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ORIGINAL ARTICLE

Improvement of some pharmaceutical properties of mycophenolate mofetil (MMF) by para sulphonatocalix[4]resorcinarene inclusion complex

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Abstract As a part of our investigations to unfold the chemistry of calixresorcinarene, we have focused on the formation of inclusion complex of a poorly soluble (43 μg ml^{-1} at pH 7) drug mycophenolate mofetil (MMF) an immunosuppressive agent and an inosine monophosphate dehydrogenase (IMPDH) inhibitor with para sulphonatocalix[4]resorcinarene (PSC4R). The complete complexation of the drug was achieved after 48 h of stirring with para sulphonatocalix[4]resorcinarene(PSC[4]R) in water and evaporation of water yield the solid complex. The interaction between para sulphonatocalix[4]resorcinarene(PSC[4]R) and MMF in solid state inclusion complexes was accomplished by aqueous phase solubility studies, Thermal Analysis, HPLC, PXRD, FT-IR, and UV–Vis spectroscopy. The results of the phase solubility experiments are in good conformity to signify the formation of 2:1 PSC4R: MMF complexes. The purpose of this study was to enhance solubility and resulting in high dissolution rate and bioavailability of this essentially water insoluble drug. The results of the in vivo study shows that there is a remarkable change in the toxicity of the pure drug MMF and complex did not produce any mortality up to 2200 mg kg^{-1}.

Keywords Para sulphonatocalix[4]resorcinarene · MMF · Phase solubility study · In vitro release · Acute toxicity · Inclusion complex

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Introduction

Calixresorcinarene, a macrocyclic compound of phenolic units, serve as a good platform for the design and synthesis of biologically active compounds [1, 2]. They are also interesting host molecules for chemical biology study purposes [3]. Their basic molecular scaffold has potential ability for molecular recognition; it is promptly synthesized in large amounts, and might be easily modified for maximizing molecular interactions toward relevant guest molecules. Calixarenes present well-defined conformational properties and cavities with molecular dimensions that enable to encapsulate guest drugs [4].

Mycophenolate mofetil (MMF), the ester prodrug of mycophenolic acid (MPA), is a potent immunosuppressive agent that is used as a part of standard immunosuppressive regimens [5, 6]. Major advantage of MMF is that it provides immunesuppression without causing major bone marrow depression and is a promising agent in treatment of inflammatory glomerular diseases. MMF administration may be sometimes associated with tolerability problems, as a result of gastrointestinal adverse effects, such as nausea/vomiting, diarrhea, abdominal pain, and gastritis. The drug is practically insoluble in water (43 μg ml^{-1}).

An increasing number of publications and patents are concerned with inclusion compounds and their improvement of solubility and bioavailability using complexing agents to form host–guest complexes [7]. Among the complexing agents available, cyclodextrins (CDs) are most widely used in drug formulations [8, 9]. It is generally accepted that CD can form inclusion complex in aqueous solution where a lipophilic guest molecule or moiety locates in the inner cavity [10]. Due to this special property, CDs have been used extensively in pharmaceutical research, which enable drugs to increase solubility,

reducing bitterness, enhancing stability and decreasing tissue irritation [11]. Furthermore, CDs can be used for stabilising flavouring agents and reducing unpleasant odour and taste in food industry, as well as stabilising chemical labile compounds in cosmetics [12]. Moreover, some in vitro and in vivo pharmacological activities of chlorogenic acid, such as hypoglycaemic, antiviral, hepatoprotective and immunoprotective activities, have also been reported [13]. There is only publication in the literature that describe the formation of complexes between drug substance and para sulphonatocalix[n]arenes which has been reported by our group [4]. However there are no report on the interaction of para sulphonatocalix[4]resorcinarene (PSC[4]R) which are cyclic oligomers synthesized by condensation of resorcinol with various aromatic or aliphatic aldehyde [14, 15]. The calixresorcinarene provide more hydrophobic environment than the calixarene, and it also has hydrophilic heads (SO_3^-) imparting to them properties of both CDs and micelles.

The present investigation is concerned with improving the solubility and dissolution rate of mycophenolate mofetil (MMF) in aqueous solution in order to modify its bio availability. This was achieved through the formation of inclusion complexe (Figs. 1, 2) with *p*-sulphonatocalix[4]resorcinarene (PSC[4]R). The interaction between para sulphonatocalix[4]resorcinarene (PSC[4]R) and MMF in solid state inclusion complexes was confirmed by aqueous phase solubility studies, Thermal Analysis, HPLC, PXRD, FT-IR, and UV–Vis spectroscopy.

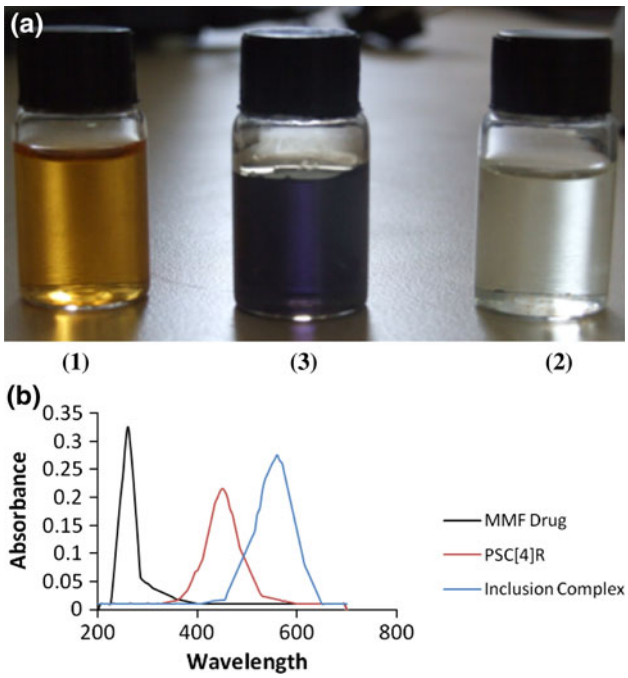

Fig. 1 **a** shows the color change after complexation (*1*) PSC[4]R (*2*) drug MMF (*3*) inclusion complex. **b** shows the UV spectra of the Drug MMF; PSC[4]R; inclusion complex

Materials and methods

Materials

Mycophenolate mofetil (MMF) was a gift sample from Intas pharmaceuticals Ahmedabad. All the chemicals used are of analytical grade supplied by Merck (India). Milli-Q water was prepared by Millipore (synergy) system and was used throughout the study. The calix [4]resorcinarene and the sulphonation of calix [4]resorcinarene has been carried out by the reported procedure [14–16] and characterized by ^{1}H NMR, ESI-MS, ^{13}C NMR. The 0.45 μm nylon filters were obtained from Millipore, Bedford, MA, USA.

Preparation of mycophenolate mofetil (MMF) PSC[4]R complex

Solid complexes of Mycophenolate Mofetil (MMF) and para sulphonato calix [4]resorcinarene (PSC[4]R) was prepared by rotary shaking. A molar ratio mixture of 1:2 MMF:PSC4R (41:224) mg) were taken into 250 mL stoppered conical flask containing 100 mL distilled water. This flask was then shaken on rotary shaker for 48 h at ambient temperature (37 °C) and then solution mixture was filtered through 0.45 μm filter. The solution was dried at 60 °C under vacuum (2–5 torr) to obtain solid complex.

Dissolution studies

The dissolution studies were performed by the USP XXIV rotating paddle method [17] at 37 °C. The dissolution rate of pure MMF, MMF:PSC[4]R physical mixture and inclusion complex was studied by mixing with placebo. Complex sample equivalent to 15 mg MMF was placed into 900 mL dissolution medium (0.1 N HCl, pH 1.2) and was stirred at 50 rpm. Aliquots (10 mL) of the dissolution mixture were withdrawn after regular time intervals, followed by immediate filtration (0.45 μm pore size filter), and analyzed by HPLC–UV. The experiment was performed in triplicate and the standard deviation was evaluated.

Phase solubility study

Phase solubility studies were carried out according to the method reported by Higuchi and Connors [18]. Excess amounts of MMF were added to 0.1 N HCl solutions (pH-1.2) containing para sulphonatocalix[4]resorcinarene in different molar concentrations and these flasks were then sonicated for 1 h and shaken at a rate of 100 strokes per min in orbital shaker incubator (Newtronic, India) for 48 h at constant temperature(25 °C). The suspensions were then filtered through 0.45 μm filter and assayed for MMF by

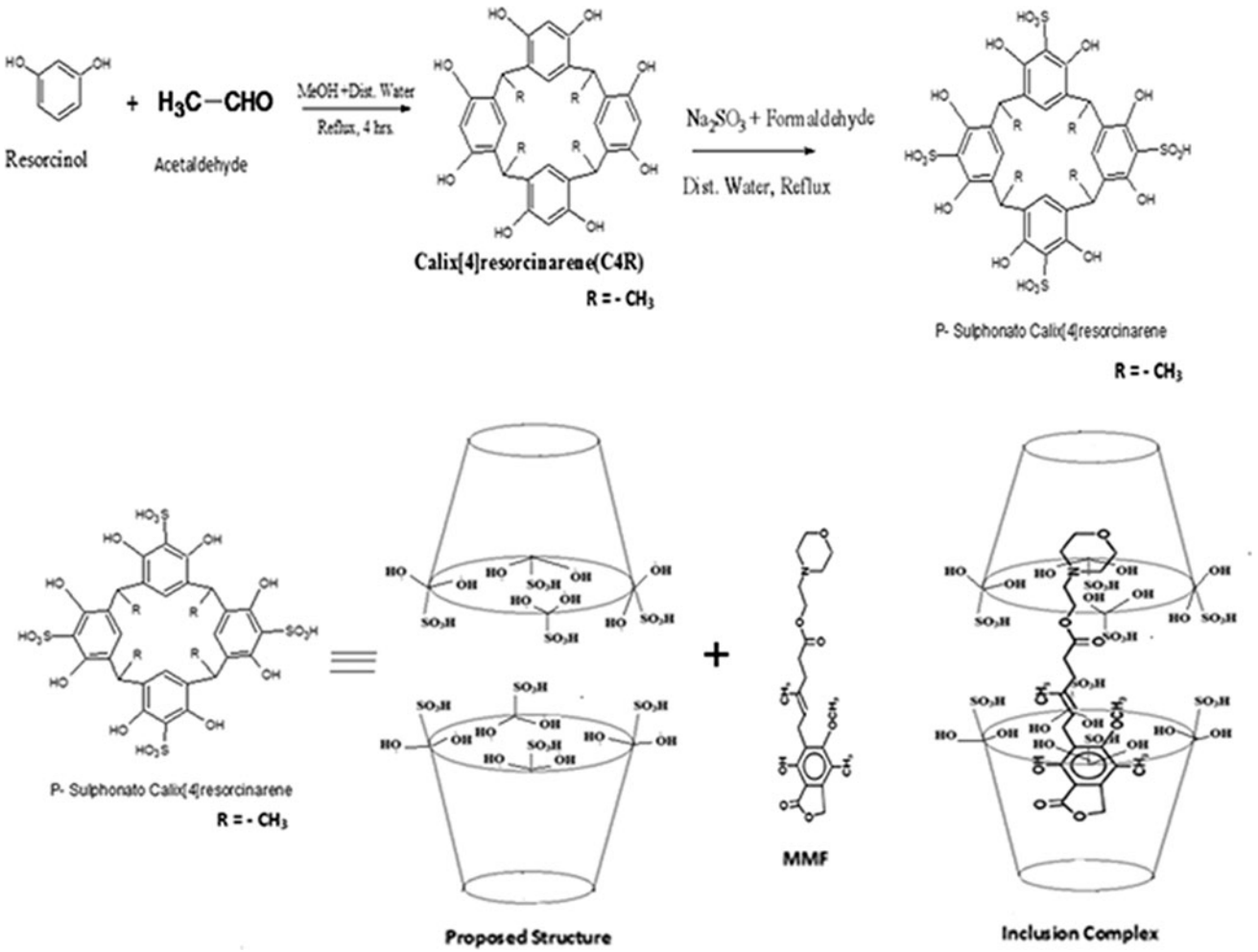

Fig. 2 Inclusion complex of MMF:PSC[4]R (1:2)

using HPLC. The solubility of MMF was also determined in water at 25 °C by the same method. The association constant (K_c) for the complex can be determined from the slope of linear portion using the following relationship, where S_0 is the intrinsic solubility of the drug under the condition studied.

$$K_c = \frac{\text{Slope}}{S_0\,(1 - \text{Slope})} \quad (1)$$

The association constant of the formed Inclusion complex was calculated by the above equation given by Higuchi et al., to define solubility characteristics [18].

Acute toxicity studies (LD_{50})

Acute Oral Toxicity (AOT) of inclusion complex of PSC[4]R was determined using Swiss albino mice. The animal were fasted for 3 h prior to the experiment and were administered with single dose of inclusion complex (doses range from 200 to 1000 mg kg^{-1} at various dose levels) dissolved in double distilled water and observed for mortality up to 48 h (short term toxicity). Based on the short-term toxicity, the dose of next animal was determined as per OECD guideline 425. All the animals were also observed for long-term toxicity (14 days). The LD_{50} of the test extract was calculated using 'AOT425' software provided by Environmental Protection Agency, USA.

Evaluation of the complex

An accurately weighed amount of the inclusion complex was dissolved in water and assayed for MMF using HPLC with UV detection. The HPLC system consisting of a Shimadzu model LC2010C isocratic pump and a Shimadzu variable wave length UV–visible detector, and output was processed and recorded by LC solution software on a Pentium computer. The analytical column used to achieve chromatographic separation was a stainless steel

(250 mm × 4.6 mm I.D., 5 μm particle size) Wakosil C18 (SGE). A JASCO V-570 spectrophotometer was used for scanning and selecting detection wavelength. The compounds were separated isocratically with a mobile phase consisting of water and methanol (50:50% v/v). Before use, the mobile phase was filtered by passing through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA) degassed ultrasonically. The flow rate was 1.5 mL min^{-1}. Chromatographic analysis was carried out at ambient temperature (25 ± 1 °C). The effluent was monitored by UV detector at 232 nm.

Since FTIR is a highly sensitive method of analysis, all spectra of complex show some or other changes from parent spectra i.e. pure drug and para sulphotonato calix[4]resorcinarene. FT-IR has previously been shown to be useful techniques for characterizing drug-cyclodextrin inclusion complexes [19]. Accordingly, we chose to employ these techniques to characterize the drug–PSC[4]R interactions. Samples were analyzed using Bruker Tensor-27 FT-IR spectrometer. Complex formation was evaluated by comparing the spectra of the solid complex, PSC[4]R and of the pure drug. Similarly, based on previous studies of drug–cyclodextrin interactions using differential scanning calorimetry (DSC) [20], we used Differential Scanning Calorimeter (Shimadzu model DSC-60) to study the solid state interaction of the drug with PSC[4]R.

Samples of the inclusion complex, pure drug, physical mixture, and PSC[4]R were heated in crimped aluminum pans over a temperature range of 50–500 °C. Nitrogen was used as carrier gas and the analysis was carried out at a heating rate of 10 °C min^{-1} and a nitrogen flow rate of 50 mL min^{-1}. The sample size was 3–5 mg. Along with DSC, we also employed powder X-ray diffraction (PXRD) to investigate the interaction between the drug and PSC[4]R. The powder X-ray diffraction studies were carried out in a SEIFERT XRD-7 X-ray diffractometer with Ni monochromated Cu Kα radiation in transmission mode. The PXRD pattern of solid complex, pure drug, and PSC[4]R were recorded between $2\theta = 5$–30° at tube power of 40 kV and 30 mA.

Result and discussion

Characterization of solid complex

The complex was characterized and evaluated by following methods.

UV–Vis study

The preliminary investigation has been carried out by the UV–Vis spectral analysis. Results showed three various peaks at the 245, 450 and 535 nm of pure drug MMF, PSC[4]R and Inclusion complex respectively which has been shown in the Fig. 1b. It is clear from the UV–Vis spectra that there is some interaction between the PSC[4]R and drug molecule.

FT-IR spectral analysis

FT-IR spectral studies were carried on FT-IR BRUKER TENSOR 27 using KBr pellets. Scanning was done from 4000 to 400 cm^{-1}. The FTIR spectra of MMF (Fig. 3c) showed a characteristic peak at 3,416 cm^{-1} (–OH stretching), 1,665 cm^{-1} (–CO–R vibration), PSC[4]R spectra (Fig. 3b) shows broad band of –OH bond peaks at 3,455, peaks at 1187, 1116 and 1056 cm^{-1} of $-SO_3$. The spectrum of solid inclusion complex (Fig. 3a) did not show any new characteristic peaks which indicates that no new chemical bond are formed in the complex.

Differential scanning calorimetry

Thermal behavior of MMF, PSC[4]R, physical mixture and inclusion complex was examined by using Differential

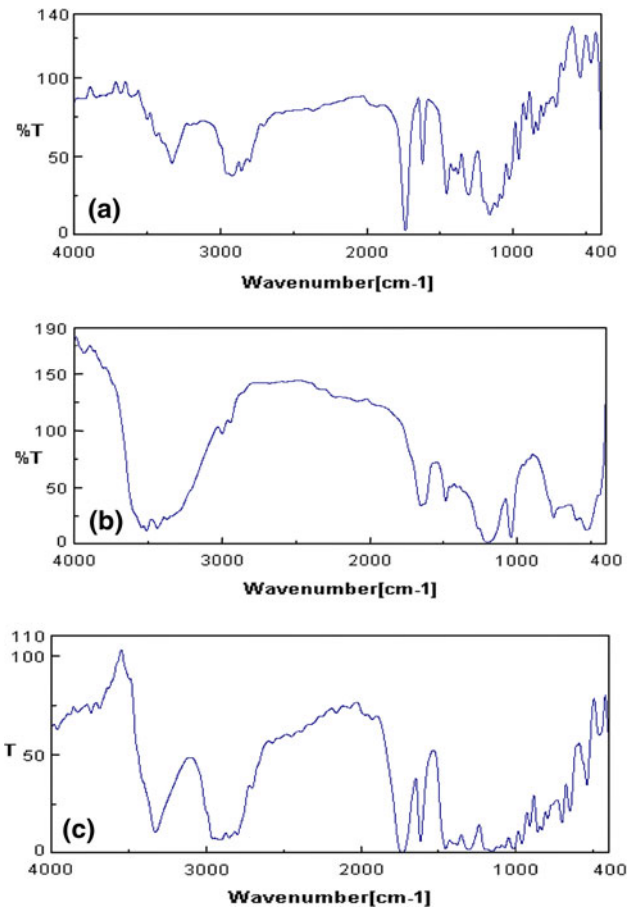

Fig. 3 FT-IR spectra of **a** inclusion complex, **b** PSC[4]R, c drug (MMF)

Scanning Calorimeter (DSC) (Shimadzu model DSC-60) nitrogen was used as carrier gas and the analysis was carried out at a heating rate of 10 °C min^{-1} and an argon flow rate of 50 mL min^{-1}. The sample size was 3–5 mg and examinations were made in the temperature intervals between 50 and 500 °C.

The DSC graph of pure MMF drug powder showed a sharp endotherm near 97 °C, which was indicative of its melting temperature, followed by an exotherm, which signified that after melting MMF decomposes (Fig. 4). The DSC graph of PSC[4]R powder showed a sharp endotherm near 235 °C, which was indicative of its melting temperature, followed by an exotherm, which signified that after melting PSC[4]R decomposes. The DSC pattern of MMF-PSC4R inclusion complex prepared by physical mixture showed the presence of peaks of both pure compounds except for a difference that the intensity of MMF melting endotherm had decreased. Thermogram of MMF-PSC4R (1:2) inclusion complex showed complete disappearance of the endothermic peaks characteristic of PSC[4]R and MMF, thus suggesting maximum/complete complex formation.

Powder X-ray diffraction studies

The powder X-ray diffraction studies were carried out in a SEIFERT XRD-7 X-ray diffractometer with Ni monochromated Cu Kα radiation in transmission mode. The PXRD pattern of solid complex, pure drug, and PSC[4]R were recorded between $2\theta = 5$–30° at tube power of 40 kV and 30 mA.

PXRD patterns for drug (MMF) PSC[4]R and the solid complex are shown in Fig. 5. The crystalline nature of MMF was clearly demonstrated by its characteristic PXRD pattern containing well-defined peaks. The drug characteristic peaks were observed at 2θ values 9.12°, 10.15°, 17.71°, 18.62°, and 20.11°. The PXRD pattern of PSC[4]R peaks were observed at 2θ values 11.03°, 18.55° and 23.19°. The PXRD diffractogram of the physical mixture constituted, appears to represent the superposition of both component although peaks attributable to MMF are remarkably reduced, indicating lower crystallinity of drug compared to pure drug. In contrast, inclusion complex showed no diffraction peaks except halo-pattern in X-ray diffractogram, suggesting an amorphous state of the drug in the inclusion complex. This decrease in the drug crystallinity was responsible for the increase in solubility as cited in literature [21].

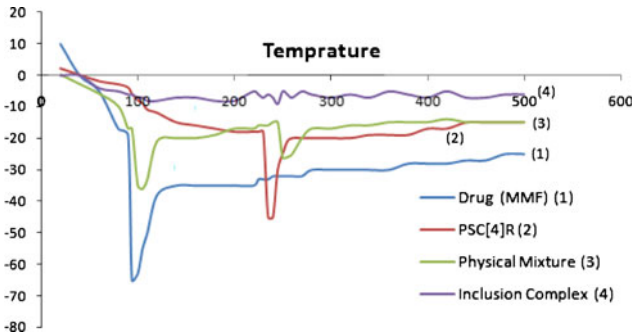

Fig. 4 DSC curves of (*1*) Drug (MmF), (*2*) PSC[4]R, (*3*) Physical Mixture, (*4*) Inclusion Complex

NMR study

The inclusion complex has also been confirmed by the NMR analysis. In PSC[4]R the aromatic hydroxyl (-OH) peak and sulphonic acid peak appeared at (9.8, s, 16H) and (8.25, s, 8H) respectively. NMR spectrum of the inclusion complex showed a change in the chemical shift values of hydroxyl and sulphonic acid group with –OH protons appearing in the range of δ 9.9–10.2 and $-SO_3H$ protons in the range of δ 8.3–8.4. The downfield shift in the protons of –OH and $-SO_3H$ groups can be attributed to the interaction of different hydroxyl and sulphonic acid protons with different ends of the drug molecule. Hence the NMR studies confirmed the results of the phase solubility studies that a 1:2 complex is formed between the drug and the receptor and moreover different ends of the drug is encapsulated in separate PSC[4]R molecules.

HPLC analysis

The HPLC analysis results are in the Table 1 below. The Fig. 6a shows the chromatogram of the pure drug MMF API having the retention time 5.4 min. Figure 6b shows the chromatogram of the PSC[4]R having the retention time 13.35 min. and Fig. 6c shows the chromatogram of the inclusion complex. Figure 6c shows the same retention time which we observed in the Fig. 6a and b for pure drug MMF and pure PSC[4]R. This results indicate the presence of MMF as well PSC[4]R in the inclusion complex.

Phase solubility study

The most common and widely used method to evaluate the ability of the inclusion complex is the phase solubility studies by Higuchi and Connors [18]. Higuchi and Connors have classified the various solubility behaviours seen during complex formation as A-type (a soluble inclusion complex is formed) or B-type (an inclusion compound of finite solubility is formed). The equilibrium binding or association constant (k) for the 1:1 complex can be determined from the slope of linear portion using the following relationship, where S_0 is the intrinsic solubility of the drug under the conditions studied.

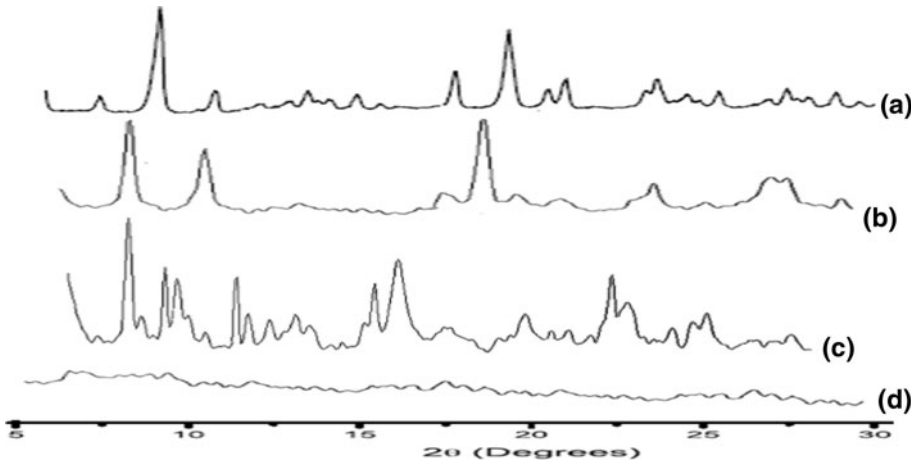

Fig. 5 X-ray diffraction patterns of: (*a*) MMF, (*b*) PSC[4]R, (*c*) MMF:PSC[4]R physical mixture, (*d*) inclusion complex

Table 1 Isocratic reverse-phase HPLC method for PSC[4]R, MMF, inclusion complex

Mobile phase	Methanol:water (50:50)
Column	C-18, 250 mm × 4.6 mm I.D., 5 μm particle size
Oven temperature	Ambient temperature (25 °C)
Flow rate	1.5 mL
Stop time	20 min.
UV	232 nm
Injection volume	20 μL
Retention time for MMF	5.4 min
Retention time for PSC[4]R	13.35 min

In the present work, complexation of MMF with PSC[4]R was carried out in an attempt to improve its solubility and dissolution rate. The phase solubility studies revealed a linear relationship (correlation coefficient > 0.99) between the aqueous drug solubility with increase in PSC[4]R concentration (Fig. 7) with the formation of soluble complexes. The equilibrium binding or association constant (k_c) for the 1:2 complex can be determined from the slope of linear portion using the following relationship (Eq. 1),

$$K_c = \frac{\text{Slope}}{S_0\,(1 - \text{Slope})} \tag{1}$$

where S_0 is the intrinsic solubility of the drug under the conditions studied. The slope of the curves observed in the regression analysis would indicate the formation of soluble complex of MMF:PSC[4]R (slope 0.47) with 1:2 stoichiometry. The extent of complexation is calculated based on the solubility diagram (Fig. 7). The solubility of MMF did not increase linearly as a function of PSC[4]R concentration and thus the solubility curve was classified as Bs type phase solubility curve indicating the formation of a 1:2 complex.). The stability constant Kc (Eq. 1) value was found to be 2285 M^{-1} for PSC[4]R indicating that the complex formed was adequately stable.

In vitro release study

Below Fig. 8 shows the dissolution profile of MMF, MMF PSC[4]R physical mixture, and MMF–PSC[4]R inclusion

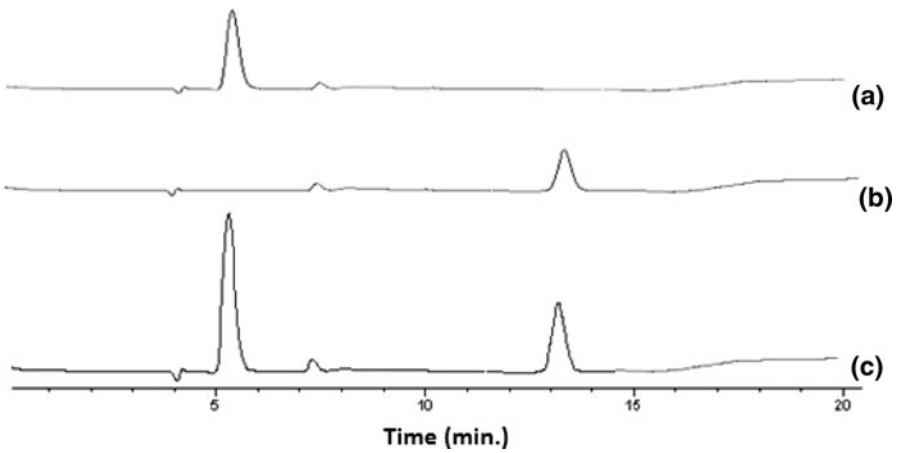

Fig. 6 Shows the HPLC chromatogram of (*a*) the pure drug MMF API (*b*) PSC[4]R (*c*) inclusion complex

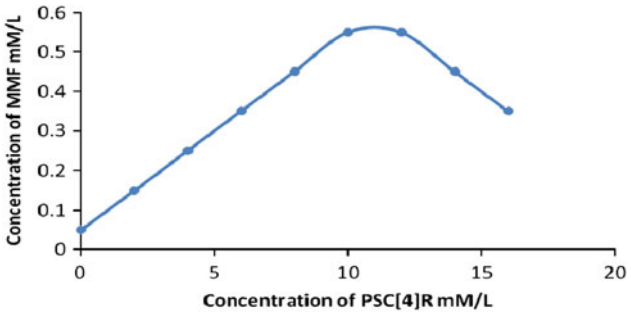

Fig. 7 Higuchi phase solubility diagram of MMF and PSC[4]R system in water at 25 °C. Each point is the mean of three determinations

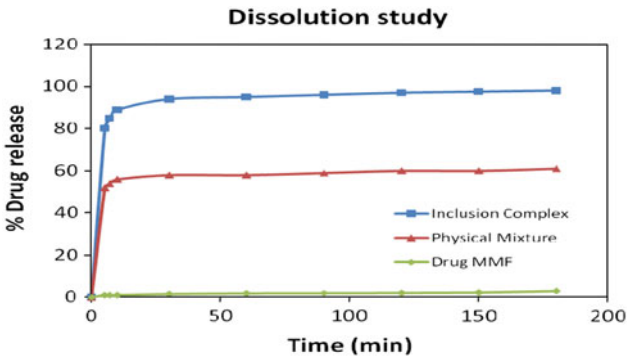

Fig. 8 Dissolution studies of inclusion complex, physical mixture and drug (MMF) system in (0.1 N HCl, pH is 1.2) at 37 °C

complex at 37 °C in acidic buffer (pH = 1.2). The dissolution rate of inclusion complex is manifestly superior to pure drug. The enhancement in the dissolution rate of the inclusion complex could be explained from increase in solubility, a marked reduction in crystallinity as confirmed by X-ray diffraction study and an improved wettability of the drug by the inclusion complexation. The physical mixtures also shows higher dissolution rate but it is medium to inclusion complex. It is assumed that in the physical mixture of PSC[4]R the dissolution is attributable to the in situ formation of readily soluble inclusion complex.

In vivo study

Animals were observed individually during first 30 min after dosing, (with special attention given during the first 4 h), and thereafter for 48 h. There were no signs of toxicity in the animals treated with prepared inclusion complex, no changes were found in skin and fur, eyes, mucous membranes, and also respiratory, circulatory, autonomic, and central nervous system, and convulsions, salivation, diarrhea, lethargy, sleep and coma. Individual weights of animals were monitored shortly before the test substance was administered and at least weekly thereafter. At the end of the test surviving animals were weighed, no significant change was observed as compared to the initial weight on 1st day. The animals were healthy and fit. Mycophenolate mofetil is the 2-morpholinoethyl ester of mycophenolic acid (MPA), an immunosuppressive agent, inosine monophosphate dehydrogenase (IMPDH) inhibitor. MMF is reported to have an oral LD_{50} of 352 mg kg^{-1} [Rat], 1000 mg kg^{-1} [Mouse], and >6000 mg kg^{-1} [Rabbit]. Results of our study revealed (Table 2) that the acute toxicity of the inclusion complex is smaller comparing with the pure drug MMF analyzed alone.

Conclusion

In conclusion, the results of the studies indicated the formation of MMF:PSC[4]R inclusion complex at 1:2 ratio in the aqueous solution with the stability constant 2285 M^{-1}, moreover no significant difference was observed in FT-IR spectra indicating absence of chemical interaction (new bond formation) between the drug and the PSC[4]R. DSC and X-ray diffractogram studies show more reduced crystallinity in inclusion complex system of PSC[4]R:MMF. This was evident from smaller characteristic peaks in the

Table 2 Survival of mice injected with inclusion complex

Animal	Time (h)	Dose (mg kg^{-1})	Survival
1	12	500	√
	24		√
	36		√
	48		√
2	12	1000	√
	24		√
	36		√
	48		√
3	12	1500	√
	24		√
	36		√
	48		√
4	12	2000	√
	24		√
	36		√
	48		√
5	12	2500	×
	24		–
	36		–
	48		–
6	12	2200	√
	24		√
	36		√
	48		√

√: Animal survive, ×: Animal not survive

X-ray diffractogram and absence of corresponding endotherm in DSC thermogram as compared to those of the corresponding physical mixture of each pure substrate. The dissolution study shows the rate of inclusion complex is manifestly superior to pure drug; these results are fascinating for their increased solubilisation in aqueous media. Our in vivo study (acute toxicity study) shows that there is a remarkable change in the toxicity of the pure drug MMF. This clearly indicates that after the complexation, the pure drug become less toxic as the LD_{50} value increases as compared to the reported LD_{50} value of MMF. The inclusion complex treated animals were observed for mortality up to 48 h (short term toxicity) and for long term toxicity (14 days). So, this inclusion complex did not produce any mortality up to 2200 mg kg^{-1}. These results recommend the described conjugates as future promising therapeutic agents.

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