A novel and multifunctional excipient for vaginal drug delivery.

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ABSTRACT

The present study explores the pharmaceutical potential of a natural organic matter (fulvic acid) for sustained release, acid buffering capacity and mucoadhesion in vaginal drug delivery. The antifungal drug, Itraconazole, was first converted into inclusion complexes with fulvic acid (1:1 & 1:2 molar ratio) and then characterized by Differential Scanning Calorimetry (DSC), X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FT IR) and Mass Spectroscopy. Results were also authenticated by conformational analysis. Solubility analysis of complexes yielded different thermodynamic parameters and explained the driving force for solubilisation when the pH was varied in an acidic range. MTT assays were also performed to assess the potential *in vitro* cell toxicity of the complexes in comparison to the neat drug. The complexes were then formulated into tablets and optimized for hardness, mucoadhesion and release profiles. The optimized tablets presented with satisfactory mucoadhesion, acid buffering and spreading ability. Moreover, the antifungal activity of the formulation was also increased due to improved aqueous solubility of the drug despite the larger size of the complex. The study also indicated the potential use of fulvic acid as a functional excipient in the preparation of a vaginal drug delivery system (VDDS).

INTRODUCTION

Fulvic acid (FA) and Humic acid (HA) are humic substances of natural organic matter and are commonly found in 'Shilajit', which can generally be translated from Sanskrit as the "destroyer of weakness" or "elixir of immortality". 'Shilajit' is found in the Himalayan ranges of the Indian subcontinent and is primarily of plant origin. It is known to have been formed during periods of major geological activity. Two fractions, namely humic acid (Figure 1A) and fulvic acid (Figure 1B) can be obtained from 'Shilajit'. Of these two, fulvic acid was used in this study as a multifunctional excipient in a vaginal drug delivery system (VDDS).

According to the definition given by the International pharmaceutical Excipients Council (IPEC), "Pharmaceutical excipients are substances other than the APIs, which have been appropriately evaluated for safety and are intentionally included in a drug delivery system" (1). However, the development and introduction of a new excipient is as complicated as the development of a new drug molecule. Excipient manufacturers tend to look

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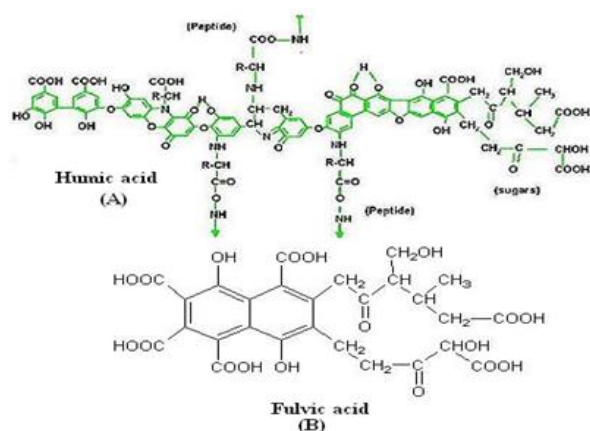
Figure 1 Molecular structures of (A) Humic acid, (B) Fulvic acid (C) Itraconazole

for value-added excipients and to date, in pharmaceutical research, humic substances have only been investigated for the preparation of inclusion complexes to enhance solubility and thus oral drug delivery (2, 3). Since fulvic acids are macromolecular, negatively charged and, together with the presence of polyfunctional groups (such as carboxylic, amino and phenolic moieties) capable of forming micelles (4, 5), they may also be suitable for other applications. Unexpectedly, a composition comprising of more than 40% (w/w) of FA was found to exhibit useful mucoadhesion properties. Based on these multifunctional properties of fulvic acid, the current research explored its potential for use in the development of VDDS, wherein mucoadhesion is a desirable attribute. A synthetic moiety, Itraconazole (Itz), was chosen as a model drug. It is a potent synthetic triazole antifungal agent exhibiting activity against a broad range of fungal species (6). Its empirical formula is $C_{35}H_{38}Cl_{2}N_{8}O_{4}$ and its molecular weight is 705.64 Da. It is a weakly basic drug, possessing extremely low water solubility (~1 ng/ml at neutral pH). The calculated log P is 6.2 (7) which is an indicator of its high lipophilicity. It has also gained a reputation as being "practically impossible to apply to the body" (8), making it a challenging molecule to develop for a topical dosage form.

Itraconazole was prepared as inclusion complexes using fulvic acid as an excipient and then compressed into tablets. Fulvic acid exhibited satisfacory mucoadhesion to the vaginal mucosa and also demonstrated an advantageous buffering potential at an acidic pH range that simulated the vaginal fluid milieu. Thus, this work shows that problems associated with a challenging molecule have almost been overcome and it was possible to develop a formulation using fewer excipients, yet having the necessary desirable functions of a topical dosage form.

EXPERIMENTS AND METHODOLOGY

"Shilajit" was provided and authenticated by Dabur Research Foundation, Ghaziabad, India. Itraconazole was received as a gift from Jubilant Organosys, Noida, India. Chemicals and reagents were of analytical reagent grade and purchased from Merck, Mumbai. Magnesium stearate and silica gel (Davisil® grade 636) were purchased from Sigma-Aldrich, India. MCC PH 102 was obtained from FMC Biopolymer, Philidelphia. Infa-V® Tablets (Lark laboratories, New Delhi), Sporanox® (Johnson & Johnson) and Candid-V® gel (Glenmark, India) were purchased from local sources. A method slightly modified from Mirza *et. al.* was used to extract the fulvic acid from 'Shilajit' (2).

Phase solubility behavior

Phase solubility studies were carried out at room temperature (25°C) in triplicate according to the method reported by Higuchi and Conners (9). An excess amount of Itz was added to distilled water containing various concentrations (0.2- 2% w/v) of fulvic acid in a series of stoppered conical flasks (100 ml) and shaken for 48 hours on a rotary flask shaker. The suspensions were filtered (0.45 μm) and analyzed for Itz by a slight modification of a validated HPLC method (10), with an accuracy of 95.5% and a precision of 6.7%. The analysis was carried out on a Waters Alliance e2695 chromatograph (Waters Co., MA, USA) using a photo diode array detector (Waters 2998) with

auto sampler and column oven. The instrument was controlled by use of Empower software for data collection. The compounds were separated using a C18 reverse phase column (25 x 4.6mm, particle size 5 µm, Merck, Germany) maintained at room temperature. The mobile phase consisted of acetonitrile, 0.05% diethylamine in deionized water (7:3, v/v), with the pH adjusted to 7.0 with phosphoric acid. The flow rate was 1 ml/min and the detection wavelength was 260 nm. 10 µl of the sample was injected into the column. Retention time (Rt) of Itraconazole was 8.25 minutes.

Preparation of Complex by Solvent Evaporation

Molar ratios of drug to complexing agent chosen were 1:1 and 1:2 based on the results from the phase solubility experiments. The calculated amount of the complex was prepared by solubilizing Itz with HCl using ethanol as a co-solvent, and then pouring this solution into an aqueous solution of fulvic acid. The solution was then stirred using a magnetic stirrer for 48 hours and then dried using a rotary evaporator. The resulting mass was powdered in a glass mortar and pestle and passed through a 100-mesh sieve to obtain a uniformly sized fine powder (3).

Characterization of the solid complexes

The complexes of Itraconazole and Fulvic acid were characterized by DSC, XRD, FT IR and Mass Spectroscopy as described in the literature (2, 3).

Differential scanning calorimeter

DSC thermograms were obtained under a nitrogen gas flow of 50 ml/min. Calibration of the DSC instrument (DSC-7, Perkin Elmer Pyris® 6 instrument, USA) was carried out using indium as a standard. Sample powders (5 mg) were crimped into aluminum pans and heated at a rate of 10 K min^{-1}. Generally, scan rates were taken between 1 and 10 K/min. Low scan rates are preferable in terms of peak resolution and investigation of the sample having peaks in

close proximity while high scan rates increase the sensitivity of the measurement as they lead to the exchange of heat within a comparatively short time period. Further, scan rates may also influence the course of temperature related processes within the sample. Samples were heated from 30°C to 350°C (taken as the highest melting point of organic substances).

FT IR analysis

The Fourier transform infrared spectroscopy (FT-IR) spectra of samples were recorded by a Perkin Elmer spectrophotometer (PE 1600 FT IR, Perkin Elmer, USA) using the potassium bromide (KBr) disc technique. 5 mg of a previously dried sample was mixed with 100 mg KBr and compressed into a pellet using an IR hydraulic press. Base line was corrected and the samples scanned from 4500 to 400 cm^{-1}.

Powder X ray diffraction

The samples were examined using an X-Ray Diffractometer (PW 1830, Phillips, Bangalore, Karnataka, India). The samples (1000 mg) on the XRD plates were rotated during data collection to reduce the orientation effects of the particles. The XRD patterns for all the samples were recorded between 5° and 70° *2θ* at 35 kV and 30 mA.

Mass spectroscopy

Samples were taken from the linear regions of the phase solubility study to evaluate the complexation. The samples were dissolved in Milli-Q water to make a stock solution of 1 mg/ml and then further diluted with a solution of Milli-Q water:Methanol (50:50). A concentration of 100 ng/ml was injected into the mass spectrometer (Synapt Mass Spectometry, Q-TOF with UPLC) on Electrospray ionization with positive mode. Capillary, sampling cone, and extraction voltages were 2.51, 21 and 5.3 units respectively. Source and desolvation temperatures were 80°C and 250°C respectively. Nitrogen gas was used as Cone

and desolvation gas at 50 and 600 liter/hour respectively. Trap collision energy was used (6.0 units). The system was from Waters bearing serial No. JAA 272 Waters, MA, USA. The software used was MassLynx V 4.1 (Waters).

Conformational analysis by computational method

The 3D-molecular structures were generated and optimized using Chem 3D-Ultra 8.0 software (Cambridge Soft Corporation, USA). All calculations used were for geometric optimization. All the energy minimizations were carried out until the root mean square (RMS) gradient was less than 0.08. Optimized molecular structures and partial atomic charges were used for the molecular modeling of FA, Itz and its complex. H-bonding analysis was based on ORTEP III (v1.0.3, L.J. Farrungia, University of Glasgow, UK).

Aqueous solubility of complexes

Saturation solubility of complexes at vaginal pH

Excess of 1:1 Itz-FA and 1:2 Itz-FA complexes were added to 10 ml of an aqueous solution (pH 4.5). They were shaken in a water bath (Ray Scientifics Instruments, India) for 5 days at 37°C, centrifuged (Tomy MX-305, Japan) at 3000 g for 10 minutes and filtered (0.45 μm). The concentration of Itz in the resulting solutions was then analyzed by the method described previously in the section "Phase Solubility Behaviour". The resulting values were the average of at least three replicates.

The standard solution Gibbs free energies were calculated using the following equation:

$$\Delta G^0_{sol} = -RT \cdot \ln X_2 \qquad \text{Eq. 1}$$

Where, X_2 is the molar fraction of Itz in its saturated solution.

The standard solution enthalpies were derived from temperature dependencies of drug solubilities expressed in molar fractions (van't Hoff equation):

$$\frac{d\ln X_2}{dT} = \frac{\Delta H^0_{sol}}{RT^2} \qquad \text{Eq. 2}$$

For use of the above equation, the following assumptions were made: (a) the activity coefficients of dissolved drugs do not deviate from unity and (b) the solution enthalpies do not depend on concentration. The solution heat capacities are considered to be constant within the studied temperature range, since the temperature dependence of solubility is described by linear equations.

Determination of driving force of solubilisation

The driving force for solubilisation of the bulk drug and complexes (1:1 and 1:2) among different thermodynamic parameters (Gibb`s free energy, Entropy and Enthalpy), was determined. Different aqueous solutions with variable pH (1, 2 and 4) were prepared by the dropwise addition of concentrated HCl into 100 ml of Milli-Q water until the required pH was confirmed using a pH meter (Decibel 1011, Chandigarh, India). An excess of each sample was added to each solution and the solubility was determined by the method described previously in the section "Saturation Solubility of Complexes at Vaginal pH". Here instead of buffer solutions, aqueous solutions of variable pH were selected. In an earlier study (11) the composition of the buffer was found to have pronounced effect in determining the thermodynamic driving force for solubility enhancement during the complexation studies.

In vitro cell toxicity studies of complexes

A MTT assay was used to assess the cytotoxicity of the free drug as well as the control and the complexes.

Table 1 Tablet composition of different batches. Regulatory status of excipients are also given. The amount used for the study was within the prescribed limits.

INGREDIENTS	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	Regulatory status of excipients for VDDS
FA:Itz complex	100	100	100	100	100	100	100	100	
Fulvic acid	320	345	320	345	320	345	320	340	
MCC PH 102	75	50	75	50	75	50	75	50	G, 3, 6, 7, 9, 10, 11, 12, 13, 14, 15, 20, 21
Silica gel	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	I
Mg stearate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22.

G: GRAS

I: Inactive ingredients guide

PHARMACOPEIAS: 1. Austrian 2. Belgian 3. British 4. Chinese 5. former Czechoslovakian 6. European 7. French 8. German 9. Greek 10. Hungarian 11. Indian 12. Italian 13. Japanese 14.Mexican 15. Netherlands 16. Nordic 17. Portugese 18.Romanian 19. Russian 20.Swiss 21. United States 22.Former Yugoslavian

The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is based on the conversion of yellow water soluble tetrazolium dye to a water-insoluble purple formazan by living cells. The amount of formazan generated is directly proportional to the number of viable cells.

MCF-7 cells (ATCC, USA through NII, New Delhi) were grown (37°C, 5% CO_2 in a water jacketed incubator shell) using DMEM media (Dulbecco's modified eagle medium for cell culture growth, Gibco Invitrogen, USA) with 10% Foetal Bovine Serum (FBS) and seeded on a single 96 well plate (Corning costar, USA) and allowed to adhere for MTT assays. Following this procedure, a concentration of free drug as well as the control and optimized formulations ranging from 20 µg/ml to 500 µg/ml having equivalent concentration were added in duplicate to a the 96 well-tissue culture-plate (Falcon Plate, Corning costar, USA). After 24 hours of treatment, the MTT assay was performed to check cell viability (12). For the MTT assay, the media was removed from all the wells, 10 µl of MTT reagent (Chemicon International, USA) per well from a working stock (5 mg/ml) was added and the plates incubated (37°C and 5% CO_2) for 2-3 hours. The reagent was subsequently removed and the crystals were solubilised using isopropyl alcohol (IPA) (Isopropanol/0.1 N HCL, supplied as a kit by Chemicon). This IPA extract was then transferred to 96 well-plates. The HCl converts the phenol red in the tissue culture medium to a yellow colour that does not interfere with the measurement of MTT formazan. The Isopropanol dissolves the formazan to give a homogeneous blue solution. The absorbance was measured at a test wavelength of 570 nm and reference wavelength of 630 nm using an ELISA plate reader, LMR-340 M (Labexim International, Austria). The value of absorbance is a measure of the number of live cells.

Preparation of Tablets (mucoadhesive, sustained release and acid buffering)

Different batches of tablets (500 mg each) were manufactured by direct compression according to the compositions presented in Table 1. Itz complex, fulvic acid and MCC PH 102 were mixed together in a mortar with a pestle. Magnesium stearate and silica gel were then added and mixed further. The tablet compositions were compressed on a 16-station rotary tablet machine using 16-mm flat-faced round punches (4-tons force for 10 seconds). Tablets T-1 to T-4 contained the 1:1 complex while T-5 to T-8 contained the 1:2 complex. Excipients other than the FA used for the formulations were approved, by various pharmacopeias or GRAS listed and suitable for use in a VDDS.

Optimization of the tablet formulation

The acid-buffering bioadhesive tablets were optimized on the basis of hardness, mucoadhesion and release profile. The releaseprofiles of the tablets were studied in 900 ml of simulated vaginal fluid containing 1% sodium lauryl sulphate using USP dissolution apparatus II at 50 rpm and 37°C. The

concentration of Itz was determined by UV spectroscopy (UV 1601, Shimadzu, Japan) at 260 nm. *In vitro* mucoadhesion studies were carried out by slightly modifying a published method (13). Briefly, an agar plate (1%, w/w) was prepared in pH 4.5 citrate-phosphate buffer. A tablet was placed at the center of the plate. After 5 minutes the agar plate was attached to a USP disintegration test apparatus (model 1901, Electronics India, India) vertically and moved up and down (10±1 cycle per minute) in pH 4.5 citrate phosphate buffer at 37°C (±)1°C. The sample on the plate was immersed into the solution at the lowest point and was out of the solution at the highest point. The residence time of the tablet on the plate was noted visually. Hardness of the tablet was determined using a Pfizer type hardness tester (KIP 2072, Kshitij Innovation, India).

Evaluation of optimized tablets

Determination of acid buffering capacity

The pH of the tablets was determined after being in contact with 2 ml of distilled water for 2 minutes. The pH of the swollen tablet was measured by touching the tip of the electrode to the wet mass of the tablet. For the measurement of the buffering capacity, one tablet was allowed to dissolve in 10 ml of 0.9% NaCl (normal saline) solution. Sodium hydroxide (1.0 N) was added in 20 µl increments while stirring constantly. The pH was measured using a standard combination electrode, 30 seconds after each addition. Stirring was stopped during the pH measurements. This procedure was repeated until the pH was greater than 7.0. The titrations were performed in triplicate for each formulation. The amount of NaOH required to bring the pH of each solution to 5.0 was taken as a measure of the buffering capacity of the tablet.

Tablet Swelling and Spreadability studies

The swelling characteristics of the bioadhesive tablets were evaluated by dynamic swelling studies. Each sample was weighed and then placed in 10 ml sodium citrate/hydrochloric acid buffers at pH 4 in a glass vial at ±37°C. The samples were periodically weighed after removing excess water from the surface with a filter paper and the swelling was calculated. For the spreading ability study, the tablets were allowed to swell in 2 ml of distilled water for 1 minute, the swollen mass was gently transferred to the center of a glass plate and compressed under several glass plates (100 g each minute). The spread diameters were recorded and compared with a marketed vaginal gel formulation used as the reference formulation (Candid-V®).

Ex Vivo Mucoadhesion studies

A modified Setnikar and Fantelli apparatus (14) was used for the study of mucoadhesion (shown in Figure 2). Excised and cleaned buffalo vaginal tube was everted on the upper and lower ends of the glass cell and crimpled using rubber bands. Water was circulated in the cell to maintain the temperature and to keep the tissue moist. The bioadhesive tablet was inserted into the tube using a pair of blunt forceps. A preload time of 5 minutes was granted to allow the formulation to adhere to the vaginal wall. Simulated vaginal fluid (15) was allowed to fall drop wise (3 ml/h) into the vertically suspended vaginal tube. The time of expulsion of the formulation from the lower end of the cell was recorded.

In Vitro antifungal studies

In vitro antifungal studies were performed against clinical isolates of *Candida albicans* in Sabouraud's agar medium by the cup plate method. The marketed formulation (100 mg Sporanax® Capsule) was suspended in 2 ml of sterilized water and transferred into the well of an agar petri dish. Optimized tablets were also put into wells in the same manner. 100 mg of Itz suspension was also prepared. 2 ml of distilled water was used as the control. All the samples were applied in triplicate. Covered petri dishes were incubated at 32°C in the BOD
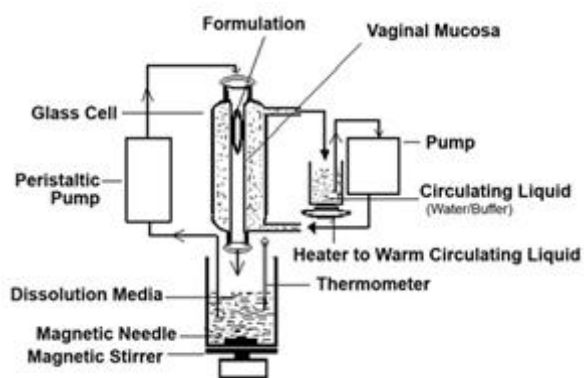
Figure 2 Modified Setnikar and Fantelli apparatus. Water flows through the crimped vaginal mucosa at fixed rate. Temperature of the media is controlled by thermometer and heating devise attached.

incubator (LHC-78-Labhospmake, India) for 40 hours. The zone of inhibition was measured after incubation.

RESULTS AND DISCUSSIONS

Phase solubility behavior

The phase solubility studies revealed a non linear relationship between the solubility of the drug and the FA concentration shown in Figure 3. The curves were characteristic A_N type (16) with $r^2 = 0.905$. The Itz:FA molar ratios selected for complexation were 1:1 and 1:2 based on the shape of the graph. Up to a concentration of 1% w/v of Itz, the relationship was linear but, nonlinear thereafter. The data also revealed that at higher concentrations of FA (1-2% w/v), the solubility of Itz was variable with noticeable deviations. The study also showed a much increased binding to commonly used chemicals than reported in some studies (17). The existence of mechanisms other than inclusion is also evident from the literature. The high molecular weight and hydrophobicity of fulvic acid favor formation of "micelle"- like structures (4, 5) with hydrophilic groups arranged on the aqueous side and the hydrophobic portion forming the inner core to entrap organic moieties (18).

A phase solubility graph was used to determine the binding constant and Gibb's free energy (19). The binding constant was calculated according to the equation:

$$K_s = \frac{slope}{S_0(1-slope)}$$ Eq. 3

where, S_0 is the solubility of Itz without fulvic acid.

The binding constant was found to be 1103.73 M^{-1}. Stability constant (K_s) values that fall between 200 and 5,000 M^{-1} are considered as most suitable for the improvement of solubility and stability of a poorly soluble drug (20). To investigate the spontaneity and feasibility of the entrapment by the thermodynamic approach, changes in Gibb's free energy (ΔG) were also calculated at constant temperature and pressure (Eq. 4). It is the net energy available for useful work.

$$\Delta G = -2.303 . RT \log \left[\frac{S_0}{S_s} \right]$$ Eq. 4

where, S_s and S_0 are the solubility of the drug in the presence and absence of fulvic acid respectively.

As ΔG becomes more negative the reaction becomes energetically more favorable. In the
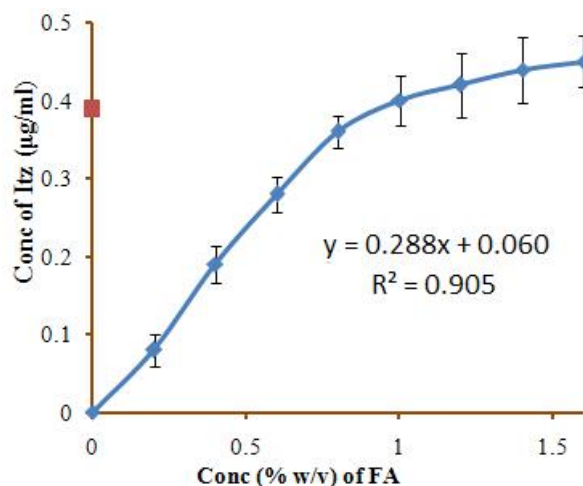

Figure 3 Phase solubility studies of Itz with FA at room temperature (25° C) in triplicate mode.

Table 2 Saturation solubility of complexes at vaginal pH (4.5). Experiment was carried out at room temperature (298K) and in a triplicate mode. Here the process appears to be entropy driven.

SUBSTANCES	SOLUBILITY (µg/ml)	X_2 (molar fraction)	ΔG^{298}_{sol} (kJ mol^{-1})	ΔH^{298}_{sol} (kJ mol^{-1})	T ΔS^{298}_{sol} (kJ mol^{-1})	ΔS^{298}_{sol} (KJ mol^{-1} K^{-1})
API (Itz)	0.13±3.7	0.0032×10^{-6}	48.46	44.5±0.8	-3.96	-0.013
1:1 FA-Itz complex	5.81±1.4	0.149×10^{-6}	38.94	48.3±0.5	9.4	0.031
1:2 FA-Itz complex	47.3±2.2	1.22×10^{-6}	39.44	47.6±1.4	8.16	0.027

present case the physical phenomena (inclusion of Itz into FA) is assumed to be a process that can be evaluated. Other thermodynamic parameters (ΔH, TΔS and ΔS) were also calculated and are presented in Table 2. The disorder of the system was lower in the case of the 1:2 complexes.

Characterization of complex

All the analyses (DSC, FT IR and XRD) confirmed the formation of complexes.

Differential scanning calorimetry

The thermograms showed a sharp peak at 170 °C for Itz and at 150°C-160°C for fulvic acid shown in Figure 4. The peak for the drug was absent in all the complexes except for some inflections. It may be inferred from the thermograms that Itz undergoes a significant crystalline to amorphous transition during complexation with FA. The details from the thermograms of Itz and its complexes are given in Table 3.

Table 3 DSC data of pure Itraconazole, fulvic acid and complexes.

SAMPLES	ΔH (J/g)	MAXIMUM PEAK (°C)	PEAK WIDTH
ITZ	105.035	170.408	6.62
FA	142.075	162.387	11.13
1:1 ITZ:FA	78.502	136.214	26.44
1:2 ITZ:FA	78.033	137.162	19.21

Melting enthalpy is characteristic of the crystal order if the influence of impurities can be ignored. For the less ordered crystal or amorphous state, the melting of the substance requires less energy than would be required to melt a completely crystalline substance. As a result, the higher melting enthalpy values should suggest a higher ordered lattice arrangement and vice versa. Another parameter that could be evaluated is peak width (i.e. difference between onset and maximum). For the different samples, the peak width was a minimum for the drug (Itz) but greater for the complexes.

This difference could be attributed to the size effect and explained by the Thomson equation (21). The particle size of unprocessed API is around 152.3 nm (diameter) while the other samples were assumed to possess larger sizes and a broader particle size distribution as they were passed through a No 100 sieve.

The endotherms of the complexes were also shifted to lower temperatures. This phenome-
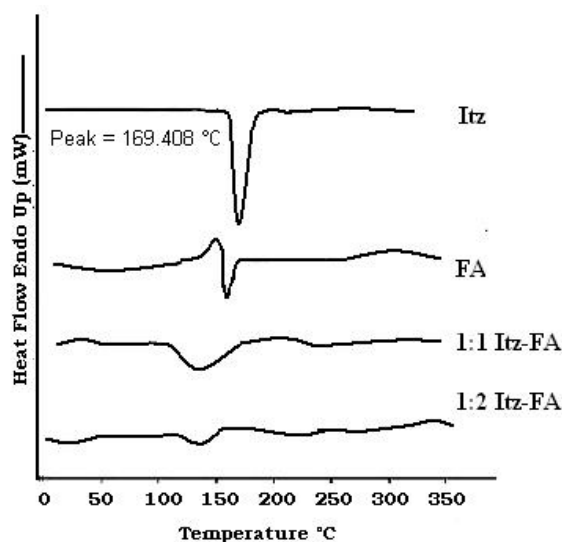

Figure 4 Representative differential scanning calorimetry profiles of Itz, FA and different complexes.

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non can be inferred from the Gibbs–Thomson equation:

$$\ln \frac{T}{T_0} = -\frac{2\gamma_{sl} V_s}{r\Delta H_{fus}}$$ Eq. 5

Where,

T   Melting temperature of a particle with radius r

T_0 Melting temperature of the bulk material at the same external pressure

γ_{sl} Interfacial tension at the solid–liquid interface

V_S Specific volume of the solid

ΔH_{fus} Specific heat of fusion

It reflects the decrease in melting temperature for a particle of given size compared to the bulk material. The sizes of the particles of the complexes are supposed to be larger than the drug molecule and are polydisperse, hence the melting transition is not only shifted to lower temperatures but is also broadened since the fractions of different particle sizes melt at different temperatures.

Fourier Transform Infrared spectroscopy

The FT-IR spectrum of the drug shows the stretching and vibrational peaks in the fingerprint region that are characteristic of the molecule (shown in Figure 5). These peaks are overlapped, diminished or dispersed in the inclusion complexes. FT-IR spectroscopy is sensitive to the structure, conformation and environment of organic compounds. The characteristic peaks of Itz occurred at 3381, 3126, 3069, 2962, 1697, 1510, 1450, and 418 cm^{-1}. The absorption due to the NH$_2$ groups are located in the bands at 3381, 3126, 3069, cm^{-1}. The first band is assigned to stretching vibrations of the free NH$_2$ of the pure drug. The peaks observed at 1609 and 1425 may be assigned to the C=N and C-N bonds respectively and the sharp peak seen at 1697

cm^{-1} is due to the C=O group of the drug. FT-IR absorption bands for fulvic acid extracted from 'Shilajit' were found to be in accordance with those reported in the literature (22). Interactions between the carbonyl peak of Itz and the carboxylic group of FA, and also between the stretching vibration of N-N of Itz and the O-H vibration of FA were observed. Olefinic and carbonyl peaks of the drug are widespread and dispersed indicating weak interaction with similar bands in the complexing agent. Peaks from the fingerprint regions (1300 – 400 cm^{-1}) are more diminished, indicating interaction between the drug and the complexing agent.

X-Ray Diffraction

A plot 2θ *vs* % Intensity can be considered a fingerprint of the crystal structure and may be used to differentiate between different crystallographic forms. These patterns are representative of the structure but do not give positional information about the atoms in the molecule. An amorphous sample will exhibit a broad hump in the pattern called an amorphous halo but crystalline substances give a characteristic pattern. Itz showed a characteristic crystalline pattern presenting intense peaks at 20.38 (100%), 20.40 (97.59%), 20.36 (96.53%), 20.34 (88.53%), 20.32 (80.66%) and
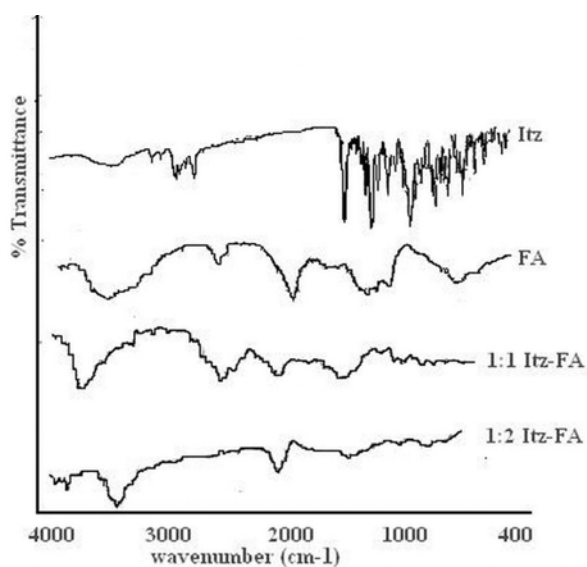

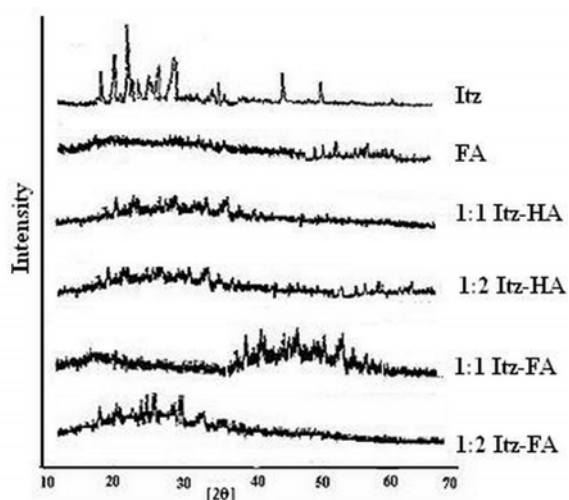**Figure 5** FT IR spectra of Itz, fulvic acid and complexes.

Figure 6 X ray powder diffraction of Itz, fulvic acid and complexes.

Figure 7 Ball and stick model (energy minimized) of Itraconazole.

17.5 (77.9%). The diffraction pattern for FA was amorphous (Figure 6). These two techniques (DSC and XRD) provide complementary information on the systems of interest and the data evaluation from these methods is usually straightforward. The XRD of inclusion complexes demonstrated their amorphous nature, indicating entrapment of drug and, peaks, if any, were appreciably diminished.

Conformational analysis by computational methods

Molecular modeling has shown that complexes of Itz-FA are stable. A ball and stick model figure of Itz is shown in Figure 7. The molecular modeling showed that FA has the ability to form inclusion complexes with Itz. The intramolecular hydrogen bonds observed for FA contribute to the stability of the molecule (figure not shown). This structure shows at least five intramolecular H-bonds. Three out of five intra-molecular H-bonds are OH-O type which means that these are strong H-bonds. These hydrogen bonds are believed to increase the stability of the molecule. Drug complex optimization with FA shows that the Itz is stabilized by a strong NH-N interaction with FA. The total potential energy of the FA
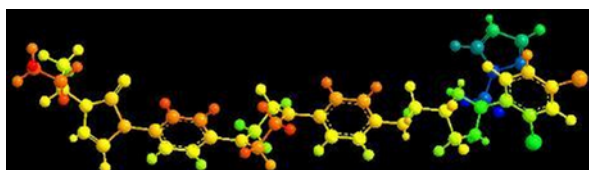
using Chem 3D-Ultra 8.0 software was calculated to be -38.8716 Kcal/mol while a complex of Itz and the FA was stabilized at -26.283, which is not significantly more unstable than Itz alone. The energy optimization of Itz yielded a potential energy of -30.84 Kcal/mol.

Mass Spectroscopy

The formation of a complex was also confirmed by mass spectroscopy. In all the mass spectra some peaks were common (with m/z variation ~ 5) that were either non-complexed drug (Itraconazole), non-complexed fulvic acid (average molecular weight ~ 1200 Da) or 1:1 an 1:2 complexes. Extensive noise was also present as shown in Figure 8. It was evident from the result that in any complex (irrespective of the ratio) some fraction of uncomplexed drug, uncomplexed fulvic acid and 1:2/1:1 complexes were present. Their relative abundances depended upon the ratio (1:1 or 1:2) taken. For the 1:1 complex the major constituent was 1:1 complex and similarly in 1:2 complex, dominating constituent was the 1:2 complex.
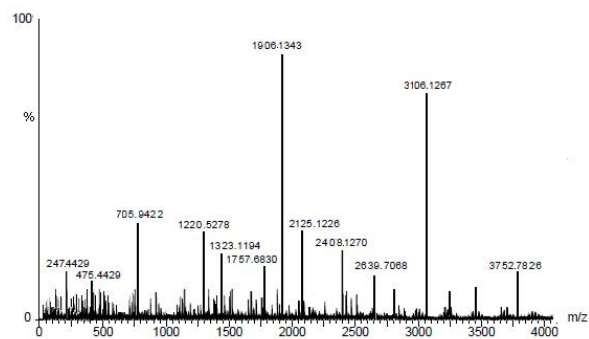

Figure 8 Mass spectra of Itz-FA complex. Peaks of uncomplexed drug, uncomplexed FA and different molar ratios are evident in the spectra obtained.

Aqueous solubility study of complexes

Saturation solubility of complexes at vaginal pH

Solutions of Itz and its complexes became saturated after day 5 (at pH 4.5) at room temperature (25°C (±)3°C). With 1:1 complexation a 44.7 fold increase in solubility was observed while with the 1:2 complexation, a 363.8 fold increase was observed. Different thermodynamic parameters of solubilisation were calculated (Table 2). The positive values of ΔH in all the cases indicate that crystal lattice energy of the substance clearly outweighs the solvation energy. The formation of complexes appears to be an entropy driven process as the values change significantly (from negative to positive) during complexation.

Determination of driving force of solubilisation of complexes

The thermodynamic data calculated for the different substances (API and complexes) indicates the different driving forces involved (shown in Figure 9). This may be due to differential interaction of Itraconazole with fulvic acid at different pH values. Itraconazole is a weak base with four ionizable nitrogen atoms. Two of the pKa values are 4 and 1.5–2 whereas the other ionizable nitrogens are not protonated between pH 2 and 10 (7). This explains the sudden drop in solubility above pH 3 and why changes in pH profoundly influenced both solubility and dis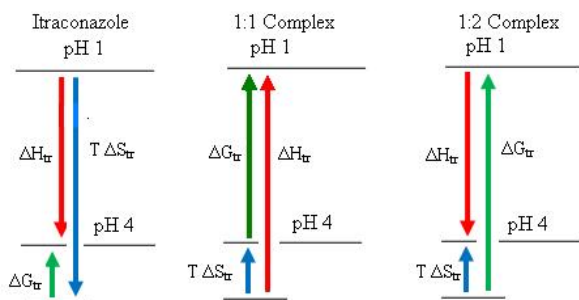solution. This is why there was no linear (ascending or descending) pattern for any thermodynamic parameter during complexation. The sudden drop in solubility of Itz above pH 3 leads to a greater hydrophobic interaction of guest and host molecules and the Itz penetrates into the host molecule thus creating Van der Waals interactions and hydrogen bonds thereafter. For the complexes and API the values of ΔG and ΔH are positive (ΔS was found to be both positive and negative). Thus it may be concluded that complexation of Itraconazole with fulvic acid becomes energetically more favorable with an increase in temperature. The enthalpy change, ΔH did not significantly affect the complexation energetics.

The same sign of enthalpy and entropic values demonstrated that the complexation was a classical model of hydrophobic interaction (23). At the same time this was not observed for the API. The change in standard entropy was positive in all cases of complexation which implied an increase in the disorder of the system during the complexation process.

The solubilization of the API in water was accompanied by a decrease in disorder. For the API, formation of a solvation shell plays a role in determining the order of a system, while in case of complexation, macromolecular inclusion and solvation shell both determine the overall order of the system. In all cases this entropic change was more noticeable when the pH changed from pH 2 to pH 1 which is very clear in the case of pure Itz. As the pH drops from 2 to 1, there is almost an 8-10 % increase in the fraction of entropy of Gibbs energy as shown in Table 4.

At pH 1 Itz molecule has a solvation shell, which consists of entropically ordered molecules of the medium in the space next to the ionized atoms, and a more disordered part of the solvation shell is located around other parts of the molecule (hydrophobic effect).

Additionally, the conformation of the molecule was found to be more linear (MacroModel,

Figure 9 Schematic representation of driving forces of solubility as the pH is varried from 1 to 4. Experiment was carried out at room temperature (298K) and in a triplicate mode.

Table 4 Thermodynamic parameters of solubilisation process of pure Itraconazole, 1:1 FA-Itz and 1:2 FA-Itz complexes in aqueous solution at variable pH at 298 K. A comparative evaluation of different variables depicts the thermodynamic driving force of the process

SAMPLES	pH	SOLUBILITY (µg/ml)	X_2 (molar fraction)	ΔG^{298}_{sol} (kJ mol^{-1})	ΔH^{298}_{sol} (kJ mol^{-1})	T. ΔS^{298}_{sol} (kJ mol^{-1})	ΔS^{298}_{sol} (J mol^{-1} K^{-1})	ϵS^a [%]
API (Itz)	1	24.17±2.3	0.618×10^{-6}	35.42	55.3±0.71	19.88	66.71	56.12
	2	0.55±4.1	0.014×10^{-6}	44.80	35.6±1.5	-9.2	-30.87	-20.53
	4	0.13±3.7	0.0033×10^{-6}	48.46	44.5±0.8	-3.96	-13.28	-8.17
1:1 FA-Itz complex	1	78.46±2.6	2.02×10^{-6}	32.48	38.3±0.6	5.82	19.5	17.91
	2	69.72±0.9	1.79×10^{-6}	32.78	35.4±0.4	2.62	8.79	7.99
	4	5.81±1.4	0.149×10^{-6}	38.94	48.3±0.5	9.4	31.4	24.13
1:2 FA-Itz complex	1	86.4±2.6	2.22×10^{-6}	32.25	40.1±0.7	7.85	26.34	24.34
	2	79.3±3.1	2.045×10^{-6}	32.46	38.6±0.2	6.14	20.60	18.91
	4	47.3±2.2	1.22×10^{-6}	39.44	33.9±1.2	8.16	27.4	20.68

$^a\epsilon S = [(T.\Delta S°) \Delta G°].100*$

version 9.7, NY 2009) at room temperature than other temperatures (-20°C, 0°C and 180°C). Thus during complexation the ordered shell portion along with some other hydrophobic portions finds its way into the cavity leaving behind disordered portions. A comparison of the 1:1 and 1:2 complexes indicated that a lower increase in entropy occurred in the 1:2 complex. This is attributed to the fact that perhaps both cavities of the fulvic acids (1:2 Itz-FA complex) entrap the hydrophobic portions of the Itz, thus leading to a more ordered structure while with the 1:1 complex only one cavity was available to entrap the molecule. This assumes that any changes in the entropy caused by differences in the solvation of the FA molecule before and after complexation are negligible due to the large size of this molecule. On the other hand, the change in entropy from pH 2 to pH 4 was nearly same or greater compared to changes occurring during pH 2 to pH 1 and a substantial difference was found between the entropy change of complexation for the ionized and unionized molecule. In this case the ionization is less and solvation shell is not highly ordered. Thus the solvated shell whether inside or outside the cavity is not stearically stabilized. The only negative values of entropies were noticed in case of pure Itz (at pH 2 and pH 4). This suggests that the solution entropies of Itz at a particular pH during the transfer of the

molecules from the solid state to the solvent, ordering of the drug-water system becomes stronger in comparison to the crystal. With other samples (1:1 complex and 1:2 complexes) these were not so strong.

In vitro cell toxicity studies of complexes

The corresponding values for optical density for the control, neat drug and the complexes are shown in Figure 10. The Itz was found to have some cytotoxic effect. However, for the complexes the toxicity did not increase at different concentrations. Only at a concentration of 500 µg/ml was some toxicity observed with the 1:2 complex. Fulvic acid is reported to be safe in cytotoxicity studies (24).
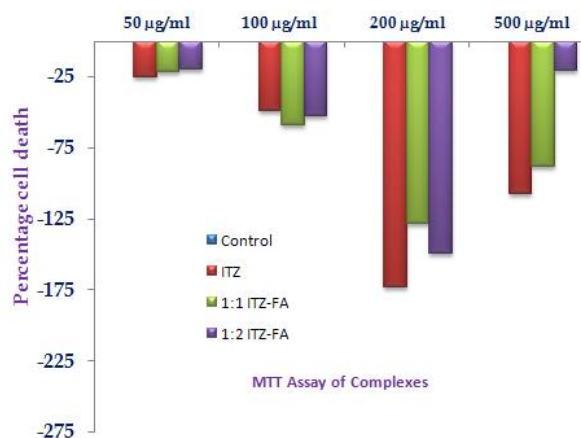

Figure 10 MTT assay results of pure drug and complexes.

Table 5 Optimization of the tablet formulations. Parameters are presented in the order of their priority. Drug release behaviour was assigned the highest priority while Hardness was assigned the lowest. The formulae which performed best in the first two tests were selected as optimized preparations.

PARAMETERS	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8
	1:1 Complex				1:2 Complex			
% drug released after 24 h	68.3± 3.2	71.2±2.6	73.5±1.9	76.1±2.4	72.4± 2.6	74.8± 4.0	77.9± 3.7	79.8 ±3.3
Mucoadhesion (s)	90±2	95±3	96±3	102±3	102±6	96±6	98±8	100±7
Hardness (Kg)	6.2±0.5	5.4±0.7	4.6±0.8	3.7±0.7	6.3±0.6	5.5±0.5	4.6±0.6	4.0±0.6

Thus, the reports of *in vitro* cell toxicity study did not indicate any additional cellular toxicity and the results obtained were, in all probability, due to the drug molecule itself.

Optimization of the tablet formulation

On the basis of hardness, mucoadhesion and release profile, T-4 and T-8 were found to be acceptable candidates for further investigation. T-8 showed a better sustained action compared to T-4. The results obtained are presented in Table 5.

Determination of acid buffering capacity

The pH of the optimized tablets was found to be acidic for T-4 (pH 4.8±0.068, n=2) and T8 (4.9±0.076, n=2), very similar to the pH of the vaginal tract (25). The pH of the reference marketed formulation, Infa-V® was also acidic

(5.19 ± 0.26, n=2). The amount of NaOH required to bring 1 g equivalent of tablets to pH 5.0 was about 0.052 mEq. This data was comparable to that found in the literature where an amount of NaOH required bringing 1 g equivalent of Advantage 24 to a pH 5 was reportedly 0.080 mEq (26).

Swelling and Spreading ability studies of optimized tablets

Increasing the amount of fulvic acid produced tablets with greater swelling rates. The equilibrium swelling of T-4 after 15 minutes was 66.2% while T-8 swelled up to 48.6%. After maximum swelling, disintegration started. Being a gel, Candid-V® spreads smoothly and covers a large area at any given point of time. T4 spread a little better than T-8 but was comparable to Candid-V® in spreading ability as shown in Figure 11.
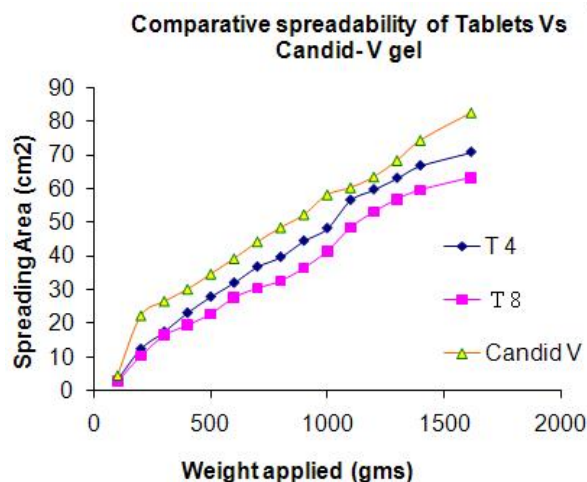
***Ex Vivo* Mucoadhesion studies**

Tablets (T-4 and T-8) swelled inside the tube and adhered to the wall in the *ex-vivo* study. They demonstrated retention times in the excess of 40 hours. Tablet T-8 showed maximum adherence (>48 hrs), while the Infa -V® (reference product) disintegrated inside the vaginal tube and started leaking after 5-8 hours.

***In Vitro* antifungal studies**

All the wells except those containing the control produced signigcantly larger zones of inhibition than Itz alone. Fulvic acid was found to possess negligible antifungal activity. Of the two, the formulation that had a 1:1 ratio created

Figure 11 Spreadibility studies of tablets when compared with marketed gel (Candid-V®)

a larger zone of inhibition (Table 6). After complexation, the solubility of the drug increased but the size of the complexed moiety was increased as well (> 1900 Da). These two factors oppose each other as regards the penetration of Itz into the agar media suggesting that some other mechanism allowed the drug molecule deeper into the agar media. Increase of aqueous solubility may be one reason for the deeper penetration. On the other hand, Sporanax® uses the same principle of complexation but with Cyclodextrin as the complexing agent. Molecular weights of cyclodextrin and fulvic acids are comparable to each other, as were their zones of inhibition.

Table 6: Zones of inhibition of Itz:FA formulations, Sporanax® and pure Itz.

SUBSTANCES	ZONE OF INHIBITION (n=3) in cm
Distilled water	--
Sporanax® 100 mg Capsule	1.6 ± 0.3
100 mg Itz	0.4 ± 0.2
1:1 complex (eq. to 100 mg Itz)	1.5 ± 0.4
1:2 complex (eq. to 100 mg Itz)	1.1 ± 0.2

CONCLUSION

Itraconazole can be complexed with Fulvic acid and formulated as a vaginal drug delivery system with an efficacy comparable to existing topical formulations on the market. The complexation was found to be largely entropy driven with the potential energy of the complex being comparable to that of Itz at pH values where the API was non-ionizable. Fulvic acid can be explored in three very new roles namely for acid buffering, mucoadhesion and sustained release in vaginal drug delivery systems. The proposed formulation used in this study suggests that further research should be carried out to evaluate fulvic acid as a potential excipient for VDDS.

ACKNOWLEDGEMENTS

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