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Investigation of a nanoemulsion as vehicle for transdermal delivery of amlodipine

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Received July 14, 2008, accepted August 5, 2008

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Pharmazie 64: 80–85 (2009) *doi: 10.1691/ph.2009.8200*

A novel oil-in-water nanoemulsion system for transdermal delivery of amlodipine was studied. Pseudo-ternary phase diagrams were developed and various nanoemulsion formulations were prepared using oleic acid (oil phase), Tween 20 (surfactant) and Transcutol P (co-surfactant). The effects of content of oleic acid and surfactant/co-surfactant ratio (S_{mix}) on skin permeation of amlodipine were evaluated through excised rat skin using a Franz diffusion cell. Highest permeation rate and permeability coefficient was found at low oil and S_{mix} concentration. On increasing the same, fluxes were further decreased, probably due to increased globule size and decreased thermodynamic activity of drug at higher surfactant mixture concentration. The optimum nanoemulsion formulation consisted of 2% oil (oleic acid), 20% surfactant (Tween 20), 10% co-surfactant (S_{mix} 2 : 1) and water exhibited highest skin permeation rate of 49.681 $\pm$ 1.98 μg/cm/h and permeability coefficient of 0.497 $\pm$ 0.056 cm^2/h. The optimized nanoemulsion was characterized for globules size, morphology, viscosity, and pH. The result suggests that nanoemulsions are potential vehicles for improved transdermal delivery of amlodipine.

1. Introduction

Nanoemulsion is defined as an O/W or W/O emulsion producing a transparent product, thermodynamically stable that has a droplet size of <0.15 nm and does not have the tendency to coalesce (Kreilgaard 2002). It consists of oil phase, surfactant, co-surfactant and aqueous phase in appropriate concentration. Nanoemulsions have received great attention in recent years for various applications, including transdermal delivery of various drugs such as propranolol (Ktistis and Niopas 1998), 8-methoxasalen (Baroli et al. 2000), methotrexate (Alvarez-Figueroa and Blanco-Mendez 2001), estradiol (Peltola et al. 2003), triptolide (Chen et al. 2004), meloxicam (Yuan et al. 2006). The primary factors influencing transdermal permeation of drugs are mobility of drug in vehicle, release of drug from vehicle, and permeation of drug into skin. It has been reported that high transdermal flux from nanoemulsion is mainly due to their high solubilization potential for lipophilic and hydrophilic drugs. This eventually produces an increased thermodynamic activity towards skin and thus high permeation rate (Kreilgaard et al. 2000; Alvarez-Figueroa and Blanco-Méndez 2001).

Conventional formulations of 1,4-dihydropyridine molecules including amlodipine have many limitations; therefore several works has been performed to develop transdermal systems for calcium channel antagonists to overcome problems of oral administration (McDaid and Deasy 1996; Krishnaiah et al. 2003).

Amlodipine is a calcium channel blocker belonging to the 1,4-dihydropyridine category and is structurally related to nifedipine. The clinical pharmacology of the antihypertensive action of amlodipine involves a direct relaxant effect on vascular smooth muscle. Amlodipine is given orally (5 mg daily) with peak plasma concentration occurring after 6–12 h, and has oral bioavailability of 60–65% only due to extensive hepatic metabolism (Williams and Cubeddu 1988).

As nanoemulsion itself has several advantages such as enhancing drug solubility, high thermodynamic stability, and ease of manufacturing. Transdermal bioavailability and sustained effect of drugs is improved compared to conventional formulations (Lawrence et al. 2000; Sintov and Shaprio 2004). Therefore, the primary aim of this work was to formulate and evaluate a nanoemulsion system for transdermal delivery of amlodipine having low surfactant concentration and high skin permeability.

2. Investigations, results and discussion

2.1. Selection of oil and surfactant

Oil for nanoemulsion has been selected on the basis of drug solubility; the solubility of amlodipine in various oils is shown in Table 1. The solubility was highest in oleic acid (40 $\pm$ 1.16 mg/ml), followed by oleic acid and triacetin mixture (1 : 1), Labrafac PG, Triacetin, isopropyl myristate (IPM) and olive oil. Therefore, oleic acid was chosen as oil phase for developing the nanoemulsion containing amlodipine. It has also been reported that oleic acid is a good permeation enhancer for transdermal delivery (Rhee et al. 2001), by increasing fluidity of intercellular lipid barriers in the stratum corneum by forming separate domains which interfere with continuity of

Table 1: Solubility of amlodipine in various oils and nanoemulsion at 25 ± 1 °C (mean ± S.D.; n = 3)

Vehicle	Solubility
Olive oil	2 ± 0.03
Ispropyl myristate	5 ± 0.07
Oleic acid	40 ± 1.16
Triacetin	10 ± 0.41
Labrafac PG	12 ± 0.35
Triacetin : Oleic acid (1 : 1)	25 ± 0.37
Nanoemulsion B1	30 ± 0.31

multilamellar stratum corneum and induce highly permeable pathways in stratum corneum (Pershing et al. 1993). Nonionic surfactants are widely used in topical formulations as solublizing agents but some recent results indicate that they may affect the skin barrier function (Lopez et al. 2000). Thus, Tween 20 was chosen as surfactant with Transcutol P as co-surfactant and deionized water as aqueous phase.

2.2. Phase diagrams studies

Figure 1 (a–e) presents phase diagrams of oleic acid (oil)–S_{mix} (Tween 20 and Transcutol P)–water system having different S_{mix} ratio (1 : 0, 1 : 1, 1 : 2, 2 : 1, 3 : 1). It can be seen that these phase diagrams contain different areas of clear and isotropic nanoemulsion region.

It can be also seen that least nanoemulsion region exists at S_{mix} ratio 1 : 0 (i.e., without co-surfactant). However, equal mixture of surfactant and co-surfactant increases the nanoemulsion region (Fig. 1b). Increasing the concentration of surfactant (2 : 1) resulted in even larger area of nanoemulsion region, along with emulsion, gel or nanoemulsion gel region (Fig. 1d). Further increasing surfactant concentration from 2 : 1 to 3 : 1 resulted in reduction of nanoemulsion region and a higher region was composed of gels (Fig. 1e). The influence of concentration of co-surfactant on the nanoemulsion region was also seen by constructing the phase diagram in the ratio of 1 : 2. It was seen that the region of nanoemulsion formation was increased but further increase in concentration of co-surfactant decreased the nanoemulsion region (Fig. 1c).

The existence of large or small nanoemulsion region depends on the capability of a particular surfactant or surfactant mixture to solubilize the oil phase. The extent of solubilization results in a greater area with clearer and homogenous solution. It was seen that when the surfactant (Tween 20) was used alone, the oil phase was solubilized to a lesser extent implying that surfactant alone was not able to reduce the interfacial tension of the oil droplets to a sufficiently low level and thus was not able to reduce the free energy of the system to an ultra low level desired to produce nanoemulsions. When a co-surfactant was added, the interfacial tension was reduced to a very low level and very small free energy was achieved which helps in larger nanoemulsion region. With further increase in surfactant from 1 : 1 to 2 : 1, further drop in interfacial tension and free energy was achieved resulting in maximum region of nanoemulsion formation. However, at S_{mix} ratio 3 : 1, clear nanoemulsion region decreased and higher gel region was observed. Thus, pseudo-ternary phase diagrams 1 : 1, 2 : 1 & 1 : 2 were selected for the formulation of drug loaded nanoemulsions. For the selection of formulations from phase diagrams, full extent of oil region showing nanoemulsion was selected with minimum surfactant showing the presence of nanoemulsion at particular oil composition.

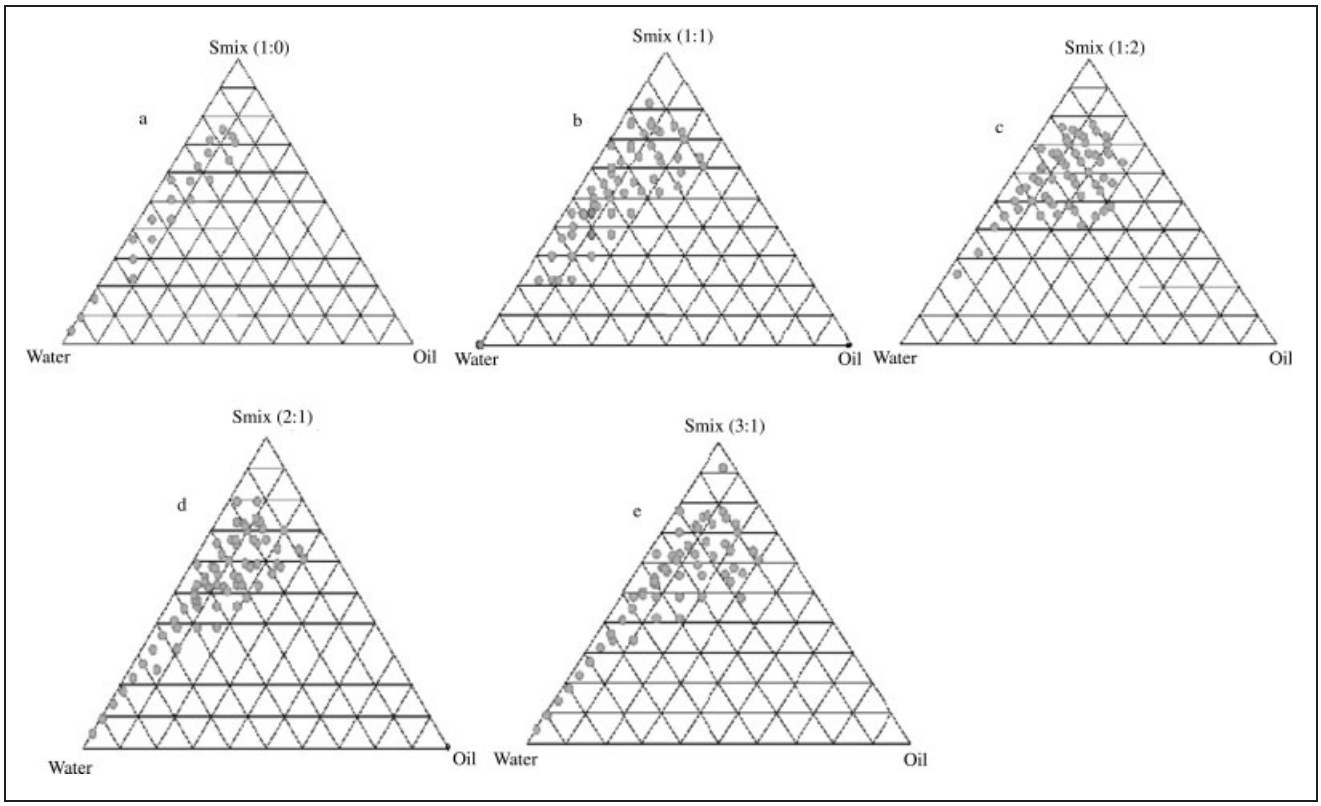

Fig. 1: Pseudo-ternary phase diagrams of nanoemulsions composed of oleic acid (oil), Tween 20 (Surfactant), Transcutol P (Co-surfactant) and water. Surfactant and Co-surfactant ratio (Smix) is varying for each pseudo-ternary phase diagram

2.3. Thermodynamic stability

Nanoemulsions are thermodynamically stable systems and are formed at a particular concentration of oil, surfactant and water, with no phase separation, creaming or cracking. The thermal stability differentiates nanoemulsions from emulsions which show kinetic instability and eventually phase separation (Lawrence et al. 2000). Therefore, to check the thermodynamic stability of a drug loaded nanoemulsion, freeze thaw cycle and centrifugation studies were performed. Physical stability such as phase separation and turbidity was continuously monitored over the period of study time. Nanoemulsions which have thermodynamic stability were taken for further studies.

2.4. Skin permeation studies

Drug loaded oil-in-water nanoemulsions were selected from the phase-diagram to explore the effect of S_{mix} ratio on skin permeation. Compositions of selected drug loaded nanoemulsions are shown in Table 2, 3 and 4. Oil component in the selected nanoemulsion was between 2–12 % v/v irrespective of the S_{mix} ratio. The permeation parameters of tested nanoemulsions are presented in Table 5. Skin permeation studies reveal that there was significant ($p < 0.05$) increase in flux of all nanoemulsion formulations over control (saturated solution) (10.285 ± 1.32 μg/cm^2/h) (Table 5). The equation of flux given by Higuchi is expressed as

$$J = (D \times Cs/h) \times (C/Cm),$$

where D is diffusion coefficient of the drug in membrane, Cs is drug solubility in the membrane, C/Cm is the extent of saturation of drug in vehicle and h is diffusion path length. C/Cm is the parameter which directly indicates the thermodynamic activity of vehicle. For high permeation rate, a saturated solution should have the highest thermodynamic activity (Higuchi 1960). However, the low solubility of amlodipine in phosphate buffer (aqueous vehicle) resulted in low permeation rate, because other factors such as D, C, Cs and Cm may influence the permeation of drug. The present work discloses that the nanoemulsion has improved permeation rate due to its high solubilizing capacity for amlodipine and high permeation coefficient.

The results in the present study are in accordance with others (Peltola et al. 2003; Chen et al. 2006). Peltola et al. (2003) which disclosed that a microemulsion increased estradiol's flux 200–700-fold over the control, the high transdermal flux of estradiol was due to 500-fold improvement in solubilization of estradiol in microemulsions formulation.

Similar results were reported by another group, in which all the selected microemulsions of ibuprofen showed a permeation rate superior to saturated solution (Chen et al. 2006). Therefore, it can be concluded that solubility plays a major role in improving permeation rate of lipophilic drugs and the nanoemulsion system provides a powerful solubilization and an increased transdermal permeation of amlodipine.

When comparing the flux of selected nanoemulsions of each oil concentration from different S_{mix} ratio (1 : 1, 1 : 2 and 2 : 1) suggests that there is no significant change in permeation rate and permeability coefficient ($p > 0.05$). However, formulation B1 showed highest rate of permeation (49.681 ± 1.98 μg/cm/h) and permeability coefficient (0.005 ± 0.056 cm^2/h) (Table 5). Enhancement ratio (ER) of formulation B1 was 4.83 ± 0.13 times that of control (saturated solution) (Table 5).

The content of oil also played an important role in skin permeation rate of nanoemulsions. Figure 2 shows the ef-

Table 2: Composition of nanoemulsions selected from phase diagram with Smix ratio 1 : 1 (Tween 20: Transcutol P)

Nanoemulsion	Oleic acid (% v/v)	Tween 20 (% v/v)	Transcutol P (% v/v)	Deionized water (%v/v)	Amlodipine (mg)
A1	2	12.5	12.5	73	10
A2	4	14	14	67	10
A3	6	16.5	16.5	61	10
A4	8	19	19	54	10
A5	10	25	25	40	10
A6	12	26	26	36	10

Table 3: Composition of nanoemulsions selected from phase diagram with Smix ratio 2 : 1 (Tween 20: Transcutol P)

Nanoemulsion	Oleic acid (% v/v)	Tween 20 (% v/v)	Transcutol P (% v/v)	Deionized water (% v/v)	Amlodipine (mg)
B1	2	20	10	68	10
B2	4	30	10	56	10
B3	6	30	15	49	10
B4	8	34	16	42	10
B5	10	34	17	39	10
B6	12	37	18	30	10

Table 4: Composition of nanoemulsions selected from phase diagram with Smix ratio 1 : 2 (Tween 20: Transcutol P)

Nanoemulsion	Oleic acid (% v/v)	Tween 20 (% v/v)	Transcutol P (% v/v)	Deionized water (% v/v)	Amlodipine (mg)
C1	2	12	23	63	10
C2	4	13	28	55	10
C3	6	16	30	48	10
C4	8	16	32	44	10
C5	10	17	33	40	10
C6	12	18	34	36	10

Table 5: Percutaneous permeation parameters of tested nanoemulsion formulations (mean S.D.; n = 3)

Nanoemulsions	Flux (μg/cm^2/h) ± SD	Permeability coefficient (K_p) × 10^2 (cm/h) ± SD	Enhancement ratio (ER) ± SD
Control	10.285 ± 1.32	0.100 ± 0.008	–
A1	39.113 ± 2.36	0.391 ± 0.012	3.80 ± 0.13
A2	44.367 ± 2.49	0.444 ± 0.011	4.31 ± 0.21
A3	37.511 ± 2.11	0.375 ± 0.009	3.65 ± 0.17
A4	27.528 ± 1.65	0.275 ± 0.010	2.68 ± 0.16
A5	24.624 ± 1.58	0.246 ± 0.005	2.39 ± 0.11
A6	24.010 ± 1.54	0.240 ± 0.012	2.33 ± 0.10
B1	**49.681 ± 1.98**	**0.497 ± 0.056**	**4.83 ± 0.13**
B2	42.921 ± 2.10	0.429 ± 0.041	4.17 ± 0.15
B3	41.409 ± 2.44	0.414 ± 0.034	4.03 ± 0.11
B4	40.301 ± 1.95	0.403 ± 0.033	3.92 ± 0.20
B5	36.104 ± 1.64	0.361 ± 0.012	3.51 ± 0.17
B6	33.595 ± 1.47	0.336 ± 0.013	3.27 ± 0.15
C1	48.713 ± 2.11	0.487 ± 0.022	4.74 ± 0.21
C2	44.367 ± 2.69	0.443 ± 0.041	4.31 ± 0.25
C3	36.104 ± 1.61	0.360 ± 0.021	3.51 ± 0.19
C4	33.595 ± 1.23	0.336 ± 0.020	3.26 ± 0.17
C5	27.528 ± 1.64	0.275 ± 0.012	2.67 ± 0.09
C6	24.010 ± 1.32	0.240 ± 0.013	2.33 ± 0.10

* $P < 0.05$ compared with the aqueous saturated solution of drug (Control sample)

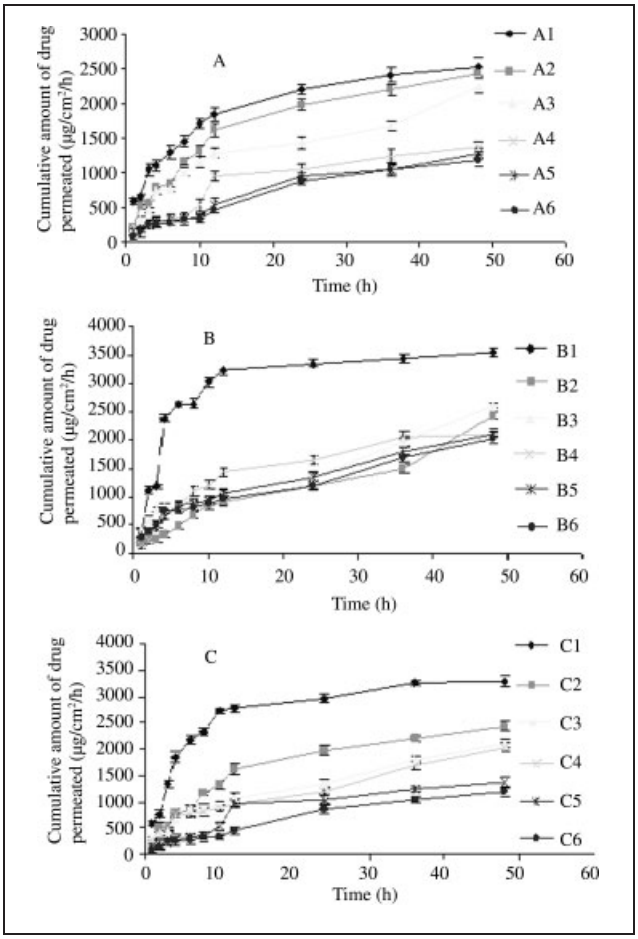

Fig. 2: Permeation profile of amlodipine through excised rat skins from nanoemulsion formulations having different Smix ratio, (A) Smix ratio 1 : 1; (B) Smix ratio 1 : 2; (C) Smix ratio 2 : 1

fect of the content of oleic acid ranging from 2% to 12% v/v on the skin permeation of amlodipine, while the content of surfactant and co-surfactant mixture S_{mix} (1 : 1, 1 : 2 and 2 : 1) ranged from 25–52% v/v. The skin permeation rate of amlodipine was high in all the cases when the percentage concentration of oleic acid was low (2–4% v/v) and enhancement ratios were 3.80–4.83 times higher than control (saturated solution). The result suggests that with increasing oil concentration at all S_{mix} ratios, a significant decrease in permeation rate ($P < 0.05$) was observed while surfactant mixture increased (Table 5). Permeation rate was not expeditiously dipped on comparing formulation B3-B6 (S_{mix} 2 : 1; Tween 20 is doubled of Transcutol P) and C3-C6 (S_{mix} 1 : 2) where the oil concentration increased significantly and surfactant concentration was kept around 50% (Tables 3–4) (Figs. 2 B and C). It was reported that oil and surfactant concentration influences the permeation of drug from nanoemulsion.

Yuan et al. (2006) reported that permeation rate decreased significantly when IPM concentration increased from 5 to 15%. This may possibly be explained by the fact that the high water content in the microemulsion could hydrate skin and cause corneous cells to swell thus making drug channels wide. Moreover, the drugs in nanoemulsions can penetrate the skin in the form of a nanoemulsion droplet and not as a free one (Yuan et al. 2006). This could be further justified by small average globule size of the amlodipine nanoemulsion (59.1 nm). It was reported that increasing the oil content leads to high average globules size and low permeation rate (Chen et al. 2004; Yuan et al. 2006). Similarly, permeation

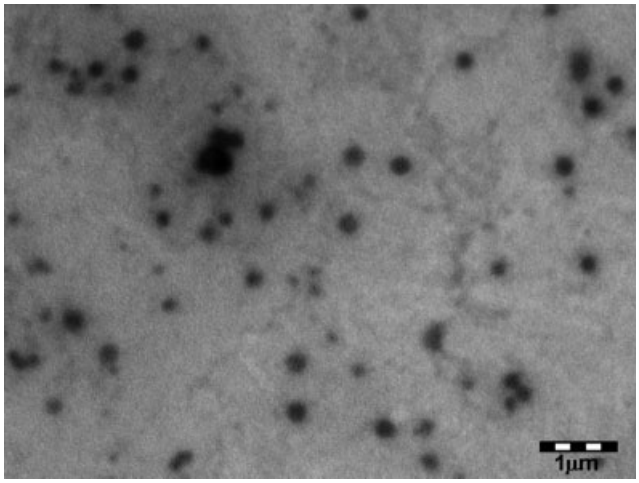

Fig. 3: Micrograph of amlodipine nanoemulsion by transmission electron microscopy (TEM)

rate of drug increased as the concentration of surfactant mixture decreased and the phenomenon might be due to an increased thermodynamic activity of the drug in nanoemulsions at lower surfactant content (Rhee et al. 2001; Yuan et al. 2006; Zhao et al. 2006).

It was also described that high oleic acid content and surfactant mixture in formulations might contribute to skin irritation. Therefore, nanoemulsion formulation B1 (2% oleic acid, 20% Tween 20 and 10% Transcutol P) was selected for further studies since it led to high skin permeation of amlodipine and was devoid of skin irritation.

2.5. Characterization of nanoemulsion

The optimized nanoemulsion B1 (2% oleic acid, 20% Tween 20 and 10% Transcutol P) was characterized for different parameters like solubilizing capacity, globule size, globule shape, viscosity, and pH. The solubility of amlodipine in nanoemulsion B1 was 30.2 ± 0.31 mg/ml. The high solubilizing capacity of the nanoemulsion may be due to surfactant mixture and small droplet size.

Morphology of amlodipine nanoemulsion was characterized by transmission electron microscopy (TEM). The image clearly shows that the shapes in the dispersed phase (oil globules) are spherical (Fig. 3). The mean globule size of the dispersed phase was 59.1 nm with a polydispersity index (PI) of 0.125 which shows the uniformity of globule size. The small globule size with narrow size distribution was justified by the very low content of oleic acid in nanoemulsion. The nanoemulsion formulation had low viscosity (91.23 ± 0.5 mPa S) with a suitable pH of 6.35 ± 0.03. The low viscosity of the nanoemusion was due to its low oil content (2% oleic acid).

3. Experimental

3.1. Materials

Amlodipine was obtained as gift sample from Torrent Pharmaceuticals, India. Oleic acid, olive oil, isopropyl myristate, Tween 20 were purchased from Central Drug House, Mumbai, India. Transcutol P and Labrafac PG were provided by Gattefosse, France. HPLC grade acetonitrile and methanol were procured from E Merck, Mumbai, India. Deionized water was obtained from Milli-Q water purification system (Millipore, Milford, MA, USA). All other chemicals and solvents were of analytical reagent grade.

3.2. Screening of oils and surfactant for nanoemulsion

In order to find an appropriate oil that had good solubilizing capacity for amlodipine, a solubility study was performed with five different oils such

as oleic acid, isopropyl myristate (IPM), olive oil, triacetin, Labrafac PG and oleic acid: Triacetin (1 : 1) mixture. One ml of different oils was taken in small vials and excess amount of drug was added. The vials were tightly stoppered and continuously stirred for 72 h at 25 ± 0.5 °C and after 72 h samples were centrifuged at 10,000 rpm for 10 min. The supernatant was separated, filtered through a membrane filter (0.45 μm) and analyzed by HPLC after appropriate dilution with methanol.

3.3. Construction of pseudo-ternary phase diagrams

Pseudo-ternary phase diagrams were constructed to determine the concentration of components that can result in large nanoemulsion region with and without drug. On the basis of solubility studies, oleic acid was selected as oil phase. Tween 20 and Transcutol P were chosen as surfactant and co-surfactant, respectively. Deionized water was used as aqueous phase. Surfactant and co-surfactant (S_{mix}) were mixed in different ratios (1 : 0, 1 : 1, 1 : 2, 2 : 1 and 3 : 1). These S_{mix} were chosen in increasing concentrations of surfactant with respect to co-surfactant for a detailed study of phase diagrams.
For each phase diagram, oil and specific S_{mix} were mixed well in different ratios. Sixteen different combinations of oil and S_{mix} (1 : 9, 1 : 8, 1 : 7, 1 : 6, 1 : 5 1 : 4, 1 : 3.5, 1 : 3, 3 : 7, 1 : 2, 4 : 6, 5 : 5, 6 : 4, 7 : 3, 8 : 2 and 9 : 1) were made so that maximum ratio could be covered for the study to delineate the boundaries of phases formed precisely in phase diagrams. Slow titration with aqueous phase was done for each ratio of oil and S_{mix} and visual observation was used for transparent and easily flowable nanoemulsion. The physical state of nanoemulsion was marked on a pseudo three component phase diagram with one axis representing the aqueous phase, the second representing the oil and the third representing the mixture of surfactant and co-surfactant at fixed ratio (S_{mix} ratio).

3.4. Thermodynamic stability studies

Centrifugation: The placebo nanoemulsion was centrifuged at 3500 r.p.m. for 30 min. Those formulations that did not show any phase separation on visual observation were taken for the freeze thaw stress test.
Freeze thaw cycle: Three freeze-thaw cycles were done for formulations between −21 °C and +25 °C. The formulations were stored for not less than 48 h at each temperature.

3.5. Formulation of amlodipine loaded nanoemulsions

After thermodynamic stability studies of placebo, drug loaded nanoemulsion was prepared by dissolving 10 mg of amlodipine in the stable nanoemulsion. Ten milligram of amlodipine were added in prerequisite quantity of oil and mixed with S_{mix} of selected ratio. Oily mixture was titrated with distilled water to obtain 1 ml formulation. The prepared nanoemulsions were tightly sealed and stored at ambient temperature.
Six nanoemulsion formulations from different S_{mix} ratios showing high nanoemulsion region (1 : 1, 1 : 2, and 2 : 1) and passed thermodynamic stress tests were selected for *in vitro* skin permeation studies.

3.6. Procedure for skin permeation studies

3.6.1. Preparation of skin for permeation studies

Albino rats (200–250 g) obtained from central animal facility at Jamia Hamdard were sacrificed by excessive ether anesthesia. The skin was excised from the abdominal region, stored at −22 °C and used within a week. One day before the permeation study hair was shaved carefully, subcutaneous fat was removed using isopropyl alcohol. Finally the skin was washed in distilled water and observed physically for any damage. The excised washed rat skins were then stored at 4 °C and used within 24 h after the skin harvest.

3.6.2. In vitro skin permeation studies

The skin permeation experiments were performed using Franz diffusion cells with excised rat skin at 37 ± 0.5 °C. The receptor cell of capacity 35 ml was filled with 5% v/v methanolic phosphate buffer (pH 7.4) and continuously stirred at 600 rpm. Drug loaded nanoemulsion (1 ml) was applied on epidermal side in donor compartment of permeating area 4.76 cm^2. In case of control amlodipine dissolved in release medium was taken to give a saturated solution of 10 mg/ml. After application of the test solution on the donor side, 0.5 ml of aliquot was collected from the receiver cell at designated time intervals (viz. 1, 2, 3, 4 5, 6, 7, 8, 10, 12, 24 and 48 h) and replaced immediately with the same volume of fresh medium maintained at 37 ± 0.5 °C. After appropriate dilution, the samples were filtered using 0.45 μm membrane filter and the amount of drug in the receptor medium was analyzed by HPLC.
The cumulative amount of drug permeated through rat skin was plotted as a function of time. The permeation rate of drug at steady-state (J_s, μg/cm^2/h) was calculated from the slope of linear portion of the plot. The permeability coefficient Kp (cm/h) was calculated using following equation:

$$Kp = J_s/Co\,, \qquad (1)$$

where, Kp is the permeability coefficient, J_s is flux of drug at steady-state and Co represents the drug concentration which remains constant in the nanoemulsion.

3.7. Characterization of nanoemulsion

3.7.1. Transmission electron microscopy (TEM)

Morphology and structure of nanoemulsion were studied using the transmission electron microscope TOPCON 002B operating at 200 KV and of a 0.18 nm capable of point to point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of nanoemulsion. In order to perform TEM observations, the nanoemulsion was diluted with water (1/100). A drop of the diluted nanoemulsion was then directly deposited on the holey film grid and observed after drying.

3.7.2. Droplet size analysis

Droplet size of nanoemulsion was determined by photon correlation spectroscopy (PCS) that analyzes the fluctuations in light scattering due to Brownian motion of particles. Nanoemulsion (0.1 ml) was dispersed in 50 ml of water in a volumetric flask and gently mixed by inverting the flask and measurement done using a Zetasizer 1000 HS (Malvern Instruments, UK). Light scattering was monitored at 25 °C at 90° angle.

3.7.3. Solubility of amlodipine in nanoemulsion

The solubility of amlodipine in optimized nanoemulsion was determined by taking excess of drug in the selected nanoemulsion. The procedure used for solubility determination was the same as reported earlier.

3.7.4. Viscosity of nanoemulsion

Viscosity of optimized nanoemulsion was determined by using a Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, USA) fitted with spindle # CPE40 at 25 ± 1 °C without diluting the nanoemulsion. The software used for the calculation was Rheocalc V2.6.

3.7.5. pH measurement

pH of nanoemulsion was determined using a digital pH meter (Mettler Toledo, Japan).

3.8. Statistical analysis

Skin permeation studies were performed three times and data were expressed as mean of three values ± SD. One-way analysis of variance (ANOVA) with Dunnett test was used for statistical comparison. The level of significance was taken as $p < 0.05$.

3.9. HPLC analysis of amlodipine

The amount of amlodipine permeated into the receptor medium was determined with a slightly modified HPLC method reported previously (Mohammadia et al. 2007). The column used was a C18 column (Supelco, 250 mm × 4.6 mm i.d., 5 μm) using a mobile phase consisting of acetonitrile-0.03 M NaH_2PO_4 buffer (pH 4.5) (55 : 45, v/v) at a flow rate of 1 ml/min and UV detection at 237 nm. After proper dilution of samples with HPLC grade methanol, 20 μl of samples were injected for quantification. All operations were carried out at ambient temperature.

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