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Inclusion Complexation of Anti-HIV Drug with β-Cyclodextrin

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Abstract

The purpose of present investigation was to investigate the effect of complexation of Nelfinavir Mesylate (NM) – an Anti-HIV drug with Beta-cyclodextrin (β-CD) on its dissolution characteristics and subsequent effect on its absorption properties and bioavailability. Phase solubility studies were conducted to find the interaction of NM with β-CD. Physical mixing and milling method were used for complexation. The inclusion complexes were characterized by X-ray diffractometry, FT-IR and NMR studies and further studied by *in-vitro* dissolution testing. The plain NM and complex was subjected to intestinal absorption studies by using Everted intestinal sac model. Data was treated statistically by Mann–Whitney *U* test. Pharmacokinetic studies were carried out in rabbits using cross over design and data was treated by Student's *t* test. Phase solubility studies confirmed 1:1 complex formation of NM with β-CD with stability constant of 204.84 M^{-1}. *In-vitro* dissolution studies of inclusion complexes of NM with β-CD prepared by milling method ($T_{90} = 60.89$ min) showed better dissolution rate kinetics in distilled water in comparison with plain NM ($T_{90} = 374.31$). The increased solubility with decreased crystallinity is attributed by inclusion of NM in the cavity of β-CD, which was further confirmed by instrumental studies. Intestinal absorption studies further supports these findings by showing 2.13 times enhancement in the absorption rate of complex as compared to plain NM. The percent relative bioavailability of complex in rabbits was 185.37 as compared to the plain NM.

Introduction

Nelfinavir Mesylate (NM) is HIV protease inhibitor exhibit good inhibitory activity against HIV-1 [1, 2]. NM is selective inhibitor of HIV protease thus helps in formation of immature, non-infectious virus particles. In combination with Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and/or other protease inhibitors it profoundly suppresses viral replication. The mean oral bioavailability of Nelfinavir ranged from 17 to 47% in various animal species [3, 4].

Intrinsic dissolution rate of NM in simulated gastric fluid is 0.09 mg min^{-1} cm^{-2} which indicates potential for dissolution rate limited absorption. The p*K*a of NM is 6.0 and $\log P_{\text{octanol/water}}$ of 4.1 [5, 6].

The recommended dosage for NM is 1250 mg (Two 625 mg tablets or Five 250 mg Tablets) twice daily or 750 mg (Three 250 mg Tablets) three times daily. It is also available as Oral Powder (50 mg/gm). Along with NM a patient has to take other medications also and thus there is heavy pill burden on AIDS patient.

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The entire research work was undertaken with broader objective of reduction of cost of anti-HIV therapy. It can be achieved by enhancement in solubility with improved dissolution rate kinetics thus helping in improved rate of absorption of drug. Thus it will help to enhance bioavailability and subsequent reduction of dose and hence the cost reduction.

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.

Phase solubility studies

The solubility of NM in presence of increasing concentration of β-CD in water was determined by the method of Higuchi and Connors [7]. The value of stability constant was also determined.

Preparation of inclusion complexes

Physical mixing
NM and β-CD were mixed in the different molar ratios (1:0.5, 1:1, 1:1.5, and 1:2). Both the ingredients were mixed thoroughly and passed through sieve # No. 40.

Milling method
NM and β-CD were mixed in the different molar ratios (1:0.5, 1:1, 1:1.5, and 1:2). Both the ingredients were mixed thoroughly and subjected to milling at 50 revolutions min^{-1} for 4, 6, 8, 12, 16, 20, 24 h. NM alone was also subjected to milling by keeping all other parameters constant for comparison.

Characterization of complexes

X-ray diffraction studies
The XRD pattern was recorded using a Philips X-ray generator (PW 1729 Eindhoven, The Netherlands) and automatic X-ray diffractometer model PW 1710 unit. The radiation used was Nickel filtered $CuK_{\alpha 1}$ radiation with a wavelength of 1.542. The scanning rate was 1.2° min^{-1}.

Fourier transform infra-red spectroscopy

β-CD, NM, physical mixture of NM and β-CD and milled complex were subjected to FTIR spectroscopic studies using KBr disc method using JASCO FT-IR-5300 Spectrophotometer.

Proton nuclear magnetic resonance spectroscopy (^1H NMR)

The ^{1}H NMR measurements were performed with a Bruker FT-NMR spectrophotometer operating at 500 MHz. Pure NM, β-CD and complexes were subjected to ^{1}H NMR studies. The solvent used was dimethylsulfoxide (DMSO-d_6).

In-vitro *dissolution testing*

Dissolution testing of powdered samples were performed according to USP 23 type II apparatus in distilled water (900 ml) rotated at 100 RPM at 37 ± 0.5 °C. The samples were withdrawn at various time intervals and analyzed spectrophotometrically (Cecil CE 2021 2000 series) at 250 nm for content of NM.

Acute toxicity studies

This study was undertaken to investigate the acute toxicity of NM–β-CD complex as compared to plain NM. OECD guideline 423 was used to study the acute toxicity in albino mice. The study was conducted with starting oral dose of 2000 mg/kg of plain NM as well as complex in different groups of animals.

Intestinal absorption studies

The rate of intestinal absorption of NM and its inclusion complex (1:1.5) was investigated by *in-vitro* rat everted intestinal sac model [8, 9]. About 1.4 ml tyrode solution was used as serosal effluent. The mucosal effluent comprised of 40 ml of 20 μg/ml solution of plain NM and complex in tyrode. At predetermined time intervals the serosal solution was removed and analyzed by developed HPLC method. The data obtained was treated statistically by Mann–Whitney U test, a non-parametric test for two independent samples.

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. $\times$ 250 mm, Kyatech Corp.). The sample volume was 20 μl at 25 °C. Mobile phase used was Acetonitrile: Potassium dihydrogen Phosphate (0.03 M), 56:44 v/v at pH 3.24. The analysis was performed at 250 nm at room temperature.

Pharmacokinetic studies

The study was undertaken to investigate pharmacokinetcs of NM and NM in the form of inclusion complex (1:1.5) in rabbits using cross over design. The dose is administered orally as NM in the form of suspension and NM–β-CD complex as colloidal solution. Blood samples were collected at 0, 1, 2, 3, 3.5, 5, 8 h. Plasma was separated immediately and the drug content was determined by developed HPLC method.

Various pharmacokinetic parameters were calculated. The oral bioavailability was calculated by comparing the mean area under the plasma concentration time curves (AUC_{0-8}). Statistical analysis was performed by using Student's t test $p < 0.05$ was considered significant.

Extraction of drug from plasma

Equal volumes of drug containing plasma was mixed with 0.01N sodium hydroxide solution. NM was extracted using Ethyl acetate: Acetonitrile (90:10). After vortexing for 7 minute the organic phase was separated and evaporated under nitrogen atmosphere. Finally it was reconstituted with 200 μl of mobile phase. 100 μl of sample was injected. The extraction efficiency was found to be 85%. The analysis was performed at 215 nm at room temperature.

Analytical procedure

The method specific for NM content as explained above was used.

Result and discussion

The phase solubility studies confirm formation of 1:1 complex forming A_L type of curve with stability constant of 204.84 M^{-1}.

The XRD pattern as shown in Figure 1 of β-CD, NM, Physical mixture and complex shows that the XRD of complex was more diffused and different than plain NM. Plain NM shows peaks at 8.80, 12.38, 18.60, 22.0, 25.5, 32.3°. Change in intensity counts is as shown in Table 1. The studies confirm formation of new solid phase.

The FT-IR records of various samples are shown in Table 2 and Figure 2. The spectra of the physical mixture of NM with β-CD showed summation effect of the peaks. The spectrum of NM and β-CD complex showed appearance of intense broad peak at 3404.4 cm^{-1} indicating possible hydrogen bonding between NM and β-CD. Shifts are seen in the peaks of –C=O, –C–C stretching, C–S stretching and –N–H as well indicating that these groups are participating in the complex formation.

The NMR studies indicate interaction at –OH positions of drug with –OH groups of β-CD lining the inner cavity. The ^{1}H NMR spectra of various samples are as shown in Figure 3. The chemical structure of NM and change chemical shifts of NM after complexation are as shown in Figures 4 and 5 respectively.

The milled samples showed a faster rate of dissolution in order of 1:0.5 < 1:1 < 1:1.5≤1:2 as shown in Table 3. The T_{90} (time required for 90% of the NM to dissolve) values of the complex containing 1:0.5, 1:1 were greater than the T_{90} value of Milled complex of 1:1.5 as shown in Table 3.

The complexes containing 1:1.5 and 1:2 had faster T_{90} value compared with T_{90} values of unmilled NM, milled NM and other milled mixtures. Comparative dissolution rate kinetics of NM and Complex (1:1.5) is as shown in Figure 6. The most important fact which was observed in *in-vitro* dissolution testing is that complex of 1:1.5 ratio shows better dissolution kinetics than complex with 1:1 molar ratio. Possible explanation for this type of phenomena could be that after complexation of NM its solubility was increased to 70%. During dissolution studies of complex of 1:1 molar ratio, the drug does not dissolve completely hence fraction of the drug remains undissolved. During dissolution studies of complex of 1:1.5 molar ratio, undissolved NM is present along with higher amount of cyclodextrin. Because of presence of more amount of cyclodextrin there could be formation of water soluble aggregates and these aggregates could be able to solubilize more amount of lipophilic water insoluble NM through non-inclusion complex formation. Thus solubility of complex of 1:1.5 molar ratio was 84.24 ± 3.33%. Hence complex of 1:1.5 molar ratio was explored for further studies.

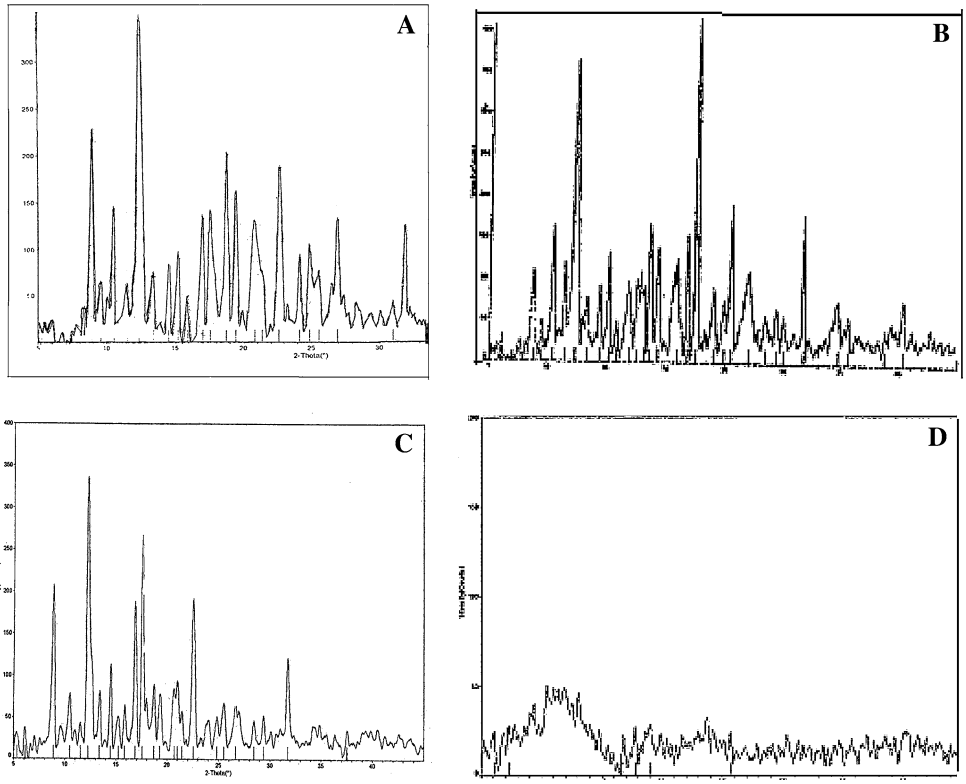

Figure 1. X-ray diffractograms. (A) β-CD, (B) NM, (C) Physical Mixture, (D) NM: β-CD complex.

Table 1. Change in intensity counts of NM after complexation

(2θ)	Counts		Δ (2θ)
	NM	Complex	
8.80	103	37	66.0
12.38	353	111	220
18.60	156	37	119
22.0	412	27	385
25.5	175	25	150
32.3	160	20	140

Table 2. FT-IR records of NM and its inclusion complex

Group assignment	Wave number (cm^{-1})		δ cm^{-1}
	NM	NM in the form of complex	
Aromatic C–C stretching	1520.0	1524.8	4.8
N–H	1430.0	1439.0	9.0
C=O	1722.0	1728	8.0
C–S Stretching	700.4	715.7	15.7

The optimum time required for complex formation was found out to be 6 h. Increase in the milling time further does not improve solubility which could be because of development of static charges which might give rise to particle agglomeration and subsequent slower dissolution. At 1:2 ratio complex there was not considerable improvement in the solubility as seen from the Table 3. Milling of plain NM showed slight improvement in the solubility. This is probably due to the fact that increase in milling time leads to size reduction with increase in surface area.

Acute toxicity studies confirms that complex is equally safe as plain NM above 2000 mg/kg in mice.

As shown in Figure 7 the rate of NM absorption from its inclusion complex with β-CD was found to be 2.13 times more than plain NM. The data was treated at $\alpha = 0.05$ with N_1 and N_2 values at 6 as shown in Table 4. The critical value for Mann–Whitney U test for two-tailed experimental design was 5. Since the calculated value of U is smaller than critical value it can be deduced that there is statistically significant difference in the absorption of drug and complex. The results of this study suggest rapid absorption of NM across the intestine when in complexed form as compared to uncomplexed NM.

The percent relative bioavailability of NM in the form of inclusion complex from AUC was 185.37 as compared to the plain NM as shown in Table 5. Mean plasma concentrations of plain NM after single oral dose administration in rabbit in comparison with NM in the form of inclusion complex formulation is represented in Figure 8.

Conclusion

We can conclude that β-CD can be used for complexation with NM for solubility enhancement. Considerable

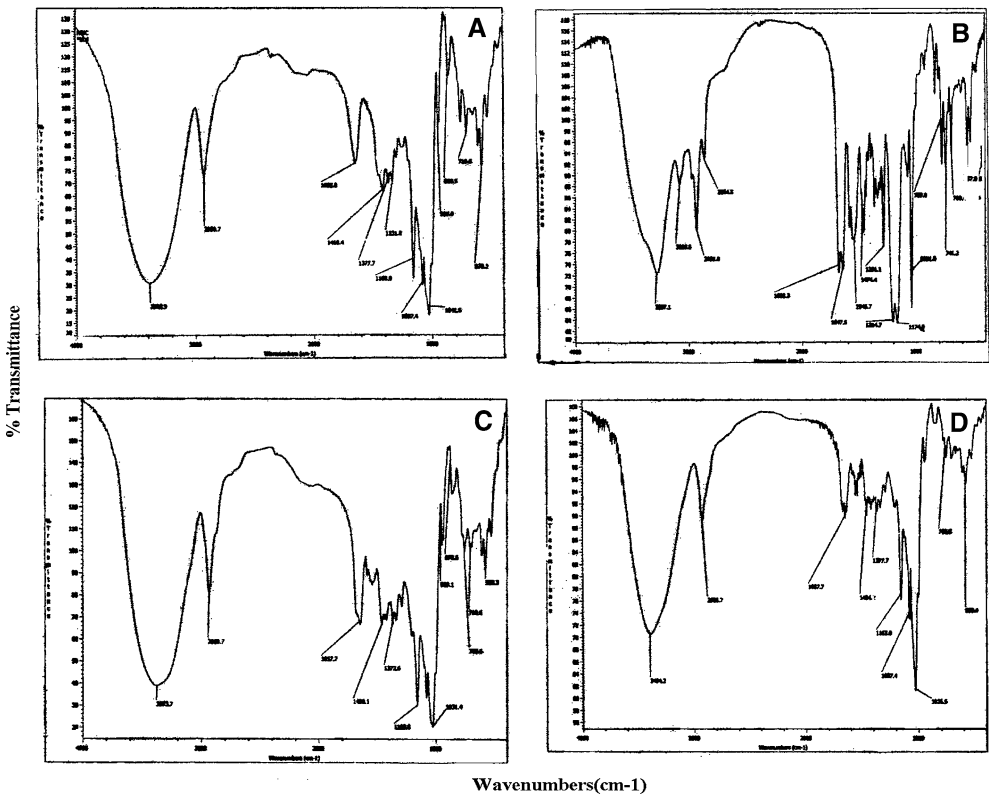

Figure 2. Fourier Transform-Infra Red Spectra. (A) β-CD, (B) NM, (C) Physical Mixture, (D) NM: β-CD complex.

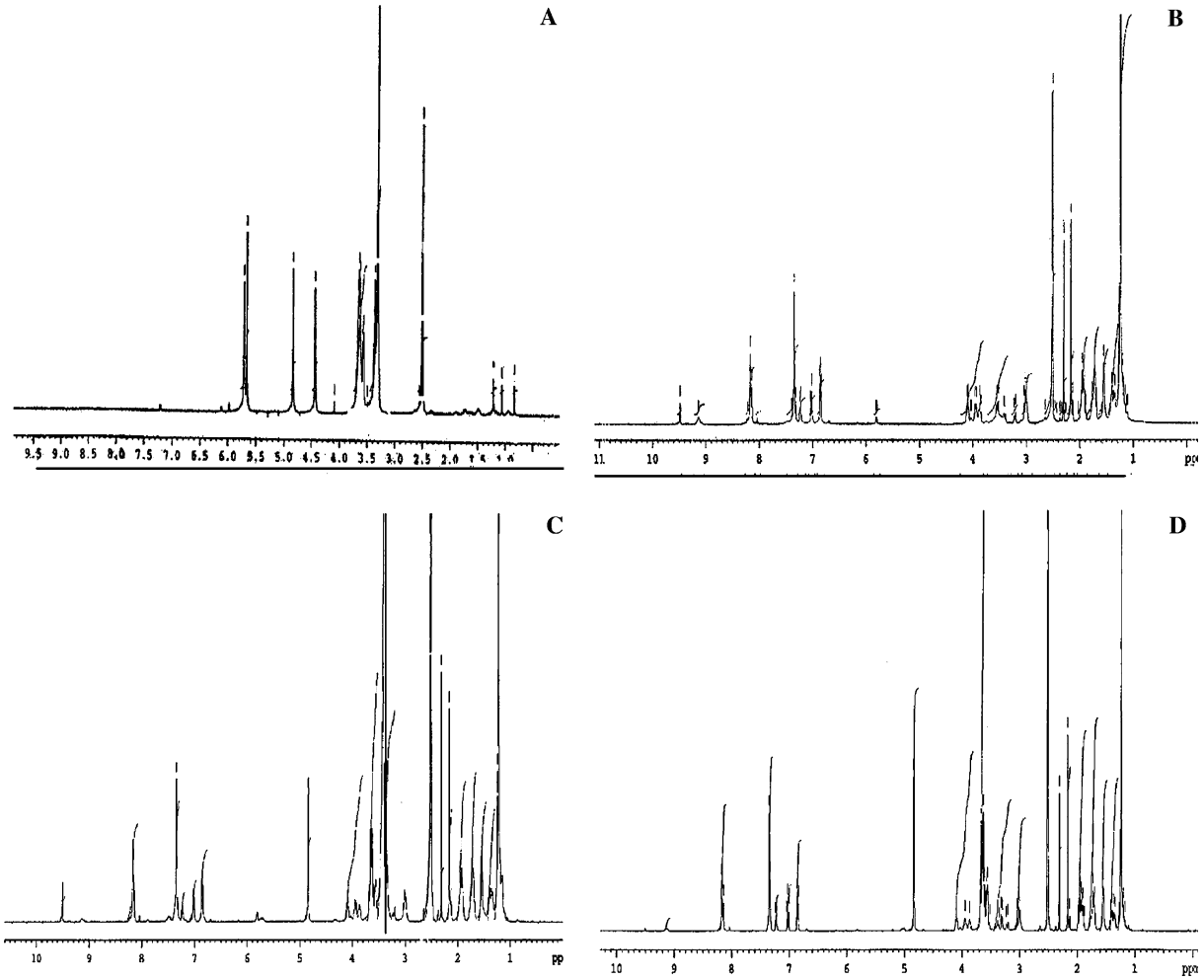

Figure 3. NMR Spectra. (A) β-CD, (B) NM, (C) Physical Mixture, (D) NM: β-CD complex.

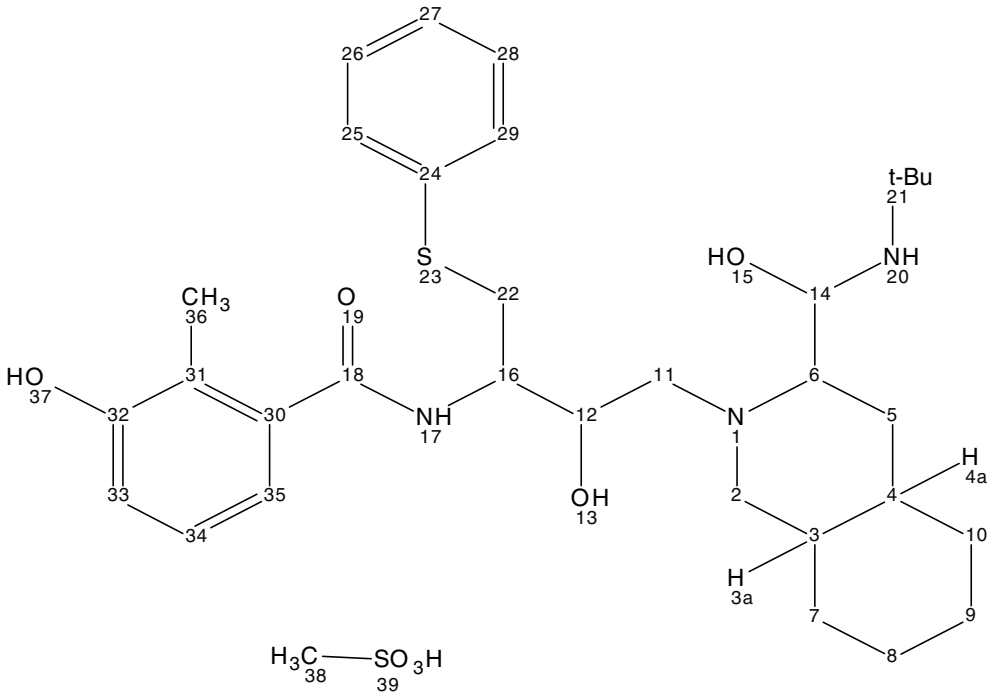

Figure 4. Chemical structure of Nelfinavir Mesylate.

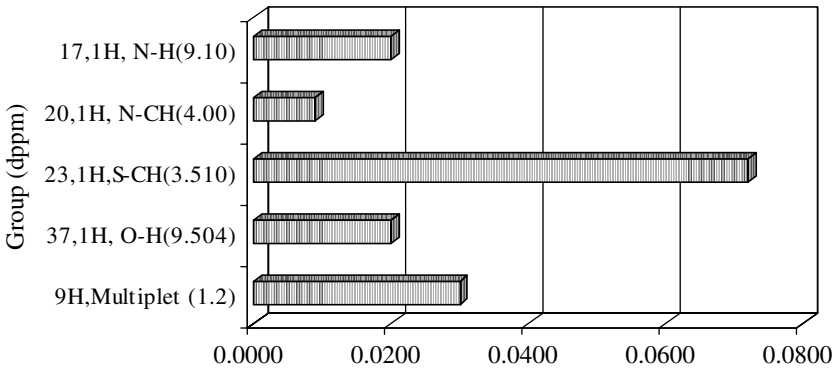

Figure 5. Change in chemical shifts ($\Delta\ \delta$ ppm) of NM in inclusion complex.

Table 3. Dissolution parameters of NM, NM: β-CD complexes of various molar ratios

Powders	T_{90}	K (r)
NM	374.31	0.0025 (0.81)
NM,*	331.04	0.0027 (0.84)
NM: β-CD, ^	235.38	0.0029 (0.89)
NM: β-CD,1:0.5,#, *	171.39	0.0048 (0.88)
NM: β-CD,1:1,#, *	85.38	0.0065 (0.84)
NM: β-CD,1:1.5,#, *	60.89	0.0062 (0.86)
NM: β-CD,1:2, #, *	77.79	0.0050 (0.80)

T_{90}: Time required for 90% of the drug to dissolve, *: Milled powder, ^: Physical Mixture, #: Milling for 6 h, Ratio indicates quantities of NM and β-CD on molar basis.

increase in bioavailability of the complex formulation can be used to reduce the dose of drug. Thus, the proposed complex formulation can be an important new means which could offer new dosage regimen thus may help to improve patient compliance by reducing pill burden which will further help to improve adherence of patient to the therapy.

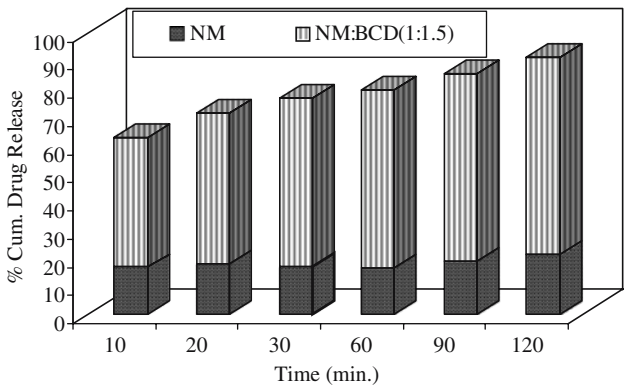

Figure 6. Dissolution Rate Kinetics of NM and complex of 1:1.5 Molar ratio in water.

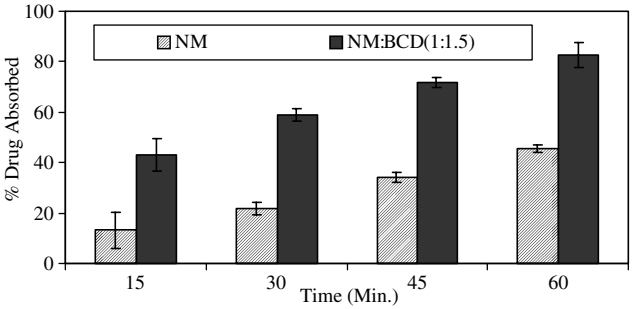

Figure 7. Rate of Intestinal absorption of NM plain and NM in the form of complex with β-CD through rat intestine ($n=6$).

Table 4. Intestinal absorption studies

Time (Min.)	NM Plain (%)	NM- β-CD Complex (%)	$U_{Calculated}$
15	29.27 ± 5.39	33.07 ± 7.85	5.20 (NS)
30	32 ± 5.88	48.12 ± 9.87	3.00 (S)
45	34.29 ± 7.11	68.79 ± 10.23	0.56 (S)
60	45.52 ± 8.56	82.60 ± 3.21	0.14 (S)

Critical value for $U_{statistics}$ for two-tailed experimental design from table is 5. S = Significant difference, NS = Non-significant difference.

Table 5. Comparison of pharmacokinetic parameters after administration of NM in the form of complex and NM plain in rabbit

Parameters	NM–β-CD Complex ± (s.d.)	Plain NM ± (s.d.)	$T_{Statistics}$ Value
C_{max} (ng/ml)	2993.24 ± 256.98	1166.74 ± 60.44	4.02 (S)
T_{max} (Hr)	2	3.5	1.76 (NS)
K_{el} (min^{-1})	0.0489 ± 0.042	0.0419 ± 0.035	0.065 (NS)
$T_{1/2}$ (Hr)	2.95 ± 0.35	2.86 ± 0.078	0.25 (NS)
AUC_{0-8} (ng·Hr/ml)	9634.63 ± 636.12	5197.29 ± 658.03	7.28 (S)

Critical T value ($\alpha = 0.05$) from statistical table is 2.3. (S = Significant difference, NS = Non-significant difference).

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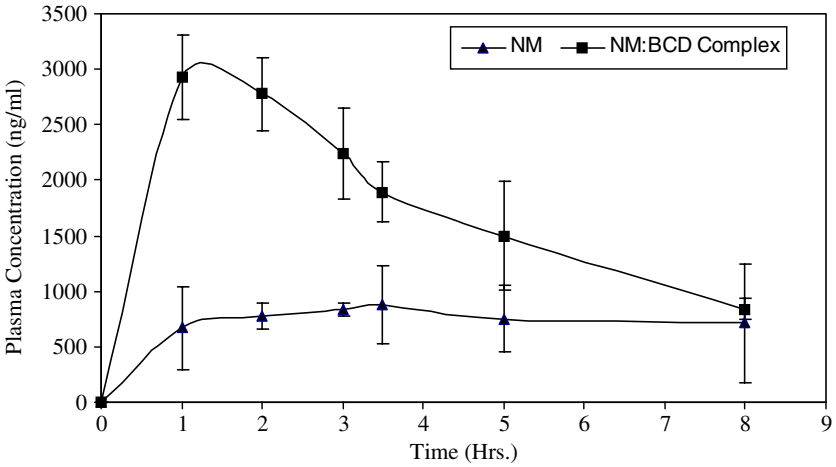

Figure 8. Mean plasma concentrations of NM and NM in the form of Inclusion complex after single oral dose administration in rabbit.

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