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DEVELOPMENT AND *IN VITRO, IN VIVO* EVALUATION OF CONTROLLED RELEASE, BIOCOMPATIBLE NANOPARTICLES

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The intention of the study was to formulate poly (D, L-lactic-co-glycolic acid) PLGA nanoparticles containing glibenclamide and to characterize by both *in vitro* and *in vivo method*. Nanoparticles were prepared by emulsion solvent evaporation technique using methanol and dichloromethane in a ratio 2:1 as solvent with (PVA/polysorbate-80) in a fixed concentration as surfactant. The prepared nanoparticles were characterized by transmission electron microscopy (TEM), differential scanning calorimetry (DSC), stability and for in vitro drug release study. The *in vivo* antidiabetic study along with biochemical and haematological study was also carried out using streptozotacin induced female albino rats. Stable glibenclamide loaded PLGA nanoparticles were successfully prepared by solvent evaporation technique without any incompatibility as indicated by DSC study. The drug release from prepared nanoparticles continued for 3 days in a controlled zero order fashion. The optimised formulation was able to produce significant antidiabetic activity and the activity continued for 7 days. No significant change in behavioural, biochemical and haematological parameters was observed during the study period.

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Keywords: poly(lactic-co-glycolic acid) (PLGA), Glibenclamide, Nanoparticles (NPs), Controlled release

1. Introduction

Nanoparticles (NPs) in the field of nanotechnology have been emerging as key mechanisms by providing a targeted approach for efficient delivery of conventional therapeutic drugs. This targeted approach leads to the delivery of drugs, in low dosages, in a controlled manner for an efficient action within the body [1-4]. NPs are nano-sized stable, colloidal or solid particles, generally made of biodegradable polymer or lipids. They entrap or encapsulate the active principal or adsorb or attach to their surface. NPs can also offer advantages like limiting fluctuation within therapeutic range, reducing side effects, decreasing dosing frequency, and improving patient compliance. In general, rapid gastrointestinal (GI) absorption is required for oral hypoglycemic drugs, in order to prevent a sudden increase in blood glucose level after food intake in patients with diabetes mellitus.

Glibenclamide (GLM) is oral antidiabetic used in the management of type II diabetes mellitus. It causes hypoglycaemia by stimulating release of insulin from pancreatic cells and by increasing the sensitivity of peripheral tissue to insulin. It is rapidly absorbed after oral administration with plasma half life 5.05 h. A slow absorption of a drug usually originates from either poor dissolution of the drug from the formulation or poor permeability of the drug across the

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GI membrane. It has a history of low bioavailability, which is attributed to poor dissolution. Glibenclamide is partially soluble in water. The formation of amorphous forms to increase drug solubility and the reduction of particle size to expand surface area for dissolution and decrease the interfacial tension with the aid of a water-soluble carrier are among the possible mechanisms for increasing dissolution rates and improving bioavailability of poor water-soluble drugs [5-14].

Nanoparticulate delivery systems, such as those based on poly(lactic-co-glycolic acid) (PLGA) polymers, have been studied extensively for many years. PLGA polymers have the advantage of being well characterized and already com-mercially used for microparticulate drug delivery systems. PLGA polymers are biocompatible, biodegradable, and bio-resorbable. Biodegradable materials with PLGA (poly DL-lactide-co-glyco-lide), have been utilized as a drug delivery system for controlling the release of various drugs. Release kinetics from PLGA and other biodegradable polymers are controlled by diffusion, erosion or a combination there of, and are dependent on the polymer (Mw, copolymer ratio and crystallinity) [15]. The primary goal of this study was to formulate characterize PLGA controlled release nanoparticles containing GLM by emulsification solvent evaporation method and to achieve a better release profile suitable for *per oral* administration and which could overcome the drawbacks of GLM delivery through conventional dosage forms.

2. Experimental

Materials

Poly (lactic-co-glycolic) acid (copolymer ratio 50:50, viscosity 0.41 dL/g) was purchased from Birmingham Polymers Inc. (Birmingham, USA) and glibenclamide (GLM) were procured as gift sample from Alembic Pharma, India. All other chemicals used in this study are of analytical grade from E Merck (India).

Methods

2.1 Methods of Preparation of Glibenclamide loaded nanoparticles NPs

Glibenclamide loaded PLGA NPs were prepared by emulsification solvent evaporation method. An attempt was made to optimize the nanoparticle formulation using various formulation parameters including varying drug/polymer ratio organic solvent (methanol/dichloromethane) ratio, stirring speed (300 – 3000 rpm) and surfactant ratio (PVA/polysorbate-80) in a fixed concentration (0.5 %w/v), using a modified emulsification solvent evaporation technique. PLGA and GB were dissolved in 25 ml of solvent mixture containing methanol to dichloromethane in a ratio 2:1, using a vortex shaker to form a homogeneous solution of drug and polymer. This homogeneous solution was added slowly to 120 ml of aqueous surfactant solution containing 0.5%(w/v) PVA, Polysorbate 80 and homogenised using a high pressure homogenizer (Ika, Japan) to prepare the emulsion [16-18]. The emulsion formed was stirred with a laboratory magnetic stirrer (Remi, India) for 5 h at 25ºC followed by centrifugation (Remi, India) for 22 min. The supernatant was discharged and the pellets obtained were washed using the same volume of distilled water as the supernatant and then centrifuged for 6 min. Washing was repeated three times and the resulting NPs were freeze-dried (Lyphlock, Labconco).

2.2 Determination of drug incorporation efficiency

Freeze-dried NPs (10gm) were dissolved in dichloromethane (50 ml), the common solvent for both drug and polymer. The amount of drug in the solution was determined using Perkin-Elmer ultraviolet spectrophotometer at 243.5 nm. Drug content (%w/w) and drug entrapment (%) were calculated using Eqs 1 and 2, respectively.

% Drug content = (M/Mr)100 .....(1)
where M is the mass of drug in the NPs and Mr the mass of NPs recovered.
% Drug entrapment = (D/Df)100 ..... (2)
where D = amount of drug in the nanoparticles

Df = Total amount of drug used for the preparation of the nanoparticles.

2.3 Particle size analysis and zeta potential measurement

To evaluate particle size, zeta potential and polydispersity index (PI), freeze dried NPs were reconstituted in distilled water. The size of the NPs was determined by Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) based on dynamic light scattering technique. The Polydispersity Index(PI) which is a dimensionless no indicating the width of the size distribution was also measured. The mean particle size of the formulation was determined by photo correlation spectroscopy with a zeta master (Malvern Instruments, UK) equipped with the Malvern PCS software. Every sample was diluted with phosphate buffered saline pH 7.4. The surface charge (Zeta potential) was determined by measuring the electrophoretic mobility of the NPs using a Malvern zeta sizer (Malvern Instruments, UK). Samples were prepared by dilution with phosphate buffer saline pH 7.4.

2.4 Differential scanning calorimetric (DSC) studies

The physical state of glibenclamide encapsulated in NPs was characterized by differential scanning calorimetry (DSC, Shimadzu, DSC-50, Japan). Each sample containing drug equivalent to 10 mg was sealed separately in a standard aluminium pan the samples were purged with nitrogen gas at a flow rate of 10ml/min, and heated at 10^0c/min from 0 to 350 °C.

2.5 Transmission electron microscopic (TEM) studies

NPs were also evaluated for size with a transmission electron microscope (Philips/FEI Inc, Barcliff, Manor, NY, USA). For this purpose, a sample of NPs was suspended in water (0.5 mg/ml) and sonicated for 30 s. One drop of this suspension was placed over a carbon-coated copper TEM grid (150 mesh, Ted Pella Inc., Rodding, CA) and negatively stained with 1 % uranyl acetate for 10 min and then allowed to dry. Images were visualized at 120 kV under the microscope.

2.6 In Vitro Drug Release Studies

The *in vitro* release of GB-loaded NPs were performed using USP type II dissolution test apparatus (Electrolab, India) in 900 ml of medium(0.1N Hydrochloric acid) for the first 2 hour and then in Phosphate buffer(PH 7.5) from 3-72h at 37± 0.5^0c and stirring rate of 100 rpm. 5ml samples were collectedperiodically and replaced with equal volume of fresh dissolution medium on each occasion. After filtration through Whatman Grade No. 41 Quantitative Filter Paper (pore size 25 µm), the concentration of GB was determined spectrophotometrically at 300 nm on UV-Visible spectrophotometer (Jasco V530, Japan). *In vitro* release profile was analyzed by various kinetic models (zero order, first order and Higuchi) in order to determine the mechanism of drug release [20-22].

2.7 Stablility Study

The selected formulations were subjected to accelerated stability studies to evaluate effect of stress conditions according to ICH guidelines. Formulations were packed on 0.44mm dilaminated aluminium foil and subjected to elevated temperature and relative humidity (RH) conditions of 40 ± 2 ºC / 75 ± 5% RH, 30 ± 2 ºC / 65 ± 5 % RH, 30 ± 2 ºC / 65 ± 5 % RH. Samples were withdrawn at the end of 1, 3 and 6 months and evaluated for physical properties, drug content and *in vitro* drug release.

2.8 *In-Vivo* studies

Meticulous analysis of the results and discussion brings us to draw the conclusion that the formulation F2 which has the desirable particle size, drug entrapment efficiency and control release rate profile is the best among all others and is chosen for carrying out *In Vivo* studies.

Study protocol

The protocol for the *In vivo* study was approved by Institutional animal ethical committee of Shree Santkrupa College of Pharmacy, Ghogaon, Shivajinagar, Karad, Maharastra) (IAEC/1110/ac/07). All ethical manners regarding the use of animals in scientific research were

carefully considered. Adult female wistar albino rats selected with average body weight of 200-250g were used throughout the study. Rats were kept in polypropylene cages. They were maintained under standard environment conditions (25±30^{0}C and 70±5% relative humidity under natural 12-h light and 12 h dark conditions). They were maintained with free access to water and standard laboratory rat pellet diet (Hindustan Lever Ltd., Bangalore). The animals were divided into five groups, each group containing 6 animals (n=6 / group).

Induction of Diabetes

Streptozotocin (Himedia) was dissolved in 100 mmol⁄L citrate buffer (pH 4.5) and 60 mg⁄ kg fresh solutions was injected intraperitoneally to overnight-fasted rats. Blood glucose levels were measured after 48 h later using Glucometer (One Touch, Johnson & Johnson, India). Each animal with a blood glucose concentration level above 250mg/dL (13.8 mM) was considered to be diabetic and used in the experiments.

Experimental Procedure

Animals were randomly divided into five groups of six rats each group. Pure Glibenclamide and optimized formulation of nanoparticles was administered orally through intragastric tube. Animals divided with five different groups were kept as follows: group I: Normal controlled rats treated with drinking water; group II: Diabetic controlled rats treated with drinking water; group III: Diabetic controlled rat administered with pure Glibenclamide(2 mg/kg body weight); group IV; Diabetic rats administered with drug loaded PLGA Nanoparticles containing glibenclamide equivalent to 1 mg/kg body weight, incorporated in a single dose; and group V; Diabetic rats administered with optimised formulation of Glibenclamide loaded PLGA Nano Particles equivalent to 4 mg/kg body weight, incorporated in a single dose. Blood glucose levels were measured in all rats using One touch®Horizon^{TM}, Blood Glucose monitoring system, Life Scan, Inc, Milpitas, USA marketed by Johnson & Johnson, India at 2 hours interval for 12hours, next 12hours upto day one and then at 24 hours interval upto 7 days of the treatment period. In all experiments, approximately 0.1 mL blood was drawn each time from the tail using aseptic procedure. Blood samples were immediately placed on the Glucostrips and blood glucose levels were determined using a glucometer. Behavioural parameters (body weight, skin colour, salivation, lacrimation, respiration, motor activity and Muscle Spasm) were studied during treatment of animals with comaprision with normal control animal. At the end of the experimental period blood samples were collected by cardiac puncture techniques and mixed well with anticoagulant. Collected samples were analysed for different Biochemical and Haematological parameters.

2.9 Statistical analysis

Results are expressed as mean ± S.D for triplicate samples. The results were statistically analyzed and significant differences among formulation parameters were determined by one-way analysis of variance using 'Graph Pad Instate®'Version 3.05 (USA), statistical analysis program. Statistical significant was considered at $p < 0.05$.

3. Results and discussion

3.1 Entrapment efficiency, drug content, particle size and TEM study

Formulation codes with entrapment efficiency, drug content and particle size were as given in table 1. It has been observed that as concentration of polymer increases entrapment efficiency, drug content and particle size also increases. Also for formulation F4 increase in drug concentration has also increased the entrapment efficiency, and particle size. Lower particle size was observed when stirring speed was increased. TEM photographs (Fig 1) indicate that NPs were spherical in shape and in the nanosize range with discrete spherical outline. DSC thermograms of native PLGA, GLM and GLM-loaded NPs showed the characteristic peaks of the respective compounds, as shown in Figure 2. An endothermic peak of PLGA occurred at approximately at 48^{0}c due to glass transition temperature. The peak of PLGA was slightly shifted in drug loaded nanoparticles compared to the native PLGA. The endothermic peak of GLM was occurred to

approximately 172.8^{0}c. In the formulation F2 such peaks were observed. . *In vitro* release profile and kinetics of GB from the NPs are shown in Figure 3 and Table 2, respectively. As polymer concentration increased, drug release decreased. Also, when the drug concentration of the NPs was increased as in case of formulation F2 (drug: polymer ratio 1:2) as compared to formulation F1(drug: polymer ratio 1:1), drug release increased. In order to determine the release pattern of GLM from prepared NPs, various *invitro* release kinetic models such as Zero order, Higuchi and First order were analysed. In all formulations highest regression co-efficient was observed in zero order equations (table 2).

Table 1: Batch codes with D (drug) P (polymer) ratio, SP (stirring speed), EE (entrapment efficiency), DC (drug content) and PS (particle size) of Glibenclamide loaded NPs.

Sr.No.	Batch code	D:P ratio	SP(rpm)	EE(%)	DC(%)	PS(nm)
1	**F1**	1:1	2500	46.32 ± 2.14*	11.21 ± 2.43*	532 ± 11.29*
2	**F2**	1:2	2500	54.24 ± 3.15	26.52 ± 3.16	562 ± 5.28
3	**F3**	1:3	2500	62.63 ± 1.87*	28.51 ± 2.29*	812 ± 14.72**
4	**F4**	2:1	2500	90.52 ± 4.52**	36.73 ± 3.16*	1453 ± 13.26**
5	**F5**	1:2	250	33.21 ±3.12**	10.28 ± 1.13*	1081 ± 23.26**
6	**F6**	1:2	1000	42.24 ± 2.57*	13.14 ± 2.12*	993 ± 14.28*
7	**F7**	1:2	1500	48.25 ± 2.21*	21.23 ± 3.31*	803 ± 11.16**

Significantly different from the value of batch F2 at $p < 0.01$ (**) and $p < 0.05$ (*)

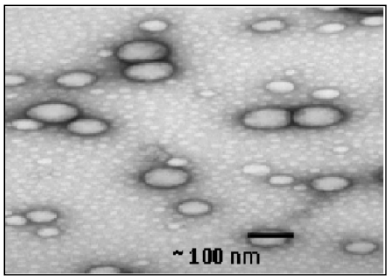

Fig. 1. Transmission electron microscopy (TEM) for batch with 1:2 drug polymer ratio

3.2 DSC Studies

DSC study shows the nature of the drug encapsulated in the NPs. This analysis was performed on native PLGA, native Glibenclamide and Glibenclamide loaded NPs. Different compounds show their characteristic peaks in DSC (figure 2). The endothermic peaks of PLGA and was found approximately at 48 ºC due to glass transition temperature (Tg) of PLGA. The peak of PLGA was slightly shifted in drug-loaded NPs as compared to that of native PLGA. The endothermic peak of native Glibenclamide was found approximately at 172.75 ºC.

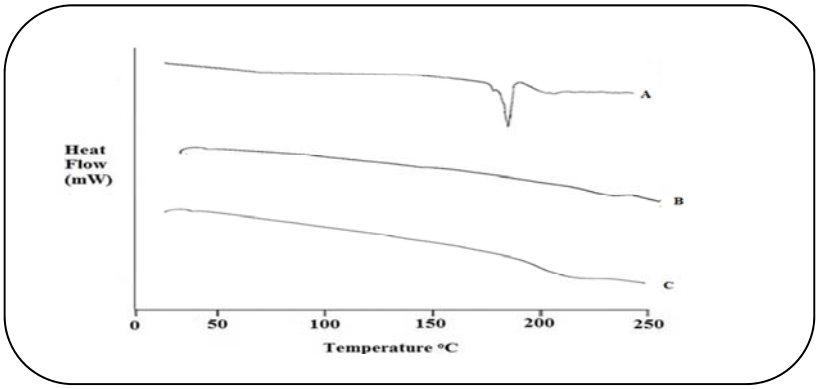

Fig. 2: DSC of Pure GLM (A), PLGA (B), Formulation(C)

3.3 In Vitro Dissolution Study

In vitro release profile and release kinetics of Glibenclamide through Glibenclamide-loaded PLGA NPs were as shown in figure 3 and table 2 respectively. It has been observed that as concentration of polymers increase drug release decreases. But for formulation with increased drug concentration drug release has increased. The in vitro release behaviour of formulations follows zero-order kinetics.

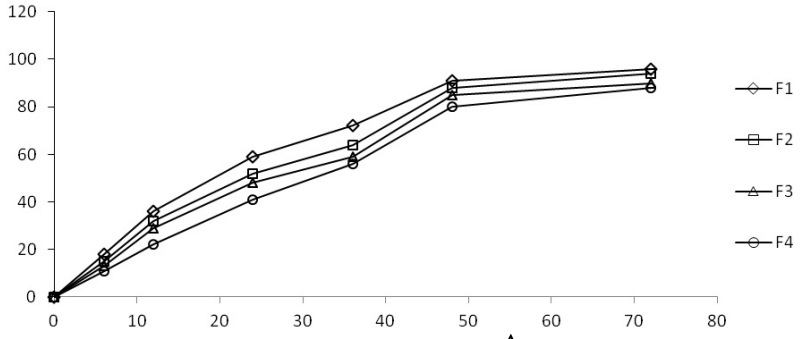

Fig. 3. In vitro release of GB from formulation F1 (◊), F2 (□), F3 (△), and F4 (○)

Table2: Release kinetics of GLM loaded PLGA NPs.

Formulation	Zero order(r^2)	First Order(r^2)	Higuchi (r^2)
F1	0.9818	0.9422	0.9413
F2	0.9812	0.9462	0.9326
F3	0.9726	0.9532	0.9524
F4	0.9831	0.9321	0.9623

3.4 STABILITY STUDY

GLM loaded PLGA NPs containing 0.5% PVA (formulation A) as surfactant was subjected to stability study. Stability studies according to ICH guidelines reveal that NPs were stable at the end of 6 months in all the test conditions. No significant changes in particle size, GLM entrapment, GLM content and release profile were observed after the end of 1, 3 and 6 months. The release kinetics was also found identical in stablility studies.

Table 3: Stability study of PVA nanoparticles

Test Duration	Test Condition ^{0}c/RH	Entrapment Efficiency	GLM Content	Mean Particle Size	Kinetics		
					Zero-order(r^2)	First-order(r^2)	Higuchi(r^2)
1	40±2^0/75±5%	54.21 ± 3.15	26.52 ± 3.14	562 ± 5.28			
	30±2^0/65±5%	56.02±2.10	25.08±1.28	560±6.24	0.9823	0.9583	0.9824
	25±2^0/60±5%	56.11±2.62	25.06±3.25	561±5.23			
3	40±2^0/75±5%	56.12±1.35	24.38±1.02	558±4.54			
	30±2^0/65±5%	56.54±2.30	25.04±2.11	557±6.13	0.9827	0.9552	0.9802
	25±2^0/60±5%	56.02±2.08	23.20±2.48	557±3.22			
6	40±2^0/75±5%	57.07±1.28	24.91±2.33	559±4.20			
	30±2^0/65±5%	57.16±2.09	25.81±2.02	558±4.22	0.9829	0.9575	0.9813
	25±2^0/60±5%	56.51±2.26	23.81±1.52	558±6.10			

3.5 In Vivo Evaluation

In vivo evaluation of the Glibenclamide loaded PLGA nanoparticles were carried out in healthy female albino rat. Measurement of blood glucose level were done after oral administration of drug loaded nanoparticles equivalent to the dose of the drug, 2 mg/kg body weight, 2 mg/kg body weight, and 4mg/kg body weight in comparison with administration of pure Glibenclamide at same dose and with double dose. The antihyperglycemic effect of formulation and pure drug in diabetic rat were assessed at different time intervals. When pure Glibenclamide solution was given orally, it was observed that the blood glucose level started to decrease from the second hour, after the sixth hour the blood glucose level reached almost normal level but after the sixth hour, blood glucose level started to increase again. On the other side, the optimized formulation of Glibenclamide loaded PLGA nanoparticles, the blood glucose level started to decrease from the second hour and this decrease continued up to 7 days [23].

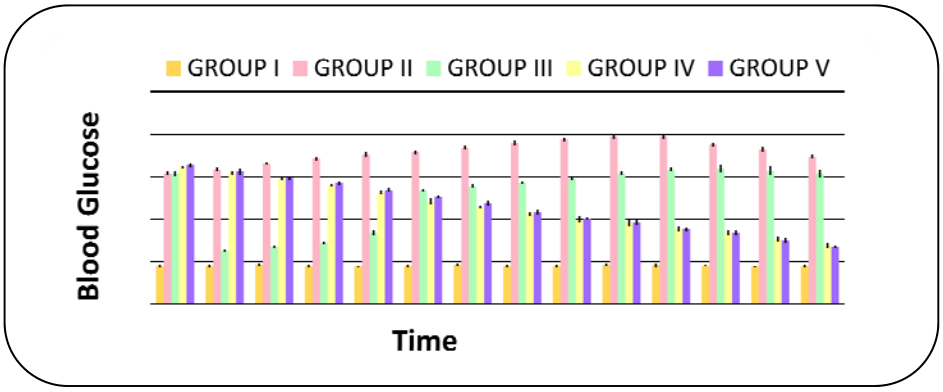

Fig. 4: Blood glucose levels in the five study groups over 24 hrs. (Group1: Control Group2: Diabetic Control Group3: 2mg/kg of PLGA loaded glibenclamide nanoparticle Group 4: 1mg/kg of PLGA loaded glibenclamide nanoparticle Group5: 4mg/kg of PLGA loaded glibenclamide nanoparticle.)

Table4: Blood glucose concentration (mg/dl) of PLGA Loaded Glibenclamide Nanoparticle compared with control rat

Time	GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
0h	88.5±2.59	307.67±4.32	307.33±6.15	323.17±2.64	327.67±4.72
2h	87.83±3.06	317±4.29	125.33±2.73	308.67±3.27	311.5±7.84
4h	91.67±3.08	331.17±2.4	135.17±2.32	295.67±4.63	296.5±5.79
6h	89.5±3.78	341.83±4.45	144.33±2.94	279.5±3.27	284.67±5.53
8h	88±1.79	352.5±5.13	167.67±6.62	263±5.83	268.83±4.26
10h	89.5±3.02	357.83±4.79	268.33±3.5	241.83±6.34	252.5±4.13
12h	91.17±3.06	368.67±5.32	277.67±3.98	228±3.63	237.17±5.63
1day	89.5±3.73	381±5.97	285.5±4.23	212.17±3.76	216.83±6.5
2days	89±4.15	387.5±5.47	295.33±4.03	199.5±6.28	201.33±4.09
3days	92.33±3.2	394.5±4.42	308.33±4.63	190.5±8.41	191.67±5.28
4days	90.67±3.27	394.33±4.37	317.5±5.39	176.83±5.34	176±4.34
5days	90.5±1.22	375.83±4.54	319.5±9.14	166.67±5.85	166.5±5.82
6days	88.5±2.07	365.33±5.72	314.17±10.35	151.83±5.78	149.67±4.93
7days	89.83±3.06	347.33±4.76	307.17±7.99	136.17±6.15	133.67±3.14

(Group I: Control Group II: Diabetic Control Group III: 2mg/kg of PLGA loaded glibenclamide nanoparticle Group IV: 1mg/kg of PLGA loaded glibenclamide nanoparticle Group V: 4mg/kg of PLGA loaded glibenclamide nanoparticle)

3.6 Behavioural Parameters

There was no significant changes were observed in physical parameters in all groups. No mortality was observed in all treatment groups throughout the experimental period. There was no significant change in the body weight of all the groups as compared with control group.

During the total treatment period the parameter like body weight, skin colour, salivation, lacrimation, respiration, motor activity and Muscle Spasm were observed and it is reported that only muscle spasm shows negative and all other parameter shows no significant changes.

Table 5: Behavioural Parameters

Parameters	Results
Body Weight	No significant change
Skin Colour	Normal
Salivation	Normal
Lacrimation	Normal
Respiration	Normal
Motor activity	Normal
Muscle spasm	Normal
Piloerection	Negative
Righting reflex	Positive

3.7 Biochemical and Haematological parameters

Levels of serum glutamate transaminase (SGPT), Serum alkaline Phosphate (SAP), serum glutamic pyruvic transaminage (SGPT), lactate, glucose, urea, cholesterol, triglycerides, were estimated in Merck semi auto analyzer by using Merck analytical kits. All the parameters level are with-in the normal range and no significant changes were observed.

Routine hematological parameters were performed on ACT diff-2 Hematology Analyzer (Beckman Coulter India, Ltd., Mumbai. Parameters like BT(sec), CT(sec), Hemoglobin (g/dL), RBC (mill/mcl), WBC (Thous/mcl), Hemoglobin Concentraion(g/dl) were studied. All the parameters are with-in the normal range and no significant changes were marked.

Table 6: Biochemical and haematological report

Parameters	Group I	Group II	Group III	Group IV	Group V
BT(sec)	71±2.1602	72.5±2.6457	75.75±3.86221	73±3.5590	71.25±3.4034
CT(sec)	49.25±2.21735	59.5±3.1091	63.75±3.0956	49.25±3.8622	50.5±1.9148
Hb (g %)	14.2±1.29	13.22±0.56	13.13±1.09	13.65±0.49	14.5±3.1091
Total RBC (mm^3)	7.29±0.22	6.49±1.67	6.95±0.62	7.59±0.40	7.25±1.2583
Total WBC (mm^3)	7.15±0.49	6.89±0.3	6.88±0.80	7.51±0.99	10±1.8257
ESR (mm/h)	6.50±1.05	6.83±0.75	7.00±0.89	7.00±0.89	6.89±0.3
SGPT (IU/L)	63.33±2.70	61.07±4.35	62.62±2.82	62.39±3.70	62.25±3.7749
SGOT (IU/L)	57.68±1.69	57.5±1.89	56.68±1.02	57.45±1.89	64.25±3.7749
ALP (IU/L)	351.17±8.35	338.5±15.93	343±6.78	340.83±12.45	157.5±3.7859
Glucose (%)	100.00±9.42	103.50±7.01	91.67±2.8	93.17±10.6	91.75±3.0956

4. Conclusion

Stable Glibenclamide loaded PLGA NPs were productively prepared by emulsification solvent evaporation method with varying the Glibenclamide PLGA ratio. Encapsulation efficiency of the prepared NPs was substantially improved with adequate drug content. Also the release of drug was sustained with the polymer concentration indicating its prospective application in drug delivery. Thus Glibenclamide loaded PLGA NPs could reduce dosing frequency leading to improve patient compliance. These NPs can be promising agents for rational drug delivery in diabetes. The *in vivo* results obtained after oral administration of nanoparticles to healthy female rats were found to be satisfactory. The developed nanoparticles are safer, and are the need of the hour for pharmaceutical industry as an alternative drug delivery system for the treatment of highly prevalent and chronic disease like type II diabetes mellitus.

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