Tuijin Jishu/Journal of Propulsion Technology
ISSN: 1001-4055
Vol. 44 No. 6 (2023)

Assessment of in Vivo Pharmacokinetics and Pharmacodynamics of Tolbutamide Nanoformulations

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***Abstract*:** Tolbutamide is an anti-diabetic drug used in the adjuvant therapy for type-II diabetes as the inhibitor of sodium-glucose co-transporter-2 in the renal tubules. The poor solubility and permeability of the drug show limitations in the formulation development and therapeutic plasma concentration. The objective of the work was to improve the solubilityand dissolution of the BCS class IV drug through surfactant stabilized nanosuspension formulation. Nanoparticle were developed by Nano precipitation-solvent evaporationmethod using Poly vinyl alcohol and Pluronic as surfactants at 1%, 3% and 5% concentration. Formulation optimized with Pluronic exhibited nano size particles (81-117nm) with monodisperse nature and high stability zeta potential. The nanosuspension prepared using 1% and 3% Pluronic F127 showed 2-fold and 5- fold increase in the drug dissolution compared to the pure drug aqueous dispersion. The optimum formulations CGF2 nanosuspension and CC1 solid dispersion were selected for the in vivo pharmacokinetic and pharmacodynamics studies to compare against the pure drug aqueous dispersion. In pharmacodynamic studies, both the nano-formulations depicted similar and significant improvement in the reduction of mean plasma glucose levelcompared to the unmodified drug. In pharmacokinetics, about 3-folds and 4.5-folds increase in bioavailability (than the pure drug) was demonstrated by the nanosuspension and solid dispersion, respectively. Histopathological analysis revealed minimal hepatotoxicity associated with the nanosuspension formulation. The results provide evidence for the prominent enhancement of the bioavailability of the BCS class IV drug, due to increase in the solubility and permeability. This study could confirm the utilization of Tolbutamide nanoformulations to replace the conventional oral dosage form administration to achieve better clinical response.

Key words: Solubility, Dissolution, Solid dispersion, Tolbutamide, Kneading

Introduction

Diabetes mellitus (type – 2) is a metabolic disorder related to glucose level in plasma and its maintenance based on the function of insulin hormone. Chronic diabetics is generally associated with long term complications such as ocular problems, microangiopathy disorders, especially the kidney diseases (Lalau et al. 2015). Combination of anti-diabetic drugs is more commonly recommended to potentiate the mechanism of action for each other.

Tolbutamide is a FDA approved anti-hyperglycemic class of medication, and a selective sodium-dependent glucose co-transporter 2 (SGLT2) inhibitor. It is recommended for the treatment of type-2 diabetes as it effectively lowers the serum glucose in an insulin-independent manner. The SGLT2 inhibitors prevent glomerular reabsorption of the filtered glucose and promote enhanced urinary glucose excretion (Kabil and Mahmoud 2018, Thrailkill et al. 2020). Adjunct therapy of oral anti hyperglycemic medications with SGLT2 inhibitors like Tolbutamide could reduce plasma glucose with lesser renal complications. However, the poor solubility and permeability of the drug imposes several challenges in the formulation strategies, drug dosing and its bioavailability.

Nano sized particles have been proposed to improve the solubility and bioavailability of several drugs due to the size and higher uptake. Different types of nano formulations could be developed using the materials like polysaccharides, synthetic polymers, lipids, etc. to obtain the products such as polymeric / lipid nanoparticles, liposomes, dendrimers, niosomes, micelles, nanoemulsion and nanosuspesions. The carriers also make possible the intended drug delivery application with improvement in solubility, permeability and physico chemical stability of the loaded drug and thereby enhancing the bioavailability through required routes of administration compared to conventional therapy (Souto et al. 2019, Nie et al. 2020)

Materials and Methods

Procurement of animals:

Wistar albino rats (male) weighing between 180 and 220 g were procured from Mass Biotech, Chengalpattu, Tamilnadu-603002. The study protocol was approved by the Institutional Animal Ethics Committee and the research experiment followed the guidelines of CPCSEA, Ministry of Environment, Forest & Climate Change, Govt. of India, New Delhi. The rats were acclimatized for one week before beginning the experiment. During an acclimatization period of one week, the animals were housed with a 12-h light/dark cycle at ambient temperature (27°C ± 2°C) and humidity (60% RH). All the rats were provided water ad libitum and a standard commercial diet.

Induction of diabetes and experimental protocol:

Diabetes was induced in the animals by giving an intraperitoneal injection of nicotinamide(110 mg/kg; prepared in normal saline) followed by administration of streptozotocin (STZ; 65 mg/kg, freshly prepared in0.1M citrate buffer, pH 4.5) after 15 min.After 6 hours of nicotinamide and STZ administration, the rats were provided with free access to glucose solution (10% w/v) for the next 24 hours. Glucose solution (10%) was poured in drinking water bottles and these bottles were inserted in the cage grills for the next 24 hours. Fasting blood glucose levels were examined using one touch glucometer (Accu-Chek Active). The animals with inception of fasting blood glucose value above 220 mg/dL after 48 hours were considered to be diabetic and used for the study (Rani et al. 2018)

Oral Glucose Tolerance Test and Assessment of Hepatotoxicity in Wistar Rats:

Male Wistar rats were divided into the following six groups, each group containing six rats.

Table 1: Study protocol for Pharmacodynamic evaluation

Groups No.	Test Protocol	Treatment
1	Normal control	Without diabetes and treatment
2	Diabetic control	Diabetic rats without any treatment
3	Standard drug control	Sitagliptin 2 mg/Kg
4	Tolbutamide aqueous dispersion	10 mg /Kg body weight
5	TolbutamideNanosuspension	10 mg equivalent dose per Kg body weight
6	Tolbutamide Solid dispersion	10 mg equivalent dose per Kg body weight

As per the animal grouping, the test samples (Tolbutamide pure, nanosuspension, solid dispersion) were orally administered to the specific group of rats at the respective dose and compared to the control groups of rats.

Glucose solution was orally administered at 2 g/kg body weight 15 min after the administration of the test compounds.

Measurement of body weight and blood glucose levels:

The body weight and blood glucose levels of the all the groups of rats were recorded during the study.Blood was collected from the tail vein into chilled tubes containing EDTA at 15 min before (-15) and 0, 10, 30, 60, and 120 min after the oral glucose administration. Plasma was separated by centrifugation and stored at -80°C until the measurement of plasma glucose (Elango et al. 2015, Oguma et al 2015).The blood glucose levels were measured using blood glucometer and strips (Accu-Chek Active).

Assessment of Hepatotoxicity

To evaluate the hepatotoxicity of different treatments in Wistar rats, histopathological examination, liver tissues are fixed in 10% formalin, embedded in paraffin, sliced into thin sections (4-5 µm), and stained with Hematoxylin and Eosin (H&E). These sections are then examined under a light microscope to identify changes such as hepatocyte degeneration or necrosis, inflammation, fatty changes (steatosis), and fibrosis. This comprehensive approach combining biochemical and histopathological analyses ensures a thorough assessment of liver damage induced by different treatments.

Pharmacokinetics study:

Adult male Wistar rats were divided into following four groups, wherein Group – I was considered as control and received water alone, Group – II was given pure Tolbutamide aqueous dispersion (10 mg/Kg body weight of animal), Group – III and Group – IV were treated with optimized Tolbutamide Nanosuspension and Solid dispersion formulation, respectively, at the drug dose equivalent to 10 mg/Kg body weight. The test samples were administered by oral gavage followed by glucose solution (2g/kg) after 15 minutes.About 200 µL of blood samples were collected from retro orbital vein at 0.5 h, 1, 2, 4, 8, 12 and 24 h after the test compound administration, into precoated anticoagulant microcentrifuge tubes (Elango et al. 2015).

Blood samples were centrifuged at 12,000 rpm for 20 minutes and plasma were separated and stored in deep freezer (-20C). For quantification of the Tolbutamidein plasma, simple protein precipitation process was carried out using methanol as the extracting solvent to separate the drug from plasma. The extracted samples were evaluated for Tolbutamide concentration by high performance liquid chromatography (HPLC) technique (Dudhe et al. 2016, Ajitha 2021). Plasma concentrations of Tolbutamide was determined and data were presented as the mean with S.D. (n=6). The obtained data was evaluated to check the relative bioavailability and other pharmacokinetic parameters.

Estimation of Tolbutamide in plasma samples by HPLC:

Quantitative analysis of Tolbutamide in plasma was performed by HPLC technique. The chromatographic separation was carried out using with WATERS (250 x 4.6 mm, 5 mm) column. Mobile phase composition was optimized with acetate buffer: acetonitrile: methanol at 30:50:20 v/v, pH 4.5 adjusted with acetic acid and filtered through 0.45 µ membrane filter under vacuum and degassed. The flow rate of mobile phase was fixed to be 1.0 mL/min and the column temperature maintained at 30°±2°C. (Dudhe et al. 2016)

Statistical analysis:

The experimental values and data comparison was expressed as mean ± SD. The experimental data were statistically analyzed using one way ANOVA followed by the Tukey test (Graph pad Prism software). Probabilities less than 5% ($P < 0.05$) were set as the basic criterion and considered to be statistically significant for each evaluation.

Results and Discussion

Selection of Optimized Nanoformulation of Tolbutamide:

Tolbutamide nanoparticles were developed by Solvent Evaporation-Nanoprecipitation technique by experimental optimization process with two different surfactants like Poly Vinyl Alcohol and Pluronic F127 at varying concentrations of 1%, 3% and 5%. Formulations optimized with Pluronic exhibited nano sized particles in the average size range of 81-117 nm with monodisperse nature and high stability zeta potential. The nanosuspension prepared using 1% and 3% Pluronic F127 exhibited 2-fold and 5-fold increase in the drug dissolution compared to the pure drug aqueous dispersion (Samed et al. 2018).

Solid dispersion formulations were processed through kneading method using hydrophilic polymers such as beta cyclodextrin and PEG 4000 in three different ratios as 1:1, 1:3 and 1:5. The obtained particles were in the nano size range of 240 – 640 nm with high colloidal stability -17 to -58 mV zeta potential. The formulation prepared with drug: beta cyclodextrin as 1:1 exhibited maximum *in vitro* drug dissolution of 75%, as performed by standard USP type II paddle method with immersed sample in dialysis bag. The formulation had displayed about 7.5-fold increase in the dissolution compared to the pure drug aqueous dispersion (Sakure et al. 2020).

Therefore among these formulations, Tolbutamide nanosuspension stabilized with 3% Pluronic F127 surfactant and the solid dispersion nanoparticles prepared with 1:1 ratio of beta cyclodextrin were selected for the *in vivo* studies (Singh et al. 2019).

Animal body weight:

The animals with weight range between 180-210 g were identified for the study and were acclimatized for the required period and maintained with normal diet and water. The individual weights of the rats were tabulated in Table 1 and the initial average weight of the animals was found to be 192 g.

Table 2: Animal body weight

Groups	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
1	192.48	193.44	180.42	192.42	192.93	200.33
2	190.46	199.5	189.65	189.02	203.83	185.69
3	188.22	192.61	183.76	187.62	184.34	189.98
4	183.67	187.19	192.92	192.07	199.69	201.58
5	192.84	186.59	187.18	191.24	209.6	190.63
6	187.05	195.97	187.66	190.03	189.68	194.59

Hepatotoxicity studies

The histopathological examination of liver tissues in male Wistar rats revealed varying degrees of liver damage across different treatment groups. The normal control group exhibited no signs of liver pathology, maintaining normal liver architecture. In contrast, the diabetic control group showed moderate hepatocyte degeneration, mild inflammation, and steatosis, indicating significant liver damage. The standard drug control group (Sitagliptin) displayed only mild hepatocyte degeneration without significant inflammation, steatosis, or fibrosis, suggesting a protective effect on the liver. Among the Tolbutamide-treated groups, the nanosuspension form demonstrated minimal hepatocyte degeneration and no significant liver damage, indicating the least hepatotoxicity. The aqueous and solid dispersion forms of Tolbutamide showed mild hepatocyte degeneration and inflammation, with no significant steatosis or fibrosis, reflecting mild to moderate hepatotoxicity.

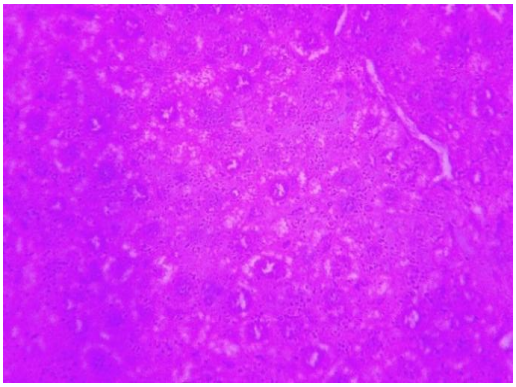

FIG -1A Liver Histology of Normal Control Rats (Group 1)

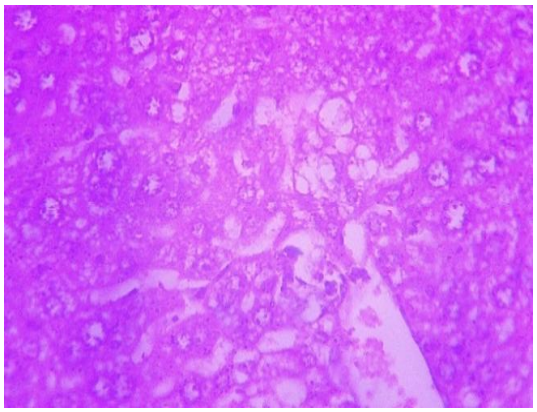

FIG -1B Liver Histology of Diabetic Control Rats (Group 2)

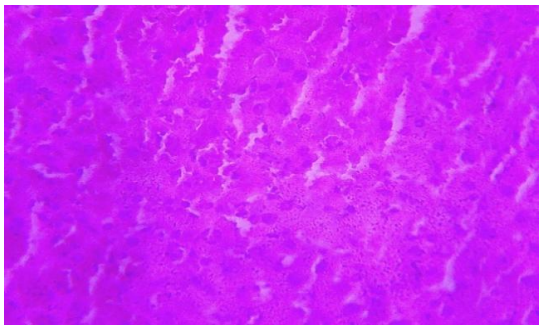

FIG -1C Liver Histology of Standard Drug Control Rats (Sitagliptin) (Group 3)

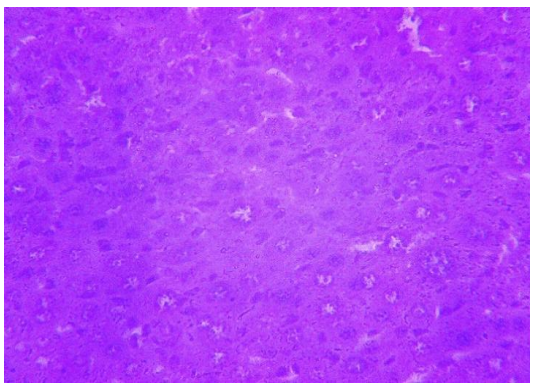

FIG -1D Liver Histology of Tolbutamide aqueous dispersion Rats (Group 4)

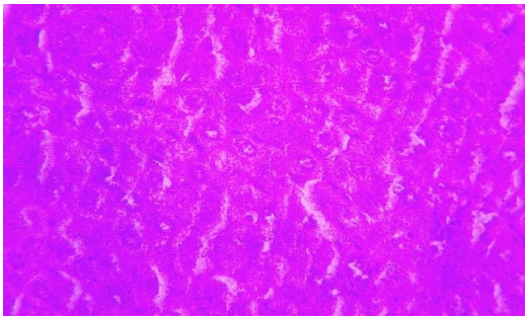

FIG -1E Liver Histology of Tolbutamide Nanosuspension Rats (Group 5)

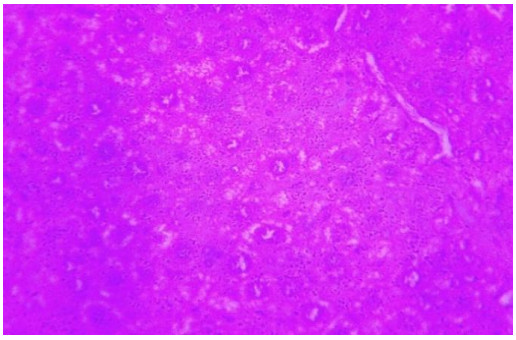

FIG -1F Liver Histology of Tolbutamide Solid Dispersion Rats (Group 6)

Pharmacodynamic studies:

The average normal glucose level of the control animals (Group I - without inducing diabetes and untreated) was 120 mg/dL. After initiating the oral glucose tolerance test, all the rats exhibited glucose levels of 330 mg/dL and 325 mg/dL at the measured time immediate to oral glucose administration (-15 min) and immediately at the start of treatment (0 min), respectively. In case of diabetic animals without any treatment (Group II), the blood glucose level elevated from 322 to maximum of 617 mg/dL. As the group III and group IV rats were treated with standard control drug Sitagliptin 2 mg/Kg and Tolbutamide pure aqueous dispersion 10 mg/Kg, the mean glucose lowering effect could be observed (Abdel-Aal et al. 2021). The optimized nanosuspension and solid dispersion formulation of Tolbutamide containing 10 mg equivalent dose of the drug had exhibited significant reduction in the glucose level from 330 mg/dL to 250 mg/dL compared to the standard drug and pure Tolbutamide. This evidenced the efficiency of the nanoformulation to improve the therapeutic efficacy of the BCS class IV drug towards the diabetic treatment.The conventional adjunct therapy of Tolbutamide with other diabetic drugs could be possible with the novel nanoformulations reported herein (Umashankar et al. 2017).

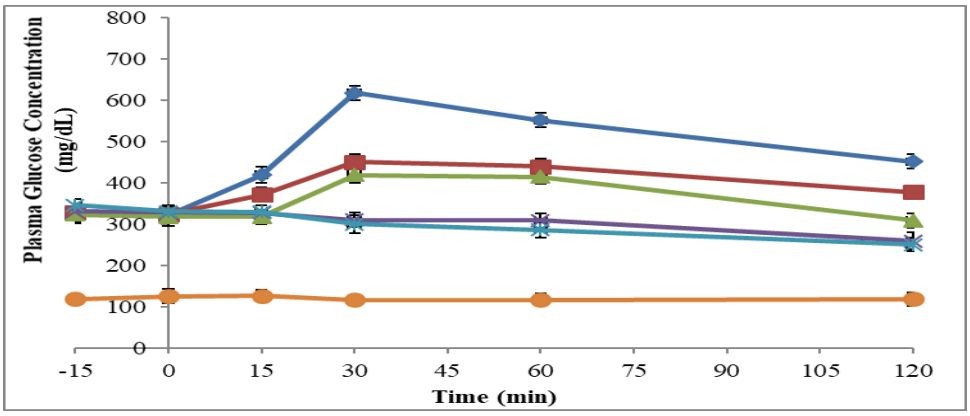

Fig 1: Effect of TolbutamideNanoformulations on Blood glucose levels

Chromatographic Method Validation:

In the HPLC analysis of plasma samples, Tolbutamide was detected at 290 nm with retention time of 5.1 min. Linearity was found to be 0.999 over the concentration range of 30 –250 ng/mL and percentage recoveries were found to be 94.68 - 103.76%. The validation was successfully performed by means of accuracy and precision, selectivity and specificity, linearity, recovery and stability under various conditions. This bio-analytical method was validated by following ICH guidelines.(Dudhe et al. 2016)

Pharmacokinetic study:

The pharmacokinetic study parameters of the optimized nanosuspension and solid dispersion formulations of Tolbutamide compared to the oral administration of pure drug aqueous dispersion are displayed in figure 2 and table 3. The plasma concentrations of unchanged Tolbutamide (10 mg/kg) increased from the time of administration (t=0) and reached the peak value (C_{max}) of 4.865 ± 0.83 µg/mL at 4 h (T_{max}), and then gradually decreased with $t_{1/2}$ values of approximately 6 h. In case of the selected nanoformulations, the pharmacokinetic parameters were found to significantly vary compared to the pure sample. The C_{max} values displayed as 7.261 ± 0.603 µg/mL and 9.16 ± 0.867 µg/mL for the nanosuspension and solid dispersion, respectively, wherein the T_{max} was also achieved within 2 hours in both samples. The bioavailability parameter based on the area under the plasma concentration vs. time profile curve was estimated to be 25.38 ± 2.25 µg.h/mL, 77.57 ± 5.51 µg.h/mL and 112.47 ± 3.59 µg.h/mL for the pure drug, nanosuspension and solid dispersion, respectively. This depicted more than 3-fold increase in bioavailability for the nanosuspension and about 4.5-fold greater availability for the solid dispersion. The relative bioavailability of these two formulations was 308% and 445% compared to the pure drug oral administration. The results were comparable to the *in vitro* studies and also could be correlated to the particle size and improvement in dissolution (Gundogdu and Yurdasiper 2014). Based on the estimation of AUMC parameter, the mean residence time of the formulations were also found be the enhanced as 8.26 h and 9.26 h for the nanosuspension and the solid dispersion, compared to the 4.67 h for the pure drug. Statistically significant ($p<0.05$) difference could be observed for the optimized formulations compared to the pure drug. The results confirmed the satisfactory application of the product for clinical application to enhance the drug release and availability of the drug *in vivo* (Patino-Herrera et al. 2019).

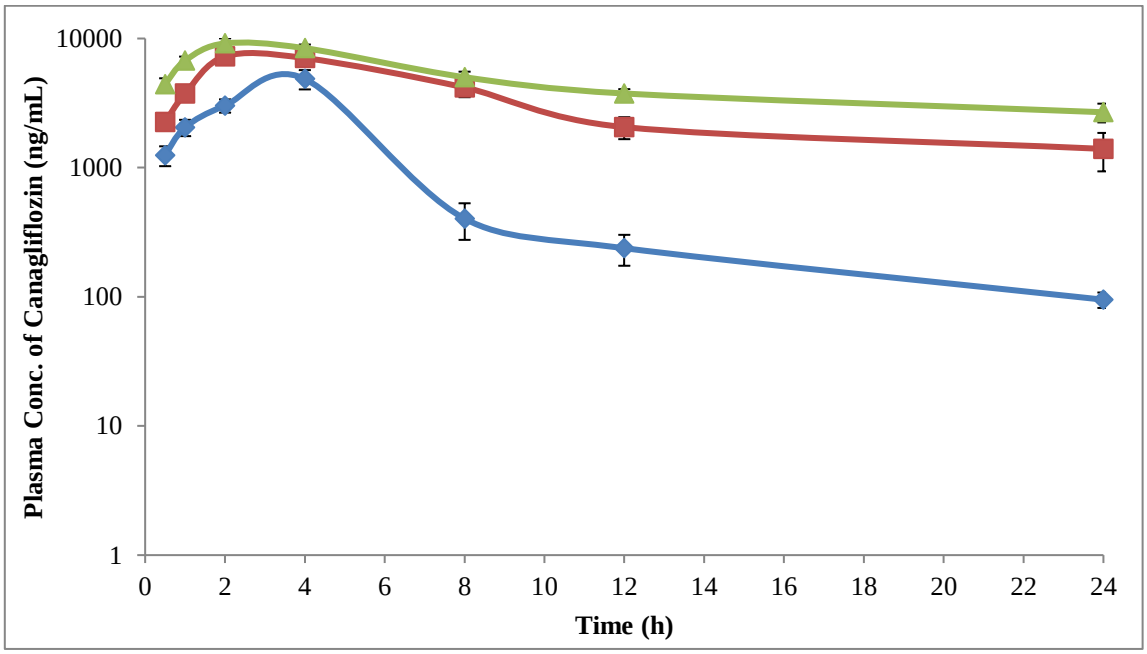

Fig 2: Plasma Concentration Vs. Time Profile of Tolbutamide Nanoformulations compared to pure drug

Table 3: Effect on Tolbutamide Nanoformulation on Pharmacokinetic Parameters

S. No.	Pharmacokinetic parameters	Unit	Pure drug	Nanosuspension	Solid Dispersion

1	C_{max}	µg/mL	4.865 ± 0.83	7.261 ± 0.603	9.16 ± 0.867
2	T_{max}	h	4	2	2
3	AUC	µg.h/mL	25.38 ± 2.25	77.57 ± 5.51	112.47 ± 3.59
4	AUMC	µg.h/mL	118.90 ± 14.62	642.75 ± 87.98	1042.28 ± 53.78
5	MRT	h	4.67 ± 0.20	8.26 ± 0.65	9.26 ± 0.23
6	Relative bioavailability	%	-	307.79 ± 28.44	445.54 ± 45.51

Conclusions

The nanoformulations of Tolbutamide were developed through two methods namely, solvent evaporation-nanoprecipitation process and solid dispersion by kneading technique using different ratio of surfactants (PVA and Pluronic F-127) and carriers (beta cyclodextrin and PEG 4000), respectively. Based on the experimental optimization and results for size, colloidal stability and drug release profile, the nanosuspension stabilized with 3% Pluronic F127 surfactant and the solid dispersion particles prepared with 1:1 ratio of beta cyclodextrin were opted for the pharmacokinetic and pharmacodynamics studies to compare against the pure drug aqueous dispersion. In pharmacodynamic studies, both the nanoformulations depicted similar and significant improvement in the reduction of mean plasma glucose level compared to the unmodified drug. In pharmacokinetics, about 3-foldsand 4.5-folds increase in bioavailability (than the pure drug) was demonstrated by the nanosuspension and solid dispersion, respectively.The results provide evidence for the prominent enhancement of the bioavailability of the BCS class IV drug, due to increase in the solubility and permeability. Histopathological examination of liver tissues in the study's Wistar rat model revealed promising results regarding the safety profile of Tolbutamide nanoformulations. The nanosuspension formulation demonstrated minimal hepatocyte degeneration and preserved liver architecture, indicating a favorable safety profile with negligible hepatotoxicity. These findings suggest that Tolbutamide nanoformulations could offer improved liver safety margins, making them promising candidates for clinical translation in the management of type-2 diabetes mellitus. This study could confirm the utilization of Tolbutamide nanoformulations to replace the conventional oral dosage form administration to achieve better clinical response.

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