JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Fabrication of β-cyclodextrin-mediated single bimolecular inclusion complex: characterization, molecular docking, *in-vitro* release and bioavailability studies for gefitinib and simvastatin conjugate

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Keywords
anticancer drug delivery; bimolecular inclusion complex; bioavailability; gefitinib; simvastatin; solubility

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Received January 8, 2017
Accepted May 7, 2017

doi: 10.1111/jphp.12769

Abstract

Objectives Introduction of multiple molecules in a single inclusion complex, albeit cheaper, lacks conclusive attempts in earlier drug delivery reports. This manuscript emphasizes simultaneous incorporation of two anticancer drugs, gefitinib (G) and simvastatin (S), in a single molecule of β-cyclodextrin for the first time to achieve effective drug delivery.

Methods The inclusion complex (GSBCD) was prepared by cosolvent evaporation technique using β-cyclodextrin (BCD) as carrier. Characterization of GSBDC was performed by Fourier transform infrared spectroscopy, COSY, differential scanning calorimetry, X-ray diffraction and dynamic light scattering analyses, which were ascribed to the complex formation inside BCD cavity, micronization of drugs and conversion to amorphous state.

Key findings The complex revealed entrapment of G and S in 3 ± 0.48: 2 ± 0.19 molar ratio and showed more than 3.5 and 10 fold increase in drug release in *in vitro* and *in vivo*, respectively. Docking and COSY studies revealed molecular alignment into BCD central cavity that been achieved *via* hydrogen bonding between certain groups of the ligands (G and S) and the polar heads of BCD. Partial incorporation of the molecular backbone inside inclusion complex suggests superficial contact with the solvent indicating slow steady release kinetics.

Conclusions This approach of forming inclusion complex with multiple molecules within a single cavity can be a landmark for further studies in drug delivery.

Introduction

The introduction of multiple molecules in a single inclusion complex can bring about synergistic effects for drug delivery. For anticancer treatment, the simultaneous incorporation of gefitinib (G) and simvastatin (S) in a single inclusion complex can be an effective method of drug delivery. Gefitinib (Figure 1a, molecular mass 446.9 g/mol) is an anticancer drug that acts by interrupting the signalling through the epidermal growth factor receptor (EGFR) in target cells by inhibiting the tyrosine kinase enzyme.[1] Simvastatin (Figure 1b, molecular mass 418.6 g/mol) is an antihyperlipidemic drug that acts by blocking the conversion of 3-Hydroxy-3-MethylGlutaryl CoA (HMG CoA) into mevalonate by inhibiting the enzyme HMG CoA reductase. Mevalonate metabolites play significant roles in the function of the EGFR; therefore, mevalonate pathway inhibitors may potentiate EGFR-targeted therapies.[1] Targeting HMG-CoA reductase using simvastatin induces a potent apoptosis in a variety of tumours.[2,3] Therefore, it gives a synergistic action when it is used along with gefitinib.[4] A randomized Phase III study showed that the

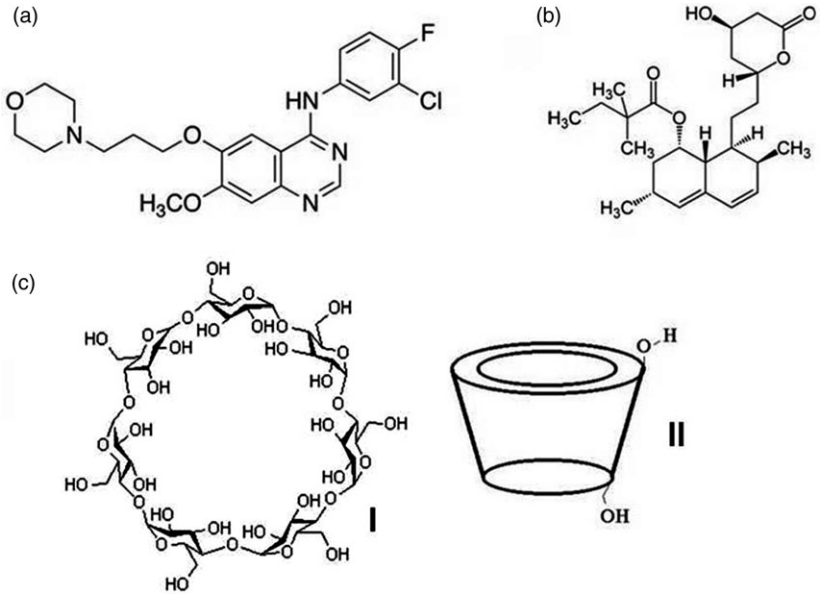

Figure 1 Structure of the compounds under study (a) gefitinib (b) simvastatin (c) β-cyclodextrin; I- a cyclic oligosaccharide with 7 glucopyranose rings; II- β-cyclodextrin as a small cylinder with a hydrophobic central cavity.

response rate was higher in case of combination compared to gefitinib alone in patients with wild-type EGFR nonadenocarcinomas.[4]

Enhanced solubility is important to achieve desired concentration of drug in systemic circulation for pharmacological response of the drug. Nearly 40% of all new pharmacologically potent molecules show poor aqueous solubility, leading to their low bioavailability.[5] The bioavailability of simvastatin after oral administration is 5% and for gefitinib is 40%.[6,7] A number of methodologies such as micronization, chemical modification, use of surfactants, pH adjustment, solid dispersion, complexation can be adapted to improve solubilization of a poor water soluble drug and further improve its bioavailability.[8–16] In this study, ternary complexation with β-cyclodextrin (BCD) (Figure 1c) has been exploited to enhance solubility of the drugs. BCD is a cyclic oligosaccharide composed of seven D-glucopyranoside units (glucose) linked by α-1,4 glycosidic bonds. It has a unique toroidal shaped structure and has a small cylinder with a hydrophobic central cavity and a hydrophilic exterior.[17] BCD is able to form inclusion complexes with lipophilic drugs and has shown to increase the dissolution of some poorly water-soluble drugs such as paclitaxel and quercetin[18,19] among others. The interior of the toroid is hydrophobic as a result of the electron-rich environment provided in large part by the glycosidic oxygen atoms. This structure allows the formation of inclusion complexes in which lipophilic compounds are non-covalently bound within the cavity.

As gefitinib with simvastatin is a budding combination and still under clinical trial, no reports are available yet for solubility enhancement of this drug couple in combination form to improve its bioavailability. Another challenge for developing this inclusion complex is the proper design of a 'combination drug inclusion complex'; because the strategic reports involving this paradigm is either lacking or involves a cross-linking polymer to stitch the two molecules of β-cyclodextrin each containing one drug molecule. However, the latter method is often found costly or needs tedious attempt for cross-linking two polymer molecules with jargonic processes. In this study, a successful attempt has been made to improve those drug's solubility and dissolution rate by incorporating both the drug molecules within a single molecule of BCD, thereby increasing their bioavailability. The drug complex with BCD was prepared *via* a simple co-evaporated dispersion technique, probably the first such attempt where a single bimolecular inclusion complex is prepared by a cost-effective and time-economic method. The inclusion complex (GSBCD) was evaluated for loading efficiency and entrapment of the molecular couple within the complex. Dynamic light scattering (DLS) was performed to determine the size of the inclusion complex while Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC) and X-ray diffraction (XRD) analyses were performed to characterize the ternary complex formed between the drug combination and BCD. Molecular docking analysis of individual drug with BCD was undertaken to introspect alignment of the molecule within

the BCD cavity together with bonding interactions of the molecules with BCD. The effect and efficacy on solubility through *in-vitro* dissolution rate study was also performed. This was further validated by kinetic modelling of the drugs' release profiles. Statistical analysis has been implemented to characterize differential elution of the drugs with or without complex. Furthermore, a comparative *in-vivo* study of the GSBCD with raw drug mixture was also carried out. Finally, pharmacokinetic parameters have been evaluated to establish the BCD's role over drugs' fate inside biological system.

Materials and Methods

Materials

Gefitinib was obtained as a gift sample from Natco Pharmaceuticals Pvt. Ltd., Hyderabad, India. Simvastatin was also obtained as a gift sample from Dr. Reddy's Laboratories Ltd., Hyderabad, India. β-Cyclodextrin was procured from Sigma Aldrich (Aldrich, St. Louis, Missouri, USA). All the solvents of HPLC grade together with Zinc sulphate ($ZnSO_4$) were purchased from Merck (Mumbai, India).

Animals

Healthy Wister rat (body weight 175–225 g) of either sex was used for the experiment. The use of animals in this study was approved by CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals). The rats were housed under standard laboratory conditions (under a 12-h/12-h light/dark cycle at a constant temperature of 22 ± 1 °C), fed with standard food pellet and water.

Inclusion complex preparation

Inclusion complex of gefitinib and simvastatin mixture with BCD (1 : 1, molar) was prepared by cosolvent evaporation technique. Solubility of gefitinib in ethanol is 4 mg/ml while that of simvastatin is 200 mg/ml (https://pubchem.ncbi.nlm.nih.gov/compound/simvastatin#section=Melting-Point). Utilizing this, BCD solution in hydroalcoholic system (20 mM) was mixed with solution of gefitinib and simvastatin mixture (1 : 1 molar ratio) in 70% ethanol. The polymer and individual drug ratio was maintained as 1 : 1 (moles/moles). Any insoluble residue afterwards was redissolved with addition of minimum lots of 70% ethanol. Then the mixture was stirred over a magnetic stirrer for 12 h followed by solvent evaporation at 40 °C with controlled agitation. Dried samples were collected, weighed and stored at 4 °C.[20,21]

Evaluation of entrapment efficiency

The entrapment efficiency was determined by simultaneous estimation method as described in Ramana *et al.*[22] involving the method proposed by Beckett and Stenlake.[23] At first two wavelengths were observed where each drug has sufficient absorption (230 and 246 nm) while BCD has null or negligible absorbance. The extinction coefficients of both the drugs at either wavelength were calculated from the slope of the calibration curves of respective drugs at either wavelengths (drug concentration range 5–30 μg/ml). These values of extinction coefficient were used to construct a simultaneous equation for determination of drug content inside the GSBCD. Afterwards, a known weight of the GSBCD was dissolved in 70% ethanol and thus a suitable dilution of the encapsulated drugs was prepared. The drugs solution was subsequently scanned at both the wavelengths. The individual drug content inside the complex was calculated using the afore-established simultaneous equation containing the known extinction coefficients and the total absorbance at both the wavelengths.

HPLC study

The liquid chromatographic system consisted of isocratic HPLC (Waters, USA) containing variable wavelength programmable UV/visible detector (model-2489) and rheodyne injector with 20 μl fixed loop. Chromatographic analysis was performed using Waters ODS C-18 column with 250 × 3.9 mm internal diameter and 4 μm particle size. The detection was performed at 246 nm. The mobile phase used in HPLC has been acetonitrile: methanol: water (60 : 30 : 10) for *in-vitro* studies and 52.5 : 30 : 17.5 for *in-vivo* samples. The flow rate and run time were 0.5 ml/min and 15 min, respectively.

Dynamic light scattering

The hydrodynamic particle size (*z*-average) and the polydispersity index of the GSBCD were measured by Photon Correlation Spectroscopy (PCS) using a Dynamic Light Scattering System (ZetasizerNano ZS; Malvern Instrument, Malvern, UK). Fresh suspensions of GSBCD were prepared by dispersing it in chloroform and subsequently suspending it in water with tween 80 as surfactant. The suspensions were further diluted with water as required and analysed at 25 °C against a 4 mw He–Ne laser beam, 633 nm and a back scattering angle of 173°. Zeta measurement was based on the particle electrophoretic mobility in aqueous medium. The measurements of particle size and zeta potential were recorded in triplicate.

Fourier transform Infrared spectroscopy

FT-IR spectra of physical mixture of gefitinib and simvastatin (3 : 2 w/w), pure β-cyclodextrin and GSBCD were obtained by Shimadzu-8400S FT-IR spectrophotometer p00-4 using potassium bromide (KBr) pellets. KBr pellets were prepared by gently mixing the sample with KBr (1 : 100). The sample was scanned from 4000 to 400 cm^{-1}.

Differential scanning calorimetry

Thermal analysis of pure gefitinib and simvastatin, physical mixture of gefitinib and simvastatin (3 : 2 w/w), and GSBCD were carried out using Differential Scanning Calorimetry method. The samples (5 mg each) were placed into pierced aluminium container. The studies were performed under inert gas atmosphere in the temperature range of 20–400 °C at a heating rate of 10 °C/min. The peak temperatures were determined after calibration with standard.

X-ray diffraction

The powder X-RD patterns of pure gefitinib, simvastatin, physical mixture of gefitinib and simvastatin (3 : 2 w/w), and the GSBCD were recorded by using Goniometer = PW3050/60 (θ/θ) scanner with filter Cu radiation over the interval 5–79°/2θ. The operation data were as follows: voltage 40 kV, current 30 mA, filter Cu and scanning speed 1°/min.

Evaluation of *in-vitro* release profile of drugs

The dissolution patterns of the complexes were compared with those of pure drug. Phosphate buffer of pH 7.2 was used as dissolution medium. 20 mg of the GSBCD containing 5.6 mg of gefitinib and 3.70 mg of simvastatin was taken in a beaker containing 50 ml of the dissolution fluid. The beaker was kept in constant temperature (37 ± 2 °C) and stirred continuously at a speed of 100 rpm. 1 ml of sample was withdrawn at specific time interval. The volume withdrawn at each time interval was replaced with fresh quantity of dissolution medium. Then the samples were evaporated on a water bath at 60 °C and the residue was reconstituted with 2 ml of methanol. A control study was also performed with raw drug mixture in the same amount as above to obtain a comparative dissolution profile of the complexed drug with respect to the control.

Statistical analysis

The statistical analysis was performed using KinetDS software[24] and Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA). The parameters calculated have been the per cent dissolution efficiency (% DE),[25] mean dissolution time (MDT)[26] difference factor (f_1) and similarity factor (f_2).[25, 26, 27] The data were analysed using a *t*-test for comparison between two different groups. The results were considered statically significant at $P < 0.05$.

Release kinetics of drug and model fitting

The release profile of the drug was fitted to conventional drug release models to elucidate the best kinetic model for demonstration of drug release pattern into the exogenous media. The models such as Korsmeyer–Peppas model (up to 60% of cumulative drug release), Weibull model, Hixson–Crowell, Higuchi, Baker–Lonsdale, Michaelis–Menten and Hill equation have been used for the study of drug release profile. Order of release kinetics has also been determined. The kinetics and modelling study have been performed by KinetDS software.

Molecular docking analysis and binding studies

3D molecular structures of gefitinib and simvastatin were prepared by CHEM OFFICE (Chembridgesoft, Chembridge, Massachusetts, UK). BCD structure was obtained from mammalian glycosyltransferase complexed with BCD (PDB id: 3CGT) obtained from protein data bank. Both gefitinib and simvastatin were considered as ligands and BCD has been taken as receptor. Docking was carried out to search for possible binding sites as global optimum over the searched space and subsequently possible ligand configurations were generated as binding conformers with the polymer (AutoDockVina, Molecular Graphics Laboratory, Department of Molecular Biology, MB-5 The Scripps Research Institute, La Jolla, CA, USA) The interactions between binding conformer and BCD binding site has been evaluated by PyMOL (Delano Scientific, San Carlos, CA, USA) and Discovery Studio 3.5 Visualizer (Molecular Graphics Laboratory Department of Molecular Biology, MB-5 The Scripps Research Institute).

Pharmacokinetics and *in-vivo* release study

The methods were applied to the investigation of pharmacokinetics of the bimolecular GSBCD in rat blood model. The study was performed in nine healthy albino rats (175–225 g) following an oral dose of 86.78 mg/kg of GSBCD containing 25.65 mg of gefitinib and 20.82 mg of simvastatin. The dose selection has been performed according to earlier reports.[4,20] Blood collection has been performed at 0, 0.5, 1, 1.5, 2, 3, 4.5, 24, 48, 72 h after dosing. The proteins have been precipitated with 5% Zinc Sulphate

solution in methanol–water system and the clear supernatant after centrifugation was injected into HPLC system for further investigation.

Results

Fourier transform infrared spectroscopy

The FT-IR spectrum of gefitinib (Figure 2a) shows characteristic O–H stretch at 3349 cm^{-1} and OCH_3 peak at 2825 cm^{-1}. It also shows peaks at 1641 cm^{-1} (aromatic skeleton), 3311 cm^{-1} (N–H stretch), 1262 cm^{-1} (aromatic C–N) and 751 cm^{-1} (2^0 NH, N–H wagging). Simvastatin, another drug molecule under investigation can also be characterized by the following IR spectrum (Figure 2c) 3368 cm^{-1} (O–H stretch), 3026 cm^{-1} (aryl C–H stretch), 1713 cm^{-1} (stretching vibration of ester and lactone carbonyl functional groups). The β-cyclodextrin (BCD) IR spectrum (Figure 2c) has been characterized by the characteristic peaks as follows: 3332 cm^{-1} (O–H stretching), 2931 cm^{-1} (C–H stretching), 948 cm^{-1} (skeletal vibration involving α-1,4 linkage). Interestingly, in the IR spectrum of GSBCD (Figure 2d), the peaks corresponding to BCD – OH group (3338 cm^{-1}), CH_2 stretching (2941 cm^{-1}) and CH_2 bending (1430 cm^{-1}) were observed leading to the inference that outer core of the polymer has remained intact. However, the peak at 1713 cm^{-1} shifted to 1688 cm^{-1} suggesting modification by GSBCD formation. Furthermore, a strong peak at 1227 cm^{-1} characteristic to C–O–C ether linkage has been notified too in the GSBCD.

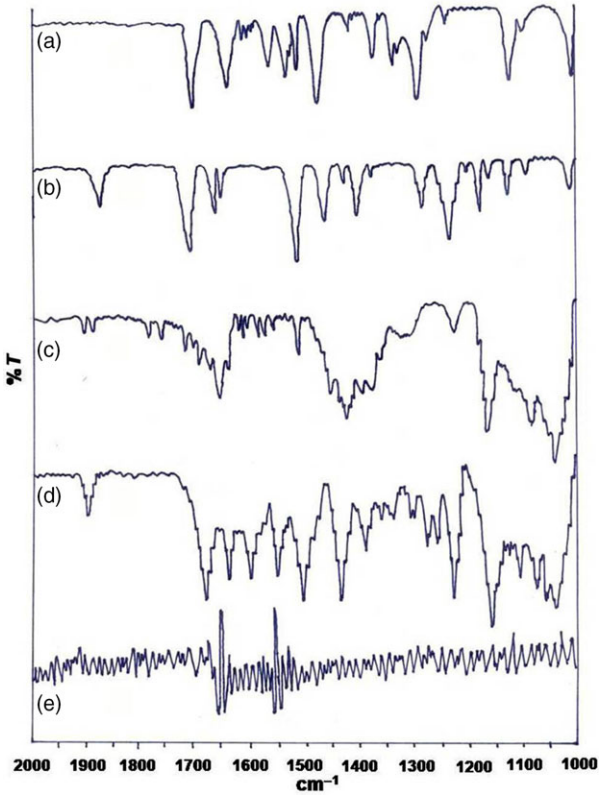

Figure 2 FT-IR Spectra of (a) gefitinib (b) simvastatin (c) β-cyclodextrin (d) inclusion complex (e) physical mixture. In the inclusion complex, the peak at 1713.95 cm^{-1} shifted to 1688.34 cm^{-1} suggesting to modification of carbonyl functional groups probably due to hydrogen bonding with the free hydroxyl groups of β-cyclodextrin. Furthermore, a strong peak at 1227 cm^{-1}characteristic to C–O–C ether linkage has been notified in the inclusion complex IR which is absent in either of the parent drug peaks.

Differential scanning calorimetry study

To evaluate the thermostability of the inclusion complex, DSC analysis was performed. We observed melting endotherms at 200 and 140.65 °C in case of pure gefitinib (Figure 3a) and simvastatin (Figure 3b), respectively. Also, in case of physical mixture (Figure 3e), a notable melting endotherm was observed at 135.14 °C together with a small endotherm at around 190 °C. The BCD showed endotherm at a significantly lower level (85 °C, Figure 3c). However, when drug molecules were formulated inside BCD, a blunt shallow endotherm was revealed in between 70 and 80 °C (Figure 3d) indicating incorporation of the drug molecules inside the polymer together with amorphous nature of microparticle formed in this process.

X-ray diffraction study

The XRD diffractogram shows substantial peak shifts or peak height appreciation or depreciation in the GSBCD (Table 1). For example, simvastatin peak ($2\theta = 7.6002$) is totally absent in the GSBCD. In addition, gefitinib peak ($2\theta = 6.9103$) has been shifted significantly in the GSBCD ($2\theta = 6.2908$) with a reduction of peak height more than half. The change of crystallinity is again depicted by alteration in the peak heights at 28.1752 and 31.7351 (simvastatin) to 28.2235 and 31.7539 (GSBCD). Interestingly, absence of gefitinib peaks such as 20.9197, 22.1277 and formation of new peaks at 12.3084 and 13.8602 in the GSBCD suggests reaction during complex formation. The XRD diffractograms have also been presented in Figures S1–S4.

Evaluation of entrapment efficiency

The percentage of total drug content of the GSBCD was 46.5% (percentage of weight of total combined drug bound per unit weight of the GSBCD). The drugs, that is, gefitinib and simvastatin were entrapped in $3 \pm 0.48 : 2 \pm 0.19$ (moles/moles) ratio, respectively (the

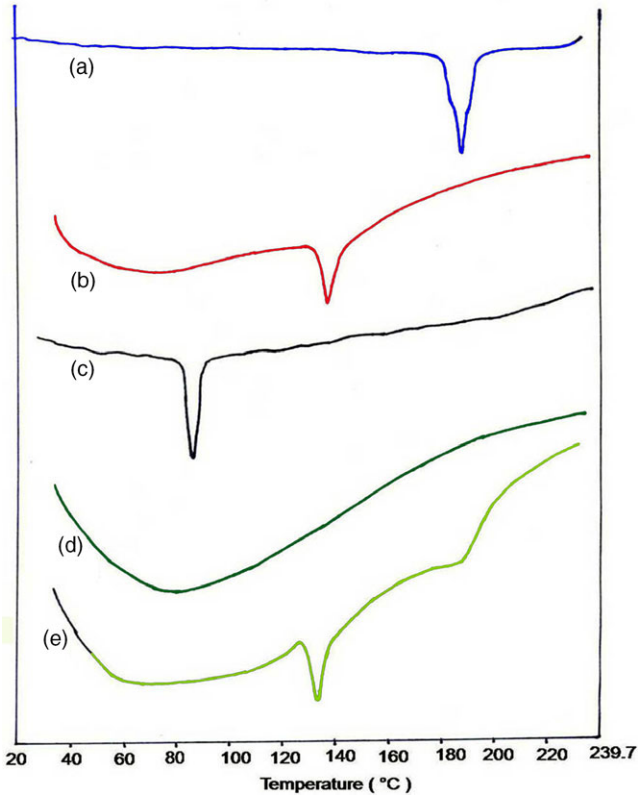

Figure 3 Differential scanning calorimetry thermograms of (a) gefitinib (b) simvastatin (c) β-cyclodextrin (d) inclusion complex (e) physical mixture. When drug molecules were formulated inside β-cyclodextrin, no significant endotherm was observed up to 227 °C indicating thermostability up to this temperature. Furthermore, appearance of relatively blunt peak for the inclusion complex in contrast to the sharp peaks for the crystalline drugs and β-cyclodextrin, suggests that the inclusion complex has been amorphous when manufactured by the cosolvent evaporation method.

Table 1 Changes in the X-ray diffractogram for the inclusion complex

Pos. [°2Th.]			Height [cts]			FWHM [°2Th.]		
GEF	SIMV	Inclusion complex	GEF	SIMV	Inclusion complex	GEF	SIMV	Inclusion complex
–	7.6002	–	–	235.98	–	–	0.2376	–
6.9103	–	6.2908	530.12	–	241.34	0.0974	–	0.1299
–	16.3643	16.4204	–	602.58	184.73	–	0.2772	0.1948
–	17.0173	17.0455	–	1438.52	402.14	–	0.3168	0.1948
–	17.4822	17.5288	–	804.73	335.37	–	0.2376	0.1624
16.3681	–	16.4204	508.63	–	184.73	0.1299	–	0.1948
20.9197	–	–	167.05	–	–	0.1948	–	–
22.1277	–	–	560.33	–	–	0.0974	–	–
–	–	12.3084	–	–	343.29	–	–	0.1948
–	–	13.8602	–	–	140.49	–	–	0.1948

FWHM, full width at half maximum.

data are represented as average ± SD of three independent replicates). The inclusion of both the drugs inside the complex suggests fabrication of bimolecular inclusion complex through a single inclusion complex development method.

Particle size, polydispersity and zeta potential measurement by DLS

The average particle size (*z*-average), polydispersity index and zeta potential of the GSBCD has been shown in

Table 2 Dynamic light scattering obtained measurements

Parameters measured	Values
Particle size distribution (*z*-average)	2–3 μm
Polydispersity Index	0.224–0.328
Zeta potentail	−15.2 mV

Table 2. The average particle size of the bimolecular complex as revealed by laser light scattering lied in between 2 and 3 μm (Figure S5) with low poly dispersity index (PDI, 0.224–0.328, Figure S6). The inclusion complex also revealed moderately high zeta potential (−15.2 mV). Thus, the data unravel that the particle size distribution is unimodel, having narrow range and a homogenous size distribution.

Evaluation of *in-vitro* release profile of drugs

HPLC methods have been used to study the dissolution profile of the drugs. Standard chromatograms of gefitinib (Figure 4a, R_t = 7.51 min), simvastatin (Figure 4b, R_t = 10.67 min) and the drug combination (Figure 4c, R_t = 7.67 and 11.41 min for G and S, respectively) have been used for qualitative and quantitative estimation of the molecules during *in-vitro* study. The release characteristics of gefitinib and simvastatin from raw drug to the GSBCD have been provided in Figures 5 and 6, respectively. It is noteworthy to mention that the gefitinib release from the BCD complex (final concentration 96.27 μg/ml; cumulative % release 85.96) was about 3.5 times higher than the raw

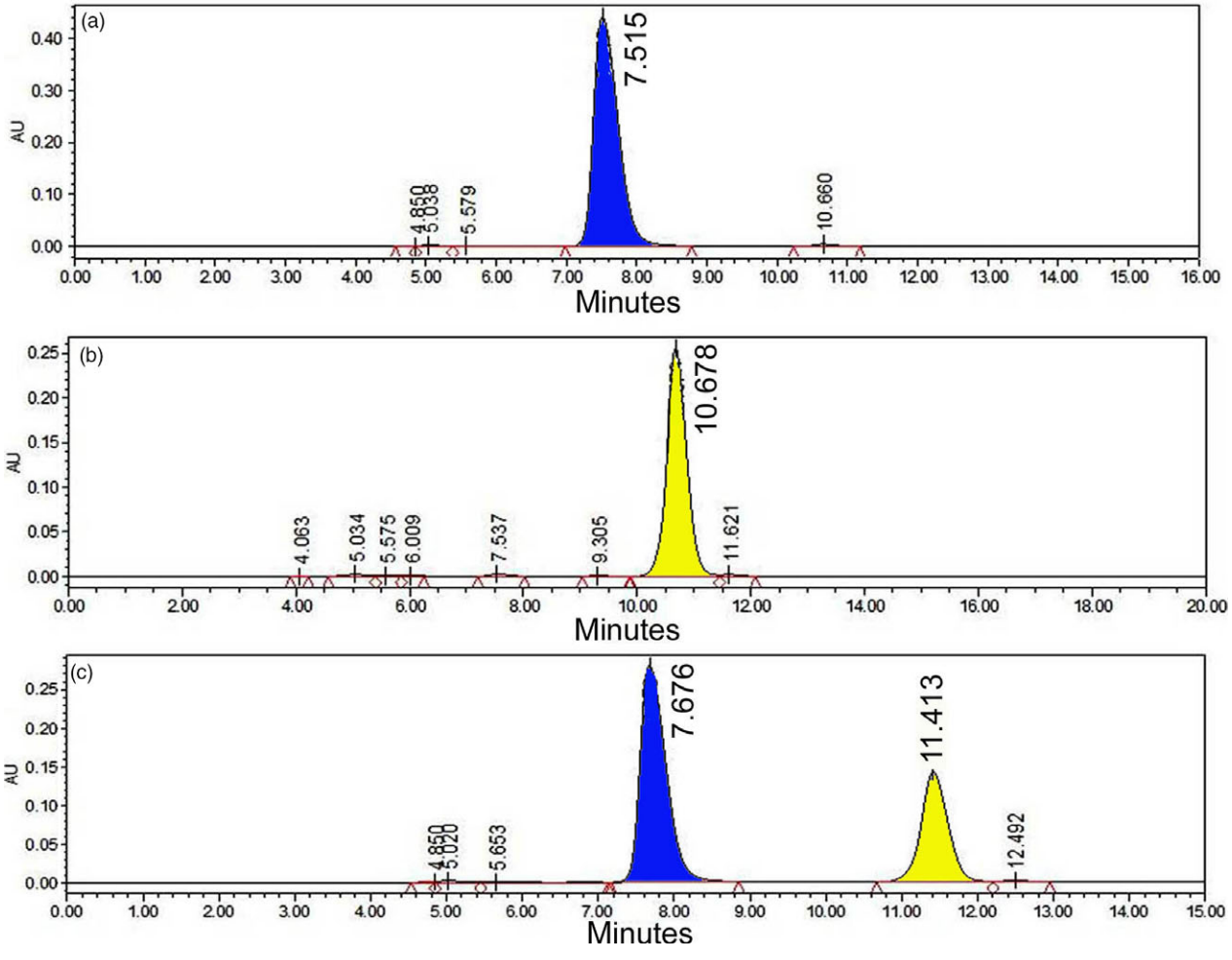

Figure 4 HPLC chromatograms of (a) pure gefitinib (b) pure simvastatin (c) released drugs from the inclusion complex within dissolution media. Standard chromatograms of gefitinib (R_t = 7.51 min), simvastatin (R_t = 10.67 min) and the drug combination (R_t = 7.67 and 11.41 min, respectively) have been used for qualitative and quantitative estimation of the molecules during *in-vitro* study.

one (final concentration 28.61 μg/ml; cumulative % release 25.55).

The release rate of simvastatin from pure drug mixture was found to be very low and could not be detected. Thus, the release improvement of formulated simvastatin was found infinitesimally higher (final concentration 36.23 μg/ml; cumulative % release 48.96) than the raw one.

Release kinetics

The best fit model for the drug release was obtained from the regression coefficient (r^2) from the model-fit data. The best regression coefficient (r^2 = 0.9255. 0.8593 for gefitinib and simvastatin, respectively) has been obtained for zero order release (Table 3) which suggests a steady prolong release of the complexed drug. Moreover, the total release of the drugs followed analogy with the Korsmeyer-Peppas model (r^2 = 0.8755–0.9622) with n value ranging from 0.09 (gefitinib) to 0.109 (simvastatin). The dissolution efficiency (DE) of the complexed gefitinib is about 3.5 times higher than the unformulated one where for simvastatin it is infinitesimally higher than that of the raw one (Table 4). Mean Dissolution Time (MDT) for complexed gefitinib is also much lower (61.77 min) compared to raw drug (75.96 min) where for raw simvastatin it could not be detected as like DE.

Docking and binding interactions

To study molecular binding interactions, docking studies were performed. The docking studies have revealed that the binding affinity of gefitinib is 6.0 kcal/mol where simvastatin may have binding affinity as low as 5.2 kcal/mol towards β-cyclodextrin. It suggests that gefitinib and simvastatin binding affinity is in the ratio 3.0 : 2.6 which is at per what we have obtained experimentally (3 ± 0.48 : 2 ± 0.19). The binding interaction of the ligands with the polymer exhibited that both the ligands

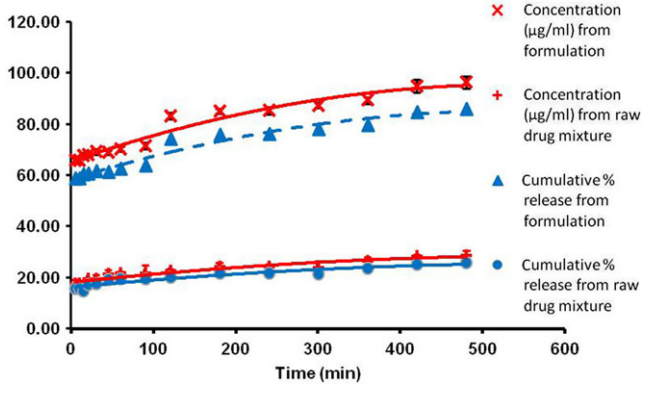

Figure 5 *In-vitro* dissolution profile of gefitinib. Gefitinib release from the β-cyclodextrin complex (final concentration 96.27 μg/ml; cumulative % release 85.96) was about 3.5 times higher than the raw one (final concentration 28.61 μg/ml; cumulative % release 25.55). All data are mean of three independent observations and the data have been represented as mean ± standard deviation. The data analysis has been performed in Microsoft Excel.

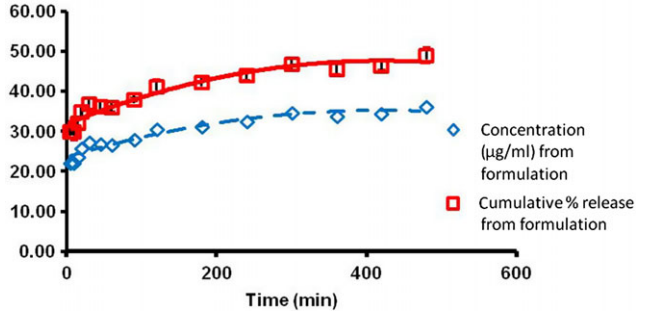

Figure 6 *In-vitro* dissolution profile of simvastatin. The release rate of simvastatin from pure drug mixture was found very low and could not be detected. Thus the release improvement of formulated simvastatin was found enormously higher (final concentration 36.23 μg/ml; cumulative % release 48.96) than the raw one. All data are mean of three independent observations and the data have been represented as mean ± standard deviation. The data analysis has been performed in Microsoft Excel.

Table 3 Determination of model order (gefitinib–simvastatin–β-cyclodextrin complex)

		Gefitinib		Simvastatin	
Model order	Equation	r^2 (regression cofficient)	RMSE (root mean square error)	r^2 (regression cofficient)	RMSE (root mean square error)
Zero order	$Q = k{\cdot}t + Q_0$	0.9255	2.62	0.8593	2.27
First order	$Q = Q_0{\cdot}e^{k{\cdot}t}$	0.9089	2.95	0.8142	2.52
Second order	$1/Q = k{\cdot}t + 1/Q_0$	0.8904	3.39	0.7624	2.88
Third order	$1/Q^2 = k \cdot t + 1/Q_0^2$	0.8708	4.06	0.7058	3.52

Q, amount (%) of drug substance released at the time t; Q_0, start value of Q; t, time; k, rate constant.

Table 4 Model independent descriptors of the drug release

Release profile	Dissolution efficiency (DE, %)	Mean dissolution time (MDT) in min
Gefitinib from complex	74.89	61.77
Simvastatin from complex	42.69	61.37
Raw gefitinib	21.49	75.96
Raw simvastatin	Not detected	Not detected

had been centrally aligned inside the central cavity of BCD (Figure 7a); however, the aromatic rings protruded out of the cavity (Figure 7b). The functional groups such as –C=O, $–OCH_3$ together with secondary amino nitrogen of the aromatic ring participated in hydrogen bonding interactions with the polar –OH heads of cyclodextrin polymer thus stabilizing the ligand inside the latter (Figure 8a and 8b). No steric hindrance has been observed between the two molecules when put together inside BCD cavity (detailed data could not be shown due to complexity of the diagram), hence the system might be considered as thermodynamically stable.

Thus, it may be concluded that the hollow inclusion space of BCD is not enough to accommodate the entire molecule of the ligands which may promote two-step release profile of the ligands from the polymer. The first one being the initial solvation of the ligands upon contact with aqueous phase, the second one is the slow diffusion of the encapsulated ligand backbone throughout the polymer matrix. This two-step release thus actually facilitates the slow but steady release pattern of BCD-drug polymer conjugate. The binding chemistry of the drug molecules was eventually elucidated by PyMOL.

In-vivo release and pharmacokinetic study of drugs

The comparative *in-vivo* release profile of gefitinib and simvastatin from the GSBCDas well as the raw drug mixture is provided in Table 5. The maximum release differentiation has been observed up to 3 h inside rat physiological system; the drug loaded microparticles showed 10 times release improvement than the raw ones under normal healthy condition.

The pharmacokinetic study of drugs (Figure 9) also revealed that the GSBCD-mediated drug release attained its maximum concentration within 3 h (3 h for gefitinib and 2 h for simvastatin). The C_{max} for gefitinib and simvastatin were found as 13.68 and 5.67 μg/ml, respectively. The

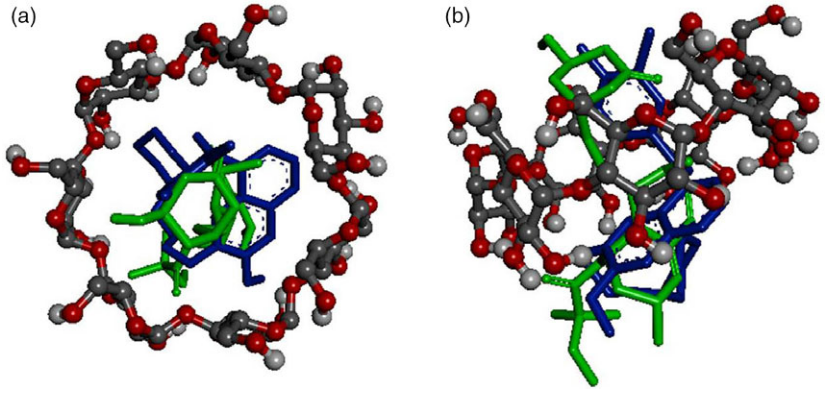

Figure 7 Fitting of gefitinib and simvastatin inside β-cyclodextrin. (a) Top view showing central alignment of drug molecules inside cavity. (b) Side view showing protrusion of aromatic heads outside the cavity while main backbones are remaining inside the cavity. The image has been constructed using Discovery Studio 3.5 Visualizer.

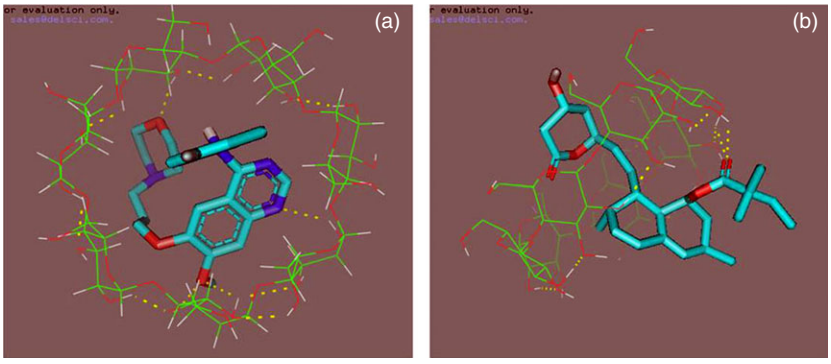

Figure 8 Binding interactions between ligands and β-cyclodextrin (a) gefitinib (b) simvastatin. Colour code: blue-nitrogen, red- oxygen, green-carbon; yellow dotted line represents hydrogen bonds. The diagram shows that hydrogen bonding interactions are predominant in ligand-polymer complexation especially in between ligand nitrogen/oxygen with polymer polar heads. The image has been constructed using standard graphics tool in PyMOL (Delano Scientific).

Table 5 Comparative *in-vivo* study of drugs

	Concentration (μg/ml) of gefitinib		Concentration(μg/ml) of simvastatin	
Time (h)	Raw drug	Inclusion complex	Raw drug	Inclusion complex
1	0.38 ± 0.15	0.80 ± 0.12	0.18 ± 0.09	0.47 ± 0.17
1.5	0.58 ± 0.11	2.65 ± 0.23	0.29 ± 0.08	1.47 ± 0.24
3	1.36 ± 0.23	13.67 ± 0.71	0.64 ± 0.18	5.69 ± 0.57

Each data is average ± SD of three independent observations.

drugs also showed a prolong residence time within rat body (16.71 and 1.97 h for gefitinib and simvastatin, respectively, Table 6).

As therapeutic efficacy depends on the release profile of concerned drug delivery system, increased drug release from our microdevice promises improved therapeutic output, faster C_{max} attainment and decreased frequency of dose administration.

Discussion

The centre of innovation of this manuscript is formation of ternary inclusion complex with two drugs. The incorporation of two drug molecules inside the BCD cavity can be proved from the characterization portfolios. For example, SBCD inclusion complex in previous reports showed complete incorporation of the drug molecule inside BCD cavity characterized by total suppression of simvastatin spectra by BCD spectra.[28] Similar observation is also obtained for GBCD spectra (data not shown). However, in our study, we obtained 1600–1700 cm^{-1} region of GSBCD IR spectrum showing partially suppressed-partially shifted peaks of both the drugs. Interestingly this corroborates with the results obtained by docking studies where protrusion of aromatic heads containing carbonyl groups have been revealed to be lying outside the cavity. This signifies partial incorporation of the drug molecules within the cavity together with hydrogen bonding. Similarly,

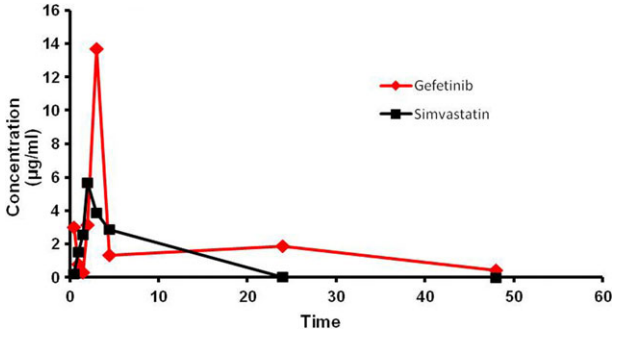

Figure 9 Pharmacokinetics study of drugs inside rat physiological system. The C_{max} for gefitinib and simvastatin were found as 13.68 and 5.67 μg/ml, respectively. The drugs also showed a prolong residence time within rat body (16.71 and 1.97 h for gefitinib and simvastatin, respectively).

Table 6 Pharmacokinetics data inside rat body

Sl no.	Pharmacokinetic parameters	Values for gefitinib	Values for simvastatin
1	Time required for maximum plasma concentration, T_{max} (h)	3 ± 0.24	2 ± 0.32
2	Maximum plasma concentration, C_{max} (μg/ml)	13.68 ± 0.67	5.67 ± 0.71
3	$AUC_{(0\rightarrow48)}$, (μg h/ml)[a]	81.00 ± 12.15	82.80 ± 5.54
4	$AUMC_{(0\rightarrow48)}$ (μg h^2/ml)[b]	1354.15 ± 121.34	163.30 ± 56.70
5	Mean residence time, MRT (h)	16.71 ± 1.30	1.97 ± 0.43

Each data is average ± SD of three independent observations. [a]AUC → area under curve. [b]AUMC → area under momentum curve.

1000–1200 cm^{-1} region of the GSBCD spectrum entirely matches with that of BCD signifying total introduction of linear backbone within the cavity. The finding again is in concurrence with the revelation of molecular docking studies where linear segment of the molecule is revealed entrapped within the BCD system.

To dissect the inclusion pattern of the guest molecules inside the cavity, solid state NMR has been performed. The formulation NMR spectra (Figure S7) suggests that it contains both the peaks of gefitinib and simvastatin. The peaks with lower chemical shifts (δ 0.754–2.495) as well as some intermediate (δ 5.184–5.965) are of simvastatin (Figure S8) while peaks like δ 3.338–3.568 are of BCD (Figure S9). The peaks of higher chemical shifts (δ 7.194–9.564) suggest the proton environment of gefitinib (Figure S10).

To further verify the inclusion of both the guest drugs (G and S) inside BCD cavity, 2D NMR of the inclusion complex has been performed. Figure 10 describes the host-guest interactions by 2D NMR (COSY). The red circles suggest proton–proton coupling between BCD and simvastatin while the blue circles represent interaction between BCD-gefitinib or gefitinib–simvastatin. It is interesting to note major interactions have occurred between BCD and simvastatin probably due to presence of –C=O– or –OH group inside simvastatin and making hydrogen bondings with the –OH groups in BCD (as shown by our docking results). Only weak interactions have been observed between gefitinib and BCD, might be due to –F or –Cl interactions with BCD. However, interactions between gefitinib and simvastatin suggest close vicinity of both molecules inside the same host cavity.

The inclusion complex also revealed moderately high zeta potential which is most likely contributed by the circumferential cyclodextrin molecules that exert physical stability on to the microparticles by local electrostatic repulsion thereby preventing aggregation. It is noteworthy to mention that the charge on to the microparticles is directly proportional to the cross-linking ratio inside the GSBCD. The stability of the formulation was also confirmed by DSC analysis that has been performed after 6 months of formulation manufacture. The formulation endotherm is also noticeable at specified region (70–80 °C) (Figure S11) which indicates the stability and amorphous nature of the formulation.

The drugs from GSBCD showed 3.5–10 times improved release profile depending on *ex-vivo* or *in-vivo* condition compared to raw mother drugs. However, can this ternary complex provide better results than the mixture (1 : 1, w/w) of individual complex of both the drugs? To evaluate that, we have performed this experiment and formed individual complexes of both the drugs with β-cyclodextrin in exactly same way as we had prepared the ternary complex. Interestingly it was found that the drug dissolution from the individual complex has been approximately the same with that of the ternary complex. However, strictly speaking, the latter has been a little bit better than the former (Figure S12). In addition, we claim that preparation of single biomolecular inclusion complex is cheaper and commercially viable compared to the preparation of individual complexes. As lower MDT and improved DE signify faster rate of dissolution, it can be deduced that drugs diffused much faster from the interface of polymer matrix into the solution than the raw powdered ones. The improved dissolution of the drug molecules could be attributed to their micronization during complex formation, or BCD is acting as a carrier to form a bridge between aqueous phase and the drug molecules for improving their release efficiency during dissolution. Furthermore, the release of the drugs followed analogy with the Korsmeyer-Peppas model ($r^2 \sim 0.8755$–0.9622) with n value ranging from 0.09 (gefitinib) to 0.109 (simvastatin). These low n values indicate slow time-dependent release of the drugs from the complex. Combining both zero order kinetics and Korsmeyer-Peppas model, the release profile could be characterized as time dependent release where the dissolution is steady but slow promising the basic characteristics of a controlled release drug delivery system.

It is noteworthy to mention that Shi *et al.*[29] reported 40% release of gefitinib from Bovine Serum Albumin conjugated carboxymethyl-beta-cyclodextrin nanoparticles in first 48 h. However, for our microparticles, about 70% release of the drug has occurred within 1 h. Again, Philip Lee[13] reported that gefitinib: HPBCD (Hydroxypropyl beta cyclodextrin) (1 : 1) complex released 50% of gefitinib within 10 min where our GSBCD discharged more than

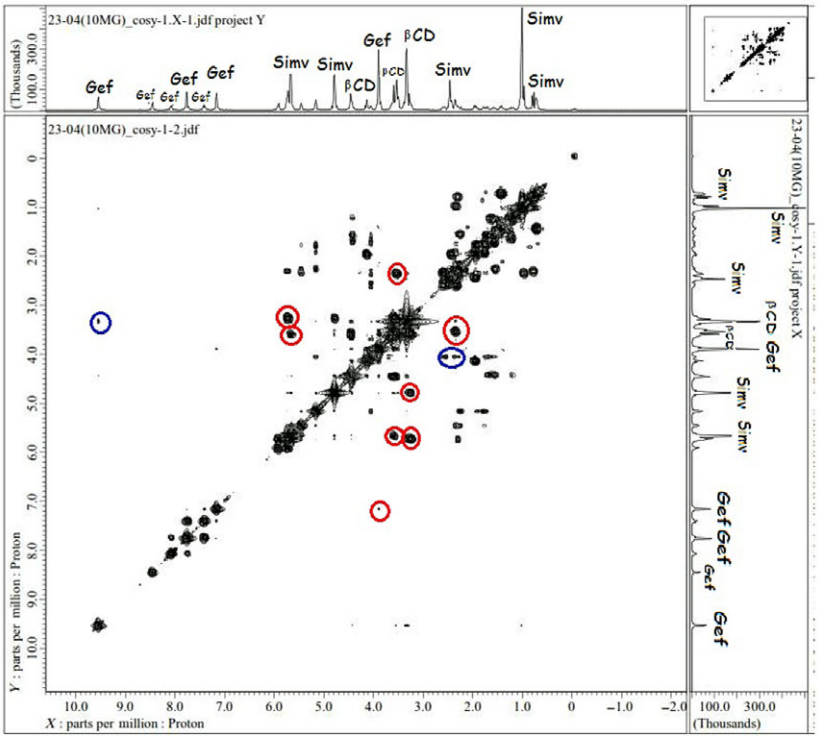

Figure 10 2D NMR (COSY) spectra of the inclusion complex. The red circles indicate β-cyclodextrin and simvastatin coupling while blue circles represent β-cyclodextrin-gefitinib and gefitinib–simvastatin coupling.

65% of gefitinib within 5 min. Furthermore, Jun *et al.*[29] prepared simvastatin loaded hydroxypropyl-β-cyclodextrin inclusion complex and observed that more than 40% simvastatin release has occurred within 5 min when prepared in normal process which is also the final steady state concentration of simvastatin. However, our inclusion complex has successfully leached simvastatin more than 48% as the final steady state concentration. Thus our GSBCD complex is either at per or superior based on its performance for improvement of the aforementioned drugs' solubility, thus can contribute significantly in delivery of those combined drugs as actual therapeutic regimen. However, Jun reported that processing the complex through supercritical antisolvent process augmented the delivery performance up to 90%. In pursuit, we are currently working on this process to upregulate both gefitinib and simvastatin release significantly through our microparticles. Although previous authors acknowledged that cross-linked β-cyclodextrins, hydrogels or nanosponges could offer value added release to drugs and that seems to be an alternate route to increase such solubilities[30–34]; these methods often fall costlier due to the expensive cross-linking polymers involved herewith. Moreover, such processes are tedious due to cross-linking reaction with the polymeric agents, maintaining its specificity and eliminating its toxic by-products. In comparison, our method is simpler, time and cost effective and thus industrially viable. Thus we propose that this method can be a promising route to increase the solubility of combination drugs.

Conclusion

Multimolecular inclusion complex formation has been the main objective of our study which has been successfully accomplished as suggested by the entrapment ratio of gefitinib and simvastatin as $3 \pm 0.48 : 2 \pm 0.19$. Earlier reports are there to formulate individual molecules of the aforementioned drugs as monomolecular inclusion complex[30–34]; however, this is the first approach to manufacture single inclusion complex containing more than one drug molecule which would be cost effective and rapid in its action than conventional method of cross-linking of BCD monomers. The characterization of the inclusion complex by different methods such as FT-IR, DSC, XRD and DLS further confirmed that there is a formation of inclusion complex of both the drugs with BCD. The *in-vitro* dissolution study of the BCD inclusion complex showed paramount improvement in drug release following zero order kinetics in comparison to the pure drug mixture. The *in-vivo* study, in pursuit, revealed a

staggering ten-fold improvement in drug release within biological system which suggested the improved efficacy of the drug device in real biochemical system. The comparative *in-vivo* study was performed for 3 h because the half-life of simvastatin is 3 h. Pharmacokinetics study of drugs revealed that there is improved plasma retention as indicated by mean residence time, of the complexed drugs. Thus this study demonstrates a promising path to improve efficacy of combination dosage form in the field of pharmaceutical sciences.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. XRD of pure gefitinib.[13]

Figure S2. XRD of pure simvastatin.

Figure S3. XRD of physical mixture of gefitinib and simvastatin.

Figure S4. XRD of β-cyclodextrin guided drug (gefitinib and simvastatin) loaded Inclusion complex.

Figure S5. DLS guided particle size measurement of the inclusion complex.

Figure S6. Zeta potential measurement of the inclusion complex.

Figure S7. $_1H^1$ spectra of GSBCD formulation.

Figure S8. $_1H^1$ spectra of simvastatin.

Figure S9. $_1H^1$ spectra of BCD.

Figure S10. $_1H^1$ NMR spectra of gefitinib.

Figure S11. DSC guided stability study of the complex.

Figure S12. Comparative dissolution profile of drugs from ternary complex and from individual complexes.

Table S1. System suitability parameters of HPLC guided drug release studies.