J Incl Phenom Macrocycl Chem (2007) 57:689–697
DOI 10.1007/s10847-006-9262-3

ORIGINAL ARTICLE

Cyclodextrin based drug delivery system of protease inhibitor—nelfinavir mesylate

S. J. Torne · J. S Torne · P. R. Vavia · S. K. Singh · N. Kishore

Received: 14 May 2006 / Accepted: 20 October 2006 / Published online: 11 January 2007

Abstract The purpose of present investigation was to understand the interactions involved in complexation of Nelfinavir Mesylate (NM)—a protease inhibitor, used in the treatment of HIV/AIDS with Beta-cyclodextrin (β-CD) and its subsequent effect on its absorption properties and bioavailability. Milling method was used for complexation. The inclusion complexes were characterized by 2D NOESY NMR and ITC studies. The feasibility of findings was further confirmed by using Cerius^2 software of Tripos Inc. using Silicon Graphics O_2. Pharmacokinetic studies were carried out in rabbits and data was treated by Student's t Test. 2D NOESY NMR studies showed very intricate behavior showing interactions amongst drug and β-CD molecule as well as amongst β-CD–β-CD molecules. This fact of formation of molecular aggregates was further confirmed by ITC studies. Computer simulation studies further supported the finding of forming shallow complex. The percent relative bioavailability of complex at the dose of 400 mg/kg in rabbits was 185.37 as compared to the plain NM at 400 mg/kg dose. The studies were conducted at low dose of 200 mg/kg of drug in the form of complex in rabbit does not show statistically significant difference in AUC, $T_{1/2}$ and Kel. as compared to plain drug at 400 mg/kg of rabbit.

S. J. Torne · J. S. Torne · P. R. Vavia (✉)
Pharmaceutical division, Mumbai University Institute of Chemical Technology, Nathalal Parikh Marg, Matunga, Mumbai 400 019, India
e-mail: vaviapradeep@yahoo.com

S. K. Singh · N. Kishore
Department of Chemistry, Indian Institute of Technology, Mumbai, India

Keywords Bioavailability · Cyclodextrins · Nelfinavir Mesylate · Aggregates

Introduction

HIV/AIDS is a global problem. There are numerous technical and formulation related issues of Anti-HIV drug that can be addressed since it is a global concern. We have identified Nelfinavir Mesylate, a protease inhibitor, and efforts have been directed to develop more soluble formulation with improved bioavailability so as to reduce dose and subsequently cost of HIV/AIDS therapy. Nelfinavir Mesylate, a protease inhibitor, is slightly soluble in water. It possess very low intrinsic dissolution rate hence it is likely to have dissolution rate limited absorption problems. The drug dose is very high of about 1.25 gm twice a day [1–3].

Nelfinavir Mesylate is one example of drugs that lose their efficacy upon reaching the lower portions of the GI tract. It is soluble in an acidic environment but insoluble in an alkaline environment. Portions of the drug that are undissolved cannot be absorbed. Portions of drug that are dissolved but not yet absorbed when they pass from the stomach into the small intestine may undergo precipitation and loss of their therapeutic benefit. This is confirmed by the fact that the presence of food in the GI tract substantially increases the extent of absorption of oral Nelfinavir. Peak plasma concentration and area under the plasma concentration–time curve of Nelfinavir are 2-fold to 3-fold greater when doses are administered with or following a meal [3].

Cyclodextrins are series of cyclic oligosaccharide containing glucopyranose units attached by α-1,

4-linkages. Cyclodextrins are also reported to form inclusion as well non-inclusion complexes forming molecular aggregate in the solution. In solution both cyclodextrins and their complexes can self associate to form two or more cyclodextrin molecules or complexes thus helps in solubilization of hydrophobic molecules [4–9].

Present research work was initiated with the objective of development of formulation of complex of Nelfinavir Mesylate with β-Cyclodextrin and its characterization with various methods to understand the phenomena of solubility enhancement. Hence a further goal of the present study was therefore to provide a means of administering the drug that will maximize their therapeutic effectiveness by increasing the solubility of drug so as to improve the bioavailability of drug which will further help to reduce the dose of drug which will further help in cost reduction.

Nelfinavir mesylate

The chemical name for nelfinavir mesylate is [3S-[2(2S*, 3S*), 3a,4ab,8ab]]-N-(1,1-dimethylethyl)decahydro-2-[2-hydroxy-3-[(3-hydroxy-2-methylbenzoyl)amino]-4-(phenylthio)butyl]-3-isoquinoline carboxamide monomethanesulfonate (salt) and the molecular weight is 663.90 (567.79 as the free base). Nelfinavir mesylate is a white to off-white amorphous powder, slightly soluble in water at pH ≤ 4 and freely soluble in methanol, ethanol, 2-propanol and propylene glycol.

Experimental

Materials and methods

β-CD (Cerestar) was supplied as gift sample by S. A. Chemicals, India. Nelfinavir Mesylate was supplied as gift sample by Hetero Drugs. Ltd., India. All chemicals and reagents were of analytical grade.

Preparation of complex

The complexes were prepared by ball milling (B.M.) method. Laboratory scale ball mill was used for this purpose. The duration of milling was optimized to 4 h and powders approximately 25 gm was milled at one time. The samples of 1:1.5 M ratio of NM:β-CD was found to give desirable dissolution characteristics like remarkable reduction in half life of dissolution. The said ratio was further explored for studies.

Characterization of powders

Nuclear Overhauser Effect Spectroscopy (NOESY) NMR studies

The 2D NOESY NMR measurements were performed on 5 mM solution of NM–β-CD complex [B.M., 1:1.5 M] in dimethyl sulfoxide (DMSO- d_6) with a Bruker FT-NMR spectrophotometer operating at 500 MHz.

Molecular Modeling Studies

The studies were conducted in order to understand the possibility of interactions. 2D NMR studies showed intricate behavior of NM–β-CD complex involving multiple interactions of NM and β-CD. To prove these the studies were conducted to find out most stable conformation of NM and β-CD molecules which was determined using Cerius^2 software of Tripos Inc. using Silicon Graphics O_2. The studies were also directed to check the feasibility of various conformations which were generated for NM–β-CD complex based on the data of various instrumental techniques.

Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry was used to understand the binding mode of the NM and β-CD [10, 11]. Addition of NM solution was automated from precision syringe in the sample cell containing β-CD solution and automatically stirred to effect rapid mixing of the reactants.

Procedure

The studies were conducted on Microcal VP-ITC Titration Calorimetry (MicroCal, Northampton, MA) and data analysis was carried out by using Microsoft Origin software. As shown in Table 1 the first set was started with the concentration of 0.004 mM of β-CD as shown for 25 injections by addition of 10 μl of

Table 1 Experimental design for isothermal titration calorimetric studies

Set no.	Molar ratio of β-CD (mM)	Molar ratio of NM (mM)
1	0.004	0.023
2	0.004 (ConCat 32)	0.023
3	0.004	0.045

0.023 mM solution of drug. In set two, sequential titration was performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand that is NM solution without removing β-CD solution from the cell. The titrations were linked together for data analysis using ConCat-32 software provided by MicroCal, Inc. Thus the second set of experimental design was repeated by addition of another 25 injections to cell. Thus set 2 consisted of total of 50 (25 from set 1 and 25 of set 2) injections of NM which were added in 0.004 mM solution of β-CD in sample cell.

Third experiment was designed with increased concentration of drug from 0.023 to 0.045. The studies were conducted for 25 injections and data analysis was performed. The heat released by dilution was corrected for actual titration of NM and β-CD and the binding mode of complexation was studied.

Bioavailability of nm

Method of study

The study was undertaken to investigate pharmacokinetics of pure NM and NM in the form of complex in Rabbits (*New Zealand White*) by Oral route. The mode of treatment is as shown bellow:

Group 1: Plain NM (400 mg/kg)
Group 2: NM–β-CD Complex, [B.M., 1:1.5 M] (400 mg equivalent of NM/kg).
Group 3: NM–β-CD Complex, [B.M., 1:1.5 M] (200 mg equivalent of NM/kg)

Initially studies were conducted in group 1 and group 2. The data was studied statistically and compared for various pharmacokinetic parameters. Based on the relative pharmacokinetic analysis of group 2, the studies were conducted in third group at reduced dose. The results of group 3 were compared with group1. Distilled water was used as vehicle for administration of compound.

About 2 ml of the blood samples were collected at 0, 1, 2, 2.5, 3.5, 5 and 8 h in heparinized tubes. The samples were immediately subjected for centrifugation at 4,000 rpm for 20 min. The supernatant plasma was separated and stored at –20°C till analysis. On the day of analysis, the samples were thawed and subjected for extraction of drug using 1 ml of plasma. Plasma levels of NM were determined by High Performance Liquid Chromatography. Graph of plasma drug concentration was plotted against time and various pharmacokinetic parameters like C_{max}, T_{max}, Kel, $T_{1/2}$ were calculated for NM and NM in the form of complex as well. Area under curve of the drug was calculated by trapezoidal rule. Elimination half life was calculated from the slope of log plasma concentration-time points. The relative oral bioavailability was calculated by comparing the mean area under the plasma concentration time curves (AUC_{0-8}) after oral dosing. Statistical data analysis was done by Student's *t* test.

Extraction of drug from plasma

Equal volumes of drug containing plasma was mixed with 0.01 N sodium hydroxide solution. NM was extracted using Ethyl acetate:Acetonitrile (90:10). After vortexing for 7 min the organic phase was separated and evaporated under nitrogen atmosphere. Finally it was reconstituted with 200 µl of mobile phase. A total of 100 µl of sample was injected. The analysis was performed at 215 nm at room temperature.

Analytical procedure

A rapid, selective high performance liquid chromatographic method was developed for the estimation of NM. The method specific for NM was developed on Jasco PU-2010 pump using Jasco PDA detector. The data integration was done by Chrompass software package V1.21. The method is developed on Hi-Q-C18 column (5 micron, spherical, pore size 100 Å, 4.6 mm i.d. × 250 mm, Kyatech Corp.). The samples volume is 20 µl at 25°C. Mobile phase used was Acetonitrile:Potassium dihydrogen Phosphate (0.03 M), 56:44 v/v at pH 3.24 at room temperature.

Result and discussion

The studies indicated strong possibility of molecular interactions between NM and β-CD molecules. The nuclear overhauser effect can be used to demonstrate that two protons or group of protons are in close proximity within the molecule and/or different molecules. Therefore it is of considerable value in the study of molecular geometry. The protons must be within 3.5 Å of each other. The 2D NOESY spectra of the complex showing intense cross peaks indicating possible interactions amongst drug and β-CD as well Inter and intra β-CD molecules is as shown in Fig. 1.

Presence of cross peaks indicates presence of those groups in the vicinity of 2–5 Å. Thus giving rise to the possibility of interactions amongst the groups.

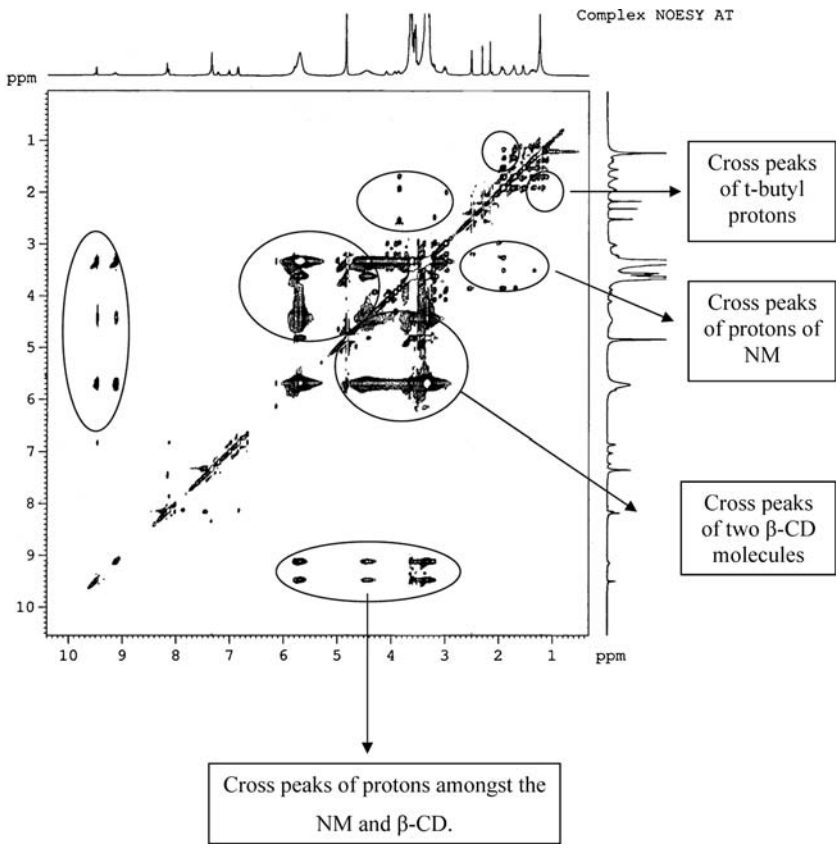

Fig. 1 2D NOESY NMR of complex

As shown in the Table 2 and Fig. 2, NM shows cross peaks with few protons of the β-CD. The cross peaks were observed for the protons of drug with protons of β-CD molecule present on both the sides. The Hydroxyl group present at 37 position showed cross peaks with OH-2 group present wider end of at β-CD and with OH-6 present at narrow end of β-CD.

The cross peaks were observed for the protons of drug present at 13-OH position of drug with protons of β-CD molecule present at two different positions. The Hydroxyl group present at 13 position of NM showed cross peaks with OH-3 group present wider end of at β-CD and with OH-6 present at narrow end of β-CD.

These observations showed that there is possibility of formation of stack like structure involving drug and β-CD molecules. These numerous conformations with specific arrangement of β-CD molecules with linking of drug molecules may further form aggregates in the solution. Figure 3 of 2D NOESY spectra showed strong interactions amongst two β-CD molecules which is interpreted and presented in Table 3.

Table 2 Cross peaks of NM and β-CD molecule

NM (δppm)	β-CD (δppm)
9.44 of OH-37	5.6 of OH-2
9.44 of OH-37	4.45 of OH-6
9.14 of OH-13	5.65 of OH-3
9.14 of OH-13	4.45 of OH-6

The drug does not show any major signal in the range of 4.0–6.0 δppm values. Hence the cross peaks observed in this range was attributed to protons of β-CD molecules. The 2-OH group present at wider end of the cavity showed interactions with 6-OH group which is present at narrow end of β-CD molecules. Thus confirms the possibility of tail to head type of orientation of β-CD molecules.

Similarly 2-OH group present at wider end of the cavity showed interactions with 4-H, protons at 4th position which is present on the sides of the cone like structure of β-CD. This interaction indicates random interactions amongst β-CD molecules.

Based on the data of 2D MNR studies the possible tail to head arrangement is as shown in Fig. 4A, B. These interactions were observed along with interactions of β-CD and NM molecules. The interactions amongst β-CD molecules further confirms the fact of formation of head to tail type of stack formation of β-CD molecules.

The possibility of interactions amongst β-CD and NM were further supported by computer simulation

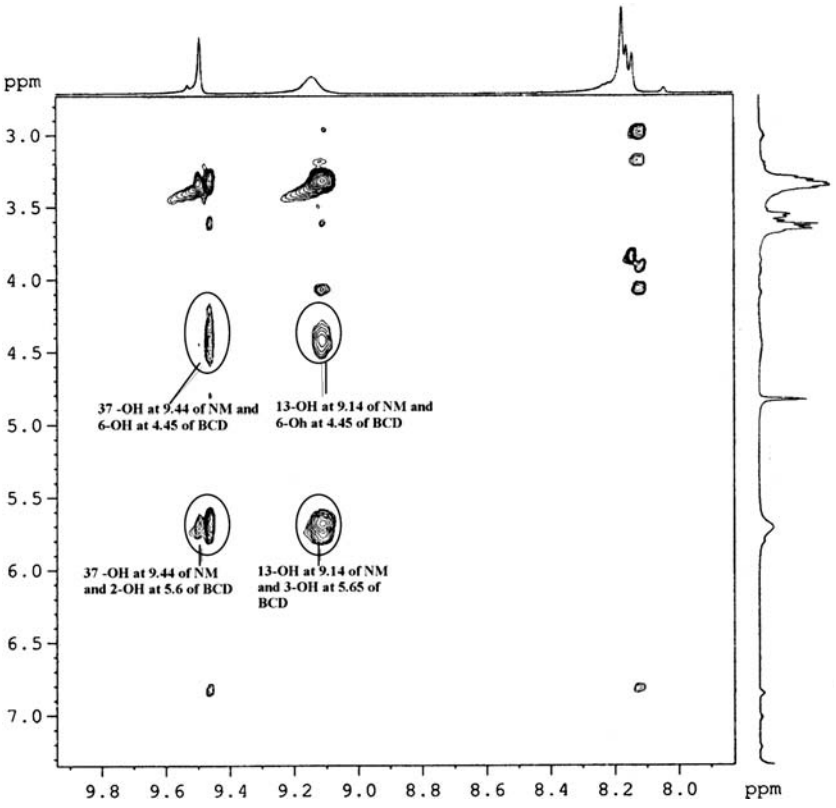

Fig. 2 NOESY spectra of complex showing cross peaks of NM and β-CD molecule in complex

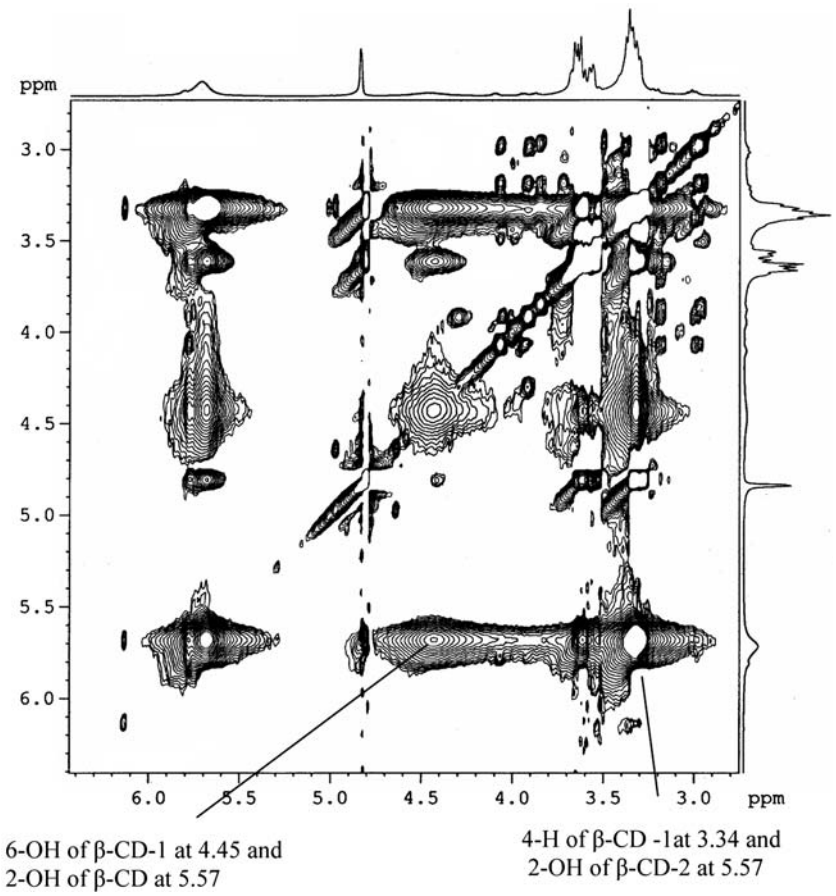

Fig. 3 NOESY of complex showing cross peaks of two β-CD molecules

Table 3 Cross peaks between two adjacent β-CD molecules

β-CD-1 (δppm)	β-CD-2 (δppm)
3.34 H-4	5.75 of OH-2
4.45 of OH-6	5.75 of OH-2

studies. The distance between OH-37 and OH-13 of NM was found out to be 6 Å as shown in Fig. 5 whereas diameter of narrow end of β-CD cavity was found to be 11 Å. Figure 5 also showed that S–Ph ring and hydroxyls groups (37-OH and 13-OH) are present

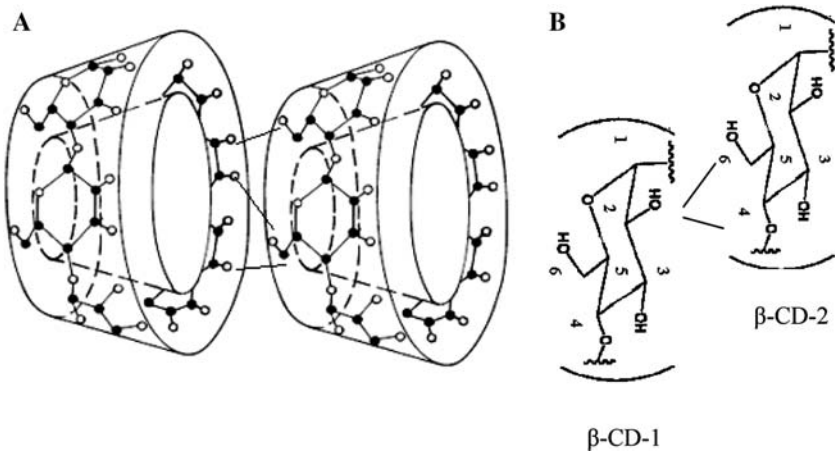

Fig. 4 Possible interactions amongst two β-CD molecules

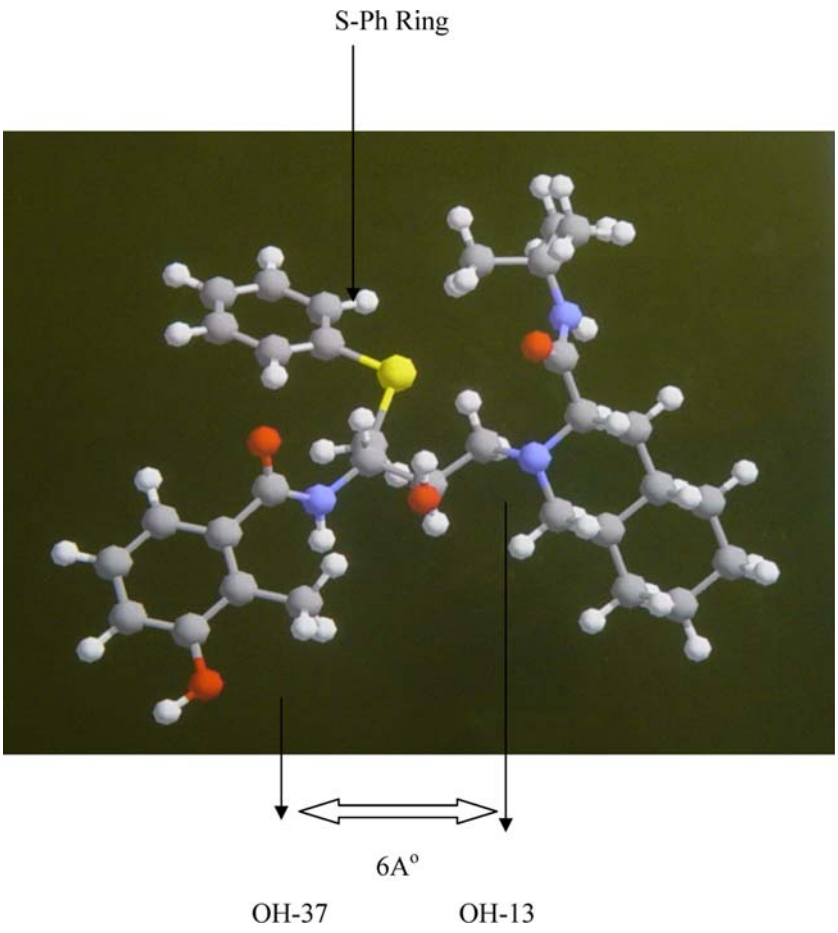

Fig. 5 Structure of NM

in different planes indicating possibility of having interactions with two different β-CD molecules.

Hydroxyl groups of NM at 37 and 13 position and S–Ph ring are present in different planes. Hydroxyl groups present in NM at 37 showed cross peaks with OH-2 and OH-6 β-CD molecule/molecules. Whereas hydroxyl group at 13 position showed cross peaks with OH-3 and OH-6 β-CD molecule/molecules. The δppm shift of C-S in NMR studies is indicative of participation in complex formation. Since these groups are located on different sides there is possibility of both hydroxyls group and S–Ph ring involved in the complexation as shown in Fig. 6.

In ITC studies, the set 1, where the titration was conducted using 0.004 mM of β-CD in sample cell and 0.023 mM of NM in syringe. After every addition of NM solution in sample cell the change in heat content occurred in the systems are plotted as μcal/s. versus time. The studies were conducted for 25 injections. The system does not come to equilibrium that is it does not reach to zero. It indicates that all binding sites of β-CD molecule are not occupied by NM.

The set 2 was conducted with additional 25 injections in the same sample cell. All 50 additions (25 injections of set 1 and 25 from set 2) were processed with ConCat 32 software. It has been observed that the system does not show equilibrium even after ConCat-32 studies. The results are as shown in Fig. 7. This gives clear indication that there are more than one binding site on β-CD molecule where drug molecule and/molecules actually interacts.

The next set of experiment was conducted where concentrations of NM in syringe was increased from 0.023 mM to 0.045 mM with the assumption that with higher concentration of drug saturation of all β-CD binding site can be achieved relatively faster. These studies also did not reach to equilibrium.

The data analysis for all three sets involved checking for the possibility of fitting the data for various models like one set of binding model, two sets of binding model and Sequential binding site model. The fitting of particular model was decided based on standard deviation observed for binding constants of the complexation. The data analysis of all three sets shows best fit with Sequential binding site model indicating possibility of more than one binding site on β-CD molecule.

As discussed in the 2D NMR studies there is possibility of forming molecular aggregates of β-CD and NM molecule in tail to head fashion. ITC studies also supported possibility of formation of molecular

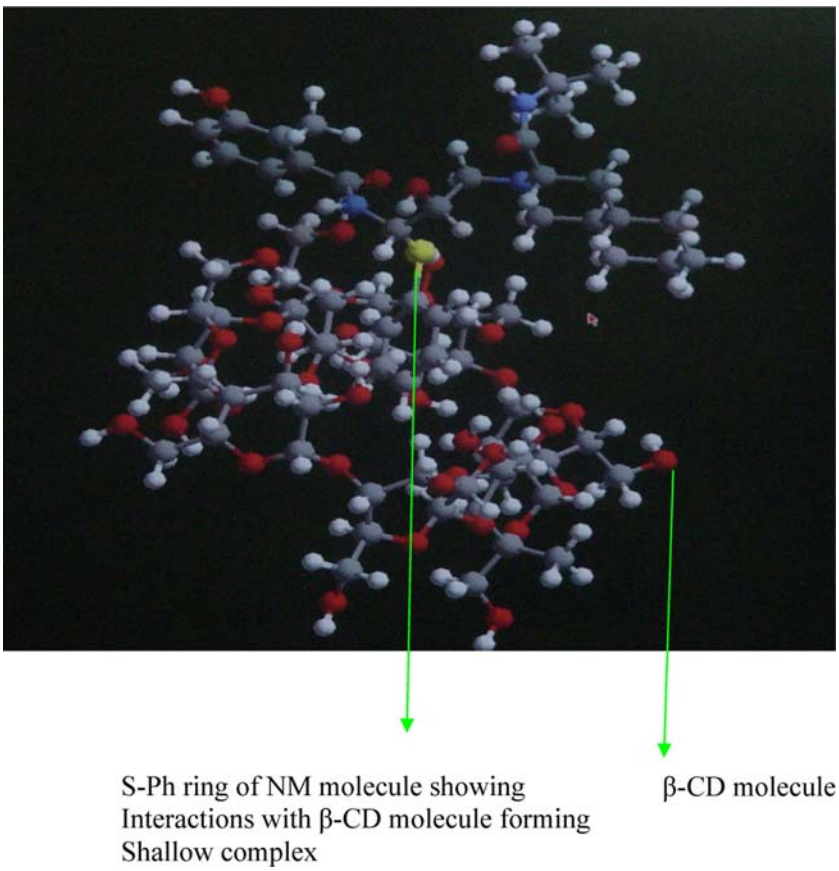

Fig. 6 Possible conformation of NM–β-CD complex indicating involvement of S–Ph ring with –OH groups of NM in complexation

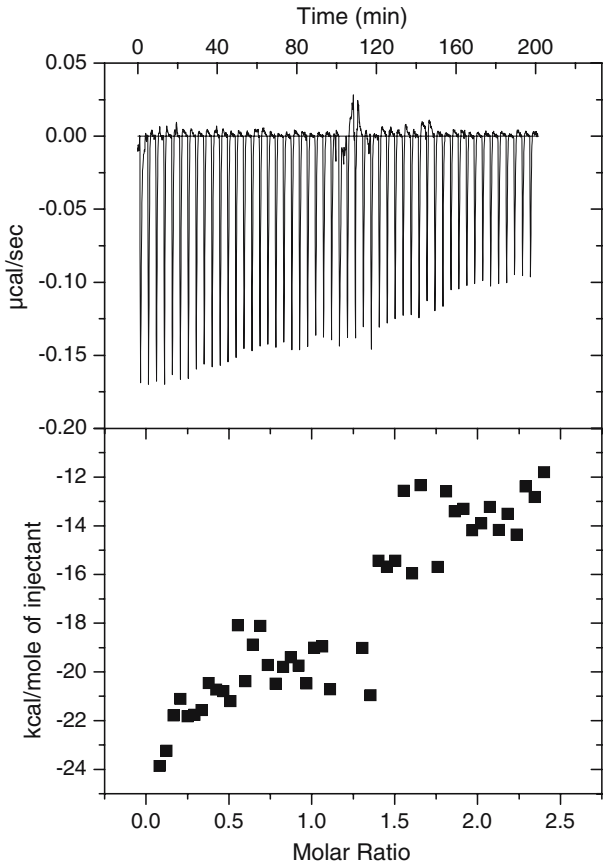

Fig. 7 Raw titration data and processed titration curve of SET-2

aggregates in solution as system is not getting saturated with respect to binding sites. The possible reason of non-equilibrium during titration could be sequential complexation and release of drug forming weak complex. Thus the process of reversible complex formation continues with formation of multiple bonding on β-CD molecule.

The solution (NMR, 2D NMR and ITC) state studies indicate possibility of presence of numerous structures giving rise to very complex geometry of the NM- β-CD inclusion complex. The multiple interactions between NM and β-CD further confirms to the fact of molecular aggregates in the solution.

The studies showed strong possibility of multiple binding on β-CD molecules thus supporting the fact that the complex exist as molecular aggregate when it goes in solution. The studies showed presence of shallow complex formation with volumetric fit of S–Ph ring of drug molecule with β-CD cavities. Thus the complex formation in solution state showed presence of more intricate behavior forming molecular aggregates.

Bioavailability of NM

Kinetic analysis of plasma levels of NM after administration of single oral dose of plain nm (400 mg/kg, group 1) and NM in the form of complex in rabbits (B. M., 1:1.5 M, 200 mg equivalent of NM/kg, group 3)

The percent relative bioavailability of NM in the form of complex from AUC was 185.37 as compared to plain drug. Hence the studies were conducted at reduced dose of 200 mg equivalent of NM in the form of complex/kg as mentioned in group 3.

The processed kinetic data of plasma levels in rabbits with statistical analysis of various pharmacokinetic parameters of group 1 (Plain NM, 400 mg/kg) and group 2 (NM in the form of complex, B.M., 1:1.5 M, 400 mg equivalent of NM/kg) are as shown in Table 4. The studies were performed up to 8 h.

The mean peak plasma concentration of complex at 200 mg/kg was found to be 1338.12 ± 133.14 ng/ml and that of plain drug was 1165.57 ± 337.16 ng/ml. These values were achieved in mean T_{max} values of 1.50 h and 5.33 h for NM in the form of complex and plain NM respectively. The AUC for NM in the form of complex and plain NM was 5419.58 ± 320.17 ng.h/ml and 5750.225 ± 1301.98 ng.h/ml, respectively.

The data analysis shows that there was not statistically significant difference in AUC, C_{max} and T_{max} values of complex at 200 mg/kg and plain drug at 400 mg/kg. The mean relative bioavailability of NM in the form of complex at reduced dose was found to be 94.24%.

Table 4 Comparison of pharmacokinetic parameters of NM plain (400 mg/kg) and NM in the form of complex (B.M., 1:1.5 M, 200 mg/kg) in Rabbits

Pharmacokinetic parameter	Plain drug (400 mg/kg)	Complex (200 mg/kg)	*T* statistics value
AUC (ng.Hr. /ml)	5750.22 ± 1301.98	5419.58 ± 320.17	2.017 (NS)
Kel (min^{-1})	0.3005 ± 0.07	0.1884 ± 0.0079	0.2801 (NS)
$T_{1/2}$ (h)	2.3948 ± 0.56	3.6796 ± 0.15	0.002579 (NS)
C_{max} (ng/ml)	1165.57 ± 337.16	1338.12 ± 133.14	0.000726 (NS)
T_{max} (h)	5.33 ± 2.51	1.5 ± 0.50	0.000125 (NS)

Critical *T* value ($\alpha = 0.05$) from statistical table is 3.18; S, Significant difference; NS, Non-Significant difference

Cyclodextrins enhance bioavailability of insoluble dugs by increasing the drug solubility, dissolution and/or drug permeability. Cyclodextrins increase the permeability of insoluble, hydrophobic drugs by increasing the amount of dissolved drug at the biological membranes. Thus dissolved drug rapidly partitions into the membranes and gets absorbed.

Thus the main aim of endeavor of reducing the dose by improving bioavailability could be successfully achieved by complexation of Nelfinavir Mesylate with β-Cyclodextrin. Significant dose reduction was achieved in animal species. Further more elaborative studies in human volunteers may help in determining the actual amount of required reduced dose thus it may help in changing the dosage regimen with reduced pill burden for the patient compliance. Since the amount of dose will be reduced this may further substantiate the potential of the new delivery system being economic, affordable and value added formulation over existing formulations.

References

1. Perry, C.M., Benfield, P.: Nelfinavir. Drugs **54**(1), 81–87 (1997)
2. Jarvis, B., Faulds, D.: Nelfinavir: a review of its therapeutic efficacy in HIV infection. Drugs **56**(1), 147–167 (1998)
3. Physicians Desk reference, 54th edn., Published by Medical economics Co., White house station, NJ, (2000)
4. Szente, L., Szetli, J., Kis, G.L.: Spontaneous opalescence of aqueous -Cyclodextrin solutions: Complex formation or self-aggregation? J. Pharm. Sci. **87**, 778–781, (1998)
5. Gonzalez-Gaitono, G., Rodriguez, P., Isasi, J.R. et al.: The aggregation of cyclodextrins asstudies by studies by photon correlation spectroscopy. J. Inclusion. Phenom. Macroc. Chem. **44**, 101–105, (2002)
6. Sigurdsson, H.H., Magnusdottir, A., Masson, M., Loftsson, T.: Self association and cyclodextrin solubilization of NSA-IDs. J. Inclusion phenom. Macroc. Chem. **44**, 213–218, (2002)
7. Loftsson, T., Sigurjonsdottir, J.F., Magnusdottir, A., Masson, M.: Self-association and cyclodextrin solubilization of drugs. J. Pharm. Sci., **91**, 2307–2316, (2002)
8. Loftsson, T., Masson, M., Sigurdsson, H.H.: Cyclodextrins and drug permeability through semi-permeable cellophane membranes. Int. J. Pharm. **232**, 35–43, (2002)
9. Duchene, D., Bochot, A., Yu, S.-C., Pepin, C., Seiller, M.: Cyclodextrins and emulsions. Int. J. Pharm. **266**, 85–90, (2003)
10. D. Liu, Y., Chen, G.S., Li, L., Zhang, H.Y., Cao, D.X., Yuan, Y.J.: Inclusion complexation and solubilization of paclitaxel by bridged Bis(-cyclodextrin)s containing a tetraethylene-pentaamino spacer. J. Med. Chem. **46**, 4634–4637, (2003)
11. Nishijo, J., Tsuchitani, M.: Interaction of L-tryptophan with -cyclodextrin: studies with calorimetry and proton nuclear magnetic resonance spectroscopy. J. Pharm. Sci. **90**(2), 134–140, (2001)