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Complexation of Furosemide with Fulvic Acid Extracted from Shilajit: A Novel Approach

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The aim of the present work was to complex furosemide (FSM) with fulvic acid (FA) extracted from shilajit with the hope of having a better understanding of the complexation behavior. The effect of FA on the aqueous solubility, dissolution rate, and permeability of FSM was investigated. Different techniques, such as grinding, freeze drying, solvent evaporation, and so forth, were used for the preparation of the complex. The complexes were prepared in molar ratios of 1:1 and 1:2 FSM:FA and were evaluated for drug inclusion, solubility, differential scanning calorimetry, Fourier transform infrared spectroscopy, X-ray diffraction, scanning electron microscopy, dissolution study, and permeation study. These methods confirm the formation of an amorphous inclusion complex of FSM with FA.

Keywords shilajit; fulvic acids; furosemide; complexation; characterization

INTRODUCTION

Shilajit, also known as salajit, shilajatu, mumie or mummiyo is a pale brown to blackish-brown exudation of variable consistency from layers of rocks in many mountain ranges of the world, especially the Himalayas and Hindukush ranges of the Indian subcontinent (Chopra, Chopra, & Handa, 1958; Ghosal, 1992). It is also found in Australia, Bhutan, China, Egypt, Mongolia, Nepal, Norway, Pakistan, Russia, and other countries, where it is collected in small quantities from steep rock faces at altitudes between 1000 m and 5000 m (Ghosal, 2002; Ghosal, 2006).

The biological effects of shilajit have been ascribed to two distinct classes of compounds. The low molecular weight bioactive organic compounds, such as oxygenated dibenzo-α-pyrones, act as the active substances, and medium molecular weight fulvic and humic acids act as carrier molecules for in vivo transportation of these bioactive molecules (Agarwal, Aqil, & Anwer, 2007; Agarwal, Khanna, Karmarkar, Anwer, & Khar, 2007). Fulvic acid (FA) and humic acid have a microporous structure. FA and humic acid are thus capable of forming complexes with nonpolar solutes and drug molecules with low bioavailability (Agarwal, Khanna, Karmarkar, Anwer, & Khar, in press). These drug molecules can be entrapped in the void so as to increase their solubility and dissolution rate, thereby enhancing their bioavailability (Ghosal, 2003; Khanna, 2006).

Furosemide (FSM) is a potent loop diuretic that is used to adjust the volume and/or composition of body fluid in a variety of situations, including hypertension, heart failure, renal failure, nephritic syndrome, and cirrhosis (Murray, Haag, Black, Hall, & Brater, 1997). FSM is practically insoluble in water (Al-Obaid, Al-Shammary, Al-Rashood, & Mian, 1989). The oral bioavailability of FSM is very poor due to insufficient aqueous solubility at gastrointestinal pH, making solubility the rate-determining step in the gastric absorption of FSM. Improvement of its dissolution properties is essential because the in vitro dissolution behavior of FSM is closely related to its bioavailability (Ammar, Ghorab, El-Nabhas, Emara, & Makram, 1999).

MATERIALS AND METHODS

Materials

An authentic sample of rock shilajit was obtained from Dabur Research Foundation (Ghaziabad, India). FSM was kindly provided as a gift sample by Modi-Mundi Pharma Ltd. (Merrut, India). All other chemicals and reagents used in the study were analytical reagent (A.R.) grade.

Extraction of FA from Shilajit

A slightly modified reported method (Ghosal, 1989) was used to extract FA. The method consisted of successive extraction of raw shilajit with hot organic solvents of increasing polarity to remove the bioactive components. The residue (marc) was dissolved in 0.1 N NaOH with intermittent shaking in the presence of nitrogen. The suspension was filtered and the filtrate was acidified to a pH of less than 3 to precipitate the humic acids. The filtrate was then shaken with macroporous

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ion exchange resin in order to adsorb the FAs, which were then eluted using 0.1 N aqueous sodium hydroxide solution. The FAs thus obtained were passed through hydrogen-saturated cation exchange resin in order to exchange the sodium ions with hydrogen ions. The resulting FA solution was concentrated and freeze dried to obtain amorphous FAs.

Preparation of the Inclusion Complexes

The complexes of FSM with FAs were prepared in molar ratios 1:1 and 1:2 by using the following methods.

Physical Mixture

Complexes of FSM and FA were prepared by grinding the mixture for 30 minutes in a clean dry glass pestle and mortar.

Freeze Drying

FSM and FA were dissolved in water with one drop of ammonia (27%) to aid dissolution of FSM and sonicated for 15 minutes to get a clear solution. The solution was frozen in an ultra freezer by keeping it for 24 hours and freeze dried for 12 hours in a Lyph-lock apparatus (Drywinner, DW-8-85 Heto Holten, Denmark). 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.

Solvent Evaporation

FSM and FA complex was also prepared by solvent evaporation by dissolving the FSM in acetone and FA in water. The FA solution was then added to the solution of FSM with stirring and then the mixture was sonicated for 2 hours. The solution thus obtained was dried in a rotary evaporator under vacuum (Hahn Shin Science Co., Hs- 2001N, South Korea).

Characterization of the Solid Complexes

Fourier Transform Infrared (FTIR) Spectroscopy

The infrared spectrum of FSM, FA and complexes was recorded on an FTS 40 (Arbro Pharmaceuticals, New Delhi, India) FTIR instrument and WIN IR software by the KBr pellet technique. Two mg of previously dried sample was mixed with 100 mg KBr and compressed into a pellet on an infra red (IR) hydraulic press. These pellets were prepared immediately prior to the recording of the spectrum. Scanning ran from 4,000 to 400 cm^{-1}.

Differential Scanning Calorimetry (DSC)

A DSC thermogram (Perkin-Elmer Pyris 6 instrument, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India) of the FSM, FA, and their complexes was recorded. For obtaining the thermogram, 2 to 3 mg of the sample was accurately weighed and heated in a closed aluminum crimp cell at a rate of 10ºC/minute under nitrogen purge with a flow rate 20 ml/minute over the temperature range of 50ºC to 400ºC.

Powder X-Ray Diffraction (XRD)

Powder XRD patterns of powdered samples of FSM, FA, and their complexes were obtained using a P analytical X-ray diffractometer (PW3719). All samples were subjected to the following specifications:

Target/Filter (monochromator)	:	Cu
Voltage/Current	:	40 kV/50 mA
Scan speed	:	4°/minute
Smoothing	:	0

Scanning Electron Microscopy (SEM)

SEM of samples was performed using a Jeol JSM-840 Scanning Microscope with a 10 KV accelerating voltage. The surface of SEM samples were made electrically conductive in a sputtering apparatus (Fine Coat Sputter JFC-1100) by evaporation of gold. A magnification of 1,500 and 3,000 was used for all samples.

High-Performance Liquid Chromatography (HPLC) Analysis

Samples were analyzed by a modified method of Bleow and Burmann's (1994). Analysis of all samples was performed with a Waters Breeze HPLC system, Waters Pvt. Ltd (India). The mobile phase (10 mM potassium hydrogen phosphate/acetonitrile, 60:40 v/v) was pumped at a flow rate of 1.0 ml/minute through a water spherisorb ODS –2, 250 × 4.6 mm column at room temperature. The injected volume was 50 µl, and the detection wavelength was 234 nm. Under these conditions, FSM retention time was 6.6 minutes.

Aqueous Solubility Determination of Solid Complexes

Excess amount of complex was kept in an amber colored bottle containing 10 ml of distilled water and stirred using a thermostated mechnical shaker (25°C) for 5 days.

Release of FSM from the Complex

Dissolution of pure FSM (40 mg) and inclusion complexes (equivalent to 40 mg FSM) was performed on a paddle-type United States Pharmacopoeia (USP) tablet dissolution apparatus in 900 ml phosphate buffer (pH 5.8) at 37.5 ± 1ºC and 50 rpm (USP, 2000). The samples were withdrawn with the help of a syringe fitted with a needle and filtered through a Millipore filter (0.22 µm). Fresh aliquots of the dissolution medium were added to compensate for the quantity of sample withdrawn. The filtered samples were analyzed by HPLC.

Permeation Study Across Rat Everted Gut Sac

In order to determine the effect of complexation on the permeability of FSM, permeability studies were carried out by the

rat everted gut sac method using FSM as a model drug (Barthe, Woodley, & Kenwarthy, 1998).

Material Required

Rat intestine (30 cm)
Complex (1:2 freeze dried FSM-FA complex)
Tissue culture medium 199 (TC199)
Normal saline (0.9% w/v)
Biological shaker

For this study, everted rat intestinal sacs of about 5 cm length were prepared in a tissue culture medium (TC199) and filled with about 3 ml of tissue culture medium. The sacs were placed in tubes containing 25 ml of tissue culture medium in which excess (equivalent to 40 mg of FSM) of either FSM alone or freeze dried FSM-FA (1:2) complex. The tubes were maintained in a shaking water bath at 37°C, continuously bubbled with oxygen and agitated at a speed of 60 rpm. The samples were withdrawn from the intestinal sac in 10-minute intervals. The samples were centrifuged for 5 minutes at 4,000 rpm and filtered with a Millipore filter (0.22 μ). The filtered samples were analyzed by HPLC.

RESULTS AND DISCUSSION

Characterization of the Solid Complexes

DSC, FTIR, XRD, and SEM studies of complexes revealed the formation of amorphous inclusion complexes, which is one of the reasons for enhanced solubility.

FTIR Spectroscopy

The FTIR spectra (Figure 1) of FSM shows characteristic absorption bands in the regions of 3,390 cm^{-1} (N-H stretching, primary sulfonamide), 3,300 cm^{-1} (N-H stretching), 1,690 cm^{-1} (C=O stretching), 1,550 cm^{-1} (C=C stretching, benzene), 1,330 cm^{-1} (S=O stretching), and 1,250 cm^{-1} (C-N stretching). FTIR absorption bands of FA extracted from shilajit were found in accordance with those reported in the literature (Schnitzer, 1972). Absorption peaks of physical mixture in the molar ratio 1:1 are present at the finger print region of FSM (1,600–400 cm^{-1}), but intensity is reduced, indicating no complexation. The spectra of the FSM-FA physical mixture complex in the molar ratio 1:2 shows that the primary sulfonamide band at 3,390 cm^{-1} in the case of FSM was overlapped with the OH band at 3,400 cm^{-1} supporting weak interaction of FSM and FA.

The FTIR spectra of the FSM-FA complex in the molar ratio 1:1 prepared by solvent evaporation showed peaks of FSM in the finger print region (1,600–400 cm^{-1}) with decreased intensity. This supports a weak interaction between FSM and FA. While FTIR of 1:2 molar ratio shows complete peak overlapping of FSM and FA at 3,900 cm^{-1}, the finger print region peaks (1,600–400 cm^{-1}) disappear, supporting complex formation.

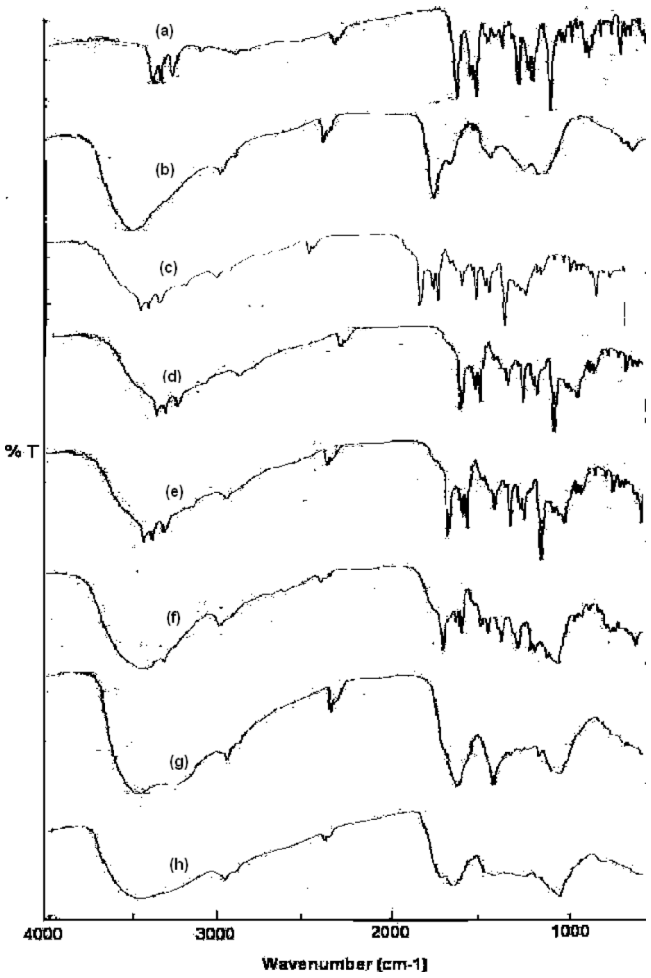

FIGURE 1. FTIR spectra of FSM-FA system prepared by different techniques: (a) FSM alone, (b) FA, (c) 1:1 physical mixture (PM), (d) 1:2 PM, (e) 1:1 solvent evaporation (SE), (f) 1:2 SE, (g) 1:1 freeze dried (FD), and (h) 1:2 FD.

The FTIR spectra of FSM-FA complex prepared by the freeze drying method in the molar ratio 1:1 showed less FSM peaks, indicating weak interaction of FSM and FA. The FTIR of FSM-FA complex prepared by the freeze drying method in the molar ratio 1:2 showed the complete absence of FSM peaks. This suggests that complex formation has taken place.

DSC

FSM exhibits (Figure 2) a characteristic, sharp exothermic peak at 219°C indicating decomposition of the drug. Two endothermic peaks could be observed near 208°C and 214°C, which are due to the degradation of the FSM. FA exhibit an endotherm heat flow near 250°C. An exothermic event could be observed at a temperature above 260°C, which could be attributed to thermal degradation (Pietro & Paola, 2004). The DSC curve of the physical mixture in the molar ratio1:1

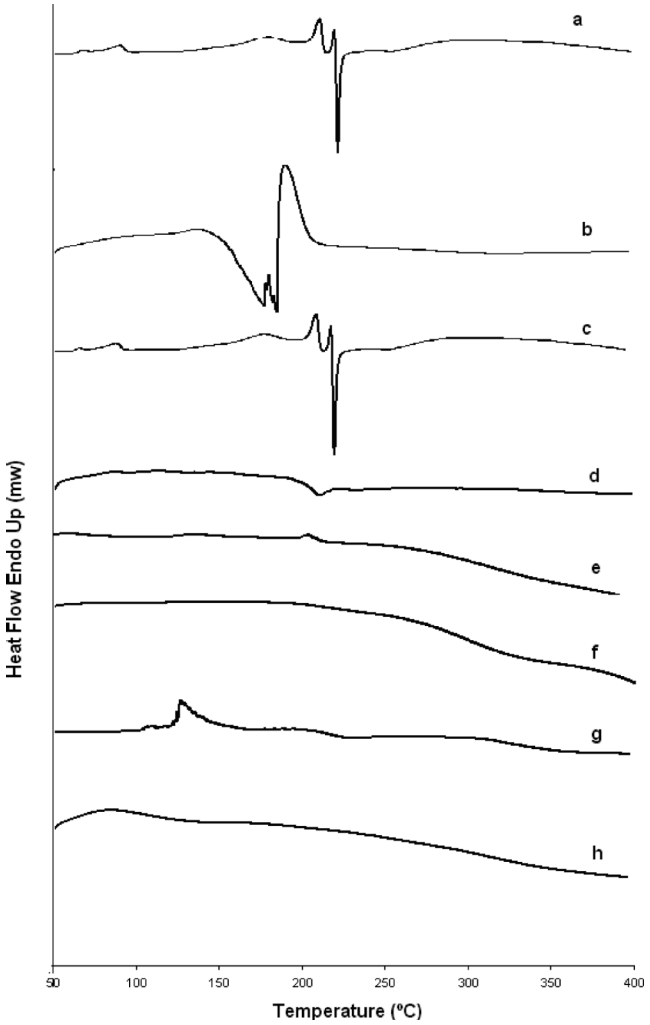

FIGURE 2. DSC thermogram of FSM-FA complexes prepared by different techniques: (a) FSM alone, (b) FA, (c) 1:1 PM, (d) 1:2 PM, (e) 1:1 SE, (f) 1:2 SE, (g) 1:1 FD, and (h) 1:2 FD.

showed an exotherm at 219°C, but the peak intensity of FSM is reduced, indicating no complex formation. The complex containing molar ratio 1:2 shows an exotherm of low intensity of FSM, indicating weak interaction between the drug and FA. The DSC of complexes, prepared by solvent evaporation and the freeze drying technique in the molar ratios 1:1 and 1:2, show complete absence of exotherm and endotherm peaks. The disappearance or shifting of endo- and exothermic peaks of the drug is a strong indication of the formation of a complex with FA.

X-Ray Powder Diffractograms

X-ray powder diffractograms of FSM, FA, and their physical mixtures, as well as their complexes, prepared by the solvent evaporation and freeze drying methods, are shown in Figure 3. The X-ray powder diffraction patterns of FSM showed various intense peaks, revealing crystallinity. Amorphous patterns, however, were observed for FA. The diffractograms of the physical mixture (1:2) was mostly dominated by the amorphous charcter of FA, while some of the FSM crystalline characteristics are noticeable. The XRD pattern of the FSM-FA complex in the molar ratio 1:2 prepared by the freeze drying technique showed complete absence of FSM peaks. This suggests that complex formation has taken place.

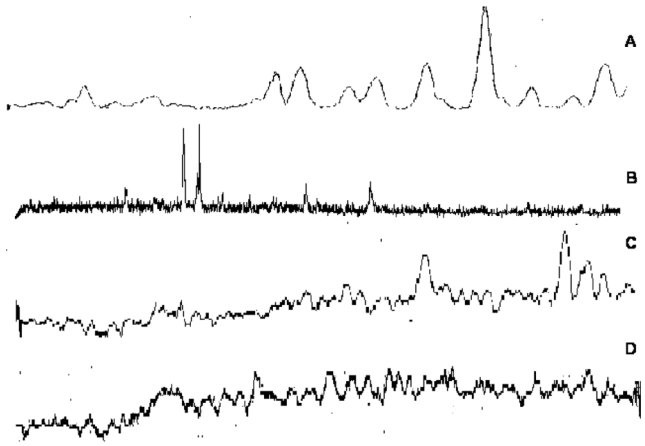

FIGURE 3. X-ray diffraction patterns of of FSM-FA complexes prepared by different methods: (A) FSM alone, (B) FA, (C) 1:2 SE, and (D) 1:2 FD.

SEM

Although SEM is not conclusive for assessing the existence of a true inclusion compound in the solid state, it can be of some utility to prove the homogeneity of solid phases. SEM (Figure 4) of FSM showed crystalline particles of regular size, indicating a crystalline nature. FA appears as fibrous material. The FSM-FA solvent evaporated (1:2) complex showed a partial crystalline nature, but the drug is not easily detectable. It appears that complex formation between FSM and FA has not taken place. The freeze dried FSM-FA (1:2) complex appears as a homogeneous amorphous mass, demonstrating a complete complex formation.

Aqueous Solubility Determination of Solid Complexes

FSM is reported to be practically insoluble in water; its saturation solubility in distilled water at room temperature was found to be 32.25 μg/ml. Complex formation of FSM with FA greatly enhanced its aqueous solubility (Table 1). Maximum aqueous solubility was enhanced 23-fold in the case of the 1:2 freeze-dried complex.

Release of FSM from Complexes

The release profile of FSM-FA systems prepared by grinding, solvent evaporation, and freeze drying method are

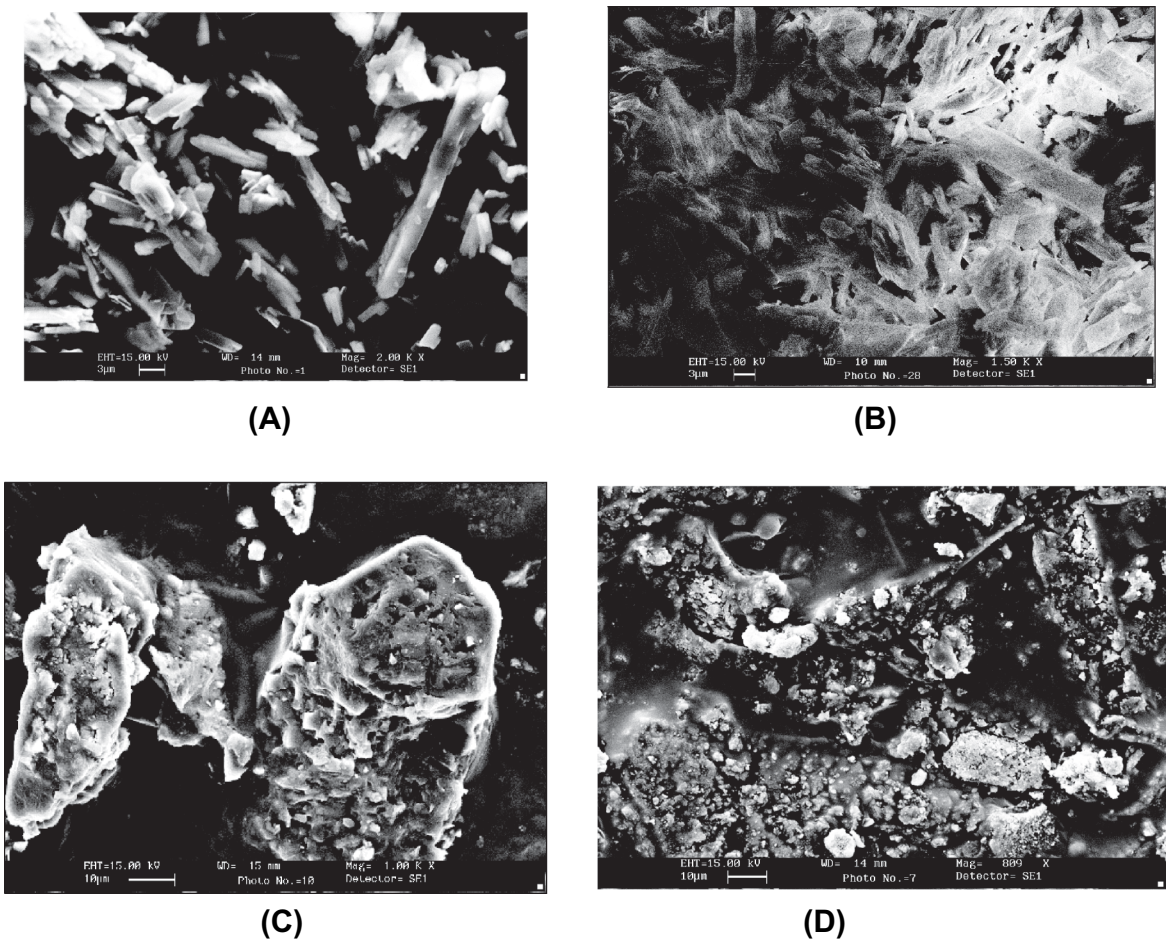

FIGURE 4. SEM of FSM-FA complexes: (A) FSM, (B) FA, (C) 1:2 SE, and (D) 1:2 FD.

TABLE 1
Aqueous Solubility Determination of FSM-FA Complexes

S. No.	Complexes	Solubility of FSM(μg/ml)	Increase in Solubility from Drug Alone (%)	Increase in Solubility (Remarks)
1	PM 1:1	157.5	488.5	≈ 5 times
2	PM 1:2	258.5	801.6	≈ 8 times
3	1:1 SE	286.8	889.3	≈ 9 times
4	1:2 SE	293.0	908.5	≈ 9 times
5	1:1 FD	525.3	1628.9	≈ 16 times
6	1:2 FD	743.3	2304.8	≈ 23 times

PM = physical mixture; SE = solvent evaporation; FD = freeze dried.

shown in Figure 5. Dissolution of FSM alone was found to be very slow, probably due to the insolubility of FSM in water. A release of 86.9% was observed in 1 hour for 1:2 FSM-FA complex prepared by freeze drying as compared to a release of only 3% from drug alone in the same period. The study clearly demonstrates that when FSM is complexed with FA, there is a significant increase in its dissolution rate.

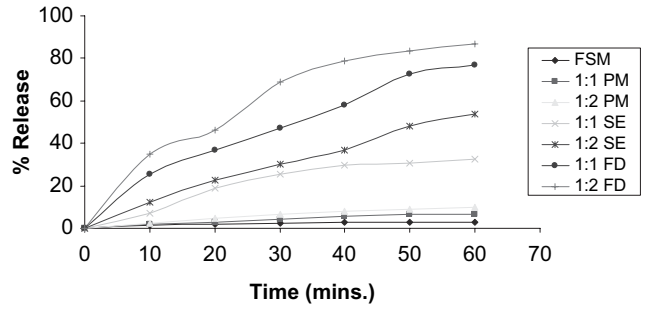

FIGURE 5. Release profile of FSM and FSM-FA complexes in a phosphate buffer pH (5.8).

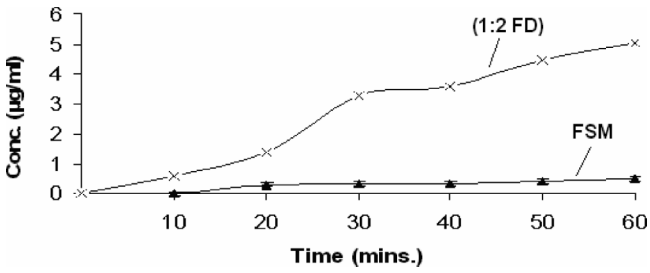

FIGURE 6. Comparative permeation study across rat everted gut sac.

Permeation Study Across Rat Everted Gut Sac

The permeability of optimized complex (1:2 freeze dried FSM-FA) across everted gut sac was significantly increased (at least 10 times) compared with FSM alone in 1 hour (Figure 6).

CONCLUSIONS

FSM is a poorly soluble drug and its oral bioavailability is very poor due to insufficient aqueous solubility at gastrointestinal pH. To enhance its solubility, complexation was tried with FA extracted from shilajit. Complexes were made in the molar ratio (FSM-FA) 1:1 and 1:2 by grinding, solvent evaporation, and freeze drying techniques. The complexes were identified by FTIR, DSC, XRD, and SEM spectral studies. Release and solubility of FSM from 1:2 freeze dried complex showed significant improvement compared with the other complex. The permeability of the optimized complex (1:2 freeze dried FSM-FA) across everted gut sac was significantly increased compared with FSM alone in 1 hour. Thus, significant enhancement of bioavailability parameters such as drug dissolution, solubility, and permeability were observed when FSM was complexed with FA. This has potential for industrial application in developing and improved dosage forms of FSM.

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