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Research Article

Eudragit-Based Nanosuspension of Poorly Water-Soluble Drug: Formulation and *In Vitro–In Vivo* Evaluation

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Abstract. The present study was performed to investigate potential of Eudragit RLPO-based nanosuspension of glimepiride (Biopharmaceutical Classification System class II drug), for the improvement of its solubility and overall therapeutic efficacy, suitable for peroral administration. Nanoprecipitation method being simple and less sophisticated was optimized for the preparation of nanosuspension. Physicochemical characteristics of nanosuspension in terms of size, zeta potential, polydispersity index, entrapment efficiency (% EE) and *in vitro* drug release were found within their acceptable ranges. The size of the nanoparticles was most strongly affected by agitation time while % EE was more influenced by the drug/polymer ratio. Differential scanning calorimetry and X-ray diffraction studies provided evidence that enhancement in solubility of drug resulted due to change in crystallinity of drug within the formulation. Stability study revealed that nanosuspension was more stable at refrigerated condition with no significant changes in particle size distribution, % EE, and release characteristics for 3 months. *In vivo* studies were performed on nicotinamide–streptozotocin-induced diabetic rat models for pharmacokinetic and antihyperglycaemic activity. Nanosuspension increased maximum plasma concentration, area under the curve, and mean residence time values significantly as compared to aqueous suspension. Oral glucose tolerance test and antihyperglycaemic studies demonstrated plasma glucose levels were efficiently controlled in case of nanosuspension than glimepiride suspension. Briefly, sustained and prolonged activity of nanosuspensions could reduce dose frequency, decrease drug side effects, and improve patient compliance. Therefore, glimepiride nanosuspensions can be expected to gain considerable attention in the treatment of type 2 diabetes mellitus due to its improved therapeutic activity.

KEY WORDS: diabetes mellitus; glimepiride; nanoprecipitation; poloxamer; sustained release.

INTRODUCTION

With the advances in pharmaceutical research, there are thousands of new compounds getting synthesized every year and out of these, 40% show solubility problem which further makes their processing difficult. Poor aqueous solubility not only produces irreproducible therapeutic response but also leads to the wastage of large amount of drugs (1,2). Also, it may lead to unpredicted and uncontrolled precipitation of drugs in aqueous biological fluid which is accompanied with bioavailability problems.

Glimepiride (GLM) is an oral sulfonylurea derivative and has been in use for the treatment of diabetes mellitus type 2 since many years ago. It is practically insoluble in water and belongs to the class II drugs of Biopharmaceutical Classification System (BCS). The reported solubility of GLM in aqueous media having pH 7.0 is 0.0012 mg/ml (2) and that in buffer of pH 6.8 is 0.00087 mg/ml (3). Thus, it shows pH-dependent solubility behavior. Due to limited aqueous solubility of class II drugs, dissolution in gastric media acts as rate-limiting step for absorption (2,4). Solubility improvement is the major challenge for formulating such drugs. It is reported in previous literatures that cyclodextrin inclusion complexes improved the solubility of GLM (1). With a view to enhance solubility, Reven *et al.* (5) investigated solid dispersions of GLM by using commercially available poly(ester amide) hyperbranched polymers, linear polymer poly(ethylene glycol), and stearoylpolyethyleneglycerides (5). Size reduction to micro/nano range is gaining more attention over the previous approaches of solid dispersion, cosolvent addition, and cyclodextrin complexation method for solubility enhancement. In this regard, Ilic *et al.* developed microspheres by spray congealing method for enhancing the solubility of GLM (3). Micronisation does change saturation (equilibrium) solubility, but in most cases the change is not significant. Therefore, improved saturation solubility of such compounds can be obtained by dramatic size reduction to nanoparticles (6,7). The superiority of nanoparticles over microparticle systems is attributed to their increased dissolution velocity as well as saturation solubility, especially when formulated below particle size of 1–2 μm (8). Hence, nanoparticles by virtue of their large surface area to volume ratio provide an alternative method to formulate

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poorly water-soluble compounds. Therefore, in this study, we are moving from micro to nanotechnology to further improve GLM saturation solubility.

Nowadays, nanoparticle technology is being exploited on commercial scale in drug delivery to overcome solubility problems. Nanoparticles, in pharmaceuticals, are generally defined as particles having size below 1 μm (6,9,10). Nanosuspensions are submicron colloidal dispersions of discrete particles that have been stabilized using surfactants, polymers, or mixture of both (11).

In addition to the improvement in the saturation solubility, nanoparticles also show increased adhesiveness to the gut wall by virtue of their small size and high surface energy, which causes enhancement of gastric residence time (8). The inherent mucoadhesive nature of nanosuspensions plays an important role in the passive absorption of drugs through gastro-intestinal tract (GIT; 6,12). Diabetic condition is associated with changes in gastric emptying time which certainly affects the absorption of drugs. GLM shows incomplete absorption in diabetic condition because of decreased gastric residence time (13). In such situations, nanoparticles are supposed to improve GLM absorption by virtue of its mucoadhesive nature and increased gastric residence time.

Nanoprecipitation method is quite simple and more economical method of preparation of nanosuspension. This method is also known as bottom-up approach as it involves controlled precipitation of drug molecules to form nanoparticles (7,11). This method was developed by Fessi *et al.* for the preparation of biodegradable nanoparticles (14). Similarly, Guterres *et al.* prepared biodegradable nanocapsules of diclofenac by utilizing above technique (15).

Since polymeric nanoparticles are considered to be more stable than solid lipid nanoparticles (16), Eudragit RLPO (ERLPO) was selected based on its capability to form nanodispersions with submicron particle size, positive surface charge, and good stability. The positive surface charge is desired for imparting mucoadhesive properties to the nanosuspension. ERLPO is an acrylic and methacrylic acid-based polymer having hydrophilic properties due to the presence of quaternary ammonium groups (QAGs). It is insoluble in water but swells in the digestive fluid, independent of the pH and become permeable. It is used mainly in film coating of tablets, granules, and other small particles and could be used in matrix formulation as well (17). Eudragit nanoparticles appear as suitable inert carriers for oral drug delivery.

Marketed formulations of GLM provide an immediate release with peak serum concentrations achieved 2–3 h after oral administration, resulting into a fluctuating release profile. Such fluctuations can be minimized by preparing its sustained and prolonged delivery systems which will be able to maintain steady state drug level in plasma. Such drugs necessitate the preparation of suitable dosage forms which can improve solubility as well as therapeutic efficacy. Thus, this study was undertaken to formulate and evaluate ERLPO-based nanosuspensions of GLM in order to improve its solubility and overall therapeutic efficacy by attaining sustained release profile, making it suitable for per oral administration.

The effects of formulation variables on the physical characteristics of the prepared nanosuspensions and the *in vitro* drug release characteristics were studied. Variables such as drug/polymer ratio and agitation time were considered during formulation. Moreover, *in vivo* kinetics and antihyperglycaemic activity of drug on diabetic rat model were investigated. Oral glucose tolerance test was also carried out by loading nondiabetic rats with glucose.

MATERIALS AND METHODS

Materials

GLM and ERLPO were obtained as gift samples from Alkem Laboratories (Gujarat, India) and Lupin Pharma Lab. (Pune, India), respectively. Poloxamer 188 (P-188) was provided by Ranbaxy Laboratories (Gurgaon, India). Streptozotocin and nicotinamide were purchased from Sigma-Aldrich, USA. High-performance liquid chromatography (HPLC)-grade acetonitrile and glacial acetic acid were procured from Spectrochem Private Limited (Mumbai, India). All other solvents and chemicals used were of analytical grade and obtained from S.D Fine Chemicals (Mumbai, India).

Preparation of Nanosuspension

Nanosuspensions were prepared according to nanoprecipitation method given by Fessi *et al.* with slight modification (14). ERLPO polymer and specified quantity of drug were dissolved in acetone at 40°C to form uniform organic solution. The prepared organic solution was then injected slowly dropwise with the help of a syringe into an aqueous phase containing 2% (*w*/*v*) P-188 kept under high-speed mechanical agitation of 8,000 rpm to get desired nanodispersion (Fig. 1a). Prepared nanosuspension was then stirred magnetically at 500 rpm at room temperature for 12 h to evaporate organic solvent. Complete evaporation of acetone was determined by spectrophotometric

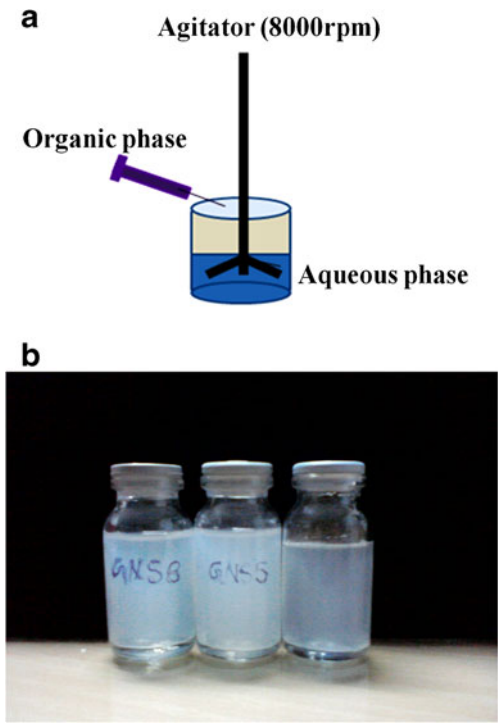

Fig. 1. **a** Setup used for the preparation of nanosuspension and; **b** *milky white appearance* of prepared formulations

method using vanillin (18). The volume was then adjusted with the addition of triple distilled water to recover loss in volume. All samples were prepared in triplicate. Drug/polymer ratio and agitation time was varied keeping other parameters constant. The batches were prepared according to the formulation design given in Table I.

Lyophilization and Redispersibility of Nanosuspension

Prepared nanosuspensions were frozen and lyophilized using lyophillizer (Decibel digital, India) for 24 h at −40°C. The freeze-dried samples were diluted to original volume with triple distilled-water, and redispersibility was observed. Freeze-dried samples were further used for solid state characterization. Calculation of the percentage nanoparticle recovery (% NR) was performed in triplicate using Eq. 1.

$$\%\ \mathrm{NR} = \text{mass of nanoparticles recovered/mass of drug and polymer taken} \times 100 \quad (1)$$

% NR refers to the percentage of nanoparticles recovered from the preparation process after lyophilization.

Characterization of Nanosuspension

Particle Size, Polydispersity Index, and Zeta Potential

All these parameters were estimated by particle size analyzer (Delsa Nano C, Beckman Coulter Counter, USA) equipped with software N4 Plus and was performed at 90° with respect to the incident beam. Nanosuspensions were analyzed for particle size and polydispersity index. Zeta potential was determined by measuring the electrophoretic mobility of particles in the electrical field using same instrument. All samples were analyzed in triplicate.

Total Drug Content and Entrapment Efficiency

An aliquot (0.5 ml) was evaporated to dryness. The residue was then dissolved in acetone and filtered with 0.45 μm filter paper. The samples were analyzed using UV spectrophotometer (Shimadzu-1700, Japan) at λ_{max} of 227 nm. Total drug content (TDC) and % TDC were calculated from Eqs. 2 and 3.

$$\mathrm{TDC} = (\text{Vol. total/Vol. aliquot}) \times \text{Drug amount in aliquot} \times 100 \quad (2)$$

$$\%\ \mathrm{TDC} = \mathrm{TDC/TAD} \times 100 \quad (3)$$

where, vol. total/vol. aliquot is the ratio of total nanosuspension volume to the volume of aliquot taken and the total amount of drug (TAD) is the total amount of drug taken for the formulation of nanosuspension (19).

For determination of entrapment efficiency (% EE), 2 ml of sample was ultracentrifuged at 15,000 rpm at 4°C (Remi Instruments, Mumbai, India) for 30 min. The supernatant was immediately analyzed for free drug content (FDC). The sediment was then washed with 0.1 N NaOH. The washings were then further analyzed for surface adsorbed drug (SAD). All drug solutions were quantified spectrophotometrically at 227 nm. All samples were evaluated in triplicate.

% EE is defined as the percentage of the drug that gets entrapped into the nanoparticles *w.r.t*, the TAD taken for the formulation of nanosuspension, and calculated using Eq. 4. (20).

$$\%\ \mathrm{EE} = \mathrm{TDC} - (\mathrm{FDC} + \mathrm{SAD})/\mathrm{TAD} \times 100 \quad (4)$$

In Vitro Drug Release

The release of GLM from nanosuspensions was evaluated over 24 h by using dialysis bag (Himedia labs, cutoff weight 12,000–14,000 Da) diffusion technique. In this technique, each bag was loaded with the formulation (equivalent to 2 mg of GLM), hermetically sealed and dialyzed against 200 ml phosphate buffer solution (pH 6.8) contained in 500 ml beaker kept on a thermostatically controlled magnetic stirrer maintaining temperature of 37±0.5°C and stirring at 500 rpm. Samples (2 ml) were collected at predetermined time intervals till 24 h, and immediately replaced with 2 ml of fresh buffer to maintain sink condi-

Table I. Formulation Design and Formulation Variables

Formulation batches	Drug (mg)	ERLPO (mg)	Drug/polymer ratio	Agitation time (min)
GNS1	20	200	1:10	15
GNS2	20	200	1:10	30
GNS3	20	400	1:20	15
GNS4	20	400	1:20	30
GNS5	20	800	1:40	15
GNS6	20	800	1:40	30
GNS7	40	200	1:5	15
GNS8	40	200	1:5	30
GNS9	40	400	1:10	15
GNS10	40	400	1:10	30
GNS11	40	800	1:20	15
GNS12	40	800	1:20	30

tion. The cumulative percent release of GLM was calculated by analyzing the samples on UV spectrophotometer at 227 nm. Microsoft Excel 2007 was used for the calculation of the release rate constants (k_x) and the determination of the correlation coefficients (R^2) for various models (zero order, first order, Higuchi model, and Korsemeyer peppas model) to understand the mechanism of drug release. The release model having R^2 value close to one was considered as best fit model.

Fourier Transform Infrared Spectroscopic Studies

Drug–polymer compatibility study is an important parameter in the development of stable solid dosage form. Compatibility of GLM, GLM-ERLPO physical mixture and nanosuspension were analyzed by using Fourier-transform infrared spectra (FTIR, SCHIMADZU, Model 8400, Japan) with Pressed pellet technique using potassium bromide. Samples were scanned in the region of 4,000–400 cm^{-1} with a resolution of 4 cm^{-1} for 20 scans.

Differential Scanning Calorimetry and X-Ray Diffraction Studies

Solid-state interaction of physical mixture of drug and excipients was studied using differential scanning calorimetry (DSC) and X-ray diffraction (XRD). DSC thermograms of GLM, ERLPO, GLM-ERLPO physical mixture, and freeze-dried nanosuspension were recorded using simultaneous differential scanning calorimeter (Mettler DSC 25, India). Each sample was scanned in hermetic pan made of aluminum at a heating rate of 10°C/min over the range of 25–300°C using empty aluminum pan as reference. Samples were heated under nitrogen atmosphere (flow rate of N_2−50 ml/min). XRD pattern of GLM, ERLPO, and freeze-dried nanosuspensions were traced by employing X-ray diffractometer (Rigaku powder X-ray diffractometer, Japan) connected to Cu-rotating anode (radiation, λ=1.54 nm) generated at 18 kW.

Microscopic Studies

Freshly prepared nanosuspensions were studied for particle size and shape by using high-resolution microscope (Nikon Eclipse e-200, Japan). Shape and surface morphology of freeze-dried nanoparticles was studied by field emission scanning electron microscopy (SEM; S-4700, Hitachi, Japan).

Stability Studies

Stability studies were carried out according to ICH guidelines Q1A (R2), by storing formulated nanosuspensions at low temperature of 5±3°C (in refrigerator) and high temperature of 40±2°C/75±5% RH (in humidity controlled oven) for 3 months. Physicochemical parameters (particle size, zeta potential, % EE, and cumulative percent release) were analyzed after storage. Freshly prepared nanosuspensions were used as controls.

***In Vivo* Evaluation**

Validation of HPLC Method

The concentration of GLM in rat plasma was determined by the method described with modifications (21). Stock solutions of GLM (1 mg/ml) were prepared by dissolving in methanol. All stock solutions were protected from light and kept at 4°C. Calibration curve in plasma was prepared by spiking stock solution of GLM with drug-free plasma and then extracting it. Extraction of drug from plasma was done by liquid–liquid extraction method using dichloromethane on vortexing for 10 s. It was then centrifuged for 20 min at 3,000 rpm and 4°C. The supernatant was withdrawn and diluted with methanol.

The supernatant was then analyzed by using HPLC method (Cecil CE4201, Cambridge, UK). Isocratic separation was achieved by injecting 20 μl of the supernatant samples in to HPLC C-18 column (Phonomenex, 250×4.60 mm, particles size 5 μm) with UV-visible detector connected to power stream software. The mobile phase consisted of 70% acetonitrile and 30% water acidified with glacial acetic acid (0.1 mM, pH 2.5). Mobile phase was deaerated by sonicator bath then filtered through 0.22 μm membrane filter. The flow rate was optimized to 1 ml/min and UV detection was done at 227 nm.

Experimental Induction of Diabetes in Rat Model

Adult Charles Foster rats of either sex weighing (150± 10 g) were used throughout the study. Rats were obtained from central animal house of Institute of Medical Sciences, Banaras Hindu University, India. The animals were housed in groups of six in polypropylene cages at an ambient temperature of 25±1°C and 45–55% relative humidity, with a 12:12 h light/dark cycle. Rats were provided with commercial food pellets and water *ad libitum*, unless stated otherwise. Rats were acclimatized to laboratory conditions for at least 1 week before using them for experiments. Body weight of rats was measured periodically. Principles of laboratory animal care guidelines (NIH publication number 85–23, revised 1985) were followed. Protocols of the study were approved by Central Animal Ethical Committee of Banaras Hindu University (approval no. Dean/2010-11/62).

Type 2 diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal (i.p.) injection of 65 mg/kg streptozotocin, 15 min after the i.p. administration of 120 mg/kg nicotinamide (22). Hyperglycaemia was confirmed by the elevated glucose level in the blood, determined at 72 h and then on day 7 after injection (23,24). Rats with consistent hyperglycaemia on the seventh day (fasting blood glucose levels, >250 mg/dl) were considered diabetic and were used for *in vivo* studies. All the rats were kept fasting before 24 h of the experiment with water *ad libitum*.

Pharmacokinetic Study

Pharmacokinetic studies were performed on a well-validated nicotinamide–streptozotocin-induced diabetic rat model. Overnight-fasted rats were randomly assigned into different treatment groups (n=6) as follows: group I, diabetic control (1 ml distilled water); group II, diabetic+suspension

(equivalent dose, ~2.5 mg/kg/day of GLM) (25); group III, diabetic+nanosuspension (equivalent dose, ~2.5 mg/kg/day of GLM). The samples were administered to the above groups using oral gavages followed by sufficient volume of drinking water. Blood samples were collected from retro-orbital venous plexus under light ether anesthesia using heparinised capillaries at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after dosing. Plasma was separated by centrifugation at 5,000 rpm for 10 min, and stored at −70°C until analysis. After drug extraction from plasma, samples were analyzed by HPLC. Noncompartmental pharmacokinetic parameters were calculated by using Kinetica 5.0 (Thermo kinetic, trial version). Maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), elimination rate constant (k), and mean residence time (MRT) were determined. The area under the plasma concentration–time curve $[AUC]_{0-24}$ and $[AUC]_{0-\infty}$ were determined by linear trapezoidal rule until last measurement point. The relative bioavailability of nanosuspension to the suspension was calculated by using Eq. 5.

$$\%\ F = [AUC]_{0-24}\text{nanosuspension} / [AUC]_{0-24}\text{suspension} \times 100 \quad (5)$$

Antihyperglycaemic Study

For antihyperglycaemic activity, the rats were divided into three groups (n=6; same as pharmacokinetic study) and then dosed for 7 days. Hyperglycaemia was confirmed from fasting blood glucose level measurement at 505 nm by glucose oxidase–peroxidase (GOD–POD) UV method (24) using glucose estimation kit (Accurex Biomedical Pvt. Ltd). Blood glucose level was measured on days 1 and 7 similar as above. Concentration of glucose was determined by Eq. 6.

$$\text{Glucose (mg / dl)} = \text{absorbance of sample / absorbance of standard} \times \text{concentration of standard (mg / dl)} \quad (6)$$

Oral Glucose Tolerance Test

Oral glucose tolerance test was performed in glucose-loaded rats. Three different treatment groups represented as nondiabetic rats (n=6), fasted overnight, and administered with distilled water (nondiabetic control), GLM suspension (nondiabetic+suspension), and GLM nanosuspensions (nondiabetic+nanosuspension). Glucose (2 g/kg body weight) was orally administered 30 min before various treatments. Blood samples were collected just prior to glucose administration followed by 30, 60, and 120 min after drug administration (23). Fasting blood glucose levels were estimated by using GOD–POD UV method at 505 nm.

Statistical Analysis

Data from different experimental groups were compared by using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. GraphPad Prism 5 and GraphPad InStat (version 3.06) software were used for statistical analysis.

RESULTS AND DISCUSSION

Formulation of Nanosuspensions

Nanosuspension technology appears to be an attractive formulation approach, especially for GLM like poorly water-soluble drugs. Nanoprecipitation method is the commonly used method for the preparation of nanosuspensions at lab scale. As it requires, minimal equipments and fewer amounts of additives it is regarded as more economical method for preparation of nanoparticles. Also, undesired adverse effects caused due to unnecessary excipients are prevented in this method. Method requires preparation of two phases: one in which drug is soluble (solvent phase) and other in which drug is insoluble (antisolvent phase). Mechanism of formation of nanosuspension involves precipitation of drug and polymer from a solvent phase (commonly organic phase) when added to antisolvent phase (aqueous phase) in a controlled manner in the presence of stabilizing agent (11). With slow addition of organic phase to aqueous phase, a quick gradient driven diffusion of acetone out of organic droplets into aqueous phase takes place. Since, polymer and drug both are insoluble in aqueous phase, they get spontaneously precipitated and forms nanosphere matrix. Supersaturated solution of drug and polymer in acetone was prepared to achieve enhanced precipitation. Final solidification into nanosphere was achieved by evaporating the organic solvent under magnetic agitation (19). Acetone (ICH class 3 solvent) was selected for the preparation of organic phase as it is less toxic in comparison to chlorinated solvents and methanol according to ICH Q3C (R4) guidelines. Also, GLM and ERLPO showed good solubility in it. In addition to this, it is volatile enough and does not form azeotropic mixture with water; hence, gets easily evaporated (boiling point −56°C) causing minimum contamination to the formulation. Therefore, formulations can be safely used for *in vivo* studies.

Figure 2 shows schematic representation of nanosuspension formation. Prepared nanosuspensions appeared translucent milky white in appearance without any visible signs of particulate matter (Fig. 1b). The degree of milky white appearance was higher in the batches containing high drug/polymer ratio which is quite obvious.

Formulated nanosuspensions were evaluated for physicochemical characteristics such as particle size, size distribution, surface charge, drug content, entrapment efficiency, release profile, solid state characterization, morphology and *in vivo* activity.

Particle Size

Particle size of prepared batches was observed in the range of 342–648 nm (Fig. 3). The smallest particle size was observed with GNS2 and largest with GNS11. Particle size was observed as a function of processing variables such as agitation time and drug/polymer ratio.

Effect of Agitation Time

Agitation time had an immense effect on particle size and its distribution, followed a proportional relation. Batches (GNS1, GNS3, GNS5, GNS7, GNS9, and GNS11) when

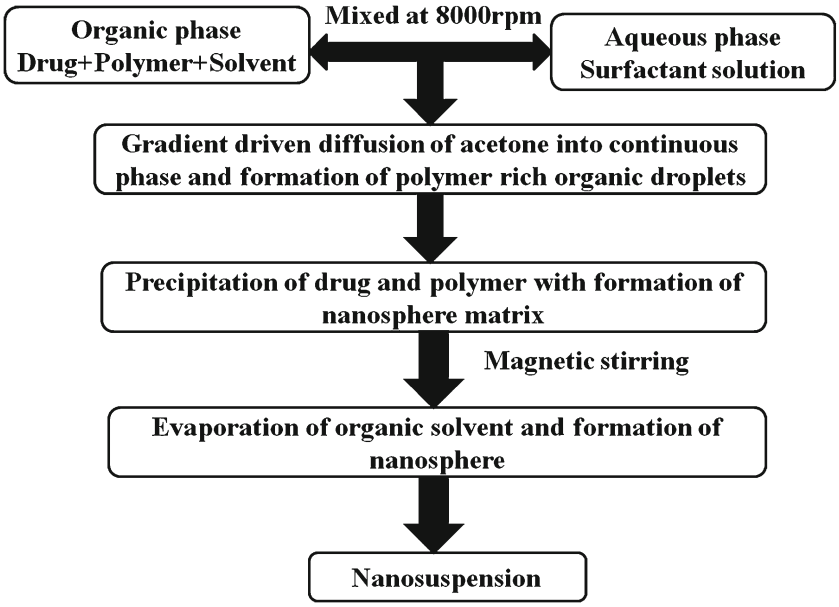

Fig. 2. Schematic representation of mechanism of formation of nanosuspension

agitated for 15 min during preparation resulted in formation of nanoparticles of larger size than those obtained at agitation of 30 min (GNS2, GNS4, GNS6, GNS8, GNS10, and GNS12). Agitation was done in order to cause dispersion of the organic phase droplets in equilibrium with the continuous phase. Results indicated that agitation for longer time produces smaller particles by imparting higher energy to break. An alternative explanation of the above results could be, longer agitation time made the solvent evaporation easier from the surface which leads to shrinkage of droplets and spontaneous rapid precipitation of polymer and drug into smaller mean sizes. These results were consistent with reported studies indicating an important role of agitation time and solvent evaporation in regulating the particle size (26).

Effect of Drug/Polymer Ratio

Although drug/polymer ratio had affected the particle size it was not the primary influencing factor. Batches (GNS3, GNS4, GNS11, and GNS12) with similar drug/polymer ratio of 1:20 had shown significant differences in their particle sizes. These differences were caused by high solid content (including both drug and polymer) of the batches GNS11 and GNS12 as compared to GNS3 and GNS4. An increase in particle size could be attributed to disperse phase viscosity as the supplied energy in the form of agitation was not able to overcome viscous forces produced by high solid content of the formulation (27). Also, high solid content promotes aggregation of particles by increasing the probability of collision between particles present in the aqueous phase (28). As a result, greater viscosity resulted into larger mean particle sizes. Overall the observed particle size data showed more correlation with agitation time than drug/polymer ratio.

Polydispersity Index and Zeta Potential

Polydispersity and zeta potential are important characterization parameters responsible for stability of nanosuspensions. Polydispersity index gives degree of particle size distribution. It ranged from 0.211 to 0.671 depending on the formulation variables (Table II). Higher value of polydispersity index indicates broad particle size distribution. A narrow

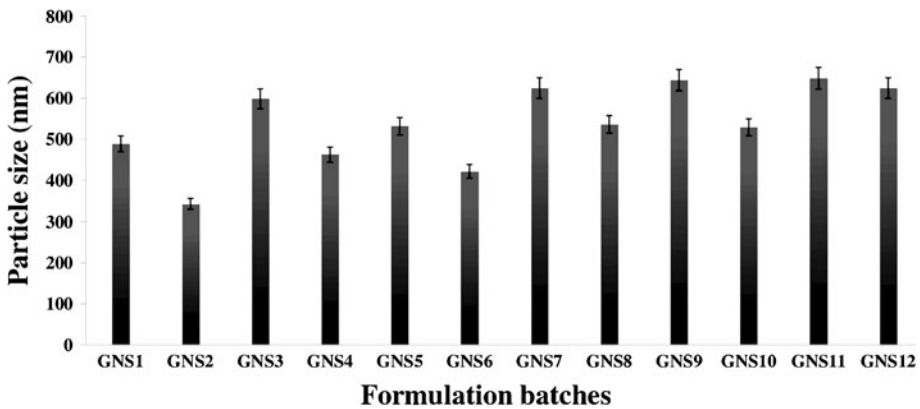

Fig. 3. Observed mean particle size of different batches. *Vertical bar* represents mean±SD (n=3)

Table II. Physicochemical Characteristics of Nanosuspensions

Batch code	Polydispersity	Zeta potential (mV)	Total drug content (%)	Entrapment efficiency (%)	Nanoparticle recovery (%)
GNS1	0.432±0.21	17.92±0.87	96.32±0.98	68.12±2.03	95.61±0.23
GNS2	0.563±1.05	15.82±2.08	95.38±1.73	66.35±3.68	98.41±0.62
GNS3	0.381±0.22	24.32±0.54	98.77±1.21	76.78±3.22	96.82±0.27
GNS4	0.258±0.24	19.43±0.86	95.71±1.32	76.29±1.67	96.31±0.51
GNS5	0.350±0.08	21.66±0.02	97.62±2.43	81.45±3.44	97.28±0.49
GNS6	0.211±0.07	20.79±0.08	98.28±3.25	79.37±5.23	95.56±1.20
GNS7	0.287±0.025	21.97±2.59	96.36±2.87	66.56±2.43	94.89±0.87
GNS8	0.513±0.15	16.98±1.69	97.45±3.64	63.13±1.56	97.77±0.39
GNS9	0.470±0.014	14.68±0.26	99.31±1.29	74.32±2.43	96.91±0.97
GNS10	0.506±2.83	13.60±0.008	96.26±1.23	71.34±3.22	98.59±0.84
GNS11	0.442±0.67	14.32±0.52	97.38±2.38	79.36±2.34	96.45±1.32
GNS12	0.671±0.23	12.37±0.045	96.56±1.36	78.34±2.64	97.63±1.18

Values are mean±SD (n=3)

size distribution is essential to prevent particle growth due to Ostwald ripening and maintaining stability of nanosuspensions (12). Batches having lower polydispersity values showed long-term stability and were preferred for studies.

Zeta potential was observed between +13.60 and +24.32 mV (Table II). High zeta potential indicates high charge on the surface of nanoparticles. This positive high surface charge produces repulsion between particles and prevents their aggregation. Apart from stability, zeta potential also gives information about mucoadhesive nature of particles. The observed positive zeta potential was based on the presence of QAGs in the backbone of Eudragit polymer. This positive zeta potential imparts mucoadhesive property to the nanosuspension as gastrointestinal mucosal layer holds anionic nature at neutral pH and therefore holds affinity for cationic molecules like Eudragit polymers (29,30). Consequently,

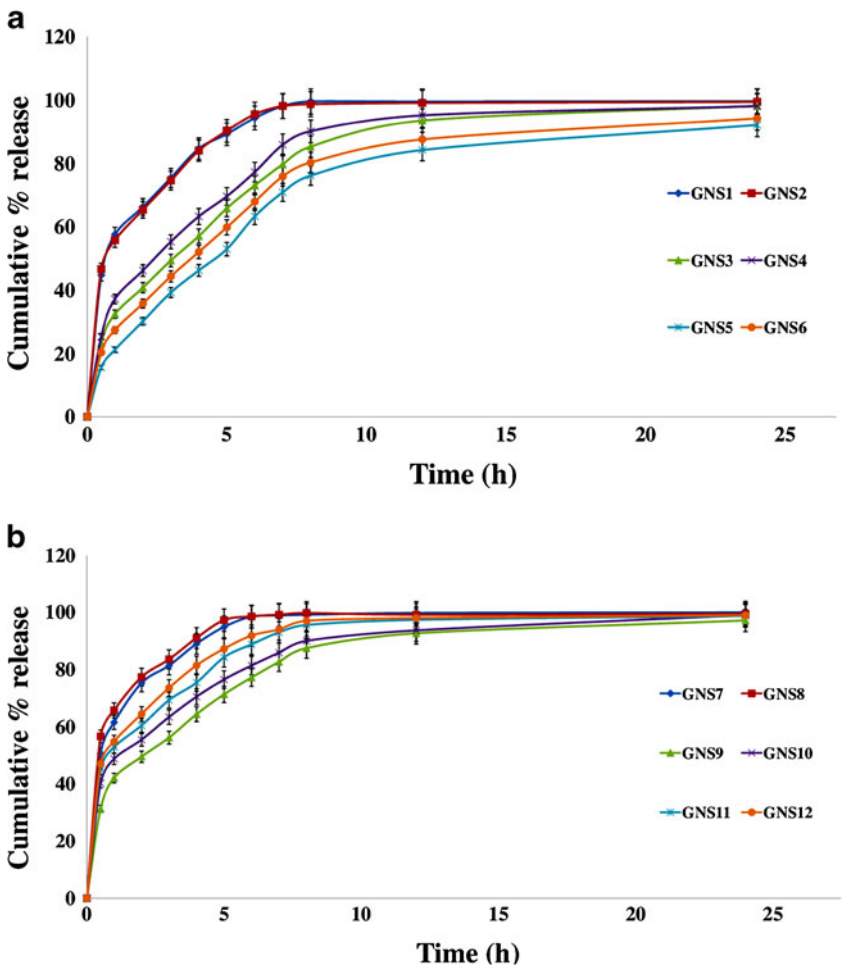

Fig. 4. **a**, **b** *In vitro* release profile of different batches in pH 6.8 buffer. *Vertical bars* represents mean±SD (n=3)

mucoadhesion would promote prolonged absorption and enhanced systemic action of drug.

Total Drug Content and Entrapment Efficiency

Table II shows TDC and % EE for the prepared batches. TDC for all batches was satisfactory and was more than 95% which indicates that loss of drug was lower during preparation process. Drug entrapment efficiency varied from 60% to 81%. Like particle size, % EE was also affected by drug/polymer ratio and agitation time. On increasing polymer concentration, viscosity of organic phase increases which further increases the thickness of droplet coming out of syringe. With this increase diffusional resistance to drug molecules moving from organic phase to aqueous phase also increases and hence more and more drug gets entrapped (27,31,32). Batches prepared with high polymer content showed more % EE as compared to batches with low polymer content. It is reported that acidic compounds such as ciprofloxacin HCl has affinity with Eudragit polymers and hence showed high entrapment efficiency (27). These findings also suggested that beyond certain concentration of drug, saturation of the polymer by drug molecule may occur. Therefore, with the increase in concentration of polymer entrapment efficiency increases but with the increase in concentration of drug it further decreases, due to saturation of polymer with drug as increase in concentration of drug is beyond the entrapment capacity of polymer. % EE can indirectly be correlated to agitation time *via* particle size. Longer agitation produces smaller particles containing lower entrapped drug and freer drug in comparison to larger particles. Therefore it can be concluded that with the increase in particle sizes % EE also increases.

Release Studies and Kinetic Analysis

Drug-loaded NPs showed a biphasic release pattern: burst release followed by a slow release (Fig. 4a, b). The first phase (burst release) was observed initially due to presence of unentrapped drug in the form of SAD and FDC, and the second phase (slow release) was due to slow diffusion (release) of GLM out of the polymeric matrix. In addition, the release during second phase was due to enhanced permeability of ERLPO matrix as it shows pH independent swelling in aqueous media. The batches were optimized and selected on the basis of minimized burst release and maintained controlled release profile in a time-dependent manner. High burst effect observed in the samples with smaller particle size and lower entrapped drug. It may be because of longer agitation time which breaks the particles and increases the free drug content. Batches with drug/polymer

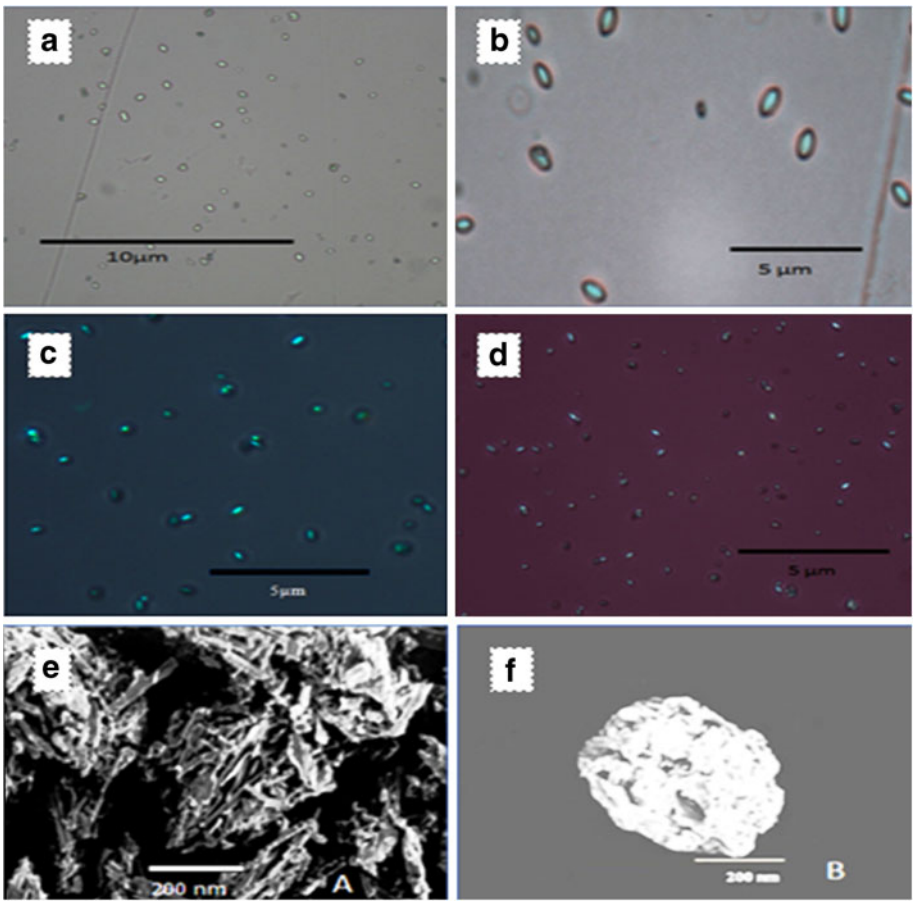

Fig. 5. **a**, **b** Mmicroscopic photographs of prepared nanosuspension; **c**, **d** phase contrast images of particles appearing bright in dark background indicating crystalline deposit on the surface of nanoparticles; **e**, **f** SEM images of lyophilized nanoparticles without cryoprotectant and with cryoprotectant, respectively

ratio 1:5 (GNS7 and GNS8) and 1:10 (GNS1, GNS2, GNS9, and GNS10) showed high burst release of about 60% within 1 h as compared to the batches with higher drug/polymer ratio (1:20 and 1:40). Formulations GNS7 and GNS8 were not considered relevant for further studies as very high burst release of 50–80% occurred within 5 h. Batches (GNS5 and GNS6) with high drug to polymer ratio of 1:40 showed sustained release profile with 95% and 96% drug release in 24 h, respectively; hence, based on more sustained profile and higher entrapped drug (81%) GNS5 was chosen for *in vivo* studies. From the above observations it can be concluded that a more sustained effect is attained with increase in concentration of polymer. *In vitro* kinetic analysis showed that drug release was best explained by Korsmeyer–Peppas equation than other models, with highest value of linearity (R^2>0.9) for all formulations (data not shown). Diffusion coefficient (n) was calculated from the Korsmeyer–Peppas model to evaluate overall mechanism of release. "n" value of all tested formulation were >0.241 and <0.518, indicating drug release following Fickian kinetics. Briefly, drug release from polymer matrix was diffusion controlled process rather than polymer erosion.

Lyophilization of Nanosuspension

Lyophilized samples without cryoprotectants appeared as off-white fluffy, sheet-like materials in aggregated form when observed by SEM (Fig. 5e). On redispersion it showed aggregations. This aggregation problem can be overcome by addition of cryoprotectants (mannitol) during lyophilization which forms white spongy, cotton-like material, having easy redispersibility in water after manual shaking (Fig. 5f). Cryoprotectants are added to improve the stability and integrity of the particles. The aggregation of nanoparticles could be attributed to an increase in the solubility of poloxamer in the bulk solution during lyophilization process (16). Enhanced solubility of poloxamer may lead to removal of protective coating layer of surfactants formed on the surface of nanoparticles causing instability of nanosuspensions. % NR indicates the efficiency of lyophilization process. More than 94% of formulation was recovered after purification and lyophilization process (Table II).

FTIR Spectra

FTIR spectra of GLM, GLM-ERLPO physical mixture, poloxamer 188 and nanosuspension are shown in Fig. 6. The IR spectra of physical mixture matched with those of GLM and ERLPO polymer when superimposed. Physical mixture spectra did not show any changes in characteristic peaks of drug. FTIR spectra of nanosuspensions showed characteristic peaks for drug in the region 2,550–1,500 cm^{-1} indicating that drug had been incorporated into nanosuspension. FTIR spectra demonstrated that no compatibility problem exists between GLM and ERLPO. These findings were further confirmed by DSC and XRD studies.

DSC and XRD Studies

Overlay of DSC thermograms (Fig. 7a, b) and XRD diffraction spectra (Fig. 8a, c) indicates GLM is crystalline (mp=212°C) in nature but ERLPO polymer exists in completely amorphous form. DSC of physical mixture of drug and polymer did not show any drug melting peak or crystallization peak (Fig. 7c). No drug peaks were observed in case of freeze-dried nanosuspension in DSC plot but a sharp peak at 58.5°C was observed (Fig. 7d). This observation can be explained by the presence of poloxamer on the surface of nanoparticles, as it exhibits a melting peak at

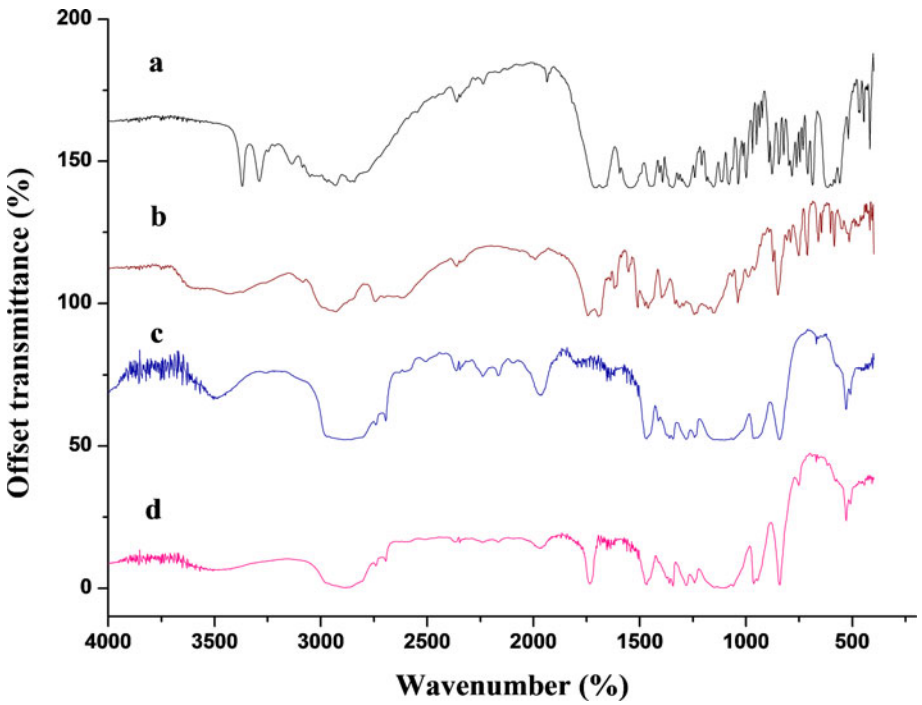

Fig. 6. FTIR spectra, **a** GLM, **b** GLM-ERLPO physical mixture, **c** poloxamer 188, and **d** nanosuspension

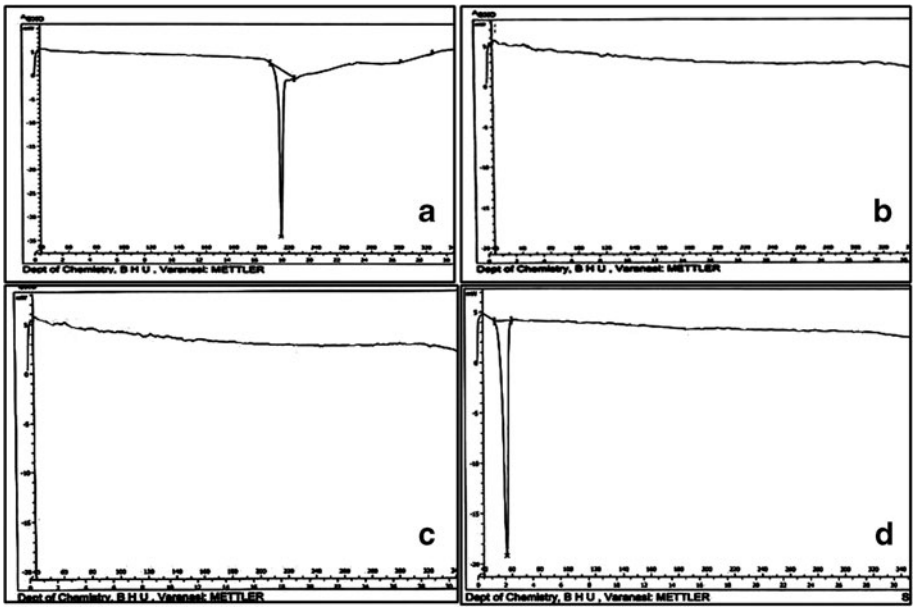

Fig. 7. Comparative DSC plot of **a** GLM, **b** ERLPO, **c** GLM-ERLPO physical mixture, and **d** nanosuspension

58.5°C (33,34). Although DSC studies do not give exact nature of interaction, but gives more valuable information on potential drug–polymer interactions. Drug–polymer interactions can be determined by changes in glass transition temperature of the polymer (35). Here, ERLPO showed flat curve (Fig. 7b) due to its amorphous nature, which can be superimposed on freeze dried nanosuspension curve (except for the presence of P-188 peak; Fig. 7d). Since there were no observable changes in transition pattern of ERLPO when compared with that of drug–polymer mixture and nanosuspension, ERLPO polymer is considered compatible with drug.

XRD studies further supported the results obtained from DSC data, considerable reduction in drug crystallinity can be observed on comparing the XRD peaks of GLM and that of nanosuspension (Fig. 8a, b). The progressive disappearance of X-ray drug signals in nanosuspension indicated a decrease in overall crystallinity of GLM. Broadening and reduction in intensity of the two major peaks (at 19.06° and 23.24°) were more likely due to reduction in crystallite size of GLM (Fig. 8b). The enhanced dissolution of GLM can be attributed to its decreased crystallinity when formulated into nanoparticles.

Microscopic Studies

Microscopic examination of the prepared nanosuspension illustrates**,** nanoparticles were more or less uniformly distributed in the formulation with no signs of aggregation (Fig. 5a–d). They were not completely spherical but slightly elongated in shape. When observed in phase-contrast microscope, they appeared bright, this may be due to the presence of small crystalline drug particles deposited on the surface. SEM surface studies showed elongated nanoparticles (Fig. 5f) with porous surface. Pores were necessarily associated with evaporation of solvent from the surface.

Stability Study of Nanosuspension

Stability studies were performed to investigate the effect of storage conditions on the physicochemical characteristics of nanosuspensions and also to find out most suitable storage condition. More significant ($p<0.05$) changes in physicochemical properties was found in case of high temperature storage (40±2°C/75±5%) than refrigerated samples (refrigerated at 5 ±3°C; Table III). On storage for 3 months, particle size increased at both storage conditions, but larger particles were

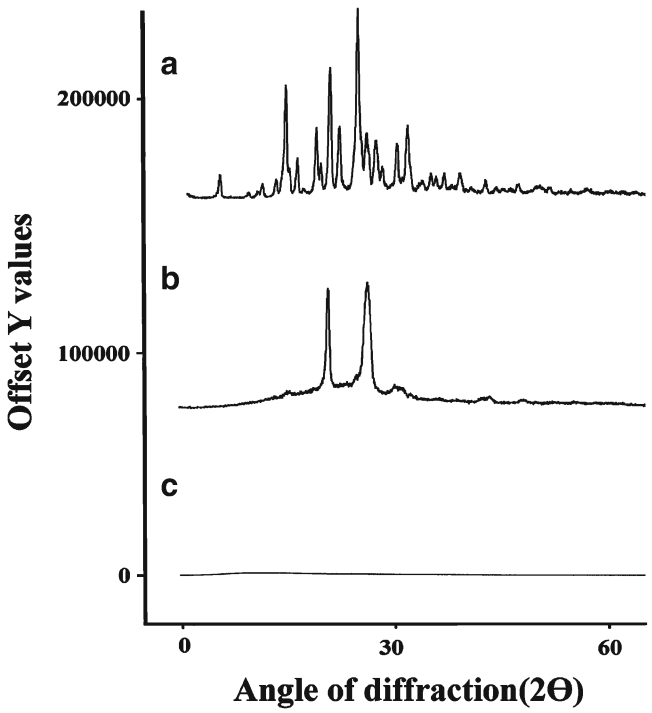

Fig. 8. XRD spectra, **a** GLM, **b** nanosuspension, and **c** ERLPO

Table III. Stability Testing Parameters of Prepared Formulation at Different Storage Conditions

Evaluation parameters (after 3 months)	Fresh formulation	Storage condition	
		Low temperature (5±3°C)	High temperature (40±2°C/75±5%)
Particle Size (nm)	531±1.26	562±1.65	658±2.21
% Entrapment efficiency	80±2.34	79.11±1.43	76.56±3.16
Cumulative % release	92.11±2.11	94.88±4.35	98±3.68

Values are mean±SD (n=3)

observed in case of high temperature (658±2.21 nm) than low temperature (562±1.65 nm) storage. Slight reduction in % EE was observed at low temperature than high temperature storage. Stored batches showed slightly higher burst release as compared to freshly prepared batches (Fig. 9). The release was, however, sustained thereafter for the whole period of the study. Reduced % EE and enhanced burst release can be attributed to leaching of drug from the polymer and increase in free drug content during. Erosion of nanoparticle may be another reason for enhanced burst release, which needs to be further, investigated (36). Deposits on the base of container formed during storage were easily redispersible on manual shaking. No changes in macroscopic properties were observed. Above results indicated that NPs showed good stability at refrigerated conditions than at higher temperature. Therefore, it is strongly recommended to store nanosuspension at refrigerated condition for maintaining the integrity of particles, safe and effective long-term use.

In Vivo Studies

Pharmacokinetic Study

GLM was detected and quantified in plasma by using HPLC method with retention time of 6.10±0.421 min (n=6). No other plasma components were eluted at the retention time of GLM. Standard calibration curve in rat plasma was found to be linear at concentrations ranging from 0 to 5,000 ng/ml (Y= 0.0318x+1.4027) with correlation coefficient of 0.9968. For intra-day precision and accuracy, three replicate samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on three different days. The results were found within limits (data not shown).

For pharmacokinetic evaluation, plasma drug concentration time profile (Fig. 10) and pharmacokinetic parameters related with GLM nanosuspension were compared to suspension following oral administration (Table IV). Nanosuspension and suspension of GLM showed significantly different (p<0.05) C_{max} of about 405.32 and 251.25 ng/ml, respectively, after 3 h of dosing. Achievement of higher C_{max} by nanosuspension can be explained by increase in saturation solubility of nanoparticles as they are absorbed without initial time consuming dissolution step. Above results are supported by Xie and Wang (37). Observed mean plasma $[AUC]_{0-24\ h}$ value (6,460.80 ng/ml h) for nanosuspension was significantly (p< 0.01) higher than drug suspension (3,172.3 ng/ml h) which indicates nearly twofold improvement in relative bioavailability of nanosuspension. Observed higher values of AUC and MRT of nanosuspension can be credited to its mucoadhesive nature imparted by positive zeta potential, hence provided longer gastric residence and extent of release (38). Plasma concentration profile of nanosuspension showed more sustained plasma drug level than suspension for 24 h. In summary, orally administered nanosuspension provided sustained and prolonged effect, which may subsequently improve patient compliance by reduction in dose and dosing frequency to a remarkable level.

Antihyperglycaemic Study

In this study, rats subjected to nicotinamide–streptozotocin challenge showed increase in plasma glucose level.

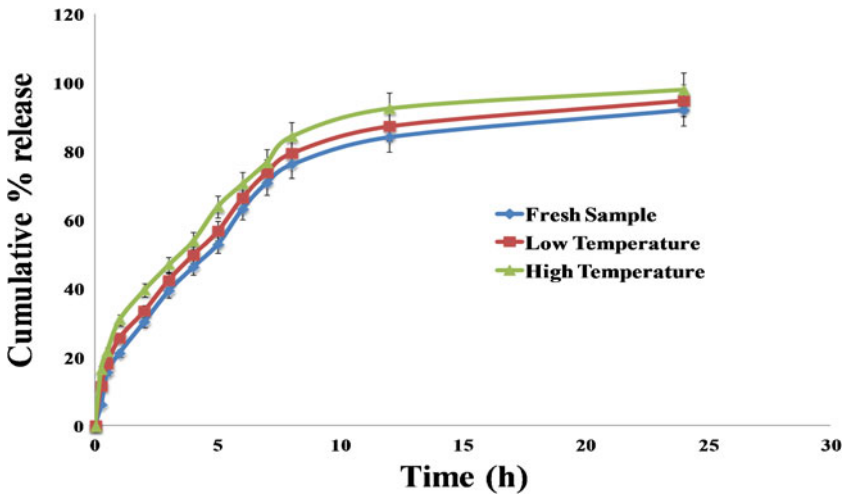

Fig. 9. *In vitro* release profile of freshly prepared and stored batches of nanosuspension. *Vertical bars* represents mean±SD (n=3)

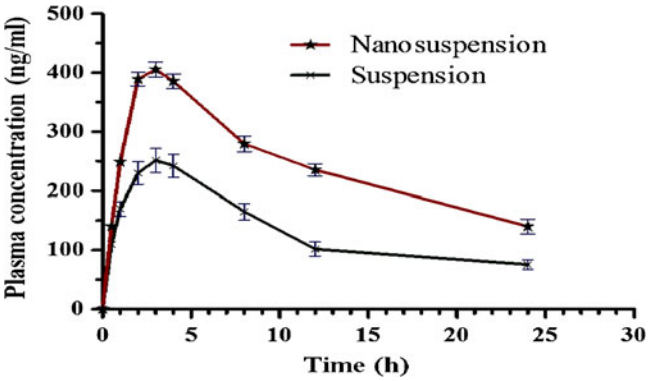

Fig. 10. Plasma concentration *vs.* time profile of GLM suspension and nanosuspension after oral administration into diabetic rats. *Vertical bars* represents mean±SD (n=6)

Nanosuspension-treated diabetic rats significantly reduced the elevated fasting blood glucose level on single-dose treatment for 1 day (Fig. 11a) and single-dose treatment for 7 days (Fig. 11b). The diabetic rats treated with nanosuspension demonstrated statistically significant (p<0.01) reduction in blood glucose levels as compared to diabetic control rats. Also, diabetic rats treated with nanosuspension demonstrated statistically significant difference (p<0.01 and p<0.001) in blood glucose levels as compared to diabetic rats treated with suspension. Hence, GLM nanosuspension showed surpassing behavior over suspension in lowering the blood glucose level and maintaining it for 7 days.

Throughout the study, body weight of animals was monitored as diabetes was found to be associated with a characteristic loss of body weight in animals. Several hypotheses have been proposed for the body weight loss in diabetic animals most important is increased muscle wasting (39,40). A significant weight loss was observed in all groups after 7 days (Fig. 12). Diabetic rats treated with suspension showed significant less weight loss than that of diabetic rats treated with nanosuspension (p<0.01) compared to diabetic control rats (p <0.001, and p<0.05), respectively. Overweight is a side effect of sulfonylurea drugs used in the treatment of type 2 diabetes mellitus. Less weight loss on day 7 was observed in case of suspension-treated groups as compared to nanosuspension of GLM (p<0.01), which may be due to GLM causing weight gain in type 2 diabetics. Here, from this observation, it can be concluded that, treatment of diabetic rats with nanosuspension of GLM demonstrated lower incidence of side effect than suspension of GLM.

Table IV. Comparative Illustration of Pharmacokinetic Parameters of GLM Nanosuspension *vs.* Suspension

Pharmacokinetic parameters	Suspension	Nanosuspension
T_{max} (h)	3.0±0.52	3.0±0.23
C_{max} (ng/ml)	251.25±0.76	405.32±0.05
$[AUC]_{0-24}$ (ng h/ml)	3,172.34±1.05	6,460.79±0.97
$[AUC]_{0-\infty}$ (ng h/ml)	4,459.82±1.30	9,034.39±1.21
MRT_{0-t}(h)	18.65±0.78	23.54±1.02
$T_{1/2}$ (h)	11.75±1.52	16.19±1.34
% F	–	203.66

Values are mean±SD (n=6)

Oral Glucose Tolerance Test

Oral glucose tolerance test was performed to measure the ability of prepared formulations to lower the blood glucose level. The test was performed on glucose loaded normal rats or nondiabetic rats. Statistical analysis observed a significant decrease in blood glucose level 60 min after glucose administration in case of non-diabetic+suspension (p<0.05) and non-diabetic+nanosuspension (p<0.001) as compared to nondiabetic control (Fig. 13). On the other hand, no significant changes in blood glucose level were observed between GLM suspension-treated and nondiabetic control rats after 120 min of glucose administration. Groups treated with nanosuspension group showed significant lowering of blood glucose levels after 30 min (p<0.05), 60 min (p<0.001), and 120 min (p<0.01) of GLM treatment in comparison to nondiabetic control. These results reveal that nanosuspension have

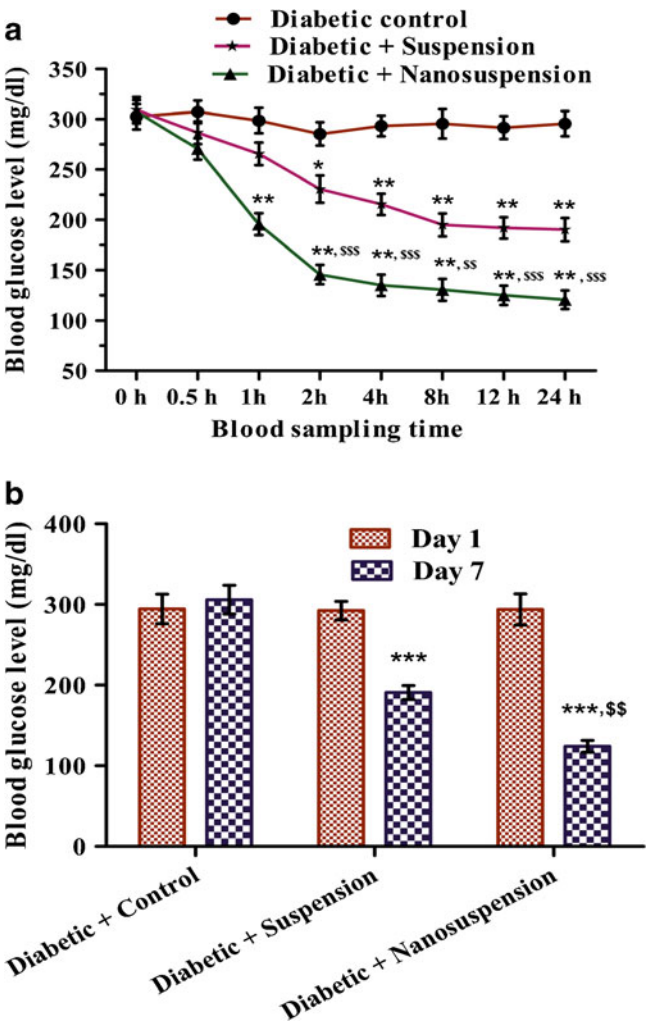

Fig. 11. **a** Showing blood glucose level different groups of rats on single dose (day 1). **b** *Bar graph* represents blood glucose level of rats as observed on days 1 and 7. *Vertical bars* represents mean±SEM, n= 6. *Superscripted asterisk* and *dollar sign* denotes statistical significant difference between treatment groups. *p<0.05, **p<0.01, and ***p< 0.001 *vs.* diabetic control; $^{\$\$}p$<0.01 and $^{\$\$\$}p$<0.001 *vs.* diabetic+ suspension (one-way ANOVA followed by Newman–Keuls multiple comparison test)

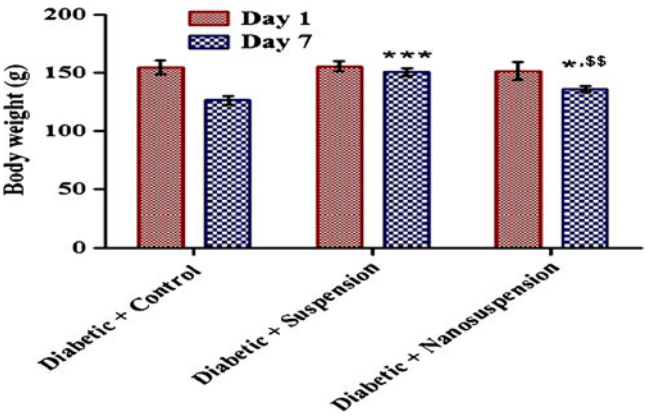

Fig. 12. Body weight comparison on first and seventh day of treatment. *Vertical bars* represents mean±SEM, $n=6$. *Superscripted asterisk* and *dollar sign* denotes statistical significant difference between treatment groups. $^{*}p<0.05$ and $^{***}p<0.001$ *vs.* diabetic control; $^{\$\$}p<0.01$ *vs.* diabetic+suspension (one-way ANOVA followed by Newman–Keuls multiple comparison test)

longer duration of action and facilitate efficient glucose utilization by significant ($p<0.05$) lowering the blood glucose level as compared to suspension.

CONCLUSION

From the above study, it is evident that ERLPO polymer successfully retarded the release of GLM, a model poorly water soluble antidiabetic drug and provided sustained and prolonged effect over 24 h. Subsequently, above results propose improved patient compliance by reducing therapeutic dose, dosing interval and systemic side effects which further needs to be established in clinical trials. The polymer was proved to be capable of entrapping high quantities of drug (up to 81%) depending on drug/polymer ratio and agitation time. DSC and XRD studies revealed that enhanced solubility of drug can be attributed to loss in crystallinity of drug. High stability was observed when nanosuspensions were stored at refrigerated condition. Prolonged GIT residence due to unique particle size and positive zeta potential was evident. *In vivo* studies showed that nanosuspensions exhibited better pharmacokinetic profile, efficiently reduced blood glucose level and maintained it to desirable level as compared to GLM suspension. *In vivo* studies showed a good correlation with *in vitro* studies. Therefore, GLM nanosuspension can be expected to gain considerable attention for improved therapeutic activity for the treatment of diabetes mellitus. Moreover, for BCS class II drugs, nanosuspensions appear as a very promising approach for enhancing solubility as well as oral bioavailability.

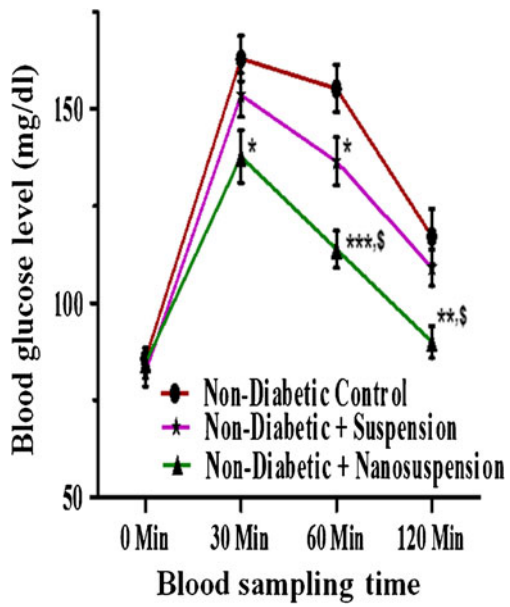

Fig. 13. Illustration of oral glucose tolerance test and effect of GLM on blood glucose level in non diabetic rats. *Vertical bars* represents mean± SEM, $n=6$. *Superscripted asterisk* and *dollar sign* denotes statistical significant difference between treatment groups. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ *vs.* nondiabetic control; $^{\$}p<0.05$ *vs.* nondiabetic+ suspension (one-way ANOVA followed by Newman–Keuls multiple comparison test)

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REFERENCES

1. Ammar HO, Salama HA, Ghorab M, Mahmoud AA. Formulation and biological evaluation of glimepiride–cyclodextrin–polymer systems. Int J Pharm. 2006;309(1–2):129–38.
2. Frick A, Moller H, Wirbitzki E. Biopharmaceutical characterization of oral immediate release drug products. *In vitro/in vivo* comparison of phenoxymethylpenicillin potassium, glimepiride and levofloxacin. Eur J Pharm Biopharm. 1998;46 (3):305–11.
3. Ilic I, Dreu R, Burjak M, Homar M, Kerc J, Srcic S. Microparticle size control and glimepiride microencapsulation using spray congealing technology. Int J Pharm. 2009;381(2):176–83.
4. Babu RJ, Pandit JK. Effect of aging on the dissolution stability of glibenclamide/beta-cyclodextrin complex. Drug Dev Ind Pharm. 1999;25(11):1215–9.
5. Reven S, Grdadolnik J, Kristl J, Zagar E. Hyperbranched poly (esteramides) as solubility enhancers for poorly water-soluble drug glimepiride. Int J Pharm. 2010;396(1–2):119–26.
6. Junghanns JUAH, Muller RH. Nanocrystal technology, drug delivery and clinical applications. Int J Nanomedicine. 2008;3 (3):295–309.
7. Kesisoglou F, Panmai S, Wu Y. Nanosizing—oral formulation development and biopharmaceutical evaluation. Adv Drug Deliv Rev. 2007;59(7):631–44.
8. Jacobs C, Muller RH. Production and characterization of a budesonide nanosuspension for pulmonary administration. Pharm Res. 2002;19(2):189–94.
9. Kipp JE. The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs. Int J Pharm. 2004;284(1–2):109–22.
10. Merisko-Liversidge EM, Liversidge GG. Drug nanoparticles: formulating poorly water-soluble compounds. Toxicol Pathol. 2008;36(1):43–8.
11. Verma S, Gokhale R, Burgess DJ. A comparative study of top-down and bottom-up approaches for the preparation of micro/nanosuspensions. Int J Pharm. 2009;380(1–2):216–22.

12. Muller RH, Jacobs C. Buparvaquone mucoadhesive nanosuspension: preparation, optimisation and long-term stability. Int J Pharm. 2002;237(1–2):151–61.
13. Reichal CR, Lakshmi JB, Ravi TK. Studies on formulation and *in vitro* evaluation of glimepiride floating tablets. J Chem Pharm Res. 2011;3(3):159–64.
14. Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int J Pharm. 1989;55(1):R1–4.
15. Guterres SS, Fessi H, Barratt G, Devissaguet JP, Puisieux F. Poly (DL-lactide) nanocapsules containing diclofenac: I. Formulation and stability study. Int J Pharm. 1995;113 (1):57–63.
16. Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev. 2006;58(15):1688–713.
17. Omari DM, Sallam A, Abd-Elbary A, El-Samaligy M. Lactic acid-induced modifications in films of Eudragit RL and RS aqueous dispersions. Int J Pharm. 2004;274(1–2):85–96.
18. Amlathe S, Gupta VK. Spectrophotometric determination of acetone using vanillin. Analyst. 1990;115(10):1385–7.
19. Chorny M, Fishbein I, Danenberg HD, Golomb G. Lipophilic drug loaded nanospheres prepared by nanoprecipitation: effect of formulation variables on size, drug recovery and release kinetics. J Control Release. 2002;83(3):389–400.
20. Mishra B, Arya N, Tiwari S. Investigation of formulation variables affecting the properties of lamotrigine nanosuspension using fractional factorial design. Daru. 2010;18(1):1–8.
21. Rabbaa-Khabbaz L, Daoud RA, Karam-Sarkis D, Atallah C, Zoghbi A. A simple and sensitive method for determination of glimepiride in human serum by HPLC. J Liq Chromatogr Relat Technol. 2005;28(20):3255–63.
22. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D, *et al*. Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes. 1998;47(2):224–9.
23. Shirwaikar A, Rajendran K, Kumar CD, Bodla R. Antidiabetic activity of aqueous leaf extract of *Annona squamosa* in streptozotocin-nicotinamide type 2 diabetic rats. J Ethnopharmacol. 2004;91(1):171–5.
24. Lott JA, Turner K. Evaluation of Trinder's glucose oxidase method for measuring glucose in serum and urine. Clin Chem. 1975;21 (12):1754–60.
25. Kuppusamy A, Muthusamy U, Thirumalaisamy SA, Varadharajan S, Ramasamy K, Ramanathan S. *In vitro* (α-glucosidase and α-amylase inhibition) and *in vivo* antidiabetic property of phytic Acid (IP6) in streptozotocin–nicotinamide-induced type 2 diabetes mellitus (NIDDM) in rats. J Complement Integr Med. 2011;8 (1):doi:10.2202/1553-3840.1483.
26. Pignatello R, Bucolo C, Ferrara P, Maltese A, Puleo A, Puglisi G. Preparation and characterization of Eudragit retard nanosuspensions for the ocular delivery of cloricromene. AAPS PharmSciTech. 2006;7(1):E192–8. doi:10.1208/pt070127.
27. Dillen K, Vandervoort J, Mooter GV, Ludwig A. Evaluation of ciprofloxacin-loaded Eudragit® RS100 or RL100/PLGA nanoparticles. Int J Pharm. 2006;314(1):72–82.
28. Murakami H, Kobayashi M, Takeuchi H, Kawashima Y. Preparation of poly(DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method. Int J Pharm. 1999;187(2):143–52.
29. Ubrich N, Schmidt C, Bodmeier R, Hoffman M, Maincent P. Oral evaluation in rabbits of cyclosporin-loaded Eudragit RS or RL nanoparticles. Int J Pharm. 2005;288(1):169–75.
30. Bayems V, Gurny R. Chemical and physical parameters of tears relevant for the design of ocular drug delivery formulations. Pharm Acta Helv. 1997;72(4):191–202.
31. Das S, Suresh PK, Desmukh R. Design of Eudragit RL 100 nanoparticles by nanoprecipitation method for ocular drug delivery. Nanomedicine. 2010;6(2):318–23.
32. Thioune O, Fessi H, Devissaguet JP, Puisieux F. Preparation of pseudolatex by nanoprecipitation: influence of the solvent nature on intrinsic viscosity and interaction constant. Int J Pharm. 1997;146(2):233–8.
33. Passerini N, Albertini B, Gonzalez-Rodriguez ML, Cavallari C, Rodriguez L. Preparation and characterisation of ibuprofen-poloxamer 188 granules obtained by melt granulation. Eur J Pharm Sci. 2002;15(1):71–8.
34. Liu D, Fei X, Wang S, Jiang T, Su D. Increasing solubility and dissolution rate of drugs *via* eutectic mixtures: itraconazole-poloxamer188 system. Asian J Pharm Sci. 2006;1(3–4):213–21.
35. Glaessl B, Siepmann F, Tucker I, Siepmann J, Rades T. Characterisation of quaternary polymethacrylate films containing tartaric acid, metoprolol free base or metoprolol tartrate. Eur J Pharm Biopharm. 2009;73(3):366–72.
36. Dandagi P, Kerur S, Mastiholimath V, Gadad A, Kulkarni A. Polymeric ocular nanosuspension for controlled release of acyclovir: *in vitro* release and ocular distribution. IJPR. 2009;8(2):79–86.
37. Xie J, Wang CH. Self-assembled biodegradable nanoparticles developed by direct dialysis for the delivery of paclitaxel. Pharm Res. 2005;22(12):2079–90.
38. Kim YI, Fluckiger L, Hoffman M, Lartaud-Idjouadiene I, Atkinson J, Maincent P. The antihypertensive effect of orally administered nifedipine-loaded nanoparticles in spontaneously hypertensive rats. Br J Pharmacol. 1997;120(3):399–404.
39. Swanston-Flatt SK, Day C, Bailey CJ, Flatt PR. Traditional plant treatment for diabetes: studies in normal and streptozotocin diabetic mice. Diabetologia. 1990;33(8):462–4.
40. Mastrocola R, Reffo P, Penna F, Tomasinelli CE, Boccuzzi G, Baccino FM, *et al*. Muscle wasting in diabetic and in tumor-bearing rats: role of oxidative stress. Free Radic Biol Med. 2008;44(4):584–93.