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Nanocarrier System for Transdermal Delivery of Glimepiride

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ABSTRACT:

The current work attempted to evaluate a nanoemulsion approach that improved percutaneous glimepiride absorption. Nanoemulsions do not require chemical enhancers and have several advantages over traditional transdermal drug delivery techniques. Pseudoternary phase diagrams were created to optimize the surfactant, cosurfactant, and surfactant : cosurfactant weight ratios (Smix). The nanoemulsion formulation comprises Labrafac and Triacetin (1:1 ratio) as an internal oil phase in an external aqueous phase, Tween 80 as a surfactant, and diethylene glycol monoethyl ether as a cosurfactant. A pseudoternary phase diagram was developed to investigate the effect of the surfactant to cosurfactant mass ratio (Smix) on nanoemulsion production, which is a transparent zone. Nine nanoemulsion formulations were chosen and characterized. The nanoemulsion formulations exhibited small droplet size, homogenous size distribution, and low viscosity. The study found that the nanoemulsion gel for transdermal systems of Glibenclamide had superior management of hyperglycaemia and alleviated diabetes mellitus problems than oral Glibenclamide treatment in wistar rats. Transdermal nanoemulsion formulations demonstrated superior enhancement in all biochemical assays compared to oral treatment. They improved tissue healing following diabetes-induced tissue damage. A novel glimepiride nanoemulsion formulation could be developed and projected to be suited for transdermal use.

Keywords: Nanoemulsions, Pseudoternary Phase-Diagram, Nanoemulsion Gel, Antidiabetic study, Transdermal Delivery.

INTRODUCTION

Nanoemulsions (NE) are quaternary systems, with four phases: oil, water, surfactant, and cosurfactant [1-3]. It is generated spontaneously and has physicochemical qualities like as low viscosity, optical isotropy, thermodynamic stability, and transparency. The particle size of stable nanoemulsion is typically reported to be in the range of 10-100 nm (100-1000 A° [4]. Due to its unique physicochemical features, NE have been shown to outperform traditional topical and transdermal medication delivery. NE formulations have been shown to improve transdermal and cutaneous properties both in vivo and in vitro [5-22]. NE have a high solubilizing power, hence they can improve the solubility profile of weakly water-soluble medicines. NE tends to raise the concentration of solute and aids medication partitioning into the stratum corneum, making nanoemulsion a best choice to improve drug permeability of transdermal drugs [23].

NE enhances lipophilic drug penetration more than macro emulsions [18]. The addition of a cosurfactant to NE reduces interfacial tension, allowing active material to move freely, as opposed to macroemulsions, which have limited mobility [3, 14]. If the mobility of drug active material is increased, it partitions and diffuses into the stratum corneum. This article describes how to formulate glimepiride for transdermal distribution utilizing an o/w NE system.

Glimeperide is an oral antidiabetic medication categorized as a second generation sulphonylurea used to treat diabetes mellitus. Glimepiride reduces blood sugar levels by boosting insulin release from pancreatic β cells and enhancing insulin sensitivity in peripheral tissues [24-25]. It has a low molecular weight (490.6 Da) and moderate lipophilicity (Log P = 1.8), making it clinically efficacious in modest dosages (5mg to 15mg) [26-27]. Percutaneous administration of the medication is preferable over oral dose type.

According to reports, oral administration of glimepiride is related with adverse effects such as hypoglycemic responses and gastrointestinal disturbances such as heartburn, nausea, vomiting, anorexia, and increased hunger. Thus, transdermal delivery of glimepiride may be preferable to oral administration in the treatment of non-insulin dependent diabetic mellitus (NIDDM) [28]. Diabetes is a chronic condition that requires lifelong control, thus patient compliance is an important element to consider. Transdermal drug delivery system (TDDS) offers advantages such as sustained release and reduced intensity of effect, making it a better option than oral medication. Because of its tiny size and ease of penetration into the skin, the NE system has the potential to be a promising vehicle for medication delivery.

MATERIALS AND METHODS

Materials

Glimepiride was procured as a gift sample from **Ranbaxy Research Laboratories Ltd, Gurgaon** India. Oleoyl macrogol-6 glycerides / glycerides (labrafil 1944 CS), Propylene glycol dicaprylate/dicaprate (labrafac PG), diethylene glycol monoethylether (transcutol P) were gift samples procured from Gattefosse SAS (France). Castor oil, oleic acid and olive oil were purchased from genuine chemicals (Mumbai, India). Triacetin (glycerin triacetate), tween 80, and tween 20 were purchased from Ozone chemicals (Mumbai, India). Polyethylene glycol 400(PEG-400), propylene glycol (PG) and n-butanol were purchased from E-Merck (Mumbai, India). Isopropyl myristatae (IPM) was purchased from S.D. Fine chemicals (Mumbai, India). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile (ACN) were purchased from Fischer chemical (Mumbai, India). Water used was obtained from Milli Q water purification system (Miliipore, MA). All other chemicals and solvents procured and utilized in the study were of analytical grade.

Preparation of Nanoemulsions

Determination of Solubility of glimepiride in Oils, Surfactants and Cosurfactants

The solubility profile of glimepiride in various oils was investigated in order to identify the most appropriate oil to serve as the oil phase in NE while also providing superior skin permeation rate. Oils utilized include oleic acid, IPM, olive oil, castor oil, Labrafill, Triacetin, Labrafac, and a 1:1 combination of Labrafac and Triacetin. The solubility of glimepiride in surfactants (sorbitanmonolaurate, sorbitanmonoleate, polyoxyethylenesorbitanmonolaurate, polyoxyethylenesorbitanmonooleate) and cosurfactants (diethylene glycol monoethyl ether, PEG 400, and propylene glycol) was also investigated. An excess amount of glimepiride was added to 2.0 mL of the selected oil, surfactant, and cosurfactant in stoppered vials (capacity 5.0 mL), followed by a few minutes of preliminary mixing on a magnetic stirrer.

The vials were stored in a mechanical bath shaker for 72 hours at 37 ± 0.5 0C. The equilibrated samples were centrifuged at 10,000 rpm for 15 minutes. The supernatant was separated and filtered before being diluted with methanol to assess solubility using validated HPLC.

Construction of Pseudo-Ternary Phase Diagram

The phase diagram and aqueous titration method was used to optimize concentration of surfactant and cosurfactant combination. The ratio of each selected surfactant to cosurfactant (Smix) was kept constant (1:1), whilst the oil to Smix ratio was 1:9. Surfactant and cosurfactant were chosen based on the number of NE points shown in phase diagrams. After selecting the surfactant and cosurfactant, the optimum concentration ranges were identified through a rigorous examination of phase diagrams using various Smix ratios (1:0, 1:1, 2:1, 3:1, 1:2, 1:3). For each Smix ratio, the oil:Smix ratio was changed. A total of sixteen distinct combinations of oil and Smix (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 1:2.3, 1:2, 1:1.5, 1:1, 1:0.7, 1:0.43, 1:0.25, 1:0.1) were made in order to cover the maximum ratios for the study to precisely identify the boundaries of phases formed in the phase diagrams.

Slow titration with an aqueous phase was performed on each weight ratio of oil and Smix, and visual observations were made for transparency, flowability, and physical state of NE. Glimepiride was added to the oil phase of the NE to prepare the drug-loaded NE.

Formulation of glimepiride loaded nanoemulsion

After identifying the nanoemulsion region using the pseudoternary phase diagram, different o/w nanoemulsion formulations corresponding to different Smix weight ratios were chosen so that the glimepiride was added to the mixture of oil, surfactant, and cosurfactant with varying component ratios as described in Table 1, and then an appropriate amount of water was added to the mixture drop by drop, and the nanoemulsion containing glimepiride was obtained by vortexing the mixture. The medication concentration was kept consistent throughout all formulations. These formulations were exposed to several thermodynamic stability tests to determine their physical durability.

Thermodynamic Stability of Nanoemulsions

To investigate the thermodynamic stability of drug-loaded NE, clarity, phase separation, droplet size, and drug content were measured before and after the stress tests described previously [30].

• Heating cooling cycle: NE formulations were subjected to six cycles of refrigeration temperatures ranging from 4°C to 45°C (storage time of at least 48 hours at each temperature). The stable formulations were next tested for centrifugation [31].

• Centrifugation: Formulations were centrifuged at 3500 rpm for 30 minutes, and those that showed no phase separation were used for the freeze-thaw stress test.

• Freeze thaw cycle: Formulations that passed the centrifugation test underwent three freeze

thaw cycles at temperatures ranging from -21 °C to +25 °C (storage time of at least 48 hours at each temperature).

Characterization of Nanoemulsions

Transmission Electron Microscopy (TEM)

Morphology of the NE was investigated using a TEM (Philips, Netherlands) operating at 200 KV and capable of point-to-point resolution. To get the TEM observations, a drop of diluted nanoemulsion was placed to a 200-mesh copper grid and left for two minutes. Following this, the grid was inverted and a drop of phosphotungstic acid (PTA) was put to it for 1 second. The NE's form and size were revealed using a combination of bright field imaging at increasing magnification and diffraction modes. To conduct the TEM observations, the diluted NE was placed on the holey film grid and studied after drying.

Droplet Size and Size Distribution

Droplet size was evaluated using a zetasizer 1000HS (Malvern Instruments, UK) and photon correlation spectroscopy, which investigated changes in light scattering caused by the particles' Brownian motion [32].The formulation (0.1 mL) was dispersed in 50 mL of water in a volumetric flask, vigorously shaken, and light scattering was measured at 25°C and a 90° angle. A solid-state laser diode was used as the light source. The polydispersity index (PI) for the formulations was calculated as the ratio of standard deviation to the mean droplet size of the formulation.

Viscosity

The viscosity was determined using Brookfield viscometer LV DV-E (Brookfield Engineering, USA) using spindle no. 2(62) in triplicate at 25 ± 0.5 °C.

Refractive Index and pH

Refractive index of NE was determined using an Abbes type refractometer ((Erma, Japan),) at 25 ± 0.5 ℃. The apparent pH of the formulation was measured by pH meter (Elico, India) in triplicate at 25 ± 1ºC.

Conductivity

The Conductivity was determined using Conductivity Meter, Testronix-15 (Microlab, Mumbai, India) in triplicate at 25 ± 0.5 ℃.

Preparation of Rat Skin

Male albino wistar rats, aged 7-9 weeks and weighing 200-250 g, were selected. Solvent ether was used to sacrifice animals. Later, the abdominal skin was meticulously removed from the underlying connective tissue with a knife. The remaining hairs on the skin were clipped away. The subcutaneous tissue was surgically removed, and the dermis side was cleaned with isopropyl alcohol to remove any clinging fat. The cleansed skin was rinsed with distilled water and stored at 21°C until ready for use.

Ex-vivo Permeation Studies

Ex-vivo skin permeation investigations were carried out utilizing the Franz diffusion cell device. The diffusion area was 0.75 cm2, and the receptor volume was 5.0 mL. The skin was warmed to room temperature, sliced and trimmed to the right size, and placed between the donor and receptor compartments of the diffusion cell, with the stratum corneum side facing upwards. The donor chambers were then clamped in place. The receptor compartment was filled with phosphate buffered saline (pH 7.4). The receptor fluid was swirled with a magnetic

rotor at 600 rpm and kept at 37 ± 1°C. After skin stabilization, one mL of NE formulations was placed in the donor compartment and sealed with paraffin film to create occlusive conditions. Samples were withdrawn at regular intervals (1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 and 48 hours), filtered through a 0.45-µm membrane filter, and evaluated for drug content by HPLS. The receptor phase was immediately replaced with an equal volume of new receptor fluid. The same study was conducted on control formulations. Each set of experiments involved three diffusion cells.

Preparation of Nanoemulsion Gel

It was easy to remove the nanoemulsion from the skin since it was not much viscous. Therefore, the optimized nanoemulsion (NE-B2) was converted into nanoemulsion gel. Nanoemulsion gel was prepared by dispersing 1 % w/w of Carbopol 934 in sufficient quantity of distilled water [22]. The dispersion was kept in dark for 24 h for complete swelling of carbopol. Then 0.005% w/w of GLPD nanoemulsion was added slowly to carbopol dispersion. Triethanolamine (0.5% w/w) was added in this mixture to neutralize carbopol. Then 35% w/w mixture of Smix (2:1) was added slowly. Then remaining quantity of distilled water was added to get the final preparation 100% w/w.

Preparation of Conventional Glimepiride Gel

Hydrogel with GLPD was utilized as a traditional dermal formulation to compare the action of glimepiride mixed into nanoemulsion gel. Carbopol 934 (0.75% w/w) was distributed in an appropriate amount of distilled water. The dispersion was kept in the dark for 24 hours to ensure complete swelling of Carbopol 934. The glimepiride was then combined with the carbopol dispersion, and 0.5% w/w triethanolamine was added to neutralize the carbopol before adding the remaining water to produce a homogeneously dispersed glimepiridein hydrogel [33-34].

Calculation of Permeation Parameters

The cumulative amount of glimepiride penetrated per unit of rat skin surface area, Qt/S (S=0.75 cm2), was shown with time (t, h). The steady-state permeation rate of glimepiride (Jss, µg/cm2/h) through rat skin was estimated using linear regression interpolation of the cumulative amount penetrated per unit area vs. time.

$Jss = \Delta Qt / S. \Delta t$ (Eq. 1)

The permeability coefficient (Kp, cm/h) was calculated according to the equation:

$Kp = Jss / Cd$ (Eq. 2)

Where Cd = concentration of drug in donor compartment (5.0mg/mL), and is assumed that under sink conditions the drug concentration in the receiver compartment is negligible compared to that in the donor compartment.

The enhancement ratio (ER) was calculated according to the equation:

ER = Flux from formulation / flux from formulation E. (Eq.3)

All skin permeation experiments were repeated three times and data were expressed as mean of three experiments ± standard deviation (S.D).

The animal protocols

The animal protocol for antidiabetic activity was evaluated and approved by the Institutional Animal Ethics Committee at Oriental College of Pharmacy Sanpada (approval no: OCP/CPCSEA/IAEC/2012/005), and their recommendations were followed throughout the investigations. The study used albino rats that were 7-9 weeks old and weighed between 150 and 220 grams. The animals were housed under regular laboratory settings (temperature: 25 ± 2 ℃, relative humidity: 55 ± 5%). The animals were housed in polypropylene cages and given

unrestricted access to a standard laboratory food (Lipton feed, Mumbai, India) and unlimited water.

Skin Irritation Test

The animals were housed in regular laboratory settings at a temperature of 25±1°C and relative humidity of 55±5%. They were housed in polypropylene cages and given free access to a normal laboratory meal (Lipton feed, Mumbai, India) and water on demand. The hairs on the abdomen side of albino Wistar rats were clipped one day before to the experiment. The skin irritation investigation involved eight rats. A single dose of 10 µL of the formulation was administered to the left ear of the rat, while the right was used as a control. The method described by Draize et al. [35] was used to track the development of erythema over six days.

Induction of Diabetes mellitus:

A single intravenous injection of streptozotocin (60 mg/kg) diluted in citrate buffer (3 mM; pH 4.5) was used to induce diabetes in overnight starved Wistar rats weighing 150-220 g [36]. After an intravenous injection of 60 mg/kg, symptoms appear within 24-48 hours, including hyperglycemia up to 800 mg%, glucosuria, and ketonemia. Histologically, the beta cells are degranulated or necrotic. After 10-14 days, the animals acquire a stable condition, which allows them to be used for pharmacological tests [37]. Blood was drawn from the tail vein at predefined intervals up to 48 hours. The blood glucose level was tested using an Accu-Check Glucometer (Roche Diagnostics, Germany).

Antidiabetic activity

The antidiabetic activity of the developed nanoemulsion gel was assessed by withdrawing blood from rats' tail veins. Twenty-four wistar rats were selected and divided into four groups (n=6). Group I was used as a control. Diabetes was induced in the other subjects (subjects II, III, and IV). The wistar rat was handled according to the following:

Group I- Control

Group II- Applied with 2.00 cm^2 transdermal system prepared with nanoemulsion gel, without drug in 0.5% carbopol gel

Group III – Glibenclamide (5 mg/kg) the oral doses were given using a round tipped stainless steel needle attached to 1 ml syringe and the dose of 5 mg/kg was selected by conducting a series of experiments with graded doses ranging between 1 to 10 mg/kg

Group IV - Applied with 2.00 cm^2 transdermal system prepared with nanoemulsion gel, containing 5 mg of drug in 0.75% carbopol gel

At time intervals between 2-48 h after treatment (acute study), blood was collected from retro-orbital plexus; blood glucose levels were determined using Accutrend Alpha Glucometer (Roche Diagnostics, Germany).

Biochemical Evaluation

At the end of the long-term experiment, blood was obtained from the diabetic wistar rat's retro-orbital plexus and serum was separated. The lipid profile (high-density lipoprotein cholesterol, triglycerides, and total cholesterol), alanine transaminase (ALT), aspertate transaminase (AST), urea, and creatinine levels in serum were measured using a Bioanalyzer [24].

Histopathological Examination

Histopathology of Skin

The abdominal skin of Wistar rats was treated with an optimized nanoemulsion gel. After 48 hours, the rat was sacrificed, and skin samples from the treated and untreated (control) areas were collected. Each specimen was kept in a 10% formalin solution in phosphate buffer saline

(pH 7.4). The specimen was sliced into vertical sections. Each segment was dehydrated in ethanol, fixed in paraffin, and stained with hematoxylin and eosin. The samples were then examined under a light microscope (Motic, Japan) and compared to the control sample. In each skin sample, three different sites were scanned and evaluated for elucidation of mechanism of penetration enhancement.

Histopathology of Pancreas

From pancreas, the formalin fixed tissue pieces were successively dehydrated in alcohol and cleared in xylene which were embedded in paraffin blocks. The micro sections (4-5 microns thick) were cut and stained in haematoxylin and eosin (H and E) using standard method and observed under a low-power microscope for histopathological changes.

RESULTS AND DISCUSSION

Screening Of Oils, Surfactant and Cosurfactants:

After screening the oils for glimepiride solubility, it was shown that glimepiride had the highest solubility in the mixture of Labrafac: Triacetin (1:1), tween 80, and transcutol-P at 18.23 mg/ml, 114.91 mg/ml, and 55.02 mg/ml, respectively (Fig. 1). As a result, labrafac : triacetin (1:1) was chosen as the oil phase. Non-ionic surfactants are frequently used as solubilizing agents in topical formulations, however new research suggests that they may alter skin barrier function. Thus, tween 80 was used as the surfactant, transcutol-P as the cosurfactant, and distilled water as the aqueous phase.

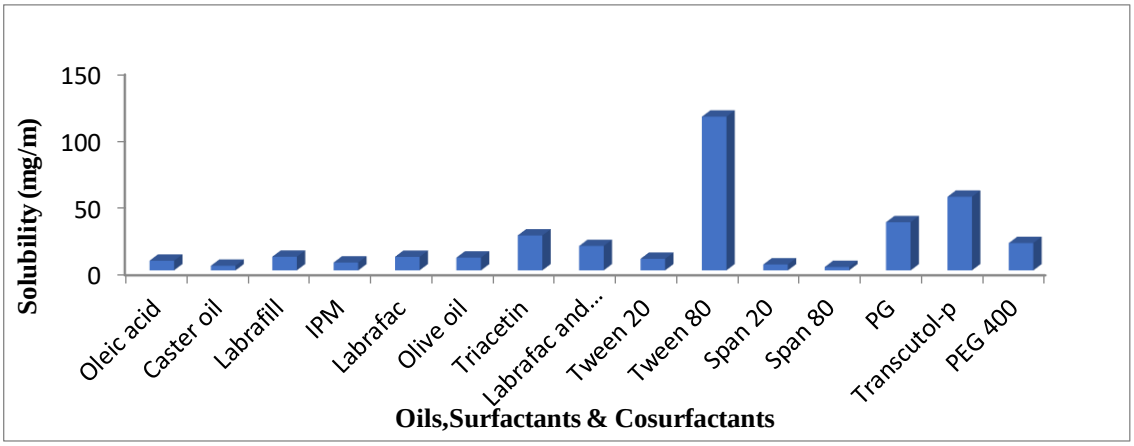


Figure 1. Solubility of glimepiride in different oils, surfactant and cosurfactant. IPM indicates Isopropyl myristate, PG Propylene Glycol and PEG =Polyethylene Glycol

Pseudo-ternary phase diagram

After selecting the proper nanoemulsion components, pseudoternary phase diagrams should be created to specify the size and nature of the nanoemulsion region as well as the adjacent two and three phase domains. The creation of pseudoternary phase diagrams began with a single surfactant, Tween 80 (1:0). It was discovered that the zone containing nanoemulsions was quite small, with the majority of the region consisting of emulsion. Along with the surfactant, the co-surfactant transcutol was added in a 1:1 ratio, and a pseudoternary phase diagram was created. It was discovered that the region of nanoemulsion existence grew significantly. Increasing the surfactant content (2:1) resulted in an even bigger area of nanoemulsion existence, as well as emulsion, gel, or nanoemulsion gels.

Increasing the surfactant concentration from 2:1 to 3:1 reduced the nanoemulsion existence area, and an increasing proportion of the area was constituted of gels. The effect of cosurfactant

concentration on nanoemulsion existence was also investigated by creating a phase diagram with a 1:2 ratio. The region of nanoemulsion production was restricted (Fig. 2).

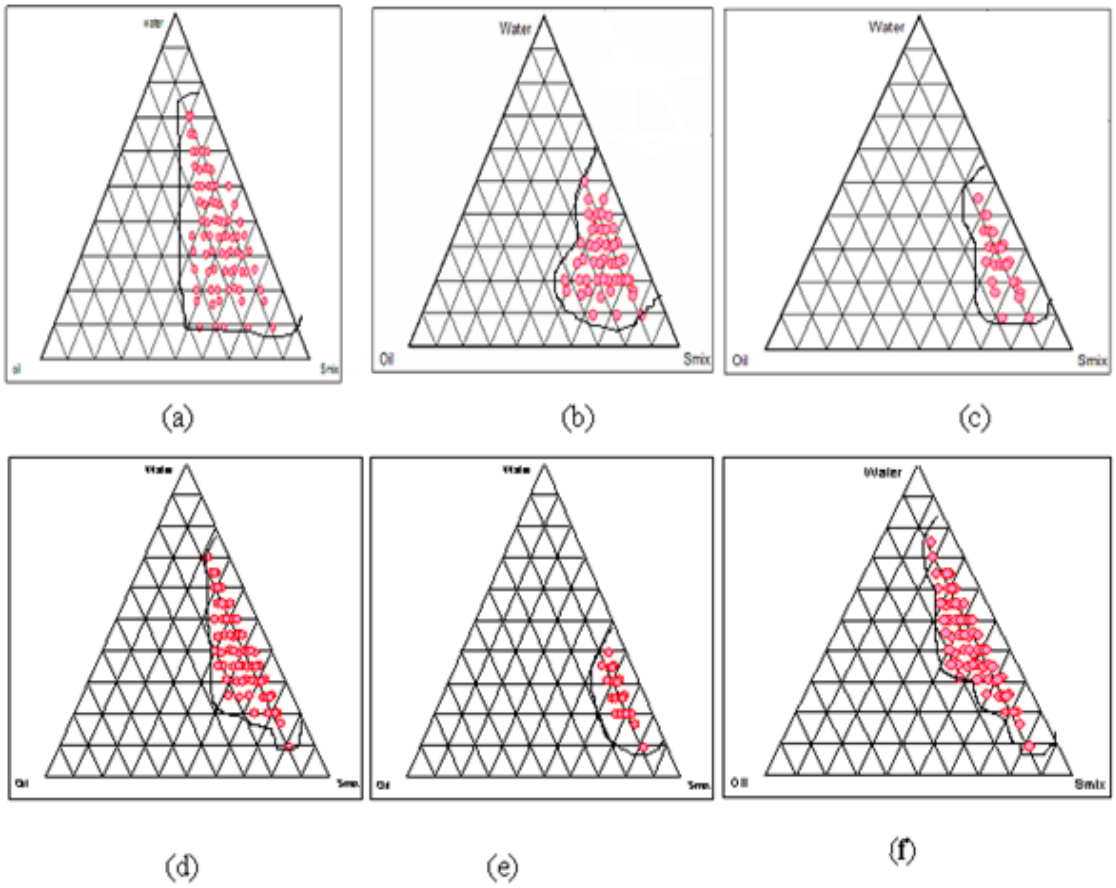


Fig. 2: Pseudo-ternary phase diagrams showing the o/w nanoemulsion (shaded area) regions of existence with Labrasol and Triacetin(1:1) (oil), Tween-80 (surfactant), Transcutol-P (cosurfactant) at Smix ratios; (**a**) 1:0, (**b**) 1:1, (**c**) 1:2, (**d**) 1:3, (**e**) 2:1, (**f**) 3:1**, (% w/w).**

Selection of Formulations from Phase Diagrams

Following criteria were chosen for the selection of formulations.

- Glimepiride was added to the mixture of oil, surfactant and cosurfactant with varying component ratio as described in Table 1, and then appropriate amount of water was added to the mixture drop by drop and the nanoemulsion containing glimepiride was obtained by vortexing the mixture at ambient temperature.
- Formulations with Smix ratio 1:0, 1:2 and 1:3 were not selected because the formulations were unstable and showed phase separation.
- Based on the phase diagrams, three Smix ratios 1:1 (NE-A), 2:1 (NE-B) and 3:1 (NE-C) were optimized. From the selected Smix ratios. NE compositions with 33 % (NE-A1, NE-B1, and NE-C1), 43 % (NE-A2, NE-B2, NE-C2) and 50 % (NE-A3, NE-B3, NE-C3) Smix ratios were selected from the region of existence (Table 1).

Table 1. Composition of Selected Nanoemulsion Formulations.

Smix Ratio [a]	**Formulation Code [b]**	**Percent w/w of Components in Formulation**		
		Oil (%)	**Water (%)**	**Smix (S + CoS) (%)**
Formulation NE-A Smix ratio = 1:1	A1	10	57	33
	A2	14	43	43

	A3	16	34	50
Formulation NE-B Smix ratio = 2:1	B1	10	57	33
	B2	14	43	43
	B3	16	34	50
Formulation NE-C Smix ratio = 3:1	C1	10	57	33
	C2	14	43	43
	C3	16	34	50

[a]Surfactant/cosurfactant ratio;

[b] Represents nanoemulsion; A, B and C represents Smix ratio 1:1, 2:1 and 3:1, respectively; Suffix 1,2 and 3 represents Smix concentration 33, 43 and 50 %, respectively.

Thermodynamic Stability of Nanoemulsions

Stress tests, including heating and cooling cycles, centrifugation, and freeze-thaw cycles, revealed that all nanoemulsion formulations, placebo and loaded, exhibited good physical stability. After three months, glimepiride was found to be stable, with recovery rates above 96% for all formulations. There was no significant change in the mean refractive index values of the formulations over the course of three months (data not shown). Thus, it may be inferred that the NE formulations were both physically and chemically stable.

Characterization of Nanoemulsions

In this work, the influence of concentration of NE components and glimepiride on the characteristics of NE was studied summarised in Table 2.

The polydispersity index (PI), which measures the uniformity of droplet size within the formulation, was also measured. Polydispersity is characterized as the ratio of standard deviation to mean droplet size. This represents the consistency of droplet size within the formulation. The higher the polydispersity, the less uniform the droplet size in the formulation [38]. Small droplet sizes are essential for medication administration because oil droplets tend to fuse with the skin, giving a route for drug delivery. All NE formulations showed a tight size distribution (PI < 0.243).When different Smix ratios were examined, NE formulations had the lowest viscosity values (31.14±2.821 to 84.00±0.857mP).

The refractive index represents the net value of the NE components and demonstrates the formulation's isotropic character. The statistics show that the mean refractive index value for all formulations was relatively identical. The conductivity data (0.131±0.16-0.262 ±0.12mS/cm) show that the NEs are oil-in-water. All of the NE formulations had pH values ranging from 6.2 to 6.74, which is ideal for topical administration. The inclusion of drugs had no significant effect on the pH levels of NEs. TEM investigation revealed that NEs droplets were spherical in shape and distinct, with sizes in the nanometer range (Fig. 3).

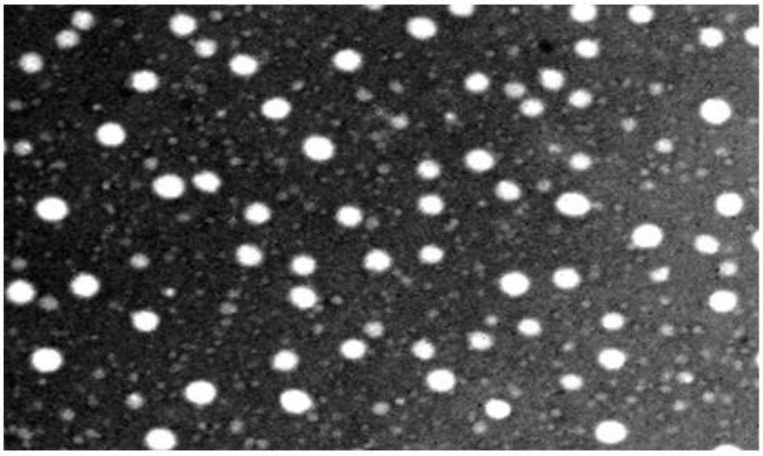


Figure 3. Transmission electron microscopic positive image of glimepiride nanoemulsion

Table 2. Physical Characteristics of Nanoemulsion Formulations (Mean ± SD, n = 3)

Formulation	Droplet Size (nm)	Polydispersity	Viscosity (mP)	Refractive Index	Conductivity (ms/cm)
A1	79.02±1.351	0.209 ±0.031	64.89±3.19	1.409±0.013	0.247 ±0.06
A2	9102±2.250	0.227±0.026	51.66±1.241	1.411±0.021	0.211 ±0.08
A3	94.03±2.322	0.233±0.021	49.00±1.034	1.412±0.009	0.143±0.04
B1	71.02±3.003	0.155±0.042	38.00±1.103	1.405±0.017	0.262 ±0.12
B2	65.00±2.351	0.137±0.037	31.14±2.821	1.401±0.008	0.224 ±0.16
B3	72.36±0.132	0.189±0.071	43.33±0.821	1.406±0.015	0.168±0.08
C1	99.67±3.481	0.219±0.110	71.66±1.213	1.411±0.003	0.237 ±0.24
C2	113.39±1.074	0.204±0.039	84.00±0.857	1.409±0.007	0.183 ±0.36
C3	119.05±2.753	0.243±0.072	67.00±0.923	1.399±0.005	0.131±0.16

Ex-vivo Permeation Studies

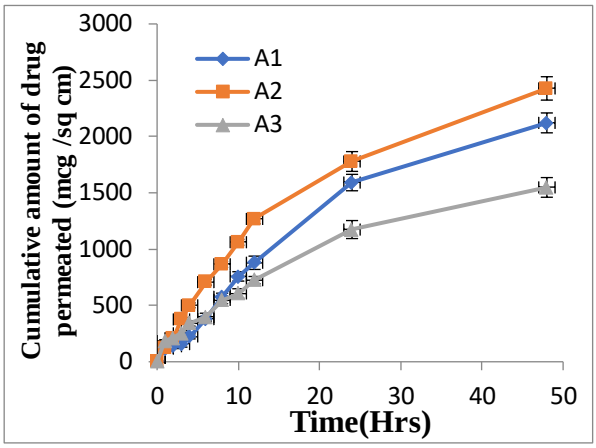
The permeation ability of the selected NE formulations was evaluated by the *ex-vivo* permeation experiments. The results demonstrate that the permeation rate and permeation coefficient of all the NE formulations through rat skin are significantly higher ($P < 0.001$) in comparison to control formulations D (Surfactant), E (Conventional Hydrogel) and Formulation F (Control) . The values of transdermal flux for different NE formulations were observed between 27.47 to 131.94 μg cm^{-2} h^{-1}. This value is approximately several fold greater than the formulation D (11.26 μg cm^{-2} h^{-1}), formulation E (7.98 μg cm^{-2} h^{-1}) and formulation F (6.39 μg cm^{-2} h^{-1}) respectively.

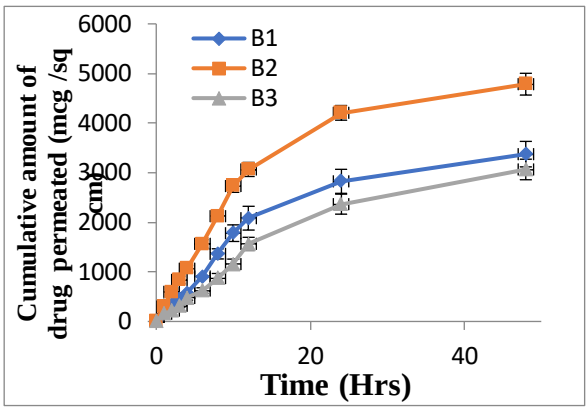
The content of Smix in the nanoemulsion formulation was found to affect the skin permeation rate of glimepiride directly. As the content of Smix increased from 33% to 55% (NE1 to NE3), the skin permeation increased from 45% then decreased up to 55 % Smix concentration and the overall flux enhancements were observed for Smix (2:1) NE-B formulations with the maximum flux (114.01 μg cm^{-2} h^{-1}) for NE-B2, at 45 % Smix (tween 80 and diethylene glycol monoethyl ether) (Fig. 4).This might be due to a decreased thermodynamic activity of the drug in the nanoemulsion at the higher content of surfactant. The thermodynamic activity of a drug in the formulation is a significant driving force for the release and penetration of the drug into the skin. In summary the different formulations can be ordered according to their descending flux values as follow: NE-B2 > NE-B1 > NE-B3 > NE-A2 > NE-A1> NE-A3 > NE-C2 > NE-C1 > NE-C3 > formulation D (Surfactant) > formulation E (Conventional Hydrogel) > formulation E (Control**)**. Comparison of the permeation of the NE formulations with corresponding droplet size and viscosity indicated that NE-B2 with smallest droplet size and lowest viscosity had highest transdermal flux and therefore selected for antidiabetic study for converting nanoemulsion gel.

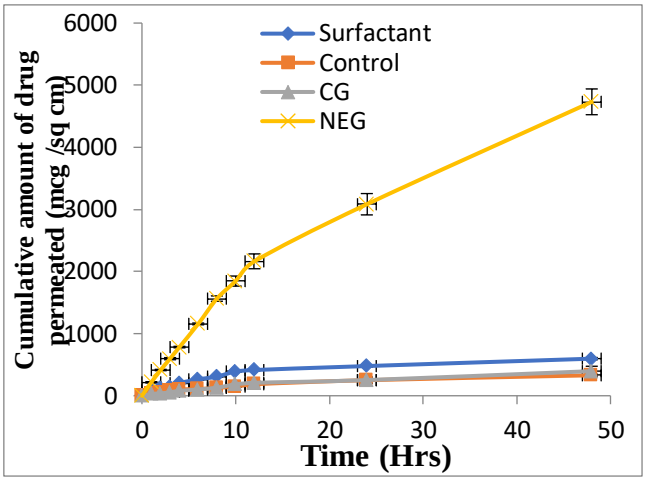
Formulation of Nanoemulsion Gel

The selected NE-B2 is then formulated into transdermal nanoemulsion gel (NEG), by

dispersing of carbopol-934 (1% w/w) showed highest viscosity, normal pH and highest gel strengths, therefore by carbopol-934 optimized for evaluation of permeation for NEG. It is determined that the flux and Pb of the TNG through animal skin are significantly greater in comparison to surfactant D, conventional gel E and Control F. The flux of NEG was found to be 114.01 (µg/cm^2/h). This flux is fifteen fold higher than the conventional gel (7.98 µg/cm^2/h) and control (6.39 µg/cm^2/h) and almost ten-fold greater than formulation D (10.65 µg/cm^2/h) respectively (Figure 21). The NEG formulation has greatest flux and Pb thus selected for antidiabetic activity study (Fig. 4).







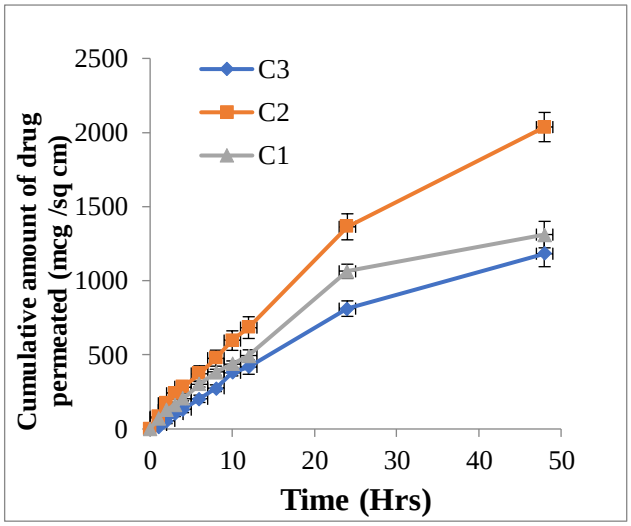


Figure 4. Permeation profiles of glimepiride through excised rat skin from nanoemulsions formulated with Smix 1:1, 2:1, 3:1 and surfactant (formulation D), conventional gel E (CG), control (formulation E) and NEG (mean ± SD, n=3).

Skin Irritancy Test

Skin irritancy test experiments were conducted to evaluate the irritant effects of nanoemulsions formulation. The skin irritation score (erythema and edema) was found to be less than 2 which is acceptable as per the method described by Draize *et al*. The formulation was found to be safe as the score.

Antidiabetic Study

Diabetes was induced effectively in animal by well-established method. The result in the Table 3. indicated that the significant diabetes was produced compared to normal animals. In the beginning the glucose levels in the control, toxic control, placebo control and formulation control were 82.51 mg/dl, 267.26 mg/dl, 271.47 mg/dl and 276.22 mg/dl respectively. There hypoglycemic effect was significantly in NE compared to control animals ($p<0.05$). The side effect of hypoglaemia is very comon with glimeperide and in case of transdermal formulation the blood glucose level decreases slowly so the chance of hypoglycaemia is very rare. The maximum hypoglycaemia was seen at 4 hours i.e. 56.96% which continue to be in same level until 48 hours. In the control group the reduction of blood glucose level was not found, and this justify the excipients the formulation has no effect on the glucose level of body.

Table 3. Reduction in blood glucose level of rats after treatment with control, placebo Control/toxic control, positive control and formulation control

Group	**Treatment**	**Initial blood glucose level (mg/dl) ±S.D.**	**Reduction in blood glucose level (mg/dl) ± S.D.**						
		0	1 Hrs	2 hrs	4 hrs	12 hrs	18 hrs	24 hrs	48 hrs
A	Control*	82.51	-	-	-	-	-	-	-
B	Placebo/Toxic control	267.26	-	-	-	-	-	-	-
C	Positive Control	271.47	167.3	151.2	161.4	171.7	245.1	262.9	274.8

D	Formulation control (NEG)	276.22	206.7	143.5	114.8	118.3	121.3	124.7	133.1

Biochemical Evaluation

Different difficulties including biochemical changes develop along with severe hyperglycemia as a significance of the metabolic imbalance in diabetes [39]. Hence, it is essential to explore the efficacy of transdermal systems in retreating these changes in diabetes associated to oral administration of glibenclamide.

The results are signified as in Table 4. The glycogen levels in the liver of diabetic wistar rat were significantly lowered compared to normal wistar rat ($p<0.05$). In the diabetic condition, the level of glycogen phosphorylase, an essential enzyme of glycolytic pathway, is increased, and consequently, liver glycogen content is decreased [24]. The oral as well as transdermal treatment of glibenclamide significantly ($p<0.05$) increased liver glycogen at the end of six weeks.

The liver protein levels of diabetic wistar rat were significantly reduced compared to normal wistar rat ($p<0.05$). Deficiency of insulin in diabetes prevents protein synthesis and causes excessive catabolism of protein, which is utilized for gluconeogenesis [24]. The glibenclamide treatment (both oral and transdermal) increased the protein levels significantly compared to diabetic untreated wistar rat ($p<0.05$).

The serum lipid profile (triglycerides, total cholesterol, and high-density lipoprotein–cholesterol) were significantly increased in diabetic control compared to normal wistar rat ($p<0.05$). In the diabetic, all the consequences of insulin that cause storage of fat are reversed [39]. The result is a significant ($p<0.05$) reduction of lipid profile after glibenclamide (transdermal/oral) treatment.

The hepatic enzyme (ALT and AST) levels were significantly ($p<0.05$) increased in diabetic control wistar rat, indicating the hepatic damage. The raised levels of AST and ALT in the liver diabetic wistar rat were significantly ($p<0.05$) decreased upon treatment. The serum urea and creatinine levels were significantly ($p<0.05$) increased in untreated diabetic wistar rat, showing the nephrotoxicity.

Table 4. Serum lipid profile (TC, TG and HDL-C), Alanine transaminase, Aspartate transaminase, Urea, Creatinine, Liver protein and glycogen levels in diabetic wistar rat after oral and transdermal administration of Glibenclamide

Treatment	**Liver Glycogen (mg/g)**	**Liver Protein (mg/g)**	**TC (mg/dL)**	**TG (mg/dL)**	**HDL-C (mg/dL)**	**ALT (IU/L)**	**AST (IU/L)**	**Urea (mg/dL)**	**Creatinine (mg/dL)**
NC	3.69±0.48	36.79±5.96	102.1±7.21	66.6±6.92	42.3±2.32	91.4±6.21	52.5±7.31	43.6±0.79	0.42±0.03
DC (0.2 mL)	1.51±0.17	12.2±1.91	312.1±4.41	183.5±6.81	102.6±3.13	164.2±8.72	92.1±8.61	51.6±3.66	0.54±0.04
NE-GLBD	3.11±0.21	33.5±3.45	127.5±7.55	81.5±6.72	55.5±7.32	103.2±5.52	54.4±5.58	45.2±2.22	0.45±0.03

GLBD	2.97±0.36	23.5±3.15	133.5±5.25	83.5±5.56	53.2±5.55	114.2±6.72	62.3±4.55	62.5±0.72	0.47±0.01

All values are expressed as Mean±SE, n=6; NC=Normal control; DC=Diabetic control; NE-GLB=NanoemulsionGlibenclamide; GLB= Glibenclamide suspension; TC=Total cholesterol; TG=Triglycerides, HDL-C= High density lipoprotein-cholesterol, ALT=Alanine transaminase, AST=Aspertatetransaminse; # significant compared to NC ($p<0.05$); *significant compared to DC ($p<0.05$).

Histopathological Examination

Histopathology of Skin

As shown in the photomicrograph, there was no significant changes in the treatment of the tween 80/transcutol and nanoemulsion gel based formulation in the skin after 3 days. Furthermore, the photomicrograph was also no significant changes in the treatment of the transdermal nanoemulsion gel based formulation in the skin after 48hrs. It was found that stratum corneum remain intact shown in **Fig. 5-6**. The results showed that the transdermal nanoemulsion gel based formulation was a safe carrier for the Transdermal delivery of glimepiride.

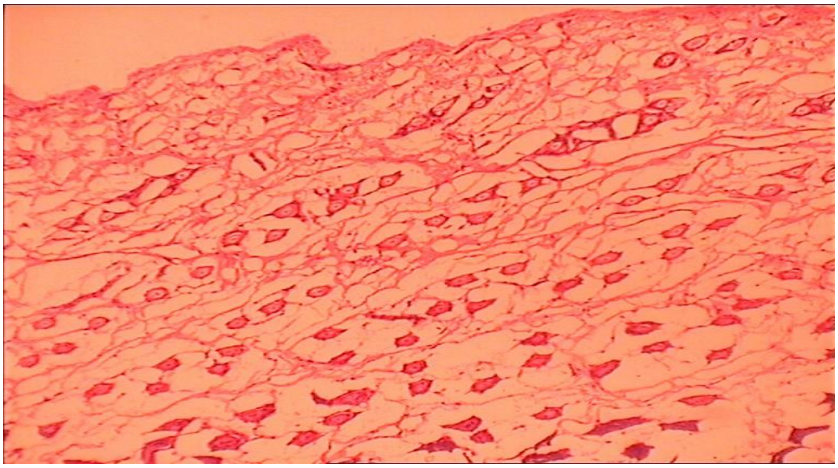


Figure 5. Light power photomicrograph of control skin

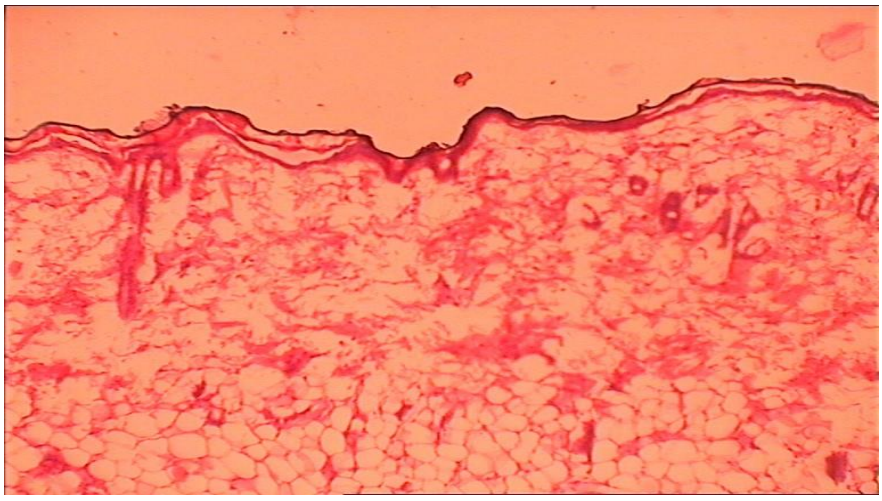
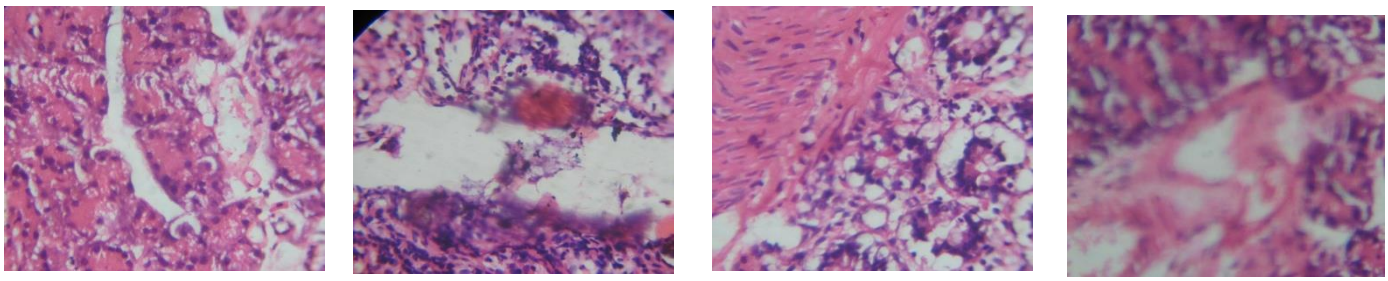


Figure 6. Light power photomicrograph of treated skin

Histopathology of Pancreas

The whole pancreas from each animal was placed in 10% formaline solution, and immediately processed by the paraffin technique. Sections of 5µm thickness were cut and stained by haematoxylin and eosin (H and E) for histological assessment. The histological study is shown in Fig. 7. Histopathological illustration showed normal acini and normal cellular population in the islets of langerhans in pancreas of vehicle-treated rats (A). General damage to the islets of langerhans and reduced dimensions of islets (B), Nanoemulsion Glibenclamide (C) was shown restoration of normal cellular population size of islets with hyperplasia. The Glibenclamide suspension was represented in partial restoration of normal cellular population and enlarged size of β-cells with hyperplasia.



(A) (B) (C) (D)

Figure 7. The rat pancreas stained by haematoxylin and eosin of (A) Normal control (B) Diabetic control (C) Nanoemulsion Glibenclamide (D) Glibenclamide suspension

CONCLUSION

An appropriate combination of the oil, surfactant, co-surfactant, and water is a major formulation consideration in nano-emulsion preparation for the transdermal drug delivery The glimepiride loaded thermodynamically stable o/w NE system were prepared and various formulation factors were evaluated to find optimized formulation which shows desirable efficacy for permeation study. The optimized formulation NE-B2, which contained labrafac and triacetin (1:1), (15 % w/w), tween 80 (30 % w/w), diethylene glycol monoethyl ether (15 % w/w) and water (30 % w/w) showed significant increase ($P < 0.001$) in the steady state flux (Jss) and permeability coefficient (Pb) compared to control or drug loaded neat components. On performing the antidiabetic activity, it was found that the formulation exhibit long lasting glucose lowering effect in controlled manner upto 48 hrs and avoids the sever hypoglycemia which is common with oral therapy and it was found significant in comparison to control and diabetic control ($P<0.05$).

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