test samples was mixed with culture medium. The diluted samples in 20 µl volume were added to the test wells so as to obtain final concentrations (at fivefold dilutions) ranging between 0.4 µg/ml to 100 µg/ml in duplicate well containing parasitized cell preparation. The culture plates were incubated at 37°C in a candle jar. After 36 to 40h incubation, thin blood smears from each well were prepared and stained with JSB stain. The slides were microscopically observed to record maturation of ring stage parasites.
Sterility testing
Sterility testing was carried out by incubating formulations for seven days at 32.5±2.5°C in the alternative thioglycolate medium (ATGM) to find the growth of bacteria and at 22.5 ± 2.5°C in soya bean-casein digest medium (SCDM) to find the growth of fungi in the formulation.

Stability study
The sealed vials of the aqueous injections were inspected every day for 30 days for color, turbidity and pH of the formulation. The chemical stability of the formulation was assessed by the estimation of the percentage drug remaining in the formulation on storage at 2–8°C in a refrigerator, room temperature, and 40°C/75% RH in humidity chamber (Zebra instrument and equipments company-Ahmadabad) at 0, 15 and 30 days interval.

Table 1: Artemisinin and Curcumin solubility and enhancement factor in various solvents at 25°C

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Artemisin</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solubility ± S.D. (mg/ml)</td>
<td>Enhancement Factor*</td>
</tr>
<tr>
<td>Water</td>
<td>0.07 ± 0.0011</td>
<td>1</td>
</tr>
<tr>
<td>Glycerine</td>
<td>0.083 ± 0.0005</td>
<td>1.18</td>
</tr>
<tr>
<td>Propylene Glycol</td>
<td>2.03 ± 0.0115</td>
<td>29.00</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.20 ± 0.010</td>
<td>45.71</td>
</tr>
<tr>
<td>PEG-400</td>
<td>10.22 ± 0.0173</td>
<td>146</td>
</tr>
<tr>
<td>Benzyl Alcohol</td>
<td>230.66 ± 0.577</td>
<td>3295</td>
</tr>
</tbody>
</table>

* = Solubility of drug in 1 ml of solvent divided by the solubility in 1 ml of water, at 25°C

Solubility of Artemisinin and Curcumin in selected binary co-solvent system

The mixed solvent system composition, namely PEG 400–water (Smix 1), benzyl alcohol (BA)–water (Smix 2), PEG 400–ethanol (Smix 3), BA–ethanol (Smix 4), and benzyl alcohol–PEG 400 (Smix 5), were used for studying the solubility of Artemisinin and Curcumin depicted in Table 2.

As the volume fraction of PEG 400 and benzyl alcohol (co-solvent) increased, in the binary mixture with water, the solubility of Artemisinin and Curcumin increased. However, it failed to achieve the desired solubility for Artemisinin (90 mg/ml) and Curcumin (180 mg/ml) when the solvents (PEG 400 or benzyl alcohol) were used in the permissible limits as per inactive ingredient guide. Hence, binary combination of PEG 400-water and benzyl alcohol-water cannot be considered for the formulation development. In PEG 400-ethanol system, as the volume fraction of ethanol increased, solubility of Artemisinin and Curcumin decreased probably because of less polarity of ethanol as compared to PEG 400. As the volume fraction of PEG 400 increased, solubility of Artemisinin and Curcumin increased. But this enhancement also failed to achieve the desired solubility for Artemisinin and Curcumin.

Solubility was also checked in the binary mixtures of benzyl alcohol and ethanol (Smix 4), and benzyl alcohol and PEG 400 (Smix 5). The solubility of Artemisinin and Curcumin was found to be higher in all of these solvent systems than other binary co-solvent systems. PEG 400 was able to dissolve more of the drugs. This may be due to favorable dielectric constant of PEG 400. The dielectric constant of PEG 400 is higher than that of ethanol. Hence, the solubility of Artemisinin and Curcumin was found to be higher in case of BA-PEG 400 co-solvent system. The enhancement in solubility also failed to achieve the desired solubility for Artemisinin and Curcumin by using the excipients in the permissible range.

Results and Discussion
Solubility of Artemisinin and Curcumin in individual solvents

The selected solvents have lower polarity as compared to water and are cleared for use in parenterals. The solubility of Artemisinin and Curcumin in different solvents at 25°C was studied and solubility data are shown in Table 1. The enhancement factor varied and the maximum observed was 42600. The enhancement factor was inversely related with solvent polarity. The selected solvents showed higher solubility as compared to water. Ethanol, PEG-400 and benzyl alcohol were found to be better solvents in case of Artemisinin and Curcumin.

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Table 2: Solubility of Artemisinin and Curcumin in binary co-solvent system

<table>
<thead>
<tr>
<th>Smix 1</th>
<th>PEG 400</th>
<th>Water</th>
<th>Solubility (mg/ml)</th>
<th>Artemisinin</th>
<th>Curcumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.40±0.00</td>
<td>0.63±0.461</td>
</tr>
<tr>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>4.32±0.0115</td>
<td>12.32±0.005</td>
</tr>
<tr>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td></td>
<td>6.86±0.0175</td>
<td>18.32±0.023</td>
</tr>
<tr>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td>7.70±0.005</td>
<td>24.20±0.011</td>
</tr>
</tbody>
</table>

Table 3: Composition of ternary co-solvent system and their refractive index

<table>
<thead>
<tr>
<th>Benzy alcohol</th>
<th>PEG 400</th>
<th>Tween 80</th>
<th>Refractive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.90</td>
<td>0.50</td>
<td>1.32</td>
</tr>
<tr>
<td>0.20</td>
<td>0.80</td>
<td>0.45</td>
<td>1.33</td>
</tr>
<tr>
<td>0.30</td>
<td>0.70</td>
<td>0.45</td>
<td>1.32</td>
</tr>
<tr>
<td>0.40</td>
<td>0.60</td>
<td>0.30</td>
<td>1.32</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>0.30</td>
<td>1.31</td>
</tr>
<tr>
<td>0.60</td>
<td>0.40</td>
<td>0.20</td>
<td>1.34</td>
</tr>
<tr>
<td>0.70</td>
<td>0.30</td>
<td>0.25</td>
<td>1.33</td>
</tr>
<tr>
<td>0.80</td>
<td>0.20</td>
<td>0.20</td>
<td>1.32</td>
</tr>
<tr>
<td>0.90</td>
<td>0.10</td>
<td>0.10</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Ternary phase diagram

Ternary co-solvent systems were prepared by using Tween 80 as a surfactant and binary co-solvent system of benzyl alcohol and PEG 400. Addition of surfactant may enhance solubility of poorly soluble drug by micelle formation and also prevent precipitation of the solutes during dilution. Six ternary co-solvent systems were tested. Ternary phase diagram was constructed for a blend of benzyl alcohol, PEG 400 and Tween 80. The refractive index of the ternary solvent system was measured. Table 3 depicts composition of ternary solvent system and their refractive index. The values of the refractive index of different co-solvent system were nearer to the refractive index of water which depicts that given composition of solvents give the single phase system. Phase diagram and the miscibility region are shown in Figure 1.
Development of parenteral injection

On the basis of solubility data obtained from the ternary co-solvent system, formulation of aqueous injection of Artemisinin and Curcumin was prepared using the selected ternary co-solvent system. This formulation contained 90 mg of Artemisinin and 180 mg of Curcumin in mixed solvent system (20% benzyl alcohol, 64% PEG 400 and 16% Tween 80). Other additives like 0.1% sodium bisulfate (antioxidant), 4% mannitol (osmogen) and water for injection quantity sufficient to prepare 3 ml of parenteral injection.

Drug–excipients compatibility studies

UV spectral studies

UV spectra of Artemisinin and Curcumin were recorded. The UV spectra of both drugs (depicted in Figure 2 and 3), show that there was no change in wavelength maxima of Artemisinin and Curcumin in the selected solvents. It can be inferred that both the drugs are compatible with the selected solvents.

Figure 2. UV spectra’s of Artemisinin in different solvents A: UV spectra of Artemisinin in Benzyl alcohol, B: UV spectra of Artemisinin in PEG 400, C: UV spectra of Artemisinin in Binary mixture of BA and PEG 400, D: UV spectra of Artemisinin in Ternary co-solvent
Figure 3. UV spectra’s of Curcumin in different solvents A: UV spectra of Curcumin in Benzy alcohol, B: UV spectra of Curcumin in PEG 400, C: UV spectra of Curcumin in Binary mixture of BA and PEG 400, D: UV spectra of Curcumin in Ternary co-solvent

FTIR study

The possible interaction between Artemisinin, Curcumin and their mixture with co-solvent system (benzyl alcohol, PEG 400, and Tween 80) was examined by Infrared spectroscopy. The infrared spectra of drugs and drug co-solvent mixture are shown in Figure 4. The IR spectra indicates compatibility between Artemisinin, Curcumin and co-solvent system. The spectra were compared with infrared spectrum of untreated drug. All the functional group frequencies were present.
Figure 4. FTIR Spectra’s of Artemisinin and Curcumin (CU) A: FTIR spectra of untreated Artemisinin, B: FTIR spectra of untreated Curcumin, C: Liquid FTIR spectra of final co-solvent system (ART+CU+BA+PEG 400 +Tween 80)

Differential Scanning Calorimetric study

DSC thermograms of Artemisinin, Curcumin and their mixture with co-solvent system are presented in Figure 5. The DSC thermogram of Artemisinin exhibited a single sharp exothermic peak at 155°C. Curcumin exhibited a single sharp endothermic peak at 180°C. Liquid DSC thermogram of the investigated co-solvent mixture exhibited the characteristic exothermic peak of Artemisinin at 155°C and endothermic peak of Curcumin at 180°C indicating the absence of interaction between the two drugs in the optimized co-solvent system.
Characterization of aqueous injection

pH, clarity, viscosity, surface tension and specific gravity

The pH of formulation was found to be 7.33± 0.0173, which is within acceptable range (pH 6.8 to 7.4). Hence, the formulation would not cause any irritation upon administration. Formulation was critically observed against black and white background which showed absence of particulate matters. Hence, clarity of formulation was found to be satisfactory. The mean viscosity of the optimized formulation was found to be 10.49 centipoise. Viscosity of parenteral formulation is a very important criteria for injectability and syringeability [23]. As the solution has less viscosity it has good syringeability and injectability. The results of viscosity, surface tension and specific gravity are shown in Table 4.

Table 4: Viscosity, Surface tension and Specific gravity of formulation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Viscosity (cp)</th>
<th>Surface Tension (dyne/cm)</th>
<th>Specific Gravity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.8379</td>
<td>72.8</td>
<td>-</td>
</tr>
<tr>
<td>Formulated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection</td>
<td>10.55</td>
<td>59.9</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>10.48</td>
<td>59.5</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>10.46</td>
<td>58.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Drug content

The drug content of Artemisinin and Curcumin was found to be 89.82 mg/ml for Artemisinin i.e. 99.8 % and 179.94 mg/ml for Curcumin i.e. 99.96 %.

Osmolarity

Osmolarity of parenteral preparation should be in range of 285- 310 mOsmol/L to avoid cellular damage. Osmolarity of the solution of the optimized formulation prepared using 4% mannitol was 297 mOsmol/L, which is within acceptable range.

Leaching/extraction test

The effect of container on pH, viscosity and drug content of the formulation was measured. From the results, it is concluded that glass vial, amber colored glass ampoule and flint glass ampoule had no significant effect on formulation characteristic as the pH of formulation remain unchanged which indicates absence of leaching of any alkali or acid material from containers. The drug content was unaltered.

In-vitro erythrocyte toxicity study
The *in vitro* erythrocyte toxicity study was carried out to check safety of prepared parenteral formulation. Benzyl alcohol, PEG-400, Tween 80 and optimized formulation showed considerably less than 10% hemolytic activity which is consider as a safe and this formulation can be used parent rally.

**In-vitro anti-malarial assay**

*In vitro* anti-malarial assay showed the concentration which inhibits the complete maturation of ring stage parasites into schizonts. The minimum inhibitory concentration (MIC) was recorded. Quinine was used as the reference drug. The parenteral injection of Artemisinin and Curcumin was taken as test sample and MIC was recorded. The MIC of quinine and marketed combination of Artemether and Lumeafantrine was also measured. The MIC of parenteral injection of Artemisinin and Curcumin was found to be 0.024 µg/ml, which is lower than the MIC of quinine (0.268 µg/ml) and marketed formulation containing Artemether and Lumeafantrine (0.033 µg/ml). It may be concluded that the prepared parenteral injection of Artemisinin and Curcumin is more efficacious than quinine and marketed formulation containing Artemether and Lumeafantrine.

**Sterility test**

The optimized formulation showed clarity in both ATGM and SCDM media after 7 days. This results ensures sterility.

**Stability study**

Formulated aqueous injection was subjected to stability testing at 5°C±3°C, at room temperature and at 40±2°C/75±5% RH. Results of the stability study of formulation showed that it remains unchanged in respect of pH and color. Precipitate formation was not observed at different storage conditions, showing appreciable physical stability. Chemical stability was also noticed.

**Conclusion**

Binary solvent systems failed to give the required solubility of both the drugs. The ternary system comprising of benzyl alcohol, Tween 80 and PEG 400 was more efficacious as compared to the binary system. Interaction was not observed between the drug and the excipients. The *in vitro* tests showed efficacy of the formulation. The aqueous injection containing the co-solvent system with 0.1% sodium bisulfate, 4% mannitol and water for injection in sufficient quantity to prepare 3 ml of aqueous injection may be considered as a promising formulation for malarial therapy.

**Author’s contribution**

Everyone who is listed as an author has made a substantial contribution to the work. Dr. Vaishali T. Thakkar, contributed on development of complete experimental set up. Ms. Rachna Dhankecha, hadworked on innovative idea and execution of experiments of research work. Dr. Mukesh C Gohel, contributed in troubleshooting and data interpretation. Dr. Purvi Shah, was involved in development of analytical method for estimation of Artimisinin and Curcumin and editing of manuscript also. Ms. Tosha Pandya, contributed in analysis of data and editing of manuscript also. Dr. Tejal Gandhi was involved in designing of experimental procedure, In vitro antimalarial experimentation and erythrocytes toxicity study of prepared formulation.

**Acknowledgements**

We would like to acknowledge Sri Ramkrishna Seva Mandal for providing the funding and research facilities to carry out this project.

**Conflict of Interest**

The authors declare no conflict of interest.

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