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Effect of Nanonization on Poorly Water Soluble Glibenclamide Using Liquid Anti-Solvent Precipitation Technique: Aqueous Solubility, *In vitro and In vivo* study

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Abstract

The aim of the present research was to improve aqueous solubility and oral bioavailability of glibencamide (GLB), a BCS class-II drug. GLB nanosuspension (NS) was prepared using liquid anti-solvent precipitation (LAS) technique and stabilized using HPMC K15M and lactose. Different in-process variables which directly affect the precipitated particle size have been thoroughly studied and optimized. The effect of cryoprotective agent that could prevent agglomeration during lyophilisation was investigated. The optimal preparations GD-H0.3d and GD-H0.4f exhibited a size range of 168.6 and 342.2 nm respectively and did not show any interaction when screened for incompatibility using FT-IR and DSC, but exhibited decrease in crystallinity. The prepared GLB NPs exhibited superior aqueous solubility and dissolution when compared to pure GLB for the same. Oral bioavailability of optimized preparation was found to exhibit 2.59, 1.67, 1.19, 2.50 and 2.40 folds of increment with respect to C_{max}, $K_{el}(hr^{-1})$, $t_{1/2,}$ $AUC_{0\text{-}24hr}$ and $AUC_{0\text{-}\infty}$ for GD-0.3d in contrast to pure GLB.

Keywords: Glibencamide; Liquid anti-solvent precipitation; Nanonization; Aqueous solubility

1. Introduction

Nearly 70% of new potential drug molecules available in today's market are classified under BCS class-II, posing difficulty in formulating oral dosage forms due to its poor aqueous solubility that ultimately affects drug bioavailability[1, 2]. Nanonization is one of the techniques which have been deeply exploited over the past few decades with an aim to achieve successful targeting as well as improving the overall drug bioavailability. Despite numerous attempts to overcome this obstacle, the designing of oral dosage forms with a desirable oral bioavailability remains a day to day challenge for the researchers[3].

Glibenclamide (GLB), 5-choro-*N*-(4-[*N*-(cyclohexylcarbamoyl)sulfamoyl]phenethyl)-2-methoxybenzamide, also known as glyburide, is an anti-diabetic drug belonging to class of sulphonylureas used for the management/control of type-II diabetes. It activates β-cells of pancreas and stimulates insulin release. However being a BCS class-II drug, aqueous solubility is the rate limiting step which consequently hinders its oral absorption and its bioavailability[4-6].

Nanonization aims at increasing the surface area to volume ratio of individual particle via particle size reduction that subsequently improves the solubility as well as dissolution of poorly water-soluble molecules[7]. Strategies to nanonize GLB and to improve its aqueous solubility and bioavailability has been previously reported by Kumar et al., 2014[8], Guan et al., 2014[9] and Salazar et al., 2013[10] using precipitation technique, supercritical fluid technology and combinative particle size reduction H 42 technologies. However, these technologies involve complicated procedures and un-convenient processes. Alternatively, Liquid Anti-Solvent Precipitation Technique (LAS) is a simple bottom up process for control over particle properties such as size, morphology as well as crystallinity[11]. Pointing out on its

advantages, LAS is a more convenient process at ambient conditions without need of any specialized equipment and is easily scalable[12].

The underlying principle behind LAS or any bottom-up techniques involve crystallization or precipitation or solvent evaporation. The particles subjected to LAS undergo supersaturation, nucleation and growth during recrystallization process before precipitating out as nano or micro particles. However, a strict optimization of process parameters is a prerequisite for preparing optimized nanocrystals and to avoid undesirable agglomeration and uncontrolled crystal growth from the solvent system comprising of the drug[13, 14]. Thorat and Dalvi, 2012[12] have highlighted the use of LAS technique for recrystallizing poorly water soluble drugs with controlled outcome of particle size, size distribution and stabilization of ultrafine particles. Shah et al., 2013[15] has previously prepared optimized GLB NPs using LAS technique in order to improvise its dissolution characteristics, and optimized the various significant parameters that could affect response variables by Plackett-Burman screening design.

The aim of the research was to optimize and study the process parameters of LAS technique that directly affect physico-chemical properties of precipitated GLB NPs and further evaluate the aqueous solubility and oral bioavailability behaviour.

2. Material and methods

2.1. Material

GLB was provided as a gift sample from Wockhard Ltd., Aurangabad, India. Hydroxypropyl methylcellulose K15M (HPMC K15M), mannitol and lactose monohydrate were purchased from Loba Chemie, Mumbai. All the chemicals and buffers used were of analytical grade.

2.2. Preformulation Studies

2.2.1. Selection of solvent

In order to select appropriate solvent for preparation of GLB NPs using LAS, various solvents and buffers were evaluated for their ability to solubilise GLB. Excess amount of the GLB was dissolved in 10 ml of selected organic solvents like acetone, methanol, ethanol and dimethyl sulfoxide (DMSO) as well as pH 1.2 HCl buffer and pH 6.8 and 7.2 phosphate buffers, under ambient temperature (25-30 ºC). Amount of drug solubilised was quantified at 238 nm by UV spectroscopy method (UV1800, Shimadzu).

2.2.2. Study and Selection of optimal Solvent to Anti-solvent ratio and drug concentration

In order to select and evaluate the effect of solvent to anti-solvent ratio (ml/ml) on particle size of GLB nanoparticles (NPs), different ratios of solvent containing 50 mg/ml equivalent concentration of GLB was added drop-wise to anti-solvent (distilled water) under constant homogenization speed (10000 rpm) using Polytron PT 1600E, Switzerland. The solvent to anti-solvent ratio used for the study were 5:5 to 1:9 ml/ml with an increment of 0.5 ml. The process time was kept constant for 5 mins. The particle size of GLB suspension was determined by photon correlation spectroscopy (PCS) using Zeta sizer Nano ZS, Malvern Instruments, Malvern, UK. The optimal ratio that precipitated minimal particle size was fixed and further considered for optimizing the drug concentration for preparation of GLB NPs.

Similarly, to select and study the effect of drug concentration on precipitated particle size of GLB, pre-fixed ratio of solvent to anti-solvent containing different amounts (10 to 60 mg/ml) of GLB was used. The method of preparation and evaluation of particle size of GLB suspension was same as mentioned above. The entire study was performed under ambient

conditions. Details of solvent:anti-solvent ratio (ml/ml) and drug concentration used for the study are further enclosed in supplementary data.

2.3. Preparation of GLB nanosuspension by LAS technique

GLB nanosuspensions (NS) were prepared by LAS precipitation technique. From the previous study, optimal solvent:anti-solvent ratio and drug concentration that produced minimal precipitated particle size were kept constant and further to understand the effect of stabilizer concentration on precipitated particle size, HPMC K15M and lactose, in different concentrations, were selected for stabilizing GLB NS. Prefixed amount of solvent containing GLB was added drop-wise to anti-solvent containing HPMC K15M/lactose (0.1-0.6% w/v with an increment of 0.1% w/v) and homogenized at 10,000 rpm for 5 mins under ambient temperature. The formed NS was centrifuged and re-suspended into fresh distilled water. This process was repeated twice before subjecting the prepared final NS to lyophilisation to obtain GLB NPs. GLB NS were re-suspended and evaluated for particle size. The formulation codes of different prepared NS batches were coded as GD-H0.1to GD-H0.6 and GD-L0.1 to GD-L0.6, representing formulations stabilized using HPMC K15M and Lactose respectively (Details of formulation codes are further reproduced in supplementary data).

Batches which reproduced lowest particle size from each stabilizer used were subjected to lyophilisation studies. Mannitol in different concentrations (0-5% w/v, with an increment of 1% w/v) was used as a cryoprotective agent. NS were filled in rubber stoppered vials and frozen using deep freezer (Remi PVD-185 D, India) at -40ºC for 24 hrs, followed by freeze drying using Ilshin Laboratory Co Ltd., Korea. The percentage yield of freeze-dried GLB NPs was calculated gravimetrically (The formulation details are presented in supplementary data).

2.4. Re-dispersibility Test

Fifty milligrams of prepared freeze-dried GLB NPs were dispersed in 1.5 ml of distilled water and vortexed for 30s under ambient condition. The samples were immediately evaluated for particle size as mentioned in previous section,[16, 17] and also visually examined and categorized under following three grade systems: Grade A) Rapidly formed NS, having a clear appearance, Grade B) Rapidly formed NS, but slightly turbid and Grade C) Particles tend to aggregate and fail to redisperse immediately.

2.5. Physico-Chemical Characterization of GLB NPs

The physico-chemical characterization of prepared freeze-dried GLB NPs and pure GLB were screened using Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), X-ray Diffraction (XRD) and studied for surface morphology.

FT-IR spectral analysis was done by employing KBr pellet press method and analysed the samples using FT-IR spectrometer, Shimadzu, Model 8033. DSC was carried out by crimping the samples in aluminium pans and analysed using DSC Dupont9900. XRD analysis was facilitated using Rigaku diffractometer coupled with copper as anode material and graphite monochromator and operated at 15mA, 30kV voltage. Scanning electron microscope (SEM) (Joel-LV-5600, USA) was used for surface morphology determination.

2.6. Evaluation of prepared GLB NPs

2.6.1. Drug Content

One hundred milligram of GLB NPs were dissolved in acetone and diluted appropriately, followed by measuring absorbance at 238 nm using UV spectroscopy method.

2.6.2. Aqueous Solubility of prepared GLB NPs

Excess amount of prepared GLB NPs and pure GLB were taken in rubber capped glass vials containing 5 ml of distilled water and shaken using mechanical stirrer for 24 hrs at ambient temperature (25-30 ºC)[18]. The resulting solution was filtered using Whatmann filter paper grade 1 and analyzed using UV spectrometer at 238 nm.

2.6.3. *In vitro* Drug release

Drug release studies of 10 mg equivalent weight of GLB NPs and pure GLB was evaluated in 900 ml of pH 6.8 phosphate buffer with 1% w/v SLS using dissolution USP XXIII attached with paddle (Electrolab, Mumbai, India) at 75 rpm and 37 ± 0.5ºC[10]. Aliquots were withdrawn at pre-determined intervals and quantified using HPLC method. Results obtained were statistically analyzed to determine the significance using paired t- test.

2.6.4. *In vivo* studies

All animals for the study were acquired after protocol approval through Institutional Animal Ethics committee, JSS University, Mysore, India (Approval No. 106/2012). The animals authorized for carrying out studies were cared under the supervision of pharmacology department, JSS College of Pharmacy, Mysore, India in compliance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines. Twelve albino Wistar rats of either sex weighing 230-260 gm were divided into two groups and monitored for one week under quarantine conditions prior to studies. The animals were fed a standard chow diet ad libitum and had free access to water.

Initially, rats were anaesthetized through intraperitoneal injection of urethane (1gm/kg) and the jugular vein was cannulated to facilitate collection of blood samples. The two pre-divided groups comprising of six in each, were labelled as test and control. Oral

administration of optimized GLB NPs and pure drug (Fixed dose of 5 mg/kg in water) to the test and control group was facilitated by stomach sonde needle respectively. 0.3 ml blood samples were withdrawn pre and post administration GLB NPs from the jugular vein at pre determined time intervals. Samples were collected in heparinised tubes, followed by centrifugation for 5 min at 10,000 rpm at 4˚C. Plasma was separated and stored at -50˚C for further analysis.

Plasma drug concentration was determined using validated HPLC method. The drug was extracted from the plasma using 2 ml of methanol and vortexed for 5 mins at 10000 rpm. The supernatant was collected and was injected into C_{18} column of HPLC and using methanol:water in 80:20 v/v as mobile phase. pH was adjusted to 3.4 with orthophosphoric acid. The samples were injected into the column at a flow rate of 1 ml min^{-1} and quantified at 238 nm. Pharmacokinetic parameters such as maximum plasma concentration (C_{max}), time to reach maximum plasma concentration(T_{max}), Half life($t_{1/2}$), absorption rate constant (K_a) and area under curve (AUC_{0-t}) were calculated from plasma-concentration time profile.

3. Results and Discussion

3.1. Development of GLB NPs

GLB NPs were developed using LAS technique with an aim to improve aqueous solubility and oral bioavailability of GLB. LAS is an attractive bottom up approach for developing NPs/Microparticles (MPs) that can be carried out under ambient temperature conditions. The underlying principal for generating NPs/MPs is precipitation. Steps involve mixing of solution (drug dissolved in suitable solvent system) and anti-solvent, generation of supersaturation, nucleation and growth by coagulation and condensation, followed by agglomeration in case of uncontrolled crystal growth[12]. This further highlights the importance of selecting suitable solvent system for rapid and high supersaturation as it has a direct effect

on the outcome such as size, morphology as well as purity. In order to bypass uncontrolled coagulation and non-uniformity of NPs, suitable polymer/surfactants are incorporated for preparing stabilized NPs. Therefore, optimizing the different process parameters is a prerequisite prior to final preparation of NPs.

3.2. Solubility Studies

In order to recognise suitable solvent/solvent system that can effectively solubilise GLB, different organic solvents and buffers were selected. The results obtained are depicted in Table 1. From the results, DMSO and acetone were found to be suitable candidates for solubilising (25gm/l and 7.3 gm/L respectively) GLB.

Beck *et al*., 2010[19] research findings concluded that higher polarity of solvent results in increased particle size and vice-versa. Considering this theory, DMSO exhibits superior solvent polarity when compared to acetone (7.2 against 5.1 respectively), making the latter a preferable candidate as solvent system for solubilising GLB, and deionised water (DI) was taken as an anti-solvent.

Table 1. Solubility data of GLB.

Solvent system	**Solubility (mg/L)***
pH 1.2 HCl buffer	5.2±0.043
pH 6.8 Phosphate buffer	18.4±0.332
pH 7.2 Phosphate buffer	23.5±0.765
Acetone	7300.94±0.798
Methanol	389.4±0.231
Ethanol	4800.07±0.11
DMSO	25100.43±0.49

***n=3**

3.3. Study on influence of various in process parameters on precipitated particle size

3.3.1. Influence of sovent:anti-solvent

Different ratios of solvent:anti-solvent (ml/ml) were screened thoroughly to identify the appropriate ratio and to understand its influence on precipitated particle size. The results obtained were depicted in Fig. 1 respectively. Pure GLB was found to be 14.3±0.8 µm in size. During the study, it was observed that by decreasing the solvent to anti-solvent ratio from 5:5 to 3.5:6.5 ml/ml, GLB mean particle size decreased subsequently from 4931.1nm to 2815.5 nm (with 1.751 fold decrease in particle size). However beyond 3.5:6.5 to 1:9 ml/ml, increase in drug mean particle size from 2815.5 nm to 5192.3 nm (with 1.844 fold increase in particle size) was observed. Even though the particle size is in the micrometer range, a 5 fold decrease in particle size was observed at 3.5:6.5 ml/ml solvent:anti-solvent ratio when compared to particle size of pure drug. In contrast to other prepared ratios, 3.5:6.5 m/ml ratio offered a rapid reduction in drug concentration which led to rapid drug precipitation upon addition of solvent to anti-solvent that ultimately resulted in least particle size[20-22].

Therefore based on the results, 3.5:6.5 ml/ml was selected and fixed as the optimal ratio for further studies. The study highlights the influence of solvent/anti-solvent ratio on the resulting particle size of the precipitated drug.

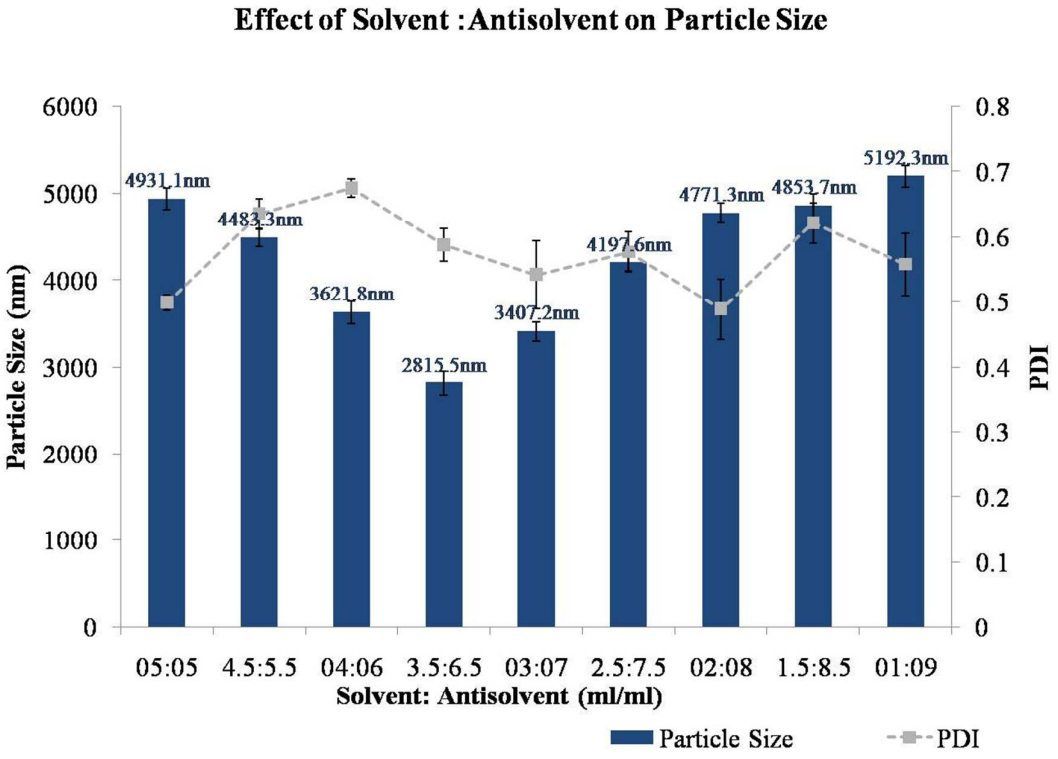

Figure 1: Effect of Sovent:Anti-Solvent ratio on Particle size.

3.3.2. Influence of drug concentration

Effect of drug concentration over precipitated particles size was carried-out by varying the drug concentrations from 10-60 mg/ml with an increment of 10 mg/ml and keeping the above 3.5:6.5 ml/ml selected solvent:anti-solvent ratio constant. The results are sketched in Fig. 2. Initially, decrease in particle size from 2815.2 nm to 2276.4 nm was observed as the drug concentration increased from 10-40 mg/ml, beyond which further increase in concentration from 40-60 mg/ml consequently increased the drug particle size from 2276.4 nm to 3089.3 nm respectively. Effect of drug concentration has previously been investigated and reported by Park and Yeo, 2010[23] for Roxithromycin (ROX) and further reviewed by Abhijit and Sanjaykumar, 2013[20]. According to the findings, drug concentration and the precipitated drug particle size are inversely proportional to one another. Decrease in

precipitated drug particle size is observed with subsequent increase in drug concentration. This further relates the dependency of nucleation rate on drug concentration in the prepared drug solution. Furthermore, degree of supersaturation alters the nucleation rate, and it directly relates to concentration of drug in the solution. By extrapolating the findings on ROX by Park and Yeo, 2010[23], the increase in particle size observed beyond 40 mg/ml concentration of GLB in drug solution might be due to agglomeration of particles together during the process of precipitation resulting in poor distribution of both size as well as shape. The observed phenomenon can be a consequence of formation of several number of nuclei at the interface with respect to influence on viscosity by drug concentration. Further, increased nuclei formation hinders diffusion from solvent to anti-solvent which ultimately leads to particle aggregation[24-26]. Solvent:anti-solvent ratio (3.5:6.5 ml/ml) and drug concentration (40 mg/ml) were considered as optimized parameters and kept constant for further course of study.

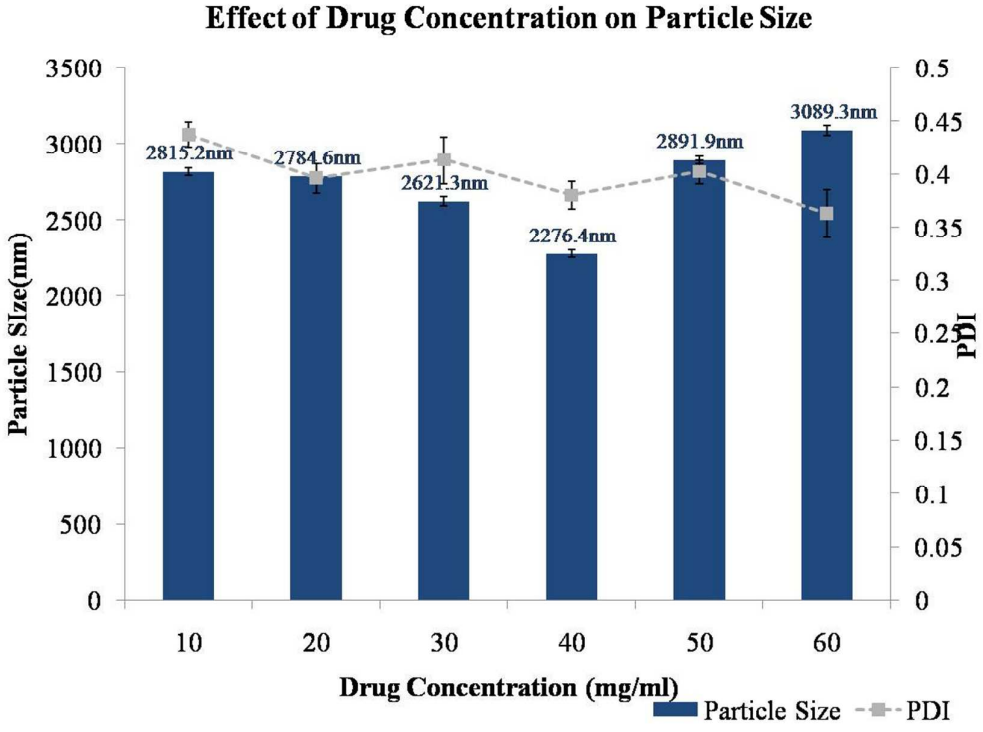

Figure 2: Effect of drug concentration on particle size.

3.3.3. Influence of Stabilizers

The theory of stabilizing of NS simultaneously with precipitation during LAS technique has been outlined by Thorat and Dalvi, 2012[12]. According to their report, adsorption strength of a stabilizer onto the drug surface depends mainly on two factors i.e., (a) amount of stabilizer adsorbed onto the surface is inversely proportional to its solubility in liquid phase and (b) stabilizer-particle interaction strength over stabilizer-solution interaction strength. HPMC K15M and lactose were screened for its ability to stabilize the GLB NS. Neutral polymers stabilize the system via hydrogen-bonding interaction with that of the drug particle surface and by reduction in solid-liquid interfacial tension[21, 27-31]. A thorough literature survey suggests that the selected stabilizers are neutral in nature [32].

The results obtained for GLB NS prepared by individually employing HPMC K15M and lactose is presented in Fig 3. From the results, formulation GD-H0.3 and GD-L0.4 containing 0.3% w/v of HPMC K15M and 0.4% w/v of lactose, exhibited least particle size of 136.1±17.9 nm and 352.9±20.9 nm respectively. At 0.3% w/v of HPMC K15M and 0.4% w/v of lactose, interaction between that of drug-polymer is superior as compared to polymer-solvent affinity, subsequently resulting in decreased precipitated particle size. The effect of particle size in relation with stabilizer can be explained by understanding the theory of interaction parameter of solvent-polymer-drug. As per the theory, favourable interaction of drug molecules with the surroundings subsequently causes quicker diffusion of stabilizer molecules towards the growing particle surface, which further inhibits addition of drug molecules and controls the precipitated particle size[12].

From the results, formulation GD-H0.3 and GD-L0.4 were considered as optimized formulations depending on its particle size and were subjected to lyophilization for developing GLB NPs.

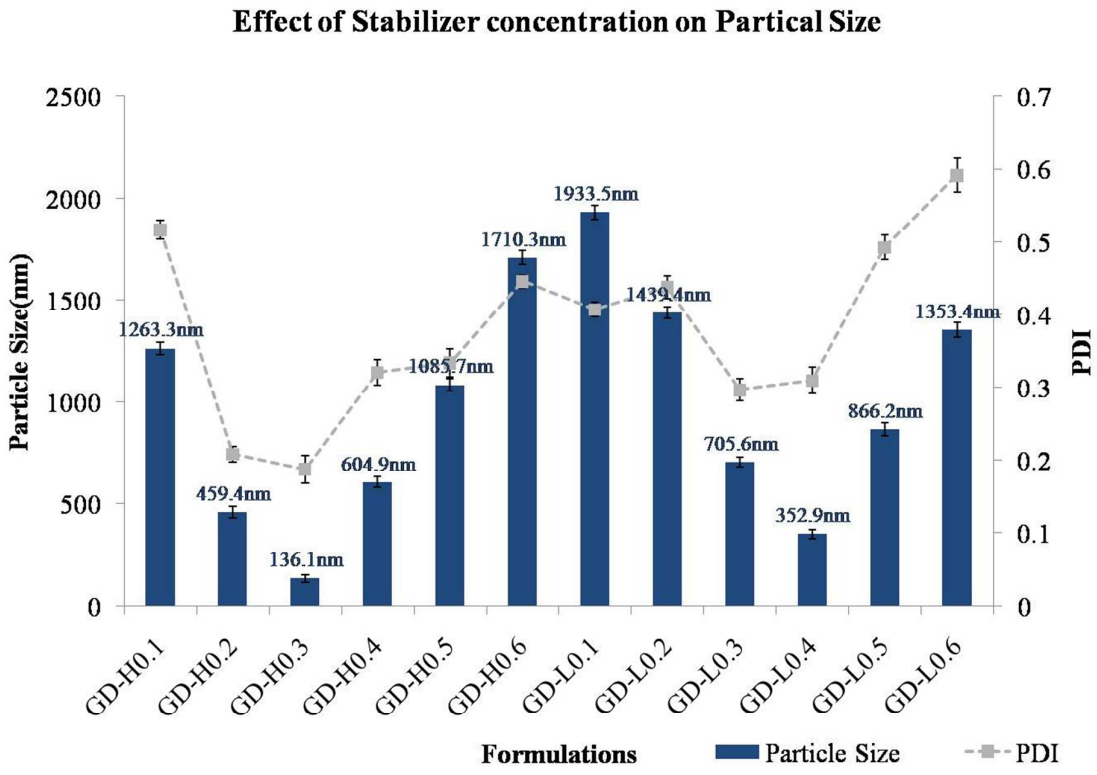

Figure 3: Effect of Stabilizer concentration on particle size.

3.4. Lyophilization of GLB NS and redispersibility test of obtained GLB NPs

During the course of lyophilisation, the precipitated particles tend to fuse with each other resulting in particles agglomeration due to the stress of freezing and dehydration encountered during the process. To prevent this, mannitol in different concentrations ranging from 0-5% w/v, with an increment of 1% w/v, was used as a cryoprotective agent to impart protection during freezing and drying stresses. In order to ensure immediate redispersibility of freeze-dried NPs, 100 mg of freeze-dried product was manually agitated for about 30s and evaluated for particle size. The results obtained were reproduced in Fig 4. From the findings, it was evident that for prepared GD-H0.3 trials, a minimal concentration of 3-5% w/v of cryoprotective agent is required for ensuring complete redispersion, whereas for GD-L0.4 trials, a minimal concentration of 5% w/v is required for the same. Results of redispersibility

test findings are given in Table 2. Below the above-mentioned concentrations and in case of cryoprotective absentia, the particles tend to aggregate and fail to redisperse upon gentle agitation. The above-mentioned concentrations of mannitol ensures redispersion through vitrification at a glass transition temperature (Tg) by immobilization of NPs within its glass matrix and preventing its aggregation to give protection against mechanical stress of ice crystals[33].

To conclude, GD-H0.3d and GD-L0.4f GLB NPs prepared using 3.5:6.5 (ml/ml) solvent/anti-solvent ratio, comprising of 40mg/ml concentration of GLB and 0.3% w/v of HPMC K15M and 0.4% w/v of lactose respectively, and followed by lyophilisation using 0.3% w/v and 0.5% w/v of mannitol as a cryoprotectant were considered as optimized freeze-dried products with an average particle size of 136.1±17.9 nm for GD-H0.3d and 342.2±26.9 nm for GD-L0.4f respectively. Even though formulations GD-H0.3e and GD-H0.3f produced desirable particle size upon redispersion, the redispersed particle size was larger than GD-H0.3d and therefore discarded for further study. The percentage yield was found to 71.8±1.8% and 73.4±2.3% for GD-H0.3d and GD-L0.4f after final gravimetric analysis with a drug content of 95.1±0.8% and 92.6±1.2% respectively.

Table 2. Redispersibility test results.

Formulation code	Grade*
GD-H0.3a	Grade C
GD-H0.3b	Grade C
GD-H3c	Grade C
GD-H0.3d	Grade A
GD-H0.3e	Grade A
GD-H0.3f	Grade A
GD-L0.4a	Grade C

GD-L0.4b	Grade C
GD-L0.4c	Grade C
GD-L0.4d	Grade B
GD-L0.4e	Grade A
GD-L0.4f	Grade A

***n=3**

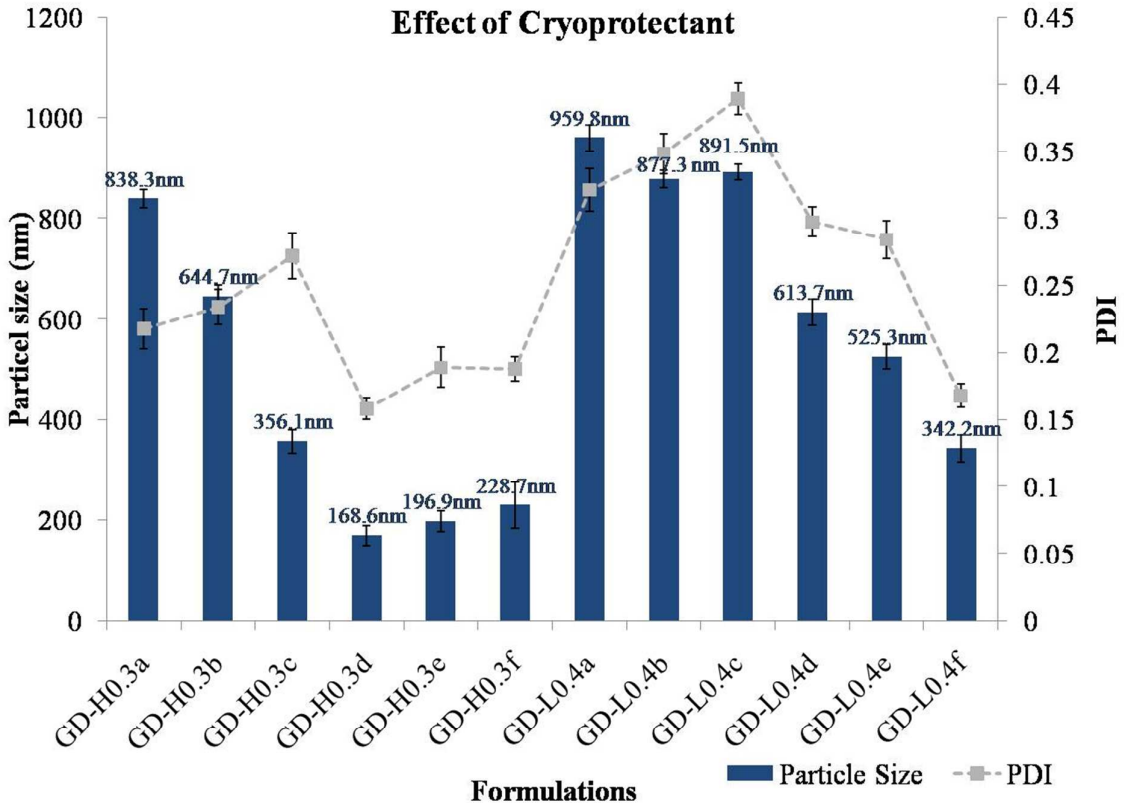

Figure 4: Effect of cryoprotective concentration over prepared GD-H0.3 and GD-H0.4 particle size.

3.5. Physico-Chemical Characterization of GLB NPs

3.5.1. FT-IR, DSC and XRD

In order to determine the compatibility, drug, NS (GD-H0.3 and GD-L0.4) and formulations GD-H0.3d and GD-L0.4f were screened for any possible interactions using FT-

IR spectrometer and thermograph investigations. The spectral peaks and DSC thermograms are sketched and compared in Fig 5a, 5b and Fig 6 respectively. GLB displays characteristic peaks at 3119.00 cm^{-1}, 2930.15cm^{-1}, 2854.56cm^{-1}, 1525.40cm^{-1} and 1157.31cm^{-1} corresponding to N-H stretching, Aliphatic C-H stretching, O-H stretching, N=O stretching and C-N stretching respectively. Similar observations with or without any modification in the characteristic peaks of GLB were seen in NS (GD-H0.3 and GD-L0.4) and formulations GD-H0.3d and GD-L0.4f respectively. The thermal property of GLB exhibits sharp endothermic peak at 175.64ºC demonstrating its crystallinity. On contrary, similar identical sharp peaks of GLB were found in GD-H0.3d and GD-L0.4f, reflecting the existence of crystalline state. Hence FT-IR and DSC confirms absence of any possible incompatibility.

The XRD data of GLB and formulations are collated in Fig. 7. The characteristic sharp and intense peaks of GLB were found at 2θ angles of 10.84, 11.70, 18.92, 20.96, 23.06 and 27.26 degrees, indicating its crystalline nature. However, formulations GD-H0.3d and GD-L0.4f exhibit peaks at similar positions of GLB but with a decreasing intensity which reciprocates to lower crystallinity/amorphous nature of GLB NPs. During the preparation of GLB NPs through LAS technique, rapid crystallization retards formation of compact crystalline structures consequently, resulting in formation of NPs with amorphous or decreased crystallinity[31].

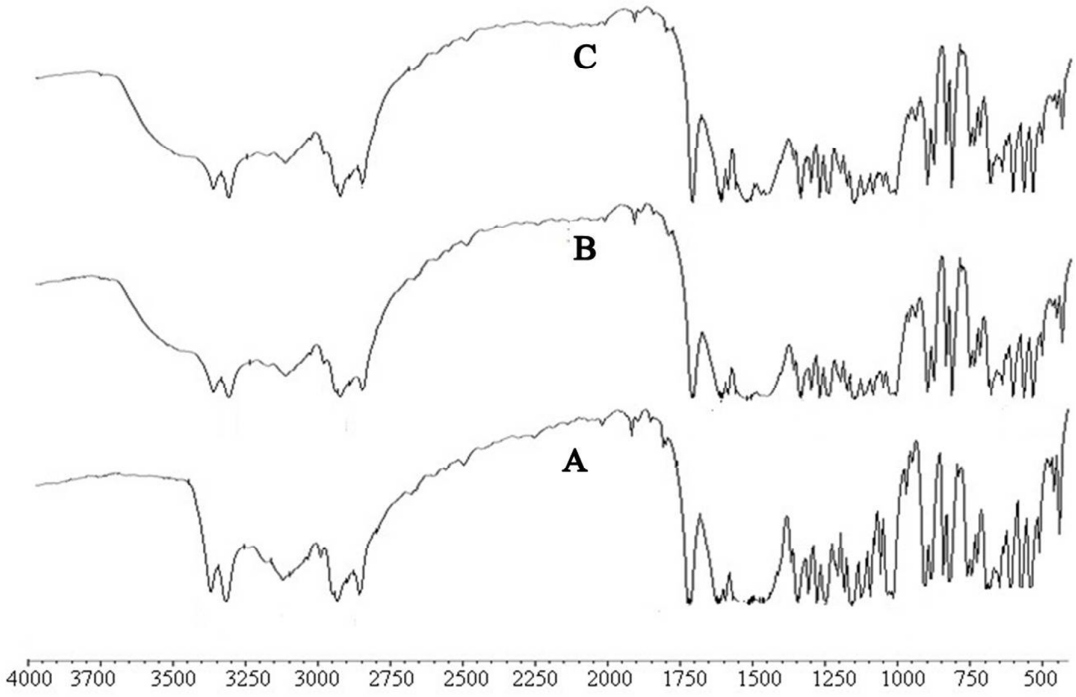

Figure 5a: FTIR spectra of A) Pure GLB, B) GD-H0.3 and C) GD-L0.4.

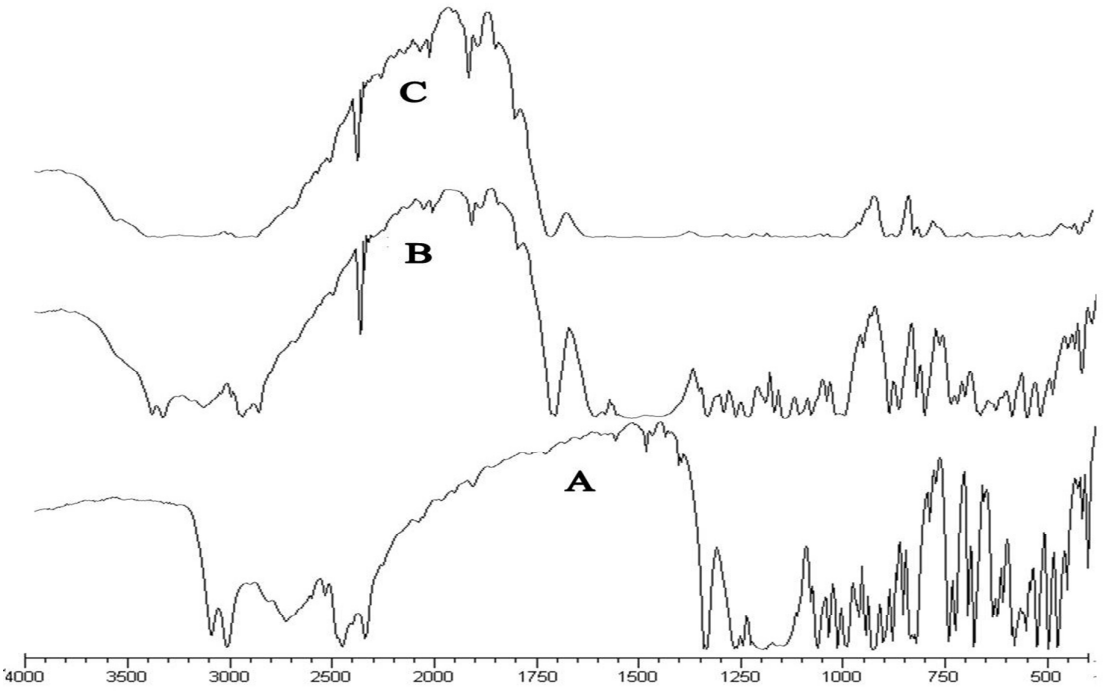

Figure 5b: FTIR spectra of A) Pure GLB, B) GD-H0.3d and C) GD-L0.4f.

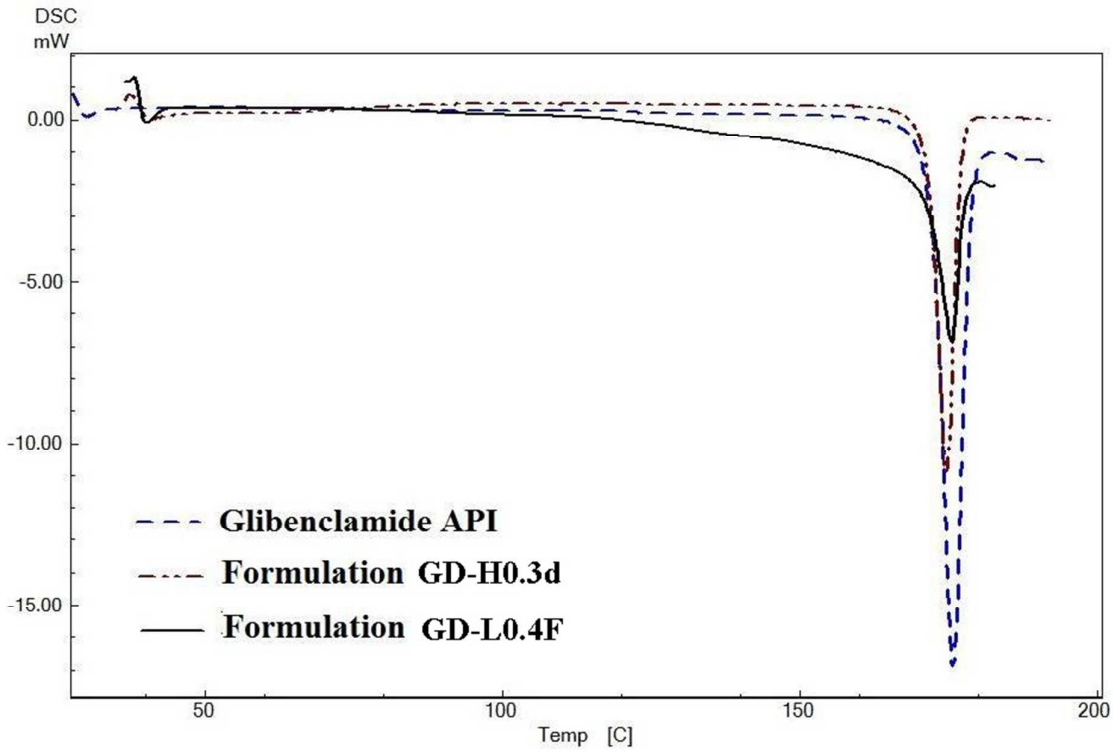

Figure 6: DSC thermograms of GLB, GD-H0.3d and GD-L0.4f.

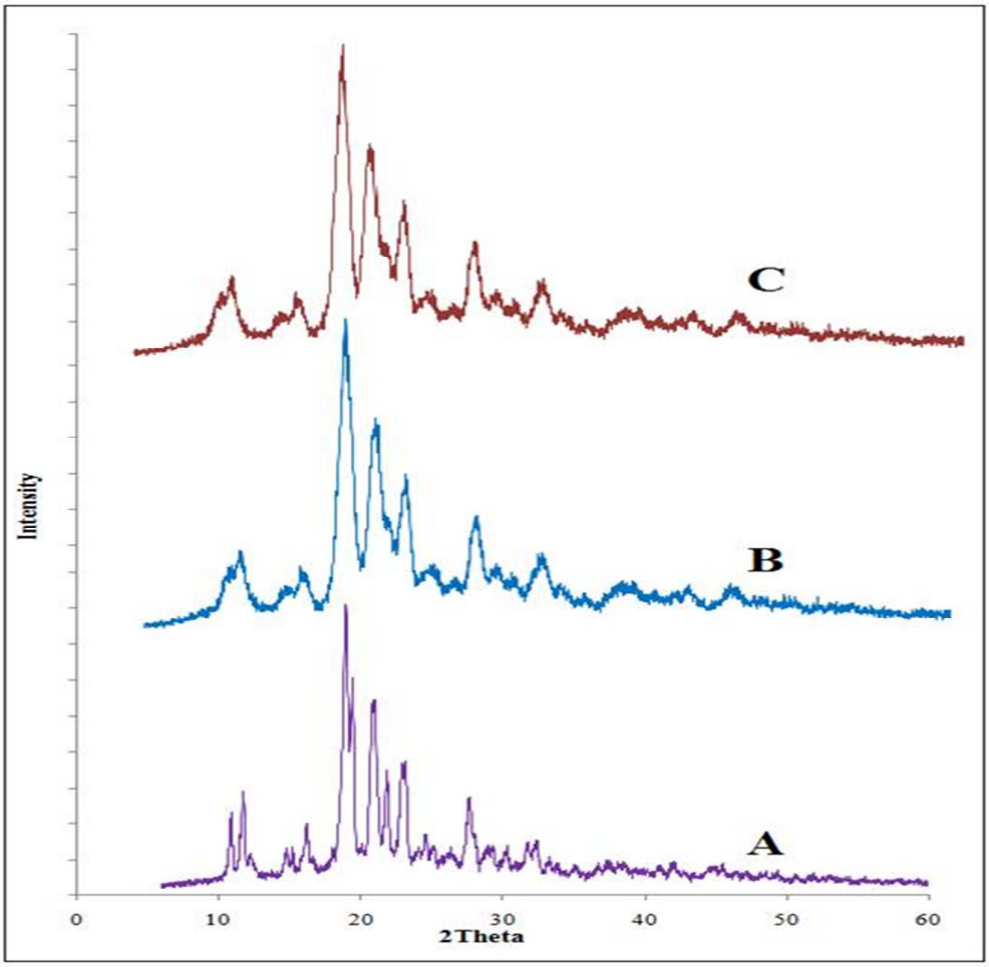

Figure 7: XRD diffractograms of: A) Pure GLB, B) GD-H0.3d and C) GD-L0.4f.

3.5.2. Scanning Electron Microscopy

The surface morphology of GLB NPs (Fig 8) reflected plate and rod shaped crystals with a smooth surface and narrow particle size distribution ranging from 100-200 nm for GD-H0.3d and 300 nm-500 nm for GD-L0.4f, correlating with its particle size as observed using Malvern zeta-sizer.

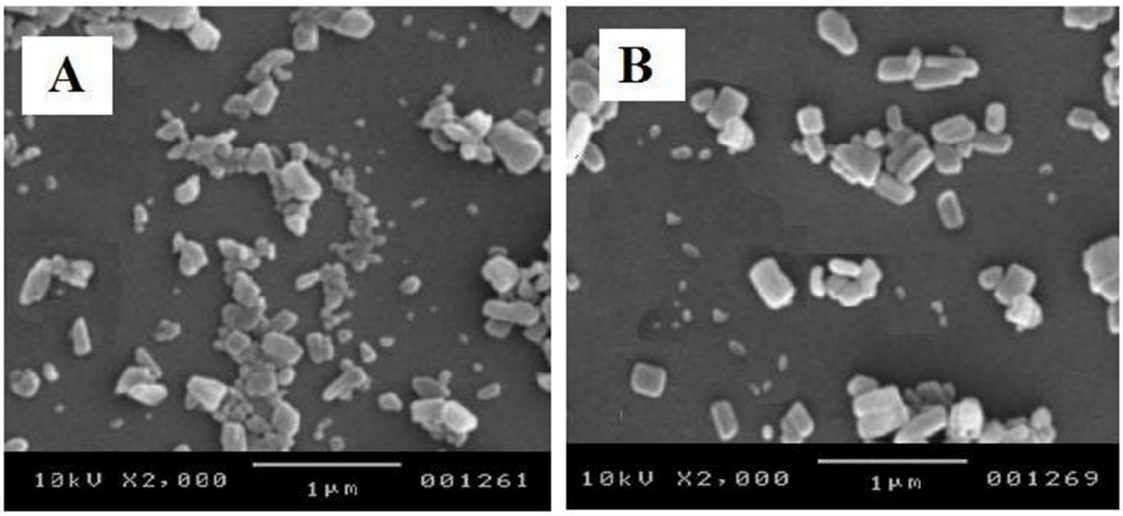

Figure 8: SEM of glibenclamide nanoparticles: (A) formulation GD-H0.3d; (B) formulation GD-L0.4f.

3.6. Evaluation of GLB NPs

3.6.1. Solubility

GLB in its pure form solubilised 24.56 mg/L in distilled water whereas GD-H0.3d and GD-L0.4f exhibited superior aqueous solubility of 157.77 mg/L and 139.03 mg/L, thereby achieving 6.4 and 5.6 folds increase in aqueous solubility respectively. This increase in solubility can be related to its amorphous or decreased crystallinity[34].

3.6.2. Dissolution studies

The dissolution studies of pure GLB and prepared GLB NPs (GD-H0.3d and GD-L0.4f) was carried out in pH 6.8 phosphate buffer with 1% w/v of SLS, and *in vitro* profile for the same is compared and presented in Fig. 9. Initially inside 5 mins, the prepared GD-H0.3d and GD-L0.4f exhibited burst release of 68.5±1.82% and 42.4±1.68% of GLB NPs, whereas for pure GLB, release was about 3.7±0.36%. Within 20 min, GD-H0.3d managed to release 94.2±1.67% of GLB whereas, it took 35 min for GD-L0.4f to release 91.8±2.42% of

GLB. Meanwhile, at the end of 120 min, only 32.25±1.19% of drug release was observed from pure GLB. About 2.96 and 2.95 fold increase in dissolution rate for GD-H0.3d and GD-L0.4f was distinguished at the end 120 min when compared to pure GLB. The increased dissolution rates for the prepared GLB NPs formulations can be attributed to transformation of physical state to highly disordered amorphous nature[35, 36]. SLS is added as a surfactant to the dissolution media to facilitate dissolution as well as drug release of poorly water soluble drugs[10].

In order to understand the influence of nanonization of pure GLB on *in vitro* drug release, release profiles of pure GLB v/s GD-H0.3d, GD-L0.4f and GD-H0.3d v/s GD-L0.4f were compared by processing the obtained data onto paired t-test. The results indicated statistical significant ($P<0.005$) difference between the pure GLB and the prepared GD-H0.3d and GD-L0.4f. However, not much significant ($P>0.005$) difference was observed within the prepared GD-H0.3d and GD-L0.4f formulations. From the above results, GD-H0.3d was considered as a superior formulation compared to its counter-part GD-L0.4f due to its rapid dissolution rate. Hence, formulation GD-L0.4f was discarded for oral pharmacokinetics study.

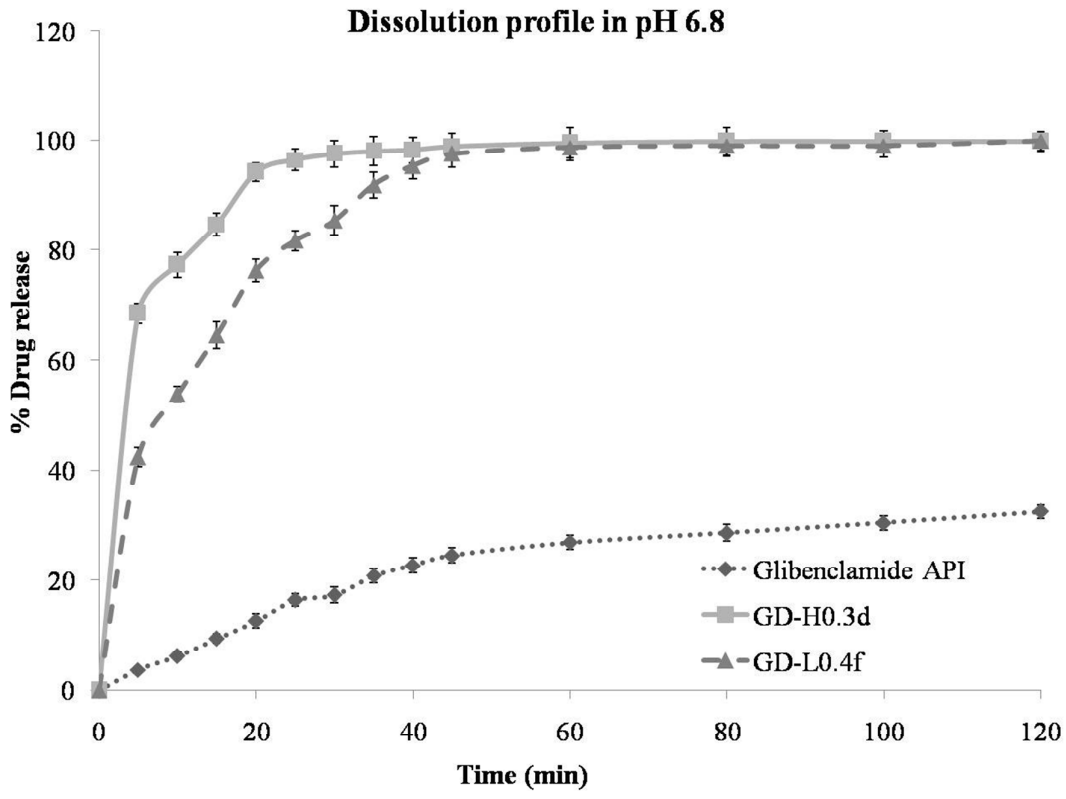

Figure 9: In vitro % drug release profile of GLB, GD-H0.3d and GD-L0.4f.

3.7. Oral Pharmacokinetics study

Plasma concentration-time profile and pharmacokinetic parameters for GD-H0.3d and pure GLB are summarized in Fig. 11 and Table 2. The study reflects that prepared GD-H0.3d shows significantly higher C_{max}, $K_{el}(hr^{-1})$, $t_{1/2,}$ $AUC_{0\text{-}24hr}$ and $AUC_{0\text{-}\infty}$, without altering $T_{max,}$ when compared to pure drug. The results indicate that particle size reduction has improved the rate and extent of GLB absorption from the GI tract. However, size reduction had no profound impact on T_{max}. A 2.59, 1.67, 1.19, 2.50 and 2.40 folds of increment with respect to C_{max}, $K_{el}(hr^{-1})$, $t_{1/2,}$ $AUC_{0\text{-}24hr}$ and $AUC_{0\text{-}\infty}$ for GD-H0.3d was found when compared to its counter-part for the same. Influence of nano-sizing of drug for improving its oral bioavailability has been previously discussed by Li *et al*., 2009[37] on rats with amorphous

fenofibrate (FF) NS. The findings observed indicated that nano-sizing of FF significantly enhanced oral bioavailability based on the particle size. These findings can be extrapolated to our present research.

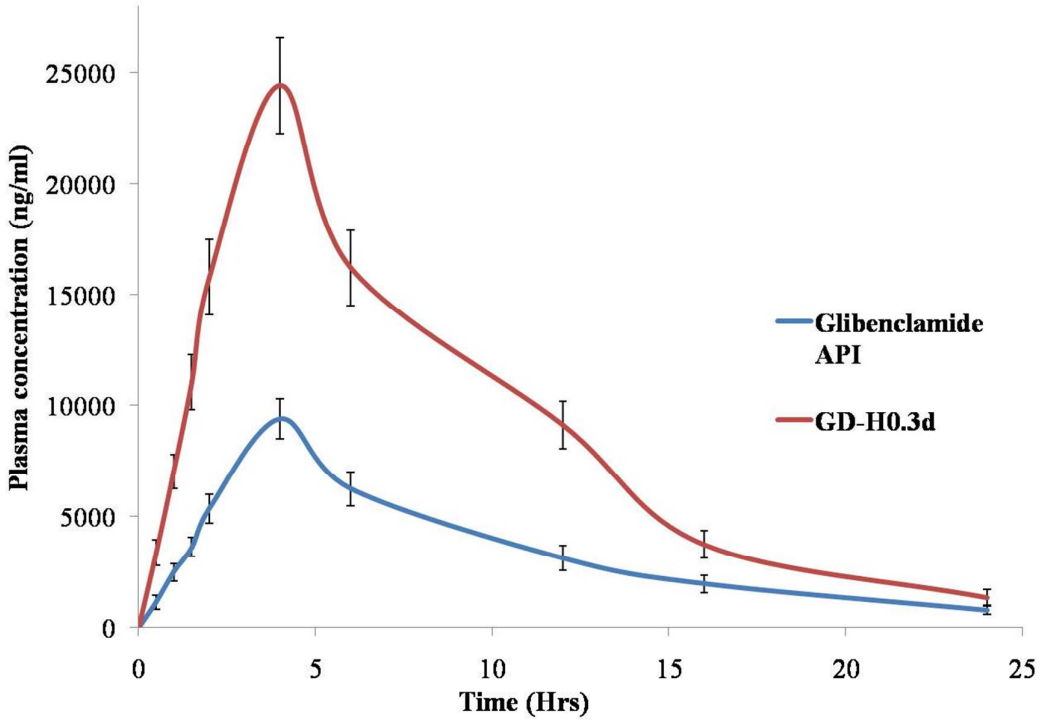

Figure 10: Plasma concentration-time profile GLB and GD-H0.3d.

Table 2: Pharmacokinetic parameters after oral administration of GLB and GD-H0.3d to rats.

Parametes*	**C_{max} (ng/mL)**	**T_{max} (h)**	**$t_{1/2}$ (h)**	**K_{el} (h^{-1})**	**$AUC_{0\rightarrow 24h}$ (ng h/mL)**	**$AUC_{0\rightarrow \infty}$ (ng h/mL)**
GLB	9428.42±8 97.8	4.0±0.0	5.73±0.42	0.1207±0.0 061	65106.41± 60.3	71680.24± 63.6
GD-H0.3d	24451.14± 2170.5	4.0±0.0	9.57±0.23	0.1438±0.0 042	162945.12 ±241.5	172383.64 ±237.2

4. Conclusion

In the present study, efforts were made to optimize the process parameters of LAS technique for developing GLB NPs, in order to enhance the aqueous solubility as well as oral bioavailability. Initially, the effect of different process parameters such as selection of solvent, ratio of solvent to anti-solvent, effect of drug, stabilizer and cryoprotectant concentration, that can directly affect the precipitated drug particle size were optimized and thoroughly discussed. The optimized GLB NPs did not show any incompatibility and was found to be readily dispersible upon gentle agitation. Further, among the optimized GLB NPs, formulation GD-H0.3d achieved 6.4 folds increase in aqueous solubility, rapid dissolution rate and 2.59, 1.67, 1.19, 2.50 and 2.40 folds of increment with respect to C_{max}, $K_{el}(hr^{-1})$, $t_{1/2,}$ $AUC_{0\text{-}24hr}$ and $AUC_{0\text{-}\infty}$ when compared to pure GLB. The results obtained summarize a platform to develop GLB NPs and its class of drugs for enhancing their oral absorption. Additionally, the work provides in depth understanding of several factors that can influence nanoprecipitation and subsequently its physico-chemical properties, aqueous solubility and oral pharmacokinetics.

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