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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 بلنبفولول هيدروكلورايد- الجزء الاول

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الخلاصة :

النبفولول هيدروكلورايد هو دواء من الجيل الثالث ذوانتقائية عالية لمستقبلات β1مع خصائص خافضة للضغط وبعمر نصف 10 ساعات تقريبا والتوافرالحيوي عن طريق الفم 12٪.

الدراسة تتضمن جزئين: الجزء الاول (الحالي) يتضمن التحضير والتشخيص والتقييم المختبري لحاملات الدهون ذات البنيه النانوية المحمله بلنبفولول هيدروكلورايد فيما يتضمن الجزء الثاني من الدراسه تحضير وتشخيص حاملات الدهون ذات البنية النانوية كقاعدة مائية هلامية باستعمال مادة الكاربوبول 934 كمادة هلامية لتحرير النبفولول هيدروكلورايد عبر الأدمة والتي سوف يتم نشرها فيما بعد.

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الدراسة الحالية تهدف لتطويرامكانية حاملات الدهون ذات البنية النانوية (NLCs) لتسليم النبفولول هيدروكلورايد عبرالجلد. توضح هذه الدراسة تأثيرنوع وتركيزالدهون المختلفة صلبة، سائلة، والسطحي/المشارك السطحي على خصائص حاملات الدهون ذات البنية النانوية مثل حجم الجسيمات، مؤشرالتشتت المتعدد، وإمكانية قياس فرق الجهد والكفاءة و تحرر الدواء.

أظهر فحص وتحليل المجهرالإلكتروني، مجهرالقوة الذرية ومجهرالإنبعاث الالكتروني أن حاملات الدهون ذات البنية النانوية تكون تقريبا كروية الشكل مع تأثيرضئيل للدهون السائلة (حمض الأوليك)على هيئة وشكل الجسيمات، فيما أظهرمسحDSC إنخفاضا في مؤشرنقطة الإنصهار والتبلور لحاملات الدهون النانوية كلما إزدادت كمية الدهون السائلة، وقد أظهرت دراسة الكفاءه ان 93% من النبفولول يتم تحميله بكفاءه عالية وأن 93٪ من النبفولول يجري تحريره من حاملات الدهون ذات البنية النانوية خلال أربع وعشرين ساعة من عمر الدراسة فيما يتم التحرير بنمط ثنائي الطوريتميزاالطورالأولي بلتحرير السريع يتبعه االتحريرالمستدام بشكل بطئ .

الكلمات المفتاحية: *النبفولول 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 classify-cation, 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)-nebivolol a potent cardioselective β1 adrenoceptor blocker) and l-isomer (RSSS)-nebivolol 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 baseis 405.4. The advantages[9,10] of NLC among others lipidcarriers 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.

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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 purchasedfrom Sigma-Aldrich, Chemie GMBH, Germany. Behenic acid was supplied by Evans Chemical ltd., England. Tripalmitin Extra pure and Cremophore EL (Polyoxy l 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 25ºC 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, behinic acid, cetyl palmitate, stearyl alcohol, tripalmitn 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 g, then shaken. The qualitative solubility of NEB in the molten lipid was estimated visually. The quantity of lipid for complete solublization 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 smallvial. The vials were tightly stoppered and were continuously stirred for 72hrs at 37ºC 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

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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 spectrophotometerically 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, transcutol P, gelot®64, and myverol™ 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 spectrophotometerically against distilled water as blank [18].

Preparation of (NEB-NLCs):

The NEB-NLC$_S$ 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 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, recrystal-lization 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 unentrapped 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:

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

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

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

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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± 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-NLC$_s$ 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 400A, 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 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 K$_V$. 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-diffractogmeter) can be used to confirm the crystalline nature of NEB-NLCs samples. The study confirmed at continuous scan range of 2θ = 5-80, the operating voltage and current were 30 kv and 20 mA, respectively[24].

Differential scanning colorimetry (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 10ºC/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

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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± 5ºC, 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 spectrophotometerically. 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-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±0.011mg/ml) since it possesses the best solublization 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 transcutol 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

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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 hydrophilihc 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 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 ($p<0.05$)

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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 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 ºC corresponding to its meltingindicating 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, the 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°, 28.487º, 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 solublization in the lipid carriers[47].

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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.

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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				

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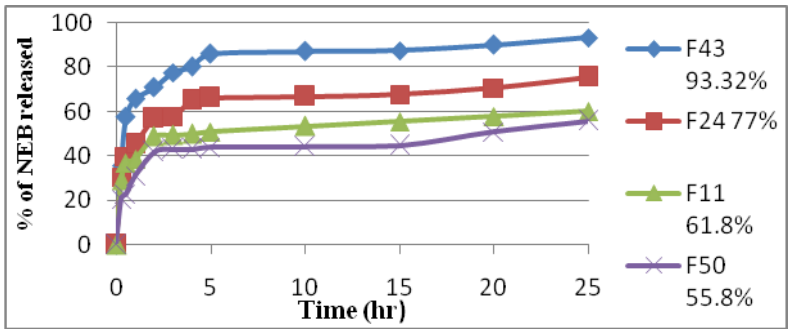

Figure-1: Nebivolol-loaded nanostructured lipid carriers (NEB-NLCs) release profile from different formulations.

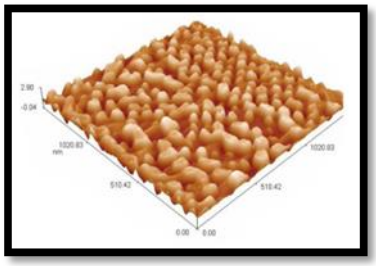

Figure-2: AFM of Optimized formula (F43).

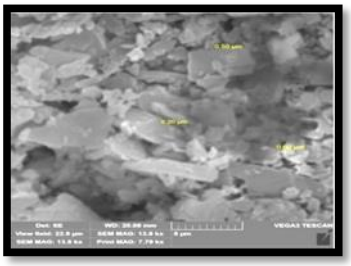

Figure-3: SEM of optimized formula (F43).

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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