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Research Article

Lyophilized Oral Sustained Release Polymeric Nanoparticles of Nateglinide

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Abstract. The objective of this study is to formulate lyophilized oral sustained release polymeric nanoparticles of nateglinide in order to decrease dosing frequency, minimize side effects, and increase bioavailability. Nateglinide-loaded poly ε-caprolactone nanoparticles were prepared by emulsion solvent evaporation with ultrasonication technique and subjected to various studies for characterization including scanning electron microscopy (SEM), Fourier transform infrared spectroscopy, photon correlation spectroscopy and evaluated for *in vitro* drug release and pharmacodynamic studies. The influence of increase in polymer concentration, ultrasonication time, and solvent evaporation rate on nanoparticle properties was investigated. The formulations were optimized based on the above characterization, and the formulation using 5% polymer, 3-min sonication time, and rota-evaporated was found to have the best drug entrapment efficiency of 64.09±4.27% and size of 310.40±11.42 nm. Based on SEM, nanoparticles were found to be spherical with a smooth surface. *In vitro* drug release data showed that nanoparticles sustained the nateglinide release for over 12 h compared to conventional tablets (Glinate 60 mg), and drug release was found to follow Fickian mechanism. *In vivo* studies showed that nanoparticles prolonged the antidiabetic activity of nateglinide in rats significantly ($p \leq 0.05$) compared to the conventional tablets (Glinate 60 mg) over a period of 12 h. Accelerated stability data indicated that there was minimal to no change in drug entrapment efficiency.

KEY WORDS: drug encapsulation efficiency; nanoparticles; poly ε-caprolactone (PCL); probe sonication.

INTRODUCTION

Diabetes mellitus (type II) is growing as a major public health problem throughout the world and is associated with increased cardiovascular mortality, so an attempt has been made towards anti-diabetic treatments. Nateglinide, 3-phenyl-2-[(4propan-2ylcyclohexane carbonyl) amino] propanoic acid, is an oral meal time glucose regulator. Nateglinide lowers blood glucose by stimulating the release of insulin from the pancreas by closing ATP-dependent potassium channels in the membrane of the β cells (1,2). In contrast to sulfonylureas, nateglinide increases pancreatic β cell sensitivity to ambient glucose without increasing basal insulin secretion. It can be used as monotherapy or in combination with metformin or thiazolidinediones. It has short half-life of 1.5 h, and peak plasma concentration reaches at 0.5–1.0 h. It is metabolized by cytochrome P-450 system to inactive metabolite and eliminated with half-life of 1.4 h.

Nateglinide is available in 60- and 120-mg immediate release tablets which is required to be administered twice or thrice a day. Nateglinide was formulated into various dosage forms such as solid lipid nanoparticles, microparticles, and matrix tablets. The rationale of this study is to formulate lyophilized oral sustained release polymeric nanoparticles of nateglinide in order to decrease dosing frequency, minimize side effects, and increase bioavailability. Lyophilized nanoparticles also stabilize the formulation by preventing particle aggregation, degradation reactions (hydrolysis), and leakage of encapsulated drug (3).

Among the various possible methods developed to prepare nanoparticles from biodegradable polymers, emulsion solvent evaporation method was selected (4). Poly ε-caprolactone (PCL) is a biodegradable and biocompatible polyester used to encapsulate nateglinide within nanospheres. The use of polymeric materials enables the modulation of physicochemical characteristics (e.g., hydrophobicity and zeta potential), drug release properties (e.g., delayed, prolonged, triggered), and biological behavior (e.g., targeting, bioadhesion, improved cellular uptake) of nanoparticles (5,6).

MATERIALS AND METHODS

Materials

Nateglinide USP was a kind gift sample from Cadila Pharmaceuticals Ltd. (Ahmadabad, India). Poly ε-caprolactone, polyvinyl alcohol, and dialysis membrane were purchased from HiMedia (Hyderabad, India). All the solvents were of high-performance liquid chromatography (HPLC) grade and purchased from Merck Chemicals.

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Chromatographic Conditions (7)

A high-performance liquid chromatography (LC-2010 C_{HT}) with SPD-M20A Prominance Diode Array detector was used. Separation was carried out on a Phenomenex C_{18} column (particle size 5 μm; 250×4.6 mm i.d) using ACN: 10 mM KH_2PO4 buffer solution [phosphate-buffered saline (PBS); adjusted to pH 3.0 with H_3PO_4] (70:30, *v/v*) as mobile phase at 203 nm, 1.0 mL/min flow rate and 25°C.

Characterization of Marketed Tablets

Glinate® 60-mg tablets manufactured by Glenmark Pharmaceuticals Ltd were chosen as reference based on the dose and wide market. Glinate® was found to have a round concave shape, 8 mm size, 3 mm thickness, 4.5 kg/cm^2 hardness, 7 min disintegration time and passed the test of friability (<1%). *In vitro* release of tablets in PBS was found to be 92.41±3.52%. Type II dissolution apparatus was used to study *in vitro* drug release at 37°C and pH 7.4 as buffer media. At pre-determined time points, 5 mL of sample was withdrawn and analyzed using HPLC. Each time, 5 mL of fresh phosphate buffer saline pH 7.4 was replaced.

Preparation of Nateglinide Nanoparticles

In this study, nanoparticles were prepared using an emulsion solvent evaporation with ultrasonication technique, a modified method used by Kim *et al*. (8,9). In this procedure, a specific amount of PCL (300 or 500 mg) was dissolved in 10 mL of solvent (methylene chloride) containing 60 mg of nateglinide. A polyvinyl alcohol (PVA, 2 g/100 mL) solution was prepared in PBS buffer. PCL solution was gradually added to 40 mL of the PVA solution. Polyvinyl alcohol was used as stabilizer (10). The total mixture was then emulsified using mechanical stirring at moderate speed for 30 min to obtain an oil-in-water emulsion and then ultrasonicated for specific time (1, 2, and 3 min) to reduce the size of globule. Another 40 mL of the PVA solution was then added to the emulsion. The final solution was flash evaporated to allow the evaporation of methylene chloride facilitating the formation of nanoparticles. The suspension was collected and centrifuged at 6,000 rpm for 45 min. The pellet was resuspended in distilled water and centrifuged twice at 1,500 rpm for 20 min each. These washing steps were performed to remove unencapsulated PVA and nateglinide. The nanoparticles were collected and freeze dried for 32 h. Lyophilization model used was BOC Edwards freeze dryer (240 sq ft). The lyophilized nanoparticles were stored at 4°C. The lyophilization conditions used are shown in Table I.

Table I. Lyophilization Conditions

	Temp (°C)	Ramp (min)	Soak (min)	Vaccum (m.bar)
Freezing	−45	120	150	0
Primary drying	−30	60	440	0.250
	−17	40	180	0.250
	−4	40	180	0.200
	10	30	200	0.200
	25	30	180	0.100
Secondary drying	38	30	240	0.050
Total time		350	1,570	

Table II. Different Ratios Used for Preparing Nateglinide Loaded Nanoparticles

Code	PCL concentration (g/100 mL)	Probe sonication time (min)
F 1	3	1
F 2	3	2
F 3	3	3
F 4	5	1
F 5	5	2
F 6	5	3

Percentage Drug Entrapment Efficiency (11)

Entrapment efficiency was determined by an extraction method. Five milliliters of HPLC grade water was added to accurately weighed freeze dried nanoparticles to make a suspension and then dissolved in 5 mL of methylene chloride. The mixture was vigorously vortexed for 1 min and sonicated for 8 min in order to extract the nateglinide into the organic solution. Then, methylene chloride was evaporated and replaced with methanol and analyzed by HPLC. Encapsulation efficiency was calculated using the ratio of the mass of nateglinide determined analytically to the mass of nateglinide added during the formation process, as shown in Eq. (1).

$$\text{Drug entrapment efficiency} = \frac{\text{mass of nateglinide determined (milligrams)}}{\text{mass of nateglinide added (milligrams)}} \times 100 \quad (1)$$

Particle Size and Poly Dispersity Index

Particle size and size distribution were determined by Zetasizer Nano ZS-90 (Malvern Instruments Ltd). For particle size analysis, a dilute suspension of nanoparticles was prepared by suspending nanoparticles in 100 mL of distilled water.

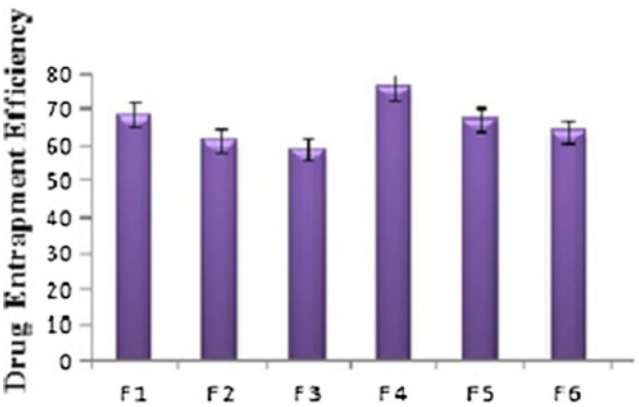

Fig. 1. Percentage drug entrapment efficiency of various formulations

Table III. Effect of Probe Sonication Time on Drug Entrapment Efficiency

Probe sonication time (min)	Particle size (nm)
1	482.19±12.29
2	361.24±9.13
3	310.40±11.42

Scanning Electron Microscopy

In order to examine the particle surface morphology and shape, scanning electron microscopy (SEM) was used. Images were taken using JSM-5200 SEM (Tokyo, Japan) at 10 kV with 750 and 2,000 magnification, and a 10-μm scale bar was used.

Fourier Transform Infrared Spectroscopy

The Fourier transform infrared analysis was conducted to verify the possibility of interaction of chemical bonds between drug and polymer. The Fourier transform infrared spectroscopy (FTIR) spectrum was performed by using Shimadzu 8400S FTIR. The samples were scanned in the spectral region between 4,000 and 400 cm^{-1}.

In vitro Drug Release Study (12)

The *in vitro* release of nanoparticulate suspension was carried out using dialysis bag diffusion method. Here, 2 mL of the nanoparticulate suspension was enclosed in a dialysis bag (cellulose acetate membrane with molecular weight cutoff value of 10,000 and suspended into a beaker containing 100 mL of release media (phosphate buffer saline pH 7.4) at 37°C. Drug release was assessed by intermittently sampling the receptor media (5 mL) at predetermined time intervals. Each time, 5 mL of fresh phosphate buffer saline pH 7.4 was replaced. The amount of nateglinide released in the buffer solution was quantified by high-performance liquid chromatography.

Kinetics of Drug Release

In order to analyze the drug release mechanism, *in vitro* release data were fitted into a zero-order, first order, Higuchi, Hixson-Crowell cube root law, Korsmeyer-Peppas model.

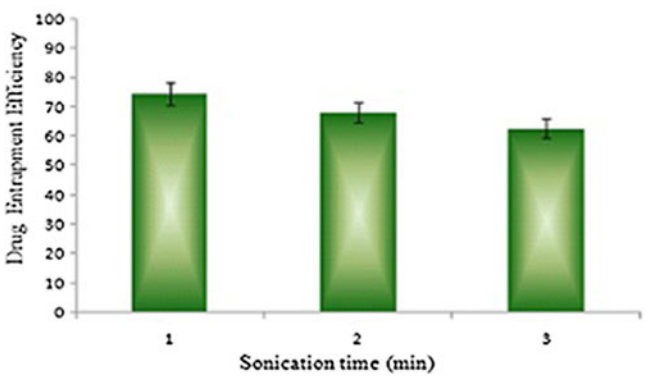

Fig. 2. Effect of probe sonication time on drug entrapment efficiency

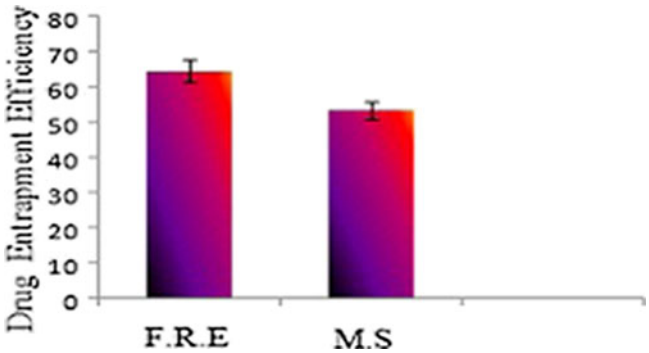

Fig. 3. Influence of organic solvent evaporation rate on drug entrapment efficiency

In Vivo Evaluation Studies of Nateglinide Nanoparticles (13–15)

Optimized sustained release formulation of nateglinide nanoparticles F6 was evaluated for pharmacodynamic activity, i.e., antidiabetic activity. The protocol was approved by the Sri Venkateshwara Institutional Animal Ethics Committee of Sri Venkateshwara College of Pharmacy and Research Center, Hyderabad (protocol number, SVCP/IAEC/2011/11). The study was conducted in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision on Experiments on Animals, India.

Animals Required

Eighteen male Wistar rats weighing about 200–250 g were required. Rats were housed for 10 days prior to study under standard environmental conditions.

Induction of Diabetes Mellitus

Procedure. Diabetes was induced in male Wistar rats weighing 220–250 g by an injection of alloxan monohydrate (120 mg/kg) intraperitoneally in normal saline 0.9% *w*/*v*. Blood glucose levels were monitored up to 48 h.

Dose Standardization. Various doses of alloxan monohydrate were injected intraperitoneally to induce diabetes in male Wistar rats ranging from 100 to 125 mg/kg.

Table IV. Particle Size and Polydispersity Index

Code	Particle size (nm)	Polydispersity index (PI)
F 1	439.14±10.26	0.407±0.21
F 2	320.70±11.98	0.312±0.36
F 3	276.60±18.65	0.389±0.59
F 4	482.19±12.29	0.296±0.19
F 5	361.24±9.13	0.372±0.44
F 6	310.40±11.42	0.269±0.53

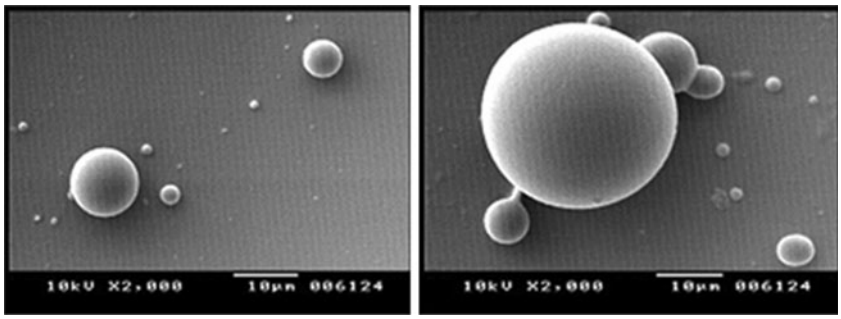

Fig. 4. SEM images of nanosuspension taken at 10 KV with 2,000 magnification

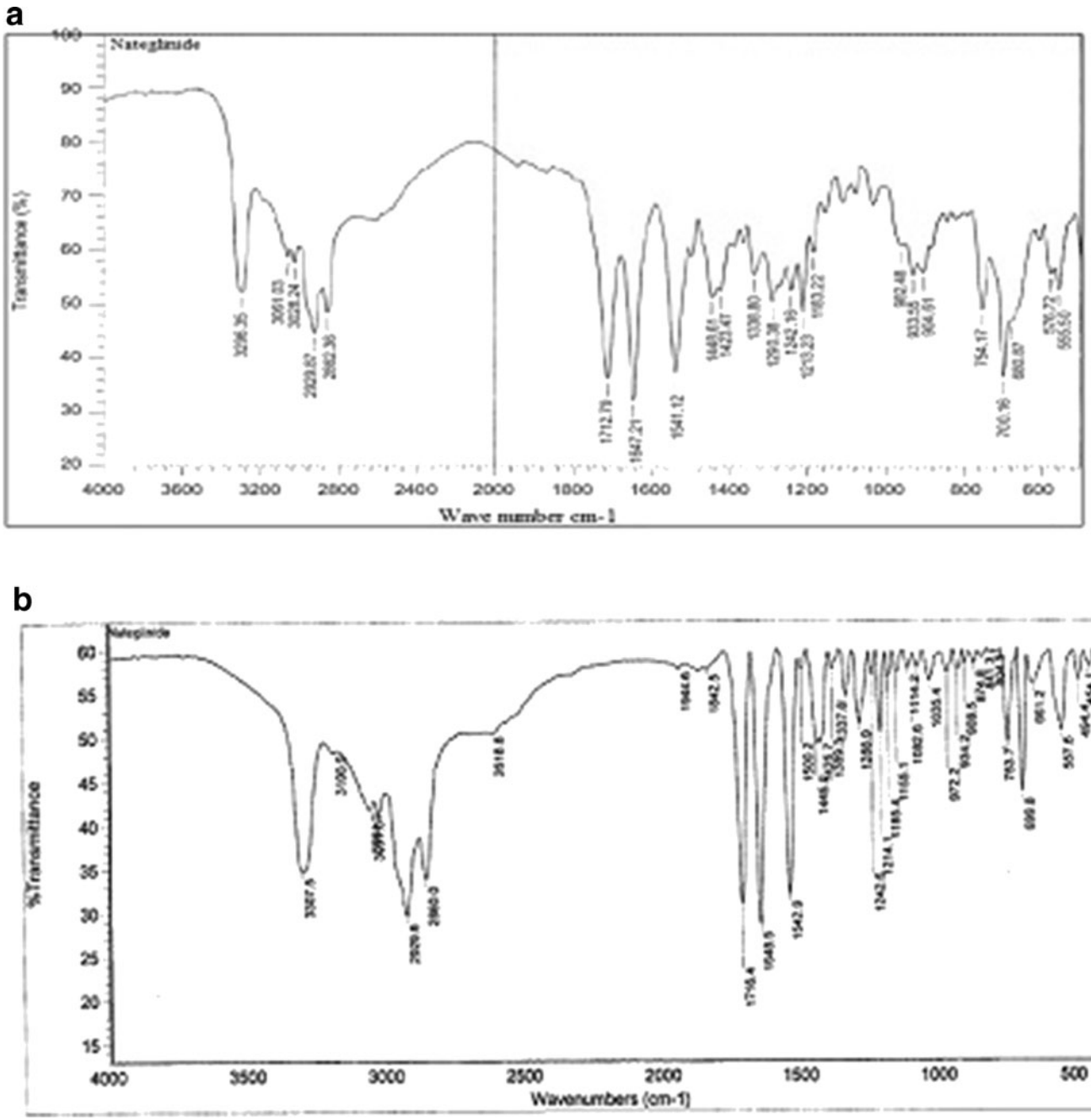

Fig. 5. FTIR spectra of **a** nateglinide and **b** nanoparticulate formulation (F6)

Pharmacodynamic Study

In order to display the biological efficacy of drug-loaded nanoparticles after oral administration, control and drug-loaded nanoparticles were given orally as a single administration to overnight fasted diabetic rats (*ad libitum*). The diabetic rats were divided into three groups (six rats in each group) for the *in vivo* uptake study. Group 1 control animals (n=6) were administered with marketed preparation (Glinate® 60 mg), group 2 test animals (n=6) were administered with oral drug-loaded nanoparticulate suspensions containing 60 mg of drug by gavage, and group 3(n=6) was kept as diabetic control. Blood samples were collected from the retro orbital plexus of the rats prior to oral administration to establish baseline glucose levels and at pre-determined time intervals after dosing. Glucose level was determined using a glucometer (Contour^{TS}).

Statistical Analysis

The data are expressed as mean ± standard error mean (SEM). The significance of differences among the group was assessed using one-way analysis of variance followed by Dunnett's test using GraphPad Prism software 5.0 at $p \leq 0.05$.

Accelerated Stability Studies (14)

Prepared lyophilized nanoparticles were subjected to accelerated stability studies at two storage conditions, i.e., at 25±2°C, 60±5% RH, and stress conditions (40±2°C, 75±5% RH) up to 3 months. Samples were collected before study and after a 3-month period and were analyzed by HPLC to determine drug entrapment efficiency.

RESULTS AND DISCUSSION

Formulation of Nanoparticulate Suspension

Nateglinide nanosuspensions were successfully prepared by oil-in-water emulsion solvent evaporation with ultrasonication technique. The method is simple, fast and reproducible for the preparation of nanoparticles. The solvent in the oil phase dissolves into the aqueous phase and then evaporates which favors the formation of nanoparticles by rigidizing the polymer. Formation of a colloidal nanodispersion can be visualized by the bluish opalescence. Nanoparticles were prepared using two concentrations of polymer, viz., 300 and 500 mg by altering the probe sonication time from 1 to 3 min (Table II)

Drug Entrapment Efficiency

Solvent extraction method was used to extract drug into methanol, which was then analyzed by HPLC in triplicate. Drug entrapment efficiency was calculated using standard graph of nateglinide in pH 7.4 PBS buffer. It was found that entrapment efficiency of nateglinide is influenced by polymer concentration and probe sonication time period (Fig. 1). In this study, entrapment efficiency was found to be 76.46±6.15% when 500 mg polymer and 1 min sonication time were used.

Effect of Polymer Concentration on Properties of Nanoparticle

In this study, two PCL concentrations, 3 and 5 g/100 mL, were selected. The encapsulation efficiency was increased from 58.71±1.83% to 64.09±4.27% by increasing PCL concentration from 3 to 5 g/100 mL. By increasing the polymer concentration in the organic phase, the viscosity of the solution was found to have increased. This may be attributed to the fact that increasing viscosity (physical examination) can decrease the drug diffusion into the aqueous phase and thus increase the drug incorporation into the nanoparticles. And it was observed that the mean particle size of nanoparticles was increased from 276.60±18.65 to 310.40±11.42 nm with the increasing polymer concentration.

Effect of Sonication Time on Drug Entrapment Efficiency

Probe sonication time is the key factor in controlling size of nanoparticles. From the data, particle mean size was found to decrease by increasing probe sonication time from 1 to 3 min. This decrease in the particle size might have attributed to the reduction in the encapsulation efficiency as seen in Table III and Fig. 2.

Effect of Organic Solvent Evaporation Rate on Drug Entrapment Efficiency

In order to verify the influence of organic solvent evaporation rate on drug entrapment efficiency, two methods of solvent evaporation were tested namely flash rota evaporator (FRE) and magnetic stirring (MS) under normal pressure. It was observed that when the former method was used, the nanoparticles presented entrapment efficiency of 64.09±4.27% (Fig. 3). This might be attributed to the fact that flash rota evaporator allows removal of solvent at a gradual yet

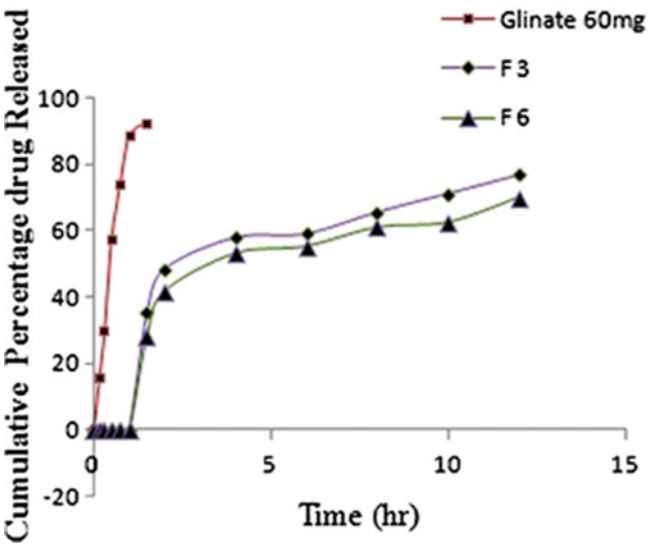

Fig. 6. Comparative cumulative percentage drug release plot for nateglinide marketed tablets (Glinate® 60 mg) and nanoparticulate formulations

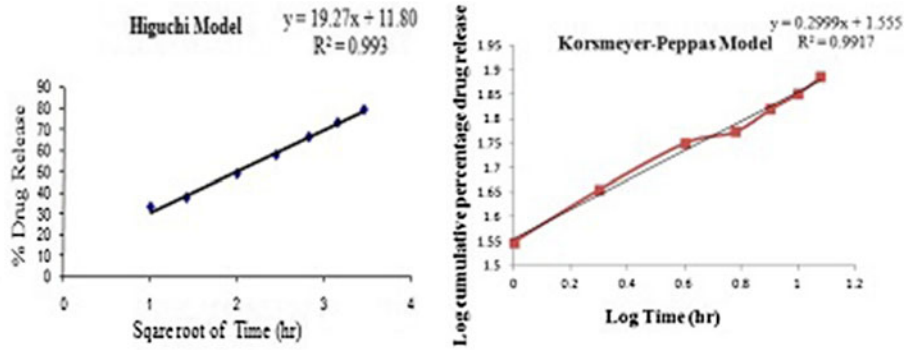

Fig. 7. Kinetics models of drug release from nanopariculate suspension

faster rate thereby minimizing the drug diffusion to the aqueous external phase.

Particle Size and Polydispersity Index

The particle size data showed that nanoparticles produced were of submicron size with relatively narrow particle size distribution. The particle size of various formulations was presented in Table IV. From the results, it is seen that the mean particle size increased with increasing polymer concentration.

Scanning Electron Microscopy

The surface morphology and shape of nateglinide nanoparticles were determined using SEM (Fig. 4). Nanoparticles were found to be distinct, spherical with a smooth surface without any crevices. The smooth surface observed in the images reveals complete removal of the solvent from the formulated nanoparticles, and it also indicates that the formulation method was efficient.

Fourier Transform Infrared Spectroscopy

FTIR of nateglinide shows the principle peaks at the wave numbers of 1,213–1,386 cm^{-1}, justifying the presence of carboxyl, carboxylate groups, and carbonyl at 1,646 cm^{-1}, C-H stretching between at 2,857 and 3,030 cm^{-1}, C=O vibration at 1,723 cm^{-1}, and NH stretching appeared at 3,296 cm^{-1}. Comparing the FTIR spectra of pure drug and formulation, it was found that characteristic peak of nateglinide at 1,386 and 3,296 cm^{-1} was slightly changed indicating that there is no significant interaction between drug and polymer. The FTIR spectrum was presented in Fig. 5.

In Vitro Drug Release Study

The nateglinide release was studied as a function of time. Nanoparticles containing 300 and 500 mg of PCL were studied in triplicate. The results over 12 h are shown in Fig. 6. The results indicate prolonged release of drug in a nanoparticle form compared to the tablet formulation (Glinate® 60 mg). About 92.41±3.52% of the drug was released from tablets after 90 min, whereas 78.79±2.89 and 74.73±3.29% of the drug was released from nanoparticles after 12 h from F3 and F6 formulations, respectively. Among F3 and F6, the formulation with 500 mg of PCL (F6) showed a slow release profile. Thus, it is evident that larger particles have a small initial burst release and a longer sustained release than smaller particles.

Kinetics of Drug Release

In order to determine the release model which best describes the pattern of drug release, the *in vitro* release data were substituted in zero order, first order, Higuchi release, and Korsmeyer and Peppas release models. Among the models tested, the drug release profiles of optimized formulation (F6) were found to be best fit with Higuchi release model (Fig. 7) based on the regression coefficient value (R^2 of 0.993). To explain the mechanism of drug release, Korsemeyer–Peppas equation, $Mt/M\infty = Kt^n$ (where $Mt/M\infty$ is a fraction of drug released at time t, k is the release rate constant and n is the release exponent) was applied, and good linearity was observed. In order to obtain n from $Mt/M\infty = Kt^n$, *in vitro* drug release data were plotted as log cumulative percentage drug release *versus* log time. The diffusion exponent (n) value was found to be 0.299 which indicated that drug release mechanism followed pure Fickian diffusion.

In Vivo Evaluation Studies of Nateglinide Nanoparticles

Dose Standardization

Among the various doses of alloxan monohydrate injected ranging from 100 to 125 mg/kg, it was found that 120 mg/kg dose of alloxan induces diabetes as blood glucose levels reached more

Table V. Effect of Marketed Preparation (Glinate 60 mg) and Nanoparticulate Formulation F6 on Blood Glucose Levels (Milligrams per Deciliter) in Alloxan-Induced Diabetic Rats

	Blood glucose levels (mg/dl)		
Time (h)	Diabetic control	Standard	Test
0	267.39±2.92	262.57±5.02	264.91±1.89
1	273.6±1.73	220.18±2.78	253.14±2.88
2	266.13±2.30	192.42±4.14	234.83±4.70
4	256.44±1.46	166.48±2.82	198.78±1.30
6	264.75±0.95	175.89±3.47	182.12±5.09
8	257.65±2.78	186.47±1.28	166.08±3.68
12	263.30±2.64	210.29±4.20	154.33±2.29
16	260.29±2.78	268.37±6.12	130.40±4.12
24	268.11±3.07	280.98±1.56	178.67±0.98

Table VI. Effect of Storage Conditions on Drug Entrapment Efficiency

Storage conditions	Percentage drug entrapment efficiency			
	Before study	After 30 days	After 60 days	After 90 days
At room temperature 25±2°C, 60±5% RH	64.09±4.27	63.79±2.45	63.21±4.73	62.93±1.32
At stress conditions 40±2°C, 75±5% RH	64.09±4.27	62.94±1.98	61.31±3.37	59.74±2.79

than 250 mg/dl after 2 days of treatment. And 125 mg/kg dose of alloxan was found to be lethal to rats. Blood glucose levels were increased after alloxan treatment as it causes degradation of β cells in the pancreas, thereby decreasing insulin production which impairs glucose metabolism in rats.

Induction of Diabetes Mellitus

Three groups (n=6) of rats were injected with 120 mg/kg dose of alloxan monohydrate intraperitoneally to induce diabetes, and blood glucose levels were monitored. Rats were considered diabetic after 2 days of stabilization as blood glucose levels reach more than 250 mg/dl. Induction rate of alloxan monohydrate was found to be 60%.

Pharmacodynamic Study

After oral administration of marketed tablets (Glinate® 60 mg) and nanoparticulate formulation to group 1 and group 2, respectively, blood glucose levels were monitored at specific time points as 0, 1, 2, 4, 6, 8, 12, 16, and 24 h. It was found that prepared nanoparticles F6 have prolonged the antidiabetic activity of nateglinide significantly ($p \leq 0.05$) compared to the conventional tablets over a period of 12 h as shown in Table V. This antidiabetic activity of nateglinide might be attributed to fact that nanoparticles might have sustained the drug release in body fluids therein regulating the blood glucose levels for a prolonged period of time.

Accelerated Stability Studies (3)

Drug entrapment efficiency was investigated before and after 30, 60, and 90 days of accelerated stability studies. The studies were carried out at 25±2°C, 60±5% RH, and 40±2°C, 75±5% RH conditions. It was found that entrapment efficiency of nanoparticulate formulation was not changed drastically, indicating the formulation was stable at specified storage condition up to 3 months (Table VI). The stability data indicate that the lyophilization of nanoparticles has contributed to the stabilization of the formulation and could be useful for improving the shelf life. This might be attributed to the fact that transformation of colloidal suspension into solid form has the advantage of preventing particle aggregation, degradation reactions (hydrolysis), and preventing the leakage of the drug.

CONCLUSION

From our investigation, it can be concluded that nateglinide nanoparticles can be prepared using emulsion solvent evaporation technique. The preparation, characterization, *in vitro* drug release, and pharmacodynamic response of nanoparticles and performance of the formulations were evaluated. Formulation F6 with 500 mg polymer, 3 min sonication time using flash rota evaporator was found to be most effective in retarding the drug release over 12 h compared to conventional tablets (Glinate® 60 mg) which was then confirmed by *in vivo* studies. So nateglinide in nanoparticulate dosage form can be considered as a suitable alternative in the treatment of diabetes mellitus (type II) especially in geriatric, pediatric, and hospitalized patient population.

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