ISSN: 0976-7126

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Development and bioavailability studies of atorvastatin nanoemulsion

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Abstract

Poor bioavailability by the oral route can be due to poor solubility, degradation in GI lumen, poor membrane permeation and presystemic elimination. Any of the approaches, which can alter these characteristics, should help in improving the bioavailability of the drugs. Atorvastatin is one of the most important hypolipidemic drug available today and circumventing the major problem of its poor bioavailability remains a bigger challenge of pharmaceutical scientists.The objective of the present study was to develop and characterize an optimal stable nanoemulsion formulation of atorvastatin (AT) with an aim to increase its bioavailability. The components selected for the nanoemulsion were of GRAS category as the safety is the major determining factor for the excipient selection. Thus, Safsol 218 and Oleic acid mixture was selected as the oil phase, surfactants namely Tween 20 and the co-surfactants, Carbitol were selected. Pseudo ternary phase diagrams were constructed using aqueous titration method. From phase diagram different concentrations of oil and surfactant, which formed nanoemulsions were selected based on the thermodynamic stability and dispersibility test. Optimized formulation was selected for *in vivo* study on the basis of higher drug release, optimum globule size, minimum polydispersity value, lower viscosity, and overall lower surfactant concentration and co-surfactant. The difference in t_{max} of nanoemulsion formulation was found to be significant ($p<0.05$) when compared to API drug suspension whereas the difference was insignificant ($p>0.05$) when compared to tablet. The difference in C_{max} of nanoemulsion was very significant ($p<0.01$) when compared with the tablet suspensions and API drug suspension. The relative bioavailability of nanoemulsion to that of conventional tablet suspensions was 356.32% whereas to that of API drug suspension was 559.86% respectively. Thus nanoemulsions could be used effectively to improve the bioavailability of poorly water soluble drugs to improve their bioavailability.

Key-Words: Nanoemulsion, Hypolipidemic, Polydispersity, Pseudo ternary phase diagrams

Introduction

Nanoemulsions being colloidal nanodispersions of oil in water (or water in oil), thermodynamically stabilized by an interfacial film of surfactant(s) and co-surfactant(s) have revealed tremendous potential in nanoengineering of various inorganic materials[1]. Droplet size in thermodynamically stable nanoemulsions is usually 10-100 nm[2]. The homogeneous systems that can be prepared over a wide range of surfactant concentrations and oil to water ratios (1:4 %) are all fluids of low viscosity. Nanoemulsions provide ultra low interfacial tensions and large o/w interfacial areas.

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Nanoemulsions have a higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions, because they can be manufactured with little energy input (Low energy emulsification techniques/heat or mixing) and has a long shelf life. The nanosized droplets leading to enormous interfacial areas associated with nanoemulsions would influence the transport properties of the drug, an important factor in sustained and targeted drug delivery[3-4]. The attraction of o/w nanoemulsion systems lies in their ability to incorporate hydrophobic drugs into the oil phase thereby enhancing their solubility[3]. Nanoemulsions have been reported to make the plasma concentration profiles and bioavailability of drugs more reproducible[5-6]. The objective of the present study

is to prepare nanoemulsion of atorvastatin to improve its solubility and bioavailability.

Material and Methods

Development of drug containing nanoemulsion formulation

The drug stock solutions in oil mixture were prepared in such a way that 10 mg dose was present in each formulation complying the oil percentage for each formula as selected from the phase diagram. This was prepared by dissolving the 1000 mg of drug individually in the 10, 15, 20 and 25 mL of oily mixture, which compiles the 10%, 15%, 20% and 25% oil compositions respectively in the formulae.

Screening of formulations on the basis of thermodynamic stability studies

Microemulsions are thermodynamically stable systems and are formed at a particular concentration of oil, surfactant: co-surfactant mixture and water, with no phase separation, creaming or cracking. It is the thermostability which differentiates micro emulsion from emulsions that have kinetic instability and will eventually phase separate[3]. The thermodynamic stability studies were performed on the basis of following tests.

Centrifugation study

The selected formulations were centrifuged (REMI, India) at the 5000 rpm for 30 mins and observed for phase separation, creaming or cracking. The formulations which showed maximum stability (no creaming, cracking, phase separation) were selected and studied for heating-cooling cycle, freeze-thaw cycles and Dispersibility tests.

Heating cooling cycles

It is used to see the stressed effect of heating and cooling on the nanoemulsion`s stability. In this study the formulations were kept at 45^0 c and at 0 ^{0}C temperature for not less then 48 h. for each temperature cycle.

Freeze –thaw cycles (Accelerated ageing)

This test was performed for accelerated stability testing of nanoemulsion formulations. In this study the formulations were exposed at two different temperatures i.e. -21^0C and 21^0C for each temperature cycles not than 24 hrs. For the better estimation of accelerated stability studies three such cycles should be run for each batch of formulation. The formulations which showed the maximum stability were selected for further study.

Characterization of the nanoemulsion

Nanoemulsion has been characterized using a wide variety of techniques. The characterization of nanoemulsion is a difficult task due to their complexity, variety of structures and components involved in these systems, as well as the limitation associated with each technique, but such knowledge is essential for their successful commercial exploitation. The rate of release of sodium salicylate from a lecithin-based nanoemulsion, is dependent upon their microstructure[7].

A complementarily of methods is generally required in order to fully characterize these systems. At the macroscopic level viscosity, conductivity and dielectric methods provide useful information.

Phase Behavior studies

Phase diagram showing the nanoemulsion region, information about of different phases as a function of composition variables can be analyzed from vigorous study of the phase diagrams.

Viscosity measurements

The viscosity of the prepared nanoemulsion formulations were determined as such without dilution by Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) using spindle # CPE40 at 25 ±0.5°C. The software used for the viscosity calculations was Rheocalc V2.6.

Refractive index

Refractive index of selected formulations was determined using an Abbe type refractrometer. It was standardized using castor oil.

Dielectric measurements

They are powerful means of probing both structural and dynamic features of nanoemulsions systems.

Electron microscopic studies

Morphology and structure of the nanoemulsion was studied using transmission electron microscopy (TEM) TOPCON 002B operating at 200 KV and of a 0.18 nm capable of point to point resolution. In order to perform the TEM, the nanoemulsion formulation was diluted with distilled water (1/100). A drop of the diluted nanoemulsion was directly deposited on the Copper holey film grid and observed after putting fixing agent and drying it in the filtered air.

Scattering techniques

Dynamic light scattering photon correlation spectroscopy (PCS) is used to analyze the fluctuations in the intensity of the scattering by the droplets due to Brownian motion.

Interfacial tension measurement

The formation and properties of nanoemulsion can be studied by measuring the interfacial tension. Spinning-drop apparatus can be used to measure the ultra low interfacial tension.

Droplet size distribution

Droplet size of the prepared nanoemulsion was determined by using photon correlation spectroscopy,

ISSN: 0976-7126

which analyzes the fluctuations in light scattering due to Brownian movement of the particles[8]. The formulation (0.1 mL) was dispersed in 50 mL (500 dilution) of distilled water in a volumetric flask and gently mixed by inverting the flask and measurement done using a Zetasizer (Nano ZS-90, UK). Light scattering was monitored at 25°C at a 90° angle.

Refractive index/ Isotropicity

Refractive index of nanoemulsions formulations was determined using an Abbe type refractrometer. It basically gives an idea about the isotropicity of the formulations. The isotropic nature of microemulsions and their optical clarity makes their study by spectroscopic techniques straightforward, particularly in comparison to conventional macroemulsions.

In-vitro drug release performance

The study was performed by using dialysis bag method[9]. The dialysis membrane used in the study was Cellulose membrane (Sigma, USA). With a molecular weight cut off of 12000 g/mole.

Table 1: In vitro dissolution study of self-nanoemulsifying pellets and marketed tablet Avas® (microlab) and Atorlip® (Cipla) in simulated gastric fluid (pH 1.2)

Time min	SNE pellet		Tablet-Microlab		Tablet-Cipla	
	CR µg/mL	%R	CR µg/mL	%R	CR µg/mL	%R
0	5	00	5	00	5	00
3	10	35.60	10	30.23	10	22.35
6	15	59.33	15	34.51	15	35.37
10	20	81.14	20	46.42	20	40.84
12	25	99.64	25	51.17	25	58.93
15	30	99.80	30	64.04	30	66.28
20	35	99.45	35	83.54	35	91.57
25	40	99.38	40	93.82	40	96.37
30	45	99.32	45	95.70	45	98.38
60	50	99.14	50	97.34	50	99.37

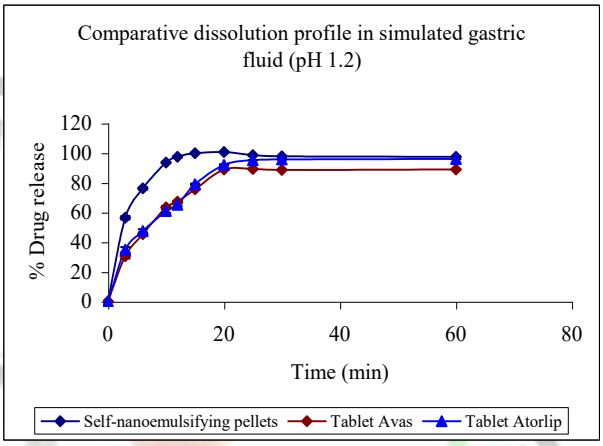

Fig. 1 Comparative dissolution profile

In vitro release test was performed in 250 ml of distilled water, which was based on USP XXIV method (Dissolution apparatus I.P. 2, at 100 rpm and 37 ± 0.5 ^{0}C). 1 ml of nanoemulsion formulation (Single dose containing 10 mg of AT Calcium) was placed in treated dialysis bag (MWCO 12,000 g/mole; Sigma, USA) and 1 mL samples was withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2,2.5,3,3.5, 4,4.5,5.5, 6,6.5,7, 8,9, 10, 12, 22 and 24 h) and same amount of distilled water was replaced[10]. The withdrawn 1 ml samples were diluted with 3 ml methanol and analyzed for the drug content by using developed RP-HPLC at 247 nm. The same method was used for the suspension containing 10 mgof AT Calcium in 1 ml distilled water. The release of drug from different selected nanoemulsion formulations was compared with drug suspension and finally selected formulation was used for the further study.

Bioavailability study of Atorvastatin nanoemulsion in rats

The ultimate test of success of a formulation depends on its in vivo performance and for the assertion of the same the in vivo studies were conducted in a suitable animal model. Approval to carry out *in vivo* study was obtained from Gyan Vihar University, Institutional Animal Ethics Committee and their guidelines were followed for the studies (173/CPCSEA). The animals used for *in vivo* experiments were adult wistar female albino rats (n=6) weighing 180-200 gm obtained from Central Animal House of Gyan Vihar University, Jaipur, India. The animals were kept under standard laboratory conditions at a temperature of 25 ± 2°C and relative humidity (55 ± 5%). The animals were housed in animal cages, six per cage, with free access to standard laboratory feed (Lipton feed, Mumbai, India) and water *ad libitum*. The nanoemulsion formulation

GM1 that showed the highest release profile of drug in *in vitro* studies was subjected to *in vivo* studies. 6 mg/kg[9] body weight of the Atorvastatin Ca was used in the study.Six tablets with label claim of 10 mg (Atorlip®) were taken, crushed in mortar, and mixed with 50 ml of double distilled water (1.2 mg/mL). Each rat in this group was given 1.0 ml using oral feeding needle. Same dose of drug suspension and nanoemulsion was administered. The blood sampling was carried out for around 48 hours and 10 to 12 samples of blood were taken from each animal in the group. Blood was withdrawn from the juglar vein after anesthesia at 0 (pre-dose), 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 6, 12, 24, 36 and 48 h in micro centrifuge tubes. Tubes were stored at room temperature, 25 ± 2°C and relative humidity (55 ± 5%) for 30 minutes. The clotted blood was then centrifuged at 5000 rpm for 30 min. The serum was separated and stored at -21 °C until drug analysis was carried out using RP-HPLC method. The collected serum was extracted with ethyl-acetate (5 ml) by using Sodium phosphate buffer (pH 7, 1 ml) as a protein-precipitating agent for analysis and centrifuged at 5000 rpm for 5 min and dried at room temperature. After evaporating the ethyl acetate solid residue was reconstituted using 100 µl mobile phase and then analyzed by RP-HPLC. The samples were injected and the area was noted down in triplicate. The mean areas obtained by HPLC are given in the Table 2.

Table 2: Working formula for nanoemulsion (GM1)

Oil : Sefsol 218+ Oleic acid , **Surfactant** : Tween 20, **Cosurfactant**: Carbitol					
Volume of components for Nanoemulsion Formulation					Code
Oil (ml)	Surfactant (ml)	Co – surfactant (ml)	Aqueous phase (ml)	Atorvastatin (mg)	
0.10	0.19	0.19	0.52	1.2	GM1

The formulations were given orally using oral feeding needle. Dose for the rats was calculated based on the weight of the rats (6 mg/ kg).

Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated by using non-compartmental analysis also called as Model independent analysis using WinNonLin version 4.0 (Pharsight Corp., Mountain View, CA). All pharmacokinetic (PK) parameters (t_{max}, C_{max}, $AUC_{0\rightarrow t}$, $AUMC_{0\rightarrow t}$ and MRT 0→t) were calculated individually for each subject in the group and the values were expressed as mean ±SD. The comparative in-vivo bioavailability profiles of nanoemulsion formulation GM1, API suspension and Tablet suspension are shown in the Table 4.

Table 4: Comparative observation table for *in-vivo* bioavailability studies in rat animal model (n=6).

Sampling time (Hours)	SNEDDS (Mean Conc. ± SD)	Tablet suspension (Mean Conc. ± SD)	API suspension (Mean Conc. ± SD)
0	0	0	0
0.25	24719.36 ± 142.54	4747.476 ± 63.76	3636.91 ± 57.56
0.5	35582.73 ± 345.54	5491.985 ± 36.87	4307.00 ± 24.87
0.75	37687.34 ± 733.16	5815.137 ± 62.97	4456.82 ± 54.89
1	41090.76 ± 531.78	6343.85 ± 64.86	4959.85 ± 86.36
1.25	43603.77 ± 632.63	6750.599 ± 145.35	5271.45 ± 75.37
1.5	37508.95 ± 456.56	5801.549 ± 48.25	4345.85 ± 86.74
6	24312.26 ± 731.84	3761.141 ± 65.36	2887.23 ± 75.36
12	12093.38 ± 234.47	1870.322 ± 124.12	1437.17 ± 44.66
24	6370.94 ± 32.47	987.3089 ± 32.85	765.24 ±75.05
36	3851.75 ± 42.74	599.0169 ± 24.86	548.16 ± 36.87
48	2811.38 ± 57.38	436.0885 ± 14.60	338.09 ± 23.21

ISSN: 0976-7126

Table 5: Relative pharmacokinetic parameters of different formulations containing AT Calcium (n = 6).

Formulation	t_{max}[a] (hrs)	C_{max}[b] (ng/ml)	$AUC_{0\rightarrow t}$[c] (ngh/ml)	Rel. BA[f] (%)
GM1	0.95 ± 0.13	43603.77 ± 632.63	383453.2 ± 2352.2	357.23± 43.3
Tablet Suspension	2.24 ± 1.74	6750.599 ± 145.35	35432.4 ± 1143.53	765.86 ±32.7
API drug Suspension	2.52 ± 1.93	5271.45 ± 75.37	68232.5 ± 6734.46	893.57 ±84.2

[a]time of peak concentration; [b] peak of maximum concentration;[e] area under the concentration time profile curve until last observation; [f]Relative bioavailability of formulations

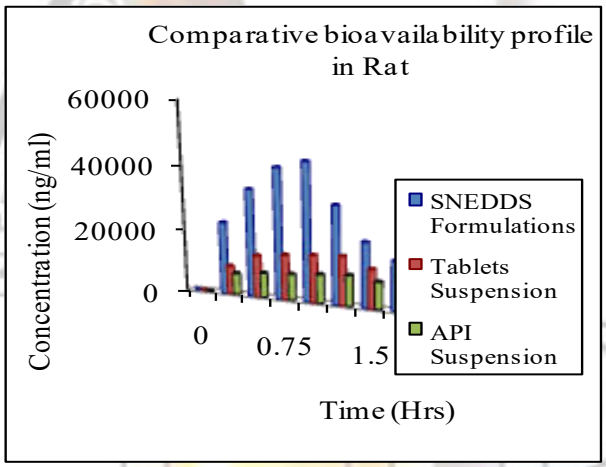

Fig 2: Comparative In-vivo absorption profile of atorvastatin using different formulations in rat model

Results and Conclusion

Poor oral bioavailability has the consequence of more variable and poorly controlled plasma concentration and drug effects. Poor bioavailability by the per-oral route can be due to poor solubility, degradation in GI lumen, poor membrane permeation and presystemic elimination. Any of the approaches, which can alter these characteristics, should help in improving the bioavailability of the drugs.

The design of effective formulation for drugs has long been a major challenge, because drug efficacy can be severely limited by instability or poor solubility in the vehicle. Nanoemulsions being a versatile technology have the potential to increase the bioavailability of drug in many ways. They act as supersolvents for poorly soluble drugs. Nanoemulsions have a higher solubilization capacity than simple micellar solutions and their thermodynamic stability offers advantages over unstable dispersions, such as emulsions and suspensions, because they can be manufactured with little energy input (heat or mixing) and has a long shelf life thus scale up technology is easy. The nanosized droplets leading to enormous interfacial areas associated with nanoemulsions would influence the transport properties of the drug, an important factor in sustained and targeted drug delivery. The droplet size of nanoemulsion is between 14 to 100 nm, and can be sterilized by filtration. The use of nanoemulsion as drug delivery systems can improve the efficacy of drug, allowing the total dose to be reduced and thus minimizing side effects.

Atorvastatin, a synthetic cholesterol-lowering agent, is a HMG-CoA (3 hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor. The calcium salt of atorvastatin is used in the treatment of primary hypercholesterolemia and dyslipidemia. Atorvastatin is highly lipophillic in nature (log*P* (octanol/water), 6.36), freely soluble in methanol and soluble in dimethylsulphoxide(DMSO) and dimethyl formamide. It is soluble in aqueous medium with a pH of less than 4.0. It is very slightly soluble in distilled water, phosphate buffer (7.4) and acetonitrile; while it is slightly soluble in ethanol. 20.4 µg/ml (pH 2.1), 1.23 mg/mL (pH 6.0). Owing to its poor water-solublity the drug, it gets metabolized in the GI tract, with absolute bioavailability of mere 14%. Atorvastatin is without question one of the most important hypolipidemic drug available today and circumventing the major problem of its poor bioavailability remains a bigger challenge of pharmaceutical scientists. With this background in mind, the objective of the present study was to develop and characterize an optimal stable nanoemulsion formulation of atorvastatin with an aim to increase its bioavailability.

The components selected for the nanoemulsion were of GRAS category as the safety is the major determining factor for the excipient selection. They offer formulative and physiological advantages and their degradation products resemble the natural end products of intestinal digestion. Thus, Safsol 218 and Oleic acid

mixture were selected as the oil phase for the development of the formulation because of the highest solubility of Atorvastatin (130.06± 2.68mg/mL) in it. The higher solubility of the drug in the oil phase is important for the nanoemulsion to maintain the drug in solubilized form. In the present study, surfactants namely Tween 20 and the co-surfactants, Carbitol were selected. AT Calcium is very slightly soluble in water at a pH below 4, therefore different buffers were used to prepare the calibration curve for preliminary studies.

The relationship between the phase behaviour of a mixture and its composition can be captured with the aid of a phase diagram. Pseudo ternary phase diagrams were constructed using aqueous titration method[10]. After taking observation, pseudo ternary phase diagrams were constructed based on the observations marked during titration. Thus while studying the phase diagrams, it could be seen that the free energy of nanoemulsion formation can be considered to be dependent on the extent to which the surfactant lowers the surface tension of the oil-water interface and the change in dispersion entropy. Therefore, nanoemulsification is specific to the nature of the oil and surfactant pair, the surfactant concentration and oil/surfactant ratio. Thus, a negative free energy of formation is achieved when large reduction in surface tension is accompanied by significant favourable entropic changes. In such case nanoemulsion formation is spontaneous and the resulting dispersion is thermodynamically stable. While going through different pseudo ternary phase diagrams, oil could be solubilized up to the extent of 25% v/v. Therefore, from phase diagram different concentrations of oil, which formed nanoemulsions, were selected at a difference of 5% (10, 15, 20 and 25%) and for each percentage of oil selected; only those formulations were taken from the phase diagram, which used least concentration of Smix, irrespective of Smix ratio used. These formulations passed the thermodynamic stability and dispersibility test showing that they can be diluted in GI fluids still maintaining the nanosized character without drug precipitation that will lead to higher solubility.

Droplet size analysis of the selected formulations was carried out. The difference in the droplet size between the formulation was not statistically significant. There was only a marginal difference in the mean droplet size of formulations but polydispersity (0.237) was minimum in the case of formulation, GM1, containing 10% of oil suggesting uniformity in the globule size (42.8 nm) of the formulation.

It was observed that the viscosity of all the formulations is less than 45 mP. Formulation, GM3, has the minimum viscosity (10.03 ± 0.91 mP), as compared to the other formulations. The viscosity of the selected formulation GM1 was found to be intermediate showing numeric value of 27.51 ± 1.01 mP.

In vitro drug release was carried out using a dialysis bag and the selected formulations were compared for the drug release with API drug suspension. The release of drug from nanoemulsion formulations was high when compared to drug suspension The drug release was found to highest (99.65±0.75%) in case of GM1 as compared to the API drug suspension (13.77±0.214) in 6.5 hrs.

Therefore, the optimized formulation, GM1, having higher drug release (99.65±0.75%), optimum globule size (42.8 nm), minimum polydispersity value (0.237), lower viscosity (27.51 ± 1.01 mP), and overall, lower surfactant concentration (19%) and co-surfactant (19%) was selected for *in vivo* study.

The *in vivo* study was performed to quantify AT Calcium, after oral administration of formulations containing drug. The plasma concentration time profiles of AT Calcium in adult female albino wistar rats following oral administration of the nanoemulsion (GM1) formulations, marketed tablet (Atorlip™ 10) suspensions and API drug suspensions formulations were compared. It was found that the C_{max} in serum for nanoemulsion formulation (45729.33 ±13689.15 ng/mL) represents greater improvement than the marketed formulations (8158.26 ±1568.84 ng/mL) or simple API drug suspension (5009.16 ±1339.59 ng/mL). It was also observed that $AUC_{0\rightarrow t}$ GM1 formulation was 394520.05 ±87932.39 ng.h/mL and thus the difference were highly significant ($p<0.01$) as compared to $AUC_{0\rightarrow t}$ (110271.4± 47224.84) and (70467.13 ± 26350.34) highly significant ($p<0.01$) tablet suspension and API suspension (Table 7.3) formulations respectively.

Statistically, the difference in t_{max} of GM1 Nanoemulsion formulation was found to be significant ($p<0.05$) when compared to t_{max} of API drug suspension where as the difference was insignificant ($p>0.05$) when compared to tablet. The difference in C_{max} of GM1 formulation was very significant ($p<0.01$) when compared with the Tablet suspensions and API drug suspension. It was also observed that $AUC_{0\rightarrow t}$ of GM1 formulation was ng.h/m L, thus the difference were very significant ($p<0.01$) as compared to Tablet suspensions and API drug suspension formulations. Both the values of GM1were very significant ($p<0.01$) as compared to drug suspension. The relative bioavailability of GM1Nanoemulsion to that of conventional Tablet suspensions was 356.32% where

as to that of and API drug suspension was 559.86% respectively.

Therefore, based on higher drug release, optimum globule size, minimum polydispersity, lower viscosity, lower surfactant concentration, higher solubility as well as higher bioavailability without variable absorption has been optimized. The composition of optimized formulation (GM1) was Sefsol 218 (5% v/v), Oleic acid (5% v/v), Tween-20 (19% v/v), Carbitol (19%v/v) and distilled water (52% v/v) as oil, surfactant, cosurfactant and aqueous phase respectively, containing 10 mg of AT Calcium.

The *in vivo* studies revealed significantly greater extent of absorption than the conventional tablet suspension and API drug suspension formulation. The absorption of AT Calcium from Tween 20 nanoemulsion resulted in 3.56 fold increase in bioavailability as compared to conventional Tablets suspension and 5.59 fold to that of API drug suspension. Furthermore, this result attributed that the presence of surfactants in nanoemulsion system might have caused changes in membrane permeability of the GI tract and the inhibition of an apically polarized efflux system, which could lead to enhancement of the oral absorption.

Acknowledgements

Author is thankful to Lupin Pharmaceuticals Pvt. Ltd. for providing drug sample. We are also thankful to Medical Superintendent, G. R. Medical College, Gwalior for kind cooperation, guidance and help during my tenure of my research work. I am also thankful to Dean Research, Gyan Vihar University, Jaipur for all the support and guidance.

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