International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2019): 7.583

Development and Evaluation of Nefidipine Loaded Nanostructured Lipid Carriers (NLCs) For Bioavailability Enhancement

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Abstract: ***Nanostructured Lipid Carriers (NLCs) are new generation of lipid Nanoparticle system, which have showed various advantages over conventional Solid lipid nanoparticles as drug carrier, such as enhanced drug incorporation and release properties. NLCs of Nefidipine were prepared and investigated to estimate the potential of NLCs as an oral drug-delivery system. In this work, solvent injection technique was used to formulate Nefidipine–loaded NLCs. The Nefidipine loaded NLCs showed smooth surface with spherical morphology under scanning electron microscope (SEM) and transmission electron microscope (TEM) analysis. The higher encapsulation efficiency observed was 90.65±0.5%. In In-Vitro release study, Nefidipine loaded NLCs exhibited a sustained release profile of Nefidipine and no burst release was observed. The oral bioavailability study was performed by using Wistar Albino rats. The relative bioavailability of Nefidipine loaded NLCs was found to be 3.9. In conclusion, the NLC formulation significantly enhanced the oral bioavailability of Nefidipine and revealed a positive aspect for oral delivery of poorly water soluble drugs.***

Keywords: Nefidipine, NLCs, Characterization, *In Vitro* Drug Release, *In Vivo* Drug Release

1. Introduction

Novel drug molecules are being investigated into the pharmaceutical industry day by day but the production of only new drugs are not sufficient to assure the advancement in drug therapy. Usually, the common issue faced is low-solubility of drug molecule which eventually leads to the low bioavailability. Therefore, it is essential to enhance the bioavailability for which the development of a drug carrier system is required that overcomes these drawbacks. The novel carrier system should have some essential properties such as no toxicity, should have adequate drug loading capacity and the feasibility of drug targeting and controlled release. The carrier system should chemical and physical stable for the encapsulated drug [1-3]. Lipid based nano-particles preparation may also increase the drug absorption by enhancing dissolution and solubility in the intestinal milieu, by decreasing gastric emptying rate and enhancement in mucosal permeability. Lipids are used to increase lymph formation and also enhance lymph flow rate [4]. NLCs are formulated by biocompatible and biologically utilizable solid and liquid lipids which have different chemical structure from the solid lipid [5]. Besides, NLCs have the usual particle diameter range of approximately 10–1000 nm. NLCs drug delivery system have many benefits like high biocompatibility, controlled drug release, high bioavailability, the possibility of large industrial scale production and no problems with multiple route of administration, such as oral, intravenous, pulmonary and transdermal administration [6-11]. However, the different types of lipid NLC components results in the imperfections type structures, amorphous state type or multiple type to adjust more drug and reduce the drug leakage during storage [12]. Poorly water soluble drugs loaded by lipid formulations have been observed for oral route and have evaluated to for enhancement in the oral bioavailability [13,14] but there are very less reports for oral administration on NLC system.

Nifedipine, a calcium channel blocker utilized in hypertension treatment. It also have antioxidant effect and is the most vascular selective dihydropyridine. Nifedipine shows high first-pass hepatic metabolism and have about 45% of bioavailability. The complete metabolism of Nifidipine takes place in the liver by cytochrome P450 3A4 to pharmacologically inactive metabolites [15]. Nifedipine has low aqueous solubility and could be classified as a BCS class II drug.

2. Materials and methods

2.1 Materials

Nifedipine was a kind gift from Balaji chemicals, Surat India. Glycerol monostearate (M.P. 52–54°C; molecular weight 358.63) was purchased from Balaji chemicals, Surat India. Poloxamer 188 (molecular weight 12.5) was purchased from Crystal Chemicals, Gangtok, India. Oleic acid was purchased from Otto Chem products, India. Other chemicals were of analytical grade.

2.2 Preparation of Lacidipine loaded NLCs

Nifedipine loaded NLCs were prepared by Solvent injection technique with slight modification [16,17]. Nifedipine, specified amount of Glycerol monostearate (GMS) and oleic acid as given in Table1 were dissolved in 4 ml of isopropyl alcohol (boiling point 81–83°C) and heated at the melting temperature of GMS. The resultant organic solution injected rapidly in 20 ml of aqueous phase having specified amount of poloxamer 188 as specified in Table1. Then, it was continuously stirred at 400 rpm for 30 min on a magnetic stirrer and then 0.1 N HCl (8 ml) was added to the dispersion. Thereafter, centrifugation was done at 10,000 rpm for 30 min at 10°C in REMI cooling centrifuge (Model C- 24BL, VACO-779, Vasai, India), and 4% poloxamer 407 (by weight) in 10 ml double distilled water was used to re-suspend the aggregates with stirring at 1000 rpm for

10min[16]. Purification of NIfedipine loaded NLCs was done by dialysis technique. In the dialysis bag re-suspended suspension was taken and sealed at both ends. The bag was then dipped into 100 ml of double distilled water having 0.2% (w/v) sodium lauryl sulphate and stirred at 100 rpm for 20 min. The un-entrapped drug was removed in 20 min. The HPLC was performed by using Younglin HPLC model equipped with Binary pump. A mixture of metanol: water (85:15 v/v) was used as mobile phase [18]. The flow rate of mobile phase, injection volume and detection wavelength were 1.0 ml/min, 20µl and 350nm respectively. Nifedipine showed linear calibration curve with R2 =0.994 in the range 20-100µg/ml.

Table 1: Formulation Design of NLCs

Formulation code	Amount of drug (mg)	Amount of oleic acid (mg)	Amount of Glycerol monostearate (mg)	Surfactant concentration (%)
F1	30	10	100	0.8
F2	30	15	100	0.8
F3	30	20	100	0.8
F4	30	20	100	0.8
F5	30	30	100	0.8
F6	30	20	100	0.8
F7	30	20	150	0.8
F8	30	20	200	0.8
F9	30	20	150	0.8
F10	30	20	150	1.0
F11	30	20	150	1.2
F12	10	20	150	1.0
F13	20	20	150	1.0
F14	30	20	150	1.0
F15	40	20	150	1.0
F16	50	20	150	1.0

2.3 Characterization of NLC

2.3.1 Particle size

The particle size of nanostructured lipid carrier formulations was measured by digital microscope (BA-310, Motic, USA). Nanostructured lipid carriers were dispersed in 10ml of water. From the dispersion of NLCs, a drop of sample was placed on glass slide and covered with cover slip. The prepared slide was then analyzed by the digital microscope of 40X magnification. The size of NLCs were also analysed by Zetasizer at 25°C at an angle of 90°, taking the average of three measurements.

2.3.2 Drug Entrapment Efficiency (DEE %)

The drug entrapment was calculated by RP-HPLC method using metanol: water (85:15 v/v) as a mobile phase. 1ml of Nifedipine loaded NLCs colloidal solution was centrifuged for 10 min at 4000rpm. After the filteration of the solution through a 0.45µm membrane filter, it was analysed by HPLC [19]. Drug entrapment efficiency (DEE) of nanostructured lipid carriers was calculated by utilizing the following equation;

$$\textbf{DEE}\ (\%) = \frac{\textbf{Total amount of drug recovered}}{\textbf{Total amount of drug added}} \times \textbf{100}$$

2.3.3 In vitro release studies

It needs to be verified *in vitro* that NLCs are able to release incorporated drug in order to achieve a biological effect. The dialysis technique was utilized for *in vitro* drug release from the NLCs [20]. Dialysis bag of cellulose dialysis membrane (MW cut- off 10,000 Da) was soaked in the distilled water overnight and then 1ml of NLCs suspension loaded by drug was taken in dialysis bag with both the ends sealed with threads. Initial studies were carried out in 100 ml of 0.1N HCl (pH 1.2) for 2 hours and then in phosphate-buffered saline (PBS) pH 6.8 at 37°C on magnetic stirrer moving at a speed of 50 rpm for 24hrs [21]. The pH was adjusted with 2N HCl or 2N NaOH. Samples were withdrawn at predetermined time intervals and replaced with fresh release media. Samples were filtered and analysed by using HPLC at λmax of 350 nm to determine the amount of drug released from the formulation at different time points [22]. All the values obtained were expressed as mean ± standard error mean (S.E.M.). Each data represents mean ± SD (n=3).

2.3.4 Scanning electron microscopy

Scanning electron microscopy was utilized to examine surface morphology of Nifedipine loaded NLC [23]. Before observation, the nanoparticles were fixed on a double-sided sticky tape which had previously been secured on aluminum stubs and then coated with gold with thickness about 450 Å by utilizing Sputter gold coater and were analyzed under scanning electron microscope.

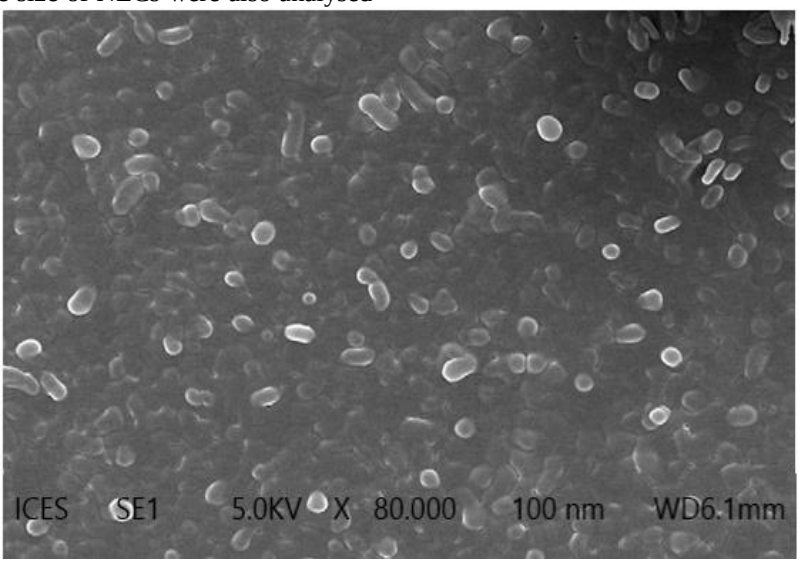

Figure 1: SEM image of nanostructured lipid carriers

2.3.5 Transmission electron microscopy

The morphology of Nifedipine loaded NLCs colloidal solution was analyzed by transmission electron microscopy. The sample was formulated by placing a drop of preparation which was diluted with double-distilled water on to copper grid coated with carbon film and followed by negative staining with 2% phosphotungstic acid. Then the sample was dried in the air before TEM observation (Figure 2).

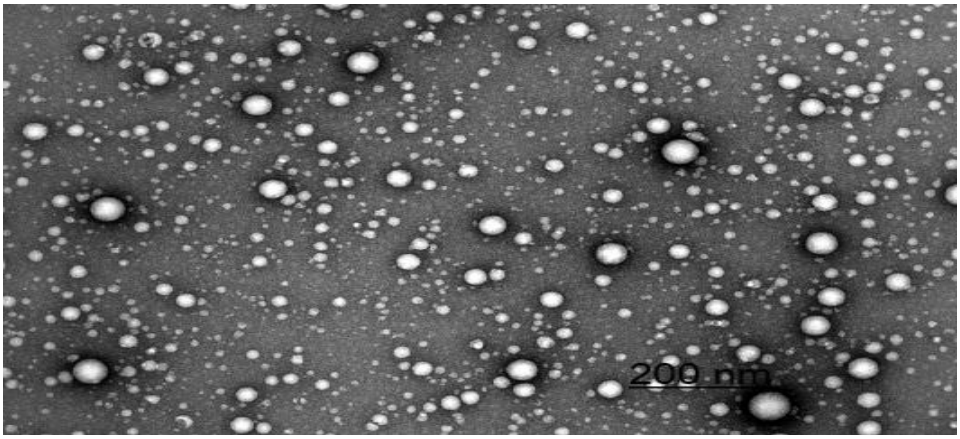

Figure 2: TEM image of nanostructured lipid carriers

2.4 In vivo pharmacokinetics

In vivo pharmacokinetic study was carried out by using male albino rats. These rats were fasted overnight with free access to water before drug administration. Nifedipine suspension and Nifedipine-NLCs suspension, these two types of formulations were administered orally to the rats. The administration dose of Nifidipine was 2 mg/kg. At defined time points (1, 2, 4, 6, 8, 12 and 24 h), the collected blood sample were centrifuged for 10 min at 4000 rpm **[18]**. The withdrawn blood sample volume was replaced immediately with an equal volume of physiological saline. The tubes were placed in a centrifuge for 20 min at 3000 rpm to separate out the serum and then serum was stored at -20°C until drug analysis was carried out by utilizing HPLC. The standard pharmacokinetic parameters were collected from each of the individual rat plasma and plasma concentration Vs. time profiles of Nifedipine were determined by non compartmental method utilizing the Win Nonlin computer program. The C_{max} and t_{max} were calculated from the plasma concentration vs. time graph of NIfedipine. The trapezoidal method was used to obtain the AUC. The $t_{1/2}$ was calculated by linear regression of log linear portion of the plasma concentration time profile. The apparent plasma clearance (CL) was determined by dividing the dose with AUC. Win Nonlin software was utilized to calculate the mean residence time (MRT). The relative bioavailability of NLCs formulations was determined by using the following formula:

$$\textbf{Relative bioavailability} = \frac{\textbf{AUC sample}}{\textbf{AUC standard}}$$

3. Results and Discussion

NLCs were successfully formulated by solvent injection technique that is control by rapid diffusion of the solvent over the solvent–lipid interfaced with the aqueous phase and this physical phenomenon is critical for the precipitation of nanosized lipid particle. The prepared small size NLCs may couple with low density of lipids. To overcome this problem, the pH was reduced to 1.5–2 to maintain the zeta potential to a level that raise the aggregation of nanoparticles. Purity of the product gained is another significant aspect in the formulation of NLCs. A feasibility of existence of free Nifedipine particles in the sediment of Nifedipine-loaded NLCs can't be refused. Both, the *in vitro* and *in vivo* release pattern of Nifedipine can affect the present free drug particles. Therefore, dialysis technique was utilized to remove out the free drug particles from the sediment of NLCs. Nifedipine have low molecular weight of 346.3 g/mol so that this technique was suitable to remove out the free drug particles. Free drug could be effectively removed by dialysis from sediment of NLCs in 20 min and was utilized throughout the experiment. Glycerol monostearate demonstrated higher bioavailability due to higher lipophillic nature of the former that is reason for the more sustained release action of the drug [24].

3.1 Analysis of dependent variables

3.1.1 Effect of formulation variables

In the preparation of nanostructured lipid carriers (NLCs), the variables such as the amount of liquid lipid, amount of solid lipid, concentration of surfactant and amount of drug may affect the size of particles and drug entrapment efficiency. The effect of formulation variables was seen on NLCs suspension to get requried particle size and maximum drug entrapment efficiency (%) and the following variables were optimized;

- Amount of liquid lipids (oleic acid)
- Amount of solid lipid (GMS)
- Surfactant concentration (Poloxamer188)
- Amount of Drug (Nifedipine)

Effect of amount of oleic acid

The amount of oleic acid (10mg, 15mg, 20mg, 25mg, and 30mg) was varied. Formulation batches from F1 to F5 were formulated to evaluate the effect of amount of oleic acid on entrapment and particle size. All other parameters in the formulations like drug amount (30mg), amount of solid lipid (100mg), concentration of surfactant (0.8%) and stirring speed (400rpm) were kept constant. Optimization

parameters such as particle size, size distribution and drug entrapment efficiency (%) were evaluated. The obtained results are tabulated in Table 2.

Table 2: Effect of amount of oleic acid

Formulation code	Amount of oleic acid (mg)	Particle Size* (nm)±S.D	Polydispersity index	Drug Entrapment Efficiency (%) ±S.D
F1	10	258.5±6.2	0.462±0.36	75.72±0.20
F2	15	257.4±9.6	0.367±0.63	86.28±0.08
F3	20	247.8±6.5	0.341±0.73	87.23±0.09
F4	25	259.8±6.5	0.361±0.62	84.24±0.10
F5	30	264.8±9.6	0.349±0.24	78.12±0.05

(Mean ± S.D.)(n = 3)

Effect of amount of solid lipid

The amounts of liquid lipid (20mg oleic acid), concentration of surfactant (poloxamer 0.8%) and amount of drug (30mg) were kept constant. The amount of Glycerol monostearate (100mg, 150mg, and 200mg) was varied. Total three batches (F6 to F8) were formulated for analysing the effect of solid lipid amount on particle size and entrapment efficiency. The obtained results are tabulated in Table 3.

Table 3: Effect of amount of solid lipid with oleic acid

Formulation code	Amount of Glycerol Monostearate (mg)	Particle Size* (nm)±S.D	Polydispersity index	Drug Entrapment Efficiency (%) ±S.D
F6	100	258.5±6.2	0.341±0.34	75.24±0.07
F7	150	256.1±7.3	0.330±0.52	82.12±0.01
F8	200	260.3±3.6 0	0.432±0.73	76.66±0.11

(Mean ± S.D.)(n = 3)

Effect of surfactant concentration

The amounts of liquid lipid (20mg oleic acid) and solid lipid (150mg glycerol monostearate) were kept constant. Different formulation batches (F9-F11) were formulated with varying amount of poloxamer 188 (0.8%, 1.0%, and 1.2%). Three batches were prepared for obtaining the effect of surfactant concentration on particle size and drug entrapment efficiency (%). The obtained results tabulated in Table 4.

Table 4: Effect of surfactant concentration

Formulation code	Surfactant Concentration (%)	Particle Size* (nm)±S.D	Polydispersity index	Drug Entrapment Efficiency (%) ±S.D
F9	0.8	251.7±5.6	0.342±0.37	87.23±0.02
F10	1.0	242.4±5.4	0.314±0.54	89.25±0.02
F11	1.2	247.8±4.2	0.381±0.44	81.90±0.18

(Mean ± S.D.)(n = 3)

Effect of drug (Nifedipine) amount

The amounts of liquid lipid (20mg oleic acid), solid lipid (150mg glycerol monostearate) and poloxamer 407 (1%) were kept constant. The amount of drug was varied from 10 mg – 50 mg and the formulation was loaded and stirring speed (400rpm) was kept constant. Five batches (F12 to F16) were formulated for evaluating the effect of drug amount on particle size and entrapment efficiency. The obtained results are tabulated in Table 5.

Table 5: Effect of drug (Nifedipine) amount

Formulation code	Amount of Drug (mg)	Particle Size* (nm)±S.D	Polydispersity index	Drug Entrapment Efficiency (%) ±S.D
F12	10	255.1±7.3	0.363±0.42	85.4±0.1
F13	20	258.2±6.9	0.385±0.73	87.34±0.2
F14	30	240.4±8.4	0.246±0.47	90.65±0.5
F15	40	245.7±6.3	0.372±0.74	86.27±0.3
F16	50	244.1±3.7	0.361±0.53	81.14±0.7

Values of the particle size of the prepared NLCs are documented in Table 2,3,4 and 5. The results obtained show that the amounts of GMS and oleic acid were key parameters in governing the particle size. After formulating NLCs, the range of particles sizes were lie between 240.4±8.4nm and 264.8±9.6nm and polydispersity was between 0.246±0.47 and 0.462±0.36. The mean particle size was least when Glycerol monostearate was 150mg and oleic acid and poloxamer188 concentration were 20mg and 1.0% (F14) and the particle size was largest when Glycerol monostearate was 150mg and oleic acid was 25 mg (F4). Thus, the oleic acid amount in the lipid matrix affects the mean particle size. The size of NLCs was evaluated by zeta sizer as shown in Figure3. The entrapment efficiency of Nifedipine within the different formulated NLCs formulations is shown in Tables 1, 2, 3 & 4. The highest entrapment efficiency of Nifedipine in all the formulated NLCs was observed in batch F14 using 150mg GMS, 20mg oleic acid, 30mg Nifedipine and 0.1% Poloxamer188 concentration. Concerning liquid lipid type, oleic acid increased the entrapment efficiency, being mixture of triglycerides of different chain length (C8, C10) form less perfect crystals with many imperfections offering space to accommodate the drug.

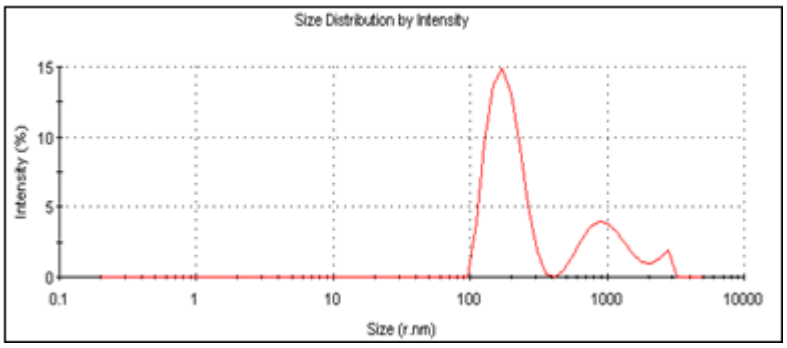

Figure 3: Particle size of nanostructured lipid carriers (Zeta sizer)

3.1.2 *In vitro* drug release

The *in vitro* drug release studies of optimized batch was observed for 2 hrs in 0.1N HCl and further upto 24 hrs in phosphate buffer (pH 6.8) by dialysis bag technique using dialysis membrane. *In vitro* release rate of nefidipine loaded NLCs is graphically presented in Figure 4. As both particle size and entrapment efficiency are determinants of drug release from a given carrier system, the *in vitro* release profile were considerably expected to vary. In 2hrs, 12hrs and 24hrs of the release study, the %CDR observed in NLCs formulated with oleic acid was 1.13±0.004%, 59.53±0.13% and 92.76±0.19 %. The formulation F14 with smallest particle size of 240.4nm and highest entrapment efficiency displayed maximum %CDR in a sustained manner.

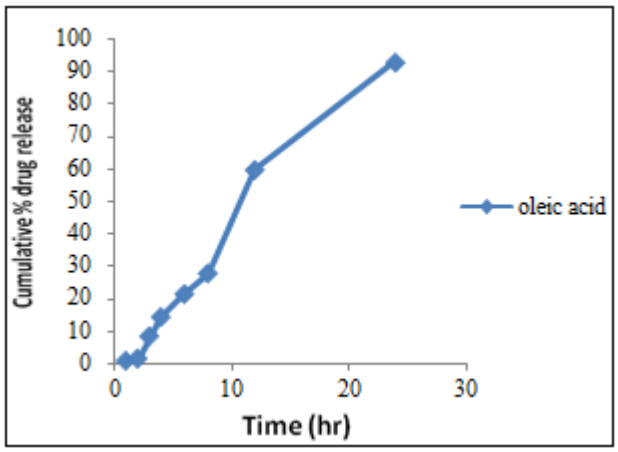

Figure 4: In vitro release profile of Nefidipine loaded NLCs

3.2 In vivo studies

Nefidipine NLCs and Nefidipine suspension were orally administrated to Male Albino rats. The Plasma concentration–time plots in rats after oral administration are shown in Figure 5. The T_{max} was 2h and the C_{max} value was 572.770ng/ml after oral administration of Nefidipine suspension. However, the T_{max} value (4.05 h) of Nefidipine NLCs was two hours later than that of Nefidipine suspension. The visible difference between T_{max} value of Nefidipine NLCs and Nefidipine suspension manifested that the rates of absorption of two formulations were not the same. Nefidipine in suspension dissolved in the intestinal tract and absorbed directly into systemic circulation. However, Nefidipine in NLC could hardly be released into the gastrointestinal tract, as was supported in the in vitro release studies. Therefore, the intact Nefidipine NLCs were directly absorbed into the blood circulation and released the drug gradually. The C_{max} value of Nefidipine NLCs was 813ng/ml which was significantly higher than that obtained with the Nefidipine suspension (572.770ng/ml). The corresponding pharmacokinetic parameters are listed in Table 6. The AUC after oral administration of Nefidipine NLCs was 8225 ng/ml/h, which was approximately 3.9 fold higher than that of Nefidipine suspension (2064.7586 ng/ml/h). The results indicated that systemic absorption of Nefidipine as significantly enhanced by incorporating into NLC compared with Nefidipine suspension. The NLCs showed a promising potential for enhancing oral bioavailability of poorly water-soluble drugs.

Table 6: Mean pharmacokinetic parameters of nanostructured lipid carriers formulations

Formulation	AUC (h*ng/ml)	C_{max} (ng/ml)	Plasma clearance (ml/hr)	MRT (h)	$t_{1/2}$ (h)	Relative bioavailability
Drug suspension	2064.7586	572.770	5392.32	2.35	1.32	1
Lacidipine loaded nanostructured lipid carriers (NLCs)	8225	813	1236.82	10.0606	4.05	3.9

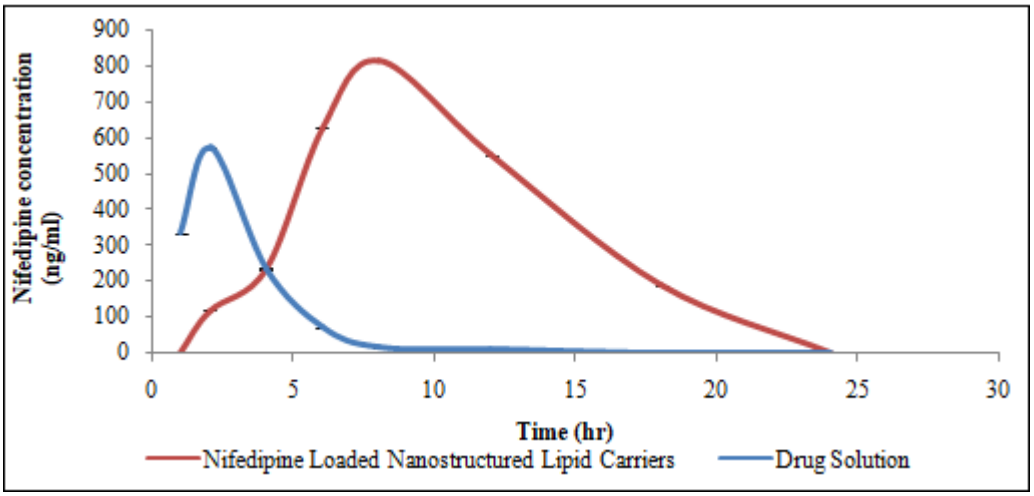

Figure 5: Plasma–drug profile curve

4. Conclusion

Nefidipine NLCs suspension successfully optimized which can be potentially useful for delivery of this drug. From *in vitro* drug release study, it was concluded that the NLCs preparation delayed the drug release for two hours and sustained drug release upto 24 hrs. The study demonstrated that the NLCs developed for oral delivery of Nefidipine possessed site specific targeting ability, better stability and higher entrapment efficiency, low recrystallization properties and easy to scale up. Pharmacokinetic study revealed prolonged T_{max}, and improved relative bioavailability (4-fold) of Nefidipine loaded NLCs to Nefidipine suspension in rats after oral administration. The results of the present investigation showed that the problems associated with the oral bioavailability of Nefidipine could be overcome by incorporating it into a new gastrointestinal drug delivery system, nanostructured lipid carriers (NLCs).

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