

Evaluation and Preparation of Crystal Modifications of Artesunate: *In vivo* Studies

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Abstract—Five crystal modifications of water insoluble artesunate were generated by recrystallizing it from various solvents with improved physicochemical properties. These generated crystal forms were characterized to select the most potent and soluble form. SEM of all the forms showed changes in external shape leading them to be different morphologically. DSC thermograms of Form III and Form V showed broad endotherm peaks at 83.04°C and 76.96°C prior to melting fusion of drug respectively. Calculated weight loss in TGA revealed that Form III and Form V are methanol and acetone solvates respectively. However, few additional peaks were appeared in XRPD pattern in these two solvate forms. All forms exhibit exothermic behavior in buffer and two solvates display maximum ease of molecular release from the lattice. Methanol and acetone solvates were found to be most soluble forms and exhibited higher antimalarial efficacy showing higher survival rate (83.3%) after 30 days.

Keywords—Artesunate, Crystal modifications, *in vivo* studies, Recrystallization.

I. INTRODUCTION

MOST of the drugs available in the market are in solid forms. The physicochemical properties of the drugs in solid-state such as melting point, solubility, true density, drug release profile, flowability and tableability are influenced by the existence of different crystalline forms [1]-[5]. The distinct crystalline forms have different processing issues and/or chemical stability which usually have direct impact on solubility and bioavailability [6], [7]. However, therapeutic effectiveness of a drug or its crystal modifications depends on the physicochemical parameters (different rates of uptake in the body, lead to lower or higher biological activity than desired) especially on the bioavailability which ultimately depends on the solubility of the drug molecules [8]. Slow drug dissolution in biological fluids, insufficient and inconsistent systemic exposure and subsequent inadequate efficacy in patients, are some of routine challenges to be coped with during the development of poorly water-soluble drug substances especially when they are administered orally [9], [10].

Of late tremendous efforts have been directed towards the prefabrication of existing drug molecules for solving predicaments related to poor water solubility, bioavailability, dosing problem, stability and toxicity [11]. One of the thriving

trends in enhancing the solubility, dissolution rate through habit recrystallization of poorly soluble drugs is to deal with crystal forms of materials which could potentially be applicable to a broad range of drugs with different crystalline habits [12].

Despite enhancements in solubility and dissolution rate and oral bioavailability of poorly water-soluble drugs with the customary pharmaceutical technologies, still there are concerns about the success of those methods in the complexities arising from the specific physicochemical nature of the drug molecule itself [13].

A number of reports in the literature show that different crystal forms are generated by using recrystallisation, lyophilisation, and slurry method. These reported methods validated the effects of variation in crystal morphology on solubility, *in vitro* dissolution rate, improvement in drug bioavailability [14], [15]. In the present study, this method was applied to investigate alternative forms of artesunate which were generated using different solvents. Artesunate is potent blood schizonticidal antimalarial drug active against *Plasmodium falciparum* strains [16]. The drug belongs to Class II of the Biopharmaceutics Classification System (BCS) that shows poor water solubility and low bioavailability [17]-[19]. As for BCS class II drugs, rate limiting step is drug release from the dosage form and solubility in the gastric fluid and not the absorption. Therefore, increasing the solubility in turn increases the bioavailability for BCS class II drugs [20], [21]. These poorly water soluble drugs are allied with slow drug absorption leading to inadequate and variable bioavailability and gastrointestinal mucosal toxicity [22]. These prepared alternate crystal forms were characterized and subjected to solubility and dissolution studies to select the appropriate form with improved solubility. Pharmacological activities of these alternate crystal forms were also evaluated as crystal modifications which have great impact on *in vivo* performance i.e. bioavailability.

II. MATERIALS AND METHODS

A. Materials

Artesunate was obtained as a gift sample from IPCA Laboratories Pvt. Ltd. (Mumbai, India) and analytical grade solvents acetonitrile, acetone, ethyl acetate, propanol, toluene, 1,4-dioxane, ethyl methyl ketone (EMK) toluene, dichloromethane and methanol (Sigma Aldrich) were used.

B. Preparation of Modified Crystal Forms

Various forms were obtained by recrystallizing the drug from various solvents ranging from polar to non polar.

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Commercial sample of artesunate was recrystallized from hot saturated solutions of acetonitrile, acetone, ethyl acetate, propanol, toluene, 1,4-dioxane, ethyl methyl ketone and mixed with acetonitrile and triply distilled water (2:1) respectively.

Besides this, two more forms were obtained by adding hot water as antisolvent to saturated solution of drug in methanol and acetone respectively. These were further categorized after DSC and XRPD studies.

C. Characterization

The prepared various crystal forms of artesunate were characterized by different analytical techniques such as DSC, FT-IR, XRPD, SEM, enthalpy of solution, solubility and dissolution studies.

1. Scanning Electron Microscopy (SEM)

A Jeol JSM-6100 scanning electron microscope was used to obtain photomicrographs of artesunate and its different forms. Samples were mounted on a metal stub with an adhesive and coated under vacuum with gold.

2. Differential Scanning Calorimeter (DSC)

DSC thermograms were obtained on DSC, Q20, TA Instruments-Waters LLC, USA. The calorimeter was calibrated for temperature and heat flow accuracy using the melting of pure indium (mp 156.6 °C and ΔH of 25.45 Jg⁻¹). A mass between 2-8mg was taken into the aluminum pan, covered with lid and sealed. DSC curves were obtained under a nitrogen purge of 50mL per minute at a heating rate of 10°C per minute with the temperature range from 50-350°C.

3. X-Ray Powder Diffraction (XRPD)

The powder diffraction patterns were recorded on an X-ray diffractometer (XPRT-PRO, PANalytical, Netherlands, Holland) with Copper as tube anode. The diffractograms were recorded under following conditions: voltage 40 kV, 35mA, angular range 5 and fixed divergence slit. Care was taken to avoid crystal changes during sample preparation. Approximately 200mg of samples were loaded into the sample holder, taking care not to introduce preferred orientation of the crystals.

4. Fourier Transform Infrared spectrometry (FT-IR)

The FT-IR spectra were obtained on FT-IR spectrometer, Mode spectrum RXI, (Perkin Elmer, England) over the range 400 – 4000cm⁻¹. Dry KBr (50mg) was finely ground in an agate mortar and sample of the drug or the solvate (1-2mg) was subsequently added and mixed gently. A manual press was used to form the pellet.

5. Solution Calorimetry

Isoperibol solution calorimeter model 4300 (Calorimetry Science Corporation, Utah, USA) was used for thermal measurements. It is a semi-adiabatic calorimeter with temperature resolution, after noise reduction, close to 1μK, which corresponds to a heat resolution of 1-4mJ in a 25ml buffer (pH 7) reaction vessel. The details are given in our previous papers [23], [24]. The performance of the system was tested by measuring enthalpy of solution of potassium chloride

(17.301kJ/mol) in triple distilled water, which is in good agreement with known enthalpy of solution of 17.322kJ/mol. The precision of any individual measurement was better than ± 0.03 kJ/mol for three consecutive experiments.

6. Aqueous Solubility Measurement

MSW-275 (Macro scientific works, New Delhi) shaker was used for measuring aqueous solubility of different forms of artesunate. Solubility studies were performed by adding 50mg of artesunate or its crystal forms in mixture of methanol and phosphate buffer (pH 7) in ratio of 2:1. The mixtures were shaken at 37°C for 24h. The aliquots were filtered through 0.45μm membrane filter and analyzed spectrophotometrically at 240nm. The standard plot of artesunate was prepared by dissolving a weighed amount of the drugs in a mixture of phosphate buffer (pH 7) and methanol, suitably diluted and $E_{1\%}^{1\text{cm}}$ was calculated.

7. Intrinsic Dissolution Study

The dissolution studies were carried for 4h, at 37±0.5°C in phosphate buffer (pH 6.8) as dissolution media. The dissolution studies were performed using paddle apparatus-12 equipped with paddles rotating at 50rpm in 500mL of phosphate buffer (pH 6.8) pre-equilibrated to 37±0.5°C. An appropriate amount of the commercial sample or the solvate was then introduced. The aliquots were withdrawn and analyzed after 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150, 180, 240 and 300 minutes. Each dissolution study was performed on duplicate batches and absorbances taken at wavelength 240 nm on a spectrophotometer.

8. In vivo Studies

Plasmodium berghei (NK 65) strain was used for evaluation of antimalarial activity *in vivo* studies and was maintained in BALB/c mice by intraperitoneal (ip) inoculation of infected blood. 4-5 Week old BALB/c mice (25-30g) were procured and maintained in the Central Animal House and were provided with standard pellet diet and water *ad libitum*. Experiments were performed as per guidelines of Committee for the Purpose of the Control and Supervision of Experiments on Animals. The experimental protocol was approved by Institutional Animal Ethics Committee (CAH/11/2010), Panjab University, Chandigarh.

D. Experimental Design

Animals were divided into 7 groups and each group comprised of 6 animals (n = 6). To monitor the efficacy and potency of prepared forms a dose 6mg/kg were administered twice to all the animals on day 1 of post inoculation (PI) for 7 days. Artesunate and its forms were suspended in 0.5% carboxymethyl cellulose. Each animal was treated with artesunate equal to 100μl. Percent parasitemia was monitored on every alternate day for up to 30 days by tail blood smear, fixed in methanol and stained with Giemsa stain by counting at least 500 cells.

E. Challenge of the Experiment Animals and Follow up of the Experimental Animals

All the mice belonging to control group were challenged with 10^6 *P. berghei* infected RBCs ip. After challenge, mean percent parasitemia, percent activities of drug and its various forms were calculated. Mean percent parasitemia was calculated for each group at different interval of time (days).

$$\text{Mean percent parasitemia} = \frac{\text{infected RBCs} \times 100}{\text{Total no. of RBCs}}$$

F. Statistical Analysis

Data was expressed as mean \pm S. D. Parasitemia of the artesunate and its forms were statistically assessed by one-way ANOVA followed by Turkey's test using Jandel sigma stat 2.0 version. Differences were considered significant at $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Scanning Electron Microscopy

Habit modification normally arises due to the change in the environmental conditions of growing crystals which leads to a change in its external shape without changing its internal structure. Artesunate and its forms with different crystal habit are given in Fig. 1. The Form I and Form II were depicted in filliform habit as sheets and tabular habit with the melting at 134.06 and 142.90°C respectively. The form III was obtained from methanol using water as antisolvent which did not show any characteristic shape. Form IV appeared as cylindrical rods when recrystallized from acetone, whereas, Form V recrystallized from acetone and water as antisolvent adopted prismatic thin plate's morphology. The crystal habit of the untreated artesunate was massive bladed-



Fig. 1 Scanning electron micrographs of artesunate and its crystal modifications

B. Differential Scanning Calorimetry

DSC in conjunction with TGA gives first hand information to differentiate pseudopolymorph from true polymorph. Desolvation/dehydration of the solid in DSC is accompanied by endothermic peak indicating that the particular form is a solvate/hydrate. Percent mass loss in the TGA determines quantitatively the loss of solvent depending upon the stoichiometry of the solvate formed [25].

The commercial sample melts at 140.05°C which is simultaneously followed by decomposition exotherm at 157.84°C. Form I and II showed a single endothermic peak at 134.06°C and 142.90°C respectively and enthalpy of fusion accompanying the melting is was found to be 66.78 J/g and 73.47 J/g respectively (Fig. 2).

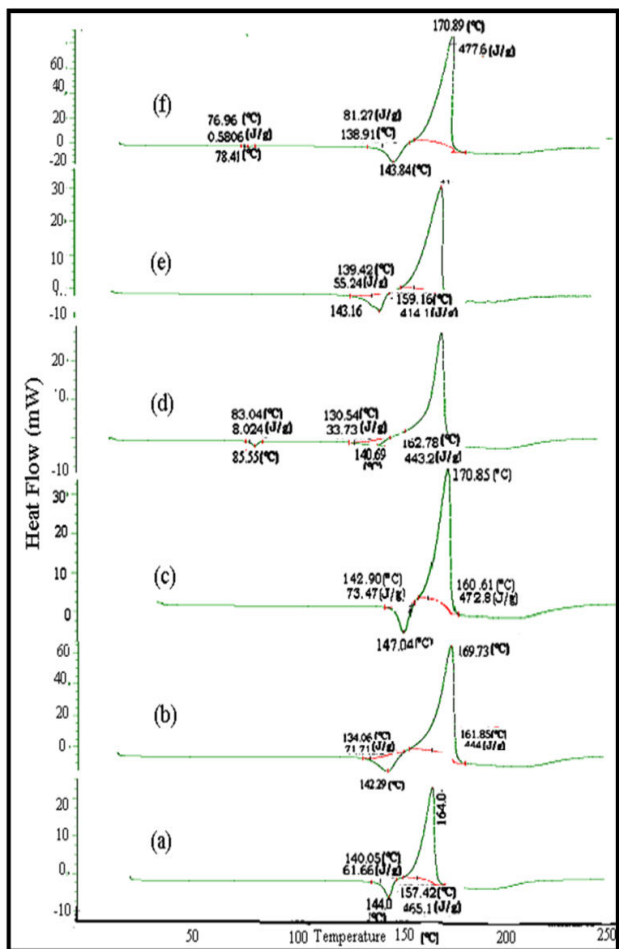


Fig. 2 DSC thermograms of artesunate and its crystal modifications; (a) commercial sample (b) Form I (c) Form II (d) Form III (e) Form IV (f) Form V

DSC scans of Form III showed a small broad peak at 83.04 °C which is followed by a melting at 130.54°C. The Form III was subjected to TGA and a weight loss of approx. 8.7% (Fig. 3) was observed in the range of 60-90°C. Calculated total weight loss suggested (96%) this form to be methanol solvate with 1:1 stoichiometry. No thermal events intervene between the solvent loss and melting.

Form IV displayed a single sharp melting endotherm at 138.83°C with no other thermal events indicating it to be phase pure form.

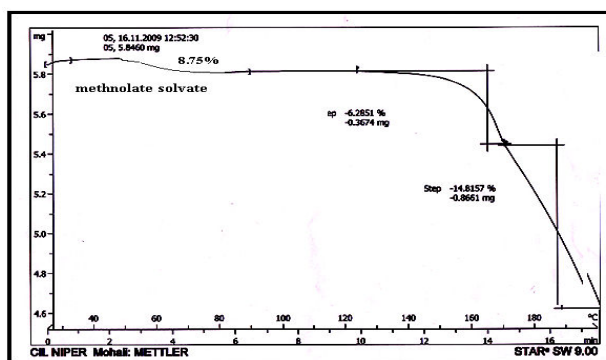


Fig. 3 (a) Thermo gravimetric analysis of Form III of artesunate

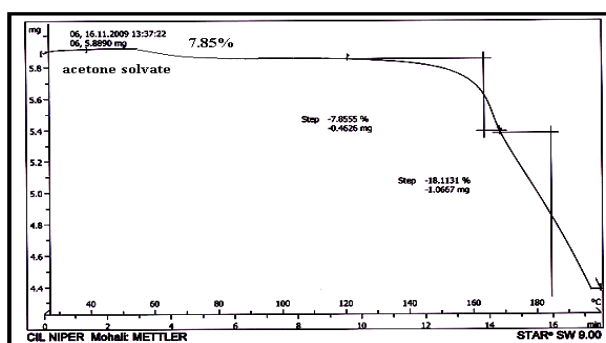


Fig. 3 (b) Thermo gravimetric analysis of Form V of artesunate

DSC scan of Form V showed a broad peak at 76.96°C prior to melting and accompanied by weight loss of 7.85% in TGA. Calculated loss of weight (7.7%) and position of the peak suggested the loss of one molecule of acetone per two molecule of drug. This indicated the form V to be acetone solvate with 1: 0.5 stoichiometry.

C. X-Ray Powder Diffraction

The commercial sample showed well defined XRPD patterns with major diffraction peaks at $2\theta = 9.458, 12.23, 12.42, 12.83, 13.04, 15.35, 15.54, 18.54, 18.64, 19.7, 19.89^\circ$. The intensity of peak in terms of no. of counts corresponding to 100% relative intensity appears at $2\theta = 13.04^\circ$ with 10203 counts. It is clear from Fig. 4 that there is not much difference in the positions of peaks present in Form I and II.

However, the peak intensities are quite different which may be attributed to either crystal orientations or the crystal habit of the sample. Form III and Form IV have shown characteristic unique peak at 7.76° and 7.57° respectively confirming these to be a different forms. Besides this, there is some shift in the position of few of the major peaks (Fig. 4). Peak corresponding to 100% relative intensity of drug is shifted to new 2θ values at 9.46° and 18.45° in form III and IV respectively indicating these to be different forms.

Similarly, in Form V, a new peak appeared at 6.78° which is not present in any of the forms and peak corresponding to 100% relative intensity was shifted to 6.78° . Also there are

some variations in positions and intensities of peaks suggesting the form to be different from other forms.

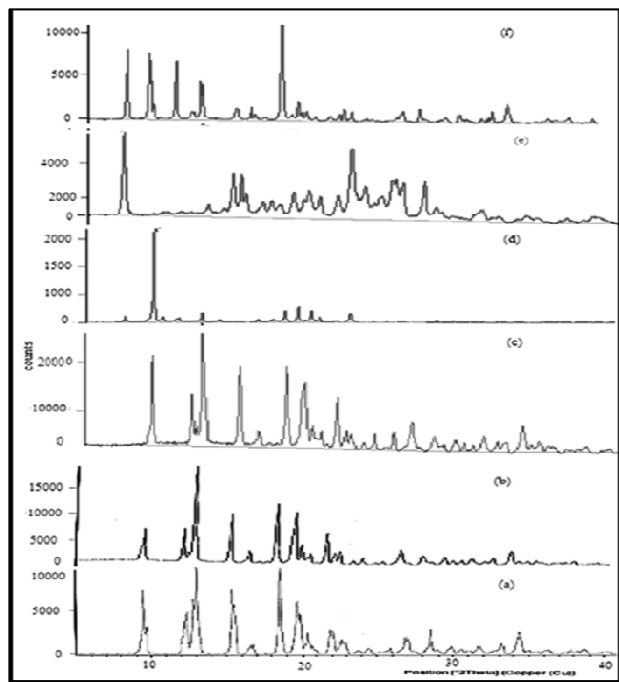


Fig. 4 XRPD pattern of artesunate and its crystal modifications; (a) commercial sample (b) Form I (c) Form II (d) Form III (e) Form IV (f) Form V

Now, to identify the final form to which the solvated form III and V are converted after desolvation, both the forms were heated at 100°C for 1hr under nitrogen gas atmosphere then cooled to room temperature in a dessicator. The PXRDP pattern of heat treated sample (Form III) shows the absence of characteristic peak at 7.76° and resembles to the original sample suggesting the conversion of methonlate to desolvated commercial sample.

The PXRDP pattern of the desolvated acetone is found to be similar to that Form IV suggesting that the removal of acetone in the solvate changes the crystal lattice (Fig. 5). These results support our DSC findings which show that desolvation is succeeded by sharp melting endotherm at 138.40°C corresponding to the melting point of form IV.

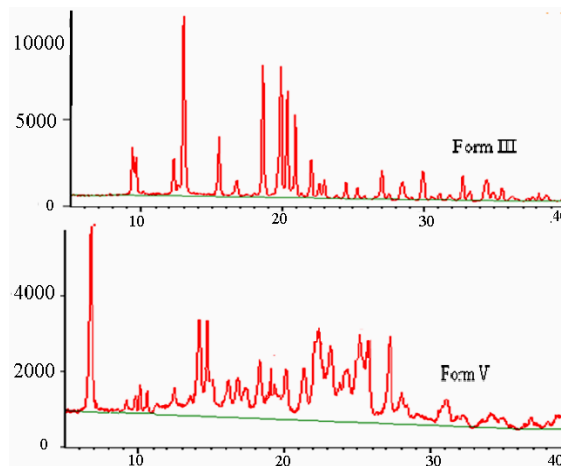


Fig. 5 XRPD pattern of after desolvation

D. Fourier Transform Infrared Spectroscopy

Differences in the FTIR spectra of artesunate and all the solvates indicate differences in their structure and therefore, provide information regarding the intermolecular interactions within the solvates.

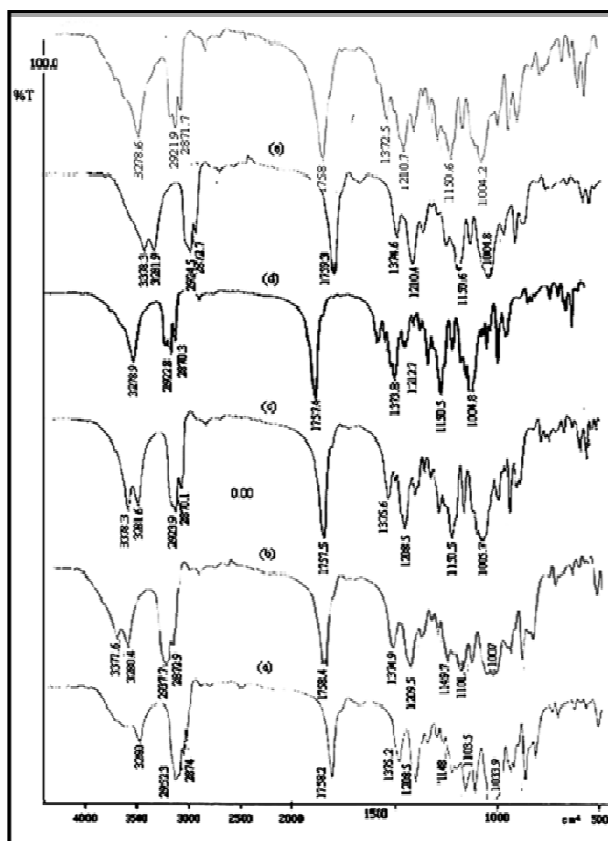


Fig. 6 FT-IR spectra of artesunate and its crystal modifications; (a) commercial sample (b) Form I (c) Form II (d) Form III (e) Form IV (f) Form V (g) Form VI

FTIR spectra of drug and its forms (Form I, II and IV) showed insignificant differences and could not be provided with more information (Fig. 6). However, shifting and appearance of new peaks were observed in Form III and V of artesunate.

E. Enthalpy of Solution

The difference in the enthalpy of solution is due to the difference in the lattice energy of the solids. All the forms exhibited exothermic behavior in the buffer (pH 6.8) but the magnitude varies from one form to another. Increased exothermic behavior of methanol solvate and acetone solvate indicates the ease of molecular release from the lattice. Molar enthalpy of solution (ΔH_{sol}) of all the forms were calculated (Table I) which followed this order: Form III > Form V > Form IV > Form I > commercial sample > Form II.

F. Solubility Studies

The solubility data (Table I) suggests that Form II was least soluble (1.18mg/10ml) while Form III was most soluble (3.55 mg/10ml) with a 2 times increase in solubility in comparison to commercial sample.

TABLE I

ENTHALPY OF SOLUTION AND SOLUBILITY OF ARTESUNATE AND ITS CRYSTAL MODIFICATIONS

Sample	Molar enthalpy of solution (ΔH_{sol}) (kJ/mol)	Solubility (mg/10ml)
Artesunate	-22.16	1.77
Form I	-33.6	2.19
Form II	-17.45	1.18
Form II	-98.89	3.55
Form IV	-40.98	2.83
Form V	-87.23	3.35

G. Dissolution Studies

Several studies in this filed have shown that exposure of diverse crystal faces determines the nature of the wettability and consequent enhancements in dissolution rate of the drugs with different crystalline shapes [26]. The powder dissolution curves of different forms showed differences in the rates of dissolution and both the solvates are less crystalline, more soluble have shown higher dissolution rates.

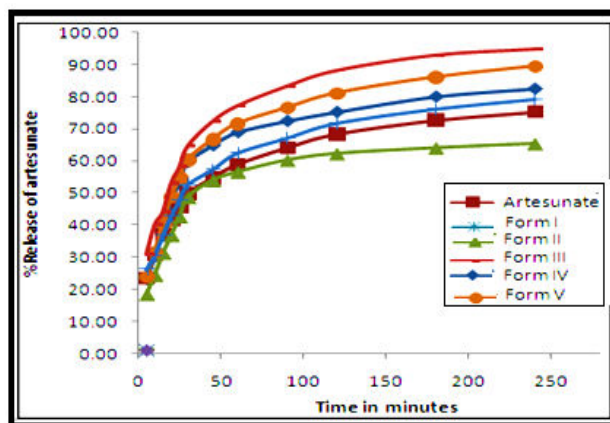


Fig. 7 Dissolution profile of artesunate and its crystal modifications

All the prepared forms followed the same pattern as revealed by the order of crystallinity, heat of solution, PXRD and solubility results. The results of dissolution studies were presented in graph and shown in Fig. 7 as the mean % release vs. time

H. In vivo studies

In vivo studies were performed to check that the most soluble solvates are more effective to eradicate the parasite from the blood. It is clear from the Table II that artesunate alone (standard group) is insufficient to prevent the mortality. However, survival time was increased (day 15-19) compared to control (day 9) but was found to be ineffective in preventing mortality. The suspensions of all the forms were administered on day 1 of PI. At the end of the treatment (day 8), when compared with control (45.56 ± 3.78), the test group 1 (32.45 ± 4.28), the test group 2 (32.45 ± 4.28), test group 3 (21.73 ± 2.016) test group 4 (18.34 ± 3.76) and test group 5 (12.45 ± 1.43) showed significantly less ($p < 0.001$) mean percent parasitaemia (Fig. 8). It is observed that percent mortality rate is minimum (16.7 %) in Form III and form V which suggested that these are physiologically more potent and effective as to commercial sample.

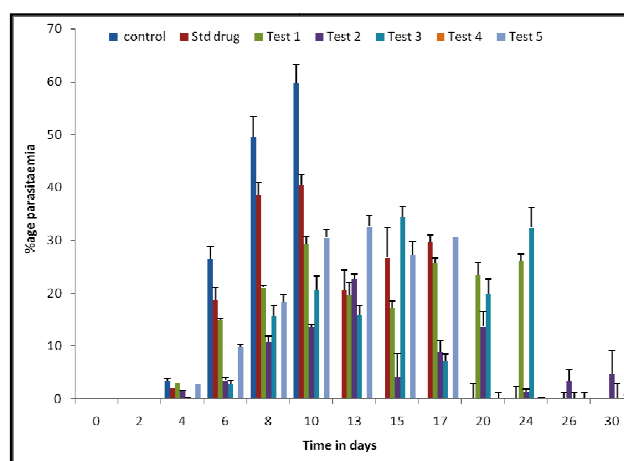


Fig. 8 Percent parasitaemia observed in *P. berghei* infected mice

TABLE II
ANTIMALARIAL ACTIVITY OF ARTESUNATE AND ITS CRYSTAL MODIFICATIONS IN P. BERGHI INFECTED MICE

S. No	Groups	Treatment	Mean % Parasitaemia* on day 8 th PI	% mortality
1	Control group	0.5% CMC solution	49.71 ± 5.04	100
2	Standard group	Artesunate ~ (6mg/kg)	31.23 ± 2.09	50.0
3	Test group 1	Form I	17.45 ± 4.28	50.0
4	Test group 2	Form II	10.54 ± 1.03	16.7
5	Test group 3	Form III	21.73 ± 3.62	33.3
6	Test group 4	Form IV	28.98 ± 4.76	33.3
7	Test group 5	Form V	36.45 ± 1.36	66.7
8	Test group 6	Form VI	12.34 ± 1.26	16.7

IV. CONCLUSION

In summary, the investigation shows that all the forms were found to be morphologically different as to commercial drug. DSC in conjunction with TGA revealed that Form III and Form V were methanol solvate and acetone solvate with 1:1 and 1:0.5 stoichiometry respectively. In addition, few new peaks were appeared in XRPD pattern of methanol and acetone solvate.

Significant changes in peaks of XRPD pattern were observed in both these solvates which revealed to be different from drug sample. Exothermic behavior was exhibited by all the forms in enthalpy of solution. These two solvates were found to be most soluble forms and less crystalline among all the forms. The increased solubility and dissolution rate of these two forms also leads to much improved in *in vivo* antimalarial activity.

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