International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 13, Issue 1, 2021

Original Article

# PRONIOSOMAL GEL MEDIATED TRANSDERMAL DELIVERY OF GLIBENCLAMIDE AND ATENOLOL COMBINATION: *EXVIVO* AND PHARMACODYNAMIC STUDIES

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*Received: 08 Oct 2020, Revised and Accepted: 12 Dec 2020*

**ABSTRACT**

**Objective:** The objective of the present work was to develop an optimized dosage form for treating comorbidity in combination and evaluate it for its pharmacodynamic performance in male Wistar albino rats.

**Methods:** Transdermal proniosomal gel for Combination of Glibenclamide (GLB) and Atenolol (ATN) was developed and optimized by Box Behnken design. This optimized combinational proniosomal gel (OCPG), which was selected by a point prediction method, was evaluated for its *ex vivo*, skin irritation studies and pharmacodynamic activities of both drugs in rats in comparison with its oral therapy.

**Results:** The *ex-vivo* permeation behavior through different skins was studied and the findings were also confirmed by the values of the steady-state flux ($J_{ss}$). The OCPG observed an increase of more than twice in the cumulative amount of impregnated drugs compared to pure drug films. The study on skin irritation revealed the non-irritability of the developed OCPG applied. OCPG significantly showed sustained hypoglycemic activity in rats ($p<0.001$), when compared to orally treat animals up to 24 h. Systolic blood pressure (SBP) lowering effect of OCPG was found to be significant ($p<0.02$), when compared to orally treat rats up to 24 h. However, the reduction was slow and sustained in the case of OPCG where a significant response was observed in the performed studies.

**Conclusion:** Overall, the results show that controlled release GLB and ATN proniosomes offer a useful and promising transdermal delivery system. Henceforth this may be an achievement in treating the diabetic hypertensive patient.

**Keywords:** Combination therapy, Atenolol, Glibenclamide, Proniosomes, Pharmacodynamic study


DOI: http://dx.doi.org/10.22159/ijap.2021v13i1.39958. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

## INTRODUCTION

Diabetes mellitus (DM) is a complex chronic disease that requires ongoing medical care and multi-factor strategies to reduce risk beyond glycemic control [1-3]. In DM, a constant increase in glucose levels can lead to chronic micro-and macro-vascular effects [4-7]. A patient with multiple comorbid conditions requires multiple medications to treat each condition, increasing side effects and treatment costs. Combined pharmacotherapies may provide additive benefits that target multiple disease processes [8]. Fixed-dose combination drugs (FDC's) were originally developed to target only one disease. However, CFDCs can also target more than one disease/condition [9, 10]. However, no CDF has been developed for diabetes and its co-morbidities, like hypertension.

Transdermal drug delivery systems offer a promising alternative to oral administration, particularly in preventing difficulties associated with this combination [11, 12]. This is particularly relevant for these products as these two classes of medications are administered at two different times (before and after the diet) when administered by the oral route [13]. Oral administration of GLB causes symptoms like headache or nausea, cold sweats, excessive hunger [14-16]. Atenolol (ATN) is widely used in the management of hypertension as monotherapy or in combination with other classes of antihypertensive agents [17]. The absorption of Atenolol upon oral administration in humans and most laboratory animal species is rapid but incomplete [18]. Thus the transdermal administration of GLB and ATN would have a better dosage form being that it increases bio-availability.

Therefore, the combination of GLB and ATN through transdermal delivery can be a better therapeutic combination for effective control of diabetes and its coexisting cardiovascular complications (hypertension). The primary objective of this research was to assess the *ex vivo* and pharmacodynamic behavior of the optimized formulation of GLB and ATN obtained by designing Box Behnken as a proniosomal gel for transdermal administration.

## MATERIALS AND METHODS

ATN and GLB were received as gift samples from Sun Pharma Ltd., Mumbai, India. Span 60 and cholesterol were purchased from SD Fine Chemicals, Mumbai, India. Phospholipid (Brand: Phospholipon 90G) was a gift sample supplied by Phospholipid GmbH, Nattermannallee, Koln Germany. The other reagents and chemicals used were of analytical grade and were procured from Merck Limited, Mumbai, India.

### Preparation and optimization of proniosomes

Coacervation-phase separation method was followed for the preparation of proniosomes, which was reported by Perrett and Vora [19, 20]. To assess the interaction impacts of surfactant, Phospholipid, and Cholesterol in the formulations; 3-factor, 3-level Box Behnken design was utilized. An absolute 17 test runs were produced by design expert Version 11 software [21]. The independent variables were surfactant (Span 60) (X1), Cholesterol (X2) and phospholipid (Phospholipon 90G) (X3) while Vesicle size (VZ) (Y1) entrapment efficiency of GLB and ATN (Y2 and Y3 respectively) were the dependent variables which are given in table 1.

### *Ex vivo* permeation study by using different skins

### Preparation of rabbit skin

The preparation of skin was as per Xi *et al.* [22]. Male, white Newzeland Rabbits weighing 2.0-2.5 kg was obtained from the National center for laboratory animal sciences (NCLAS) after approval from the Institutional Animal Ethics Committee (IAEC No.: IAEC/ANCP/2018-19/02). All the animals used in the study were caged and maintained according to the guidelines of CPCSEA or principles established for the care and use of laboratory animals. After the rabbit was anesthetized with urethane (20 %, w/v), the skin was made hairless by applying hair removal cream (Veet® depilatory cream). The side of stratum corneum was extracted after the rabbit was sacrificed and the sub-dermal tissue was carefully removed. The side of the stratum corneum was cleaned with distilled water. The skin was washed with phosphate buffer saline (PBS), wrapped in aluminum foil and stored in a deep freezer at-20 °C till further use (used within 2 w of preparation) [23-25].

**Table 1: Independent and dependent variables used in box–behnken design for the development and optimization of proniosomal combination**

| Variables | Levels | | |
|---|---|---|---|
| **Independent variables** | **-1** | **0** | **+1** |
| X1=Span 60 (mg) | 450.00 | 675 | 900.00 |
| X2= Cholesterol (mg) | 150.00 | 200 | 250.00 |
| X3= Phospholipid (mg) | 900.00 | 1050 | 1200.00 |
| **Dependent variables** | **Constraints** | | |
| Vesicle Size (Y1) | Minimum | | |
| EE% of GLB (Y2) | Maximum | | |
| EE% of ATN (Y3) | Maximum | | |

**Preparation of rat skin**

Wistar albino male rats were obtained from the National center for laboratory animal sciences. All rats were kept under standard laboratory conditions in the 12-h light/dark cycle at 25 °C±2 °C provided by pellet diet (Lipton India Ltd., Bangalore) and water ad libitum. The animals were selected after superficial examination of the skin surface for abnormalities. The hair on the skin of Wistar albino male rat was clipped and subcutaneous tissues were removed, and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. The skin was washed with PBS, wrapped in aluminum foil and stored in a deep freezer at-20 °C till further use (used within 2 w of preparation) [26].

**Goat skin**

The hair on the skin of slaughtered goat was removed carefully and separated from the underlying cartilage with a scalpel. After separating the full-thickness skin, the fat adhering to the dermis side was removed using a scalpel and isopropyl alcohol [27].

**Skin permeation studies**

The diffusion of GLB and ATN from the prepared gel, either contain pure drugs or OCPG, was carried out utilizing the same conditions for the method previously reported [28, 29]. A two-chamber horizontal Franz diffusion cell (volume of 12.5 ml and surface area of 3.14 $cm^2$) was utilized and the system was kept up at 37±0.5 °C. OCPG was spread on the stratum corneum side and two cells were clipped together. The skin was mounted over the diffusion cell in contact with the receptor compartment, which was cleaned before use. The receptor compartment was filled with diffusion medium (PBS, pH 7.4) and this receptor phase was continuously stirred at 600 rpm with a Teflon-coated magnetic bar. Serial sampling was performed at specified time intervals (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h) by removing the contents of the receptor compartment and replacing it with the fresh medium. The sample was examined utilizing the HPLC. From the obtained data, flux is calculated by using the below formula [30].

Steady state flux ($J_{ss}$) = Slope of the linear portion of the curve/Surface area of diffusion cell

Permeability Coefficient ($K_p$) = flux/initial concentration of the drug in donor chamber = $J_{ss}/D_0$

Enhancement Ratio (ER) = $J_{ss}$ of proniosomes/$J_{ss}$ of control

**Skin irritation studies**

The study was performed by using healthy Male Newzeland Rabbits (2.5-3.0 kg) which were fed with food and water for 1 w to adapt to the environment before the study. For the skin irritation study, the rabbits were divided into three groups. Group 1 served as control. Group 2 received blank Proniosomal gel and Group 3 received OCPG on the previously shaven (24 h before the study) dorsal side of rabbits. Skin irritation study was performed as per the Draize scoring system (table 2) [31, 32]. The responses scored were noted approximately at 1, 2, 3and on the 7th day after the removal of the standard irritant and test formulations, rabbits were examined for signs of erythema and edema. The untreated skin of each rabbit was treated as a control for the respective test formulations [33, 34].

**Table 2: Primary dermal irritation study of OCPG in rabbits: draize score evaluation criteria for skin reactions**

| Value | Erythema | Value | Edema formation |
|---|---|---|---|
| 0 | No erythema | 0 | No edema |
| 1 | Very slight erythema (barely perception), edges of area not well defined | 1 | Very slight edema (barely perceptible. Edges of area not well defined) |
| 2 | Slight erythema (pale red in color and edges definable) | 2 | Slight edema (edges of the area well defined by definite raising) |
| 3 | Moderate to severe erythema (defined in color and area well defined) | 3 | Moderate edema (raised approximately 1 mm) |
| 4 | Severe erythema (beet to crimson red) to slight scar formation (injuries in depth) | 4 | Severe edema (raised more than 1 mm and extending beyond the area of exposure |

**High-performance liquid chromatography (HPLC) of GLB and ATN**

The concentration of the combination of GLB and ATN in the samples was analyzed by using HPLC method (Water 2690 composed of PDA-2996 detector) with BDS C18 250 × 2.1 mm, 1.6 µ columns. Data acquisition, recording, and chromatographic integration were performed by Empower 2 software. The mobile phase consisting of 0.01N potassium dihydrogen orthophosphate (pH 4.8) and acetonitrile taken in the ratio 55:45 with an injection volume of 1.0 ml in gradient mode with column oven temperature maintained at 30 °C. The flow rate was 1.0 ml/min and the detector was set at 235.0 nm. Method validation including recovery, specificity, and within-and between-day precision of HPLC was carried out, which was reported in our earlier work Anitha *et al.*, [35].

**Pharmacodynamic activity of OCPG**

Animal experiments were done as per the standards and guidelines set by the institutional animal ethical committee (IAEC No.: CBLRC/IAEC/13/01-2019). All wistar albino rats (150-180 g) were fed ad libitum and housed in light and dark cycle in an ambient temperature-controlled environment. They were placed into the rat holder for 5–6 d for a period of 10–20 min. By repeating the exercise, the animals taught to remain in the rat holder calmly and they became acquainted with the experimental conditions [36-39].

**Antidiabetic activity of OCPG in rats**

**Studies in normal rats**

Male Wistar albino rats (n=6) were fasted overnight only water was given. The hair in the neck region of the animals was removed using

a hair removal cream and OCPG was applied. The rats were left in individual cages, blood was withdrawn periodically from the orbital sinus at the interval of 0, 2, 4, 6, 8, 12, and 24 h and blood glucose levels were measured. GLB was administered orally as a suspension to another group of six rats after overnight fasting and blood glucose levels were estimated at intervals mentioned above. In both the cases each animal served as its own control and the hypoglycemic response was calculated by taking the difference in glucose levels at the 0 hour and subsequent hours. For the untreated group (n=6) of animals, after overnight fasting, the glucose levels were estimated by taking blood samples at 0, 6, 12 and 24 h [40].

**Studies in diabetic rats**

The rats were fasted for 30 h and later rendered diabetic by an intraperitoneal injection of streptozocin (50 mg/kg body weight) in pH 4.5 citrate buffer. The blood glucose was measured after 24 h and rats with blood glucose levels>250 mg/dL were selected. The experimental protocol used in normal rats was followed in the assessment of hypoglycemic activity. The rats were divided into 3 groups (n = 6). The rats were treated as follows, Group-I–oral administration of suspension of the marketed GLB, Group-II–GLB (single drug) Proniosomal gel (PNG), and Group-III–OCPG (ATN and GLB combination) was applied after application of Dermaroller on the previously shaven dorsal side of rats. An adhesive tape was rolled over the gel formulation to fix it securely to the site of application. The blood glucose level of each rat was measured at the interval of 0, 2, 4, 6, 8, 12, and 24 h using the One Touch glucometer [41].

**Antihypertension activity of OCPG in rats**

Methyl prednisolone acetate (20 mg/kg/week) for 3 w was administered via the subcutaneous injection to 24 Wistar rats (n = 6) for the induction of hypertension, and the SBP of these rats was measured by tail-cuff method (NIBP system IN125/R; AD Instrument Pvt. Ltd., Australia**)** and rats with the systolic pressure (SBP)>130 mmHg were then selected for the experiment [42, 43]. Hypertension was successfully induced in all rats. The rats were treated as follows, Group-I–oral administration of suspension of the marketed ATN, Group-II–ATN (single drug) Proniosomal gel, and Group-III–OCPG (both drugs–ATN and GLB) was applied after application of Dermaroller on the previously shaven dorsal side of rats. An adhesive tape was rolled over the gel formulation to fix it securely to the site of application. SBP levels were measured just before and at 0, 2, 4, 6, 8, 12, and 24h using the tail-cuff system [44, 45].

**Data analysis**

Data were expressed as the mean±standard deviation of the mean, and statistical analysis was carried out by employing the one-way analysis of variance (ANOVA). A value of $p<0.05$ was considered to be statistically significant.

**RESULTS AND DISCUSSION**

In this study, various formulations were prepared containing GLB and ATN combination given in the design. The optimum formulation of Proniosomal gel was chosen dependent on the criteria of achieving the desirable value of vesicles size, entrapment efficiency of GLB and entrapment efficiency of ATN by applying numerical point prediction method. The OCPG formulation gives the experimentally observed values of vesicles size of 559 nm and entrapment efficiency of GLB is 97.037±1.43% and entrapment efficiency of ATN is 97.230±1.62%. These experimental values of vesicles size, entrapment efficiency of GLB and entrapment efficiency of ATN yielded by the OCPG Formulation were found in agreement with the predicted value of vesicles size (562±17.223 nm), entrapment efficiency of GLB (97.13±1.47%) and entrapment efficiency of ATN (97.42±1.85%) respectively created by design expert software, recommending that the optimized formulation was rational and reliable. The formulation composition with Surfactant (Span 60) (830.272 mg), Cholesterol (191.219 mg) and Phospholipid (900.00 mg) were found to satisfy the essentials of an optimized combination proniosomal gel (OCPG) formulation. This formulation was successful in permitting the transdermal permeation of GLB and ATN.

***Ex vivo* permeation study by using different skins**

Following the preparation of proniosomal gel loaded with OCPG; the *ex-vivo* permeation behavior through different skins was studied. The release profile from the transdermal gel loaded with OCPG was superior to normal patches. More than 2-fold increase in the cumulative amount of drug permeated was noticed from the OCPG when compared to pure drug films, which is evident from the values given in table 3. This finding was also confirmed by the values of the steady-state flux ($J_{ss}$). It is obvious that OCPG formulation demonstrated the greatest flux over the rabbit skin and rat skin (0.221±0.04 mg/cm$^2$/h and 0.583±0.02 mg/cm$^2$/h, 0.229±0.06 mg/cm$^2$/h and 0.588±0.04 mg/cm$^2$/h) of GLB and ATN respectively compared with goatskin (0.186±0.03 mg/cm$^2$/h and 0.516±0.02 mg/cm$^2$/h), which is similar to findings of Imam *et al.* [46] and Alsarra *et al.* [47]. The permeability coefficient ($K_p$) valuations for OCPG were 0.0221 cm/min and 0.0233 cm/min, 0.0229 cm/min and 0.02352 cm/min of GLB and ATN respectively from rabbit and rat skin which is greater than goatskin (0.0186 cm/min and 0.0206 cm/min). The permeation profiles of two drugs from different skins were shown in fig. 1 and 2. The permeation of GLB and ATN from the prepared gel either contains a pure drug or OCPG, was in favor of the Higuchi-diffusion model. According to the Korsmeyer-peppas model, the calculated n-values were greater than 0.45 but less than 0.89, indicating non-Fickian or anomalous release, which refers to a combination of both diffusion and erosion controlled-drug release mechanism. These results are in good agreement with previous work for a combination of drugs that have been loaded with a transdermal delivery system [45, 48].

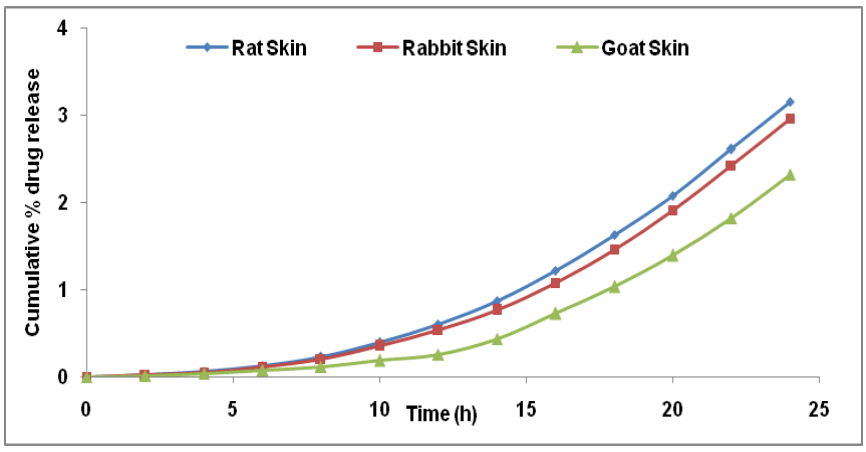


**Fig. 1: *In vitro* skin permeation profile of GLB from OCPG using different skins**

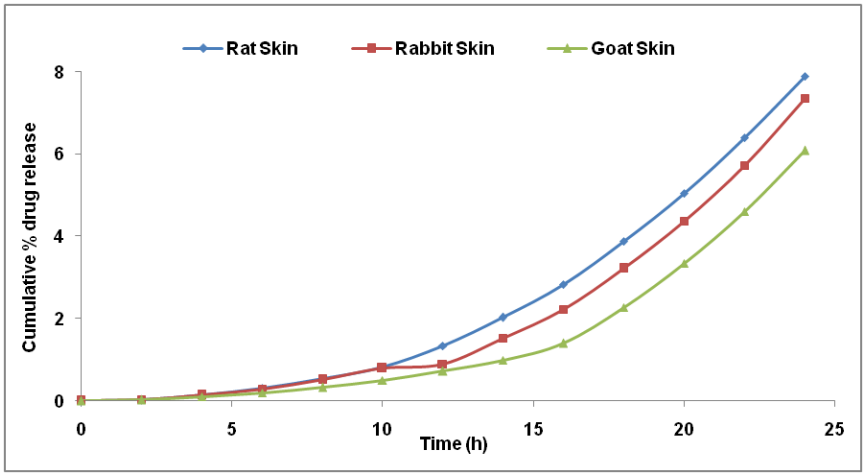


**Fig. 2: *Ex vivo* skin permeation profile of ATN from OCPG using different skins**

**Table 3: *Ex vivo* permeation data of OCPG for different skin**

| S. No. | *Ex vivo* permeation | Flux (mg/cm²/h) | | n-values | |
|---|---|---|---|---|---|
| | | GLB | ATN | GLB | ATN |
| 1 | Rabbit Skin (OCPG) | 0.221±0.04 | 0.583±0.02 | 0.608 | 0.650 |
| 2 | Rat Skin(OCPG) | 0.229±0.06 | 0.588±0.04 | 0.575 | 0.642 |
| 3 | Goat Skin (OCPG) | 0.186±0.03 | 0.516±0.02 | 0.595 | 0.493 |
| 4 | Control (Patches) | 0.175±0.03 | 0.399±0.02 | 0.686 | 0.590 |

Data for each response is presented in mean±SD (n=3)

**Skin irritation studies**

Based on the results, the skin irritation investigation depicted the non-irritancy of the developed OCPG applied. Results showed that the prepared OCPG was safe to be used for transdermal route. No obvious erythema, edema or inflammation was observed on the Male Newzeland Rabbit skin after one week of application of the selected formulation [49].

**High-performance liquid chromatography (HPLC) of GLB and ATN**

An isocratic LC method, coupled with PDA detection, was developed for the simultaneous determination of ATN and GLB. Chromatogram A and chromatogram B represents the blank mobile phase and an average retention time of 2.322 min for GLB and 3.260 min for ATN, with no interfering peaks, respectively in fig. 3. According to ICH guidelines (International Council for Harmonisation), this method was validated. The validation characteristics were addressed in our earlier work Anitha *et al.*, [35].

**Pharmacodynamic activity**

**Antidiabetic activity of OCPG in rats**

**Studies in normal rats**

Hypoglycemic effect was significant in oral and OCPG applied group when compared with the untreated group (fig. 4). The untreated group of animals did not show any noticeable hypoglycemia. Oral GLB produced a decrease of 40.7±5.4% in blood glucose levels after 2 h. In the case of OCPG, the maximum response was observed after 8 h (30.4±5.4%) and thereafter remained stable up to 24 h (42.4±5.6%) and the hypoglycemic response was gradual. The blood glucose levels declined after 8 h and were only 5.5±4.3% after 24 h in an orally treated group, which is similar to findings mentioned in Sridevi *et al.*, [39].

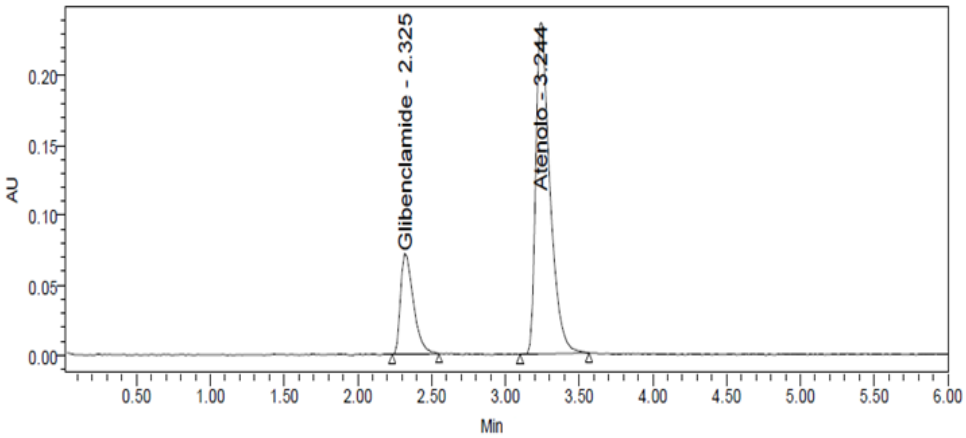


**Fig. 3: HPLC chromatograms of mobile phase containing 10 µg/ml GLB and 25 µg/ml ATN (chromatogram B)**

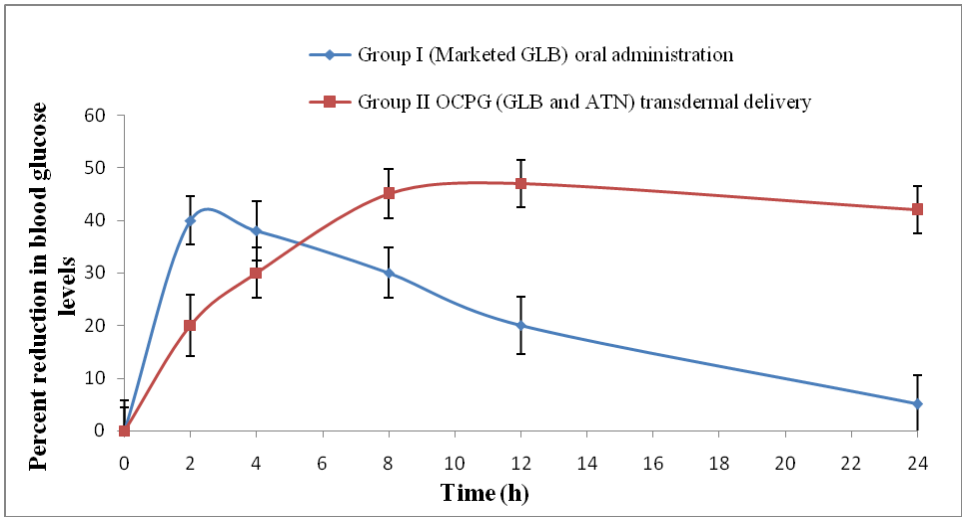


**Fig. 4: Percent reduction in blood glucose levels after oral and OCPG administration in normal rats (n = 6, mean±SD)**

### Studies in diabetic rats

Results obtained from the diabetic rats after application of OCPG, GLB PNG and GLB oral administration are shown in (fig. 5). Blood Glucose levels obtained by from diabetic rats showed significant and almost similar hypoglycemic activity up to 24 h. The hypoglycemic effect produced by OCPG and GLB PNG in the rats is significantly less when compared to oral administration in the initial 4 h. The observed effect was found to be significant in case of OCPG treated animals (P<0.001), when compared to orally treat animals up to 24 h. The GLB (oral) produced a decrease in blood glucose level up to 90.12±9.23 mg/dL (P<0.05) for 4 h. In case of OCPG and GLB PNG, the hypoglycemic response was gradual. A maximum hypoglycemic response was observed after 8 h and remained stable up to 24 h (fig. 5). The plasma insulin level was elevated to the maximum in oral, transdermal proniosomes (OCPG and GLB PNG) treated groups at 4 h and 12 h, respectively as similar to the findings mentioned in Vijayan *et al.*, [50].

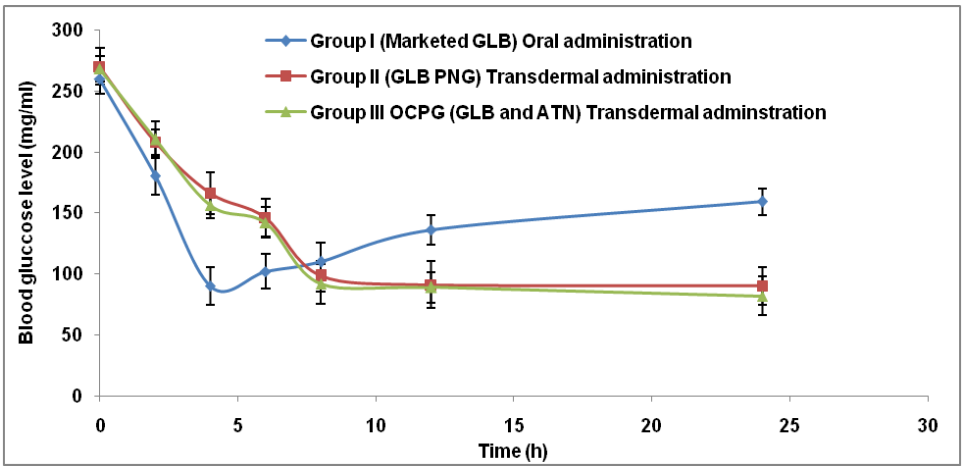


**Fig. 5: Showing changes in blood glucose levels after oral administration of GLB suspension, GLB proniosomes and OCPG in streptozocin-induced diabetic wistar albino rats. *P<0.001, significant difference in blood pressure with respect to oral treated group (n = 6, mean±SD)**

### Antihypertension activity of OCPG in rats

During antihypertensive study, hypertension was induced in all rats in a successful manner after subcutaneous injection of methyl prednisolone acetate (20 mg/kg/week for 3 w). There is a significant difference in SBP values in pre-and post-treatment with methylprednisolone acetate in Wistar albino rats. The changes in systolic blood pressure (SBP) (mm Hg) after oral administration of marketed ATN, ATN PNG and OCPG in methylprednisolone acetate-induced hypertensive wistar rats are presented in fig. 6. An early drug action was observed in Group 1 after oral administration of ATN; hypertension was significantly reduced to normal value with the maximum reduction in SBP (110.67±3.33 mm Hg) observed at 2 h. After 6 h of oral administration, SBP progressively started to rise and reached up to 125.50±4.62 mm Hg at 12 h and reached 140.83±3.12 mm Hg at 24 h (P<0.05) after oral administration. The observed effect was found to be significant in the case of OCPG treated rats (P<0.02), when compared to orally treat rats up to 24 h. On the other hand, ATN PNG and OCPG steadily decrease the SBP and maximum action of the drug observed after 4 h after transdermal application (SPB 115.50±4.26 mm Hg) in hypertensive rats, which is similar to findings in Abdul Ahad *et al.*, [51]. The SBP lowering effect of proniosomal gel formulation was sustained and maintained up to the complete duration of study i.e. 24 h. It is concluded that the developed OCPG formulation released the drug gradually and efficaciously controlled the SBP in rats up to 24 h.

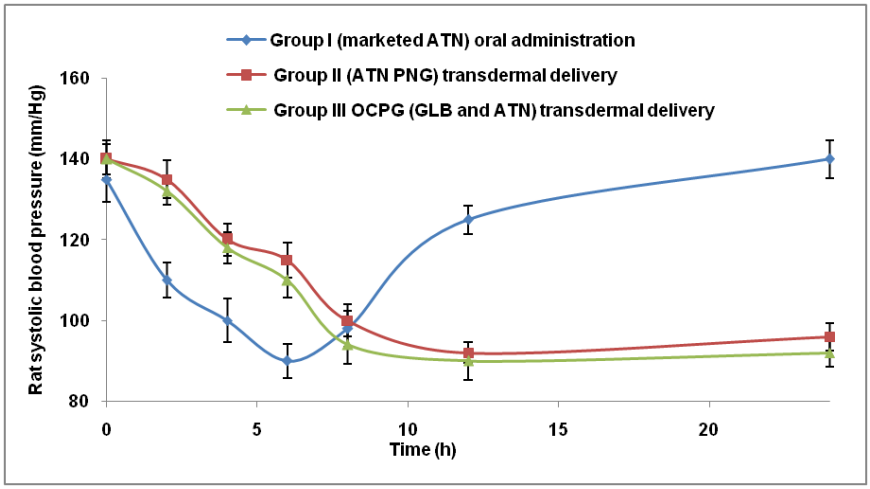


**Fig. 6: Showing changes in systolic blood pressure (mm Hg) after oral administration of ATN suspension, ATN proniosomes and OCPG in methyl prednisolone acetate-induced hypertensive wistar albino rats. *P<0.02, significant difference in blood pressure with respect to oral treated group (n = 6, mean±SD)**

## CONCLUSION

Transdermal route not only effectively maintained normoglycemic levels in contrast to the oral group but also an effective approach for treating its Comorbidities i.e., hypertension in combination, which produced remarkable hypoglycemia and controlled SBP, which is an indication that a similar episode might be prevented in diabetic hypertensive patients. This study showed that proniosomal transdermal administration of a combination of GLB and ATN showed better control of high blood sugar and hypertension, in addition to reversing complications of diabetes mellitus more efficiently than oral administration in rats. However, ex vivo assessment, pharmacokinetics and pharmacodynamics of these systems in human volunteers is required to confirm these results.

## FUNDING

Nil

## AUTHORS CONTRIBUTIONS

All the author has contributed equally.

## CONFLICTS OF INTERESTS

Declared none

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