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DEVELOPMENT AND *IN-VITRO* EVALUATION OF NANOSUSPENSION OF NEBIVOLOL HYDROCHLORIDE TO ENHANCE ITS SOLUBILITY FURTHER LOADED IN A MOUTH DISSOLVING FILM

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Abstract

The aim of this study was to develop and characterize Nebivolol hydrochloride (NBH)nanosuspension to enhance its dissolution rate and oral bioavailability. Nanosuspensions were prepared by the nanoprecipitation method. The effects of parameters such as the concentration of Nebivolol in the organic phase, the precipitation temperature, the solvent-antisolvent ratio and the time period of stirring on the particle size of nanosuspensions were investigated systematically, and the optimal value was 20 mg/ml, at room temp,1:30 and 15 min, respectively. The average particle size and zeta potential of nanosuspension were 197.6nmand−34.2mV (±6.01 mV), respectively. The morphology of nanosuspension was found to be ofregular morphology and rectangular in shape by scanning electron microscopy (SEM) observation. The X-ray powder diffraction (XRD) indicated that there was change in the nanosuspension compared with raw crystals i.e. crystalline drug changes into almost amorphous nature. FT-IR studies there is no chemical interaction between drug and excipients. The in-vitro dissolution rate of nebivolol was significantly increased by reducing the particle size.Ex-vivo studies shows adequate results.Selected formulation of film give suitable results in case of surface PH, folding endurance,disintegration test, drug content and drug release.Comparison studies show nanosuspension film gives faster and more drug release(93%).Stability studies also shown the acceptable results.

Key Words: Nebivolol hydrochloride, Nanosuspension, Nanoprecipitation, in-vitro dissolution, ex-vivo dissolution studies.

Introduction

Nebivolol is chemically well-known as 1-(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)-2-{[2-(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)-2-hydroxyethyl] amino}ethan-1-ol], which is high-selective β1-adrenergic blocker (along-acting) with nitric oxide mediated vasodilatory actions, favorable upshots on vascular endothelial function and utilized in the controlling of hypertension [1,2]. It reduces heart rate, rate of myocardial contractility and systemic blood-pressure, while increasing diastolic pause. β-blockers are useful prophylactic agents in stable and unstable types of angina. Nebivolol is a racemate (combination) of two enantiomers, SRRR-nebivolol (*d*-Nebivolol) and RSSS-nebivolol (*L*-Nebivolol). It combines two pharmacological activities, such as (a) a competitive and selective β1-recepter antagonist which is attributable to the *d*-enantiomer, and (b) mild vasodilating properties, possible owing to an interaction with the *L*-arginine/nitric oxide pathway. It shows the vasodilating action that lacks of intrinsic sympathomimetic and membrane stabilizing efficiency.

NBH having very low water solubility, which results into poordissolution rates.[1]Its bioavailability through oral route is just 12%.The main objective of this work was toinvestigate the possibility of improving the solubility and dissolution rate of NBHby preparing nanosuspensions by nanoprecipitation method.[2,3]According to the Noyes–Whitney equation, the dissolution rate could be increased by reducing the particle size at the micro or nano-scale to increase the interfacial surface area.[4]The nanoprecipitation technique is a more facile, less complex, less energy consuming as well as widely applicable technique without any additives for the manufacturing of defined nanospheres.[5] This technique is based on the interfacial deposition due to the displacement of a solvent with the non-solvent.[6]

Nanoparticle formation via nanoprecipitation is assumed to be due to the nucleation of small aggregates of macromoleculesfollowed by aggregation of these nuclei.[7] The aggregation stops assoon as the colloidal stability is reached. A theory describing thenucleation in supersaturated solutions into nanodispersion isproposed by Lamer.[8]

In contrast to emulsion/solvent diffusion,no surfactants are necessary that might influence the surface characteristics or cause toxic effects. Moreover, a broad variety of benign solvents, such as DMSO or acetone, can be used.[9] The size and shape of the particles can only hardly be predicted but it is comparatively easy to influence the particle formation by changing concentration, solvent-antisolvent, andpreparation technique.[10]

In this study we evaluated new colloidal system (nanosuspension) loaded in fast dissolving film for immediate release, better dissolution and to increase its bioavailability.[11] Nanosuspensions were made here by varying the different organic volatile solvents, changing solvent-antisolvent from 1:10 to 1:50 and concentration of the drug in the solvent.[12] Finally accepted NBH A-20 formulation undergone for characterization such as Particle size, Zeta potential, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffractrometry (XRD) and Scanning Electron Microscopy (SEM).[13]Acceptance of NBH A-20 formulation done by evaluating the product yield, particle size,drug content, solubility and in-vitro dissolution studies compared with other formulations.This NBH A-20 then further loaded in fast dissolving film of HPMC E-50.[14]Film was made by casting method.[15]Then this film was evaluated by various characterization processes such Surface pH,folding endurance,thickness,drug content, FTIR, in-*vitro* and ex-*vivo*drug release studies.[16]

Materials and Method

Materials

Nebivolol hydrochloride was received as a gift sample from UniSpeed PharmaceuticalsPvt.Ltd. Baddi,Solan(H.P.).Acetone used was of laboratory reagent grade and purchased from Qualikems, Vadodara. Hydroxypropylmethylcellulose(HPMC E-50) was also received as a gift sample from6Colorcon Asia Pvt.Ltd, Goa. PEG 400 and Tween 80 were purchased from S.D Fine –Chem Limited, Mumbai and Molychem, Mumbai respectively.Distilled water was prepared by ELGA LabWater laboratory water purification system.

Method

Formation of nanosuspension

NBH nanosuspension were prepared by the nanoprecipitation method.[17] Raw NBH powder (20 mg) was dissolved in a 1ml solvent (acetone - as a saturated solution) and fill it in the syringe of size 0.55 x 25 mm and then nanosuspension was formed by adding this solution through syringe in an anti-solvent (distilled water) containing 0.1ml of tween 80 which act as stabilizer at controlled and uniform speed. Drug particles in the nanosuspension were obtained by desolvation and then quick evaporation of the solvent, followed by drying of the particles by placing in hot air oven at 50°C. The effects of parameters like drugconcentration in solvent, and solvent to anti-solvent ratio wereinvestigated. The

drug concentrations used were 5, 10, 15 and 20 mg/mLand the solvent to antisolvent (S-AS) ratios were varied to 1:10,1:20,1:30,1:40 and 1:50 (by volume).

Formation of FDF

Then final preparation which was selected on the basis of product yield, drug entrapment, kinetic solubility and drug release was loaded in a fast dissolving film of HPMC E-50 by casting method.[18]First 0.1ml of PEG 4000 and 0.1ml of Tween 80 dissolved in 10 ml warm water,it was stirred by mechanical stirrer at 1000 rpm for 15 min.Then 100mg HPMC E-50 added in it againstirred for at 1000 rpm for 15 min. at last 5mg of NBH A-20 formulation was added to this solution again stirred on same criteria. Then this solution poured in the petri dish,placed it in the hot air oven at 60□C till it get dried, formed film was taken for evaluation.

Table-1: (A) Different formulation and their percentage yield and (B) Different formulations of Mouth dissolving films loaded with nanosuspension. (A)

Formulation code	Drug conc. (mg)	Solvent	Solvent-antisolvent ratio	Yield (%)	Saturation solubility (%)
N1	5	Acetone	1:10	84	82.02
N2	5	Methanol	1:10	49	52.24
N3	5	Ethanol	1:10	60	64.95
N4	5	DMSO	1:10	-	-
N5	5	DMF	1:10	-	-
NA2	5	Acetone	1:20	72	56.06
NA3	5	Acetone	1:30	85	90.38
NA4	5	Acetone	1:40	49	43.44
NA5	5	Acetone	1:50	74	42.42
NE2	5	Ethanol	1:20	60	75.12
NE3	5	Ethanol	1:30	48	58.6
NE4	5	Ethanol	1:40	50	48.43
NE5	5	Ethanol	1:50	44	43.35
NBH A-10	10	Acetone	1:30	79	62.41
NBH A-15	15	Acetone	1:30	70	50.97

NBH A-20	20	Acetone	1:30	83	96.73
NBH A-30	30	Acetone	1:30	85	95
NBH E-10	10	Ethanol	1:20	80	89.41
NBH E-15	15	Ethanol	1:20	70	50.97
NBH E-20	20	Ethanol	1:20	83	96.73
NBH E-30	30	Ethanol	1:20	82	95

Note: Green highlighted formulation taken for drug release studies.

(B)

Ingredients	NF1	NF2	NF3	NF4
Nanosuspension (mg)	5	5	5	5
HPMC E-50 (mg)	120	-	-	100
PVA (mg)	-	50	-	-
PVP (mg)	-	40	50	-
Sodium alginate (mg)	-	-	100	-
PEG (ml)	0.1	5	-	-
Glycerol (ml)	-	-	20	5
Tween 80 (ml)	0.1	0.1	0.1	0.1
Distilled water (ml)	10	10	10	10

Characterization

For NBH nanosuspension

Particle size and Zeta potential measurement

Particle size and Zeta potential of prepared nanosuspension was determined by photon correlation spectroscopy using Zetasizer Version. 7.0(Malvern Instruments,Malvern, UK).[19,20] Each measurement was performed in triplicate.The Mie theory (dispersant refractive index = 1.33;real particle refractive index = 1.59; imaginary part of theparticle refractive index = 0.001) was used for particlesize calculation. The samples were diluted with distilledwater to obtain an appropriate obscuration. Particle sizeswere expressed by the volume-based 10% (d 0.1), 50% (d0.5) and 90% (d 0.9) diameter percentiles.

SEM

Particle morphology was observed usingSEMJSM-6360LV (JEOL Inc., Japan).[21]The samples, an appropriate amount of powder or a glass slide with a small drop of the suspension, were fixed on an SEM stub using double-sided adhesive tape and coated with Au at 50mA for 6min through a sputter-coater (KYKY SBC-12, Beijing, China). A scanning electron microscope with a secondary electron detector was used to obtain digital images of the samples at an accelerating voltage of 10 kV.

Kinetic solubility or Saturation solubility

Kinetic solubility of pure drug, plain andprepared nanosuspension was determined by adding predetermined amount to each 10 ml of distilled water.[22] Samples were shaken for 48 hours on a mechanical water bath shaker at an ambient temperature.The solutions were then centrifuged at 5000 rpmfor 15 min. Supernatant was filtered through membranefilter (0.45μm). Filtered solution was suitably diluted andanalyzed spectrophotometrically at 281nm.

FTIR

FTIR spectra of pure drug andprepared nanosuspensions were recorded using Bruker, FTIR-ATR, Alpha -E instrument.[23] About 2–3 mg of sample was placed and the sample was scannedthrough the wave number range of 4000–400 cm^{-1}.

X-ray diffraction studies (XRD)

The XRD patterns were recorded on an X-ray diffractometer(XPERT-PRO), PANalyticals, Netherland.[24] The sampleswere irradiated with monochromatized CuKα radiation(1.5406 A) and analyzed between 5 and 50° 2θ. The voltageand current used were 45 kV and 40 mA, respectively.The range and the chart speed were 1 × 104 CPS and 10mm/°2θ, respectively.

***In-vitro* dissolution studies**

The dissolution studies of pure drug and prepared nanosuspension were performed usingUSP type - II dissolution test apparatus (LabIndiaDS-8000, India).[25]Samples equivalent to 5 mg ofwere raw NBH and other formulation placed in the dissolution vessel containing 900 mlof 0.1 N Hcl solution maintainedat 37±0.5°C and stirred at 50 rpm. The aliquoted samples were filtered with 0.45 μm filter, which was retained in thedissolution medium throughout the

dissolution studies.Samples were collected periodically with the help ofpipette and replaced with a fresh dissolution medium. Concentration was determined spectrophotometrically at 281 nm.

Stability studies

The physical stability of the nanosuspension was evaluated after storage for 3 months under room temperature condition.[26]Nanosuspensions were wrapped in aluminium foil paper and then placed in well closed containers and placed at room temperature 20°C to 25°C and away from direct light. Sample taken after an interval of 3 months for evaluation i.e. particle size, zeta potential and dissolution studies.

For Mouth dissolving film

Appearance of Film

Appearances of films were evaluated by visual observation such as transparency or opaqueness, color.

Thickness

The thickness of each film was measured using micrometer (thickness tester) at different positions of the film and the average was calculated.[27]

Folding endurance

The folding endurance of the films was determined by repeatedly folding one film at the same place till it broken, which is considered satisfactory to reveal good film properties.[28]This test was done on all the films.

Fourier Transform Infrared Spectroscopy Studies

Drug–polymer interactions have been investigated mainly using Fourier-transform infrared spectroscopy using Bruker, FTIR-ATR, Alpha -E instrument. About 2–3 mg of sample of pure Nebivolol hydrochloride,HPMC E-50, and their physical mixture was placed and the sample was scanned through the wave number range of 4000–400 cm^{-1}.

Surface pH

The films were allowed to swell in closed Petri dish at room temperature for 30 min in 10 mL of distilled water. Solution was placed under digital pH meter, to determine the surface pH.[29]

Drug content

Film (size of 3×2 cm) was taken fromdifferent areas of the film formed and placed in a 10 ml volumetric flask, in it 10ml of methanol was added and kept aside till the film dissolves completely. From this

solution, 0.1 ml was pipetted out and diluted to 10 ml with methanol.[30]The solution was analyzed by UV–Visible spectrophotometer at 282 nm.

Disintegration Time

Disintegration time provides an indication about the disintegration characteristics of the film. The required size of the film (3 ×2 cm) was put in 25 ml phosphate buffer pH 6.8 at 37±2°C. The medium was kept mildly agitated by swirling every 10 s.[31]Time taken by film to break and dissolve was measured as in vitro disintegration time. It was also performed on disintegration tester (LabIndia, DT 1000) following same above procedure.

***In-vitro* drug release**

In vitro dissolution study for all the formulations was performed for 5 min in USP type -I basket apparatus(LabIndia DS-8000, India). The dissolution medium consisted of 200 ml of phosphate buffer pH 6.8 was kept at 37±0.5°C and baskets were rotated at 50 rpm.[32]The samples (4 ml) were withdrawn regular interval of time and the same volume of fresh medium was replenished. The solution was analyzed by UV–Visible spectrophotometer at 280 nm.

***Ex-vivo* drug release**

The *ex-vivo*buccal permeation of NBH nanosuspension throughthe porcine buccal mucosa was performed using a modifiedFranz glass diffusion cell. Porcine buccal mucosa wasobtained from a local slaughterhouse and used within 2 hoursof slaughter. Freshly obtained porcine buccal mucosa wasmounted between the donor and receptor compartments. Thefilm was placed on the smooth surface of mucosa by gentlepressing and the compartments were clamped together. Thedonor compartment was moistened with 1 ml of phosphate buffer (pH 6.8) and the receptor compartment was filled totouch the membrane with a mixture of 30 mlof phosphate buffer (pH 6.8).[33]The fluid motionin the receptor compartment was maintained by stirringwith a magnetic bead at 50 rpm. The temperature wasmaintained at (37±0.2) °C by water jacket surrounding thechamber. At predetermined time intervals, a 1 ml samplewas withdrawn (replaced with fresh medium) and analyzedspectrophotometrically at 280 nm.

Stability studies

The physical stability of the nanosuspension loaded film was evaluated after storage for 3 months under different temperature conditions.[34] Films were wrapped in aluminium paper and placed at room temperature 20°C to 25°C and at

2°–6°C (refrigerator) away from direct light.Samples taken after an interval of 45 days for determination of drug content and physical appearance.

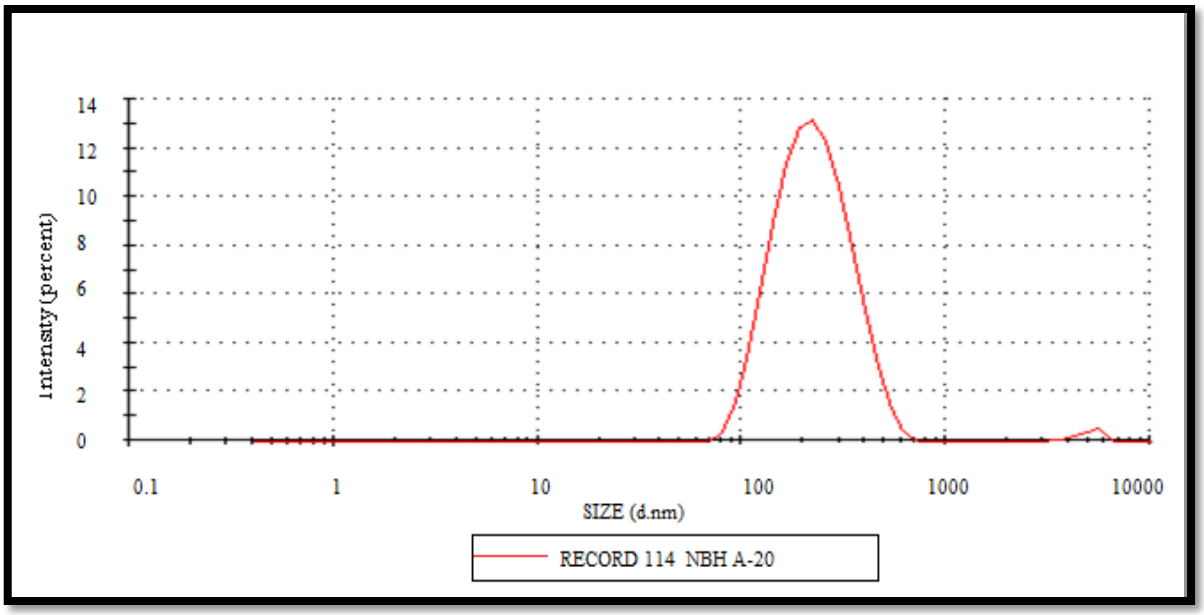
Result and Discussions

Formulation of NBH nanosuspension

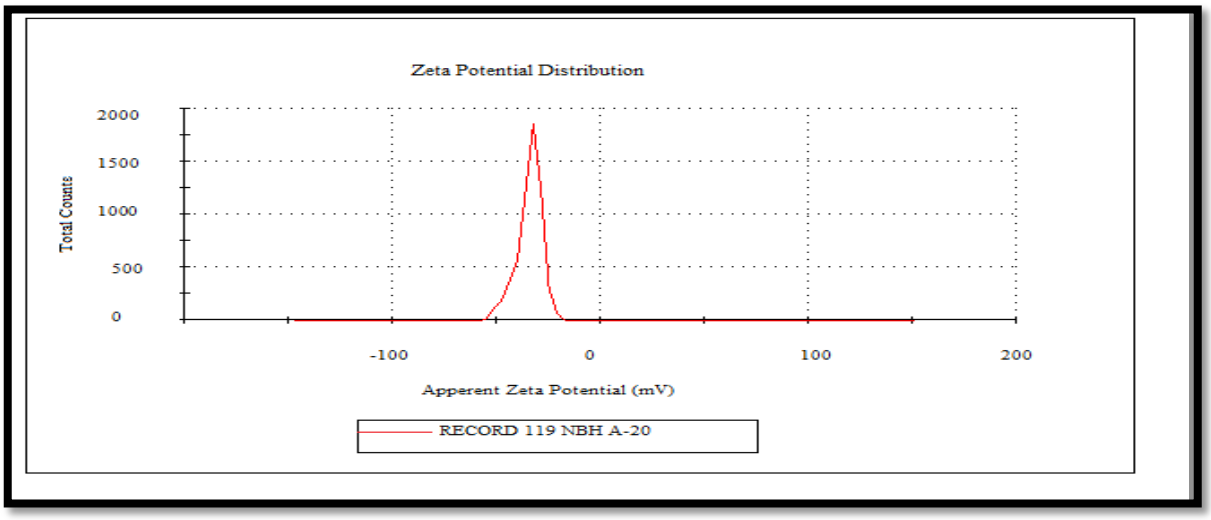
Nanoprecipitation methods are able to create nanosuspension of poor water soluble drugs. Precipitation from solution can offergreater flexibility for altering the crystalline form of the API as well for achieving high drug loadings. The API in organic solution may be mixed with an aqueous antisolvent solution in the presence of stabilizing surfactants to formultrafine particles.[35]Hydrophilic groups in the surfactants lead to rapid wetting of the high-surface-area particles in aqueous media. Using this technique, nanoparticle suspensions were designed with different types of surfactants, as a means to control the particle size and surface charge of the prepared nanoparticles. Surfactants were chosen from a selection of excipients proven to be safe for human use in certain concentrations.[36]

Particle size, morphology and zeta potential

The particle size and Zeta potential of finally selected NBH nanosuspension were shown in Table.3Themean particle size of NBH suspension was 197.6± 9.82 nm with a polydispersity index of 0.210 and zeta potential is -34.2± 6.01 mV.[37]SEM photos (Fig.5) showed that after nanosizing, NBH large crystals were transformed into round or cubic nanoparticles.



(A)

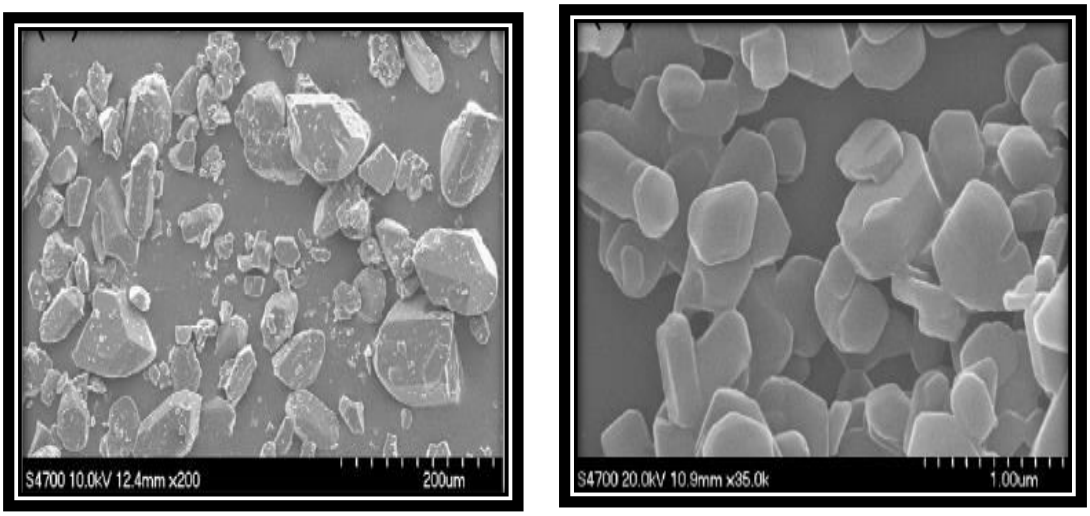


(B)

Fig.1: Graphical representation of particle size distribution and zeta potential distribution of NBH A20 formulation

Scanning electron microscopy (SEM)

The surface morphology of the pure drug and selected formulation powder was investigated by scanning electron microscope (SEM).[38]Samples were fixed on a brass stub using double-sided adhesive tape and were made electrically conductive by coating with a thin layer of gold, and SEM images were recorded at 10 kV accelerating voltage.



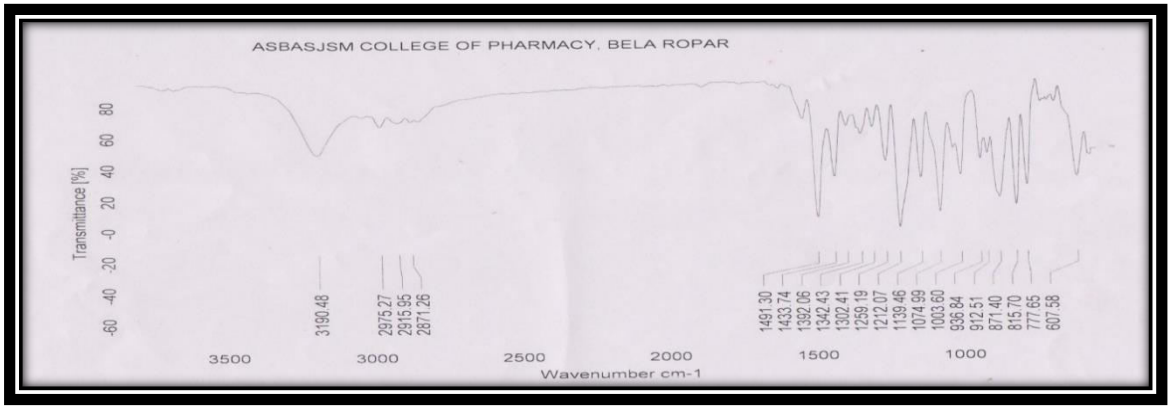
(A) **(B)**

Fig.2: SEM images of (A) pure nebivolol hydrochloride (B) NBH A-20

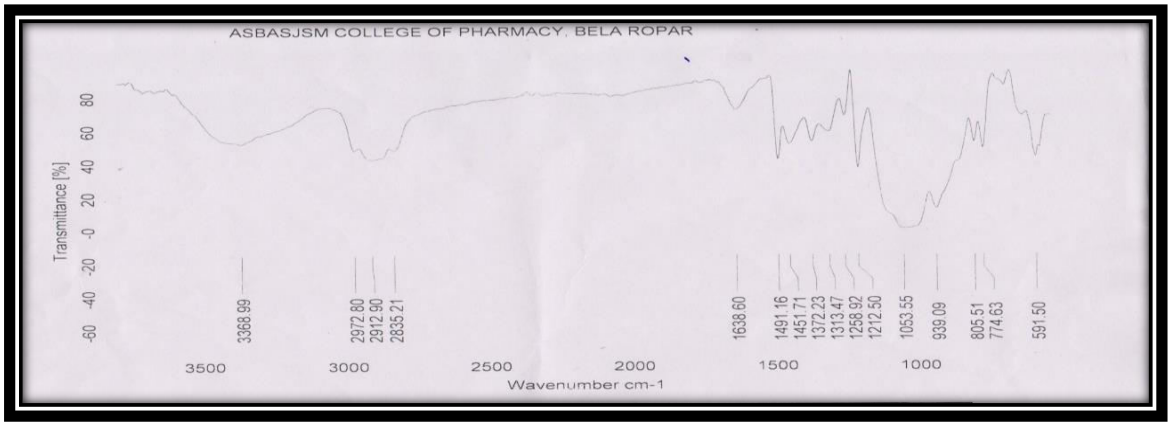
Morphology of pure NBH and NBH nanosuspensions are compared in Fig.3 Pure NBH performed irregular shape, with a mean particle diameter of 50µm whereas the NBH A-20 exhibit regular morphology, they are rectangular in shape, and the average size was less than 200 nm.

FTIR Spectroscopy

State of pure drug (nebivolol hydrochloride) with solvents was determined using FT-IR. Fig.5 shows FT-IR spectra of pure nebivolol hydrochloride and final formulation NBH A-20 i.e.nanosuspension respectively.FT-IR spectra of nebivolol hydrochlorideand NBH A-20 shows peaks at suitable range, so it is clear that there was no chemical change occur in the pure state of the drug.



(A)



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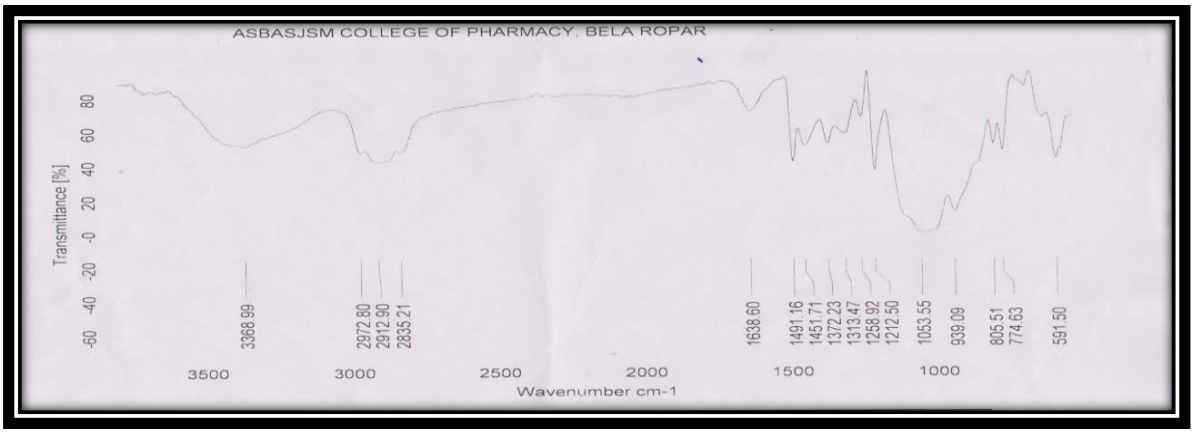
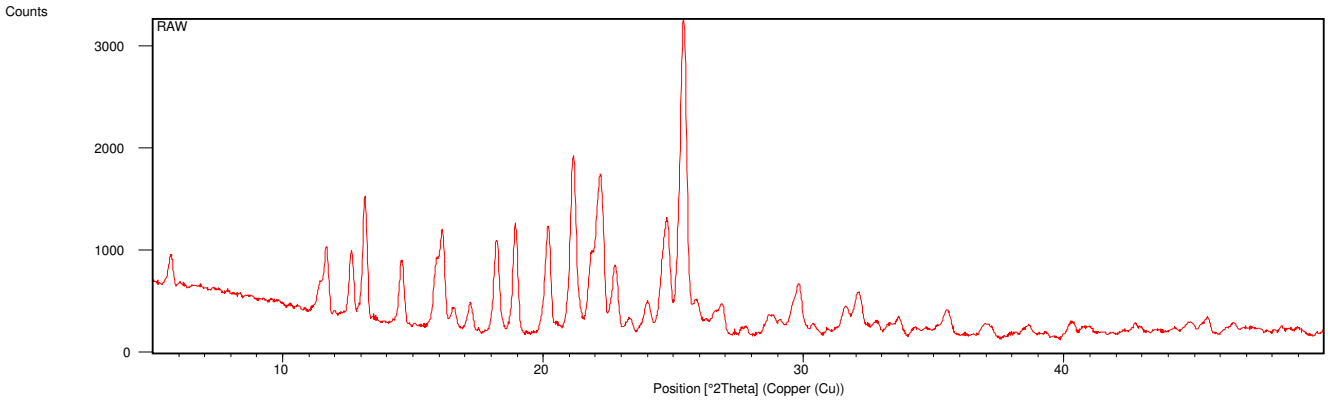


(C)

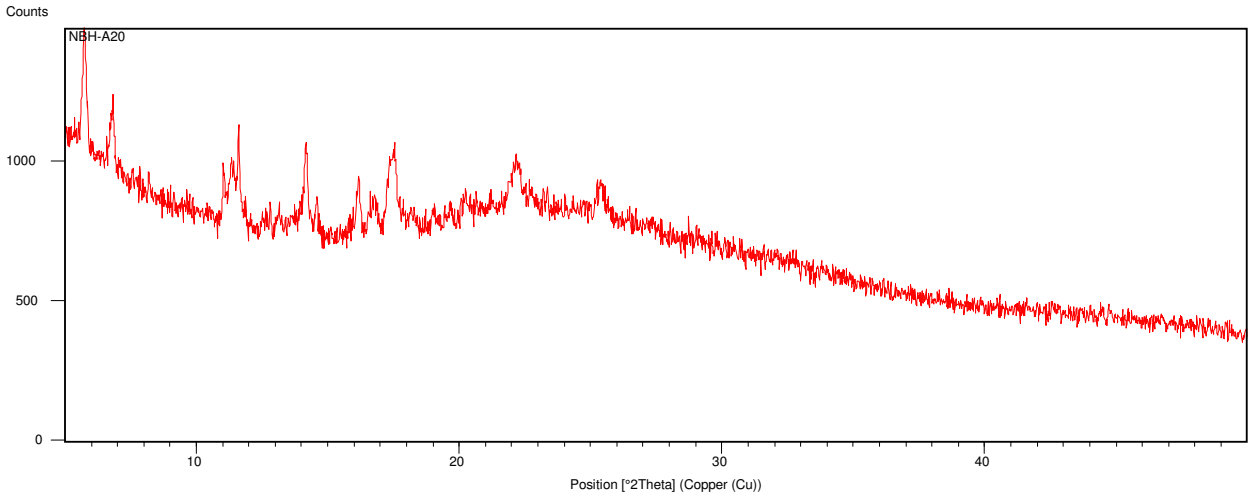
Fig.3: FTIR spectra of pure drug (A), NBH A-20 (B) and (C) mixture of nebivolol Hcl with MDF Excipients.

XRD analysis

The XRD patterns of the pure NBH and final nanosuspension formulation were as shown in Fig.4 (A) and (B).The XRD of pure NBH exhibited peaks at 2θ of 13.2°, 22.0°, 23.6°and intense peak at 25.6° indicative of its crystalline nature. Whereas the profile for final formulation dried powder was almost flat with no intense peaks but have few shorter peaks indicating an essentially amorphous nature.



(A)



(B)

Fig.4: XRD pattern of (A) pure drug and (B) NBH A-20.

Saturation or kinetic solubility: Different formulations were made and further parameters varied to enhance the saturation solubility of the drug.Table 1 shows the results of the different formulations and also of optimized formulations.

In-vitro **dissolution:** Samples equivalent to 5 mg of were raw NBH and other formulation placed in the dissolution vessel containing 900 ml of 0.1 N Hcl solution maintained at 37±0.5°C and stirred at 50 rpm.The samples aliquoted at predetermined interval and these samples were filtered with 0.45 µm filter, which was retained in the dissolution

medium throughout the dissolution studies. Samples were collected periodically with the help of pipette and replaced with a fresh dissolution medium. Concentration was determined spectrophotometrically at 281 nm. Fig.6 shows the drug release of selected formulation.

Stability studies

This is done on the basis of particle size and drug release after equal interval of 45 days. The sample were wrapped in aluminium foil paper further placed in closed tight container and then are kept at room temperature for 3 months. Results shows there was very slight change hence almost no effect on the formulation due to environmental factor. Fig.6 shows the stability results.

Evaluation of mouth dissolving film

FTIR Spectroscopy: State of finally selected dried nanosuspension with hydrophilic polymer (HPMC E-50) was determined using FT-IR. Fig.3 show FT-IR spectra of drug, HPMC E-50 and other excipients. FT-IR spectra of pure was not changed in its combined mixture with excipients.

Visual inspection

All the prepared fast dissolving film showed homogenous and smooth surfaces properties but the films prepared by HPMCE-50 are transparent and colorless while those prepared by using sodium alginate and kollidon combination of are white in color. Other films were not properly formed.

Evaluation results of NF1 formulation

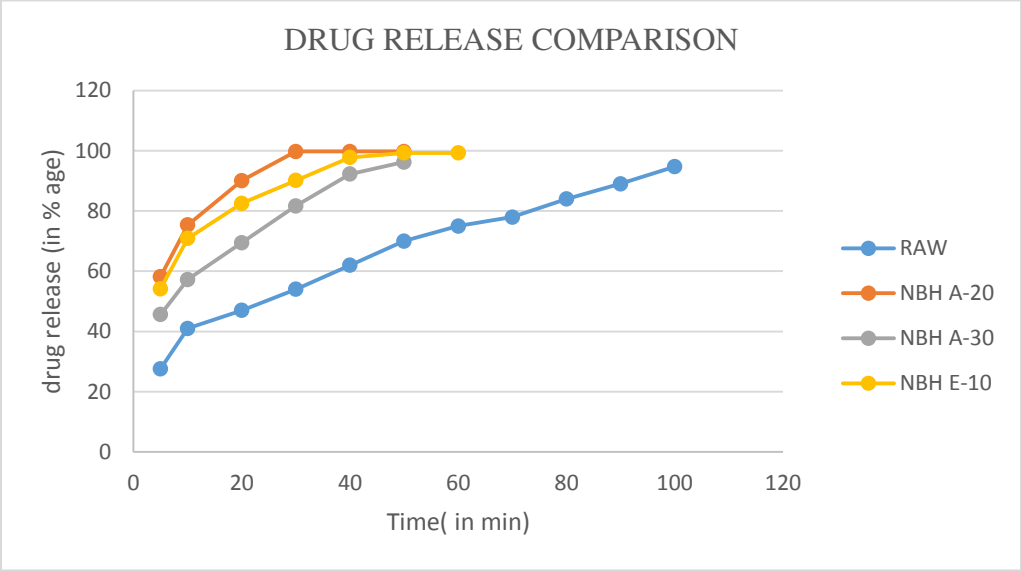
- **Thickness (mm):** 0.09±0.02
- **Folding endurance:** 100 times
- **Surface pH:** 7.1±0.2
- **Disintegration time (sec):** 40±2
- **Drug content (%):** 94

Dissolution studies

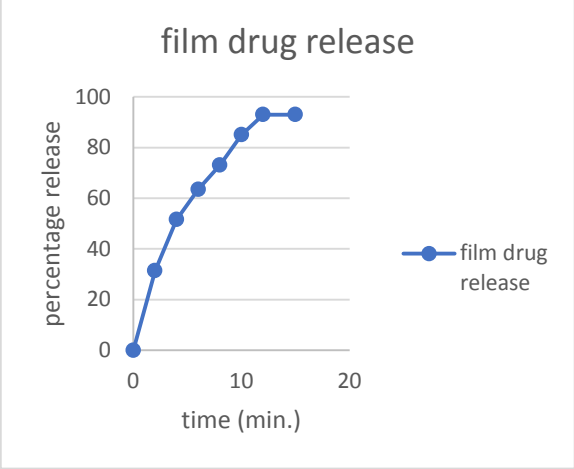
Finally NF1 film formulation selected for drug release.Samples taken at predetermined specific interval. This film gives more than 93% drug release within 12 minutes. Fig.5 (B) shows the drug release at specific time intervals.

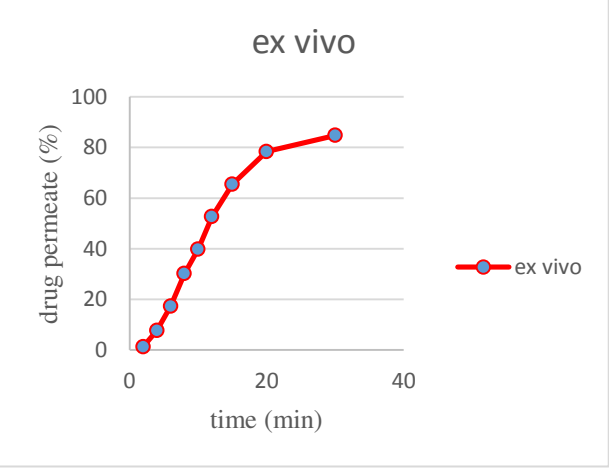
Ex-vivo drug release

More than 84% of the drug permeate from the porcine buccal mucosa within half an hour.Fig.5 (C) shows the results of drug permeation.



(A)





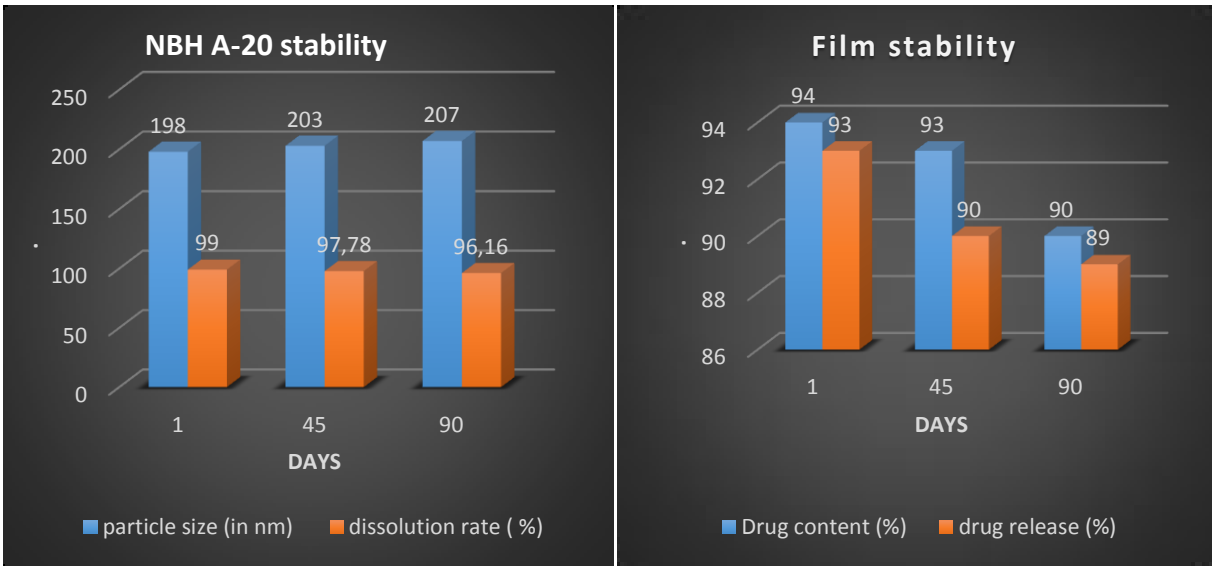
(B) **(C)**

Fig.5: (A) Drug release from mouth dissolving film and (B) Drug permeation through porcine buccal mucosa.

Stability studies

This was done on the basis of physical appearance, drug content and drug release after equal interval of 45 days. The sample was wrapped in aluminium foil paper and are kept at room temperature and at freezing conditions temperature range 2-8°C for 3 months. Results shows there was very slight change in appearance and almost no effect on the drug

release film kept at freezing conditions but film kept at room temperature become sticky in nature, means storing condition for this is freezing condition. Fig. shows the stability results.

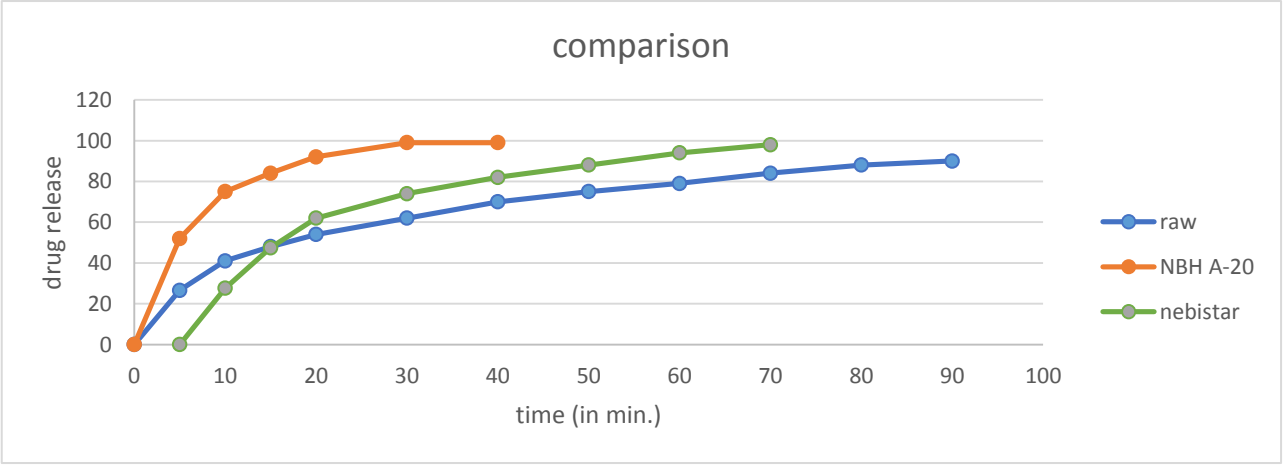


(A) **(B)**

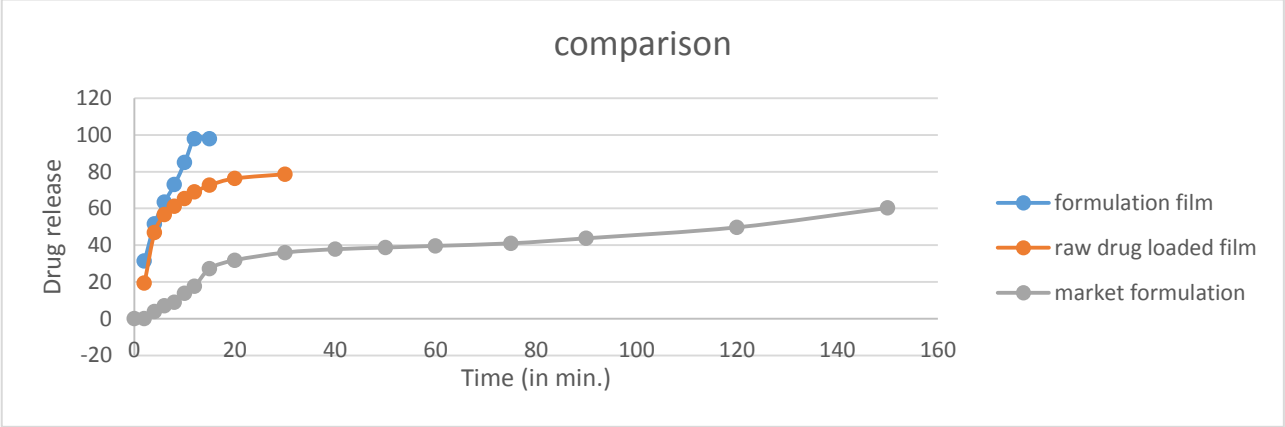
Fig.6: (A) Stability comparison of NBH A-20 formulation and(B) Stability comparison of NF1 film after regular intervals

***In-vitro* comparison with Marketed product.**

Results of the release profile showed that the marketed product had taken approximately 1.5 hour and 6 hours dissolution time in 0.1 N Hcl and phosphate buffer (pH-6.8) respectively, whereas optimized batch NBH A-20 had taken just 30 min. and NBH A-20 loaded film took 12 min for complete dissolution in 0.1 N HCL and phosphate buffer (pH-6.8) respectively.Here,nanosuspension of Nebivolol hydrochloride was more efficient dosage form than marketed product Nebistar*shown in fig.7.



(A)



(B)

Fig.7: (A) Comparison between raw drug, NBH A-20 formulation and market preparation and (B) Comparison between nanosuspensions loaded film with market preparation and pure drug loaded film.

Conclusion

This studydemonstrated that the nanoprecipitation technique is able to prepare NBH nanosuspension with significantly higher percent dissolution in water. The method developed is very cost effective, easy to operate and can be easily scaled for industrial production of drug nanoparticles. . The formulations were optimized by varying parameters like solvent – antisolvent ratio, drug concentration, temperature etc.Nanosuspension mean particle size is 198 nm.The XRD analysis suggested that NBH changes to amorphous from crystalline nature when it was turned to nanosuspension form. Further, nanosuspension formed transferred to MDF by applying casting method. The result showed the optimized NBH A-20 containing filmwas disintegrated in water within 40seconds. The optimized NBH A-20 containingfilm exhibits much faster drug release rates compared to NBH purematerial film and marketed product. Therefore, this novel NBH A-20containing film may provide a potential opportunity for oral delivery of drugs with poor water solubility. Nanosuspension produced by nanoprecipitation method would have high potential for delivery in much smaller doses compared with commercial preparation containing the normal form of the drug.

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Conflicts of interests

All authors have none to declare.

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