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Research Article

CHARACTERIZATION, THERMODYNAMIC PARAMETERS, MOLECULAR MODELING AND *IN VIVO* STUDIES OF INCLUSION COMPLEXES OF PYRIMETHAMINE WITH NATIVE β-CYCLODEXTRIN AND ITS DERIVATIVES

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ABSTRACT

Inclusion complexes of pyrimethamine with β-cyclodextrin (β-CD) and its derivatives (methyl and hydroxypropyl) were prepared with an aim to increase its solubility as well as to boost its antimalarial activity. Phase solubility studies and mass spectrometry indicated a 1:1 stoichiometry for pyrimethamine cyclodextrin (CD) complexes which were confirmed by solution calorimetry. Stability constant (K) and other thermodynamic parameters determined by solution calorimetry indicated that the inclusion of drug is exothermic process accompanied by negtive value of enthalpy (ΔH^o) and small positive value of entropy (ΔS^o). Molecular modelling calculations performed with *Fast Rigid Exhaustive Docking* acronym for drug cyclodextrins depicted the existance of two types of complexes differing slightly in energy of binding. The experimentally determined Gibb's free energy (ΔG^o) is found to be closer to complex-2 where chlorobenzene ring snugly fits into the cavity and is supported by 2D NMR. Efficiency of the encapsulated drug was evaluated by *in vitro* drug release and lyophilized complexes of pyrimethamine with M-β-CD found to be best. The *in vivo* studies were performed to predict the pharmacological activity and have shown significantly higher survival rate for β-CD (50%), M-β-CD (83.3%) and HP-β-CD (67.3%) complexes respectively as compared to the commercial drug (16.7%).

Keywords: Pyrimethamine; β-cyclodextrins; Inclusion complexes; Stoichiometry; *In vivo* studies; Dissolution efficiency

INTRODUCTION

Pyrimethamine, an antiprotozoal drug is a folic acid antagonist which reversibly binds to enzyme dihydrofolate reductase in protozoa and prevents the nucleic acid and amino acid biosynthesis[1-2]. However, its inherent poor water solubility (0.12 mg/mL)[3-5] demands high doses to provide a satisfactory inhibition of parasite proliferation which is associated with aggravated side effects[6-7]. The potency of this therapeutically useful molecule can be improved by complexing it with the water soluble cyclodextrins. Encapsulation of the drug with cyclodextrins (CDs) has become an increasingly successful approach to enhance the bioavailability and is very well documented in literature[8-14]. The key factor for deciding the solublization efficacy of the complexes is to compare their stability constants (K)[15-19]. Thus, the determination of K along with other thermodynamic parameters is pre-requisite for formulation development. The present work report the characterisation of the inclusion complexes of pyrimethamine (pyri) with β-CD, M-β-CD and HP-β-CD. Molecular modeling calculations providing complementary evidences for inclusion mode and stiochiometry of the complex formation are also reported. The pharmacological activity of encapsulated complexes was also performed to evaluate their efficacy which has been correlated with the complexing abilities of different CDs. Three reports emerged during literature survey have demonstrated the effect of HP-β-CD and α-CD on the solubility of the drug[19-21]. However, the thermodynamic parameters accompanying the encapsulation as well as *in vivo* studies are completely lacking. Moreover, the studies involved only the HP-β-CD and no attempt was made to compare the complexing abilities of other suitable derivatives of β-CD with native β-CD. Therefor, the focus of the present study is tilted towards the determination of stability constant and other thermodynamic parameters associated with host guest interaction between the above mentioned molecules.

MATERIAL AND METHODS

Materials

Pyrimethamine was obtained as gift sample from Ipca Laboratories ltd. Mumbai. India. β-CD, M-β-CD and HP-β-CD were obtained from Sigma Aldrich. The other analytical grade chemicals such as sodium hydroxide, potassium hydrogen phosphate, sodium dihydrogen phosphate were procured from SD fine Chemicals. *In vivo* experiments were performed as per guidelines of committee for the purpose of the control and supervision on experiments on animals (CPC-SEA). The experimental protocol was approved by Institutional Animal Ethics Committee (A. I. E. C.).

Preliminary Studies

Phase solubility studies

Phase solubility diagrams of pyrimethamine with various CDs in phosphate buffer (pH 6.8) were obtained according to Higuchi and Connors[22]. An excess of drug was added to 10 mL buffer or CD buffered solutions (0.002 to 0.01 M) in 20-ml glass vials. The suspensions were sealed and shaken in water-bath shaker MSW-275(Macroscientific works, Delhi) at 37±0.5 ^oC for 24 h to ensure equilibrium. After equilibration, aliquots of the supernatant were withdrawn, filtered through 0.45 μm milipore filter paper, and the pyrimethamine content (after suitable dilution), was determined spectrophotometerically at λ 272 nm (UV/VIS. Spectrophotometer (Perkin Elmer Lamda 15, USA). The presence of CDs did not interfere with the spectrophotometric assay of the drug.

Preparation of CD formulations

Various Pyri-CD formulations were prepared in a 1:1 molar ratio on the basis of the results obtained from preliminary phase solubility studies by following methods

(a) **Physical mixing**: Physical mixtures were prepared by simple mixing of drug with different CDs in mortar and to ensure uniform mixing, the vial was subjected to vortex mixing for 5 min.

(b) **Kneading**: Drug and CDs were blended together in mortar with water; a paste was obtained and kneaded for 90 min. The product was then dried under vacuum at 40 ^oC for 48 h and passed through a 150 μm mesh and stored in glass vials in vaccum dessicator.

(c) **Freeze-drying method**: the required 1:1 stoichiometric quantity of drug was added to aqueous solution of different CDs and solution in presence and absence of CDs were agitated on magnetic stirrer for 24 hours. The resulting solutions were frozen at (-80 ^0C) in deep freezer for overnight. These were then lyophilized under 17.2 m Torr for 48 hours. The sample was transferred immediately into a vacuum desiccator and dried over silica gel under vacuum for at least 24 hours.

Characterization

These complexes were characterized both in solid and solution phases by the following methods:

Differential scanning calorimetry (DSC)

DSC thermograms of pyrimethamine, pure CDs and their inclusion complexes 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}). The temperature range was from 25-350 °C with a heating rate of 10° C per minute.

X- ray powder diffraction (XRPD)

Powder diffraction pattern of pyrimethamine and their inclusion complexes were recorded using an X-ray diffractometer (XPERT-PRO PANanalytical, Netherlands, Holand) Cu as tube anode. The diffractograms were recorded under following conditions: voltage 40 kV, 35 MA, angular range 5, fixed divergence slit.

Mass spectrometry

ESI-MS were performed using a Q-ToF quadruple time of flight mass spectrometer (Waters) equipped with an electrospray source. The sample was introduced via a syringe pump at a flow rate of 5 µL/min. High flow rate nitrogen gas was employed as the nebulizing gas as well as the drying gas to aid desolvation. The sheath gas flow rate was 0.5 $\mu L/min^{-1}$. After optimization of the MS parameters, the spray voltage was set to 2.5 kV in the positive mode, and the heated metal capillary temperature was set at 80 ^{o}C. The mass scale was calibrated by using the standard calibration procedure and compounds provided by manufacturer.

Fourier transforms infrared spectrometry (FT-IR)

The FT-IR spectra of Pyrimethamine and their inclusion complexes forms were recorded on FT-IR spectrometer, Mode spectrum RXI, Perkin Elmer, England over 400-4000 cm^{-1} range. Dry KBr (50 mg) was finely ground in mortar and samples of drug and their complexes (1-2 mg) were subsequently added and gently mixed. A manual press was used to form the pellets.

Solution calorimetry

Isoperibol solution calorimetry (ISC) (Calorimetry Science Corporation, UTAH, USA) model 4300 was used for thermal measurements. The calorimeter consists of a constant temperature bath held at 37 °C (±0.005ºC) and heater assembly. The drug was filled into batch adaptor of volume 0.9 mL, sealed on both sides with 'O' rings and cover glass. The batch adaptor holding the drug was inserted into the Dewar flask containing buffer (25 mL). The combined unit was then lowered in the calorimeter bath. The glass stirrer was rotated at 100 revolutions / min. and was allowed to equilibrate for 90 min. The ampoule was shattered automatically by means of plunger and temperature change noted. The performance of the system was checked using KCl, which has known enthalpy of solution and a good agreement (±0.03 kJ/mol) was found with published value.

Proton nuclear magnetic resonance (^{1}H-NMR), ^{13}C NMR and 2D COESY spectroscopy

^{1}H-NMR, ^{13}C NMR and 2D COESY spectra in d_6DMSO of pyrimethamine and inclusion complexes were recorded with a Brucker AC 300 ^{o}C NMR spectrometer apparatus operating at 300 MHz using tetramethylsilane as an internal standard. For 2D COESY experiments, samples were equilibrated for at least 24 hrs.

***In Vitro* dissolution studies**

The dissolution rate studies of the formulation were performed in 900 mL of 0.1 N HCl using USP (12) apparatus at pre-equilibrated temperature 37± 0.5 ºC and at a stirring rate of 50 rpm. Drug and its Inclusion complexes each containing 200 mg of drug were filled in hard gelatin capsules. Samples was withdrawn at different intervals for a period of 2 hr and analyzed spectrophotometrically at λ 272 nm. The results were evaluated on the basis of the dissolution efficiency parameter at 90 min (DE_{90min}). The dissolution parameters were calculated from the area under the dissolution curve and expressed as a percent of the area of the rectangle described by 100% dissolution in the same time period.

Molecular Modeling studies

Computational Details

The computational studies were carried out on an Intel Xeon based system with the Linux Enterprise OS. The structure preparation, simulations and analysis were carried out with *Schrodinger Suite 2010*, (*Schrödinger LLC, New York, NY, 2010*). The docking studies were carried out with *Fast Rigid Exhaustive Docking* acronym (*FRED version 2.2.5, Open Eye Scientific Software, Santa Fe, USA*)[23-24]. The 3D structure of β-CD was retrieved from the Protein Data Bank[25] while pyrimethamine from PubChem (4993). The structures were geometry optimized following atom type and charge assignments based on the OPLS2005 force field.

Docking studies

The β-CD molecule was subsequently imported into the program *FRED-RECEPTOR* (*version 2.2.5*) for docking. First the active site box where the ligand or guest is expected to bind is defined and the shape of the active site is described by two shape potential contours, referred to as the inner and outer contour. It is essential that the docked host guest poses fit within the shape of the outer contour and ensures that the center of at least one heavy atom of any docked pose touches the inner contour. Following this protocol setup, the '*host*' with the defined active site box and shape is utilized for docking of the '*guests*'. During the rigid body docking of the guest molecule into the host, the intrinsic scoring functions were used for the ranking of docking solutions. From a set of 1000 solutions the best 100 were saved. Based on the scoring function two orientations of pyrimethamine were identified in the β-CD cavity, which were subsequently taken up for MD simulations in *Desmond (version 2.4, DE Shaw Research NY, 2010)*.

MD Simulations

Initially the complexes of pyri-β-CD were solvated with TIP3P waters[26] to form a water shell 10 Å^{o} thick around the pyri-β-CD complex. The solvated protein-ligand system was simulated for a period of 5 ns with the '*NPT relaxprotocol*' in *Desmond*. During the protocol execution the system was relaxed to relieve the energetic strain complex due to solvation using a set of short MD simulations of 12–24 ps in sequential NVT and NPT ensembles with the Langevin thermostat and barostat[27]. The temperature was maintained by coupling to an external 300 K bath based on the Langevin algorithm[27]. The pressure was isotropically restrained to 1 bar with the Langevin barostat. High-frequency vibrations were removed by applying the SHAKE algorithm[28] by constraining all bonds to their equilibrium values. Initial velocities were generated randomly from a Maxwell distribution at 300 K in accordance with the masses assigned to the atoms. The trajectories and corresponding energies were sampled every 5 ps. No constraints were applied on the pyri-β-CD system during the simulations, so as to avoid introduction of any artifacts in the ligand conformation in the binding site.

***In Vivo* Studies**

Evaluation of pharmacological antimalarial activity of pyrimethamine in mice

Plasmodium berghei (NK 65) strain was used for evaluation of antimalarial activity *in vivo* studies and was maintained in BALB/c mice by intraperitoneal (i/p) inoculation of infected blood. 4- 5 weeks 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 libtum*.

Experimental design: Animals were divided into 5 groups and each group comprised of 6 animals (n=6). These were treated orally with single dose therapy (6 mg/kg) two times a day on 1 day of PI for 7 days to monitor the efficacy and potency of prepared lyophilized complexes.

1. Control group — treated with 0.5 % carboxymethyl cellulose (CMC) suspension;
2. Standard group—administered pyrimethamine in 0.5 % CMC suspension;
3. Test group 1— treated with pyri-β-CD complex in 0.5 % CMC suspension;
4. Test group 2— treated with pyri-M-β-CD complex in 0.5 % CMC suspension;
5. Test group 3— treated with pyri-HP-β-CD complex in 0.5 % CMC suspension;

Preparation and administration of doses: Pyrimethamine and its complexes were suspended in 0.5 % carboxymethyl cellulose (CMC). Each animal were treated with 100 µl pyrimethamine and its various lyophilized complexes.

Challenge of the experiment animals and Follow up of the experimental animals

All the mice belonging to control group was challenged with 10^6 *P. berghei* infected RBCs i/p. After challenge mean percent parasitaemia, percent activities of various complexes of pyrimethamine along with animal survivality were monitored. Mean percent parasitaemia was calculated for each group at different interval of time (days).

Mean percent parasitaemia = infected RBCs x 100/ Total no. of RBCs

Percent Parasitaemia

Percent parasitaemia was monitored on every alternate day for up to 30 days by tail blood smear, fixed in methanol and stained in Giemsa stain by counting at least 500 cells.

Statistical analysis

The differences between multiple groups of dissolution efficiency data (DE_{90min}) were assessed by analysis of variance followed by Turkey's post test to determine the level of significance between different groups. Mean differences with $P < 0.05$ were considered to be significant.

Data was expressed as mean ± S. D. and parasitaemia of the pyrimethamine and its inclusion complexes were statistically assessed by one-way ANOVA followed by Turkey's test using Jandel sigma stat 2.0 version. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Phase solubility analysis

The phase solubility method is useful for providing solubilizing ability of host molecules. The linear host-guest correlation (A_L-type system) with slope less than one suggested the formation of first order soluble complexes. M-β-CD shows the maximum solubilizing capacity (Figure 1).

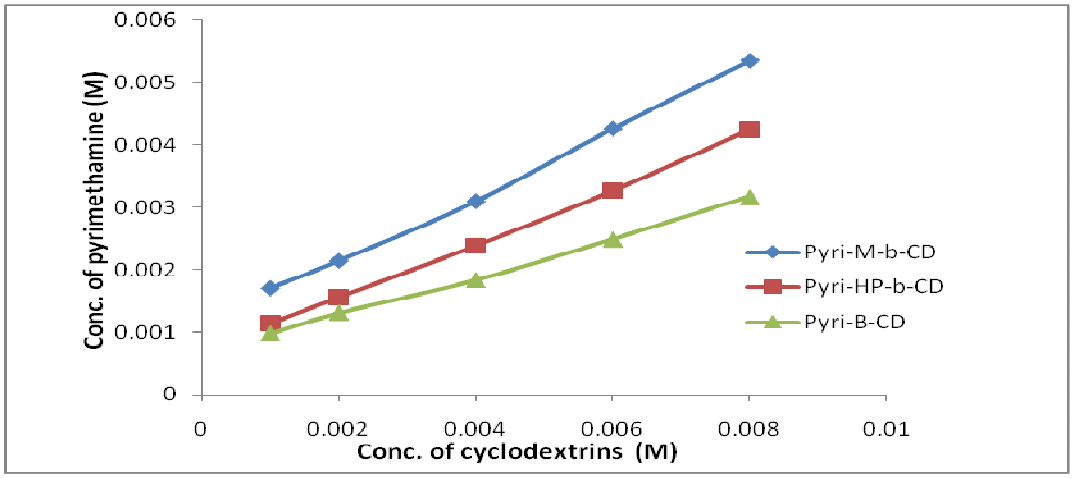


Fig. 1: Phase solubility curve of pyrimethamine with β-CD and its derivatives at 37⁰C

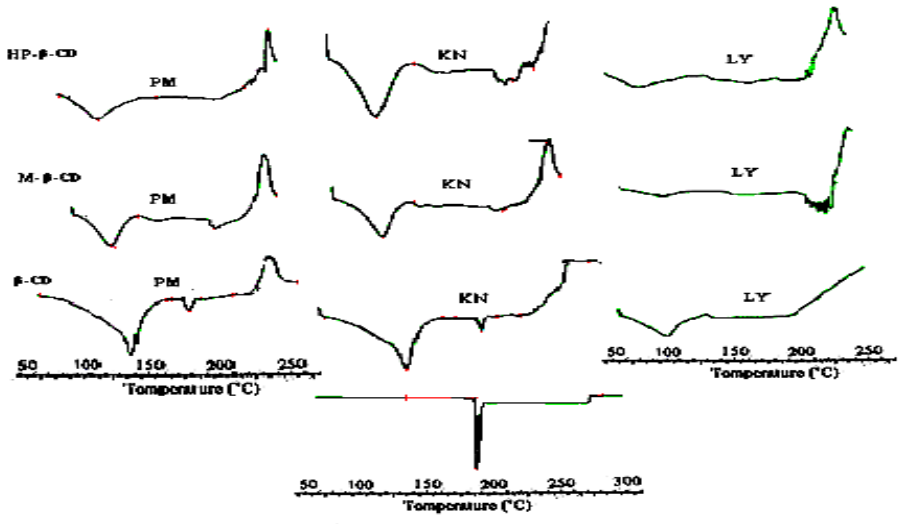


Fig. 2: DSC thermograms of pyrimethamine-CD solid systems: Pyri, β-CD, M-β-CD and HP- β-CD, (PM) physical mixture, (KN) kneaded complex and lyophilized complex (Ly).

Differential scanning calorimetry (DSC)

DSC was used to provide first hand information about the physical state of the drug in the complexes (Figure 2). The complete disappearance of endothermic peak of pyrimethamine (241.49°C) for kneaded and lyophilized systems of drug with M-β-CD and HP-β-CD indicate inclusion phenomenon. It is clear from the figure that thermal profile of pyrimethamine remains recognizable in their physical mixtures.

X-ray powder diffraction (XPRD)

The diffraction patterns of the complexes should be clearly distinct from those of each component if a real inclusion complex has been formed. The diffraction pattern of the lyophilized products with β-CD, M-β-CD and HP-β-CD show hollow pattern with complete disappearance of characteristic diffraction peaks of drug (Figure 3) indicating true inclusion. Presence of drug characteristic peaks with reduced intensity in kneaded complexes indicates incomplete inclusion process.

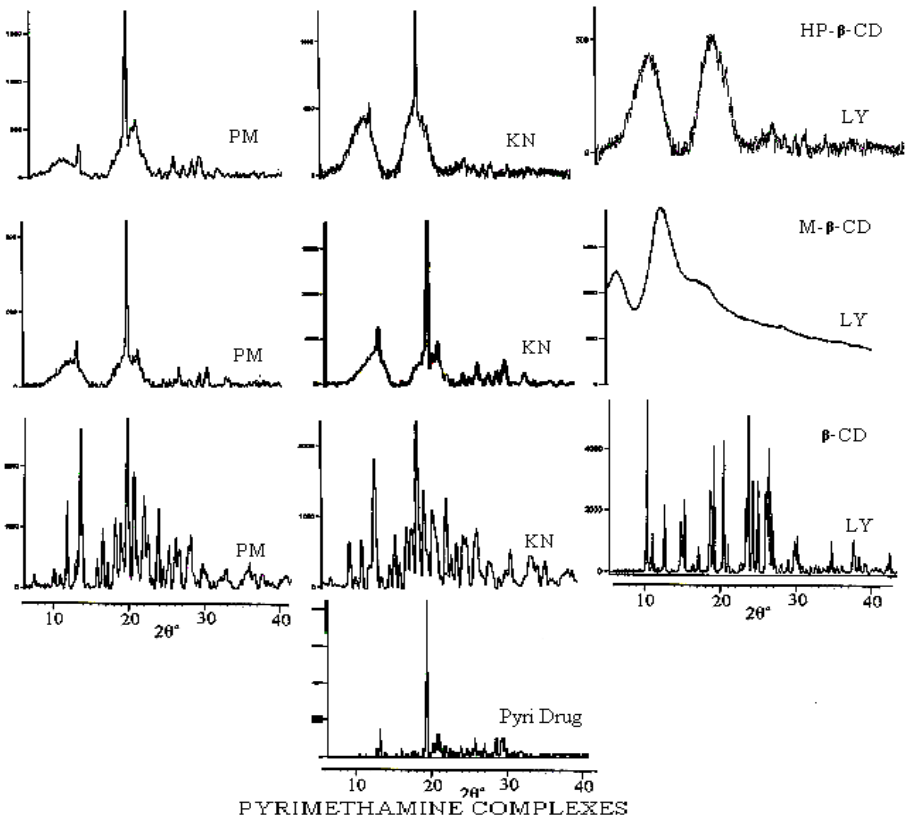


Fig. 3: PXRD diffraction pattern of pyrimethamine-CD solid systems: (Pyri) pyrimethamine, β-CD, M-β-CD and HP-β-CD, (PM) physical mixture, (KN) kneaded complex and lyophilized complex (Ly).

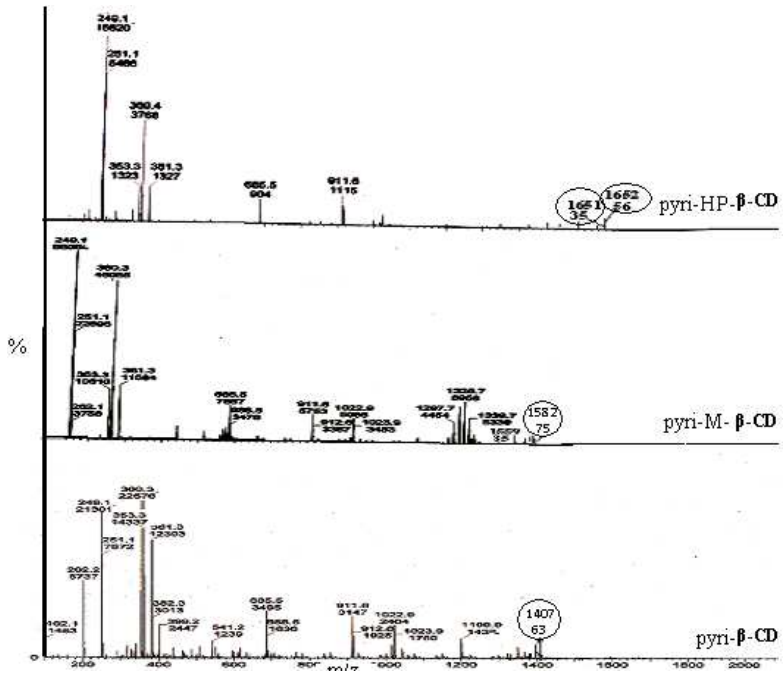


Fig. 4: Mass spectra of lyophilized complexes of pyrimethamine

Mass spectrometry

The use of ESI-MS for characterizing the stoichiometry and strength of interactions between synthetic or biological hosts and guests is a growing area of research[29-30]. The presence of peaks at m/z 1407, 1582, and 1652 for [Pyri + β-CD + Na + H]$^+$, [Pyri+ M-β-CD +Na + H]$^+$, [Pyri + HP-β-CD + Na + H] $^+$ respectively supports 1:1 stiochiometry (Figure 4). The presence of another peak at m/z 1651 is probably due to [Pyri + HP-β-CD + Na] $^+$.

Fourier transforms infrared spectroscopy (FT-IR)

FT-IR spectra of the inclusion complexes are quite similar to the drug & the corresponding CD (Figure 5) because of concurrent absorption of both the host and guest molecules in whole of the spectral regions. However, the small shifts in characteristic bands of drug at 3467 cm^{-1}, 1629 cm^{-1}, 1561 cm^{-1}, and 1439 cm^{-1} undoubtedly confirm the presence of drug in complexes.

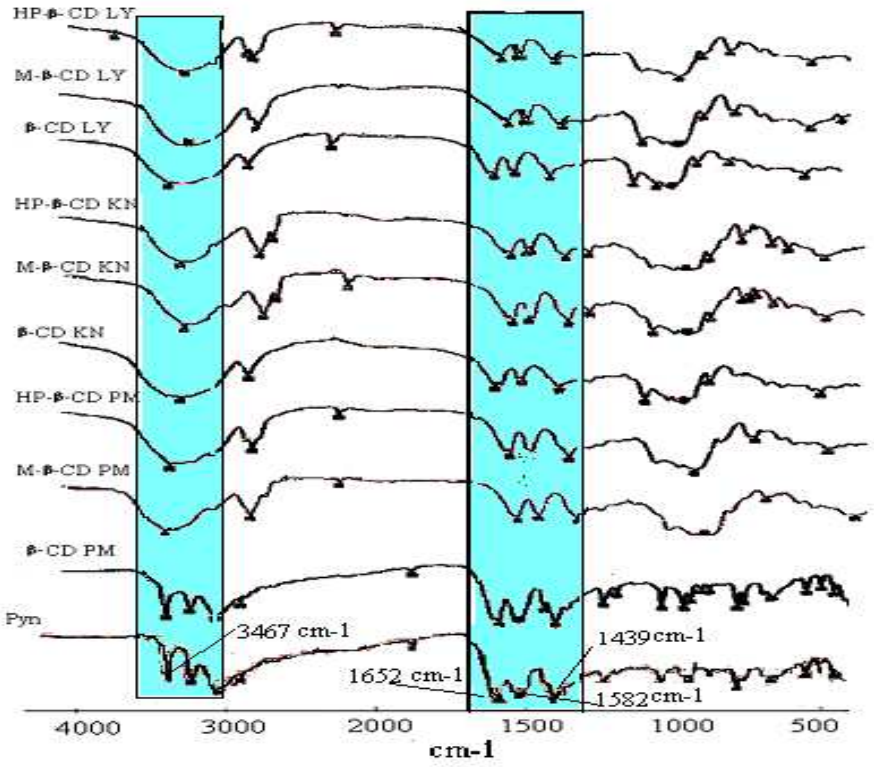


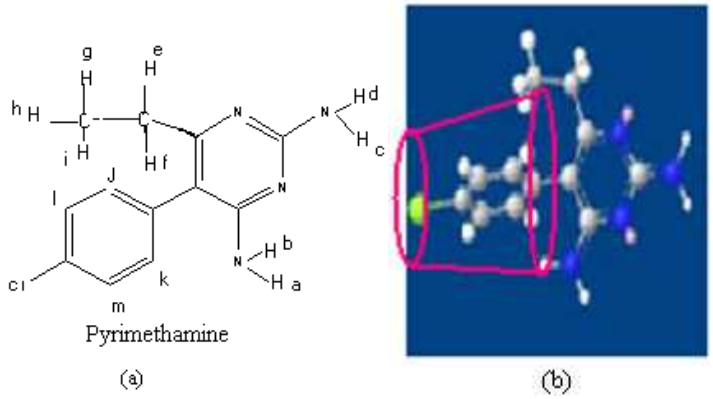
Fig. 5: FT-IR spectra of pyrimethamine-CD solid systems: (Pyri) pyrimethamine, β-CD, M-β-CD and HP- β-CD, (PM) physical mixture, (KN) kneaded complex and lyophilized complex (Ly).

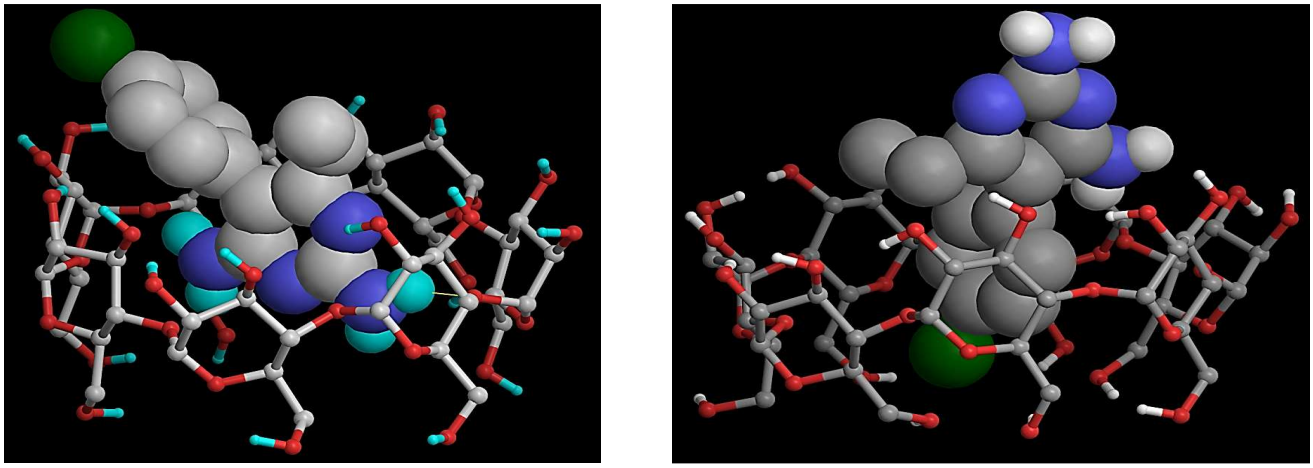
Proton NMR spectroscopy

Proton and ^{13}C NMR spectroscopy was used to ensure the existence of complexes in solution. The intermolecular insertion of pyri into hydrophobic cavity of β-CD, HP-β-CD and M-β-CD resulted in modification of NMR shifts (Table 1). Chemical downfield shift observed in protons

H-j, k, l, m indicate the insertion of cholorbenzene ring inside the cavity. The possibility of drug to enter the cavity through pyrimidine ring with two amino groups is ruled out because of its lesser hydrophobic nature. However, the downfield shift in H-a, H-b protons indicate that amino protons attached to pyrmidine ring form strong hydrogen bonding with exterior hydroxyl groups of cyclodextrin (Figure 6). The proposed inclusion mode can be further explained on the basis of the size. Distance between H-e, and H-f protons of ethyl group and H-a and H-b protons of amino group is approx. 6.57A^o which will not allow this part of the molecule along with the chlorobenzene ring to enter into the cavity (6.5 A^o).

In the ^{13}C NMR spectra of all the complexes, the signals of carbons attached to chlorobenzene ring shifts to lower frequency whereas no shift in signals of pyrimidine carbons as well as ethyl carbons was observed.





Complex-1 **Complex-2**

Fig. 6: (a) Chemical structure of pyrimethamine (b) Inclusion mode of pyrimethamine with β-CDs (c) Space-fill model to depict the interaction of pyrimethamine (guest) with β-cyclodextrin (host) in two possible orientations

Two-dimensional (2D) COESY spectra were used to support the proposed geometry of the complex as it provides the information about the spatial proximity between host and guest atoms by observing intermolecular cross-relations. The off-diagonal peaks are displayed between the H-3 and H-5 protons of cyclodextrins (present on the wider side) and phenyl protons of chlorobenzene ring (Figure 7). Similar cross peaks were found in M-β-CD and HP-β-CD however, peaks could not be clearly identified due to random substitution. Our proposed geometry is in agreement with the results reported by de Araujo et al who studied the complexation of HP-β-CD with pyrimethamine[14].

Table 1: Values of ^{1}H chemical shifts before and after Inclusion of drug inside CD cavity

	Δδ = δ (complex) - δ (Free)		
Pyrimethamine	**$\Delta\delta_{bCD}$ (ppm)**	**$\Delta\delta_{M\text{-}B\text{-}CD}$(ppm)**	**$\Delta\delta_{HP\text{-}B\text{-}CD}$(ppm)**
H-a, H-b	+0.2479	+0.1001	+0.1186
H-c, H-d	+0.0041	+0.0015	+0.0016
H-e, H-f	-0.04396	-0.0449	-0.0481
H-g, h,i	-0.03573	-0.03506	-0.0367
H-j	+0.0124	+0.0093	+0.0078
H-k	+0.0135	+0.0106	+0.0089
H-l	+0.0464	+0.0421	+0.0420
H-m	+0.0493	+0.0452	+0.0447

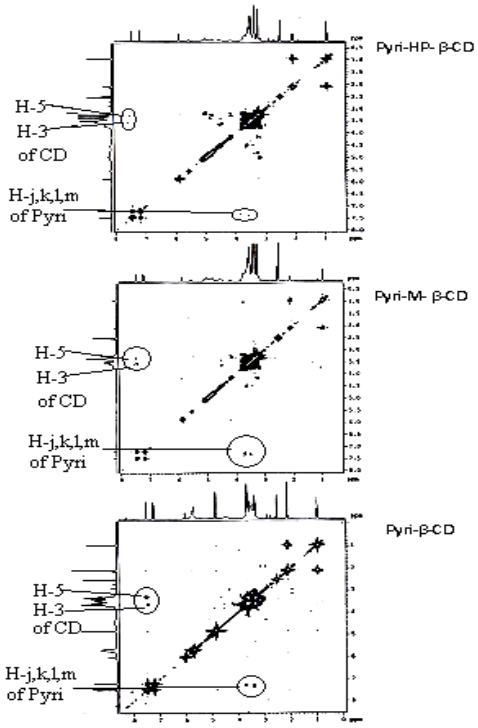


Fig. 7: COESY spectra of inclusion complexes of pyri with β-CD, M-β-CD and HP-β-CD

Micro calorimetric studies of inclusion complexes

In order to study the structural effect of β-CDs on their inclusion efficiency, stability constant and other thermodynamic parameters, were calculated by determining their enthalpy of solution of the drug in absence and presence of CDs. The enthalpy of solution of drug was found to be exothermic (0.04 kJ) in phosphate buffer (pH 5). Enhanced exothermic behavior was exhibited by the drug in presence of CDs in solution which is attributed to interaction between drug and the cyclodextrin. Enthalpy of interaction was calculated from the difference of the enthalpy of solution of drug in cyclodextrin from that in pure buffer. The enthalpy of interaction was calculated by the equation:

$$\Delta_{sol}H_{int(exp)} = \frac{\Delta_{sol}H_{(CD)} - \Delta_{sol}H}{v(l)} \quad (1)$$

where $\Delta_{sol}H_{int,(exp)}$ is enthalpy of interaction between drug and cyclodextrin per liter of solution, $\Delta_{sol}H$ *and* $\Delta_{sol}H_{(CD)}$ are enthalpy of solution of drug in buffer and in buffered aqueous solution of cyclodextrin respectively, *v (l)* = volume of sample cell in liters (0.025 L). Enthalpy of interaction per mole of drug and M-β–cyclodextrin $(\Delta_{sol}H_{int(M)})$ were calculated from equation (2) and their values are presented in (Table 2). Similar results were obtained for β–CD and HP-β-CD.

$$\Delta_{sol}H_{int(M)} = \frac{\Delta_{sol}H_{int(exp)}}{a+b} = \frac{\Delta_{sol}H_{(M)(CD)} - \Delta_{sol}H_{(M)}}{1+x_2/x_1} \quad (2)$$

Where a and b are initial molar concentration of drug and cyclodextrin, x_1 and x_2 are apparent mole fractions of the drug and cyclodextrin ignoring the concentration of buffers.

Table 2: Interaction enthalpy of inclusion complexes of pyrimethamine with M-β-CD at pH 5 in phosphate buffer

X_2	M_D (a) (mM)	M_{CD} (b) (mM)	$\Delta_{sol}H_{(CD)}$ (J x 10^{-2})	$\Delta_{sol}H_{int\,(exp)}$ (J/l)	$\Delta_{sol}H_{int(M)}$ (kJ/mol)
0.946	0.205	3.596	12.484	-2.498	-0.657
0.885	0.304	2.340	29.501	-3.436	-1.299
0.824	0.415	1.939	34.640	-4.457	-1.894
0.767	0.445	1.464	34.181	-4.211	-2.206
0.686	0.805	1.758	34.967	-6.219	-2.426
0.539	0.512	0.599	54.414	-2.897	-2.607
0.603	0.391	0.593	44.355	-2.659	-2.703
0.501	0.338	0.339	73.364	-1.878	-2.776
0.403	0.444	0.299	40.103	-1.778	-2.393
0.302	0.839	0.363	63.671	-2.616	-2.176
0.247	0.761	0.249	30.116	-1.973	-1.955
0.184	0.706	0.159	61.245	-1.262	-1.459
0.109	1.440	0.176	126.889	-1.794	-1.110

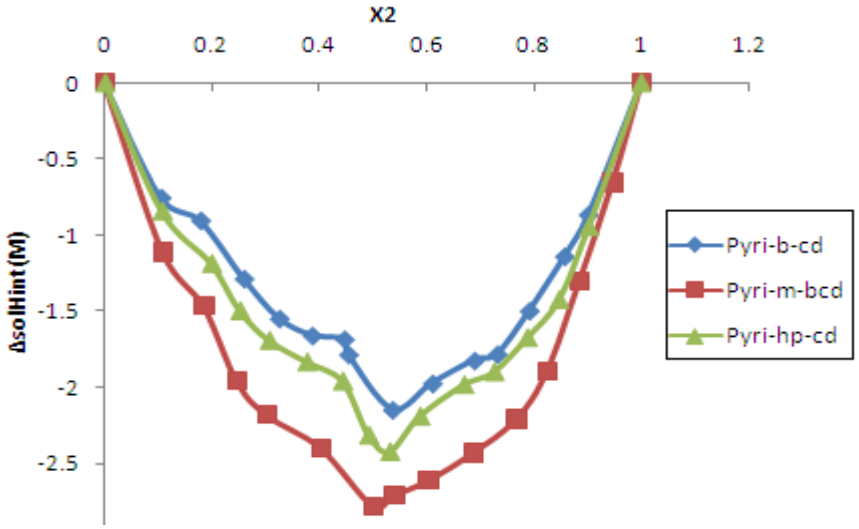


Fig. 8: Plot between $\Delta_{sol}H_{int(M)}$ vs X_2 of β-CD, M-β-CD and HP-β-CD of Pyrimethamine ' (Pyri) at pH5 in phosphate buffer

The stoichiometry of the complex was ascertained utilizing continuous variation method (Job's plot) [31] by plotting ($\Delta_{sol}H_{int(M)}$) versus (x_2) (Figure 8). It can be seen that the minimum occurs at x_2 = 0.5. This indicates that complex has 1:1 stoichiometry and supports its determination by other techniques.

The binding constant K and enthalpy of binding ($\Delta_{sol}H^0$) were computed from the experimentally determined enthalpy of interaction ($\Delta_{sol}H_{int(exp)}$). The calculations were done by our computer program utilizing an iterative non-linear least square regression method to minimize the value of $\Sigma(\Delta_{sol}H_{int(exp)} - \Delta_{sol}H_{int(calc)})^2$ and are given in (Table 3).

The values of free energy of inclusion (ΔG^0) and entropy of inclusion (ΔS^0) were calculated from the following equations:

$$\Delta G^0 = - RT\ln K \quad (3)$$

$$\Delta S^0 = (\Delta H^0 - \Delta G^0)/T \quad (4)$$

The numerical values of ΔH^o and ΔG^o are used as a measure of the "depth" of penetration of guest molecule in CD cavity. The numerical value of K in complexation is an indicative of host guest affinity. The comparison of K values confirms that M-β-CD has best complexation capability (1885 M^{-1}) with pyrimethamine (Table 3). Lower value of K

in case of HP-β-CD can be attributed to the fact that the hydroxyl group makes the CD cavity partially hydrophilic. The enthalpy-entropy compensation theory[32-35]; where the increased enthalpic benefits of tighter binding result in increased entropic cost of restriction of relative motion of the associating entities are reflected in our results. Large negative value of ΔH^o and somewhat low positive value of ΔS^o in all the complexes indicates that van der Waals's interaction play a more important role than hydrophobic ones in the formation of complex with 1: 1 molar ratio. Moreover, the highest value of ΔH^o is accompanied by the lowest positive value of ΔS^o in M-β-CD indicating that favorable enthalpy brought about by structural tightening is only partially compensated for by a more unfavorable entropy.

Table 3: Thermodynamic parameters of pyrimethamine with β-CD, M-β-CD and HP- β-CD at pH 5 in phosphate buffer, determined using solution calorimetry

System	K (M^{-1})	ΔH^o(kJ mol^{-1})	ΔG^o(kJ mol^{-1})	ΔS^o($Jmol^{-1}K^{-1}$)
Pyri + β-CD	1080± 0.005	-9.80 ± 0.004	-18.00 ± 0.001	26.46 ± 0.004
Pyri +M- β-CD	1885± 0.011	-13.10 ± 0.006	-19.39 ± 0.001	20.31 ± 0.005
Pyri + HP-β-CD	1552±0.008	-11.66 ± 0.005	-18.94 ± 0.001	23.47 ± 0.006

***In Vitro* dissolution studies**

The dissolution of pyri and the binary system was performed to select the most appropriate system for the animal studies as comparative release of active material is strongly affected by the method of formulation.

The dissolution properties were assessed using dissolution rate (Figure 9) and dissolution efficiency at 90 min (DE_{90}) (Table 4). All the binary systems have shown significantly improved ($P<0.05$) dissolution rate as compared to the pure drug However, lyophilized complexes are most effective. The improvement in the dissolution rate may be attributed to the amorphous state of the active material, together with the increase in wettability and the solubility of the drug as well as hydrophilic effect of CDs, which can reduce the interfacial tension between the poorly soluble drug and dissolution medium. It is also clear from results of dissolution studies and dissolution efficiency that release rate is faster for M-β-CD followed by HP-β-CD and β-CD.

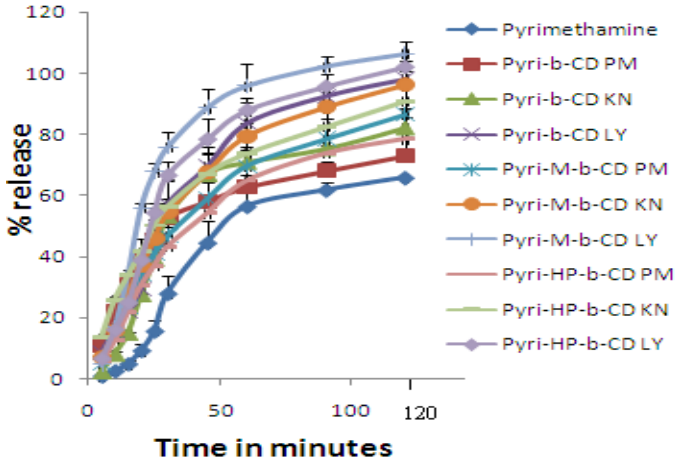


Fig. 9: Dissolution profile of pyrimethamine and their complexes

Table 4: Mean ± SD values of dissolution efficiency at 90 min (DE_{90min}) of pyrimethamine and its complexes with β-CD, M-β-CD and HP- β-CD at 37°C in phosphate buffer at pH 6.8.

Sample	*DE_{90min}
Pyri	51.97 ± 1.3
Pyri-+ β-CD PM	57.54 ± 2.42
Pyri + β-CD KN	64.20 ± 3.3
Pyri-+ β-CD LY	68.97 ± 9.28
Pyri-+M-β-CD PM	62.38 ± 2.72
Pyri +M-β-CD KN	72.12 ± 1.83
Pyri + M-β-CD LY	79.36 ± 3.93
Pyri-+ HP-β-CD PM	58.20 ±1.83
Pyri + HP-β-CD KN	66.50 ± 1.78
Pyri-+ HP-β-CD LY	74.84 ± 2.78

*DE_{90min} was calculated as described in the text from area under the dissolution curve at 90 min expressed as a percentage of the area of the rectangle described by 100% dissolution in the same time.

Molecular Modeling Studies

Pyrimethamine was docked into the cavity of β-CD using FRED which is a fast and rigid body docking technique that places the guest into the active cavity using a set of rotational and translational motions. Using different intrinsic scoring functions two orientations of pyrimethamine were identified. One (complex-1) in which the aminopyrmidine ring is inside the cavity and chlorobenzene group is hanging outside the cavity. Whereas, in complex-2 the chlorobenzene is snug fit into the cavity and aminopyrmidine ring is remaining outside the cavity (Figure 6a). The two docking solutions were subsequently taken up for the MD simulation.

Post simulation, it was observed that the complexes maintained their integrity relevant from the RMSD computed for the trajectory profile. The RMSD for the trajectory frames was calculated for heavy

atoms using frame zero as the reference. For the complex-1 the RMSD remained steady between 1.0 and 2.0 over a 3 ns period and subsequently it increased to 3.0 for next 1000 ps and finally returned to 2.0 over the last 1000 ps. In comparison to complex-1, the RMSD for complex-2 remained steady between 1.0 and 2.0 over the entire trajectory. The mean RMSD for the two complexes was determined as 1.87 and 1.40. The average Coulombic and vander Waals interaction energies and H-bond count were computed to be –2.83, -22.43 kcal/mol for complex-1 and -2.38 and -23.46 kcal/mol for complex-2 and –30.93 kcal/mol and 0 – 3 respectively (Table 5). The extent of binding was determined by following equation.

$$\Delta G_{binding} = \Delta G_{complex} - (\Delta G_{host} - \Delta G_{guest})$$

The mean binding energy computed for pyri-β-CD complexes are –5.57 and –4.58 kcal/mol for the complex-1 and complex-2 respectively. The experimentallly determined ΔG° (-18.00 kJ/mol) is closer to the calculated the binding energy for complex-2. Higher H-bond count and slightly lower binding energy favors the snug fit of chlorobenzene ring into the β-CD. It is also evident from NMR data that chlorobenzene protons (complex-2) involved stronger correlations with β-CD protons (H-3, H-5) suggesting the most favorable mode of inclusion between pyrimethamine and β-CD (Fig. 6c).

In vivo efficacy of inclusion complexes

In vivo antimalarial activity and efficacy against *P berghei* infection of the pyrimethamine and its inclusion complexes was performed and the results are summarized in (Table 6). The lyophilized complexes of all the CD were selected due to their higher dissolution rate and dissolution efficiency. It is clear from the table that pyrimethamine alone (standard group) is insufficient to prevent the mortality. However, survival time was increased (day 11-16) compared to control (day 9) but was found to be ineffective in preventing mortality. The suspensions of the lyophilized complexes were administered to compare and differentiate them on the basis of their protective efficacy and potency. At the end of the treatment (day 8), when compared with control (45.56 ± 15.78), the test group 1 (4.45 ± 5.28), the test group 2 (0.0725± 1.127) and the test group 3 (5.73 ± 11.016) showed significantly less (P<0.001) mean percent parasitaemia. It is observed that percent mortality rate decreases from 83.3% (Standard group) to 67.3%, 33.3%, 16.7%, for β-CD complex, HP-β-CD complex and M-β-CD complex respectively. It is clear from percent moratlity rate that M-β-CD complex has shown better antimalarial activities as compared to drug alone and its complexes with HP-β-CD and β-CD. ANOVA have also shown significant (P< 0.05) antimalarial activity of all complexes as to pyrimethamine (Fig.10).

Table 5: Interaction and Binding energies (Coulombic, vander Waals), H bond counts and RMSD (w.r.t. frame zero in simulation) computed over the MD trajectory for two BCD-pyrimethamine complexes.

S. No.	Detailed Energetics	Complex - 1		Complex - 2	
		Interaction Energies (kcal/mol)	Binding Energies (kcal/mol)	Interaction Energies (kcal/mol)	Binding Energies (kcal/mol)
1.	Max. Total energy	–	29.97	–	29.75
2.	Min. Total energy	–	-49.78	–	-41.73
3.	Mean. Total energy	–	-5.57	–	-4.58
4.	Max. Coulombic energy	3.05	37.66	2.36	30.83
5.	Min. Coulombic energy	-15.24	-54.05	-10.12	-48.65
6.	Mean Coulombic energy	-2.83	-7.56	-2.38	-6.25
7.	Max. van der Waals energy	-8.96	31.50	-11.59	25.62
8.	Min. van der Waals energy	-28.49	-27.21	-28.84	-24.91
9.	Mean. van der Waals energy	-22.43	1.99	-23.46	1.67
10.	H-bond count	0 – 3		0 – 3	
11.	Max. RMSD	2.92		2.37	
12.	Min. RMSD	0.00		0.67	
13.	Mean RMSD	1.87		1.40	

#energy values in kcal/mol

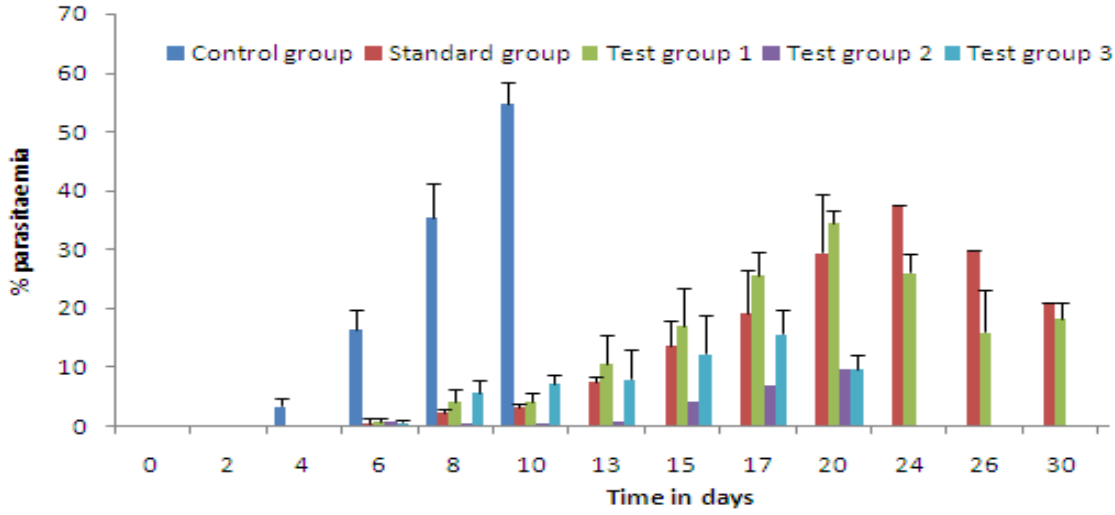


Fig. 10: Percent parasitaemia observed in *P. berghei* infected mice "(n=6)".

Table 6: Antimalarial activity of complexes of Pyrimethamine in *P. berghi* infected mice

S. No.	Groups	Treatment	Mean % Parasitaemia* on day 8th PI	% mortality (n=6, t= 30 days)
1	Control group	0.5% CMC solution	45.56 ± 15.78	100
2	Standard group	Pyrimethamine ~(6mg/kg)	5.78 ± 2.46	83.7
3	Test group 1	Pyri-β-CD	4.45 ± 5.28	50
4	Test group 2	Pyri-M-β-CD	0.0725± 1.127	16.7
5	Test group 3	Pyri-HP-β-CD	5.73 ± 11.016	33.3

* Values were expressed as mean ± SD (standard deviation)

t= no. of days; n =no. of animals per group; PI= post inoculation

Pyri -β-CD = Pyrimethamine-β-cyclodextrin complex

Pyri - M-β-CD = Pyrimethamine-methyl-β-cyclodextrin complex

Pyri -HP-β-CD =Pyrimethamine-hydroxypropyl-β-cyclodextrin complex

CONCLUSION

The present study concludes that the solubilizing efficiency of drug-cyclodextrin complexes obtained from phase solubility studies can be quantified by correlating it with the stability constant determined by solution calorimetry. The inclusion of chlorobenzene ring into the cyclodextrin cavity was found to be more favorable as to aminopyrmidine ring that have slightly higher mean binding energy. This mode of inclusion is depicted by docking studies and supported by 2D NMR. Numerical value of stability constant increases in the order M-β-CD> HP-β-CD>β-CD and is supported by the *in vitro* dissolution rate and dissolution efficiency which was found to be maximum for M-β-CD lyophilized complexes. Significantly less mean percentage parasitaemia and better antimalarial efficacy of CD complexes observed in *in vivo* studies conclude that the encapsulation of pyrimethamine by cyclodextrin is a successful approach for improving its pharmacological activity.

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