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Valsartan inclusion by methyl-β-cyclodextrin: Thermodynamics, molecular modelling, Tween 80 effect and evaluation

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ABSTRACT

The rationale of present study is to investigate the effect of Tween 80 on encapsulation ability of valsartan (VAL) by methyl-β-cyclodextrin (M-β-CD) and to determine the exact mode of binding. Phase solubility studies indicated 1:1 stoichiometry between VAL and M-β-CD both in the presence and absence of Tween 80. The NMR and molecular modelling studies indicated the insertion of both aromatic and aliphatic regions of VAL into the M-β-CD cavity suggesting the coexistence of two 1:1 complexes in equilibrium with each other. The stability constants, K_1 (aromatic) and K_2 (aliphatic), were enhanced in the presence of Tween 80 as evident by higher value of stability constants (K_1 1992.0 M^{-1}, K_2 1864.0 M^{-1}) for ternary system in comparison to binary system (K_1 1741.6 M^{-1}, K_2 1499.2 M^{-1}). Efficacy of ternary complex was established by significant decrease in the systolic blood pressure of deoxycorticosterone acetate (DOCA) induced hypertensive rats.

1. Introduction

Hypertension, most common cardiovascular disease and a major public health issue has affected approximately one billion people worldwide (NIH Publication No. 03-5233, 2003). In the last 20 years antihypertensive therapy has gained recognition in abating the cardiovascular complications with the introduction of new antihypertensive agents (Pardell, 1990). One of the classes of newer antihypertensive agents is of Angiotensin receptor blockers which act as antagonists of the AT_1 receptor subtype. This receptor subtype is responsible for the well known effects of angiotensin II such as vasoconstriction, aldosterone and adrenaline release, water intake and cellular proliferation (Criscione et al., 1990; Herblin et al., 1991; Smith et al., 1992). VAL a potent, highly selective, and orally active antagonist at the angiotensin II AT1-receptor, is currently holding the largest market share. Unfortunately it is poorly water soluble which is responsible for its low bioavailability and consequently hampers its therapeutic efficacy (Criscione et al., 1993; Siddiqui et al., 2011). Among various formulation strategies to improve the dissolution profile of lipophilic drugs, cyclodextrins complexation has proved to be very effective (Grillo et al., 2008; Hassan, Al-Marzouqi, Jobe, Hamza, & Ramadan, 2007; Loftsson, Brewster, & Masson, 2004; Tommasini et al., 2004). Various desirable attributes like absorbability, biodegradability and encapsulation ability of wide variety of guest molecules, has put cyclodextrins on a status of important element of formulation development (Araujo et al., 2007; Chao & Zhang, 2007; Martin Del Valle, 2004).

Cyclodextrins are macromolecules in which several D-glucose units are linked by α-1,4 glycosidic bonds providing hydrophobic cavities to accommodate appropriately sized guest molecules either whole or the non-polar part through non-covalent interactions, which results in improved physicochemical parameters of the guest. Beside this the systems are expected to provide chemical stability and protection from degradation to guest molecule (Jullian, Moyano, Yanez, & Olea-Azar, 2007; Loftsson & Dominique, 2007). It should be emphasized, however, that in pharmaceutical formulations the amount of cyclodextrin incorporated should as little as

Abbreviations: VAL, valsartan; M-β-CD, methyl-β-cyclodextrin; DOCA, deoxycorticosterone acetate; HP-β-CD, hydroxypropyl-β-cyclodextrin; DSC, differential scanning calorimeter; XRPD, X-ray powder diffractrometry; FT-IR, Fourier transform infrared; NMR, nuclear magnetic resonance; ROESY, rotating frame overhauser effect spectroscopy; DP, percentage of VAL dissolved; RDR, relative dissolution rate; CMC, carboxy methyl cellulose; NaCl, sodium chloride; CPCSEA, control and supervision on experiments on animals; IAEC, Institutional Animal Ethics Committee.

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possible, due to the inherent toxicity as well as the cost associated with cyclodextrin (Loftsson & Brewster, 1996). Therefore, in situation where the complexation efficiency is low, the success of this approach is dependent on the use of larger amount of cyclodextrin than that acceptable for solid or liquid dosage forms, which may lead to problems of formulation bulk or potential toxicity. Under these circumstances, addition of third component such as water soluble polymer offer an alternative and potentially fruitful method for enhancing the complexation efficiency (Chadha, Arora, Gupta, & Jain, 2011; Chowdary & Srinivas, 2006; Loftsson & Frioriksdottir, 1998; Mura, Maestrelli, & Cirri, 2003; Ribeiro, Carvalho, Ferreira, & Veiga, 2005; Savolainen et al., 1998; Valero, Esteban, Pelaez, & Rodrıguez, 2004).

Therefore, the present results describe the interaction of VAL with M-β-CD in the absence and presence of Tween 80 both in solution (phase solubility, ^{1}H NMR, ROSEY, solution calorimetry) and solid (DSC, XRPD, FT-IR) phases. The favourable effect of cyclodextrins complexation on VAL solubility and dissolution rate has also been demonstrated previously (Cappello, Clelia, Lervolino, & Ageness, 2005; Nalawade, Aware, Dand, Kadam, & Hirlekar, 2009). However, the effect of third component on inclusion complexation, binding parameters as well as geometry of inclusion have not been reported. Therefore the study is focused on determining the thermodynamic parameters of binary and ternary complexes, exact stoichiometry as well as the geometry of the complexation using molecular modelling. The superiority of ternary complex over binary complex is illustrated by performing the *in vivo* studies.

2. Experimental

2.1. Materials

VAL and poloxamer were obtained as gift samples from Ranbaxy (Gurgaon, India). M-β-CD, polyvinylpyrrolidone K30, hydroxypropyl methylcellulose 4000 and DOCA were purchased from Sigma–Aldrich, Co. (St. Louis, USA). Tween 80 and carboxymethylcellulose were obtained from LOBA Chemie and Merck specialities Pvt. Ltd. (Worli, Mumbai), respectively. All other chemicals used were of analytical reagent grade.

2.2. Preparation of complexes

The inclusion complexes (binary and ternary) were prepared by freeze drying method. For binary complex, the aqueous solutions containing VAL and M-β-CD in 1:1 molar ratio was obtained by dissolving 0.435 g of VAL in 30 mL triple distilled water containing 1.310 g of M-β-CD. The mixture was stirred for 24 h at room temperature, then filtered through 0.45 μm filter and the clear solution was frozen at −80 °C and subsequently freeze dried for 24 h at −55 °C.

For ternary system, drug (0.453 g) and cyclodextrin (1.310 g) were mixed in 30 mL triple distilled water and to this 1.148 mL of Tween 80 corresponding to 0.5% (v/v) of 30 mL was added. This solution was autoclaved at 120 °C for half an hour. The autoclaving is important as water soluble polymers enhance drug solubilization only when activated by heating (Cappello, Carmignani, Iervolino, La Rotonda, & Saettone, 2001; Loftsson & Järvinen, 1999; Ribeiro, Loftsson, Ferreira, & Veiga, 2003). The autoclaved solution was frozen at −80 °C and subsequently freeze dried for 24 h at −55 °C. After lyophilization in both binary and ternary systems the amount of white dried products obtained was found to be 1.61 (yield: 92%) and 1.67 g (yield: 92%), respectively. The lyophilized complexes were passed through 60 mesh and observed under optical microscope (eclipse i90, Nikon) for particle size uniformity before packing in airtight container.

2.3. Equilibrium phase solubility studies of VAL in presence and absence of Tween 80

Equilibrium phase solubility studies were carried out according to the method described by Higuchi and Connors in unbuffered aqueous solutions. The unbuffered triple distilled water was used as media for phase solubility studies because VAL belongs to BCS class II; therefore by increasing its apparent solubility in water its bioavailability can be improved. Therefore, an excess amount of VAL (50 mg) was added to series of unbuffered triple distilled water solutions (10 mL each) containing increasing amount of various cyclodextrins (α-CD, β-CD, HP-β-CD and M-β-CD) over the concentration range (from 0 to 8 mmol) or Tween 80 [from 0% to 1% (v/v)] in the presence or the absence of a fixed amount of Tween 80 (0.5%, v/v) or M-β-CD (2 mmol). The mixtures were firstly dispersed by vortex shaker for 2 min, and then oscillated by rotary shaker (MSW-275 Macro scientific works, Delhi) at 37 °C for 24 h and the samples withdrawn after 24 h were filtered through 0.45 μm membrane filter and analyzed spectrophotometrically (Perkin Elmer precisely, Lambda 25, UV/vis spectrophotometer) at λ_{max} (252 nm). The presence of cyclodextrin and or Tween 80 did not interfere with the spectrophotometric analysis of VAL.

2.4. Computational studies

In order to scrutinize the mechanism of complexation between VAL and M-β-CD computational studies were carried out on an 9-node High Performance Computing cluster (HCL Infosystems Ltd. India) with Intel Xeon processor and CentOS Enterprise Linux 5.4.

2.4.1. Structure preparation

The 3D structures of M-β-CD and VAL were retrieved from the Protein Data Bank (Berman et al., 2000) and Pub Chem Compounds, compound ID: 60846 (http://pubchem.ncbi.nlm.nih.gov) respectively. The structures were prepared for docking in Schrodinger Suite 2012 (Schrödinger LLC, New York, NY, 2012).

2.4.2. Docking studies

FRED 2.2.5 (Open Eye Scientific Software, Santa Fe, USA) was used to perform docking studies (McGann, Almond, Nicholls, Grant, & Brown, 2003; McGaughey et al., 2007). Expected binding site for docking of VAL in the cavity of M-β-CD was defined by a grid box (Chadha, Gupta, Pathak, et al., 2011; Chadha, Gupta, Shukla, Jain, Pissurlenkar, et al., 2011). The docking poses which were achieved by placement of the rigid conformation of VAL over combinations of rotational and translational motions within the grid box in M-β-CD, were scored on the basis of ChemGauss3 scoring function. The diverse docking solutions for each molecule were then considered for MD simulations which were performed using Desmond (version 3.0, DE Shaw Research NY, 2012).

2.4.3. MD simulations

The VAL:M-β-CD complexes were soaked into TIP3P waters (Mark & Nilsson, 2001) such that a 10 Å water shell was formed around the complex. The solvated complexes were relaxed with restrains on solute and subsequently equilibrated at 300 K and 1 atm pressure by NVT and NPT ensembles (Chadha, Gupta, Pathak, et al., 2011; Chadha, Gupta, Shukla, Jain, Pissurlenkar, et al., 2011).

After this the system was simulated for a period of 10 ns under NPT ensemble to obtain energy and trajectory profile of the solvated system to compute the free energy of binding. The trajectories and corresponding energies were sampled after every 10 ps. The average binding energy of the guest molecule (VAL) with the host (M-β-CD) was determined using Eq. (1) which calculates the

difference between the bound and unbound M-β-CD and drug molecules over the entire 10 ns trajectory.

$$\Delta G_{binding} = \Delta G_{complex} - (\Delta G_{host} - \Delta G_{guest}) \quad (1)$$

2.5. Characterization of inclusion complexes in solid state

2.5.1. Differential scanning calorimetry (DSC)

DSC measurements were carried out on DSC (Q20, TA Instruments Waters LLC, USA) which was calibrated using pure indium. The samples were weighed (3 mg) in aluminium pans and scanned at heating rate of 10 °C in the temperature range from 50 to 250 °C. Dry nitrogen with flow rate of 50 mL min^{-1} was used as purge gas.

2.5.2. Thermogravimetry

Samples of VAL, M-β-CD, binary and ternary complexes; weighing about 2.5 mg were placed in alumina pan and heated from 0 to 700 °C at a rate of 10 °C min^{-1} under a nitrogen flow of 50 mL min^{-1} in a TA Instruments-SDT Q600 V20.9.

2.5.3. X-ray powder diffraction (PXRD)

Powder diffraction patterns were recorded on an X-ray diffractometer (XPERT-PRO, PANalytical, Netherlands) with Cu as tube anode. The diffractograms were recorded under the following conditions: voltage 40 kV, 35 mA, angular range five and fixed divergence slit.

2.5.4. Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectra of VAL, its binary and ternary complexes over the range 2000–1000 cm^{-1} were obtained on FT-IR spectrometer (Mode spectrum RXI, Perkin Elmer, England) applying 16 scans. The samples were prepared by using the potassium bromide (KBr) disc technique (3 mg sample in 297 mg KBr). The narrow range was used because the characteristic peaks of VAL and shifts in their position on inclusion in the CD cavity are in this range. A manual press was used to make the pellets of the samples. The FT-IR spectra of the inclusion complexes were compared with their pure M-β-CD, VAL and physical mixture.

2.5.5. Elemental (CHN) analysis

The amount of nitrogen in the binary and ternary complexes was verified with elemental analysis. CHN analysis of samples was performed on a Thermo Scientific FLASH 2000 Organic Elemental Analyzer.

2.6. Characterization in solution state

2.6.1. Nuclear magnetic resonance (NMR) spectroscopy

One dimensional ^1H NMR spectra were recorded at 300 K on a Bruker Avance III 400 instrument in unbuffered D_2O solution. Rotating-frame Overhauser Effect Specroscopy (ROESY) spectra were acquired in the phase sensitive mode using the same spectrometer at 300 K with a 5 mm probe and standard Bruker parameters (pulse program roseyphsw). Each spectrum consisted of a matrix of 2 K (F_2) by 256 (F_1) points covering a spectral width of 8012.820 Hz. Spectra were obtained from the samples solutions prepared for the ^1H NMR studies, using a spin-lock mixing time of 200 ms, relaxation delay 2 s, and 4 scans were recorded.

2.6.2. Microcalorimetric study

Isoperibol solution calorimeter (ISC) model 4300 (Calorimetry Science Corporation, UTAH, USA) was used to determine the enthalpy of solution. It is a semi-adiabatic calorimeter consisting of 25 mL silvered Dewar flask in a constant temperature bath held at 37 °C (±0.0001 °C). The sample is filled into ampoule which is shattered automatically by plunger and temperature change noted. Complexation thermodynamics of VAL with M-β-CD was determined by measuring the enthalpy of solution of drug in pure buffer (pH 7) and in buffered solution (pH 7) of cyclodextrins with and without 0.5% Tween 80. The solutions of cyclodextrin were prepared over a concentration range of 0.001–0.01 M for binary and ternary system.

2.6.3. Dissolution study

The dissolution profiles of the VAL, its binary and ternary complexes were obtained using USP XII apparatus equipped with paddle type tribune at 37 ± 0.5 °C. Dissolution media consisted of 1000 mL of phosphate buffer (pH 6.8), previously filtered, degassed, and maintained at 37 ± 0.5 °C. The stirring speed was set at 100 rpm. The amount of inclusion complex added was equivalent to 40 mg of VAL. The aliquots of 5 mL were withdrawn at regular time intervals and analyzed till the absorbance of the solution attains a constant value. At each sampling time, an equal volume of fresh medium was added and the correction for the cumulative dilution was calculated. Each dissolution study was performed 198 on duplicate batches.

2.6.4. Permeability study

The permeation study was performed through a semi-permeable cellulose membrane (dialysis tubing, high retention seamless cellulose tubing, 12000 Dalton, Himedia, Mumbai, India). The cellulose membrane was placed in Franz type diffusion cells; the surface area of membrane in the diffusion cells was 1.77 cm^2. The receptor phase (20 mL) consisted of phosphate buffer (pH 6.8). The membrane of the diffusion cell was kept at 37 °C by circulating water through an external jacket. The donor phase consisted of 5 mL of aqueous suspension or solution of 1 mg VAL or its equivalent amount of VAL:M-β-CD complexes with or without Tween 80 (0.5%, v/v). Samples of receptor fluid (0.5 mL) were withdrawn at various intervals up to 6 h and replaced with fresh buffer solution and analyzed spectrophotometrically at 252 nm.

2.7. Pharmacological studies

2.7.1. Antihypertensive activity evaluation

The worth of binary and ternary inclusion complexes of VAL was determined by performing pharmacodynamic antihypertensive study in DOCA salt induced hypertensive rats. The experimental protocol for the study was approved by Institutional Animal Ethics Committee (IAEC) and all the experiments were performed as per the guidelines of committee for the purpose of the control and supervision on experiments on animals (CPCSEA).

2.7.2. Animals and their maintenance

A colony of 200–300 g female Wistar rats 4–5 weeks old (Panjab University Central Animal House, India) fed on rodent pellet diet and water *ad libitum*, was used. Animals were housed at controlled room temperature (23 ± 0.5 °C), relative humidity (65 ± 2%) and maintained under an alternating 12 h light/12 h dark cycle (light on at 6:00 am).

2.7.3. Groups

Animals were divided into 6 groups and each group comprised of 6 animals ($n = 6$).

2.7.3.1. Groups I and II: control rats. These rats were vehicle treated. They were orally fed with distilled water daily and cotton seed oil (0.1 mL/100 g) was injected subcutaneously twice a week for 6 weeks. After 6 weeks M-β-CD (Blank for binary system) and M-β-CD:Tween 80 (0.5%, v/v) (blank for ternary system) suspended in 0.5% (w/v) sodium carboxymethyl cellulose (CMC) suspension

were administered orally by gavage to group I and group II rats, respectively.

2.7.3.2. Group III: DOCA plus 1% saline-treated rats. To this group 15 mg/kg of DOCA dissolved in cotton seed oil was subcutaneously injected, twice weekly (Monday and Friday) for a period of 6 week, for the induction of hypertension. The systolic blood pressure was recorded before and once weekly after the DOCA treatment. At three separate occasions recordings were made to establish the base line blood pressure. After the induction of hypertension (6 weeks) this group received a single oral dose therapy of M-β-CD:Tween 80 (0.5%, v/v) suspension in CMC for seven days orally by gavage.

2.7.3.3. Group IV: DOCA plus 1% saline followed by VAL-treated rats. Similar to group III, this group received DOCA and 1% NaCl solution for 6 weeks. After the induction of hypertension (6 weeks) this group received daily a single dose therapy of 1.14 mg/kg of VAL in 0.5% CMC suspension orally by gavage for seven days.

2.7.3.4. Group V: DOCA + 1% saline followed by VAL:M-β-CD-treated rats. Similar to group IV, this group received DOCA and 1% sodium chloride solution for 6 weeks followed by a daily single dose therapy of VAL:M-β-CD equivalent to 1.14 mg/kg VAL orally by gavage for seven days after induction of hypertension (6 weeks).

2.7.3.5. Group VI: DOCA + 1% saline followed by VAL:M-β-CD:Tween 80-treated rats. Similar to group IV and V, group VI received DOCA and 1% sodium chloride solution for 6 weeks followed by a daily single dose therapy of VAL:M-β-CD:Tween 80 (0.5%, v/v) equivalent to 1.14 mg/kg VAL orally by gavage for seven days after induction of hypertension (6 weeks).

In all the above mentioned groups after induction of hypertension (6 weeks), treatment was given daily to overnight fasted rats as single dose therapy orally by gavage for seven days.

The *in vivo* antihypertensive efficacy of VAL, VAL:M-β-CD, VAL:M-β-CD:Tween 80 (0.5%, v/v) in DOCA-salt induced hypertensive rats was evaluated by comparing the percentage decrease in systolic blood pressure after daily single dose oral administration by gavage of VAL (1.14 mg/kg) and the binary and ternary systems equivalent to 1.14 mg/kg of VAL. The changes in systolic blood pressure of all the groups were recorded at 10 min time intervals, by the indirect tail cuff method (Kh, Khullarb, Kashyapb, Pandhi, & Uppala, 2000) using the blood pressure recorder Cat. 8006, UgO (Basile, Biological Research Apparatus, Italy) to monitor the efficacy and potency of prepared VAL binary and ternary complexes in comparison to the groups I, II, III and IV. Heart rate was recorded from the digital recording displayed by the instrument.

2.7.4. Statistical analysis

Data was expressed as mean ± SD. One way analysis of variance (ANOVA) was used for each parameter, followed by Tukey's test. $P < 0.01$ is considered statistically significant.

3. Results and discussion

The inclusion complexes were characterized in the solid as well as in the solution phase and the mode of interaction is also proposed by *in silico* studies.

3.1. Phase solubility studies

3.1.1. Interaction of VAL with various cyclodextrins

The analysis of phase solubility diagram is one of the essential techniques to explain the host–guest relationship and has become the preliminary method to evaluate the inclusion complexation (Higuchi & Connors, 1965). The complexation ability changes with the type of cyclodextrin, so various cyclodextrins (α-CD, β-CD, HP-β-CD and M-β-CD) were tested to select the most suitable for the present study. The aqueous solubility of VAL increased linearly as a function of cyclodextrin concentration, indicating the formation of soluble inclusion complexes with 1:1 stoichiometry, however M-β-CD showed superior solubilizing and complexing ability towards VAL. This might be attributed to the easier access of guest molecules to the enlarged opening and extended lipophilic central cavity of the modified CD (M-β-CD) due to the presence of methyl groups (Upadhye et al., 2010). Furthermore, the strong intramolecular hydrogen bond network in native β-CD structure destroyed due to the introduction of methyl substituents, making it more prone to interaction with a lipophilic drug. This led to the selection of M-β-CD for further experimental work.

3.1.2. Screening of the third component

The multi-component technology as a strategy for significant reduction in amount of cyclodextrin in desired formulations is well established, however, a few reports are also available showing the adverse effect of the polymer on solubility (Bakshi, 2007). Therefore the screening of possible third component for ternary complex is a vital step, and Tween 80 (0.5%, v/v) was found to be most suitable out of the various additives (polyvinylpyrrolidine, hydroxylpropyl methyl cellulose, polyethylene glycol and poloxamer) for increasing the solubilizing efficacy of cyclodextrin. Therefore, it seemed of interest to further prepare the ternary complex and subjecting it to characterization and evaluation.

3.1.3. Solubility of VAL in binary and ternary systems

The solubility of VAL in unbuffered triple distilled water was found to be 0.124 mg/mL in comparison to 0.178 mg/mL L^{-1}, 0.17 g/mL and 0.085 mg/mL as reported by Nalawade et al. (2009), Martin Del Valle (2004), and Cappello et al. (2001, 2005), respectively. The difference may be due to slight variation in experimental conditions by different workers. The solubility of VAL was found to increase from 0.286 mmol L^{-1} to 1.73 mmol L^{-1} in the presence of 8 mmol L^{-1}. M-β-CD indicating a 6-fold increase in solubility and is characterized by an AL type diagram. At same concentration of M-β-CD, the solubility of drug in presence of Tween 80 (0.5%, v/v) is found to be 4.66 mmol L^{-1}. This leads to ~2.7-fold increase in solubility of VAL on switching over from binary system to ternary and overall there is 16.3 times increase in solubility of VAL in drug:M-β-CD (8 mmol L^{-1}):Tween 80 (0.5%, v/v) system in comparison to solubility in unbuffered water. This suggests that when M-β-CD and Tween 80 (0.5%, v/v) are present together in solution, increase in solubility is greater (Fig. 1a) as compared to when they are used separately indicating that the increase in solubility upon addition of Tween 80 (0.5%, v/v) to binary system is synergistic than simply additive. To further confirm the synergistic effect of Tween 80 (0.5%, v/v), the solubility studies for VAL were performed in the presence of pure Tween 80. Fig. 1b depicts the effect of increasing concentrations of Tween 80 on the solubility of VAL in unbuffered triple distilled water either with or without 2 mmol M-β-CD. In VAL-Tween 80 binary systems, the equilibrium plot shows a continuous increase in apparent drug solubility up to about two folds that of VAL alone in the presence of 0.5% (v/v) and after which plateau is reached. It is interesting to note that in ternary system, at 2 mmol L^{-1} of M-β-CD and 0.5% (v/v) of Tween 80 concentration a comparable solubility (1.55 mmol L^{-1}) of VAL has been achieved which in case of binary system VAL-CD was observed at 8 mmol L^{-1} (1.73 mmol L^{-1}). Thus, this approach made it possible to achieve the targeted solubility with minimum amount of cyclodextrin. The linear phase solubility curve in the presence of 0.5% (v/v) of Tween 80 was also found to be AL type. The addition of polymer does not seem to change the stoichiometry in all the complexes (1:1) as the slope

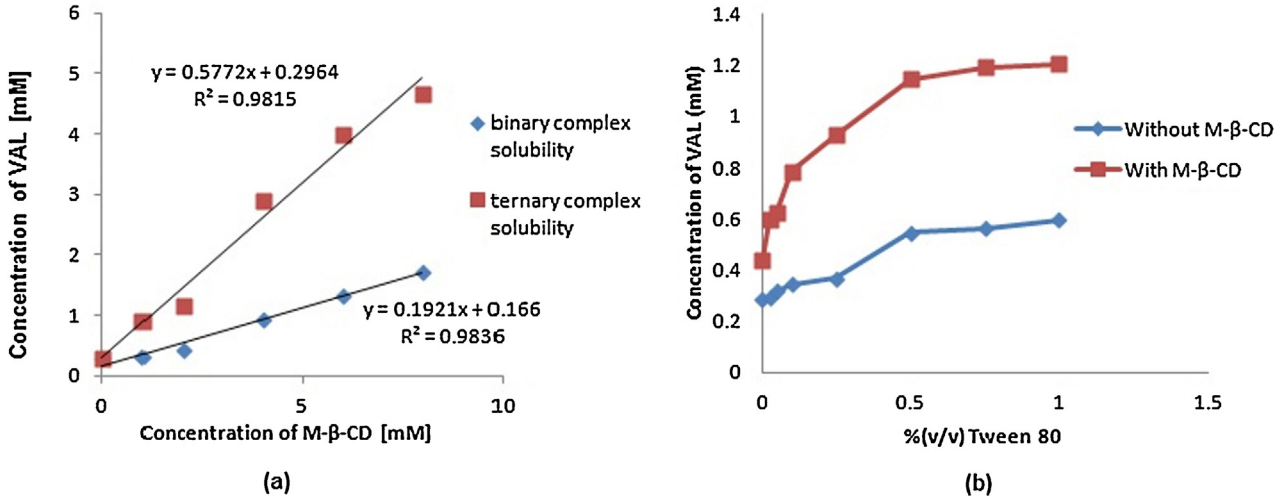


Fig. 1. Phase solubility diagram of VAL (a) in aqueous solutions of Tween 80 with or without 2 mmol M-β-CD binary and ternary complexes (b) in aqueous solutions of M-β-CD with or without Tween 80 (0.5%, v/v).

still continues to be less than one. This led to the preparation of ternary inclusion complexes containing the drug and cyclodextrin in the presence of 0.5% (v/v) Tween 80.

3.2. Molecular modelling studies

The molecular modelling studies have been performed with intent to scrutinize the mechanism of complexation between VAL (Fig. 2a) and M-β-CD. Based on the pK_a of VAL different ionization states were identified; bearing charge +1, charge 0 (zwitterion) and charge −1. All three ionization states were respectively docked into the cavity of M-β-CD using fast and rigid body docking technique, which places guest into the active cavity using a set of rotational and translational motions. Diverse docking solutions were identified for VAL based on the ionization states and orientations of the molecules inside the M-β-CD cavity and only one charge state of −1 was chosen according to its lowest energy and is further used for MD simulations to achieve realistic conformations for VAL:M-β-CD complexes. During the docking studies, it was observed that the VAL molecule oriented itself inside the M-β-CD cavity in two different ways. In mode-1, the aromatic ring of VAL is buried in the M-β-CD cavity with hydrogen bond interaction seen between the tetrazole basic nitrogen and the secondary hydroxyl group (2-OH) of M-β-CD molecule β-CD-glucose sub-unit (Fig. 2b). In mode-2, the aliphatic chain is embedded in the M-β-CD cavity while the rest of the molecule remains outside the cavity on secondary face of M-β-CD (Fig. 2c). Hydrogen bonded interactions that stabilize the complex are seen to exist between the carbonyl of amide and carboxylate functions of VAL and secondary hydroxyl groups (2-OH and 3-OH) on M-β-CD-glucose sub-units. The binding energies (−17.77 kJ mol^{-1} for aromatic region and −17.22 kJ mol^{-1} for aliphatic region) determined from MD simulation over 10 ns time length revealed that both sides of VAL form inclusion complex of almost similar stability. The possibility of 1:2 and 2:1 complexes has been ruled out because of high instability of formed complexes due to steric impediment. Thus the molecular modelling studies predicted the existence of two types of complexes with 1:1 stoichiometry and equal stability which can coexist in solution phase. Therefore, the binary and ternary complexes for characterization in solid phase were prepared keeping in mind the 1:1 stoichiometry.

3.3. Characterization in solid state

3.3.1. Differential scanning calorimetry (DSC)

The qualitative investigation of thermal behaviour of VAL, VAL:M-β-CD (physical mixture), and the lyophilized samples from

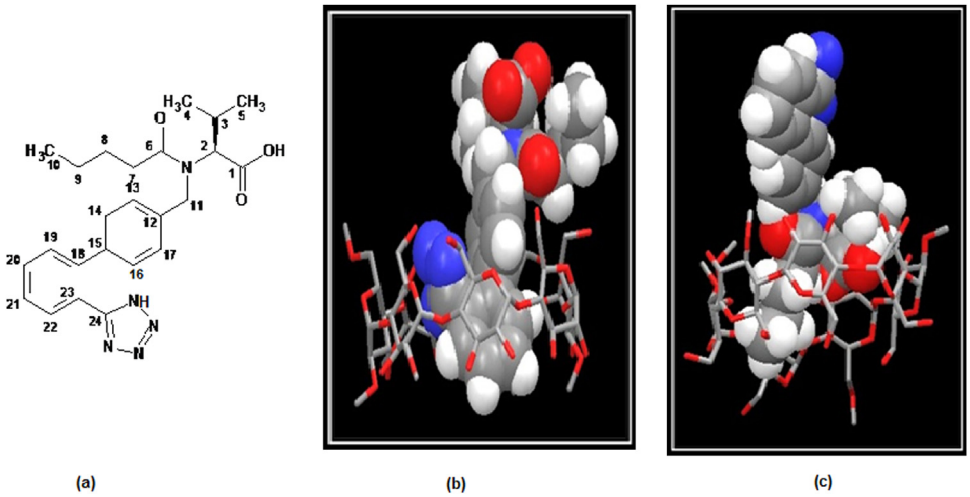


Fig. 2. (a) Chemical structure of VAL (b) orientation of complex on inclusion of VAL aromatic ring into M-β-CD (c) orientation of complex on inclusion of VAL aliphatic chain into M-β-CD.

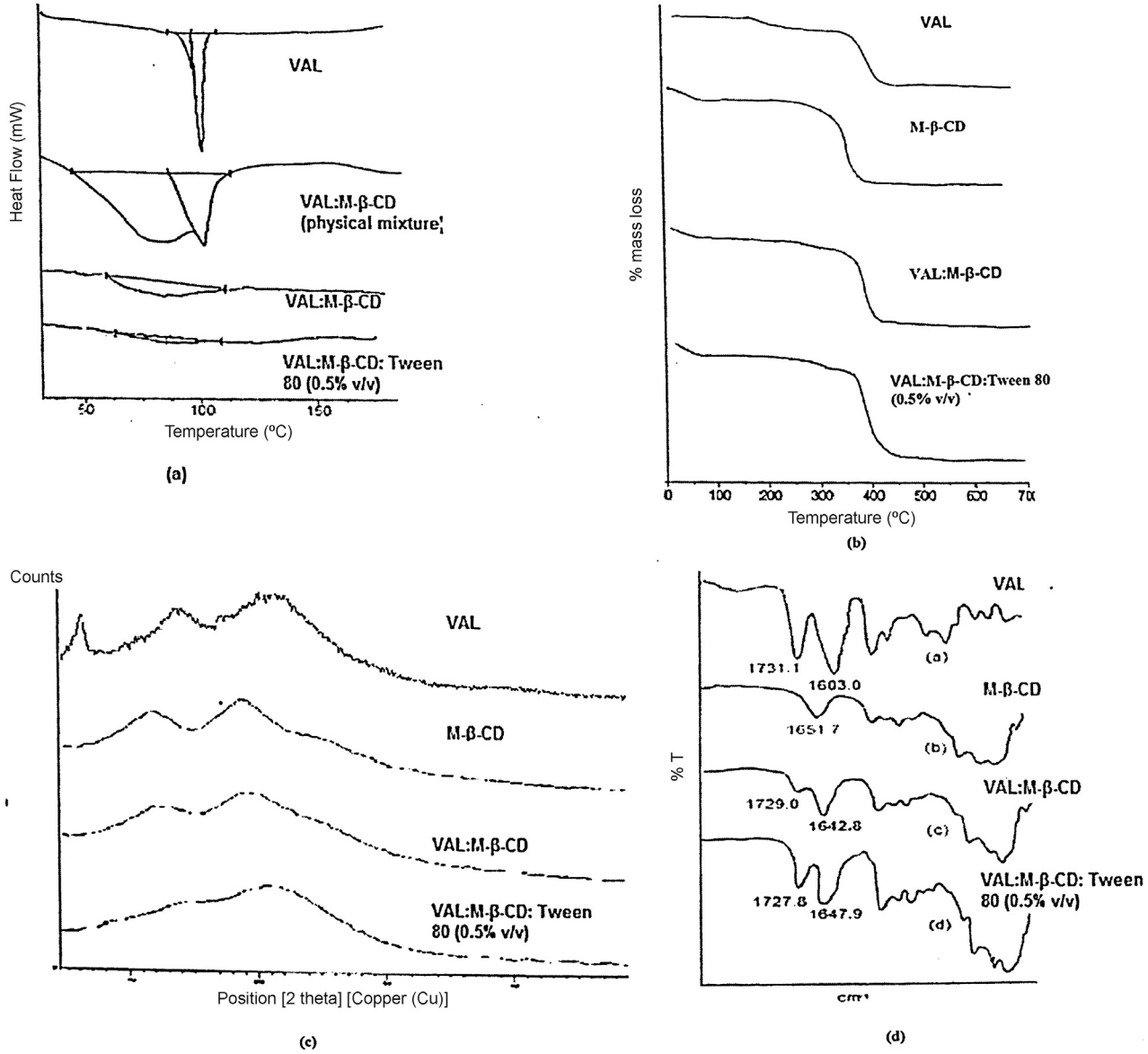


Fig. 3. (a) DSC curves of VAL, VAL:M-β-CD (physical mixture), VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) (b) TGA curves of VAL, VAL:M-β-CD, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) (c) X-ray diffraction pattern of VAL, M-β-CD, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) (d) FTIR spectra of VAL, M-β-CD, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v).

DSC was done to characterize the binary and ternary systems. In Fig. 3a the DSC trace of VAL exhibited an endotherm at 104.03 °C corresponding to its melting. DSC profile of the physical mixture showed broadening as well as displacement of endothermic peak towards lower temperatures which is ascribed to incomplete inclusion with some interaction. On contrary, the melting peak completely disappeared in both binary as well as in the ternary lyophilized system. As mentioned above that the melting point of drug is 104.03 °C and there may be possibility of merging of drug endotherm with dehydrating peak of M-β-CD in the complex. Thus at this point it is difficult to conclude the confirmation of true inclusion complexation on the basis of DSC alone and other techniques (PXRD and FTIR) were used to confirm the formation of inclusion complex in solid state.

3.3.2. Thermogravimetry (TG)

The TGA curves for VAL and methyl-β-cyclodextrin shows two mass loss regions. For VAL, first mass loss occurs between 150–260 °C (12.5%) and the second between 260–500 °C (70.27%) due to thermal decomposition of VAL while for M-β-CD first occurs below 90 °C due to loss of water and the other at temperature above 300 °C due to its decomposition. Fig. 3b shows TGA results for the binary and ternary complexes, in both these cases TGA evidences the loss of water below 90 °C and the onset temperature for thermal decomposition of VAL shifted to higher temperature probably due to improved thermal stability on complexation with M-β-CD. This shift in temperature is more in case of ternary complex as compared to binary complex. Moreover mass loss of 79.20% above 300 °C in case of ternary complex as compared to 82.06% in binary complex shows formation of much stable ternary complex (Serafini et al., 2012).

3.3.3. X-ray powder diffractometry (XRPD)

The XRPD patterns of both (VAL and M-β-CD) are nearly same (Fig. 3b) and lack sharp distinct peaks except one characteristic peak of VAL at 6.6476. This characteristic peak of VAL is found to be absent in binary and ternary systems which may be due to the

Table 1
Variation of ^{1}H chemical shifts before and after inclusion.

Position	δ_{free} (ppm)	$\delta_{complex}$ (ppm)	$\Delta\delta$ (ppm)	Shift
H-7	2.4898	2.0020	−0.4878	Upfield
H-8	2.0448	2.0838	0.0390	Downfield
H-9	1.2954	1.3617	0.0663	Downfield
H-10	1.1452	1.1499	0.0047	Downfield
H-13 and H-17	6.9729	6.9683	−0.0046	Upfield
H-14 and H-16	7.0236	7.0294	−0.0058	Upfield
H-19 and H-22	7.5439	7.5589	0.0150	Downfield
H-20 and H-21	7.6516	7.6692	0.0176	Downfield

$\Delta\delta$ (ppm) = $\delta_{complex} - \delta_{free}$.

inclusion of the drug into the CD cavity, indicating the formation of a true inclusion complex.

3.3.4. Fourier transform infrared spectroscopy (FT-IR)

In Fig. 3c distinct changes were observed in VAL characteristic IR peaks. The peaks at 1731.0 cm^{-1} for carboxyl carbonyl and 1603.0 cm^{-1} for amide carbonyl have shifted to 1729.0 cm^{-1} and 1642.8 cm^{-1} respectively, in binary complex. This reduction in the peak intensity and the shift in the peak position were more prominent in the ternary complex (1727.8 cm^{-1}, 1647.9 cm^{-1}), which confirms that Tween 80 enhances the complexation efficiency of cyclodextrin with drug.

3.3.5. Elemental (CHN) analysis

The results of CHN analysis have shown the amount of nitrogen in binary complexes to be 4.03% (Theoretical: 4.01%). The percentage of nitrogen present in ternary complex is approximately the same as expected because the Tween 80 is present only in 0.5% (v/v).

3.4. Characterization in solution phase

3.4.1. Nuclear magnetic resonance spectroscopy (NMR)

The 1D and 2D NMR have been utilized to confirm the interaction and to predict the direction of penetration of guest molecule into the cyclodextrin cavity (Veiga, Fernandes, Carvalho, & Geraldes, 2001). The encapsulation has been ascertained by the changes in the chemical shifts (δ) of the protons in the complex, relative to free VAL (Table 1). The structure of VAL suggests that both the aromatic ring and the aliphatic chain have the potential for entering the CD cavity. On comparing the values of chemical shifts of protons attached to aromatic ring and aliphatic chain of VAL, occurrence of appreciable changes in the chemical shifts of guest molecule indicated the interaction of identified regions of VAL with the cyclodextrin cavity (Table 1). The protons H-8, 9, 10 showed appreciable downfield shifts indicating that the aliphatic protons are located close to the hydroxyl groups present on the rim of the cyclodextrin cavity. Also, as a consequence of interaction between aromatic ring and cyclodextrin, the H-19, 20, 21 and 22 protons shifted to a higher frequency (deshielding) indicating that this part of the drug molecule is also involved in complexation. The upfield shift of H-7 proton of aliphatic chain and H-13, 14, 16, 17 of aromatic ring is attributed to the variation in the local polarity indicating a weaker interaction with the hydrogen atoms positioned just outside the cyclodextrin cavity. The most significant upfield shift was observed for the inner cavity proton H-3′ (−0.0482 ppm) of M-β-CD while relatively low upfield shift was observed for H-5′ proton (−0.0093 ppm), suggesting that VAL enters from the wider side of the cyclodextrin cavity. The above observations suggest deep inclusion of both aromatic ring as well as the aliphatic chain into the cyclodextrin cavity.

These 1D NMR results are further supplemented by 2D ROESY NMR which shows (Fig. 4a) the presence of NOE cross-peaks between protons of VAL and M-β-CD molecule and providing important conformational information. Two intermolecular cross peaks were found to be of interest, the first peak between H-3′ and H-5′ protons of M-β-CD with aromatic protons and the second one between H-3′ and H-5′ protons of M-β-CD with H-8, 9, 10 of aliphatic chain. These correlations indicate the spatial contacts between wider side of the cyclodextrin cavity with the aliphatic as well as aromatic ring of VAL.

1D and 2D NMR studies revealed that there are two distinct regions of VAL which could be included in M-β-CD; however, the stoichiometry of 1:1 has been established by phase solubility studies. To unravel the incongruity in the results of phase solubility, NMR and molecular modelling studies, the solution calorimetric analysis has been performed to confirm the stoichiometry as well as to calculate the stability constant.

3.4.2. Thermodynamic parameters

Solution calorimetry was used to determine the magnitude of stability constant and enthalpy associated with binding by determining the enthalpy of solution (Chadha, Jain, Aggarawal, Singh, & Thakur, 2007). The enthalpy of solution of VAL ($\Delta_{sol}H$) was determined in buffered aqueous solution showing exothermicity. The molar enthalpy of solution ($\Delta_{sol}H_{(M)}$) is found to be −2.01 kJ mol^{-1}. The enthalpy of solution of VAL ($\Delta_{sol}H_{(CD)}$) in the presence of cyclodextrins ($\Delta_{sol}H_{(M)(CD)}$) was found to be more exothermic which is attributed to interaction between drug and cyclodextrins. The enthalpy of solution was calculated by using Eq. (2):

$$\Delta H_{int(exp)} = \frac{\Delta_{sol}H_{(CD)} - \Delta_{sol}H}{v(l)} \quad (2)$$

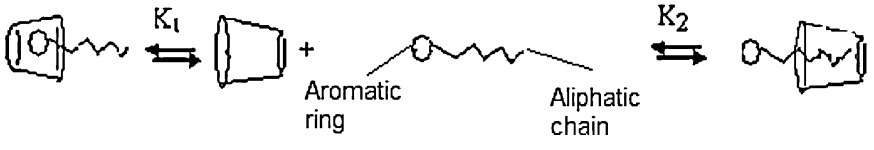
$\Delta H_{int(exp)}$ = enthalpy of interaction between drug and cyclodextrin per litre of the solution. $\Delta_{sol}H$, $\Delta_{sol}H_{(CD)}$ are the enthalpies of solution of VAL in buffer and in buffered aqueous solution of M-β-CD, respectively. $v(l)$ = volume of sample cells in litres (0.025 L).

Enthalpy of interaction per mole of drug and cyclodextrin calculated using Eq. (3):

$$\Delta H_{int(M)} = \frac{\Delta H_{int(exp)}}{a+b} = \frac{\Delta_{sol}H_{(M)(CD)} - \Delta_{sol}H_{(M)}}{1 + x_2/x_1} \quad (3)$$

Here a and b are the initial molar concentrations of the VAL and M-β-CD in the solution. x_1 and x_2, apparent mole fractions of the drug and cyclodextrin ignoring the concentration of buffers.

The stoichiometry was ascertained utilizing continuous variation method (Job's plot) by plotting ($\Delta H_{int(M)}$) against (x_2) for both binary and ternary systems (Fig. 4b). It is clearly indicated that the minima occurs at x_2 = 0.5 confirming the 1:1 stoichiometry as indicated by phase solubility. As discussed above NMR as well as molecular modelling data indicate two potential regions in VAL, therefore, a two class binding model was utilized to determine the concentration of drug: CD complex which suggested that two types of 1:1 complexes co-exist in the solution. Now the binding constants are calculated assuming the following two equilibra.



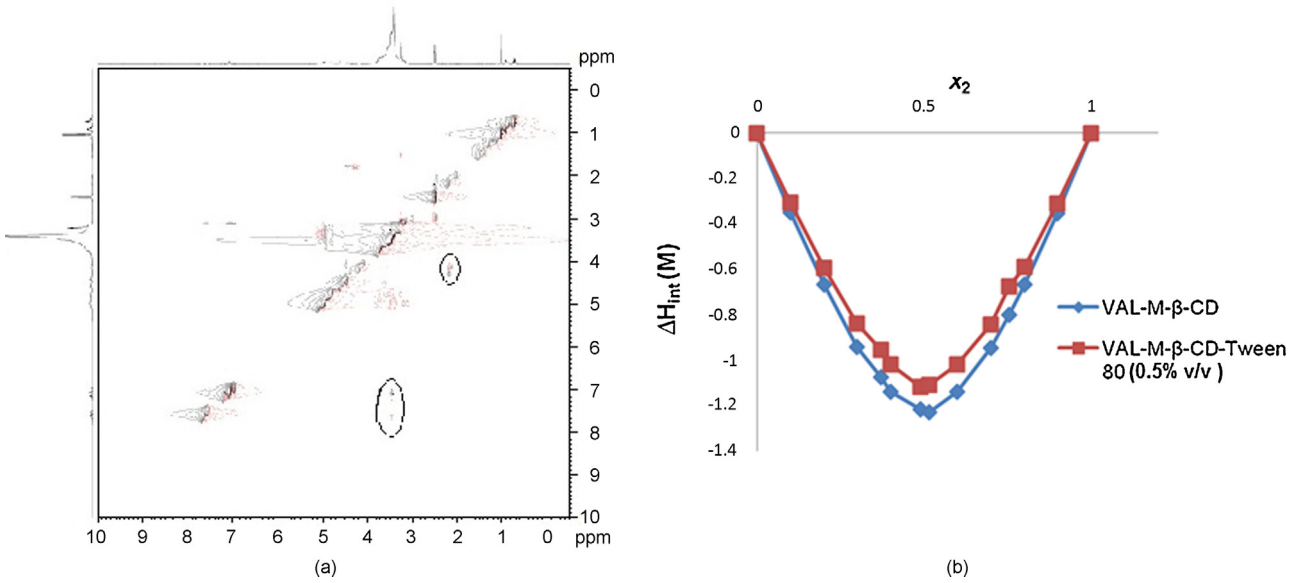


Fig. 4. (a) ROESY spectrum of VAL with M-β-CD (b) plot of molar enthalpy of interaction ($\Delta_{sol}H_{int(M)}$) of VAL with M-β-CD in the presence and absence of Tween 80 (0.5%, v/v).

K_1 is the equilibrium constant for one type of complex where aromatic ring enters the cyclodextrin cavity.

K_2 is the equilibrium constant for second type of complex where the aliphatic chain enters the host cavity.

The experimentally calculated enthalpy of interaction ($\Delta H_{int(exp)}$) is proportional to the sum of the product of molar concentration of VAL:M-β-CD complex in the solution at equilibrium and associated molar enthalpy of binding.

$$\Delta H_{int(calc)} = [\Delta H_1 \times K_1 (C)(D)] + [\Delta H_2 \times K_2 (C)(D)] \quad (4)$$

where ΔH_1 and ΔH_2 are molar enthalpy of binding of complex 1 and complex 2, respectively. C = free concentration of M-β-CD, D = free concentration of VAL.

Successive iteration was used to compute the interaction parameters ($K_{(i)}$ and $\Delta H_{(i)}$) for the drug-CD system.

The values of K_1 and K_2, ΔH_1 and ΔH_2 were evaluated by using a self consistent iterative non-linear least square regression programme after successive iteration to minimize the values of $\sum\{\Delta H_{int(exp)} - \Delta H_{int\,L(calc)}\}^2]$.

Further the values of C and D were calculated by iteration until their values within selected limits were obtained by putting any reasonable initial values of K_1 and K_2. The details are given in our previous paper (Chadha, Gupta, Shukla, Jain, & Singh, 2011). The values of free energy of inclusion (ΔG_1° and ΔG_2°) and entropy of inclusion (ΔS_1° and ΔS_2°) for VAL have been calculated from the following equations and are given in Table 2.

$$\Delta G_1^\circ = -RT \ln K_1 \quad (5)$$

$$\Delta G_2^\circ = -RT \ln K_2 \quad (6)$$

$$\frac{\Delta S_1^\circ = (\Delta H^\circ - \Delta G_1^\circ)}{T}$$
$$\frac{\Delta S_2^\circ = (\Delta H^\circ - \Delta G_2^\circ)}{T} \quad (7)$$

The same method was utilized to determine the stability constant and other thermodynamic parameters in the presence of Tween 80 (0.5%, v/v). The enthalpy of solution of VAL in the presence of Tween 80 (0.5%, v/v) and M-β-CD was found to be more exothermic. This is attributed to positive interaction among all the three components (VAL:M-β-CD:Tween 80) in the complex.

The above mentioned thermodynamic parameters, K, ΔH, ΔG and ΔS, of the binding process are essential for determining the driving forces of interaction of VAL with M-β-CD. The values of the binding constants thus determined for both binary and ternary systems are given in Table 2. The higher value of K for ternary system reflects the higher efficiency of VAL to be included into the cyclodextrin cavity. This synergistic effect shows that Tween 80 facilitates the inclusion of VAL into M-β-CD cavity. The negative values for free energy for both binary and ternary complexes means that the binding process is a spontaneous process and thermodynamically favoured. The entropic terms, $\Delta S > 0$, for the complexation are also found to be favourable for complexation.

3.4.3. Dissolution studies

The percent release of VAL from physical mixture, VAL complexes with M-β-CD in the presence and absence of Tween 80 was compared by using two relative parameters. At time t = 2 min the two relative parameters of the dissolution process, *i.e.* the percentage of VAL dissolved (DP) and the relative dissolution rate (RDR) for pure drug, VAL:M-βCD (physical mixture), binary and ternary system, respectively, were calculated and followed the order as

Table 2
Thermodynamic parameters associated with the inclusion of VAL with M-β-CD in the absence and presence of Tween 80.

System	K_1 (M^{-1})	ΔH_1° (kJ mol^{-1})	ΔG_1° (kJ mol^{-1})	ΔS_1° (J mol^{-1} K^{-1})	K_2 (M^{-1})	ΔH_2° (kJ mol^{-1})	ΔG_2° (kJ mol^{-1})	ΔS_2° (J mol^{-1} K^{-1})
VAL:M-β-CD	1741.6	−9.35	−19.23	31.87	1499.2	−8.82	−18.85	32.35
VAL:M-β-CD:Tween 80	1992.0	−13.44	−19.58	19.81	1864.0	−12.38	−19.41	22.67

Table 3
Percent of VAL dissolved (DP) and relative dissolution rate (RDR) at $t = 2$ min of VAL and its physical mixture, binary system and ternary system with M-β-CD.

Sample	Percentage of VAL dissolved (DP) at $t = 2$ min	Relative dissolution rate (RDR)
VAL	14.2	1
Physical mixture	28.7	2.0
VAL:M-β-CD	79.7	5.6
VAL:M-β-CD:Tween 80	86.2	6.1

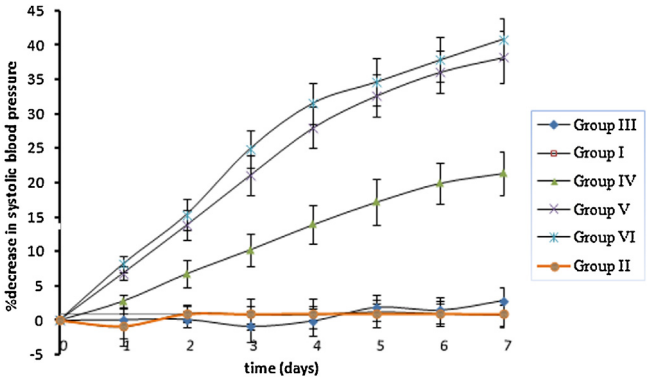


Fig. 5. Antihypertensive activity of VAL, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) in DOCA induced hypertensive rats as compared to VAL ($n = 6$).

VAL:M-β-CD:Tween 80 (0.5%, v/v) > VAL:M-β-CD > physical mixture > VAL (Table 3). There is 6-folds increase in VAL dissolution rate from ternary complex as compared to that from pure drug.

3.4.4. Permeability studies

The assessment of cyclodextrin induced bioavailability of VAL and effect of Tween 80 (0.5%, v/v) on it was also done by measurement of drug permeability through cellulose membrane as barrier model. In this study permeation was performed on VAL, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) lyophilized complexes. A significant increase in value of permeation flux was observed on moving from pure VAL (0.126 mg/cm^2/min) to VAL:M-β-CD complex (0.147 mg/cm^2/min). A further increase in permeation flux (0.167 mg/cm^2/min) occured when a surfactant was present as the ternary component. Thus, it can be concluded that the permeation improved significantly when VAL is complexed with cyclodextrin, and this improvement is more prominent in the presence of Tween 80 (0.5%, v/v).

3.5. Pharmacological studies

Efficacy of binary and ternary complexes was determined by performing *in vivo* study. A gradual noticeable increase of approximately 50–90 mm Hg in mean systolic blood pressure over a period of 6 weeks was observed in DOCA treated rats (groups III, IV, V and VI) contrary to control rats (groups I and II). After the oral administration of M-β-CD and M-β-CD:Tween 80 (0.5%, v/v) in CMC suspension to groups I and II, respectively, changes in the systolic blood pressure were recorded and expressed as percentage decrease in the systolic blood pressure. No noteworthy change in the blood pressure levels of rats during the experimental period was observed (Fig. 5). During the experimental period after six weeks the variation in systolic blood pressure caused by oral administration of VAL, VAL:M-β-CD, VAL:M-β-CD:Tween 80 (0.5%, v/v) to groups IV, V and VI is depicted in Fig. 5. Significant lowering of elevated systolic blood pressure was observed in hypertensive rats as compared to vehicle treated DOCA control rats. VAL 3 has reduced the systolic blood pressure in group IV but was not able to normalize whereas the VAL:M-β-CD, VAL:M-β-CD:Tween 80 (0.5%, v/v) had normalized the increased blood pressure values in V and VI. After seven day treatment with VAL, VAL:M-β-CD and VAL:M-β-CD:Tween 80 (0.5%, v/v) percent decrease in the elevated systolic blood pressure values was 21.39%, 38.25% and 40.86%, respectively. These results supports the efficacy of VAL:M-β-CD:Tween 80 (0.5%, v/v) ternary complex as compared to binary complex.

The experimental data depicted in Fig. 5 was statistically analyzed by ANOVA and significant decrease in systolic blood pressure as compared to the control, DOCA control and the pure drug was observed ($P \leq 0.001$).

4. Conclusions

The complexation efficiency of M-β-CD with VAL was found to increase on addition of 0.5% (v/v) Tween 80. NMR experiments and molecular modelling studies indicate that aliphatic as well as aromatic rings fit well into the cyclodextrin cavity, suggesting simultaneous existence of two types of complexes which was confirmed by solution calorimetry. The higher values of stability constants were obtained when Tween 80 (0.5%, v/v) is present as the third component and also the negative enthalpy and positive entropy of binding determined by solution calorimetry indicated the formation of stable system. The dissolution rate for ternary was found to be higher than binary system and pure drug. The permeation flux was highest for the ternary system and maximum decrease in systolic blood pressure in DOCA induced rats was observed for this system, indicating the improvement of pharmaceutical potential of VAL in the form of VAL:M-β-CD:Tween 80 (0.5%, v/v) complex.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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