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Synthesis, characterization and anti-tubercular activity of ferrocenyl hydrazones and their β-cyclodextrin conjugates

Prasad Dandawate[a,1], Kiranmayi Vemuri[b,1], Ejazuddin M. Khan[a], Manjula Sritharan[b,**], Subhash Padhye[a,*]

[a] ISTRA, Department of Chemistry, Abeda Inamdar Senior College, University of Pune, 411001, India
[b] Department of Animal Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

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ABSTRACT

A series of ferrocenyl hydrazones and their β-cyclodextrin (CD) inclusion complexes were prepared and evaluated for antitubercular activity under low and high iron conditions. The inclusion complexes were characterized by Fourier Transform Infrared (FTIR), Differential Scanning Calorimetry (DSC), Powder X-ray Diffraction (PXRD), ^{1}H NMR, Scanning Electron Microscopy (SEM) and Cyclic Voltammetric (CV) studies. The inclusion complexes exhibited improved aqueous solubility as well as enhanced hydrolytic and thermal stability. They were also found to exhibit greater antitubercular activity than the parent ferrocenyl hydrazones against *Mycobacterium tuberculosis* under high iron conditions. When grown under low iron conditions these compounds exhibited lower activity suggesting requirement of iron-dependant peroxidase activation.

1. Introduction

Tuberculosis (TB) has afflicted humanity since ancient times and still remains a major public health concern worldwide as it ranks the second leading cause of death from infectious diseases worldwide, after the human immunodeficiency virus (HIV). In 2011 there have been 8.7 million incident cases of TB (13% co-infected with HIV) and 1.4 million deaths due to the disease (WHO Global Tuberculosis Report, 2012). Isoniazid (INH) is the major first-line drug used for the treatment of TB, although emergence of resistance toward this drug has raised serious concerns regarding its continued use in future (Wade & Zhang, 2004). Resistance toward INH in humans has been explained by two mechanisms, the first involving enzymatic acetylation by *N*-acetyltransferase (NAT) enzyme leading to impaired serum concentrations and reduced therapeutic activity (Augustynowicz-Kopeć & Zwolska, 2002), while the other implicating mutation or inactivation of mycobacterial catalase-peroxidase enzyme known as KatG to convert pro-drug INH into an isonicotinoyl radical, which then couples to NAD1/NADH forming an isoniazid-NADH adduct that ultimately confers antitubercular activity (Rozwarski, Grant, Barton, Jacobs, & Sacchettini, 1998; Zhang, Heym, Allen, Young, & Cole, 1992). The mutation or inactivation of KatG has been shown to lead to INH resistance (Rouse, Li, Bai, & Morris, 1995; Zhang et al., 1992). The revival of INH as an anti-tubercular drug seems to depend upon whether it can be modified suitably which can address these two issues. We have hypothesized that since NAT metabolizes INH in humans by means of acetylation reaction at N2-center in the hydrazinic chain, it is desirable to modify the hydrazinic chain at N2 with a functional group that can block acetylation (Fig. S5) and hence maintain strong antimycobacterial activity (Hearn & Cynamon, 2004; Hearn et al., 2009). In addition it may be possible to append INH with agents that can generate isonicotinoyl radical, independent of KatG which can then retain its anti-tubercular activity in spite of KatG mutation. In the present work we have attempted to address both these issues by modifying the N2-center in the hydrazinic chain by condensing it with ferrocenyl moiety and at the same time introducing the ferrocene/ferricenium redox couple in the drug moiety which can help generate the isonicotinoyl radical.

Nguyen, Quémard, Broussy, Bernadou, and Meunier (2002) have reported that INH can be quickly oxidized by the stoichiometric amounts of manganese (III)-pyrophosphate, which in the presence of the nicotinamide co-enzyme can lead to

* Corresponding author at: ISTRA, Department of Chemistry, Abeda Inamdar Senior College, University of Pune, Pune 411001, India. Tel.: +91 8390025533.
** Corresponding author.
E-mail addresses: sbpadhye@hotmail.com, bhash46@hotmail.com (S. Padhye).
[1] These authors contributed equally to this work.

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the formation of INH-NAD (H) adducts which in turn leads to *in vitro* inhibition of the enoyl-acyl carrier protein reductase, *viz.* InhA, which is the target of INH in the biosynthetic pathway for mycolic acids. The *Mycobacterium tuberculosis* strain H37Rv carrying the wild-type katG allele, also exhibits increased susceptibility to INH when used in combination with superoxide radical generating compounds like Plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) as judged from the decrease in both IC_{50} and MIC values of INH in the presence of such reagents (Bulatovic et al., 2002), suggesting that superoxide-dependent mechanism appears to be important for *KatG*-mediated INH activation in INH-resistant strains.

There have been literature reports describing Ferrocene, as a source of superoxide anions due to the ferrocene/ferricenium redox couple active under biological conditions (Top et al., 2001, 2003). Consequently drugs incorporating ferrocenyl moiety have been found to exhibit enhanced biological activity. For example, incorporation of the ferrocenyl moiety into chloroquine scaffold has been found to exhibit more potent anti-malarial activity than chloroquine alone even against the resistant strains of *Plasmodium* (Biot, Glorian, Maciejewski, & Brocard, 1997). Recently some ferrocenyl hydrazones have also been found to be active against *M. tuberculosis* (Maguene et al., 2011; Mahajan et al., 2011). It thus seems logical to explore ferrocenyl conjugates of INH and other similar antitubercular drugs as novel agents against resistant mycobacterial species which can exert antitubercular activity even in the absence of KatG (Fouda, Abd-Elzaher, Abdelsamaia, & Labib, 2007).

In the present work, we have described the synthesis and structural characterization of ferrocenyl conjugates of INH and analogous hydrazides and have evaluated their anti-mycobacterial activity under high and low iron conditions since these conditions are known to influence the levels of catalase and peroxidase enzymes in the mycobacterial species. In our group, we have studied the response of mycobacterial species like *M. tuberculosis, Mycobacterium bovis, M. bovis* BCG, *Mycobacterium fortuitum, Mycobacterium kansasii* and *Mycobacterium smegmatis* under low and high iron conditions wherein it was observed that when sufficient iron was present in the growth medium, significant levels of both catalase and peroxidase enzymes were observed while a dramatic decrease (10–13 fold) in the peroxidase activity was observed in all mycobacterial species under iron limitation condition (Yeruva, Sundaram, & Sritharan, 2005). The latter condition thus represents the inactivation of KatG reflected by high MIC value of INH leading to resistance. This provides the logical basis for evaluating anti-tubercular activity of new isoniazid analogs under high and low iron conditions.

Several literature reports have suggested that hydrophobic anti-tubercular drugs when molecularly encapsulated into β-cyclodextrin (CD) can lead to higher solubility and stability. For example, the cyclodextrin conjugate of clofazimine exhibited better water solubility and inhibited growth of MAC inside macrophages with similar efficacy (Salem, Steffan, & Düzgünes, 2003). Similarly the pre-clinical testing of the Nitroimidazopyran derivative PA-824 formulated with cyclodextrin/lecithin at 100 mg/kg reduced the bacterial load below 500 CFU in the lungs and spleen on long term administration (Lenaerts et al., 2005). Consequently an inclusion complex of different drugs is a favored strategy for pharmaceutical applications (Buriez et al., 2008; Loftsson & Brewster, 1996; Rajewski & Stella, 1996). The hydrazone derivatives are prone to hydrolysis at physiological pH of the body (Jamadar et al., 2012), whereas the advantage offered by the cyclodextrin conjugation is in improving the hydrolytic stability of guest molecule (Tomren, Másson, Loftsson, & Tønnesen, 2007). Consequently in the present work, we have evaluated the hydrolysis behavior of ferrocenyl hydrazones at physiological pH.

2. Materials and methods

2.1. Phase solubility analysis

The phase solubility study was carried out by the method reported by Higuchi and Connors (1965). Different concentrations of β-cyclodextrin (CD) solutions from 0 to 20 mM were prepared in distilled water and filled in screw capped bottles. Excess quantities of ligands (FIHZ, FNHZ, FSHZ and FBHZ) were added to these solutions to attain saturation. Each bottle was capped and shaken for 72 h in a constant temperature water bath at 30 ± 2 °C. Following equilibrium, these solutions were filtered using 0.45-μm nylon disk filter, diluted, and assayed for the total dissolved ligand content by UV analysis at 286.4, 291.2, 296.0, 291.2 nm respectively. Each sample was determined in triplicate and the samples were protected from light. The phase solubility diagram was constructed by plotting concentrations of the dissolved ligands against β-cyclodextrin (CD) concentration. The binding constant, K_s, was calculated from the slope of phase solubility plot (Higuchi and Connors, 1965); $K_s = [\text{slope}/(S_0(1 - \text{slope}))]$, where S_0 is molar solubility of ferrocenyl hydrazones in the absence of cyclodextrin.

2.2. Solubility studies

Excess quantities of present ligands and their β-cyclodextrin inclusion complexes were dispersed in 25 mL of distilled water in screw-capped bottles to get a super-saturated solution. The bottles were shaken continuously for 2 h at ambient temperature until equilibrium was attained. Super-saturated solution was filtered through a 0.22-μm nylon filter and further diluted with methanol followed by measuring the absorbances at 286.4, 291.2, 296.0, 291.2 nm respectively.

2.3. Hydrolytic stability study

The hydrolytic stability study was carried out by previously reported method (Jamadar et al., 2012; Tomren et al., 2007). The hydrolytic stability of FIHZ was studied in buffered 10% (w/v) CD solution at pH 7.4 in phosphate buffer (PBS) at 30 °C. The Stock solution of FIHZ was prepared in methanol at a concentration of 1.5 mg mL^{-1}. A 100 μL of this solution was added to 10 mL of the buffered 10% (w/v) CD solution and the phosphate buffer respectively. The samples were drawn at regular time intervals (0–96 h) and analyzed by recording their absorption spectra.

2.4. Infra-red studies

The infra red spectra of all ligands, CD alone and their β-cyclodextrin inclusion complexes were recorded on Shimadzu Fourier Transform Infrared Spectrophotometer (FTIR-8700) by potassium bromide disk method. Samples were mixed with dry powdered potassium bromide and compressed into transparent disk under high pressure using special dies. The KBr disks were placed in IR spectrophotometer using sample holder and spectra were recorded over the range of 4000–400 cm^{-1}.

2.5. Differential scanning calorimetric studies

Differential scanning calorimetric studies of all ligands, CD and their β-cyclodextrin inclusion complexes were performed on differential scanning calorimeter METTLER-TOLEDO DSC1 instrument. Samples were weighed (2.50–5.00 ± 0.5 mg) and placed in sealed aluminum pans. Liquid nitrogen was used the coolant. The samples were scanned at 10 °C/min from room temperature to 300 °C and corresponding thermograms were recorded.

2.6. X-ray diffraction studies

X-ray diffraction patterns of all ligands, CD alone and their β-cyclodextrin inclusion complexes were determined using a diffractometer on Bruker, AXS D-8 Advance machine equipped with a rotating target X-ray tube and a wide-angle goniometer. The X-ray source was Cu, at the wavelength of 1.5406 Å.

2.7. Scanning electron microscope studies

Surface morphology of all ligands, CD alone and their β-cyclodextrin inclusion complexes were examined by scanning electron microscope SEM, Jeol JST-6390LV. The samples were dispersed onto carbon tabs (double-adhesive carbon coated tape) adhered to aluminum stubs. They were coated with a thin layer (30E) of gold by employing Jeol JFC-1600 auto fine coater. Samples were subsequently examined by SEM and photographed under various magnifications with direct data capture of the images onto a computer.

2.8. Cyclic voltametric studies

Electrochemical studies for all ligands, CD alone and their β-cyclodextrin inclusion complexes were carried out in DMSO solvent using Bioanalytical System Epsilon (BASi) electrochemical analyzer. All experiments were carried out at room temperature by using DMSO as solvent and in the presence of tetrabutylammonium perchlorate (TBAP) as supporting electrode. The three-electrode system consisted of Platinum disk as the working electrode, Ag/AgCl as the reference electrode and platinum wire as the auxiliary electrode. nitrogen gas was purged in the solution for 30 min before commencement of the measurements. The cyclic voltammetric (CV) profiles were recorded in the potential range of +800 and 0.00 mV, at 100 μA current, quiet time of 2 s and the scan-rate of 50 mV/s respectively. The software program provided by BASi was used to plot the graph of current (μA) Vs potential (mV).

2.9. Nuclear magnetic resonance spectroscopy

All NMR spectra were recorded in DMSO-d6 using BRUKER AV III (^{1}H NMR 500 MHz, ^{13}C NMR 100.63 MHz) spectrophotometer. Chemical shifts (δ) as given in terms of parts per million (ppm) are referenced to the residual solvent DMSO, ^{1}H NMR – 2.50 ppm.

2.10. Antitubercular activity

2.10.1. Mycobacterial strain and growth conditions

M. tuberculosis ATCC 27294 was grown in Middlebrook 7H9 broth medium (pH 7.0, Difco, Detroit, MI, USA) containing 10% (v/v) albumin-dextrose-catalase (ADC; Difco) enrichment and 0.2% glycerol and maintained with shaking at 150 rpm at 37 °C.

2.10.2. Preparation of the compounds for screening

Stock solutions of the ferrocenyl hydrazones and their CD inclusion complexes were prepared in DMSO (HPLC grade) solvent (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 10 mg mL^{-1}. These stock solutions were filter-sterilized (0.22 μm filters, Sartorius) and stored at −80 °C.

2.10.3. Screening of the compounds

The compounds were initially screened in the MB/BacT^{TM} 240 Mycobacteria Detection System (BioMerieux/Organon Teknika, France) followed by the determination of the MIC values by Microplate Alamar Blue assay (MABA). The MIC values were confirmed in the MB/BacT^{TM} 240 system by growth at MIC and sub-MIC concentrations of the compounds tested. These compounds were screened for antimycobacterial activity in the MB/BacT^{TM} 240 Mycobacteria Detection System. The detection system uses standard bottles of 10 mL culture media to which 0.5 mL of the cell suspension of mycobacteria (prepared as detailed above) was added. The system also included the 'Proportion control' that was inoculated with 0.5 mL of the cell suspension diluted as 1:100. The system was standardized for the antimycobacterial activity testing by using INH at 0.1–0.5 μg/mL final concentration. All the compounds were added at 256 μg/mL and evaluated for their efficacy against *M. tuberculosis* as described earlier (Jamadar et al., 2012; Sritharan, Yeruva, Sivasailappan, & Duggirala, 2006). Briefly, it included testing of the compounds in 96 clear bottomed, sterile, microtitre plates (Corning, New York, USA). The outer perimeter wells were filled with 200 μL of sterile, distilled water to prevent evaporation from the experimental wells during incubation. An aliquot of 100 μL of 7H9 medium was added to all wells except those of column 2. Similarly 100 μL of the compound(s) to be tested was added to column 2 which represented the highest concentration of the compound(s). An aliquot of 100 μL of the compound(s) was added to column 3 followed by serial dilutions of the compounds from columns 3–10. Column 11 served as the control without the addition of drug. The 100 μL of the cell suspension (1:50 dilution of the cell suspension prepared above) was added to all wells and the plate was incubated at 37 °C for 5 days. 50 μL of Alamar Blue [Invitrogen Corporation, Carlsbad, CA, USA; prepared as a 1:1 (v/v) mixture with 10% Tween-80 (Sigma)] was added to the first control well and observed after 12 h. If the color changed from blue to pink, Alamar Blue was added to all the wells and visual grading of the color change was made 6 h after the dye addition. The MIC of the specific compound was taken as the lowest concentration at which there was no change of the blue color of Alamar Blue indicating lack of any viable bacilli. Once the MIC value was calculated by MABA, the MICs of the compounds were confirmed by adding them at MIC and sub-MIC concentrations in the MB/BacT^{TM} 240 system.

3. Results and discussion

3.1. Synthesis of and characterization ferrocenyl hydrazones

The ligands (**FIHZ**, **FNHZ**, **FSHZ** and **FBHZ**) were synthesized by following a procedure described by Chohan and Praveen (1999) and Maguene et al. (2011) with slight modification. Briefly, the synthesis involved condensation of equimolar quantities of acetyl ferrocene and selected hydrazides in methanol in the presence of trifluoroacetic acid as a catalyst with continuous stirring at 60 °C for 2 h (Fig. 1). The precipitated compounds were dried under vacuum and purified by column chromatography using chloroform: methanol (9.5:0.5 v/v) as eluent. The compounds were subjected to characterization by spectroscopic (UV-, FTIR-, ^{1}H/^{13}C -NMR-, Mass-spectroscopy), thermal (Differential Scanning Calorimetry) and electrochemical methods (Cyclic Voltammetry) (Supplementary data).

3.2. Preparation and characterization of β-cyclodextrin conjugates

Kneading method (Dandawate et al., 2012) was used for the preparation of cyclodextrin inclusion complexes of the ligands wherein the constituents in 1:1 molar ratio were mixed in a mortar for 1 h with methanol and distilled water mixture to get slurry-like consistency. The paste was dried in a hot air oven at 45 °C for 24 h. The dried complexes were pulverized into fine powders and sifting through sieve No. 80.

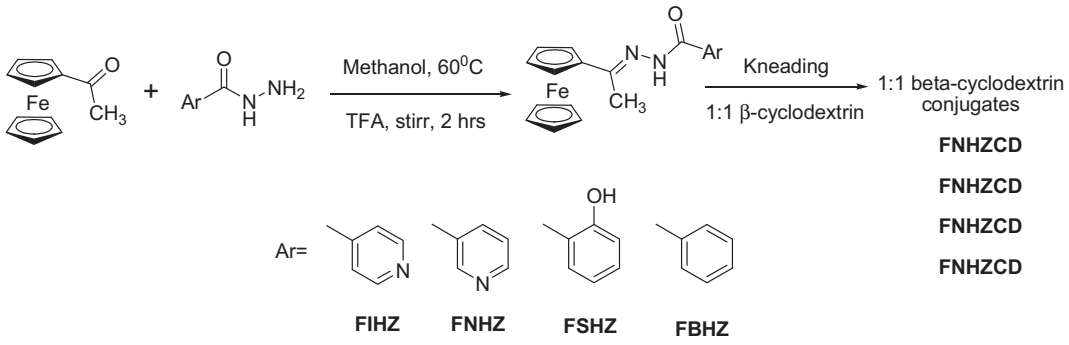

Fig. 1. Schematic representation of synthesis of ferrocenyl hydrazones and their β-cyclodextrin inclusion complexes.

3.3. Phase solubility studies

The phase solubility diagrams for the complex formation between all ligands and β-cyclodextrin are shown in Fig. S1 (Supplementary data). These plots show that aqueous solubility of the ligands increases linearly as a function of cyclodextrin concentration showing linearity (Jullian et al., 2008). It is clearly observed that the solubility diagram of compounds in the presence of β-cyclodextrin can be classified as A_L type according to Higuchi and Connors (1965). This may be attributed to the formation of soluble 1:1 ferrocenyl hydrazone-β-cyclodextrin inclusion complexes. Some previous studies have also reported that Schiff base ligands usually form 1:1 inclusion complex with cyclodextrin (Hadjoudis et al., 2011; Linares, de Bertorello, & Longhi, 1997; Pistolis, Gegiou, & Hadjoudis, 1996). The stability constants ($K_{1:1}$) of FIHZ, FNHZ, FSHZ and FBHZ was found to be 1894.78, 2235.45, 1072.90 and 636.42 M^{-1} respectively. It was previously reported that the stability constant (K_s) values ranging between 200 and 5000 M^{-1} has been considered as most suitable value for the enhancement of solubility and stability of poorly water soluble drugs (Garg, Gupta, Prakash, & Singh, 2010; Vandelli et al., 2000). Thus, it may be concluded that the cyclodextrin complexes are capable of enhancing the solubility and stability of ferrocenyl hydrazones.

3.4. Solubility studies of ferrocenyl hydrazones-β-cyclodextrin inclusion complexes

Complexation with β-cyclodextrin has been known to enhance the aqueous solubility of therapeutic compounds (Carrier, Miller, & Ahmed, 2007). In the present study, the aqueous solubilities of FIHZ, FNHZ, FSHZ and FBHZ ligands were found to be 8.13, 5.12, 7.90 and 9.84 μg/mL respectively, which on complexation with β-cyclodextrin showed considerable enhancement, the values being raised to 26.54, 19.24, 20.17 and 18.00 μg/mL for the four synthesized conjugates.

3.5. Hydrolytic stability study

Hydrazones were prone to hydrolysis at physiological pH of body (Jamadar et al., 2012), while cyclodextrin inclusion complexes were found to improve hydrolytic stability of compounds (Tomren et al., 2007). Hence, in order to study the hydrolysis of FIHZ, the hydrolytic stability study was carried out with and without 10% (w/v) CD in PBS solution. PBS is isotonic with human plasma and was previously used for studying the hydrolysis of related hydrazones (Buss & Ponka, 2003). The analysis of the UV spectra of FIHZ suggested the gradual hydrolysis of compound over a period of 96 h in PBS. Hydrolysis was evidenced by a decrease in intensity of the absorption band, while the significant hydrolysis of FIHZ was observed at 72 h in PBS (Fig. 2). The FIHZ was found to be resistant to hydrolysis in PBS containing 10% CD, while there was slight decrease in absorption spectra over a period of 72 h as compared to spectra of FIHZ in PBS without CD. The study suggested that presence of CD improved hydrolytic stability of hydrazone class of compounds.

3.6. Infra-red studies

The IR-spectral assignments of the parent ligands and their-β-cyclodextrin inclusion complex are shown in Fig. S2 and Table S1 (Supplementary data). The FTIR spectra of all ligands exhibited absorption bands in the region 3255–3171 cm^{-1} corresponding –NH stretches, while appearance of a band at 1631–1649 cm^{-1} (C=N) confirmed the formation of Schiff base moiety (Chohan & Praveen, 1999). Other sharp absorption bands were seen at 2929–2912 cm^{-1} (C—H), 1668–1741 cm^{-1} (C=O), 1024–1078 cm^{-1} (N—N) respectively (Chohan & Praveen, 1999). The FTIR spectrum of β-cyclodextrin exhibited intense peaks at 3389–3331 and 2924 cm^{-1} due to O—H and C—H stretching vibrations, while skeletal vibrations involving α-1,4 linkage of glucose moiety and cyclodextrin rings corresponding to (C—C—H), (C—O—H), (H—C—H), (C—O), (C—C), (C—O—C) were seen at 1334.78, 1155.40 and 1078.24, 1030.02, 945.15 cm^{-1} respectively. In the FTIR spectra of β-cyclodextrin inclusion complexes, all sharp peaks of β-cyclodextrin were observed along with few peaks of the ligands with lower intensity as observed by other researchers (Wu, Liang, Yuan, Wang, & Yan, 2010; Yallapu, Jaggi, & Chauhan, 2010). On the other hand, the β-cyclodextrin bands were found to be shifted to lower or higher wave numbers upon complex formation with the hydrazone ligands (Wu et al., 2010). The intense peak of phenolic hydroxyl group was found to be shifted from 3389–3331 to 3373–3250 cm^{-1}. These results confirmed the intercalation of the ligands in β-cyclodextrin cavity. Such small differences between values in the FTIR peaks of β-cyclodextrin and the present hydrazone ligands before and after inclusion complex formation also suggest that there are no chemical bond formed between these ligands and β-cyclodextrin moiety (Song, Wang, Guo, & Bai, 2008).

3.7. Differential scanning calorimetric (DSC) studies

Thermal analytical techniques are useful to study the physical state of the drug molecule present in the inclusion complexes, polymers or nano-particulate systems (Lemarchand et al., 2005; Uner, 2006). The formation of cyclodextrin inclusion complexes have been commonly studied by using DSC studies based on the absence/shifting of endothermic peaks indicating a change in the crystal lattice, melting, boiling or sublimation temperature point (Horvath et al., 2008; Loukas, Vraka, & Gregoriadis, 1997). Consequently such studies can be employed for obtaining qualitative and quantitative information on the drug present in the cyclodextrin

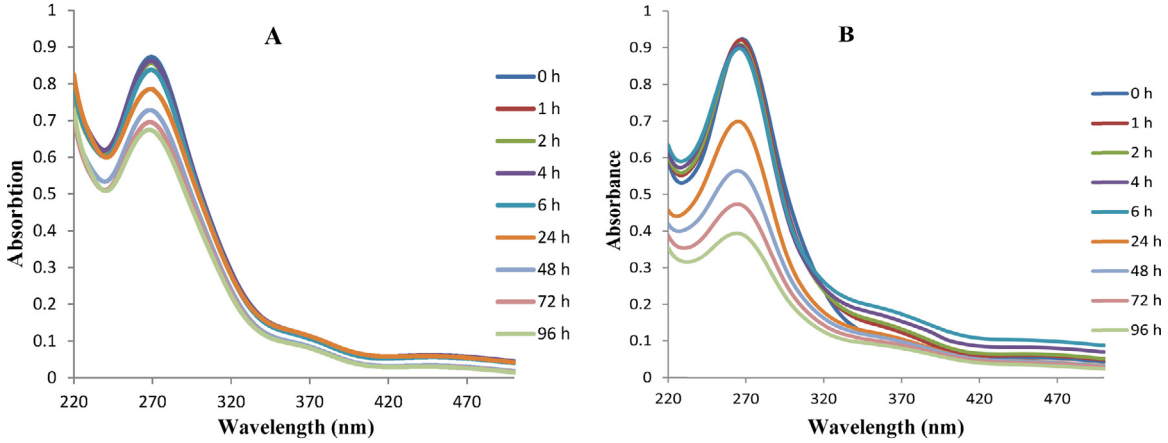

Fig. 2. Hydrolytic stability study of FIHZ in PBS with (A) and without (B) 10% CD solution over a period of 0–96 h.

inclusion complexes. In present study, we have studied the thermal properties of the parent ligands, β-cyclodextrin and its inclusion complexes with the ferrocenyl hydrazone ligands by using DSC technique (Fig. 3). The parent β-cyclodextrin ligand was found to exhibit endothermic peaks at 100.01 °C (with onset at 55.77 and endset at 136.78 °C), while the ferrocenyl hydrazone ligands yielded sharp endothermic peaks at 85.05 °C (with onset at 81.67 and endset at 89.05 °C for FIHZ), 190.30 °C (with onset at 181.52 and endset at 193.36 °C for FNHZ), 236.24 °C (with onset at 233.77 and endset at 238.47 °C for FSHZ) and 173.07 °C (with onset at 169.60 and endset at 175.17 °C for FBHZ) respectively, which were ascribed to their melting temperatures. On other hand, β-cyclodextrin inclusion complexes exhibited diminished melting peaks of the ligands, along with shifting of the β-cyclodextrin peak from 100.01 to the range of 75.90–89.90 °C respectively. These results indicated that the ligand molecules have been included into the cavities of β-cyclodextrin undergoing stronger solid state interactions between the ferrocenyl hydrazone ligands and β-cyclodextrin. Thus, DSC analysis has suggested successful formation of inclusion complex between β-cyclodextrin and ferrocenyl hydrazone ligands.

3.8. X-ray diffraction studies

X-ray diffraction technique has been used to evaluate crystalline and amorphous nature of the drug and drug-polymer inclusion complexes (Franco et al., 2001). We have employed the PXRD technique for studying the effect of β-cyclodextrin complexation on crystalline nature of the present ligands. The powder X-ray diffraction patterns of the ligands, β-cyclodextrin and their inclusion complexes are shown in Fig. S3 (Supplementary data). The presence of numerous distinct peaks in PXRD of the present ferrocenyl hydrazone ligands suggests their crystalline nature, while β-cyclodextrin exhibited few crystalline peaks. The β-cyclodextrin inclusion complexes in general showed reduced intensity of peaks indicating that the crystalline nature of the present ferrocenyl hydrazone ligands was significantly lost upon cyclodextrin complexation. These observations seem to suggest the change in the crystal structure or modification of the unit cell of β-cyclodextrin upon after formation of the inclusion complexes (Veiga & Ahsan, 2000) possibly through H-bonding interactions between the ligands and β-cyclodextrin polymer (Jun et al., 2007).

3.9. Scanning electron microscopic (SEM) studies

The technique of SEM was used to study the bulk morphology of the surface of present ligands, β-cyclodextrin and their inclusion complexes (Figs. 4 and 5). The SEM pictures have revealed that β-cyclodextrin exhibit crystalline flake-like structure, while the ligands showed needle or rod like clumps (FIHZ), irregular spherical (FNHZ), rod-like (FSHZ) and rounded spherical crystals (FBHZ) respectively. The inclusion complexes of the ligands were found to contain neither crystalline structures nor needle like spherical-crystals, but exhibited irregular-shaped micron sized particles. This change in morphology has largely been attributed to inclusion of the ligands into the β-cyclodextrin cavity leading to its inclusion complexes.

3.10. Cyclic voltammetric studies

Cyclic voltammetry (CV) technique has generally been applied for evaluating the interaction between cyclodextrin and guest molecules (Kolivoska, Ga'l, Hromadova, Valasek, & Pospisil, 2011; Komura, Yamaguchi, Noda, & Hayashi, 2002). In the present investigation, we have used CV studies for analyzing the inclusion complexes of ferrocenyl hydrazone ligands (Fig. 6, Table 1). The electrochemical profiles of the ferrocenyl hydrazones showed reversible redox peaks attributable to the ferrocenyl moiety present in the ligands. The electrochemical profiles of β-cyclodextrin inclusion complexes were found to be similar to the parent ligands with minor shifting of peak positions toward positive potentials and decreased peak current (Kolivoska et al., 2011; Srinivasan, Kayalvizhi, Sivakumar, & Stalin, 2011). The decrease in current is attributed to slower diffusion coefficient from bulk layer to electrode surface of the β-cyclodextrin inclusion complexes than the ligands. On the other hand, it was observed that the electrochemical oxidation and reduction of β-cyclodextrin inclusion complexes was more difficult than the parent ferrocenyl hydrazone ligands perhaps due to their entrapment in the hydrophobic cavity of β-cyclodextrin resulting in the shifting of peak potentials (Srinivasan et al., 2011). Thus, the electrochemical studies provide additional evidence for the formation of the inclusion complexes and their enhanced stability in the mixed-aqueous solvents.

3.11. Nuclear magnetic resonance spectroscopy

NMR spectroscopy is the most powerful tool for the study of formation of inclusion complexes between cyclodextrins and a variety of guest molecules, since the chemical and electronic environments of protons affected during complexation is reflected through changes in the NMR signal peaks (Garnero, Zoppi, Genovese, & Longhi, 2010). In the present work, we have evaluated inclusion of the ligands into the β-cyclodextrin cavity by analyzing the changes observed in NMR signals of the protons in the complex in

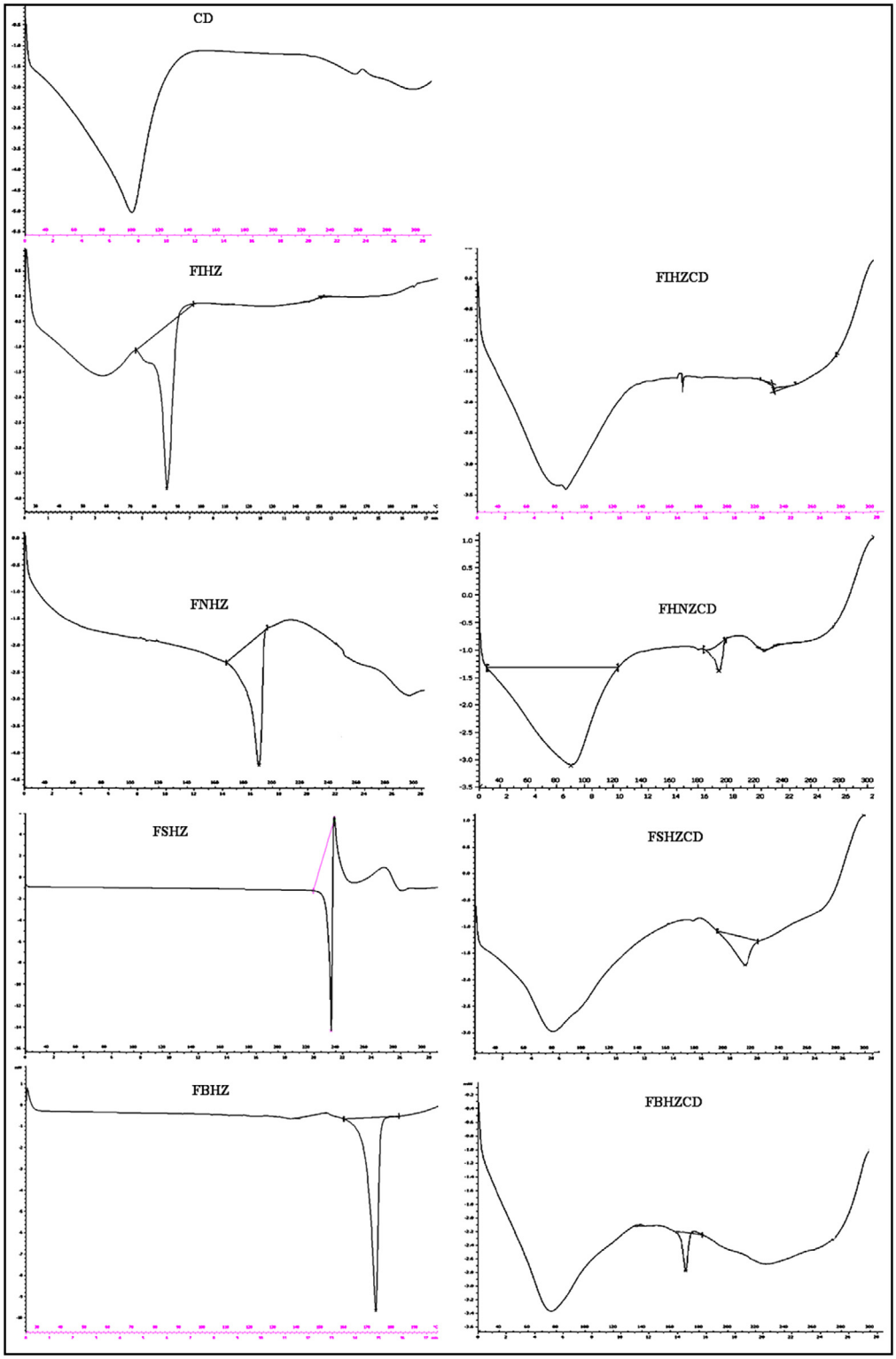

Fig. 3. Differential scanning calorimetric (DSC) spectra of CD, FIHZ, FNHZ, FSHZ, FBHZ and their inclusion complexes.

comparison to the signals for free ligands and β-cyclodextrin (Fig. S4, Supplementary data). The spectral assignments of β-cyclodextrin protons before (δ_{free}) and after formation of inclusion complexes along with signal shift index ($\Delta\delta$) are indicated in Table S2 (Supplementary data). Generally it was found that the resonances of the protons of β-cyclodextrin located within or near the cavity (H-3, 5, 6) showed larger shifts compared to H-1, 2, 4 located on the exterior of β-cyclodextrin (Inoue, 1993). In presence of the ferrocenyl hydrazone ligands, the protons of β-cyclodextrin undergo appreciable downfield shift (higher ppm) as a result of weak interactions (van der Waals forces) between the ferrocenyl hydrazone ligands and β-cyclodextrin in the interior of the cavity. Shift to higher fields of the protons located within the drug and β-cyclodextrin cavity suggest that a hydrophobic interaction was predominant between the drug and the CD (Fathy & Sheha, 2000; Sun, Li, Qiu, Liu, & Yin, 2006). Such shifts of β-cyclodextrin protons

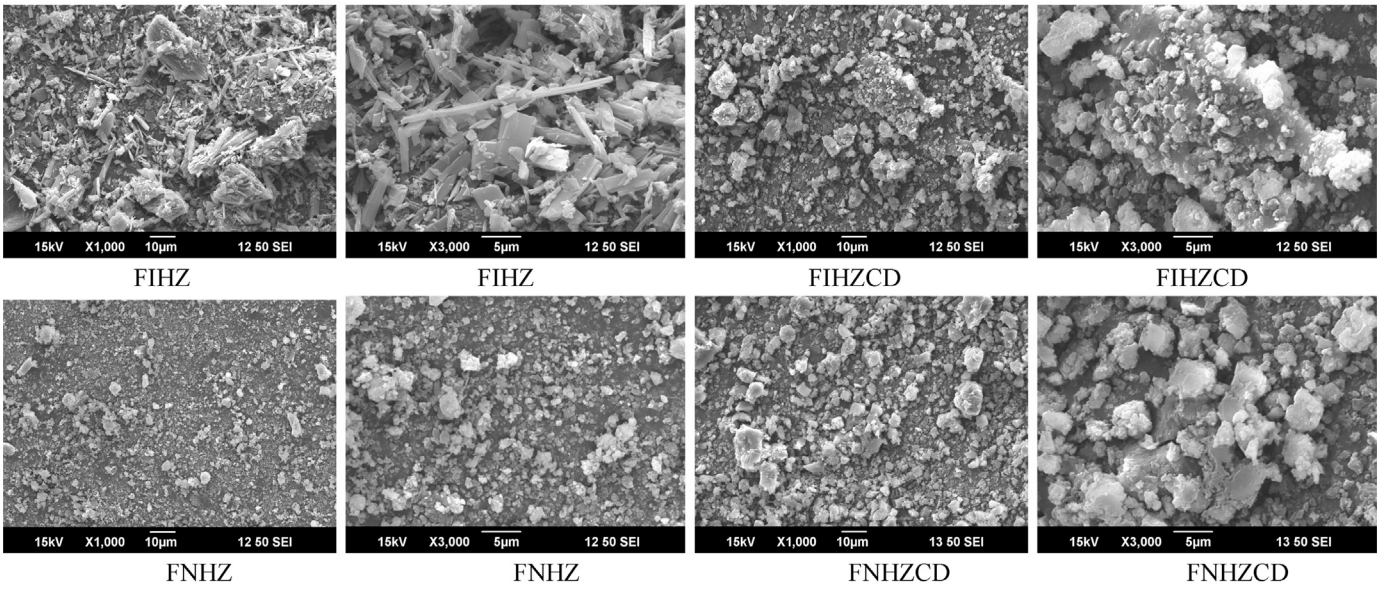

Fig. 4. Scanning electron microscopic images of FIHZ, FNHZ and their inclusion complexes.

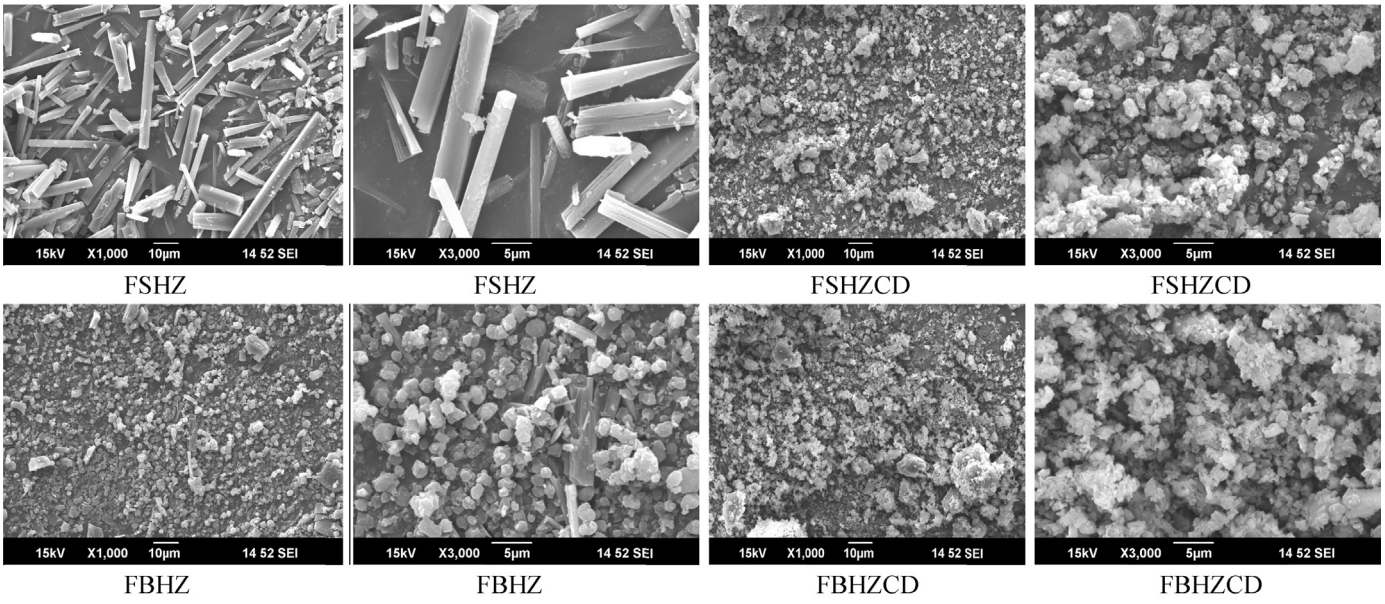

Fig. 5. Scanning electron microscopic images of FSHZ, FBHZ and their inclusion complexes.

also provide evidence of the formation of partial or complete inclusion of the ligand. The aromatic ring protons of the ligands appeared in the range of 6.8–9.2 ppm which upon complexation with β-cyclodextrin was shifted to the up-field region (lower ppm) suggesting that these protons are involved in the complex formation with β-cyclodextrin. This shift in the aromatic region after complex formation with CD was minor in case of FIHZ and FNHZ, but a peak broadening was observed in the spectra of FIHZCD and FNHZCD compounds indicating successful formation of inclusion complexes (Lai et al., 2003). The ^{1}H NMR spectroscopic

Table 1
Cyclic voltammetric assignments of FIHZ, FNHZ, FSHZ, FBHZ and their cyclodextrin inclusion complexes.

I_{pa}	I_{pc}	E_{pa}	E_{pc}	$E_{1/2}$	I_{pa}	I_{pc}	E_{pa}	E_{pc}	$E_{1/2}$	δI_{pa}	δI_{pc}	δE_{pa}	δE_{pc}	$\delta E_{1/2}$
FIHZ					FIHZCD					Difference (δ)				
3.6469	2.1729	646	544	595	2.1210	0.7294	659	574	616.5	1.5259	1.4435	13	31	21.5
FNHZ					FNHZCD									
2.9847	2.5025	680	585	632.5	2.8168	1.3458	676	577	626.5	0.1679	1.2367	4	8	6
FSHZ					FSHZCD									
5.9754	4.0955	653	558	605.5	2.7588	1.8830	657	564	610.5	3.2166	2.2125	4	6	5
FBHZ					FBHZCD									
3.6957	1.6693	669	565	617	2.4964	0.9979	657	565	611	1.1993	0.6714	12	0	6

I_{pa}: anodic current (μA), I_{pc}: cathodic current (μA), E_{pa}: anodic peak potential (mV), E_{pc}: cathodic peak potential (mV), $E_{1/2}$: half wave potential (mV).

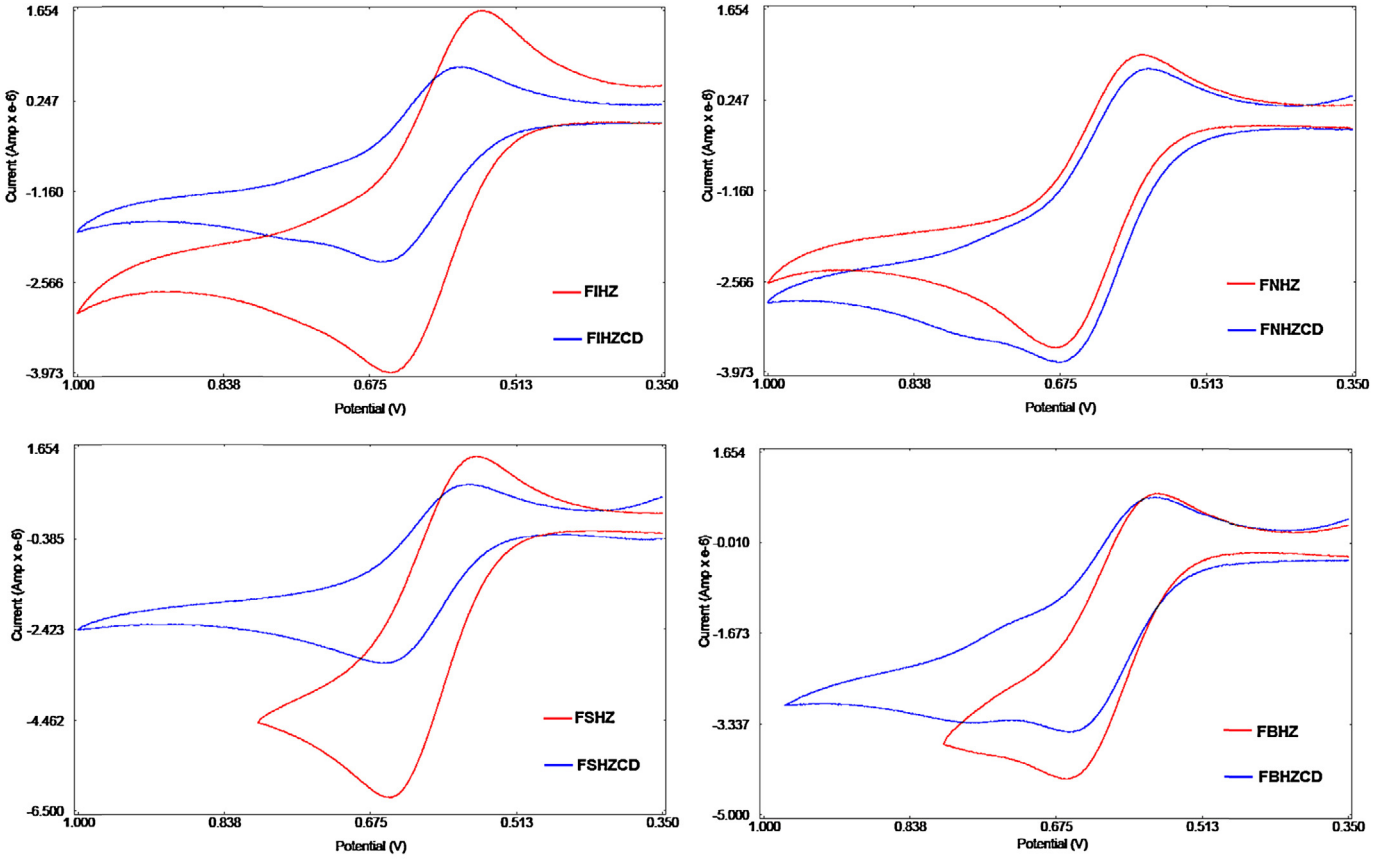

Fig. 6. Cyclic voltammetric spectra of FIHZ, FNHZ, FSHZ, FBHZ and their inclusion complexes.

Table 2
Influence of iron concentrations in the growth medium on the minimum inhibitory concentrations (MICs) of **FIHZ, FNHZ, FSHZ, FBHZ** and their cyclodextrin inclusion complexes.

Code	MIC (μg/ml) 7H9 medium	MIC value in high iron conditions (μg/ml)	MIC value in low iron conditions (μg/ml)
FIHZ	1.0	16	256
FIHZCD	0.5	4	256
FNHZ	256	NT	NT
FNHZCD	128	NT	NT
FSHZ	64	NT	NT
FSHZCD	32	NT	NT
FBHZ	32	1	256
FBHZCD	16	0.5	256
INH	0.25	0.125	32

NT = not tested. Low iron: 0.02 μg Fe/mL; high iron: 8 μg Fe/mL, $FeSO_4$ as iron source.

results thus indicate successful formation of inclusion complexes of ferrocenyl hydrazone ligands.

3.12. Anti-tubercular activity

The ferrocenyl hydrazone ligands and their cyclodextrin inclusion complexes were screened against *M. tuberculosis* under both low and high iron conditions. INH was used as the reference drug. The MIC values of the ligands were in the range of 1.0–256 μg/mL, while their cyclodextrin inclusion complexes exhibited lower MIC values (0.5–128 μg/mL) against 7H9 medium (Table 2). It has been shown that INH is a pro-drug that needs activation by mycobacterial KatG for activity. Under low iron condition, there is lowering of the peroxidase activity leading to reduced activity of KatG and ultimately higher MIC values of INH (Sritharan et al., 2006; Yeruva et al., 2005). The present ferrocenyl hydrazonates exhibit similar trend under low iron conditions. The potent compounds of the series especially FIHZ, FBHZ and their β-cyclodextrin inclusion complexes were selected for further screening against high and low iron conditions. The most potent compound of the series FBHZ and its β-cyclodextrin inclusion complex exhibited remarkable inhibitory activity under high iron condition with MIC values of 1.0 and 0.5 μg/mL indicating the advantages of cyclodextrin formulations. Under low iron conditions, however, the compounds exhibited higher MIC values (256 μg/mL).

4. Conclusions

In the present investigation, we have successfully synthesized and characterized novel ferrocenyl hydrazones as well as their cyclodextrin inclusion complexes and have evaluated their anti-tubercular activities. The β-cyclodextrin inclusion complexes of the ferrocenyl hydrazones were found to possess higher water solubility, improved hydrolytic stability and greater anti-tubercular activity. The differential response of these inclusion complexes under low and high iron conditions suggests structural

modifications of existing anti-tubercular drugs while targeting mycobacteria. Present study thus highlights the importance of cyclodextrin conjugates in the formulations of antitubercular drugs especially under differential iron status.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.03.006.

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