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Effect of Fulvic Acid on Oral Delivery of Carbamazepine

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Shilajit has been used in traditional system of medicine since antiquity which is also a rich source of humic substances (Humin, Humic acid and Fulvic acid). These least explored macromolecular humic substances possess several unique properties. Here the Complexation property of Fulvic acid with Carbamazepine is explored for the first time and different *in vitro*, *ex vivo* and pharmacodynamic parameters are evaluated. Complexes were evaluated by Differential scanning calorimetry, Fourier transform infra red spectroscopy, X-ray diffraction, solubility analysis, release profile study, computational method and molecular modeling. Optimized complexes were undergone, permeation study across intestine, anticonvulsant activity (Maximal Electro Shock) and biochemical estimations (TBARS and glutathion). These studies confirmed the entrapment efficiency of the fulvic acid. Increased solubility (2268.75%), better release profile (~81% in 60 min), rat intestinal permeability (>3 times), biochemical and pharmacodynamic studies conferred the best to freeze dried (ratio; 1:2) and kneading (ratio; 1:2) complexes.

Keywords: Epilepsy, Shilajit, Complexation, Solubility, Intestinal Permeability.

RESEARCH ARTICLE

1. INTRODUCTION

Carbamazepine (Fig. 1(A)) is an anticonvulsant drug, widely used in the treatment of simple and complex seizures, trigeminal neuralgia, and bipolar affective disorder.[1] This drug is a first-choice for the treatment of simple and complex partial seizures. It is practically insoluble in water and lies in class II of Biopharmaceutical Classification System.[2] Its absorption is dissolution rate limited.[3] It exists in about four polymorphic forms that contribute to variable absorption. Low aqueous solubility and poor wettability of the drug further contribute to the variability in absorption and hence bioavailability.[4,5] Generally, carbamazepine (CBZ) is available in conventional tablet dosage forms that yield peak plasma concentrations during 4 to 32 h. Improvement of dissolution characteristics could result in increased rate and extent of absorption that will contribute in enhancing the therapeutic efficacy of CBZ. Dissolution characteristics can be improved by complexing with a suitable complexing agent such as Fulvic acid (FA). Thus, the project was designed to investigate the effect of FA on CBZ in enhancing the aqueous solubility, dissolution, bioavailability and permeability across intestine.

FA is one of the humic substances obtained from Shilajit,[6] a brown colored exudates coming out from steep mountain rocks of Himalayan and Hidukush ranges of Indian subcontinent.[14] Favorable altitude for the occurrence of Shilajit is 1000–5000 m. It also occurs in small quantities in different regions like Australia, Norway, China, and Russia etc. Humic substances extracted from Shilajit (Fig. 2) are Humin, Humic acid and FA.[7] Humic substances are the ubiquitous components of natural organic matter, and are macromolecular, negatively charged polyelectrolytes that contain mainly carboxylic and phenolic functional groups but structural, conformational and physico-chemical differences between them are well reported. FA (Fig. 1(B)) is micro porous in structure having hydrophilic exterior and hydrophobic interior[8] having average molecular wt 1200. Thus low water soluble drug molecules can form inclusion complexes with them, resulting in increased solubility and bioavailability.[9] As CBZ is the drug of choice in treatment of pediatric seizures,[10] a re-constituable suspension is highly recommended.

2. MATERIALS AND METHODS

2.1. Materials

An authentic sample of rock shilajit was obtained from Dabur Research Foundation, Ghaziabad, India. Carbamazepine was kindly provided as a gift sample by Novartis Pharmaceuticals Ltd., India. All other chemicals and reagents used in the study were A.R. grade.

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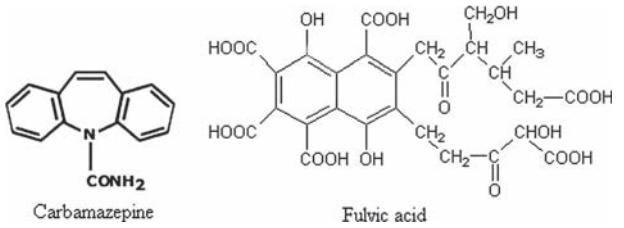

Fig. 1. Chemical structure of (A) Carbamazepine and (B) Fulvic acid.

2.2. Method for Obtaining Fulvic Acid from Rock Shilajit

A slightly modified method[11] was used to extract FA. The method consisted of successive extraction of rock and powdered shilajit with hot organic solvents of increasing polarity to remove the bioactive components. The residue (marc) was dissolved in 0.1 N NaOH[12] 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 out the humic acids. The filtrate was further shaken with macroporous ion exchange resin in order to adsorb the FAs, which were then eluted using 0.1 N aqueous sodium hydroxide solutions.

2.3. Phase Solubility Behavior and Development of Complexes

Phase solubility studies were carried out at room temperature (25 °C) in triplicate according to the method reported.[13] Excess amount of CBZ was added to distilled water containing various concentrations (0.2–2% w/v) of FA in a series of stopper conical flasks and shaken for 48 h on a rotary flask shaker. The suspensions were passed through membrane filter (0.45 μm) and analyzed for CBZ using a UV spectroscopy (Shimadzu, UV 1601) at 285 nm against blanks prepared using same concentration of FA in distilled water.

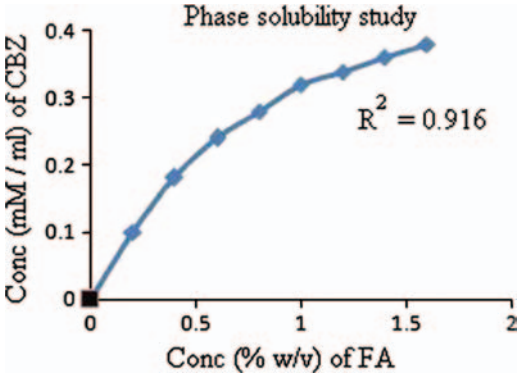

Fig. 2. Phase solubility study of carbamazepine and Fulvic acid.

2.4. Preparation of the Inclusion Complexes

Complexes of CBZ were prepared with FA extracted from shilajit by using different techniques in two different molar ratios 1:1&1:2 (Drug: FA). 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.[14] Equal amount of drug was also under gone to check the process effect.

2.4.1. Physical Mixture (PM)

Complexes of CBZ and FA were prepared by grinding the mixture for 60 min in a clean dry glass pestle and mortar and the resulting mass was passed through a 100-mesh sieve to obtain a uniformly sized fine powder.[14]

2.4.2. Freeze Drying (FD)

Weighed amount of CBZ was dissolved in water by using co-solvency (ethanol) and also aqueous FA solution was prepared. Both the solutions were mixed and stirred (200 rpm, 60 min) and then sonicated for an *h*. The solution was frozen for 24 hrs in a Lyph-lock apparatus and then freezes dried (Dry winner, DW-8-85 Heto Holten, Denmark) for 12 hrs. Sucrose solution was (2% w/v) added as a cryoprotectant. The resulting mass was then powdered in glass mortar and pestle and passed through 100-mesh sieve to obtain a uniform size fine powder.[14]

2.4.3. Solvent Evaporation (SE)

Calculated amount of drug was dissolved in water with the help of few drops of ethanol and poured into aqueous solution of FA. The solution was then sonicated for an hour. The solution thus obtained was dried in a rotary evaporator under vacuum (Hahn shin science Co Hs-2001N, South Korea) and passed through 100-mesh sieve to obtain a uniform size fine powder.[14]

2.4.4. Kneading (KD)

Solid complexes of CBZ-FA were prepared in 1:1 and 1:2 molar ratios by following kneading method.[13] Weighed amount of CBZ and FA were triturated for 15 min in a clean dry glass pestle and mortar. During the process, the water content of the paste was empirically adjusted by ethanol and triturated to maintain the consistency of the paste. Trituration was continued until the product started drying on the walls of mortar. The products were further dried in the hot air oven at 60 °C for 30 min, powdered, passed through 100-mesh sieve and stored in desiccators.[14]

2.5. Characterization of the Solid Complexes

2.5.1. Differential Scanning Calorimetry (DSC)

For Differential scanning calorimetry (DSC) study samples of the solid complex, pure drug, and FA (10 mg) were taken in a flat-bottomed aluminum pans and heated over a temperature range of 50–350 °C at a constant rate of 10 °C/min with purging of nitrogen (50 ml/min) using alumina as a reference standard in a differential scanning calorimeter (DSC-7, Perkin Elmer Pyris 6 instrument, USA).

2.5.2. Fourier Transforms Infra-Red Spectroscopy (FT-IR)

The FT-IR spectra of CBZ, FA and inclusion complexes were recorded on the Perkin Elmer using the potassium bromide (KBr) disc technique. Five mg of previously dried sample was mixed with 100 mg KBr and compressed into a pellet on an IR hydraulic press. Base line was corrected and scanning was done from 4000–400 cm^{-1}.

2.5.3. Powder X-Ray Diffraction (XRD)

X-ray diffraction of CBZ, FA their inclusion complexes were studied by using X-Ray diffractometer (PW 1830, Phillips, Japan). The samples (1000 mg) were rotated during data collection to reduce orientation effects. PXRD pattern of solid complex, pure drug, and FA were recorded between $2\theta = 10$ to 70° at 35 kV and 30 mA.

2.6. Conformational Analysis by Computational Method

The 3D-molecular structures were generated and optimized with Chem 3D-Ultra 8.0 software. While all calculations used are for geometric optimization. All the energy minimizations were carried out till the RMS gradient is less than 0.08. Optimized molecular structures and partial atomic charges were used for the molecular modeling FA and its complex. H-bonding analysis was based on ORTEP III (v1.0.3).

2.7. HPLC Analysis of Carbamazepine *In Vitro*

The concentration of CBZ in *in-vitro* samples was determined by reproduced HPLC method[15] using a Shimadzu LC2010 system (Kyoto, Japan) consisting of quaternary LC-10A VP pump, SPD-10AVP column oven, variable wavelength programmable UV/VIS detector (285 nm), SCL 10AVP system controller, Rheodyne injector fitted with a 20 μL loop, degasser and a data processor. Chromatographic separation was achieved using a LiChrospher® 100 reversed-phase C-18 column (250 × 4.6 mm) which was packed with 5 μm particles with a mobile phase consisting of water and acetonitrile (60:40::water:acetonitrile). The mobile phase was pumped at a flow rate of 1.0 ml/min at an ambient temperature (25 ± 2 °C). The eluent was monitored by ultraviolet absorbance at a wavelength of 285 nm with retention times for CBZ was 4.8 ± 0.35 min.

2.8. Aqueous Solubility Determination of Solid Complexes

Excess amount of complex was kept in amber colored bottles containing 10 ml of distilled water and stirred on thermo stated mechanical shaker (Grower enterprises, New Delhi, India) at 25 °C for 5 days. Suspensions were filtered through 0.22 μ "*Millipore*" filter, adequately diluted with distilled water and analyzed by reported HPLC at λ 285 nm.

2.9. Release of Carbamazepine from Complex

Drug release study of API (50 mg CBZ solution) and inclusion complexes (equivalent to 50 mg CBZ) was performed using USP II dissolution apparatus (Hanson Research SRS, USA) in 900 mL of distilled water at 37.5 ± 0.5 °C (75 rpm, 60 min). The study was carried out by putting the constituted suspension (5 ml) in dialysis bag (Spectra-Por dialysis bag, Sigma Aldrich, St. Louis, MO with cutoff 12 000–14 000 Da). The concentration of the drug in solution at various time intervals was analyzed by HPLC at 285 nm. All dissolution studies were carried out in triplicate.

2.10. *In Vitro* Everted Intestinal Sac Permeation Study

Rats were anesthetized by ether sprinkled to a piece of cotton wool in a glass container equipped with a lid. After making a midline incision in the abdomen, the small intestine was cut at two positions, at about 18 cm distal to the stomach[16] and at about 30 cm (being the medial jejunum). This segment was then removed and ligated with silk thread to one end of a glass rod and carefully everted on the rod, rinsed with saline solution and then cut and secured to the tip of a 1 ml disposable syringe barrel. The gut sac was filled with the modified KRPB buffer solution and was then placed inside the bath containing 100 ml of test solution continuously bubbled (95% O_2 and 5% CO_2).[17] After stabilization 3 ml (equivalent to about 10 drug) CBZ (API), 1:2 freeze dried and 1:2 kneading complex solution were added into the sac. The tubes were maintained at 37 °C and shaken continuously at 60 rpm with bubbling oxygen supply. 100 μl Samples were withdrawn at an interval of 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24 hrs from the dissolution medium and centrifuged at 4000 rpm for 5 min. After filtering through Millipore filter (0.45 μ) these were analyzed by HPLC.[15]

2.11. MES (Maximal Electro Shock Seizure) Induced Convulsion

Swiss albino mice with average body weight (20–30 g) of either sex were used for the experiment. Animals were reared in the Central Animal House for 2 weeks in polypropylene cages and fed on standard animal feed and water. The animals were divided into six groups i.e., control, pure CBZ, 1:2 freeze dried, 1:1 freeze dried, 1:2 kneading, 1:1 kneading with 6 animals each with an average group weight of 25 g in the maximum electroshock seizure (MES) experiment. Pure CBZ (30 mg/kg body weight of mice) gives 100% protection to animal in MES study[18] and amount of FA-CBZ complexes were taken as per the calculation that contains the said (Table II) amount of CBZ. The control and different dosage of complexes 30 min before the induction of MES were given to separate group of mice. Then, the stimulus train was applied via ear-clip electrode (50 mA, 0.2 sec, average voltage 200–250 V) through Electroconvulsiometer (Techno India). The incidence and duration of extensor tonus were noted. The duration of seizures (tonic-clonic convulsions) was recorded.[19] Solutions (q.s to 5 ml) of drug and complexes were prepared in glycerin. A 0.2 ml of these solutions was given per oral to the mice. All animal experiments were carried out in accordance with Jamia Hamdard animal ethical committee.

2.12. Picrotoxin Induced Oxidative Stress

2.12.1. Thiobarbituric Acid Reactive Substances (TBARs)

1 ml of suspension medium was taken from the 10% tissue homogenate. 0.5 ml of 30% TCA was added to it, followed by 0.5 ml of 0.8% TBA reagent. The tubes were then be covered with aluminium foil and kept in shaking water bath for 30 minutes at 80 °C. After 30 minutes tubes will be taken out and kept in ice-cold water for 30 minutes. Then these were centrifuged at 3000 rpm for 15 minutes.

The absorbances of the supernatant were read at 540 nm at room temperature against appropriate blank. Blank consist of 1 ml distilled water, 0.5 ml of 30% TCA and 0.5 ml of 0.8% TBA. The concentration of MDA will be read from standard curve prepared by using TEP.[20]

2.12.2. Tissue Glutathione (GSH)

A 10% tissue homogenate of brain was prepared in 0.02 M EDTA and 4 ml of cold distilled water was added to it. It was mixed well with intermittent shaking for 10 minutes using Vortex mixer and the contents will then be transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6000 rpm for 15 minutes. After that, 2 ml of supernatant was mixed well with 4 ml of tris buffer (0.4 M, pH 8.9) and 0.1 ml of 0.01 M DTNB was added to it. The absorbance was read within 5 minutes of the addition of DTNB at 410 nm against a reagent blank with no homogenate.[21]

3. RESULTS

3.1. Characterization of Solid Complexes

The Phase solubility studies revealed a non linear relationship between the aqueous drug solubility with increase in FA concentration ($R^2 = 0.916$). The phase solubility diagram showed its characteristics AL type (Fig. 2), according to Higuchi and Connors. Up to concentration of 1% w/v of FA the relationship was linear but nonlinear afterwards. Thus, molar ratio we opted for complexation were 1:1 (steep rising portion) and 1:2 (since it was more inclined towards X-axis).

3.1.1. Differential Scanning Calorimetry

DSC of pure CBZ shows a sharp exothermic peak at 189 °C, which is in accordance with the melting point reported in literature.[22] FA shows blunt endotherm and exotherm in the region of 100–200 °C which is due to thermal degradation.[23] Complete or nearly complete linear thermo grams of complexes were observed in all the results (Fig. 3).

3.1.2. Fourier Transforms Infra-Red Spectroscopy

The FT-IR spectrum (Fig. 4(a)) shows characteristics peaks of CBZ peaks at 1752 cm^{-1} (C=O stretching), 3460 cm^{-1} (N–H vibration) and 1550 cm^{-1} (C=C stretching of phenyl).[19] FT-IR absorption bands (Fig. 4(a)) of

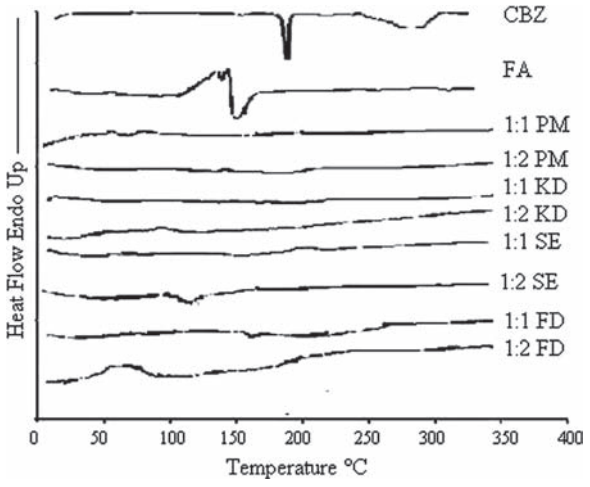

Fig. 3. DSC thermogram of carbamazepine-fulvic acid (CBZ-FA) complexes prepared by different techniques: (a) CBZ alone, (b) FA, (c) 1:1 PM, (d) 1:2 PM, (e) 1:1 KD, (f) 1:2 KD, (g) 1:1 SE, (h) 1:2 SE, (i) 1:1 FD and (j) 1:2 FD.

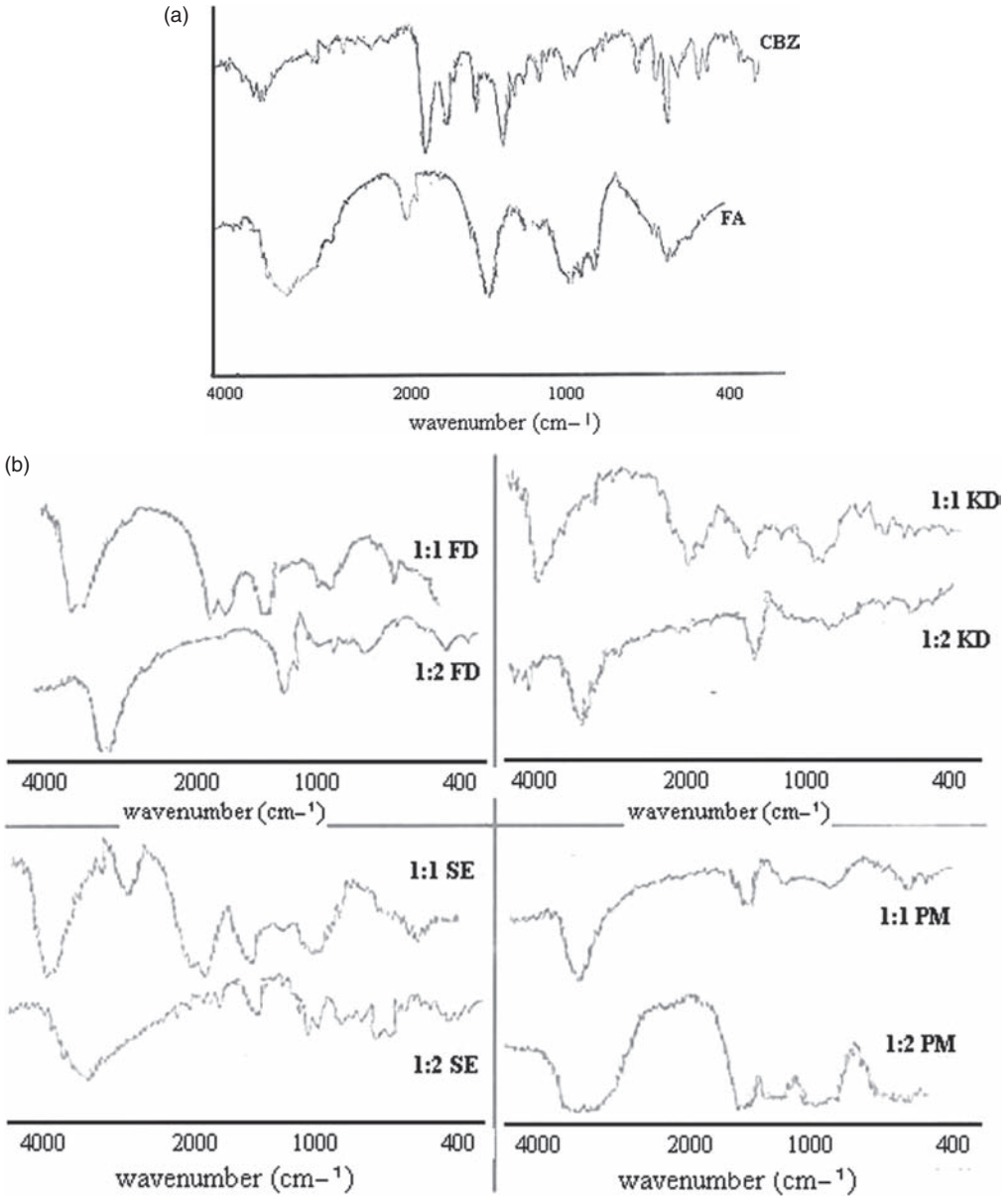

Fig. 4. (a) FT-IR spectra of CBZ and FA. (b) FT-IR spectra of CBZ-FA complexes prepared by different techniques.

FA extracted from shilajit were found in accordance with those reported in literature.[24] All the FT-IR spectra of complexes are exhibited in Figure 4(b). The result obtained further corroborates the inferences of DSC.

3.1.3. X-Ray Power Diffractograms

XRD of CBZ shows various peaks at different angles with most intense peaks at 15.41° (100%) followed by 13.18° (83%), 27.84° (66%), 27.32° (60%), 27.66° (56%) respectively (Fig. 5), revealing the crystalline nature of CBZ. The X-ray diffraction patterns of FA show almost amorphous nature but some diminished peaks at angles 39° and 47°. All the complexes were showing almost amorphous nature.

3.2. Conformational Analysis by Computational Method

Molecular modeling has shown that complexes of CBZ-FA are stable. It revealed that FA has ability for inclusion complexation with CBZ. Intermolecular hydrogen bonds observed contributes to the stability of the molecule. In the case of CBZ as shown in Figure 6 amide hydrogen is oriented away from the carbonyl group but is approaching towards one of the aromatic moiety. Figure 7 shows the energy minimized structure of FA. 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 supposed to increase the stability of the molecule. While a

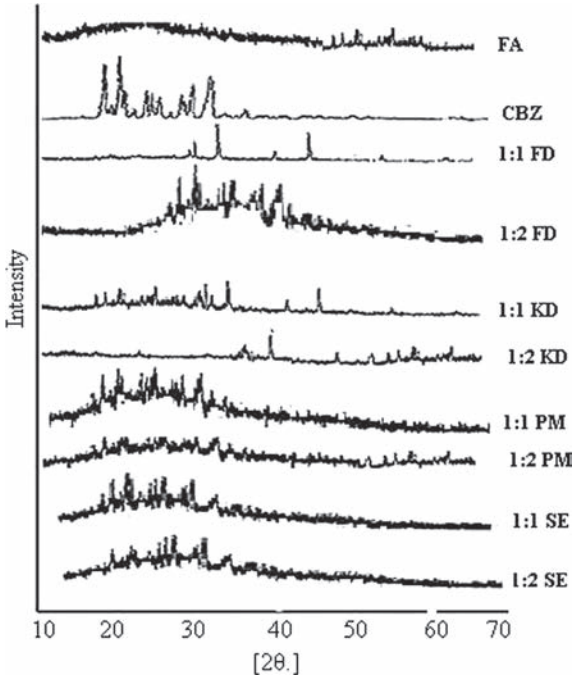

Fig. 5. X-ray diffraction pattern of complexes, fulvic acid and carbamazepine.

drug complex optimization with FA shows that the CBZ is stabilized by a strong NH–N interaction with FA (Fig. 8). Total potential energy of the FA using Chem 3D-Ultra 8.0 software comes to around −38.8716 Kcal/mol while a complex of CBZ and the FA is stabilized at −22.584, which is more stable as CBZ alone. Energy optimization of CBZ resulted in −6.84 Kcal/mol.

3.3. Aqueous Solubility of Complexes

CBZ is practically insoluble in water, aqueous saturation solubility of CBZ was found to be 12.65 μg/ml. Complexation of CBZ with FA greatly increased the solubility (Table I). Maximum percentage increase in solubility was 2268.75 ± 29.73 in freeze dried (1:2) complex while minimum was 648.30 ± 16.19 in 1:1 PM.

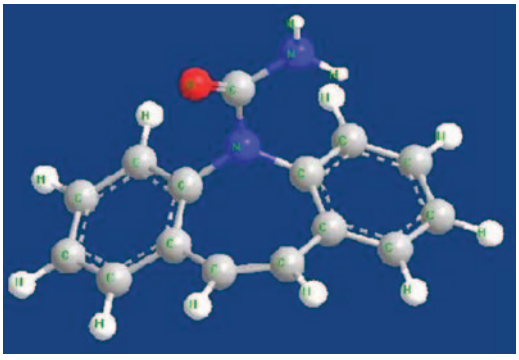

Fig. 6. Energy minimized structure of Carbamazepine.

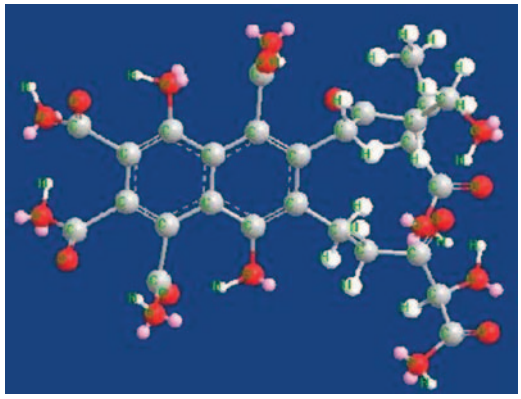

Fig. 7. Energy optimized structure of fulvic acid.

3.4. Release of Carbamazepine from Complex

The release profile of pure CBZ and complexes prepared by different methods are shown in (Fig. 9). The best release profiles were exhibited by complexes (1:2) developed by Freeze drying and kneading (∼81% in 60 min) where as minimum release in case of 1:1 PM complex.

3.5. Permeation Study Across Rat Gut Sac

The permeability of optimized complexes (1:2 freeze dried and Kneading complexes) across gut sac were significantly increased (∼2.9 to 3.7 times) as compared to CBZ suspension in water in 24 h (Fig. 10). The permeation profile of complex shows two patterns i.e., in initial 5 hrs there was a sharp increase in permeation but after that a plateau was observed.

3.6. Comparison of Anticonvulsant Activity

From all the previous mentioned studies it was very much obvious that the complexes developed by kneading and freeze-drying methods were showing promising results. So, these were chosen for further pharmacodyanamic

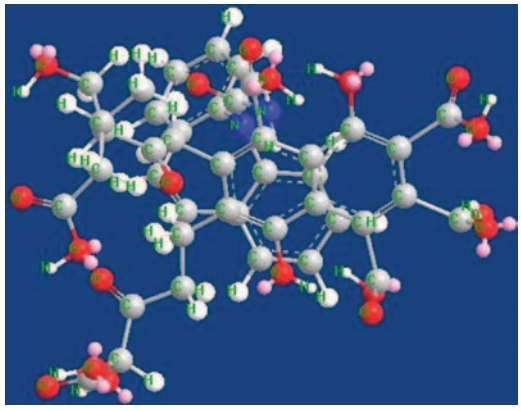

Fig. 8. Carbamazepine complexed with fulvic acid.

Table I. Solubility of carbamazepine (CBZ) and CBZ—FA complexes in water at room temperature.

Complexes	Solubility of Carbamazepine (μg/ml)	% increase in solubility from drug alone
1:1 PM	94.66 ± 9.63	648.30 ± 16.19
1:2 PM	102.47 ± 10.21	710.03 ± 2.17
1:1 SE	119.32 ± 4.19	843.24 ± 15.38
1:2 SE	137.36 ± 19.93	985.84 ± 19.73
1:1 KD	156.65 ± 5.01	1138.34 ± 21.13
1:2 KD	187.33 ± 13.23	1380.67 ± 21.19
1:1 FD	198.15 ± 29.73	1466.43 ± 14.2
1:2 FD	299.65 ± 1.35	2268.75 ± 29.73

% increase in solubility of complex (S_{comp}) = Solubility of $CBZ^{complex}$ × 100/Solubility of CBZ^{API}. # Physical Mixture (PM), Kneading (KD), Solvent evaporated (SE) and Freeze dried (FD).

study. The amount of CBZ was chosen as per the recommended dose of the drug.[25] The amount of humic substances present in a dose of suspension was also within the permissible limit i.e., 512 mg kg^{-1} body weight. Study of MES activity showed that due to complexation there was threefold increase (CBZ:FA::1:2) and 1.5 fold (CBZ:FA::1:1) in potency of CBZ in freeze-dried and kneading complexes respectively when compared to pure drug (Table II).

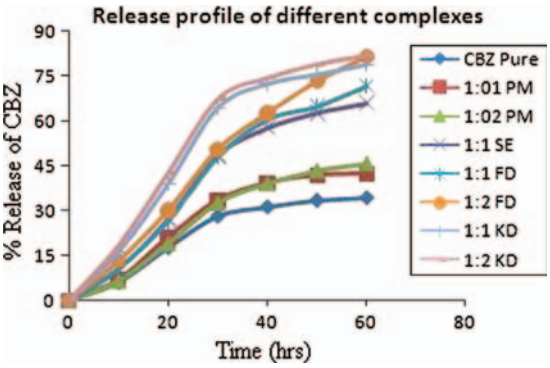

Fig. 9. Release profile of carbamazepine (CBZ) and CBZ–FA complexes in distilled water.

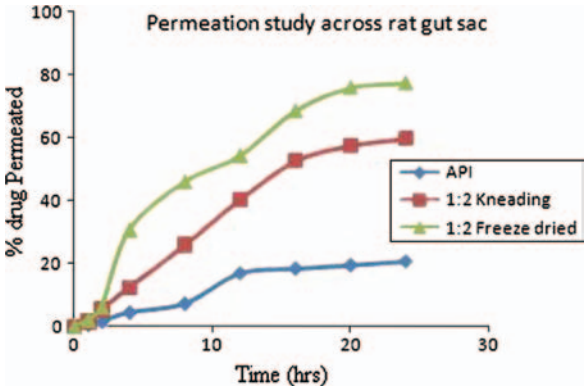

Fig. 10. Permeation of carbamazepine (CBZ) and CBZ–FA complexes across everted rat gut sac.

Table II. Comparative anticonvulsant activity of carbamazepine and CBZ–FA complexes in swiss albino mice.

Substance	Dose	% inhibition
CBZ	30 mg/kg body wt	100
FA	94 mg/kg body wt	0
KD (1:1)	Eq. to 10 mg/kg body wt of CBZ	75
KD (1:2)	Eq. to 20 mg/kg body wt of CBZ	100
FD (1:1)	Eq. to 10 mg/kg body wt of CBZ	75
FD (1:2)	Eq. to 20 mg/kg body wt of CBZ	100

% inhibition = Animals showing positive response × 100/Total animals.

Table III. Picrotoxin induced oxidative stress studies.

Group treatment	TBARS(μmol MDA/mg protein)	GSH(μmoles/mg protein)
1. Normal Saline (NS, 10 ml/kg, i.p.)	1.13 ± 0.064^a	97.29 ± 1.99^a
2. PTX (3.5 mg/kg, s.c)	4.49 ± 0.14^b	29.24 ± 1.12^b
3. CBZ(30 mg/kg, p.o)	2.1 ± 0.06^a	90.96 ± 1.59
5. CBZ-FA (1:2)-KD (30 mg/kg, i.p.)	1.36 ± 0.03^a	99.79 ± 1.74^a
7. CBZ-FA (1:2)-FD (30 mg/kg, i.p.)	1.62 ± 0.04^b	94.96 ± 1.34^a
8. FA *per se* (335 mg/kg body wt.)	1.15 ± 0.07	99.79 ± 1.30

3.7. Picrotoxin Induced Oxidative Stress

3.7.1. Thiobarbituric Acid Reactive Substances (TBARs)

TBARS levels were significantly elevated in picrotoxin (PTX) treated group (1.13 ± 0.064 vs. 4.49 ± 0.14) ($p < 0.01$). TBARS levels were also significantly elevated in CBZ treated group. TBARS level were significantly decreased to the normal in both the groups treated with the carbamazepine complexes (group 5, 7). {F (8, 45) = 245.21} (Table III).

3.7.2. Tissue Glutathione (GSH)

GSH PTX treatment resulted in significant decrease in glutathione level when compared with the saline control group and CBZ treated group (97.29 ± 1.99 vs. 29.24 ± 1.12) ($p < 0.01$). GSH level were significantly normalized with the treatment with CBZ complexes (group 5, 7) (Table III).

4. DISCUSSIONS

4.1. Characterization of Complexes

Molar ratio opted from phase solubility studies were 1:1 and 1:2 (CBZ:FA). Another finding we could conclude from the data is that at higher concentrations of FA (1–2% w/v), solubility of CBZ exhibits much variable solubility as the deviations are much noticeable. Existence of some other mechanism other than inclusion is also evident from the data obtained. High molecular weight and basic

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hydrophobicity of humic substances favour formation of "micelle"-like structures[26, 27] with hydrophilic groups on the water side and the hydrophobic nucleus inside the core. That makes it possible to entrap organic moieties inside.[28] There were nearly negligible effects of different complexation process parameters on pure drug.

4.2. Differential Scanning Calorimetry

Absence of any sharp peak in DSC thermo gram of FA may indicate either the existence of amorphous structure (or presence of impurity). Nearly all complexes were showing phenomena of complexation. This disappearance or shifting of peaks of drug is a strong indication of the formation of complex with FA. Also with rigorous trituration, interaction with different functional groups to develop complexes has been established.

4.3. Fourier Transforms Infra-Red Spectroscopy

In Physical Mixture complex there is almost complete absence of peaks in fingerprint region (Fig. 4(b)). There is interaction of carbonyl peak of CBZ with the carboxylic group of FA, stretching vibration of N–H (3460 cm^{-1}) is interacting with O–H vibration of FA indicating weak interaction. In 1:1 PM, peaks in the fingerprint regions are present but not very much prominent. Even peaks due to C=C are present, although blunt. All these interaction leads to a lesser degree of complex interaction. Factor that strengthens the idea of complexation in this complex is blunt and broad interaction of N–H vibration (3460 cm^{-1}) with O–H stretching and C–H stretching of FA. Spectrum of 1:1 KD complex shows blunt and diminished peaks in fingerprint region. Olefinic and carbonyl peaks of the drug are wide spread and dispersed indicating weak interaction with similar bands in the complexing agent. In kneading complexes extent of complexation is more than physical mixture and solvent evaporated. Peaks of the fingerprint regions (1300–400 cm^{-1}) are more diminished, indicating greater interaction between the drug and complexing agent. Some characteristic peaks of the drug like C=O at 1752 cm^{-1} and N–H at 3460 cm^{-1} are absent in 1:2 ratio but present in diminished amount in 1:1 ratio. Peaks of the freeze dried kneading complexes appear the best developed complexes 1:2 complex is the best between the two ratios.

4.4. X-Ray Power Diffractograms

XRD analysis confirms the inference obtained by DSC analysis and augurs that the FA is amorphous in nature rather having substantial impurity. Complex developed by Kneading and lyophilized showed complete absence of peak at 15.41°, 13.18° which were characteristics of CBZ. These results showed the formation of complex. Solvent evaporation and physical mixture method developed complexes showed few characteristic peaks of drug but in much reduced form, indicating formation of complex.

4.5. Aqueous Solubility of Complexes

The variation in solubility profile may be due to variable entrapment inside the host molecule that could also be seen in different characterization spectra. Results also depict better performance of 1:2 ratios in every method compared to 1:1. The reason behind it indicates the existence of some other mechanism despite complexation, like micelles formation. Because in a similar study[29] CMC of humic substance was found to be forming micelles at a concentration of 2 gm/Lit. This work also reports the amount of drug solubilized by per gram of humic substances which is in accordance with our findings. Further, Humic substances offer both type of interaction, like metal ion interaction due to presence to various functional group and inclusion of hydrophobic moieties.[30, 31]

4.6. Release of Carbamazepine from Complex

Any drug has an intrinsic dissolution rate that is dependent on its solubility and particle size[32] which was 34% in 60 min here and attaining plateau then after. Physical mixture (1:1) complex shows the least release but more than pure drug. In every method opted for complexation, 1:2 ratio exhibited comparatively better release profile than 1:1. The result corroborates the data obtained from solubility analysis and different instrumental analysis (Figs. 3, 4 and 5). We could conclude that better complexing interaction results into more sustained release profile.

4.7. Comparison of Anticonvulsant Activity

Better performance of 1:2 complexes could be correlated with higher proportion of FA. Thus, existence of some solubulizing mechanism other than inclusion complex cannot be ignored. This may be micelle formation, as humic substances are well known for aggregation properties.[33] FA was also given to mice to check the antiepileptic activity (amount equivalent to FA used in 1:2 ratios of complexes) which showed zero percent inhibition (Table II). Our optimized complexes were exhibiting better performance in crossing blood brain barrier (BBB) barrier which may be attributed to increased solubility and passive diffusion gradient but formation of aggregates in humic material is well known.

4.8. Permeation Study Across Rat Gut Sac

Reason for plateau could be understood by physical mechanisms. Here two opposing forces (Concentration gradient and aggregation of humic substances) act against each other. The one which predominates influences the result.

Initially, permeation increased steeply because there was an increasing concentration gradient across the sac but after some time (5 h) it attains plateau. Reason for plateau may be the aggregation of complexed and free FA.

4.9. Picrotoxin Induced Oxidative Stress

4.9.1. Thiobarbituric Acid Reactive Substances (TBARs)

Picrotoxin treatment significantly enhances the TBARS level compared with the saline control group. This finding is in agreement with earlier findings which point to the development of oxidative stress in epilepsy. Enhanced lipid peroxidation as a result of oxidative stress produces convulsion which leads to inactivation of glutathione synthase and thus permitting abnormal build up of excitatory neurotransmitter glutamic acid. CBZ treated group significantly decreased TBARS level compared to the pictotoxin (PTX) group. This decrease in the TBARS levels was further augmented upon complexation of CBZ with the complexing agent. Augmented reduction in TBARS level could be attributed to the enhanced bioavailability of CBZ. This is in consistent with the previous findings which reported enhanced bioavailibity upon complexation with humic substances.[14]

4.9.2. Tissue Glutathione (GSH)

Ptx treatment leads to significant lipid peroxidation as evident from enhanced TBAR level and this leads further leads to generation of free radicals which are quenched by the glutathione. This was observed in picrotoxin treated group which significantly reduces glutathion level compared to the normal. While CBZ treatment significantly enhanced glutathione compared to the picrotoxin treated group. Complexation of CBZ with FA further enhanced the glutathione level.

This could be attributed to the enhanced bioavailability of CBZ as a result of complexation. Further there are reports[34] of free radicals scavenging activity of HA and FA and thus the enhanced antioxidant activity of CBZ complex can also be explained by synergistic activity of FA.

5. CONCLUSION

CBZ has a poor bioavailability because of its poor water solubility. Thus, to increase the solubility of the drug, it was complexed with FA in the molar ratio 1:1 and 1:2 by different methods like physical mixture, kneading, solvent evaporation and freeze drying. Complexes were characterized by several techniques like XRD, DSC, and FT-IR. These analyses confirmed the formation of complexes and indicated kneading and freeze drying as the optimized methods. Release profile of 1:2 freeze drying complex was the best. *Ex vivo* permeation study of Kneading and freeze drying betoken a good pharmacodyanamic response. Further MES evaluation and biochemical estimation on mice favors our previous findings. So, Complexation of CBZ with FA could be opted as a promising tool for oral drug delivery and need to be evaluated clinically.

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