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RESEARCH ARTICLE

Design and development of nanoemulsion drug delivery system of amlodipine besilate for improvement of oral bioavailability

Gulshan Chhabra[1], Krishna Chuttani[2], Anil K. Mishra[2], and Kamla Pathak[1]

[1]*Department of Pharmaceutics, Rajiv Academy for Pharmacy, Mathura, India, and* [2]*Government of India, Ministry of Defense, Institute of Nuclear Medicine and Allied Sciences, Delhi, India*

Abstract

Nanoemulsion (NE) of amlodipine besilate (AB) was developed by spontaneous emulsification method with the aim to enhance the solubility and oral bioavailability of AB and to achieve localized delivery of drug at target site. Pseudoternary phase diagrams were constructed to identify the NE region. The selected formulations from NE region were subjected to droplet size analysis, partitioning study and *in vitro* drug release. The partition coefficient was calculated and correlated with percent dissolution efficiency as a tool to predict *in vitro* drug release from NEs. The release of drug from NEs was significantly higher ($p < 0.01$) than the marketed tablet formulation. The optimal formulation contained 15% Labrafil M, 35% [Tween 80: ethanol (2:1)], and 50% by weight aqueous phase (NE3) was characterized by transmission electron microscopy (TEM) and for thermodynamic stability. The pharmacokinetics and biodistribution studies of the optimized radiolabeled formulation (^{99m}Tc-labeled) in mice (p.o.) demonstrated a relative bioavailability of 475% against AB suspension. In almost all the tested organs, the uptake of AB from NE was significantly higher ($p < 0.05$) than AB suspension especially in heart with a drug targeting index of 44.1%, also confirming the efficacy of nanosized formulation at therapeutic site. A three times increase in the overall residence time of NE further signifies the advantage of NEs as drug carriers for enhancing bioavailability of AB.

Keywords: Amlodipine besilate, bioavailability, biodistribution, nanoemulsion, pharmacokinetics, phase diagram, solubility

Introduction

The lipid-based formulation approach has attracted wide attention to improve the oral bioavailability of poorly water-soluble drugs and to deliver the drug at target site. The most popular approaches are the incorporation of the active lipophilic component into inert lipid vehicles such as oils, surfactant dispersions, self nano- or micro-emulsifying formulations, solid emulsions, and liposomes[1]. The drug can be loaded into the inner phase of these systems and delivered by lymphatic route, bypassing the enzymes in the gastrointestinal tract (GIT) and reducing the presystemic clearance and hepatic first-pass metabolism. Nanoemulsions (NEs) due to higher drug solubilization capacity, better thermodynamic stability, long self-life, rapid onset of action, and reduced intersubject variability form a promising technology to achieve optimum targeted drug delivery[2].

Mechanistically, in an NE, the drug is partitioned between dispersed and continuous phase and when the system comes into contact with semipermeable membrane, the drug can be transported through barrier due to enhanced permeability[3]. The nanosized droplets lead to enormous interfacial area and would influence the transport properties of the drug, which remains a crucial parameter for enhancement of bioavailability[4,5].

Amlodipine besilate (AB), 3-ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methylpyridine-3,5-dicarboxylate,

Address for Correspondence: Gulshan Chhabra, Assistant Professor, Department of Pharmaceutics, Rajiv Academy for Pharmacy, Mathura (U.P.)– 281001, India. Tel.: +915652913018; Fax. +915652825050. E-mail: gulshan_chhabra2003@yahoo.com or gulshan.chhabra2008@gmail.com

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monobenzenesulphonate is a dihydropyridine calcium antagonist that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle[6]. The absorption of AB is nearly complete after oral administration, and it is more slowly absorbed[7]. Poor aqueous solubility (slightly soluble) and low permeability through GIT[8] seems to lower the bioavailability (60–65%)[9] and hence limits the access of drug to their therapeutic targets like heart and cardiac smooth muscles.

On the basis of cited literature, till date there is no research report available on oral bioavailability enhancement of AB by NE, to the best of our knowledge. Hence, the aim of present investigation was to maximize the therapeutic efficacy of AB by developing oil-in-water (o/w) NE to increase the solubility of slightly soluble drug and consequently enhance the oral bioavailability and to deliver the drug at therapeutic site. Criterion for selection of NEs as targeted drug delivery system was based on facts that these act as a supersolvent for both hydrophilic and lipophilic drugs[10,11] and can increase the solubility of AB. Additionally, NEs can improve the bioavailability of AB by enhancing the absorption of drug via surfactant-induced permeability changes of GI membrane[12] and also they are known to cross the gut lumen by paracellular pathway and enter the blood circulation, thus demonstrating a systemic effect and also circumventing the hepatic first pass effect[13].

Materials and methods

Chemicals

AB was obtained as a gift sample from Cadila Pharmaceuticals Limited, Ankleshwar, India. Capmul MCM and Captex 100 were obtained as a gift sample from Abitech Corporation Limited, Janesvile, WI. Labrafil M 1944 CS (oleoyl macrogolglycerides), labrafac CC (dicaprylocaprate), labrasol (caprylocaproyl macrogolglycerides), and transcutol P (diethylene glycol monoethyl ether) were obtained as a gift sample from Colorcon Asia Private Limited, Goa, India. Oleic acid and iso propyl myristate were purchased from Sigma Aldrich chemicals, Riedstr, Germany. Olive oil, Tween 80 (polyoxyethylene sorbitan monooleate), propylene glycol, poly ethylene glycol, methanol, and ethanol were purchased from S.D. Fine chemicals, Mumbai, India. Span 80 (sorbitan monooleate) was purchased from G. S. Chemicals Lab, Mumbai, India. Stannus chloride dihydrate was purchased from Sigma Chemical Co., St. Louis, MO. Sodium pertechnetate was purchased from Regional Center for Radiopharmaceutical Division, Board of Radiation and Isotope Technology, Delhi, India. All chemicals were of analytical grade.

Preparation of NEs

Screening of components by solubility studies

An excess amount of AB was added to different oily phases (Capmul MCM, labrafac CC, labrafil M 1944 CS, captex 100, olive oil, oleic acid, iso propyl myristate), surfactants (Tween 80, Span 80, labrasol) and cosurfactants (PEG-400, propylene glycol, transcutol and ethanol) and shaken in digital water bath shaker (HICON, Delhi) for 72 h at 37°C. At the end of test period, samples were left to stand for 10 min to allow equilibrium, then centrifuged at 4000 Relative Centrifugal Force (RCF) (1073*g*) for 20 min, filtered the supernatant (except oily samples) through membrane filter (0.45 µm) and analyzed the drug in filtrate (and in supernatant for oily samples) by UV spectrophotometer at 238 nm ($y = 0.0308x + 0.0837$). The study was done in triplicate.

Construction of pseudoternary phase diagrams

The pseudo ternary phase diagrams consisting of oil, S_{mix} (surfactant–cosurfactant mixture) and distilled water were developed using the aqueous titration method[14–16]. Surfactant and cosurfactant mixed in different weight ratios (0.5:1, 1:1, 1.5:1, and 2:1) were selected on the basis of increasing concentration of surfactant with respect to cosurfactant. For each phase diagram, oil and a specific S_{mix} ratio were mixed properly in different weight ratios from 0.5:9.5 to 9.5:0.5 in separate glass vials. Ninteen different combinations of oil and S_{mix}, 0.5:9.5, 1:9, 1.5:8.5, 2:8, 2.5:7.5, 3:7, 3.5:6.5, 4:6, 4.5:5.5, 5:5, 5.5:4.5, 6:4, 6.5:3.5, 7:3, 7.5:2.5, 8:2, 8.5:1.5, 9:1, and 9.5:0.5 were made, so that maximum ratios were covered, in order to define the boundaries of phases specifically formed in each phase diagram. Slow titration of each weight ratio of oil and S_{mix} was performed with aqueous phase and visual inspections were made for assessing transparency (aqueous phase was added drop wise till the nanoemulsified blend is clear to the naked eyes, after that further addition of aqueous phase was stopped) and ease of flow of various o/w NEs formed. The shaded area (NE area) in each phase diagram was plotted and the wider region indicated better nanoemulsifying efficiency[17]. No heating was conducted during the preparation. Phase diagrams were developed by using CHEMIX School Ver. 3.50 software (MN) (www.standones.no/chemicals/english/purchase1.htm).

Optimization of NE

Considering the amount and solubility of drug to be incorporated in NE, certain oil–S_{mix}–water mixtures within the NE region were prepared and final composition of NE was optimized based on transparency, dilution characteristics, globule size, zeta potential, and polydispersity index (PDI). For optimization of process, NEs were prepared by varying the stirring speed and stirring time and globule size was taken as response. Seven different formulations encoded as NE1–NE7 were prepared to identify optimum formulation by varying the percentage of S_{mix} and water.

Characterization of NEs

pH, viscosity, and percentage transmittance

The pH was measured at 25 ± 2°C using calibrated pH-meter (HICON, New Delhi, India) and the viscosity measurements were made using Brookfield viscometer DV-II+ Pro (Brookfield Engineering Laboratories, Inc.,

MA) coupled with S-94 spindle at 100*g* at 25 ± 2°C. For measurement of percentage transmittance (%T), NEs were diluted 10 times with distilled water and %T was checked against distilled water using UV–Visible spectrophotometer at 630 nm.

Globule size, PDI, refractive index, and zeta potential determination

The globule size of NEs was determined using a photon correlation spectrometer (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK) based on laser light scattering phenomenon, which analyzes the fluctuations in light scattering. Helium–neon gas laser having intensity of 4 mW was the light source. Light scattering was monitored at 25°C at a 90° angle. Properly diluted samples of NEs were used for droplet size analysis. Electrophoretic mobility (μm/s) was measured using small volume disposable zeta cell and converted to zeta potential[18] by in-built software using Helmholtz–Smoluchowski equation. Average droplet size, PDI, and zeta potential were determined. Refractive index value of the formulations was also recorded. All the quantitative determinations were done in triplicate.

Partitioning studies

The study was done by shake flask method[19] in which NE and *n*-octanol (1:1 ratio by vol.) were initially presaturated with counterpart and then shaken for 30 min in digital water bath shaker at 37 ± 0.5°C. The mixture was allowed to stand for 5 min and NE layer was collected, diluted properly, and analyzed for drug concentration before and after partitioning. The *n*-octanol–NE partition coefficient; $K_{(O/NE)}$ was calculated for each formulation by dividing concentration of drug in *n*-octanol by concentration of drug in NE.

In vitro *drug release study*

In vitro drug release was performed using dialysis bag method. Four milliliters of the NE placed in dialysis bag (HIMEDIA dialysis membrane-150) was subjected to release study in 500 mL of dialyzing media (phosphate buffer pH 7.4) stirred at a speed of 100*g* and temperature 37 ± 0.5°C. Aliquots of 5 mL samples were withdrawn at regular time intervals (0, 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 h) from the dialyzing medium and volumes withdrawn were replaced with the fresh medium each time. The samples were analyzed at 238 nm and percentage cumulative drug release was calculated. The model-dependent and -independent parameters were calculated using PCP-Disso-V2.08 software, Pune, India.

Thermodynamic stability study

The optimized NE was centrifuged at the 4000 RCF (1073*g*) for 30 min and observed for phase separation, creaming, or cracking. Then, it was subjected to heating and cooling cycle. Six cycles between the refrigerator temperature (4°C) and 45°C temperature were performed with storage at each temperature for not <48 hr. Finally, it was subjected to freeze thaw cycle. In this, formulation was exposed for three freeze thaw cycles between –21°C and +25°C with storage at each temperature for not <48 h to check the thermodynamic stability of the formulation.

Transmission electron microscopy

Surface morphology of NE was studied with the help of transmission electron microscope operating (HITACHI, H-7500, Tokyo, Japan) at 200 KV, capable of point to point resolution. A drop of NE was allowed to deposit directly on the microscopic carbon-coated grid and observed after drying. Combination of bright field imaging at increasing magnification and of diffraction modes were used to determine the form and size of the NE.

In-vivo biodistribution and pharmacokinetics studies

Radiolabeling of formulations

The optimized formulation, NE3 (ABNE), and AB suspension (ABS) were radiolabeled using technetium-99 (^{99m}TC) by direct labeling method[14–16]. ABS containing 1.25 mg/mL of AB was prepared by dispersing the required amount of drug in 2% w/v sodium carboxymethylcelllulose mucilage. Two milliliters of ABNE and ABS formulations were taken in separate vials in which 400 μg of stannous chloride dihydrate (2 mg/mL in 10% acetic acid) was added and pH was adjusted to 6.0–6.5 using 50 mM sodium bicarbonate solution. To the resultant mixture (filtered through 0.22 μm nylon 66 membrane) required volume of ^{99m}TC-pertechnetate (2 mCi/mL) was added with continuous mixing, such that the resultant mixture has a radioactivity of 2 mCi/mL and was incubated at 30 ± 5°C for 15 min.

The radiochemical purity of ^{99m}TC-ABS (^{99m}TC-labeled ABS) and ^{99m}TC-ABNE were determined using instant ascending thin layer chromatography, silica gel-coated fiber glass sheets, and solvent systems consisting of methanol as mobile phase. The effect of incubation time, pH, and stannous chloride concentration on labeling was studied to achieve optimum reaction conditions. The *in vitro* stability of radiolabeled formulations was evaluated both in 0.9% w/v sodium chloride and in mice serum[20].

Biodistribution and pharmacokinetic studies

The protocol for animal experimentation was approved by the Institutional Animal Ethics Committee (IAEC) of Rajiv Academy for Pharmacy, Mathura (IAEC/RAP/2553/2009). Swiss albino mice (male, aged 4–5 months) weighing between 25 and 30g were selected for the study. Three mice for each formulation per time point were used in the study. Radioloabeled complex of ^{99m}TC-AB formulations, ABNE and ABS (200 μCi/100 μL) containing 4 mg/kg of AB were administered with the help of 16# gauge oral feeding cannula. The mice were anaesthetized using chloroform and sacrificed at 0.5, 1, 2, 4, 6, and 24 h postadministration, and blood was collected using cardiac puncture method. Subsequently, organs/tissues (heart, liver, spleen, kidney, and intestine) were dissected, washed twice using normal saline, made

free from adhering tissues/fluid, dried between absorbent paper folds, placed in preweighed plastic tubes, and weighed. Radioactivity present in each tissue/organ was measured using shielded well-typed gamma scintillation counter. Radiopharmaceutical uptake per gram in each tissue/organ was calculated as a fraction of administered dose using equations:

%Radioactivity/gm of tissue = (Counts in sample × 100) (Weight of sample × total counts administered)

Various pharmacokinetic parameters (C_{max}, t_{max}, AUC, Cl_T, V_d, K_{el}, and $t_{1/2}$) were calculated for both ABS and ABNE formulations using QuickCal software (Developed by Dr. Shivprakash, Plexus, Ahmedabad, India). The percent relative bioavailability (% F_r) of NE to AB suspension was calculated as:

$$\% F_r = [AUC_{NE(0\rightarrow\infty)}/AUC_{ABS(0\rightarrow\infty)}] \times 100 \ldots.$$

The drug targeting index (DTI) was calculated to evaluate the targeting efficacy of radiolabeled ABNE in heart of Swiss albino mice. It was calculated from the ratio of AUC at target (pouch) and systemic site (venous blood).

Statistical analysis

All data are reported as mean ± SEM, and the differences between the groups were tested using Student's *t*-test at the level of $p < 0.05$. More than two groups were compared using ANOVA and differences greater at $p < 0.05$ were considered significant.

Results and discussion

Screening of components for NE

Selection of suitable oil, surfactant, and cosurfactant is a very critical parameter in development of an appropriate NE system. Higher solubility of AB in oil phase is more imperative for the NE formulation rather than drug solubilization in surfactant or cosurfactant. This is because dilution of NE in GIT will lead to lowering of solvent capacity of surfactant or cosurfactant causing risk of drug precipitation[1,21]. The solubility of slightly soluble AB was higher in the oily phases as compared with water with maximum solubility in oleic acid followed by labrafil M (Figure 1). However, labrafil M produced wider NE region in the ternary phase diagrams than oleic acid, thus it was selected as oily phase for preparation of NEs.

Use of nonionic surfactants in NE generally warrants less toxicity along with lower critical miceller concentration as compared with their ionic counterparts. Further, o/w NEs based on nonionic surfactants are also likely to offer better *in vivo* stability[4]. In our study, the nonionic surfactant Tween 80 displayed maximum solubility that was definitely advantageous as one of the important criterion for selection of surfactant is Hydrophilic Lipophilic Balance (HLB) value >10[4]. Tween 80 with an HLB value of 15 was thus a good excipient as a surfactant. Transient negative interfacial tension and fluid interfacial film are rarely achieved by the use of single surfactant, hence short-to-medium-chain length alcohols are necessary as cosurfactants. The cosurfactant also ensures that the interfacial film is flexible enough to deform readily around each droplet as their intercalation between the primary surfactant molecules decreases both the polar head group interactions[4,14–16]. Thus, cosurfactant ethanol with an HLB value of 4.2 was selected for the study as it showed highest drug solubility due to shorter chain length and less viscosity than rest of the cosurfactants[22].

Construction of pseudo-ternary phase diagrams

Ternary phase diagrams were constructed by varying Tween 80: ethanol (T: E) ratios as 0.5:1, 1:1, 1.5:1, and 2:1 (Figure 2A–2D). The shaded areas of phase diagrams show the NE regions, whereas the nonshaded area displays the emulsion region. Ternary phase system with T: E (2:1) was selected for optimization of NE, because it exhibited maximum area for NE formation (Figure 2D). The wider region indicated better nanoemulsifying efficiency of the developed formulation and better interaction among oil phase, S_{mix}, and aqueous phase[10]. It may be due to higher HLB value of labrafil M than the other

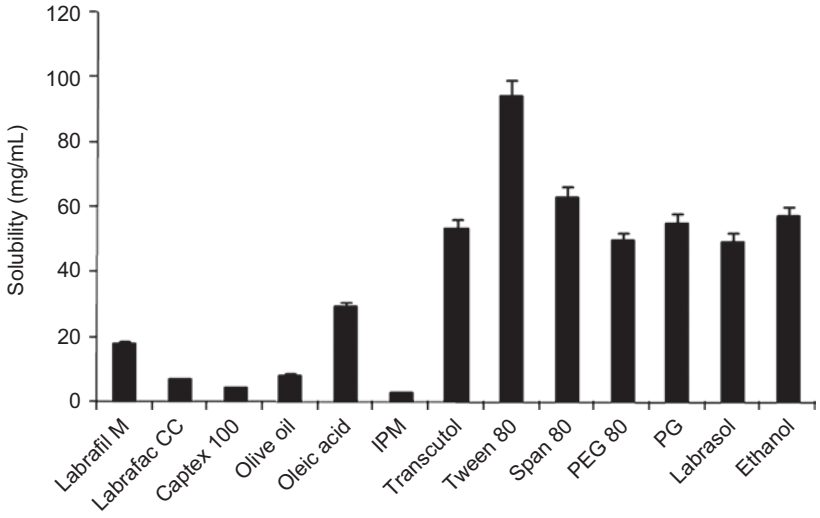

Figure 1. Comparative solubility profile of amlodipine besilate in various oily phases, surfactants, and cosurfactants.

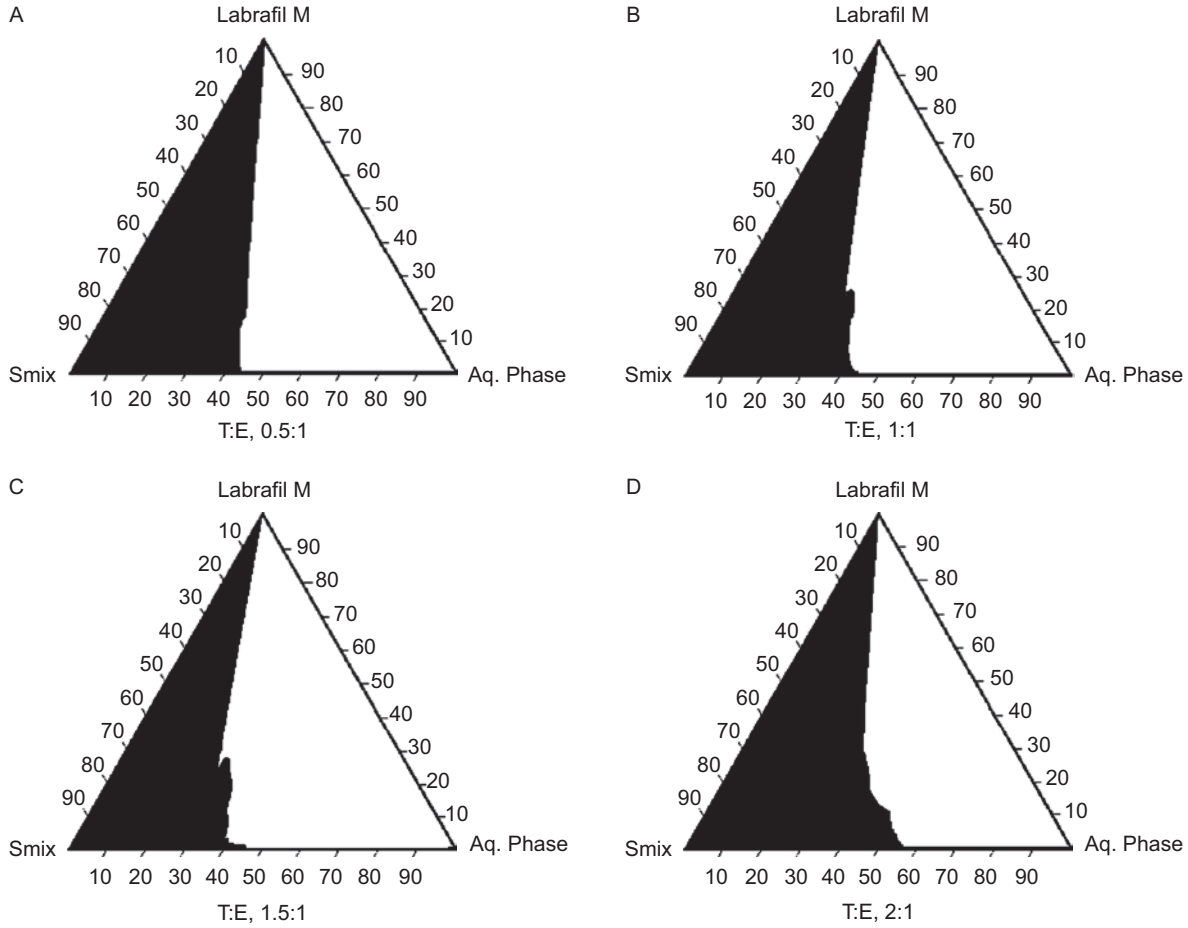

Figure 2. Pseudoternary phase diagrams of the formulations composed of Labrafil M:Tween 80:Ethanol dispersed with water at 37°C. S_{mix} ratio = 0.5:1 (A), 1:1 (B), 1.5:1 (C), 2:1 (D).

oils and oily mixtures favoring the formation of o/w NE. It is an oleoyl macrogol glyceride that offers more interaction sites for drug to interact with oil phase[23]. In addition, shorter chain length and less viscosity of the cosurfactant effects on the fluidity of the interfacial film and interdroplet interactions by which it reduces the bending stress of the interface and allow sufficient flexibility to the interfacial film to take more curvature, resulting wider NE region[2].

Apart from the ternary phase diagrams, globule size determinations were also performed as it could provide supportive evidence for the selection of phase diagram 2D. It was clearly evident that an increase in the concentration of Tween 80 resulted in a decrease in globule size. Thus, at the lowest concentration of surfactant globule size was 129.6 nm, whereas at the highest concentration of Tween 80, it reduced to 32.6 nm (data not shown), hence the S_{mix} ratio of 2:1 (Tween 80: ethanol) was selected for further optimization studies.

Optimization and characterization of NEs

The optimization of NEs with S_{mix} of 2:1 was carried out on the basis of globule size, zeta potential, and PDI. According to solubility study of AB in labrafil M, a minimum of 15% by weight of oily phase was required to fulfill the dose requirement. Seven different AB-loaded NEs (NE1–NE7) were formulated and characterized (Table 1). A speed of 800*g* for 15 min was selected as optimum process parameters to obtain drug-loaded NEs. NEs form spontaneously without the aid of high shear equipment and their microstructures were independent on the order of addition of excipients. All formulations had narrow globule size range of 29.6–34.6 nm, which was very well evident from the low PDI values. PDI that is the ratio of standard deviation to mean droplet size; hence, it indicates the uniformity of droplet size within the formulation. Higher the PDI, lower the uniformity of the droplet size in the formulation[24].

This was indicative of NEs approaching a monodispersed stable system that could effectively deliver the drug effectively owing to larger surface area. Neither phase separation nor creaming upon centrifugation of the ABNEs indicated stability of the prepared system. The conductivity measurements (0.133–0.249 mS/cm) indicated the nature of NEs to be of oil-in-water type. The refractive indices of formulated NEs ranged from 1.330 to 1.429, the least being for NE3. It was observed that viscosity of the NE formulations generally was very low. This was expected, because one of the characteristics of NE formulations is of lower viscosity[1] and low viscosity values ensure easy handling, packing, and smooth administration of formulations.

Table 1. Formulation optimization and characterization of amlodipine besilate loaded Nanoemulsions (NE1–NE7).

NE Code	Drug (mg/mL)	Labrafil M (%w/w)	Smix (%w/w)	Water (%w/w)	Zeta potential (mV)*	Globule size (nm)*	PDI*	%Drug content*	pH*	Viscosity (cP)*	Conductivity (ms/cm)*	%T (DF = 10)	Refractive index
NE1	1.25	15	30	55	−26.75 ± 1.27	102.6 ± 4.25	0.489 ± 0.08	96.42 ± 1.13	5.94 ± 0.22	254 ± 3.4	0.249 ± 0.04	88.67	1.432
NE2	1.25	15	32.5	52.5	−32.8 ± 1.36	82.3 ± 3.42	0.421 ± 0.06	98.21 ± 0.58	5.98 ± 0.25	271 ± 3.2	0.228 ± 0.03	92.75	1.389
NE3	1.25	15	35	50	−45.1 ± 2.58	29.6 ± 2.52	0.220 ± 0.04	99.47 ± 0.22	6.10 ± 0.24	286 ± 2.8	0.216 ± 0.03	99.78	1.334
NE4	1.25	15	37.5	47.5	−36.5 ± 1.75	34.5 ± 2.68	0.251 ± 0.06	98.73 ± 0.35	6.04 ± 0.29	292 ± 2.7	0.189 ± 0.02	98.74	1.345
NE5	1.25	15	40	45	−28.7 ± 1.31	67.3 ± 1.95	0.376 ± 0.05	95.94 ± 1.23	6.12 ± 0.18	319 ± 1.8	0.168 ± 0.04	96.88	1.373
NE6	1.25	15	42.5	42.5	−25.9 ± 1.42	62.04 ± 2.18	0.318 ± 0.07	97.83 ± 0.68	6.18 ± 0.23	328 ± 2.4	0.147 ± 0.03	97.47	1.362
NE7	1.25	15	45	40	−38.4 ± 1.96	38.6 ± 2.08	0.368 ± 0.02	98.45 ± 0.87	6.14 ± 0.27	360 ± 2.1	0.133 ± 0.02	98.69	1.349

*Each value in the table is the mean ± SEM of three estimations.

Table 2. Comparative $K_{(O/NE)}$, *in vitro* drug release, % dissolution efficiency, and release kinetics of different formulations (NE1–NE7).

NE Code	$K_{(O/NE)}$	% Cumulative drug release after 12 h#	% Dissolution efficiency#	Cumulative amount of drug released after 12 h (mg)v	Release kinetics
NE1	1.05	69.77 ± 2.12	46.82 ± 1.24	3.62 ± 0. 16	Higuchi's Model
NE2	1.11	71.58 ± 1.56	49.03 ± 1.58	3.71 ± 0. 24	Higuchi's Model
NE3	2.29	94.85 ± 2.30	72.30 ± 2.15	4.68 ± 0.18	Higuchi's Model
NE4	1.92	86.54 ± 1.81	63.78 ± 1.91	4.54 ± 0.26	Higuchi's Model
NE5	1.15	76.72 ± 1.72	52.94 ± 1.46	3.95 ± 0.19	Higuchi's Model
NE6	1.34	78.49 ± 1.84	53.80 ± 1.74	4.06 ± 0.23	Higuchi's Model
NE7	1.57	80.56 ± 1.82	55.16 ± 1.63	4.25 ± 0.27	Higuchi's Model
Marketed formulation	—	54.86 ± 0.85	37.73 ± 0.92	2.84 ± 0.13	—

#Each value in the table is the mean ± SEM of three estimations.

The formulation NE3 (oil:S_{mix}:water, 15:35: \50) was selected as optimized formulation as it displayed optimum characteristics such as 99.78% optical transparency, least globule size (29.6 nm), polydispersity of 0.220, and zeta potential to the tune of –45.1 mV. A high percentage transmittance value indicated clear dispersion. The presence of zeta potential to the tune of –45.1 mV on the globules conferred physical stability to the system. Zeta potential (ζ) is the main indicator of emulsion stability and for ζ values within ±30 mV (–30 to +30 mV), the emulsion tends to destabilize. Visually, all the formulations were transparent in nature but NE3 with a refractive index of 1.330 a value close to that of water (1.334) indicating maximum transparency and isotropic nature of NE3.

In vitro drug release study

Dissolution studies by dialysis bag method were performed to compare the release of drug from seven different optimized NE formulations (NE1–NE7) against marketed formulation having same quantity (5 mg) of AB. The release of drug from all NEs was much faster and higher in phosphate buffer pH 7.4 than the marketed formulation. NE3 showed highest 94.85 ± 2.38% drug release in contrast, the marketed formulation released 54.86 ± 0.85 % of the drug in 12 h due to low aqueous solubility. The comparative drug release profile is depicted in Figure 3. The percentage dissolution efficiency (%DE) and cumulative amount of drug delivered from NEs after 12 h were calculated using PCP-Disso-V2.08 software, Pune, India. Reduction in the droplet size leads to higher surface area and complete dissolution of AB in oily phase of NE eventually permitted drug release at faster rate from NEs showing the significance of the nanosizing of the oils globules[25]. The *in vitro* drug release profiles were analyzed by fitting dissolution data to kinetic models using PCP-Disso-V2.08 software, Pune, India, in order to evaluate release mechanisms of AB NEs (Table 2). All the developed NEs followed Higuchi model (matrix type).

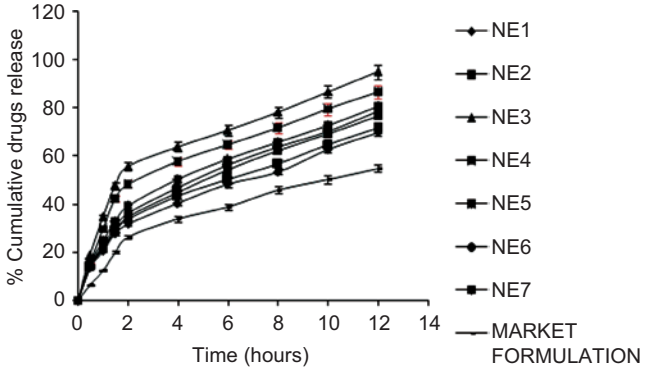

Figure 3. Comparative *in vitro* dissolution profile of amlodipine besilate from different NE formulations and marketed formulation in phosphate buffer pH 7.4.

Partitioning studies

Partitioning study was done to find out the partitioning behavior of drug between NE and the lipophilic *n*-octanol that has similar properties to biological membranes[19]. Highest $K_{(O/NE)}$ value for NE3 suggested that the drug is more liable to be transported across the membranes as compared with the rest of the formulations (Table 2). An attempt was also made to correlate the $K_{O/NE}$ with % DE by linear regression analysis to evaluate the possibility of using $K_{(O/NE)}$ as a single point predictive parameter for evaluating the *in vitro* drug release from NEs. It was observed that higher the $K_{O/NE}$ more was the %DE and a r^2 of 0.9925 (Figure 4) indicated sufficient linearity between the physical and performance parameter of the designed system. However, its extrapolation to other systems needs intensive investigations.

Thermodynamic stability study

NEs are thermodynamically stable systems and formed at a particular concentration of oil, surfactant, and water with no phase separation, creaming, or cracking. It is the thermostability that differentiates nano- or microemulsions from macroemulsions thata are kinetically unstable that eventually lead to phase separation. Thus, the optimized formulation NE3 was subjected to different thermodynamic stability stress tests by using centrifugation cycle, heating cooling cycle and freeze-thaw cycle[5]. The formulation was stable during each stress cycle; hence it can be said as thermodynamically stable.

Transmission electron microscopy

TEM revealed that the lipid NE droplets were almost spherical in shape, discrete, appearing dark with an amorphous core (Figure 5). Some droplet sizes were measured and the droplets in nanometer range varied from 30 to 60 nm.

In-vivo biodistribution and pharmacokinetics studies

The optimized NE formulation (NE3) encoded as ABNE was selected for biodistribution and pharmacokinetics studies. AB was effectively labeled using technetium (^{99m}Tc), and both ABS and ABNE were optimized for

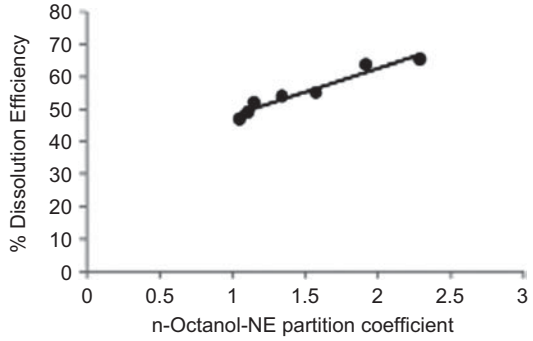

Figure 4. Correlation curve between %dissolution efficiency and *n*-octanol–NE partition coefficient showing linear relationship.

maximum labeling efficiency, radiolabeled complex were found to be stable for 24 h. The radiochemical purity achieved for ABS and ABNE was 98.64 and 98.51%, respectively, when evaluated for reduced/hydrolyzed ^{99m}Tc and free ^{99m}Tc. A pH range of 6.0–6.5 and 400 µg of stannous chloride with 30 min of incubation time for ABS and ABNE were selected as the conditions for the optimum radiolabeling. Biodistribution studies of ^{99m}Tc-AB formulations following oral administration in Swiss albino mice were performed by estimation of radioactivity at different time intervals of up to 24 hr[26]. The AB concentrations in heart and blood following the oral administration of ABNE were found to be significantly higher ($p < 0.05$) at all the time points as compared with ABS (Table 3).

Pharmacokinetic parameters of AB obtained by one-compartment open model given by extravascular route are mentioned in Table 4. NE formulations led to pronounced alteration in the pharmacokinetics of AB. The C_{max} and $AUC_{0\to\infty}$ of the ABNE formulation was significantly higher than that of conventional tablet suspension ($p < 0.05$). The AUC and C_{max} increased by 4.78 and 2.2 folds, respectively, for ABNE when compared with ABS. The percentage relative bioavailability ($\%F_r$) was found to be 475% as compared with AB suspension (Figure 6), indicating the influence of nanosizing of oil droplets on the bioavailability.

The biodistribution studies revealed a significantly higher uptake of AB from ABNE than ABS in almost all the tested organs especially in heart ($p < 0.05$) that is the site of drug action (L-type myocardial Ca^{+2} channels) demonstrating the passive targeting efficacy of the nanosized formulation. The %A/g was 11.8 and 13.6 folds higher in heart at 0.5 and 1.0 h, respectively, from ABNE in comparison with ABS. This is many folds higher than the %A/g in blood at the same time points (7.25 folds at 0.5 h and 5.35 folds at 1.0 h). Thereafter, the %A/g almost equaled to the blood till 24 h.

DTI was also calculated to evaluate the targeting efficacy of NE. It was calculated from the ratio of AUC at target, that is, pouch (7.021 h%/g) and systemic site,

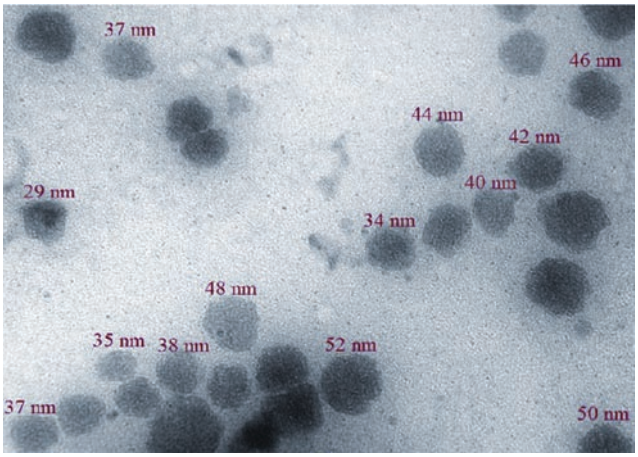

Figure 5. Transmission electron microscopic positive image of amlodipine besilate NE showing size of few oil droplets.

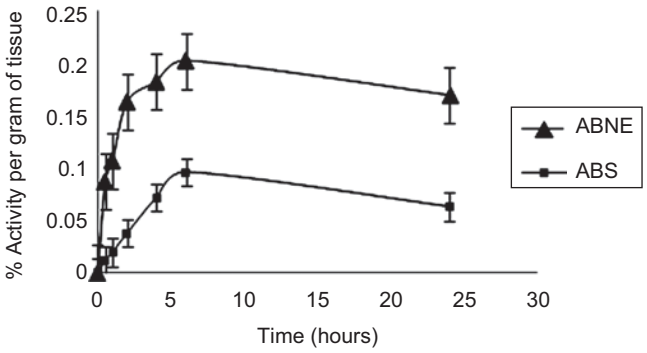

Figure 6. Comparative bioavailability plot of radiolabeled ABNE and ABS in Swiss albino mice after oral administration.

Table 3. Comparative biodistribution of ^{99m} Tc labeled ABS and ^{99m} Tc labeled ABNE at different time intervals in normal Swiss albino mice obtained after oral administration*.

		Percentage activity per gram of organ/tissue (%A/g) ± SEM					
Organ/tissue	Formulation	0.5 h	1.0 h	2.0 h	4.0 h	6.0 h	24.0 h
Blood#	ABS	0.012 ± 0.002	0.02 ± 0.003	0.038 ± 0.004	0.072 ± 0.005	0.096 ± 0.008	0.063 ± 0.006
	ABNE	0.087 ± 0.005	0.107 ± 0.009	0.163 ± 0.015	0.182 ± 0.012	0.202 ± 0.016	0.169 ± 0.014
Heart#	ABS	0.003 ± 0.001	0.005 ± 0.002	0.066 ± 0.003	0.042 ± 0.004	0.091 ± 0.007	0.024 ± 0.004
	ABNE	0.035 ± 0.003	0.068 ± 0.006	0.091 ± 0.009	0.078 ± 0.010	0.124 ± 0.012	0.067 ± 0.005
Liver	ABS	0.019 ± 0.001	0.038 ± 0.002	0.086 ± 0.004	0.102 ± 0.008	0.181 ± 0.013	0.127 ± 0.012
	ABNE	0.081 ± 0.003	0.097 ± 0.007	0.127 ± 0.08	0.123 ± 0.010	0.201 ± 0.018	0.154 ± 0.013
Spleen	ABS	0.082 ± 0.004	0.087 ± 0.004	0.113 ± 0.009	0.198 ± 0.014	0.263 ± 0.017	0.095 ± 0.004
	ABNE	0.063 ± 0.005	0.177 ± 0.012	0.265 ± 0.015	0.496 ± 0.018	0.507 ± 0.024	0.081 ± 0.007
Kidney	ABS	0.025 ± 0.002	0.043 ± 0.003	0.087 ± 0.005	0.096 ± 0.005	0.139 ± 0.012	0.086 ± 0.004
	ABNE	0.051 ± 0.002	0.085 ± 0.003	0.101 ± 0.005	0.136 ± 0.007	0.204 ± 0.008	0.099 ± 0.002
Intestine	ABS	0.38 ± 0.032	4.23 ± 0.49	7.08 ± 0.78	6.35 ± 0.67	18.65 ± 1.05	8.31 ± 0.81
	ABNE	0.71 ± 0.25	1.23 ± 0.42	2.47 ± 0.65	12.27 ± 0.96	21.95 ± 1.58	7.52 ± 0.85

*The mice were administered with 200 µCi ^{99m}Tc-AB and the radioactivity was measured in percentage per gram of tissue of the administered dose. Each value is the mean ± SEM of three estimations. Radioactivity was measured at 0 hr and all the measurements were performed using 0 hr sample of the corresponding tissue/organ as blank sample.
#The % Activity per gram of organ/tissue (%A/g) data is significant at $p < 0.05$.

Table 4. Pharmacokinetic data of different AB Formulations at different time intervals in normal Swiss albino mice following one-compartment open model obtained by QuickCal software.[1]

Pharmacokinetic parameter	Formulation data[1]	
	ABNE	ABS
C_{max} (%A/g)	0.202 ± 0.042	0.096 ± 0.015
T_{max} (hrs)	6.0 ± 0.32	6.0 ± 0.43
$AUC_{0\rightarrow 24h}$ (h %/g)	4.25 ± 0.97	1.75 ± 0.32
$AUC_{0\rightarrow\infty}$ (h %/g)	15.19 ± 1.26	3.19 ± 0.51
Cl_T (L/h)	0.263 ± 0.04	1.75 ± 0.28
V_d (L)	28.83 ± 1.14	17.05 ± 1.05
K_E (h^{-1})	0.015 ± 0.003	0.043 ± 0.009
$T_{1/2}$ (h)	44.87 ± 1.42	15.98 ± 0.76

[1]The mice were administered with 200 μCi ^{99m}Tc-AB and the radioactivity was measured in percentage per gram of tissue of the administered dose. Each value is the mean ± SEM of three estimations.

that is, venous blood (15.91 h%/g). DTI was found to be 0.441 (44.1%). These data conclude the efficacy of nanosized globules as therapeutically effective carrier for drug targeting. It is the passive drug targeting mechanism by which NE more approached to the heart and this is supported by the biodistribution profile of drug in experimental animals. The reasons contributing to the effect are drug in the solubilized form as nanolipid globules is expected to bypass enzymatic degradation in GIT while being sequestered through lymphatic route and substantially circumventing hepatic first-pass metabolism[2]. The proposed mechanism is supported by the biodistribution of the drug in liver. The %A/g in liver and kidney from ABNE was higher than ABS at all time points but it was statistically not significant ($p > 0.05$) and also was very less as compared with %A/g from ABNE in heart. ABNE improved the bioavailability of AB by enhancing the absorption of drug via surfactant-induced permeability changes of GI membrane[27,28] and these are also known to cross the gut lumen by paracellular pathway and enter the blood circulation, thus demonstrating a systemic effect and also circumventing the hepatic first pass effect[29]. Moreover, nanosized preparation ensures greater surface area and also the presence of Tween 80 as a surfactant in the NE formulation might modulate the membrane permeability through apically polarized efflux system leading to enhanced oral bioavailability[30], hence permitting more amount of drug to be delivered at the therapeutic site.

Conclusion

ABNE that contained 15% oil, 35% Smix, and 50% water by weight displayed best pharmacotechnical properties and higher organ/tissue biodistribution after oral administration than the AB suspension. An overall three times higher residence of NE compared with ABS signifies the advantage of NEs as drug carriers for AB in enhancing the bioavailability and facilitating passive delivery of AB to heart. However, clinical benefits to the risk ratio of the formulation developed in this investigation will decide its appropriateness in the clinical practice for the treatment of hypertension and angina pectoris.

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Declarations of interest

The authors report no declarations of interest.

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