

Nebivolol Hydrochloride Loaded Nanostructured Lipid Carriers as Transdermal Delivery System: Part 1: Preparation, Characterization and In Vitro Evaluation

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Abstract:

Nebivolol hydrochloride (NEB) is a 3rd generation highly selective β_1 -blocker with antihypertensive properties, the elimination half-life is about 10 hrs and the oral bioavailability is about 12%.

The study was aimed to develop nanostructured lipid carriers (NLC) for transdermal delivery of NEB.

The study involves two separate parts, part 1 (current) involves preparation and characterization of NEB loaded NLCs (NEB-NLCs). Part 2 of the study, NEB-NLCs based gel was formulated using gelling agent carbapol 934 as transdermal delivery system using rat skin. Part 2 of the study will be presented separately in the forthcoming issue.

The current investigation describes the effect of type and concentration of different solid lipids, liquid lipids, and surfactant/co-surfactant on the characteristics of NLC such as particle size, polydispersity index, zeta potential, drug entrapment efficiency, and drug release profile. Transmission electron microscopy, scanning electron microscope and atomic force microscope revealed nearly spherical shape NLC with negligible effect of liquid lipid (oleic acid) content on the particle morphology. The differential scanning calorimetry demonstrated depression in the melting point and crystallinity index of the NLCs with increasing the amount of liquid lipid. The in vitro drug release studies demonstrated that 93% of the drug was released over 24hrs. The NEB-NLCs possessed a biphasic release pattern characterized by a rapid initial release followed by a sustained release.

Keywords: *Nebivolol hydrochloride; Nanostructured lipid carriers; Transdermal delivery.*

التحضير والتشخيص والتقييم المختبري لحاملات الدهون ذات البنية النانوية المحملة بنيفولول هيدروكلورايد- الجزء الاول

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الخلاصة :

النيفولول هيدروكلورايد هو دواء من الجيل الثالث ذوانتقائية عالية لمستقبلات β_1 مع خصائص خافضة للضغط وبعمر نصف 10 ساعات تقريبا والتوافر الحيوي عن طريق الفم 12%. الدراسة تتضمن جزئين: الجزء الاول (الحالي) يتضمن التحضير والتشخيص والتقييم المختبري لحاملات الدهون ذات البنية النانوية المحملة بنيفولول هيدروكلورايد فيما يتضمن الجزء الثاني من الدراسة تحضير وتشخيص حاملات الدهون ذات البنية النانوية كقاعدة مائية هلامية باستعمال مادة الكارببول 934 كمادة هلامية لتحرير النيفولول هيدروكلورايد عبر الأدمة والتي سوف يتم نشرها فيما بعد.

الدراسة الحالية تهدف لتطوير امكانية حاملات الدهون ذات البنية النانوية (NLCs) لتسليم النيفولول هيدروكلورايد عبر الجلد. توضح هذه الدراسة تأثير نوع وتركيز الدهون المختلفة صلبة، سائلة، والسطحي/المشارك السطحي على خصائص حاملات الدهون ذات البنية النانوية مثل حجم الجسيمات، مؤشر التشتت المتعدد، وإمكانية قياس فرق الجهد والكفاءة و تحرر الدواء.

أظهر فحص وتحليل المجهر الإلكتروني، مجهر القوة الذرية ومجهر الإنبعاث الإلكتروني أن حاملات الدهون ذات البنية النانوية تكون تقريبا كروية الشكل مع تأثير ضئيل للدهون السائلة (حمض الأوليك) على هيئة وشكل الجسيمات، فيما أظهر مسح DSC إنخفاضا في مؤشر نقطة الإنصهار والتبلور لحاملات الدهون النانوية كلما إزدادت كمية الدهون السائلة، وقد أظهرت دراسة الكفاءة ان 93% من النيفولول يتم تحميله بكفاءة عالية وأن 93% من النيفولول يجري تحريره من حاملات الدهون ذات البنية النانوية خلال أربع وعشرين ساعة من عمر الدراسة فيما يتم التحرير بنمط ثنائي الطور يتميز الطور الأولي بتحرير السريع يتبعه التحرير المستدام بشكل بطيء .

الكلمات المفتاحية: النيفولول هيدروكلورايد، حاملات الدهون ذات البنية النانوية، التحرير عبر الادمة.

Introduction:

In last decade, the prefix “nano” has an increasing application to different fields of knowledge. Nanoscience, nanotechnology, nanomaterials or nanochemistry; are only a few terms that occur frequently in scientific reports, popular books as well as in newspapers and have become familiar to a wide public, even of non experts. International system of units is used to indicate a reduction factor of 10^9 times^[1].

Transdermal delivery systems (TDDS_s) have been classified into different generations. According to the classification, the first generation dealt mostly with small, lipophilic and uncharged molecules that can be delivered in therapeutic range by passive diffusion alone^[2].

Nanostructured lipid carriers (NLCs), is the second generation of lipid nanoparticles technology after the solid lipid nanoparticles. The NLCs are produced by using blend of solid lipids and liquid lipids (oils). Nanostructured lipid carriers are colloidal particles that typically range in size from 100-500nm. They have been successfully multi-functionalized to capture a payload of drugs, to target specific cells, release the entrapped drug in the controlled manner, and they are able to enhance the chemical stability of compounds sensitive to light, oxidation and hydrolysis^[3].

Nebivolol hydrochloride (NEB) is a lipophilic β_1 -blocker, devoid of intrinsic sympathomimetic and membrane

stabilizing activity. Clinically, NEB is administered as a racemic mixture of equal proportions; d-isomer ((SRRR)-neбиволол a potent cardioselective β_1 adrenoceptor blocker) and l-isomer (RSSS)-neбиволол with favorable hemodynamic profile^[4].

The enantiomers have unequal potency with regard to β -receptor blocking activity and nitric oxide mediated vasodilation, so the combination has greater antihypertensive activity than either enantiomer alone^[5,6]. Nebivolol hydrochloride is an official drug in British and Indian Pharmacopoeia^[7,8].

The M.Wt of NEB is 441.9 and for the free base is 405.4. The advantages^[9,10] of NLC among others lipid carriers could be ascribed to the nature of the NLC, which include and not limited to the following advantages; improve physical stability and benefit/risk ratio, ease of preparation, scale-up and sterilize, controlled particle size and increase dispersability in an aqueous medium, efficient carrier system in particular for lipophilic substance, however, high entrapment of both lipophilic and hydrophilic drugs, it is one of the carriers of choice for topically and transdermally applied drugs because of the small size of lipid particles which ensures close contact to the stratum corneum, thus enhancing drug penetration, increase of skin hydration, elasticity and occlusion, extended release of the drug, more affordable and less expensive than polymeric/surfactant based carriers.

Materials and Methods:

Materials:

Transcutol P and NEB were purchased from Provizer Pharma, India. Oleic acid was supplied by Riedel De Haen AG Seelze, Honnover, German Soyabean oil, Castor Oil, Cotton Seed Oil and Olive Oil were supplied by Fluka AG. Chem Loba Chemie Pvt. Ltd. Mumbai, India. Lecithin (Phosphatidylcholine purity 72.7%) and Poloxamer 188 (Pluronic F-68) Lutrol® were purchased from Sigma-Aldrich, Chemie GMBH, Germany. Behenic acid was supplied by Evans Chemical Ltd., England. Tripalmitin Extra pure and Cremophore EL (Polyoxy 1 35 Castor oil) were purchased from HiMedia Lab Pvt. Ltd, India. Polyoxyethylene sorbitan monooleate (Tween 80), Tween 60, Tween 20, Span 20 and Span 80 were provided by Hopkin and Williams LTD, England. Gelot-64® and Myverol™ 18-04K were purchased from Gatteefosse France. Potassium dihydrogen phosphate, Disodium hydrogen phosphate, Diethyl ether and Mannitol were provided by BDH Chemicals Ltd., Poole, England.

Solubility determination:

Solubility of NEB was measured in D.W and phosphate buffer pH 7.4 solution. briefly, an excess amount of NEB powder was added to the vehicle in small glass vial, then whole mixture was incubated in a shaking water bath maintained at 25°C for 24 hrs until equilibrium is reached after filtration using 0.45µm Millipore filter. The supernatant was diluted suitably prior to UV-analysis. The solubility determination was carried out in triplicate^[11-13].

Screening of starting materials:

Solid lipid selection:

The selection of solid lipid was based on the solubility of NEB to give visibly clear solution in lipid. Palmitic acid, stearic acid, behenic acid, cetyl palmitate, stearyl alcohol, tripalmitin and glyceryl monostearate (GMS) were investigated. Ten mg of NEB was dispersed in test tube containing solid lipids which was added gradually up to 0.5

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g, then shaken. The qualitative solubility of NEB in the molten lipid was estimated visually. The quantity of lipid for complete solubilization of the drug was calculated, the experiment was conducted in triplicate^[14].

Partitioning behavior of NEB in various solid lipids:

NEB (20mg) was dispersed in a mixture of melted lipid (2gm) and hot water (2ml). The mixture was kept on a hot water bath shaker maintained at temperature 10°C above the melting point of concerned lipids, and shake for 30 min. Then the mixture was centrifuged and the aqueous phase was separated and filtered through 0.45µm Millipore filter. Then, NEB content was analyzed spectrophotometrically. Thereafter, log P (log drug concentration in the lipid phase/drug concentration in the water phase) was calculated^[15].

Screening of liquid lipid (oils):

Solubility of NEB in different liquid lipids (oils) including; oleic acid, castor oil, soyabean oil, cotton seed oil, and olive oil were determined after addition of excess amount of NEB to (2 ml) of different oils in small vial. The vials were tightly stoppered and were continuously stirred for 72hrs at 37°C using mechanical water bath shaker. The mixture was centrifuged at 6000 rpm for 20 min. The supernatant was separated, filtered and suitably diluted prior to UV-analysis for determining the amount of NEB. Blank was prepared by dissolving respective oil in methanol with same dilution as for the samples^[16]. The solubility studies were done in triplicate and the results were reported as Mean±SD.

Screening of surfactant (emulsification study):

Poloxamer 188, cremophore EL, tween 80, tween 20, tween 60, span 20 and span 80 were screened for their emulsification ability in selected oil phase. Surfactant selection was done on the basis of % transparency (%T) and ease of emulsification. Briefly, (0.5 ml) of

surfactant was added to (0.5 ml) of the selected oil phase, mixed thoroughly, then the mixture was heated at 50°C for homogenization. Each mixture was then diluted with (50 ml) D.W. in glass stopper conical flask. The emulsions were allowed to stand for 2 hrs, then (%T) was measured spectrophotometrically using D.W. as a blank. In addition, emulsion was further observed visually for any turbidity or phase separation^[17].

Screening of co-surfactant:

Lecithin, transcutool P, gelot[®]64, and myveroI[™] were screened for their emulsification ability. For this, (40µl) of surfactant was mixed with (20µl) of co-surfactant (surfactant:co-surfactant ratio 2:1). The selected oil (65µl) was added to the mixture then the blend was gently heated in a water bath shaker to ensure proper mixing. Ten µl was diluted with D.W. and the screening was done on the basis of %transparency and ease of formation of emulsion. To produce uniform emulsion, the number of inversions required was monitored. The mixtures were set aside for two hrs before analyzing spectrophotometrically against distilled water as blank^[18].

Preparation of (NEB-NLCs):

The NEB-NLCs were prepared by melt-emulsification and low temperature solidification method with slight modification^[19]. A binary lipid mixture composed of solid and liquid lipid were blended and melted at 70±0.5°C, along with (100mg) of NEB to form a uniform and clear oil phase. The aqueous phase consisting of dispersing surfactant or co-surfactant in distilled water was also heated to 70±0.5°C. Pre-emulsion was prepared by slowly dispersing the melted lipid phase to the above surfactant solution under mechanical stirring at 1500rpm maintained at 70±0.5°C for 20min, then sonicate for 10min to ensure further reduction in size to obtain microemulsion. Homogenization for 15min at 6000 rpm using (T-25 digital Ultra-Turrax[®]), high-speed stirring was introduced so that the

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microemulsion breaks into ultrafine nanoemulsion droplets. In order to prevent recrystallization during homogenization, production temperature was kept at least 5°C above the lipid melting point. Ultimately, after homogenization, the resulting hot o/w nanoemulsion was cooled at 4±0.5°C for 15-20 minutes, recrystallization of the lipid and NEB-NLCs dispersion were generated. Furthermore, centrifugation of aqueous dispersion at 45000 rpm for 30 min at 25°C for separation of the nanoparticles. Deposited nanoparticles were re-dispersed in little amount of distilled water. The resultant NEB-NLCs with 5% mannitol (as cryo-protectant agent) was frozen for 24 hrs then lyophilized for 48hrs under vacuum at temperature of -40°C and used for further tests in the study. Fifty six formulas were prepared to study the effect of different factors on the NLCs properties. A fixed amount (100 mg) of NEB, as well as a fixed ratios of solid:lipid (5:5, 6:4, 7:3, 8:2 and 9:1) were used in the study.

Characterization of NEB-NLCs:

Drug loading capacity and percent entrapment efficiency:

The percent entrapment efficiency (EE%) was determined by measuring the concentration of free NEB in the dispersion medium. The untrapped NEB was determined by adding (0.5ml) of NEB-loaded nanoparticle to (9.5ml) methanol, and then the dispersion was centrifuged at 9000 rpm. The supernatant was filtered through Millipore membrane filter (0.2µm), suitably diluted, then analyzed spectrophotometrically for un-encapsulated NEB at 281nm. The EE% and drug loading percent (DL%) were calculated using the following equations:

$$EE\% = \frac{W_{initialdrug} - W_{freedrug}}{W_{initialdrug}} \times 100 \text{Eq.(1)}$$

$$DL\% = \frac{W_{initialdrug} - W_{freedrug}}{W_{lipid}} \times 100 \text{..Eq.(2),}$$

Where, ($W_{initialdrug}$) is the weight of initial drug used, ($W_{freedrug}$) is the weight of free drug detected in the supernatant after centrifugation of the aqueous

dispersion, and (W_{lipid}) is the weight of lipid used^[20].

Particle size and particle size distribution:

The aqueous NEB-NLCs were dispersed in a fixed amount of filtered distilled water (1:50), dilution of all formulations was made and placed in 1cm diameter disposable cuvette to yield a suitable scattering intensity. From the analysis, the mean particle size and polydispersity index PDI of NEB-NLCs were calculated using Brookhaven Instruments Corp90 PLUS (ZetaPlus Particle Sizing, NY, Software, Version 5.34). The measurements were carried out in triplicate, and the mean \pm SD were calculated at a fixed scattering angle of 90° at room temperature^[21].

Zeta potential:

Zeta potential of NEB-NLCs was measured using the (NanoBrookZeta-PALS) using phase analysis light scattering technique. The NLC suspensions were diluted with D.W. to get a uniform dispersion prior to analysis. The conductivity of the diluted sample was measured to choose the detection model. The whole measurement was carried out at 25°C^[21].

Microscopic evaluation:

Visualization by scanning electron microscopy (SEM):

The particle shape and surface morphology of NEB-NLCs were determined by a scanning electron microscope (3rd generation VEGA3-SEM). For conventional imaging in SEM, NEB-NLCs specimens were dusted onto double-sided tape on an aluminum stub and coated with gold in an argon atmosphere for 10 min using a cold sputter coater in SEM chamber to a thickness of 400Å, then photomicrographs were captured by operating at an accelerating voltage of 10Kv electron beam^[22].

Visualization by atomic force microscope (AFM):

The size and surface morphology of NEB-NLCs were confirmed by

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atomic force microscopy after drying the formula. All results were recorded under ambient laboratory condition and scanning frequency of 2Hz. Particle size, 3D-dimension graph and histogram of particle size distribution were obtained^[23].

Visualization by transmission electron microscope (TEM):

The size and morphology of the selected formula was examined by TEM (PHILIPS CM 10) with an accelerating voltage of 100 Kv. One drop of sample was placed on a copper grid coated with a formvar carbon film and allowed to stand at room temperature for 90 seconds to form a thin film.

Evaluation of crystalline state:

Powder X-ray diffraction analysis (PXRD):

The x-rays diffraction patterns (inel-diffractometer) can be used to confirm the crystalline nature of NEB-NLCs samples. The study confirmed at continuous scan range of $2\theta = 5-80$, the operating voltage and current were 30 kv and 20 mA, respectively^[24].

Differential scanning calorimetry (DSC):

The change in the structure of NEB and lipid used during the method of preparation can be predicted by using DSC. Accurately weighed samples (5mg) were placed in non-hermetically aluminum pans and heated to the rate of 10°C/minutes against an empty aluminum pan as a reference covering a temperature range of (40-300°C) under a nitrogen atmosphere^[25].

In-vitro release study:

In-vitro release study of NEB-NLCs was performed using a modified dialysis membrane diffusion technique^[26]. Dialysis membrane (Hi-media, Mumbai, India) with molecular weight cut off between 12,000–14,000Da. Dialysis membrane previously soaked overnight with distilled water, was tied to one end of a specially designed glass cylinder (open at both ends). Five ml of NEB-NLC formulation was accurately placed into this

assembly. The cylinder was attached to a stand and suspended in (100ml) dissolution medium which was freshly prepared phosphate buffer pH 7.4 maintained at $37 \pm 5^\circ\text{C}$, so that the membrane just touched the receptor medium surface. The dissolution medium was stirred at low speed using magnetic stirrer. An aliquot of (5ml) sample was withdrawn from the receiver compartment at pre-determined time intervals (0.25, 0.5, 1, 2, 3, 4, 5, 10, 15, 20 and 24 hrs), and replenished equivalent volume of fresh dissolution medium to maintain constant volume. Samples were analyzed spectrophotometrically. A cumulative amount of drug released was calculated. All the operations were carried out in triplicate^[27].

Results and Discussions:

Solubility determination:

The saturated solubility of NEB in water was found to be 59.5mg/100ml. The solubility of NEB in phosphate buffer solution pH 7.4 was found to be 63.75mg/100ml that maintain a sink condition of the receptor medium in both release and permeation studies^[28].

Screening of starting materials: High solubility of the drug in the melted lipid is substantial for achieving high entrapment efficiency. Nebivolol showed maximum solubility (6.8mg/gm) in GMS compared to other investigated lipids. Drug partition coefficient is a requisite since it affects EE as well as the release of the drug from NLCs^[29]. Accordingly, NEB showed significantly higher partitioning ($p < 0.05$) in GMS than stearic acid, followed by behenic acid. So GMS was selected as the solid lipid for the formulation because it has more potential to solubilize NEB as compared to the other two lipids. On the basis of solubility studies, NEB exhibits poor solubility in the oils of natural sources that have been tested. This may be attributed to the fact that unmodified edible oils are unable to dissolve large dose of lipophilic drug and less efficient self-

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emulsification^[30]. Oleic acid is classified as a monounsaturated omega-9 fatty acid, long chain triglyceride^[31]. Oleic acid exhibits significant influence ($p < 0.05$) on NEB solubility (7.89 ± 0.011 mg/ml) since it possesses the best solubilization capacity among the various hydrophobic oils. So it was chosen as a liquid lipid for the formulation of NEB-NLC. In general, lipophilic drugs are much more soluble in liquid lipids than in solid lipids^[30].

Screening of surfactant and co-surfactant (emulsification study):

Surfactants, being amphiphilic in nature, can dissolve relatively high amounts of hydrophobic drug compounds^[32]. The basis for the selection of surfactant or co-surfactant was mainly dependent on the emulsification efficiency rather than the ability to solubilize NEB. Ease of emulsification was judged by the number of flask inversions required to yield homogenous mixtures and it was assessed visually. As depicted in (Table-2), oleic acid exhibit high emulsification properties and maximum transmittance with transcutool P ($T\% = 99.6$) followed by cremophore EL ($T\% = 99.3$), since they required a few number of inversions to produce a homogeneous formulation. Poor emulsification properties were found with other surfactants (Tween 20, 60) and (Span 20) despite higher transmittance values as they require higher number of flask inversions.

Entrapment efficiency and drug loading:

It was observed that changing the type of solid lipids (GMS and stearic acid) and the type of surfactant used had a significant influence ($p < 0.05$) on the % EE of the prepared formulas. This may be attributed to the solubility of NEB in the lipid phase since a prerequisite for successful entrapment of a drug into NLCs formulation is its solubility or miscibility with the lipid^[33]. With increasing the amount of GMS, % EE is bound to increase because of the increased concentration of mono-, di-, and triglycerides that act as solubilizing agents

for highly lipophilic drug^[34]. The use of one surfactant may hardly achieve transient negative interfacial tension and fluid interfacial film. Thus, adding co-surfactant is crucial since it yields NLC with more homogeneous appearance, lower tendency to form macroscopic particles and subsequently results in lowering the interfacial bonding stress and predispose the interfacial film to occupy enough flexibility to yield various curvatures required for nanoemulsion formulation. Lipophilic surfactants such as span 80 were used as an emulsifier in order to provide better emulsification^[35].

In the present study, combination of hydrophilic-lipophilic surfactants/co-surfactants were used (F22-F56) since it is known that the employment of two surfactants of lipophilic and hydrophilic nature yields better stability of the dispersed system^[36]. Hydrophilic surfactants, poloxamer 188 (F1-F22, F35-F38), tween 80 (F23-F34) and cremophore EL (F40-46) were selected for the reason that each surfactant by virtue of its properties may make a poorly water-soluble drug, more soluble than its inherent solubility^[37]. The effects of (lipid ratio, type of lipid, type and concentration of surfactant) on EE% and loading capacity are illustrated in (Table-3).

Polydispersity index and particle size distribution:

As the lipid concentration increase, more particles were aggregated resulting in an increased particle size. The increase in particle size due to increasing the amount of GMS can be justified in terms of tendency of lipid to coalesce at high lipid concentration. According to Stoke's law, this behavior can be explained by difference in density between internal and external phase^[38]. It is expected that the crystal order in the inner core is greatly disrupted by incorporating a liquid lipid (oleic acid) despite that the carrier remains solid as in (F7-F10, F23-F26) with the ratio of solid to liquid lipid of (6:4). Thus, the addition of a liquid lipid tends to

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promote the formulation of a small particle population as result of the higher molecular mobility of the matrix^[39]. In general, PDI for prepared NEB-NLCs formulas were ≤ 0.3 , with no significant difference ($p > 0.05$) among formulas with various lipid ratios indicating good uniformity of particle size distribution after dilution with water^[40]. In the present study, various surfactants and co-surfactants were optimized in terms of the particle size and PDI of NEB-NLCs obtained (Table-4). It was found that span 80 and cremophore EL give the best results over other surfactants in terms of particle size.

Zeta potential analysis:

All formulations produced negative ZP values (Table-4), however, it is clear that the values were as negative as those recommended for NLC formulations (ZP values of ≤ -30) to be considered stable. Such evidence may be because of a shift in the shear plane of the NLC. However, it is important to note that this rule applies only to colloidal systems that are stabilized by electrostatic interactions alone^[41].

Release profile of selected NEB-NLCs:

According to EE%, PS, PDI and ZP, four formulas (F11, F24, F43 and F 50) were introduced to the release study. The release profiles showed initial burst release followed by gradual release of the NEB. The initial burst may be related to the presence of un-entrapped NEB in the NLC dispersion. Another reason for the initial burst could be attributed to that most of the liquid lipid (oleic acid) is located in the outer shell of the nanoparticles. The oleic acid-enriched outer layers possessed a soft and considerably higher solubility for lipophilic drugs, which ultimately increase the loading of the drug which could be easily released by diffusion or matrix erosion^[42]. Nevertheless, burst release can be useful to improve the penetration of the drug, while sustained release supplies the drug over a prolonged period of time. The rate of release of NEB from the NLCs was significantly ($p < 0.05$)

affected by using different lipid concentrations and surfactants, with the following order, F43> F24> F11> F50 (93.32, 77, 61.8, 55.8%), respectively.

Another factor subscribing to the rapid release of NEB-NLC43 was its smaller particle size. It is known that, the small size should create a larger total surface area, and consequently the release rate would be expected to be elevated^[43]. There is close relationship between EE% and %drug release. In the present study, it was found that %EE and % drug release of optimized NEB-NLC were 93.2%±0.02 and 93.3±0.06, respectively. Accordingly, F43 was chosen as the optimized formula. Such reduction in solid lipid re-crystallization was due to the presence of liquid lipid (oleic acid) which maintained the subsaturation condition of GMS by preventing supersaturation. The liquid lipid content affects the EE and drug release to a great extent by providing enough space for large amount of drug to lodge (and thereby release) in the imperfections^[44].

SEM, AFM and TEM studies:

The obtained SEM images of the optimized formula F43, reveals almost all spherical shapes and the size of NEB-NLCs were within the nanometer range. As seen in figure-2. While, the morphological and particle size analysis of F43 performed by AFM showed spherical shaped lipid nanoparticles with size of 57 nm. The micrographs of NEB- NLCs examined by TEM revealed that they were almost spherical with smooth morphology, well dispersed and separated on the surface. Such analysis results are in agreement with the results produced by SEM and AFM. The average droplets size was less than 200nm^[45], no phase separation was observed^[46].NLCs examined by TEM revealed that they were almost spherical with smooth morphology, well dispersed and separated on the surface. Such analysis

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results are in agreement with the results produced by SEM and AFM. The average droplets size was less than 200nm^[45], no phase separation was observed^[46].

Differential scanning calorimetry (DSC) study:

Thermal analysis of DSC thermogram of NEB profiles show sharp endothermic peak at 221.43 °C corresponding to its melting indicating its crystalline anhydrous state^[47]. The DSC curve revealed that the NEB and GMS mixture (1:1) shows no extra peak when compared to NEB and GMS alone, so they are compatible. However, there is no characteristic endothermic peak, corresponding to NEB melting was observed in the optimized formula (F43) owing to decreased crystallinity in NLCs and/or solvation of NEB in the lipid carriers before reaching its fusion temperature. The GMS entrapped in the NLC is solidified perfectly since the melting point is higher than 40°C which is preferred for skin formulations^[48].

X ray diffraction (XRD) study:

The XRD pattern of pure NEB show high crystalline nature as indicated by the numerous distinctive peaks with major characteristic diffraction peaks appearing at a diffraction angle of 2θ at 2.903°, 16.220°, 28.487°, 38.34° and 45.321°. The diffraction pattern of GMS was significantly differ (p<0.05) from NEB-NLCs.

The GMS showed diffraction peaks at 2θ values of 5.5°, 7.4°, 19.5°, 20.6°, and 23.4°. Whereas, the optimized NLC showing absence of NEB constructive peaks indicating conversion of NEB from crystalline to amorphous state or molecularly dispersed structure, which may be attributed to the NEB solubilization in the lipid carriers^[47].

Table-1: Preparation of different formulas of Nebivolol - Loaded Nanostructured Lipid Carriers (NEB-NLCs).

| Formula code | Amount of Drug (mg) | Solid Lipid (mg) | | Liquid Lipid (mg) | Surfactant | | | Co-Surfactant (%) | | | | | |
|--------------|---------------------|------------------|-----|-------------------|------------|---------------------|----------------------|---------------------|-----|-----|-----|-------|------|
| | | SA | GMS | | OA | T ₈₀ (%) | F ₆₈ (mg) | S ₈₀ (%) | TCp | Le | CR | Ge-64 | Myv |
| F1 | 100 | 300 | | 200 | | 150 | | | | | | | 0.26 |
| F2 | 100 | 350 | | 150 | | 200 | | | | | | | 0.26 |
| F3 | 100 | | 250 | 250 | | 150 | | 5 | | | | | |
| F4 | 100 | | 250 | 250 | | 200 | | 5 | | | | | |
| F5 | 100 | | 250 | 250 | | 250 | | 5 | | | | | |
| F6 | 100 | | 250 | 250 | | 300 | | 5 | | | | | |
| F7 | 100 | | 300 | 200 | | 150 | | 5 | | | | | |
| F8 | 100 | | 300 | 200 | | 200 | | 5 | | | | | |
| F9 | 100 | | 300 | 200 | | 250 | | 5 | | | | | |
| F10 | 100 | | 300 | 200 | | 300 | | 5 | | | | | |
| F11 | 100 | | 350 | 150 | | 150 | | 5 | | | | | |
| F12 | 100 | | 350 | 150 | | 200 | | 5 | | | | | |
| F13 | 100 | | 350 | 150 | | 250 | | 5 | | | | | |
| F14 | 100 | | 350 | 150 | | 300 | | 5 | | | | | |
| F15 | 100 | | 400 | 100 | | 150 | | 5 | | | | | |
| F16 | 100 | | 400 | 100 | | 200 | | 5 | | | | | |
| F17 | 100 | | 400 | 100 | | 250 | | 5 | | | | | |
| F18 | 100 | | 400 | 100 | | 300 | | 5 | | | | | |
| F19 | 100 | | 450 | 50 | | 150 | | 5 | | | | | |
| F20 | 100 | | 450 | 50 | | 200 | | 5 | | | | | |
| F21 | 100 | | 450 | 50 | | 250 | | 5 | | | | | |
| F22 | 100 | | 450 | 50 | | 300 | | 5 | | | | | |
| F23 | 100 | | 300 | 200 | 0.6 | | | | 0.4 | | | | |
| F24 | 100 | | 300 | 200 | 0.8 | | | | 0.4 | | | | |
| F25 | 100 | | 300 | 200 | 1 | | | | 0.4 | | | | |
| F26 | 100 | | 300 | 200 | 2 | | | | 0.4 | | | | |
| F27 | 100 | | 350 | 150 | 0.6 | | | | 0.4 | | | | |
| F28 | 100 | | 350 | 150 | 0.8 | | | | 0.4 | | | | |
| F29 | 100 | | 350 | 150 | 1 | | | | 0.4 | | | | |
| F30 | 100 | | 350 | 150 | 2 | | | | 0.4 | | | | |
| F31 | 100 | | 350 | 150 | 0.8 | | | | 0.6 | | | | |
| F32 | 100 | | 350 | 150 | 0.8 | | | | 1 | | | | |
| F33 | 100 | | 350 | 150 | 0.8 | | | | 1.2 | | | | |
| F34 | 100 | | 350 | 150 | 0.8 | | | | 2 | | | | |
| F35 | 100 | | 300 | 200 | 1 | | | | 5 | | | | |
| F36 | 100 | | 350 | 150 | 1 | | | | 5 | | | | |
| F37 | 100 | | 400 | 100 | 1 | | | | 5 | | | | |
| F38 | 100 | | 450 | 50 | 1 | | | | 5 | | | | |
| F39 | 100 | | 300 | 200 | | | 2 | | | 2 | | | |
| F40 | 100 | | 300 | 200 | | | 1.4 | | | 2.6 | | | |
| F41 | 100 | | 300 | 200 | | | 1 | | | 3 | | | |
| F42 | 100 | | 300 | 200 | | | 0 | | | 4 | | | |
| F43 | 100 | | 350 | 150 | | | 2 | | | 2 | | | |
| F44 | 100 | | 350 | 150 | | | 1.4 | | | 2.6 | | | |
| F45 | 100 | | 350 | 150 | | | 1 | | | 3 | | | |
| F46 | 100 | | 350 | 150 | | | 0 | | | 4 | | | |
| F47 | 100 | | 350 | 150 | | 200 | | | | | | | 0.10 |
| F48 | 100 | | 350 | 150 | | 200 | | | | | | | 0.20 |
| F49 | 100 | | 350 | 150 | | 200 | | | | | | | 0.26 |
| F50 | 100 | | 350 | 150 | | 200 | | | | | | | 0.30 |
| F51 | 100 | | 300 | 200 | | 200 | | 6.4 | | | 5 | | |
| F52 | 100 | | 300 | 200 | 5 | 200 | | 6.4 | | | 7.5 | | |
| F53 | 100 | | 300 | 200 | 5 | 200 | | 6.4 | | | 10 | | |
| F54 | 100 | | 350 | 150 | 5 | 200 | | 6.4 | | | 5 | | |
| F55 | 100 | | 350 | 150 | 5 | 200 | | 6.4 | | | 7.5 | | |
| F56 | 100 | | 350 | 150 | 5 | 200 | | 6.4 | | | 10 | | |

NEB: Nebivolol hydrochloride, SA: Stearic Acid, GMS:Glyceryl monostearate, OA: Oleic Acid, T80: Tween 80, F68: Poloxamer 188, S80: Span 80, TCP: Transcutol P, Le: Lecithin, CR:Cremophore EL, GE-64: Gelot64, MYV:Myverol.

Table-2: Solubility of NEB in different surfactants and co-surfactants.

| Type of Surfactant/co-surfactant | No. of Inversions | %T mean±SD, n=3 | Type of Surfactant/co-surfactant | No. of Inversions | %T mean±SD, n=3 |
|----------------------------------|-------------------|-----------------|----------------------------------|-------------------|-----------------|
| Tween 20 | 14 | 96.4 | Myverol | 8 | 98.5 |
| Tween 80 | 5 | 99.1 | Gelot-64 | 10 | 97.3 |
| Span 20 | 22 | 94.5 | Trancutol P | 3 | 99.6 |
| Span 80 | 7 | 96.7 | Poloxamer | 3 | 90.1 |
| Cremophore EL | 2 | 99.3 | Lecithin | 7 | 92.7 |

Table-3: Entrapment efficiency percent and drug loading percent of different Nebivolol Loaded Nanostructured Lipid Carriers.

| No. | EE% | DL% | No. | EE% | DL% | No. | EE% | DL% | No. | EE% | DL% | No. | EE% | DL% |
|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|-----|------|-----|
| F1 | 41.8 | 1.2 | F13 | 80.2 | 3.9 | F25 | 65.3 | 6.9 | F37 | 80.5 | 1.6 | F49 | 76 | 4.8 |
| F2 | 49.7 | 10 | F14 | 80.1 | 3.9 | F26 | 67.8 | 6.4 | F38 | 83.5 | 3.8 | F50 | 92.1 | 4.5 |
| F3 | 51.4 | 9.7 | F15 | 84.6 | 3.5 | F27 | 68.2 | 6.3 | F39 | 81.3 | 3.1 | F51 | 76.6 | 4.6 |
| F4 | 52.3 | 9.2 | F16 | 88.7 | 2.2 | F28 | 68.8 | 6.2 | F40 | 87.5 | 1.9 | F52 | 76.8 | 4.6 |
| F5 | 53.6 | 9.4 | F17 | 84.6 | 3 | F29 | 83.6 | 3.2 | F41 | 89.5 | 2.1 | F53 | 79.5 | 4.1 |
| F6 | 53.7 | 9.2 | F18 | 84.4 | 3.1 | F30 | 82.3 | 1.4 | F42 | 86 | 2.8 | F54 | 76.8 | 4.6 |
| F7 | 54.5 | 9 | F19 | 83.2 | 3.3 | F31 | 82.4 | 3.5 | F43 | 93.2 | 7.3 | F55 | 77.5 | 4.5 |
| F8 | 58.4 | 8.3 | F20 | 85.9 | 2.8 | F32 | 83.6 | 3.2 | F44 | 94.5 | 5.4 | F56 | 79.2 | 9.1 |
| F9 | 58.5 | 8.4 | F21 | 84.2 | 3.1 | F33 | 83.3 | 3.3 | F45 | 95.7 | 4.2 | | | |
| F10 | 59.8 | 8 | F22 | 83.9 | 3.2 | F34 | 83.8 | 3.2 | F46 | 99.7 | 4.6 | | | |
| F11 | 89.7 | 3.6 | F23 | 60.1 | 7.9 | F35 | 84.5 | 1.9 | F47 | 90.1 | 5.9 | | | |
| F12 | 81.4 | 3.7 | F24 | 91.9 | 7.6 | F36 | 81 | 1.8 | F48 | 75.4 | 4.9 | | | |

Table-4: Particle Size, polydispersity index and zeta potential of different NEB-NLCs formulations.

| No. | PS | PDI | ZP | No. | PS. | PDI | ZP | No. | PS | PDI | ZP | No. | PS | PDI | ZP |
|-----|-------|------|-------|-----|-------|------|-------|-----|-------|------|-------|-----|-------|------|-------|
| F1 | 645.3 | 1 | -39 | F16 | 426.8 | 0.5 | -31 | F31 | 496 | 0.33 | -31 | F46 | 316 | 0.25 | -30 |
| F2 | 520.2 | 0.7 | -38 | F17 | 501 | 0.41 | -31 | F32 | 490.8 | 0.3 | -30 | F47 | 405.1 | 0.26 | -44 |
| F3 | 299 | 0.2 | -32 | F18 | 517.6 | 0.5 | -34 | F33 | 515.8 | 0.31 | -33 | F48 | 413.1 | 0.29 | -45 |
| F4 | 304.1 | 0.21 | -31.7 | F19 | 655 | 0.41 | -32 | F34 | 460 | 0.3 | -30 | F49 | 450.1 | 0.3 | -44.2 |
| F5 | 320.2 | 0.2 | -30 | F20 | 713.1 | 0.1 | -32.5 | F35 | 620.2 | 0.31 | -30 | F50 | 418.1 | 0.27 | -43 |
| F6 | 345.5 | 0.22 | -31 | F21 | 690.2 | 0.08 | -30 | F36 | 642.6 | 0.42 | -37.7 | F51 | 433.5 | 0.31 | -30 |
| F7 | 419 | 0.5 | -31.2 | F22 | 672.6 | 0.07 | -30.1 | F37 | 658.7 | 0.45 | -33 | F52 | 439.8 | 0.33 | -32 |
| F8 | 443.2 | 0.53 | -31 | F23 | 340.1 | 0.2 | -31 | F38 | 711 | 0.44 | -32 | F53 | 447.6 | 0.52 | -33 |
| F9 | 468.4 | 0.6 | -30 | F24 | 300 | 0.23 | -30.2 | F39 | 791.2 | 0.4 | -30 | F54 | 481 | 0.45 | -38 |
| F10 | 492.9 | 0.6 | -30 | F25 | 348.5 | 0.21 | -30.2 | F40 | 299.6 | 0.46 | -34 | F55 | 485.7 | 0.5 | -30 |
| F11 | 288.2 | 0.26 | -30 | F26 | 350.6 | 0.23 | -30 | F41 | 294.1 | 0.32 | -32 | F56 | 487.2 | 0.52 | -38 |
| F12 | 398 | 0.2 | -34 | F27 | 420.4 | 0.2 | -30.1 | F42 | 297.1 | 0.24 | -36 | | | | |
| F13 | 405.6 | 0.26 | -30 | F28 | 446 | 0.3 | -33 | F43 | 256.3 | 0.22 | -30 | | | | |
| F14 | 411 | 0.29 | -30.1 | F29 | 460 | 0.31 | -30 | F44 | 304.7 | 0.24 | -30.1 | | | | |
| F15 | 420.3 | 0.51 | -32 | F30 | 466.9 | 0.33 | -30 | F45 | 312.6 | 0.28 | -30 | | | | |

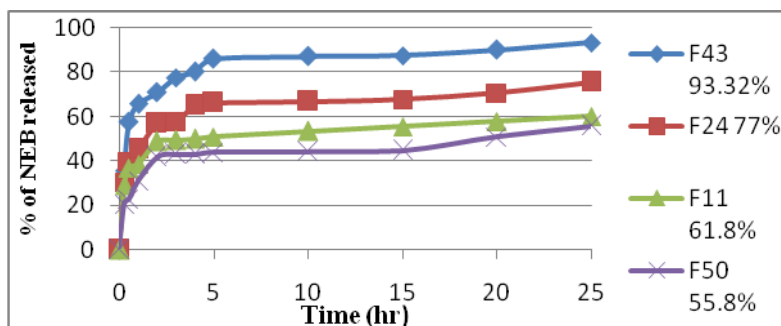


Figure-1: Nebivolol-loaded nanostructured lipid carriers (NEB-NLCs) release profile from different formulations.

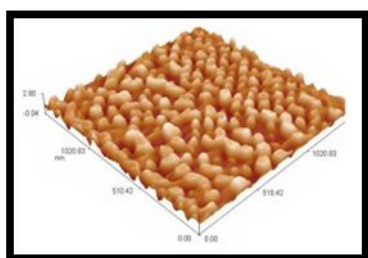


Figure-2: AFM of Optimized formula (F43).

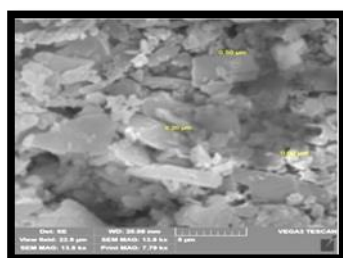


Figure-3: SEM of optimized formula (F43).

Conclusions:

In the present investigation (part 1 of the study), Nebivolol nanostructured lipid carriers (NEB-NLCs) were prepared using different concentrations and types of lipids (solid and liquid) and were stabilized by different surfactants/co-surfactants combination. It was found that melt emulsification and low temperature solidification is simple and efficient method for NLCs preparation which could be modified for better result. The NEB-NLCs prepared by using glyceryl monostearate as solid lipid and oleic acid as liquid lipid had smaller particle size and higher entrapment efficiency than those prepared from stearic acid as solid lipid. The release of NEB from NLCs displayed

biphasic release pattern with burst release at the initial stage followed by sustained release. The results indicated that NLCs 43 is a suitable carrier of NEB with improved loading capacity and controlled release properties. The advantage of NLCs formulation is that, the scale-up of the proportions is easy since the system is thermodynamically stable. Therefore, NLCs 43 was selected and NEB-NLCs based gel was formulated by using gelling agent carbapol 934 in part 2 of the study which will be published soon.

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