Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2013, 5 (6):151-163
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Enhancement of bioavailability of nebivolol hydrochloride through liquisolid formulations: *In Vitro* and *In Vivo* evaluation

Pravala K, *Vijay Kumar Nagabandi, Ajmeera Divya

St. Peter's Institute of Pharmaceutical Sciences, Hanmakonda, Andhra Pradesh, India

---

ABSTRACT

Nebivolol hydrochloride {α, α'-[iminobis (methylene) bis [6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-methanol] hydrochloride} is a selective β_1- antagonist falls under class II biopharmaceutical classification system. In this present study liquisolid technique was applied to improve the solubility and dissolution properties thereby enhancing the oral bioavailability of nebivolol hydrochloride. Different formulations were developed by dissolving the drug in various non-volatile liquid vehicles like PEG 400, Propylene glycol, Tween 80 and Glycerine using Avicel PH 102, Lactose Monohydrate and Syloid 244 FP as carriers and Aerosil as coating material. In vitro drug release profiles of liquisolid compacts shown enhanced drug release when compared to pure drug as well as marketed formulation and the formulation containing PEG 400 (25%w/w) as solvent with syloid 244 FP and Aerosil (30:1) shown maximum dissolution rate compared to other formulations. The in vivo studies indicated that the enhanced bioavailability in case of liqisolid formulations which might be due to increased wetting properties and more surface of drug available for dissolution. XRD and FTIR studies conformed that there was no interaction between drug and excipients used. From this study we can conclude that liquisolid technique is one of the promising alternative techniques to improve the dissolution rate of class II drugs.

Key words: Nebivolol hydrochloride, liquisolid compacts, PEG 400, Syloid 244 FP, Avicel PH 102.

INTRODUCTION

Solubility is an important parameter for absorption of drugs especially for those which are water insoluble or poorly soluble. Dissolution of such drugs limits their absorption through oral route. Till to date oral route is a major route of administration for majority of drugs. Due to poor solubility and limited dissolution rate, class II drugs suffer less bioavailability thereby decreased therapeutic effect. Several techniques have been reported to improve the solubility and dissolution properties of poorly soluble drugs which intern can improve absorption and bioavailability [1-2].

Nebivolol hydrochloride is a class II drug that selectively blocks β1 receptor with therapeutic applications as antihypertensive and can also used as monotherapy for initial management of uncomplicated hypertension. In this study Nebivolol Hydrochloride was taken as a model drug and a novel dissolution enhancement technique, Liquisolid compaction was adopted to improve its oral bioavailability. Liquisolid medications are those in which liquid lipophilic drugs, drug suspensions or solutions of water insoluble drugs in suitable non- volatile solvent systems were formulated into dry, non volatile adherent, free flowing and readily compressible powder admixture by blending with selective carrier and coating materials [3].

This technique involves the conversion of water insoluble drugs into immediate release forms by simple blending with suitable carriers and coating materials [4]. This method is simple and requires very less time as it does not involve any drying or evaporation steps. After administration of liquisolid compacts, the active drug present in solid dosage form undergoes dissolution before absorption takes place in the gastrointestinal tract. The solubility is

enhanced by means of increased aqueous solubility of the drug, improved wetting properties and increased drug surface area [5].

MATERIALS AND METHODS

Materials

Nebivolol hydrochloride(α,α' – [iminobis(methylene)]bis[6-fluoro- 3,4-dihydro – 2H-1-benzopyran-2-methanol]hydrochloride was kindly provided by AET Labs, Hyderabad. Lactose monohydrate, sodium starch glycolat and colloidal silicon dioxide were provided by Finar Chem. Ltd. PEG400 and Propylene Glycol were provided by Sd fine chemicals Avicel PH 102and Syloid 244FP were purchased from Qualikems fine chem.Pvt Ltd and Grace Divis, India respectively.

Methods

1. Saturation solubility studies

Solubility of Nebivolol hydrochloride in different non-volatile solvents was estimated by conducting saturation solubility studies. Excess amount of drug was added to 10 ml of solvent in vial and subjected to continuous shaking using a Rotary shaker for 72h at 25^0 c. Then each solution was filtered and the filtrates were analyzed for drug content and solubility using UV- spectrophotometer at 281nm [6].

2. **Micromeritic properties of pure drug**[7]

2.1. Bulk density

The drug powder was weighed and transferred to a measuring cylinder and the volume occupied by the powder was noted. The bulk density was calculated using the formula

$$B.D = \frac{Powder\ mass}{Bulk\ volume}$$

2.2**. Tapped density**

The drug was weighed and transferred to a measuring cylinder. Then it was tapped on a Bulk density-Tapped density apparatus for 500 taps and the final volume was noted. The tapped density of the drug powder was calculated usin the formula

$$T.D = \frac{Powder\ mass}{Tapped\ volume}$$

2.3. Carr's index

The carr's index was calculated using the formula

$$C.I = \frac{T.D - B.D}{T.D} \times 100$$

2.4. Hausner's ratio

Bulk density and Tapped density were measured and Hausner's ratio was calculated using the formula

$$H.R = \frac{T.D}{B.D}$$

2.5. Angle of repose

The angle of repose of the pure drug powder was determined using fixed funnel method. The powder was allowed to flow through the funnel fixed to a stand at a definite height (h) and the radius(r) of the heap formed by the powder was measured. The angle of repose was calculated using the formula

$$tan\,\emptyset = h/r$$

3. Determination of liquid loading factor (Lf)

The concept extracted from Ajit S. Kulkarni et al., 2010 was utilised to caliculate Lf. This gives the amount of carrier required. Lf was calculated for 3 different carrier materials in the selected solvent using formula

$$Lf = \frac{W}{Q}$$

Where, '*Lf*' is the liquid loading facter, '*W*' is the weight of the liquid medicament and '*Q*' is the weight of carrier material [4]

4. Preparation of liquisolid compacts

Nebivolol hydrochloride liquisolid compacts were prepared by using 3 different carriers (Lactose, Microcrystalline cellulose and Syloid 244FP in three different ratios(10,20and 30%w/w) and the drug concentrations were 25 and 50%w/w in PEG 400. Total number formulations prepared were 18 and the composition for each formulation was given in table-1. Accurately weighed quantities of drug and PEG400 were transferred in to a glass beaker and mixed well. The resulted liquid medicament was incorporated into calculated quantities of carrier and coating material by blending in a mortar. The liquid/powder admixture was then evaluated for flow properties, drug content and dissolution. Based on the results obtained an optimum formulation was selected and compressed into tablets using 6mm single punch tablet compression machine. Before the compression step, 5% sodium starch glycolate was added to the blend as disintegrating agent [8, 9].

5. Pre-compression Evaluation studies for Nebivolol hydrochloride liquisolid formulations

All the formulations were estimated for the flow properties, drug content and dissolusion based on standard procedures.

5.1. Flow properties

Bulk density, Tapped density, Carr's index, Hausner's ratio and Angle of repose for all the formulations were estimated and calculated as per the procedures followed for the pure drug.

5.2. Drug content

Each formulation was taken in dose equivalent amounts of Nebivolol hydrochloride and dissolved in methanol. The solutions were filtered and filtrates were analyzed for drug content using UV-spectrophotometer at 281nm.

5.3. Dissolution

Dissolution studies of all the formulations were done using USP Dissolution apparatus II and the dissolution medium used was 500ml of 7.4pH phosphate buffer. The temperature and speed of rotation maintained were 37 ± 0.5^0c and 50rpm respectively. 5ml samples were collected and the equal volumes of fresh dissolution medium were replaced at predetermined time points and analyzed using UV-spectrophotometer at 281nm.

6. Post compression evaluation studies for optimised formulation

Based on the results obtained from precompression evaluation tests, optimum formulation was selected and subjected to evaluation other required parameters [10-12].

6.1. Hardness

Hardness of the prepared liquisolid compacts was measured using Monsanto hardness tester

6.2. Thickness

Thickness of prepared liquisolid compacts was measured using Digital micrometer

6.3. Weight variation

20 liquisolid compacts were randomly selected and individual weight was measured using electronic weighing balance and the total weight there by average weight was calculated. The % weight variation was calculated using the formula

$$\%\ weight\ variation = \frac{individual\ weight - average\ weight}{average\ weight} \times 100$$

6.4. Friability

Six liquisolid compacts were randomly selected, weighed and friabilated for 100 revolutions using Roche friabilator for 4min at 25 rpm. The % friability was calculated using the formula

$$\%\ Friability = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100$$

6.5. Assay

Six liquisolid compacts were randomly selected and grinded to powder. The powder equivalent to dose of Nebivolol hydrochloride was dissolved in methanol and filtered. The filtrate was analyzed for drug concentrations using UV-spectrophotometer at 281nm.

6.6. Disintegration time
Six liquisolid compacts were randomly selected and placed in 6 baskets of USP disintegration apparatus and run the apparatus for 10 min. Then each liquisolid compact was checked for complete disintegration.

6.7. *In vitro* drug release
The release of Nebivolol hydrochloride from liuisolid compacts was studied using USP Dissolution apparatus II and the dissolution medium used was 500 ml of 7.4pH phosphate buffer. The temperature and speed of rotation maintained was 37±0.5^{0}c and 50 rpm respectively. 5ml samples were collected and the equal volumes of fresh dissolution medium were replaced at predetermined time points and analyzed using UV-spectrophotometer at 281nm [13].

6.8. *In vivo* drug release
Animals were fed with standard diet throughout the study as there was no impact of diet on drug absorption. 18 rats weighing 210-260g were divided in to 3 groups of six in each. Group 1 was administered with pure drug, group2 was administered with marketed formulation (nebistar 5mg) and the group 3 was administered with optimum formulation at 2mg/kg dose through oral route. Blood samples were collected from retro orbital vein at predetermined time points upto 8h and were centrifuged at 5000 rpm for 5 min. Plasma was collected and stored at -20 ^{0}c until analyzed. The plasma samples were analyzed using RP-HPLC and plasma drug concentrations were determined using standard calibration curve. All possible pharmacokinetic parameters ($AUC_{(0\text{-}t)}$, C_{max}, t_{max}, $t_{1/2}$) were calculated for each formulation and each subject [14].

6.8.1. Analysis of variance
A two way ANOVA was applied (at 99% confidence interval and 0.01level of significance) to the AUC values to determine whether there is any significant difference in the bioavailability between formulations or between the subjects. If the obtained F value is lesser than the table value at concerned degree of freedom, there is no significance difference and vice versa.

7. X - Ray Powder Diffraction (XRD) studies
X-Ray powder diffraction studies were conducted for pure drug, Lactose, Avicel pH 102, Syloid 244FP, physical mixture and optimised formulation using Phillips PW 3719, Netherlans. All the samples were exposed to Cu- Kα radiation at a scan rate of 2^o/ min over the 2Ø range of 3-4 ^{o}C.

8. Fourier Transform Infrared Spectroscopy (FTIR)
FTIR studies were performed on pure drug, excipients and the optimised formulation using V5300 FT- IR (Tokyo, Japan). Samples were analysed between wave numbers 4000 and 400 cm^{-1}.

RESULTS AND DISCUSSION

9.1. Saturation solubility studies
Saturation solubility studies of nebivolol hydrochloride in different solvents were performed and the results obtained were represented as a bar graph in fig 1. Among all the solvents tested, nebivolol hydrochloride was found to be more soluble in PEG 400.

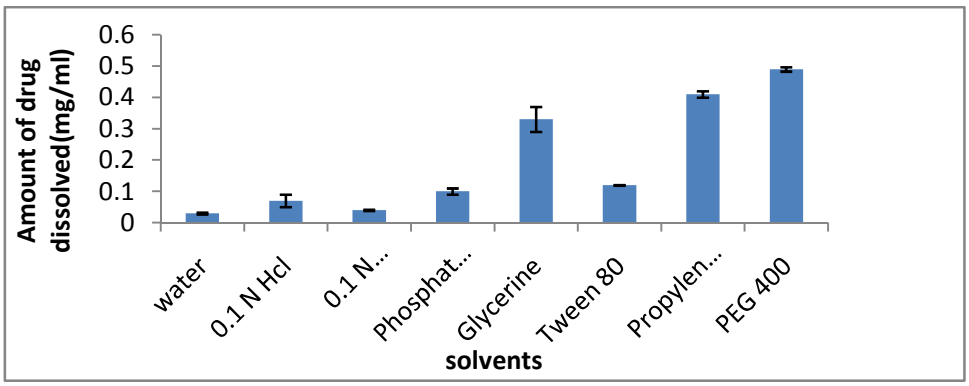

Fig 1. Saturation solubility studies of nebivolol hydrochloride in different solvents

9.2. Micromeritic properties of pure drug

Nebivolol hydrochloride was shown poor flow with an Angle of repose >45^{o}, Carr's index of >25% and Hausner's ratio of >1.25.

9.3. Determination of liquid loading factor (L_f)

From the saturation solubility studies PEG 400 was selected as solvent of choice and Lf for 3 different carrier materials was calculated. The values obtained were given in the table 1.

Table1.Results of Liquid loading factor for different carrier materials

Carrier material	Percentage drug dissolved in PEG 400(%)	L_f
Lactose	50	0.41
	25	0.47
Micro crystalline cellulose	50	0.52
	25	0.58
Syloid 244 FP	50	0.60
	25	0.64

9.4. Pre compression studies

Flow properties of all the formulations were evaluated and the results are shown in table 2. The values of Angle of repose (31.2± 0.2 - 39.2±0.3), Carr's Index 13.1±0.2 - 18.4±0.4), and Hausner's ratio (>1.25) in all the formulations corresponding to good flow. Flow ability of nebivolol hydrochloride liquisolid formulations was enhanced compared with pure drug.

Table2. Pre compression evaluation of Nebivolol hydrochloride liquisolid formulations

Formulation	Angle of repose(Ø)	Carr's index (%)	Hausner's ratio	Drug content (%)
F1	34.2±0.3	15.6±0.2	1.18	98.4±0.5
F2	35.3±0.5	15.3±0.3	1.18	97.5±0.2
F3	35.5±0.3	15.8±0.4	1.18	98.8±0.1
F4	31.80±0.6	15.12±0.2	1.18	97.2±0.3
F5	35.2±0.2	16.2±0.22	1.17	98.5±0.2
F6	35.0±0.8	16.1±0.3	1.19	98.2±0.4
F7	36±0.8	18.4±0.8	1.19	96.8±0.2
F9	34.4±0.4	17.7±0.01	1.22	99.2±0.5
F11	36.9±0.3	18±0.61	1.21	97.6±0.4
F13	31.2±0.2	13.1±0.2	1.21	97.2±0.3
F15	33.4±0.2	15.1±0.4	1.17	98.2±0.4
F17	31.8±0.3	14.5±0.3	1.17	97.2±0.3
F18	32.23±0.1	13.2±0.6	1.15	99.8±0.3

Data represents mean ±S.D (n=3)

Table3. *In vitro* release data of nebivolol hydrochloride liquisolid formulations

Formulation	% drug release (15min)	% drug release (30mins)	% drug release (45min)	% drug release (60mins)	% drug release (90mins)
F1	28.64±0.4	34.15±3.6	36.92±4.3	39.51±1.09	44.87±0.98
F2	47.64±2.5	50.07±3.2	52.79±6.92	54.46±10.2	56.2±9.95
F3	50.97±1.1	53.33±0.5	55.6±1.3	58.4±0.5	59.51±0.6
F4	30.43	33.53±0.3	37.69±0.27	40.12±0.46	49.61±0.48
F5	50.9±1.1	52.04±0.53	54.64±0.19	55.74±1.27	65.4±0.65
F6	49.89±3.3	56.46±0.8	59.6±1.51	64.71±2.8	69.53±0.65
F7	18.2±0.86	26.48±0.8	30.92±0.39	31.17±0.5	32.51±0.5
F8	39.1±1.3	50.04±2.1	50.9±0.76	51.98±1.23	53.23±3.1
F9	42.30±0.51	46.30±2.0	52.21±3.4	52.43±2.1	55.12±1.8
F10	25.12±5.4	41.84±4.5	53.82±0.3	55.66±0.69	58.76±0.48
F11	40.51±2.4	49.04±2.1	52.15±4.0	56.20±6.8	68.64±1.0
F12	54.5±0.9	57.2±3.33	62.07±2.3	66.97±1.21	74.23±0.62
F13	37.38±0.70	57.28±4.5	62.07±0.3	66.97±0.69	55.74±0.9
F14	38.23±3.04	49.02±2.8	52.15±4	56.20±6.8	58.41±0.6
F15	46.61±1.90	41.84±3.3	53.82±2.3	55.66±1.21	68.53±2.7
F16	34.89±0.6	48.90±0.26	50.09±0.98	51.97±0.76	54.43±0.43
F17	48.58±1.6	60.66±0.23	62.23±1.77	64.15±1.2	71.16±1.3
F18	101.02±3.9	101.28±0.44	101.56±0.5	101.79±0.04	101.8±0.4

9.4.1. Drug content

Drug content was estimated for all the formulations of nebivolol hydrochloride by recording the absorbance at 281 nm by using UV-Visible spectrophotometer and the results were given in table no. It was found that all the

formulations were passed for drug content as the results obtained within the acceptable limits and are given in table2.

***9.4.2. In vitro* release studies**

Dissolution studies were conducted for the formulations F1 to F18. The formulations containing 75% liquid vehicle (F6, F12 and F18) shown high dissolution rate and among these three formulations F18 showing highest dissolution rate within a less time period (<15min). This could be achieved because of high surface area provided by Syloid 244FP. Hence F18was selected as optimized formulation and compressed into tablets using a 6mm single punch compression machine after adding 5% sodium starch glycolate as disintegrating agent. These compacts were further evaluated. The dissolution profile was given in table3.

10. Post compression evaluation of optimised formulation F18 as tablet

The mean hardness of optimized formulation was determined and proved that it had acceptable hardness. Nebivolol hydrochloride liquisolid tablets and acceptable friability as none of the tablet had percentage loss in tablets weights that exceed 1% also, no tablet was cracked, split or broken. Results of post compression evaluation parameters of optimized formulation were shown in table4.

Table4. Results of post compression evaluation parameters

parameter	Hardness (kg/cm^2)	Weight variation	Friability (%)	Assay (%)	DT (sec)	%DR (15min)	%DR (30min)
Result	4.4±0.2	342.5±1.5	0.5±0.07	98.2±0.5	180±5	100.25±3.5	101.29±1.2

Data represents mean ±SD (n=3), DT= Disintegration time, DR= Drug release

10.1. Comparison of optimized formulation with pure drug and marketed product

Comparative *in vitro* drug release studies were performed to pure drug, marketed product and optimised formulation. F18 as a tablet was showing highest dissolution rate (100.2%) when compared with marketed product (31.235) within 15 min. *In vitro* drug release data was given in table5 and dissolution profiles were given Fig 2.

Table5. *In vitro* drug release data of pure drug, marketed drug and optimised formulation

Formulations	**% drug release (15min)**	**% drug release (30min)**	**% drug release (45min)**	**% drug release (60min)**	**% drug release (90min)**
pure drug	15.64±0.29	18.5±0.23	23.60.3	26.280.23	37.70.1
marketed product	31.23±0.2	33.87±0.08	34.0.07	35.20.1	39.40.3
optimized formulation(F18)	100.25±1.0	101.1±0.4	101.8±0.2	102.08±0.3	102.4±0.7

Data represents mean ±SD (n=3)

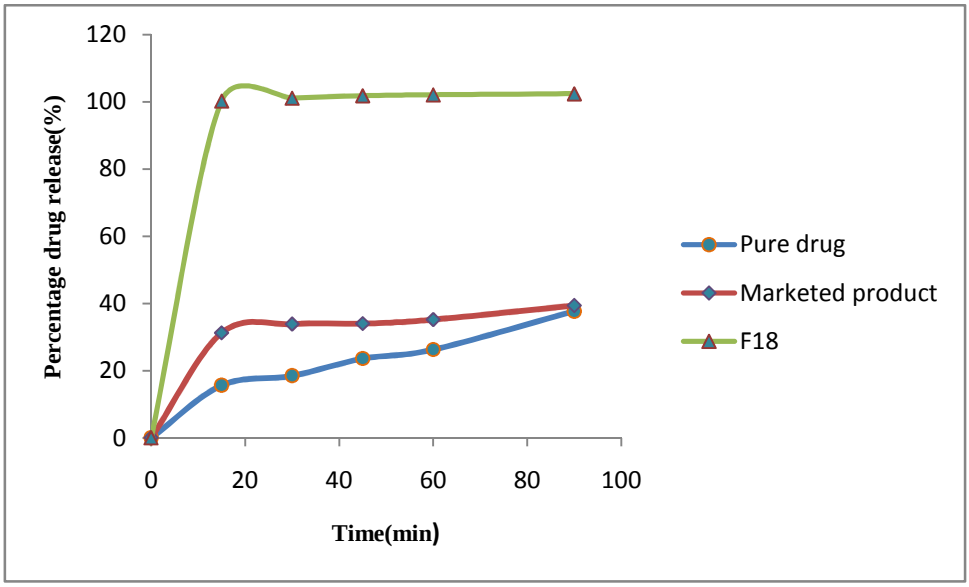

Fig2. Comparative Dissolution profiles of Pure drug, Marketed product and optimized formulation

10.2. *In vivo* studies

In vivo studies revealed that the bioavailability of optimized formulation is high compared to pure drug and marketed product. The plasma concentration data was given in table6. The pharmacokinetic parameters were calculated and listed in table6.1

Table6. Plasma Conc-time data of pure drug, marketed product and optimised formulation F18

Time(h)	Plasma conc of pure drug (µg/ml)	Plasma conc of Marketed product(µg/ml)	Plasma conc of Optimised formulation(µg/ml)
0.5	5.67±0.4	5.85±1.7	6.64±3.0
1	8.32±2.1	8.55±0.91	9.1±1.2
2	12.11±3.72	15.63±1.3	19.93±2.5
4	7.72±1.43	7.9±2.73	8.2±1.2
8	2.65±1.4	2.17±0.5	3.27±0.8

Table6.1. Pharmacokinetic parameters of pure drug, marketed product and optimised formulation(F18)

Pharmacokinetic parameter	Pure drug	Marketed product	Optimised formulation (F18)
AUC(mcg.hr/ml)	35.05	38	46.1
Cmax	12.11	15.63	19.93
Tmax	2	2	2

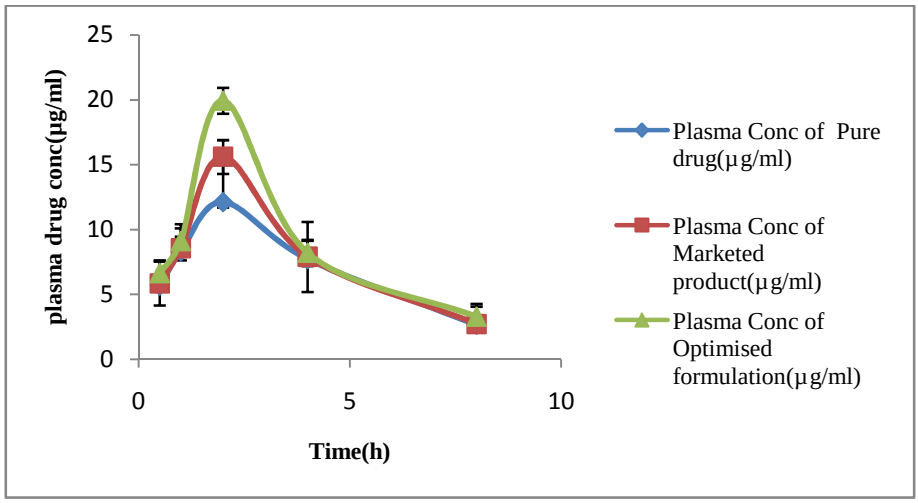

Fig3. Plasma drug conc-time profile of nebivolol hydrochloride liquisolid compact, pure drug and marketed product

11. X-Ray Diffraction studies

X Ray Diffraction (XRD) is a technique to recognise different polymorphic forms of a compound and as well to identify the solvated and unsolvated form of a compound. X-Ray diffraction studies in fig 4(a) showed sharp, distinct peaks at 5.9º, 11.9º, 12.2º, 16.3º, 18.4º, 21.4º, 22.4º, and25.67º confirms that the pure drug is in the crystalline state. Whereas the fig4(g) showing absence of nebivolol hydrochloride constructive peaks indicating conversion of nebivolol hydrochloride from crystalline to amorphous state in optimized formulation mainly because of solubilisation in the liquid vehicle which is further absorbed in to the carrier, Syloid244FP and adsorbed onto the coating material, Aerosil.

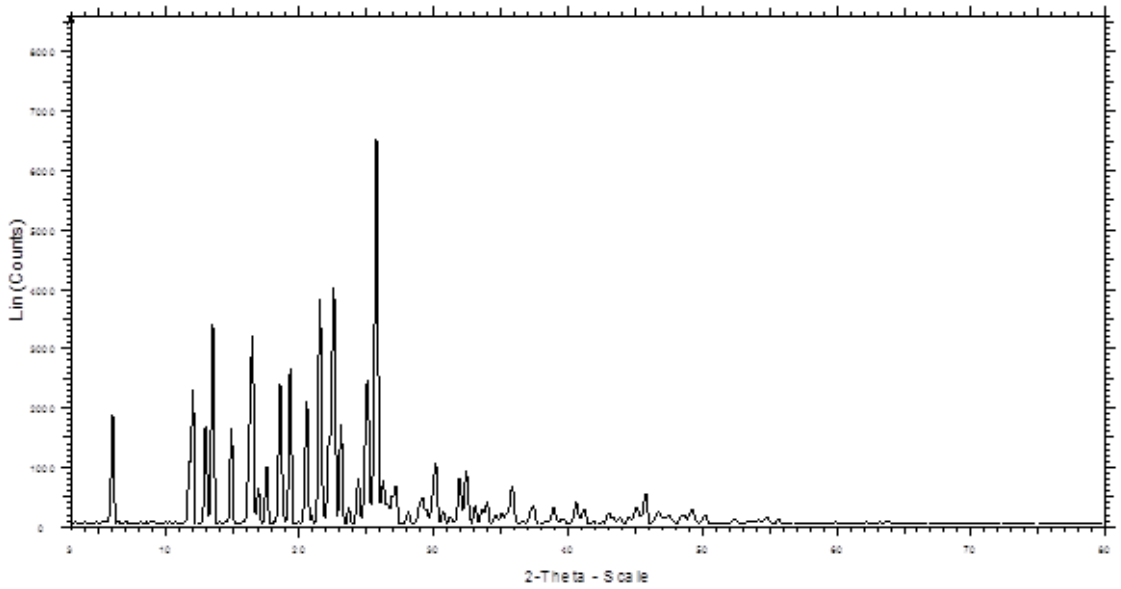

Fig4(a). x-Ray diffraction of nebivolol hydrochloride

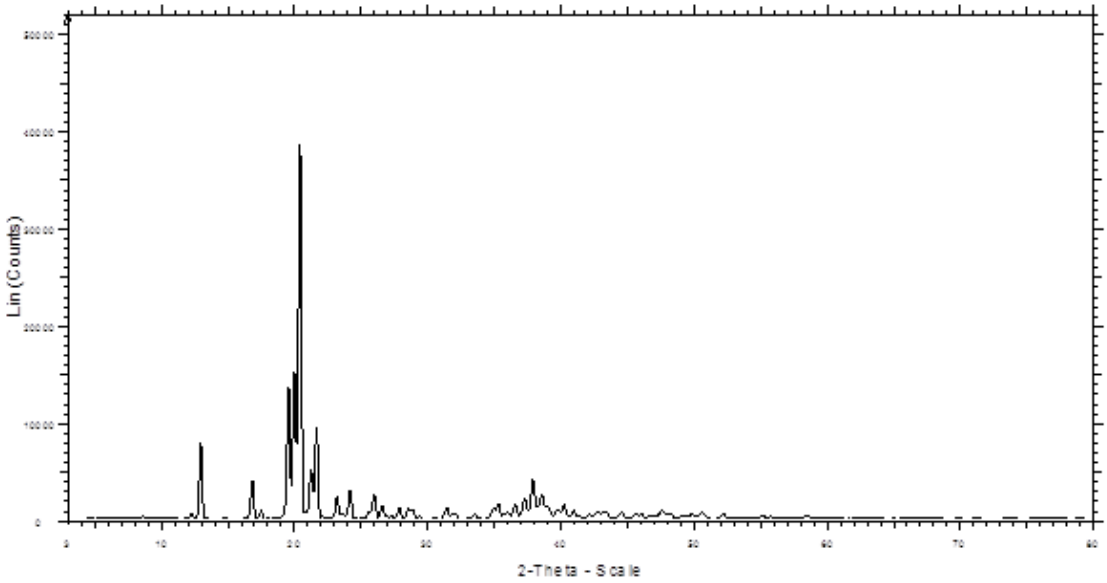

Fig4 (b). X-Ray difratction of Lactose

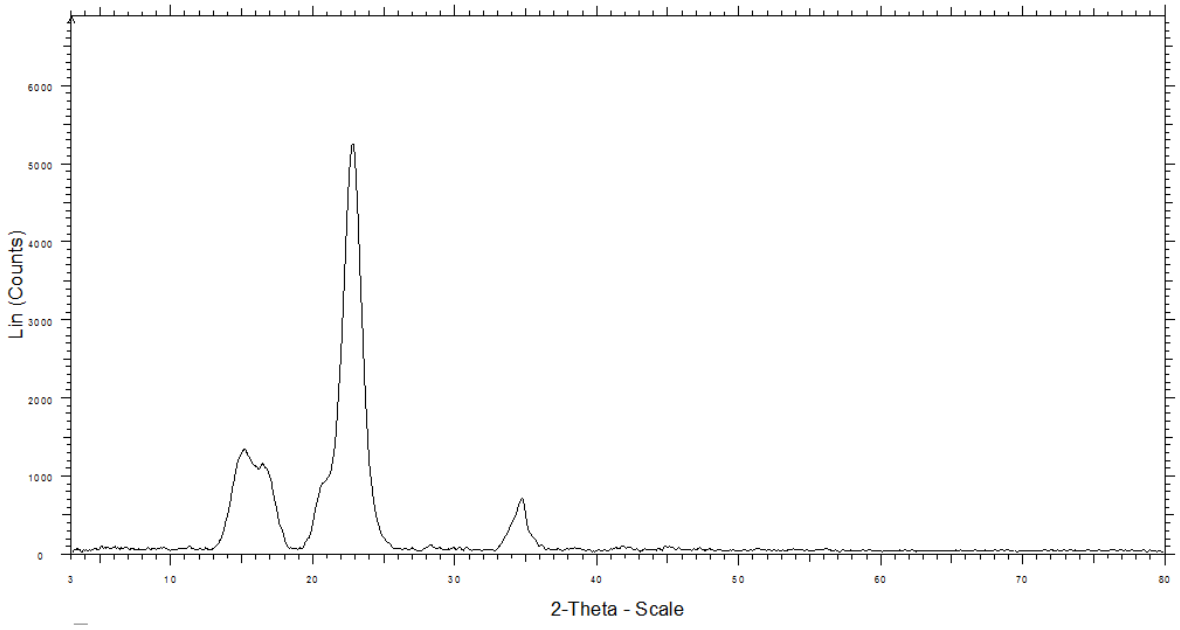

Fig4(c). X-Ray diffraction of Avicel pH 102

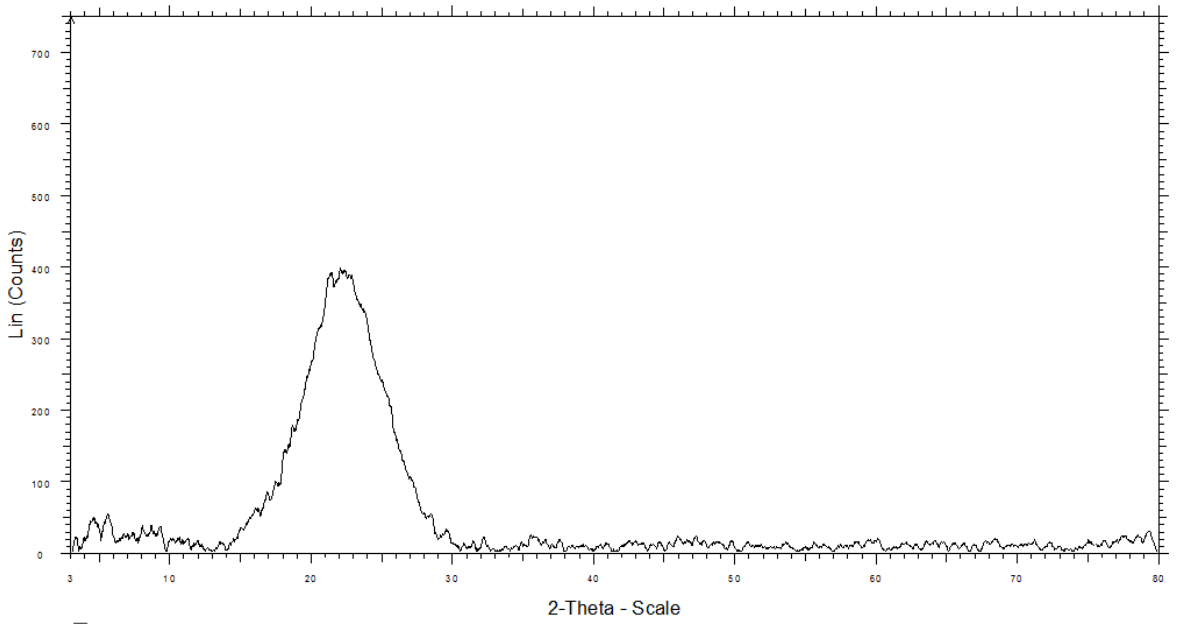

Fig4 (d). X-ray diffraction of Aerosil

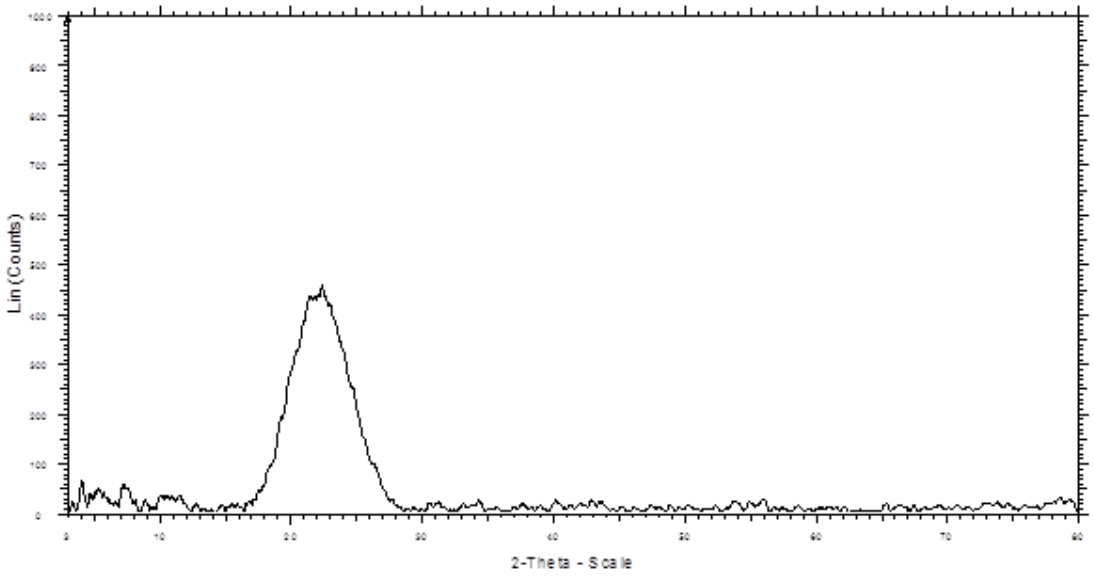

Fig4 (e). X-Ray diffraction of Syloid 244FP

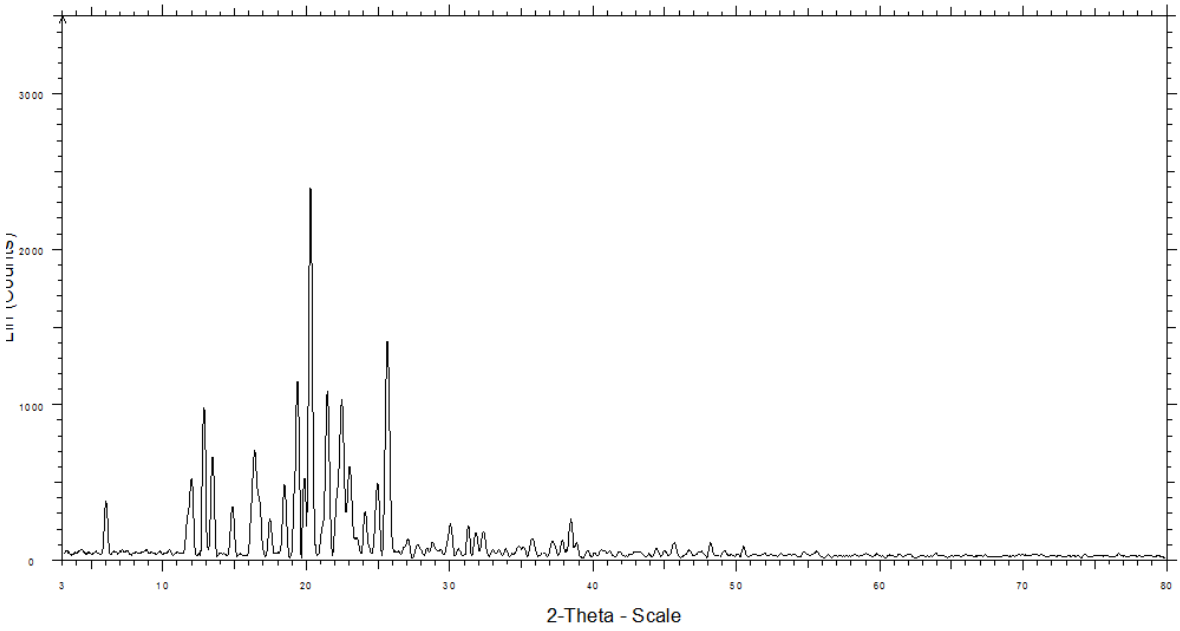

Fig4 (f). X-ray diffraction of physical mixture

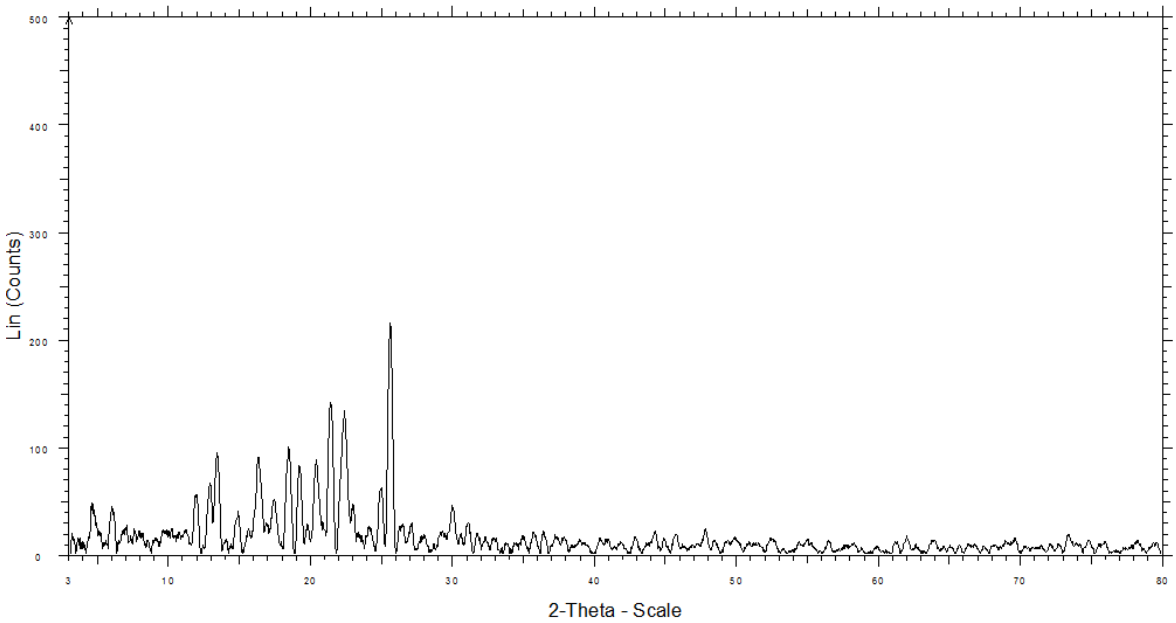

Fig4 (g). X-Ray diffraction of optimised formulation (F18)

12. Fourier Transform Infrared Spectroscopy (FTIR)

Chemical interaction between drug and the polymeric material was studied by using FTIR. FTIR results of nebivolol hydrochloride and optimised formulation was shown in fig 5(a) and 5(b) respectively. Functional group stretching of nebivolol hydrochloride and optimised formulation was shown in table 7. There is no difference between the IR patterns of nebivolol hydrochloride as pure drug and in optimized formulation.

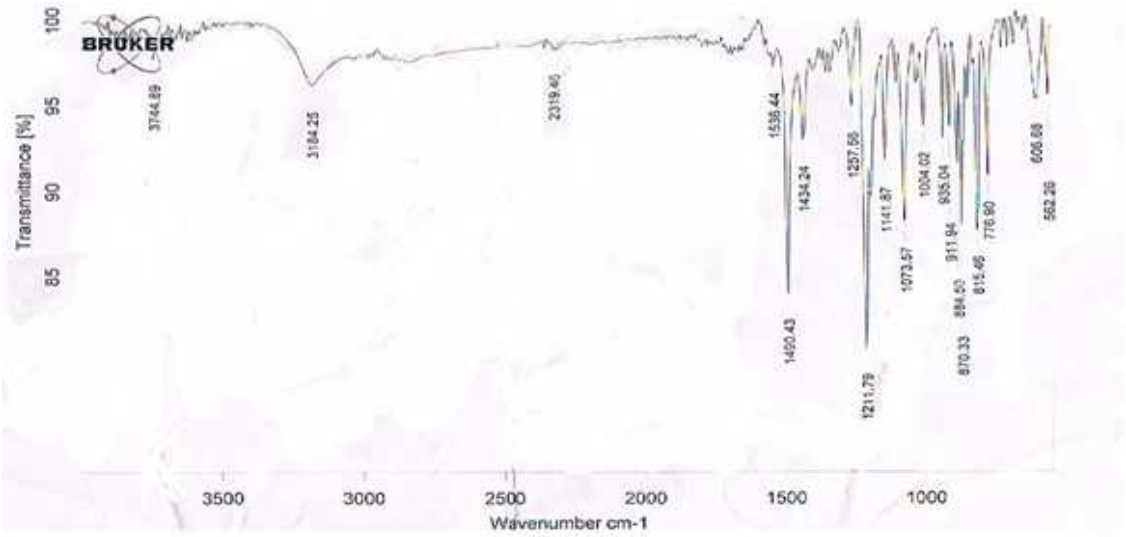

Fig5(a). FTIR of nebivolol hydrochloride

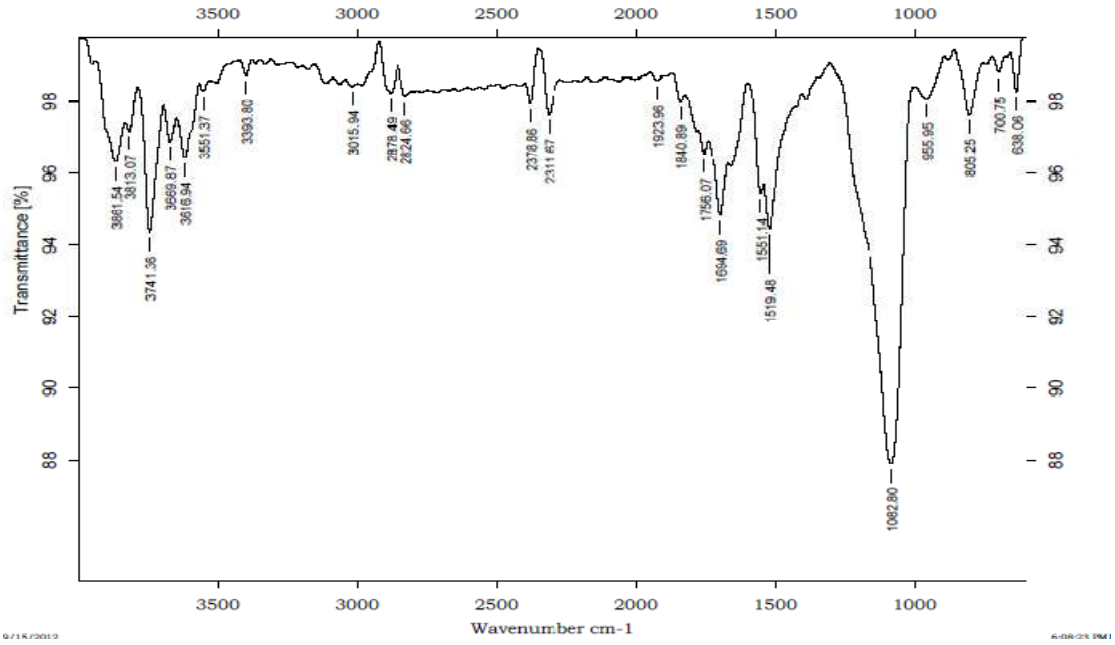

Fig5(b). FTIR of optimised formulation (F18)

Table7. Functional group stretching of nebivolol hydrochloride and optimised formulation

Functional group stretching	Nebivolol hydrochloride	Optimised formulation (F18)	Inference
N-H	3184.25cm^{-1}	3015.94cm^{-1}	No change in wave length
C=C	2319.46cm^{-1}	2378cm^{-1}	No change in wave length
C=O	1536.44cm^{-1}	1551.15cm^{-1}	No change in wave length
C-H	1490cm^{-1}	1519cm^{-1}	No change in wave length
C-N	1073cm^{-1}	1082cm^{-1}	No change in wave length

CONCLUSION

Liquisolid technique changes the properties of poorly water soluble drugs like nebivolol hydrochloride simply by dispersing the drug particles in suitable non volatile liquid vehicle, which in turn increases the wetting property and surface area of the drug particles. present study has proven that the liquisolid technique is one of the promising alternative technique for improving bioavailability of poorly water soluble drugs. The liquisolid formulation developed with PEG 400 at drug concentration of 25%w/w with syloid 244 FP and aerosil (30:1) shown highest dissolution rate and high bioavailability compared with pure drug as well as marketed formulation.

REFERENCES

[1] Majid Saeedi, Jafar Akbari, Katayoun Morteza Semnani, Ala Soleymani, *Int. J. Pharm. Res*., **2011**, 10, 25-35.
[2] Naveen Chella, Nalini Shastri, Rama Rao Tadikonda, *Acta Pharm. Sin. B*, **2012**, 5, 502-508.
[3] Spireas S and Sadu S, *Int. J. Pharm*., **1998**, 166, 177-188.
[4] Ajit S Kulkarni, Nagesh H. Aloorkar, Madhav S. Manne, Jayasree B. Gaja, *Int. J. Pharm. Sci. Nanotech*., **2010**,3,793-802.
[5] Ali Nikhodchi, Roya Aliakbar, Sandip Desai, Yousef Javadzadeh, *Colloids and Surfaces B: Biointerfaces*, **2010**, 79, 262-269.
[6] Kavitha K, Kotha NS, Lova Raju, *Der Pharmacia Lett*, **2011**, 3, 71-83
[7] Powder flow; *United States of Pharmacopoeia*; **1174**
[8] Hentzschel CM, Alnaief M, Simivnova I, Sakmann A, Leopord CS, *European J. Pharm. Biopharm*., **2012**, 80, 130-135
[9] Hitendra S Mahajan, Manoj R Dhamne, Surendra G Gattani, Ashwini D Rasal, Hannan T Shaikh, *Int. J. Pharm. Sci. Nanotech*., **2011**, 4, 1205-1213
[10] Javadzadeh Y, Baharak jafari N, Nokhdochi A, *Int. J. Pharm*., **2007**, 341, 26-34
[11] Khalid K. A, Asiri Y. A, EI Y. M. Sayed, *Int. J. Pharm*., **2001**, 222:1-6
[12] Rania h. Fahmy, Mohemmed A. Kaseem, *European J. Pharm. Biopharm*., **2008**, 69, 993-1003
[13] Vijaykumar Nagabandi, Rama Rao T, Jayaveera KN, *Int. J. Bio. Res*., **2011**, 2, 530-41

[14] Shannon ReaganShaw, Minakshi Nihal, and Nihal Ahmad, *The FASEB Journal*, **2007**, 22, 659-661