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RESEARCH ARTICLE

AGGRANDIZED TRANSDERMAL DELIVERY OF GLIMEPIRIDE VIA TRANSFERSOMES: FORMULATION, EVALUATION AND STATISTICAL OPTIMISATION

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ABSTRACT:

The aim of the study was to prepare and statistically optimize transdermal formulation of antidiabetic drug Glimepiride (Glmp). In the present investigation Protransfersome gel (PTG) of glimepiride was prepared by modified coacervation phase separation technique and characterised for various parameters like vesicles shape, vesicles size and size distribution, entrapment efficiency and stability. Box-Benkhen model was chosen as optimization design. Three factors (amount of phospholipids, amount of surfactant and amount of drug) were varied at three levels Box-Benkhen statistical experimental design. These factors were found to have significant effects on the vesicular size (296.6±1.2nm), PDI(0.241±0.4) and drug loading(71.90±4.8%) and were optimized based on the desirability of the responses. The skin permeation studies were performed for 24 hours on pig ear skin using Franz diffusion cell. The flux value obtained from PTG (5.129±1.24 μg/cm^2/h) was greater as compared to the drug suspension (0.430 μg/cm^2/h). PTG formulation showed good stability at 4±1^{0}C and after 3 months of storage there was no change in liquid crystalline nature, size of vesicles, drug content and other characteristic parameters observed. In vivo pharmacokinetic study of PTG showed significant drug release as compared to plain transdermal patch of the drug. Hence, present study reveals that PTG generates a new breakthrough for the transdermal delivery of Glimepiride with higher bioavailability, negligible gastrointestinal and hepatic side effects and increased patient compliance.

Keywords: Glimepiride, protransfersome gel, optimization

INTRODUCTION:

Diabetes mellitus (DM) is a serious world health problem defined as group of metabolic disease characterised by hyperglycemia resulting from defects in insulin secretion or insulin activity or both. Type 2 DM is associated with obesity and insulin resistance, together with defects in beta cell function[1].

Glimepiride is a medium to long acting 3rd generation sulphonylurea antidiabetic drug which is indicated to treat type 2 DM. It acts as an insulin secretagogue. It lowers blood sugar by stimulating release of insulin by pancreatic beta cells and by inducing increased activity of intracellular insulin receptor[2]. It is available in the form of tablet and suspension in the market with the dose of 2, 4, 8 mg per day.

Direct delivery of antidiabetic drug glimepride using oral approach is not suitable because it produces hypoglycaemia during initial hours of oral administration and irregular bioavailability due to low solubility in water and poor patient compliance. It is also associated with gastrointestinal side effects and hepatic side effects (like cholestatic jaundice) [3]. Thus, this necessity improved drug delivery via transfersome approach which enhanced delivery of this drug via the skin barrier, significantly improving bioavailability as well as patient compliance due to its ultradeformabilty property. The concept of ultradeformable or highly elastic vesicles was introduced in 1992 first by Gregor Cevc as new generation of vesicles, known as transfersomes, composed of phospholipids and surfactants called as edge activators[7,8]. The edge activator refers to the single chain surfactants which causes destabilization of the lipid bilayer. In addition, it also increases the vesicle-elasticity or fluidity. Later in 1999, the second generation of the elastic vesicles were introduced by Van den Berg[7,8]. These vesicles consist of a micelle forming and a bilayer forming surfactant as destabilizing and stabilizing portion, respectively. In order to overcome the stability problem liquid crystalline pro-ultraflexible lipid vesicles "Protransfersome" were proposed, that will be converted into ultraflexible lipid vesicles transfersomes also known as elastic liposomes, *in situ* by absorbing water from the skin[4,5]. Protransfersomes provide higher stability and better skin penetration ability than the traditional lipid vesicles, e.g. liposomes, niosomes, etc[6]. The proposed Pro-T gel is a liquid crystalline gel in which the drug is intercalated within phospholipids[9].

MATERIALS AND METHODS

Glimepiride was procured from Panacea Biotec, Delhi, India. L-α- phosphatidylcholine and sodium deoxycholate from Sigma Aldrich Co., St. Louis, U.S.A. Disodium hydrogen phosphate, Hydrochloric acid and sodium hydroxide were purchased from RFCL limited, New Delhi, India. All reagents used in this study were of analytical grade.

Preparation of ProTransfersomal Gel

Protransferosomal gel (ProT-gel) was prepared with slight modifications as reported by Perrett *et al* [10]. Soya phosphatidylcholine, sodium deoxycholate, drug (Glmp) and alcohol were weighed and collected in an amber colored vial. All the components were then mixed with the help of a magnetic bead at a temperature of 60-70^{0}C, while keeping the open end of the vial closed, in order to prevent the loss of solvent. Then 100 mL phosphate buffer saline (pH 7.4) was added at the same temperature with continuous stirring, which lead to the formation of less viscous translucent liquid. This liquid composition was converted into the ProT-gel with overnight cooling at room temperature[10].

Preparation of Transfersomes from ProTransfersome

ProT-gel (100 mg) was hydrated using 10 mL of phosphate buffer saline (pH 7.4) with manual shaking to produce transferosomal formulation for in-vitro evaluations.

Optimization

Response Surface Methodology – The Box-Behnken Model

Optimization was carried out with response surface methodology (RSM) by the means of Box–Behnken model. Factors such as amount of soya phosphatidylcholine, amount of sodium deoxycholate, and amount of drug (Glmp) were found to be significantly affecting the encapsulation efficiency of the drug, and size of the carrier, estimated by a series of hit and trial formulations. Hence, these three factors were varied at 3 levels while keeping the other factors such as type of alcohol, amount of alcohol, amount of buffer, stirring speed, and stirring temperature constant. The independent variables X_1, X_2, X_3 are listed in Table1. Based on the results of hit and trial formulations and literature study, appropriate ranges of the components were chosen as listed in table. Design Expert software was used to optimize the formulation and to develop the mathematical (quadratic) equations[11].

Table 1: Independent Variables and Their Levels in Box–Behnken design

Independent Variables	Level	
	-1	+1
X_1 = Amount of soya phosphatidylcholine (SPC) (mg)	375	475
X_2 = Amount of sodium deoxycholate(SDC) (mg)	25	125
X_3 = Amount of drug (Glimepiride) (mg)	1.25	5.0

Where, -1 signifies minimum amount and +1 signifies maximum amount

Characterization

Vesicle Size and Shape Analysis

Size (Z-Average diameter) and the polydispersity index (PDI) of transfersomes derived from ProT-gel were analyzed by dynamic light scattering technique (DLS), using Zetasizer Nano ZS (Malvern Instruments Ltd., USA) at 25^{0}C, and at an angle of 173^0. The PDI relates with the width of vesicle size distribution and its small value (< 0.1) points towards homogeneity, while a high value (> 0.3) indicates high heterogeneity. All the measurements were conducted in triplicate[10].Shape, size, and surface morphology of the transfersomes formed after hydration were confirmed by high resolution TEM at 300 kV (FEI, Technai G2 30 UTWIN). Samples were obtained by dispersing the ProT-gel (hydrated transfersomes) (0.1 mL) into 15 mL of double distilled water (diluted up to 150 times). A drop of prepared dispersion was stratified over a carbon-coated copper grid for approximately 15 min. Excess dispersion was removed with a filter paper and the sample was subsequently dried in a desiccator for 60 minutes. Sample was then loaded on the probe and observed under transmission electron microscope.

Encapsulation Efficiency (%)

Hydrated ProT-gel formulation (1mL) was centrifuged for 35 min at 12,000 rpm and 4˚C using an ultracentrifuge (Hettich, Germany). The supernatant was analysed to determine the unentrapped fraction of drug (Glmp) using UV spectrophotometer (Shimadzu, USA)[12].

The EE (%) was calculated by using the following:

$$EE\% = \frac{\text{Amount of Drug Encapsulated}}{\text{Amount of Drug Used}} \times 100.$$

Drug Loading (%)

The DL (%) was calculated by using the following:

$$DL\% = \frac{\text{Amount of Drug Encapsulated}}{\text{Total weight of the particles in the formulation}} \times 100.$$

Drug Release Study

The kinetics of drug (Glimepiride) release from the ultradeformable transferosomal preparation formulated as ProT-gel was determined by modified diffusion cell using pig ear skin as a membrane. The values were put into various kinetic equations and respective graphs were plotted such as: Zero order release kinetics, first order release kinetics, and Higuchi model. Release profiles were further evaluated using Korsmeyer-Peppas equation[15] to study the transport mechanism of the preparation.

***In vitro* Skin Permeation Studies**

The pig skin was carefully cleaned with distilled water and mounted on a Franz diffusion cell assembly (Orchid Scientific, India) with an available diffusion area of 1.43 cm^2. Fluid in diffusion cells was maintained at 37±0.5^{0}C under continuous stirring[13]. The stratum corneum of skin confronted the donor section, and the dermis confronted the receptor section and the apparatus was assembled. Equal amounts of drug (Glmp) loaded Pro-T-gel formulation (optimised result by Box Benkhen statistical design) as well as drug (Glmp) suspended in hydroxyl propyl methyl cellulose (HPMC) gel (as control) were applied to the donor cells facing the topical side of the skin. Isotonic phosphate buffered saline (PBS, pH 7.4) having small amount of 40% (v/v) PEG 400, was added to the receptor chamber to mimic the transdermal water gradient across the epithelial and stratum corneum faced upward to the donor compartment (sink condition). Aliquots (1.0mL) of receptor solution were withdrawn periodically for 48 h and replaced with the same volume of pre-warmed receptor solution[14]. Samples were analysed by validated HPLC method at λ_{max} (228 nm), and amount of drug permeated was calculated. Each preparation was studied in triplicate and the results represent the average value. All the measurements were conducted in triplicate.

Stability Studies

Optimized ProT-gel formulation was divided into 2 groups and stored at 4 ± 1^{0}C and room temperature, respectively for 3 months in screw capped amber colored glass bottles. Formulations were evaluated for drug crystals under digital microscope, drug content remaining, vesicle size and PDI after every 30 days. Stability studies of each formulation were carried out in triplicate.

In *vivo* pharmacokinetic studies

For in vivo studies, 6-8 weeks male albino wistar rats (245-265g) were used as experimental animals. Animals were kept in plastic cages in climatically controlled rooms and fed with standard rodent food pellet diet. The studies were carried out as per the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt of India and in accordance with the protocol duly approved by Institutional Animal Ethical Committee of Delhi Institute of Pharmaceutical Science and Research, New Delhi, India (Approval No. IAEC/2015/05 Dated: 20/11/2015). Hair on backside of male wistar rat was removed on previous day of experiment. Dose of Glmp was selected by conducting hypoglycemic studies with dose of 1-10 mg/kg[16]. Following an overnight fast, rats were divided in to 3 groups (n=3) and were treated as follows:

GroupI(Control): Patch containing Gel without drug

GroupII: Patch containing Gel loaded with micronised drug

GroupIII: Patch containing drug in Protransferomal Gel

After the administration, blood samples (0.2mL) for pharmacokinetic analysis was collected from lateral tail vein in heparin tubes at time intervals between 1-48 hrs after treatment. Plasma was separated by centrifugation and then stored at -20ºC until assayed.Plasma samples were subjected to HPLC analysis, using Shimadzu UFLC system. Glimepiride concentration was calculated from linear regression calibration curves of peak height with respect to different plasma samples obtained after centrifugation.

Statistical Analysis

Data analysis was implemented with the software, GraphPad Prism (5.0). The data were reported as mean ± S.D. (n = 3 or 6) and multiple comparisons of means (one way ANOVA, Dunnettpost test). Student's t-test was performed for comparing two samples. Results were said to be significant at ≥ 95% confidence interval, $p < 0.05$ and are shown as the mean ± SD.

RESULT AND DISCUSSION

Optimisation

Optimisation of Glmp loaded PTG was done by using Response Surface Methodology (RSM) : The Box-Behnken Model. RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM, which includes factorial design and regression analysis, helps in evaluating the important factors, building models to study the interactions between the variables, and selecting the optimum conditions of variables or desirable responses[11]. Box–Behnken statistical design was used to statistically optimize the significant formulation factors and evaluate their quadratic effects on size, PDI, zeta potential, and drug loading. The values of remaining parameters were kept constant throughout the Box-Behnken method. A design matrix comprising of 17 experimental runs with 5 centre points was constructed by varying 3 factors over 3 levels. The possible 17 combinations generated by the Design Expert (Version 9, Stat-Ease Inc., Minneapolis, MN) were run and responses were recorded. The independent variables and the responses for all 17 experimental runs are given in Table 2.

Table 2: Box Behnken Experimental Design with Measured Responses

Run	X_1	X_2	X_3	Size (nm)	PDI	Drug Loading (%)
1	425	75	3.125	279.11	0.353	70.20
2	425	125	5	499.21	0.331	68.38
3	375	25	3.125	721.31	0.584	52
4	475	25	3.125	819.75	0.666	54.9
5	375	75	5.00	292.01	0.279	66
6	475	75	5.00	394.45	0.343	61.9
7	425	75	3.125	287.32	0.35	71.21
8	425	125	1.25	380.69	0.302	45
9	425	75	3.125	288	0.351	72.41
10	425	75	3.125	287.26	0.353	70.02
11	375	75	1.25	265.66	0.266	39.12
12	375	125	3.125	545.25	0.281	64.5
13	475	75	1.25	332.25	0.323	41.87
14	425	75	3.125	286.92	0.355	71.11
15	425	25	1.25	642	0.633	36.62
16	425	25	5.00	685	0.642	61
17	475	125	3.125	619	0.309	57

X1: amount of soya lecithin, X_2: amount of SDC, X_3: amount of drug

The dependent variables i.e. Size, PDI and drug loading obtained at various levels of 3 independent variables (X_1, X2, X3) were subjected to multiple regression to yield second order polynomial equation. A positive value represents an effect that favours a better value of a given response, while a negative value indicates an antagonistic relationship between the factor and the response. The following regression equations were given by the design:

Regression Equations

Size = + 5972.08299 - 22.42457X_1 - 19.79207X_2 + 39.67556X_3 - 3.00800E - 003X_1X_2 + 0.098107X_1X_3 + 0.027787X_2X_3 + 0.030638$(X_1)^2$ + 0.12768$(X_2)^2$ - 12.18500$(X_3)^2$

This indicates that amount of soya lecithin and SDC has unfavourable effect while amount of drug has significant favourable effect on size. The positive coefficient for interaction between 2 variables (X_1X_3, X_2X_3) indicates favourable effect on size if combined together.

PDI = -1.93453 + 0.012255X_1- 9.68217E - 003X_2 + 0.02182X_3 - 5.40000E - 006X_1X_2 + 1.86667E – 005X_1X_3 + 5.33333E - 005X_2X_3 - 1.33300E - 005$(X_1)^2$ + 5.63700E - 005$(X_2)^2$ - 4.64356E - 003$(X_3)^2$

Amount of SDC has unfavourable effect while amount of drug and soya lecithin has favourable effect on PDI. The negative coefficient for interaction between 2 variables (X_1X_2, X_1X_3, X_2X_3) indicates unfavourable effect on PDI if combined together.

Drug loading (%)= -599.30903 + 2.71778X_1+ 1.00024X_2 + 37.40711X_3 - 1.10000E - 003X_1X_2 - 0.021333X_1X_3 - 2.98483E - 017X_2X_3 - 3.03660E - 003$(X_1)^2$ - 3.03460E - 003$(X_2)^2$ - 3.51047$(X_3)^2$

The above equation for drug loading indicates that the amount of soya lecithin, SDC, and drug, positively increase drug loading (%) in the ProT-gel.

From the above result the final optimised formulation was prepared which gave the result as given in Table 3.

Table 3: Box-Behnken design results for optimized Glimepiride loaded ProT-gel

Amount of SPC (mg)	Amount of SDC (mg)	Amount of Drug (mg)	Size	PDI	Drug Loading	Desirability
406.266	92.494	2.122	296.848	0.300	72.4	0.954

Size, PDI and Entrapment Efficiency

To determine the vesicles shape Transmission Electron Microscopy (TEM) study was carried out (Figure1). Vesicles size and size distribution was determine by optical microscope. The size was found to be 296 nm with the PDI of 0.25 and the maximum entrapment efficiency of vesicles was found to be 68%.

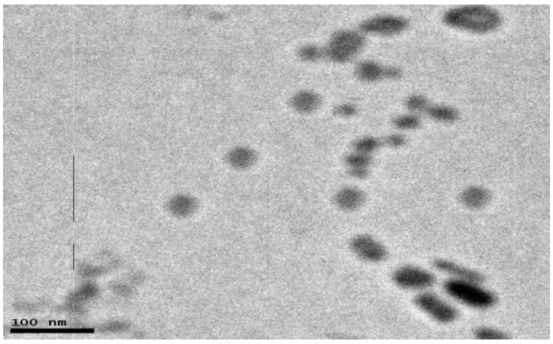

Figure 1: Photomicrograph [TEM at 300 kV] of transfersomes reconstituted from ProT-gel formulation.

In vitro Skin permeation studies

In vitro skin permeation studies were carried out on the optimized formulation and were compared with the drug suspension. Significant augmentation in the skin permeation of the drug has been observed (Figure 2) with vesicular formulation as the percent cumulative drug permeated through the skin was 9.00±2.2 % for drug suspensions whereas the amount of drug permeated through the ProT-gel formulation was 82.50±9.8%. ProT-gel system provides a greater flux (5.129±1.24 μg/cm^2/h) as compared to the drug suspension (0.430 μg/cm^2/h) for Glimepiride (Figure 2). Reduction in lag time for Glimepiride was observed with the ProT-gel system. After spontaneous transformation of ProT-gel into transferosomal vesicles (due to water permeation from the receptor compartment to the skin under non-occlusive conditions) two mechanism, (a) penetration enhancing effect and (b) the intact vesicular permeation through the skin, might simultaneously prevail the permeation of drug depending upon the vesicular composition, characteristics, and physicochemical properties of entrapped drug. Other reasons for enhanced permeation profile from ProT-gel carriers could be (a) better EE% of drug in transfersomes, (b) completely encapsulated drug within the vesicles, (c) alcohol providing penetration enhancement, and (d) membrane elasticity of the developed vesicular system. Due to spontaneous transformation of ProT-Gel into transferosomal vesicles, rapid drug permeation was detected for Glimepiride initially. Suspension demonstrated minimal to negligible permeation of drug over a period of 48 h. However, a sustained effect was observed with the ProT-gel formulation.

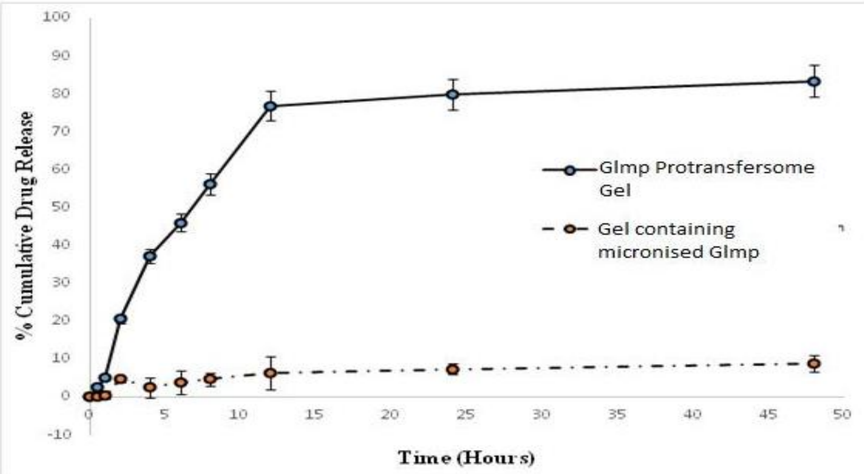

Figure 2: Cumulative drug release (%) from optimized ProT-gel formulation and micronized drug patch, through the ear skin of pig (Data are mean ± SD; n = 3)

Table 4: Permeation rate (μg/cm$^{2/}$h) at steady state along with the permeation enhancement ratio (Er)

Formulation	Drug flux (permeation rate) at steady state (μg/cm^2/h)	Enhancement ratio (Er)
Glmp loaded Protransfersome gel	5.129	11.927
Gel containing micronized Glmp	0.430	-

Release study

The release from ProT-gel formulations was subjected to different model dependent kinetics (zero order, first order, Higuchi model release kinetics and Korsmeyer-Peppas model, Fig.4 (a), (b), (c) and (d) respectively). The release profile exhibiting maximum R^2 value was found to obey Higuchi model release kinetics for ProT-gel formulations (Table 4). Hence, ProT-gel formulations follow Higuchi model drug release mechanism which means formulation exhibits an initial burst release followed by sustained drug release.

Korsmeyer-Peppas kinetics model was also analysed to see whether it was following Fickian diffusion (n= 0.5), anomalous transport (0.5< n<1.0), case-II transport (n= 1), or super case-II transport (n>1). A graph was plotted between the percentage cumulative release and log time (h). Release exponent (n), and correlation coefficient was analysed (Fig. 4 d). The release exponent (n) for the optimised ProT-gel formulation was found to be 0.8588. Therefore, the formulation followed Higuchi and anomalous transport for drug release.

Table 5: Release rate constants (k), correlation coefficients (R^2), and release exponent (n) of optimized ProT-gel formulation

Zero order		First order		Higuchi		Korsemeyer-Peppas	
n	R^2	n	R^2	n	R^2	n	R^2
7.67±0.812	0.599	0.277±0.058	0.695	18.69±0.101	0.9700	0.8588	0.897

Stability Studies

The storage stability of the colloidal carriers is the foremost restriction in the preparation of clinically appropriate marketed formulations. The influence of aging on EE% was not too significant in the case of refrigerated temperature (Table 5). Reason for increased consistency of ProT-gel might be due to a possible molecular interaction of surfactant's polar head groups with the solvent and permeation of solvent into bilayer. Vesicle size increased whereas PDI decreased indicating complete swelling of bilayer as the solvent diffused into the bilayers completely with time and hence more uniform vesicles were formed upon storage.

Table 6: Effect of storage on vesicle size, PDI, and Encapsulation Efficiency

	Time (months)	Temp. (4±1°C)	Temp (Ambient Conditions)
Vesicle size (nm)	0 day	310.5±1.2	
	1	394.2±1.2	340.1±2.3
	2	408.6±0.8	550.9±0.3
	3 s	520.6±1.1	610.8±0.9
Poly Dispersibilty Index (PDI)	0 day	0.258±0.4	
	1	0.230±0.6	0.394±3.1
	2	0.242±1.9	0.326±0.7
	3	0.241±0.5	0.298±0.4
Encapsulation Efficiency (%) of Glmp	0 day	68.180±8.3	
	1	66.421±9.1	58.960±4.6
	2	65.910±4.0	46.224±1.6
	3	64.011±6.8	43.190±7.3

In vivo kinetic studies:

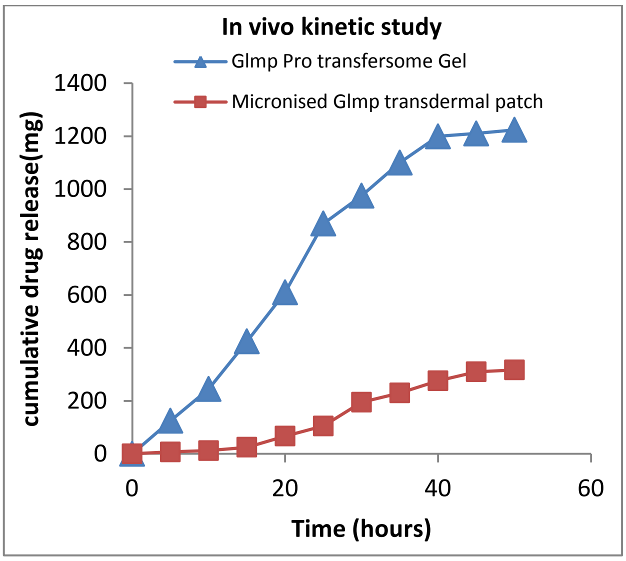

Figure 3: Comparison in drug release from micronised drug patch and protansfersomal gel formulation

In vivo studies in male wistar rats revealed that very low concentration of drug in plasma was achieved when micronized drug in gel was applied whereas when optimised Glmp loaded pro-transfersome patch was applied, it showed extensive increase in the drug release which was maintained over 48 hours (Figure 3). These results established the sustained and prolonged delivery of Glimepiride from pro-T gel formulation and ability to maintain constant drug concentration over 48 hours. Based on in vivo results, it can be concluded that investigated carrier system provided accentuated delivery through alongwith controlled and prolonged delivery of Glimepiride.

CONCLUSIONS

Results of present study established that introduction of ProT-gel generates a new breakthrough for the transdermal delivery of Glimepiride and exhibited improved release kinetics. It anticipate numerous advantages for instance bypassing first pass metabolism, counting a higher bioavailability, negligible gastrointestinal side effect, less long term effects due to smaller amounts of drug administered, and most importantly increased patient compliance (with availability of patch system) which is foremost in treatment of such a life-time disorder.

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