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INFLUENCE OF TOAD PAROTID GLAND SECRETION FROM INDIAN TOAD (*BUFO MELANOSTICTUS)* IN DIABETIC RATS: AN EXPERIMENTAL EVIDENCE OF P-GLYCOPROTEIN INHIBITION

ABSTRACT:

The study was conducted to improve the oral bioavailability of glyburide (GLY) with Indian Toad Parotid Gland Secretions (TPGS). P-glycoprotein is an efflux transporter cellular protein and effluxes xenobiotics and drugs to the outside of cells lead to decreased concentration of drugs at the target site. P-gp inhibitors essentially increase the levels and there is a need for new P-gp inhibitors to develop for the improvement of the oral bioavailability of P-gp substrate drugs because the existing inhibitors have serious side effects. This study was aimed to describe the P-gp inhibitory action from TPGS, *Bufo melanostictus*, in diabetic rats by using glyburide as p-gp substrate. Acute toxicity studies showed 300mg/kg as toxic dose and 50mg/kg was selected as study dose according to OECD 423. LC-HRMS study conducted to identify the new compounds. Apparent permeability (P_{app}) was estimated by non-everted sac method (I*n Vitro*) with rat jejunum and ileum to confirm the P-gp inhibitory activity of TPGS by using fexofenadine (FEX) as P-gp substrate. In *in-vivo* protocol rats grouped into 4 groups (n=6), the first one is normal, second diabetic, third GLY 30mg/kg, and fourth group GLY+ TPGS, 50mg/kg for the single and multiple-dose treatment study. The spectrometric analysis revealed the new compounds, and TPGS P_{app}($X10^{-6}$ cm/s) in rat jejunum and ileum was significantly increased from 2.0±0.1 to 6.4±0.3 and 1.2±0.3 to 3.0±0.3 respectively. Blood glucose concentration in rats more than 250 mg/dl were considered as diabetic and in single, multiple-dose interaction studies (SDI, MDI) the concentrations decreased from 140.0±2.0 and 122.0±2.2 µg/dl respectively. The pharmacokinetic parameters like C_{max}, Cl and in SDI, MDI and significant increase of C_{max} and AUC_t and decrease of Cl was observed. The above results conclude that TPGS had the potential P-gp inhibitory activity and improved the oral bioavailability of GLY significantly. Subsequent experimentation is warranted to chemically characterize the compounds from TPGS as potential new P-gp inhibitors.

Keywords: ToxinousTPGS, LC-HRMS, *Bufo melanostictus*, P-gp, Glyburide.

1. INTRODUCTION

Inoculation of the venom system was not developed by the toads, even though they are treated as venomous animals as they secrete highly toxic venomous emanations from their skin. Toads contain alveolar mucus and acinar granular glands with different types of functions [1, 2]. Frogs and toads skin, glandular secretions contain bioactive host-defensive molecules from Australian anurans and, there are several types of glands available for the secretion of these compounds [3-5]. These natural compounds with biological activities like anti-bacterial, anti-inflammatory and anti-cancer activities [6-11] are reported surprisingly there are no studies on Indian Toad secretions. Therefore, as these secretions are unique resources for novel drug development we conducted this study for the first time with Indian Toads. The secretions from mucus gland maintain to make the skin slippery as well as prevent its mechanical damage by some materials [12, 13] and mucus control the body-surface P^H and also maintain skin moisture [14]. These secretions are bacteriostatic, can trap microbial and fungal pathogens and also protect from an adverse effect of prolonged contact with water and slow down the evaporative loss of water. *Xenopuslaeris*, a type of African frog contain different types of mucins with different functions [15]. The well-developed parotid glands in toads are present in *Buffo* and some other species on the skin and secrets mucins[16].Surprisingly different types of compounds produced from toads and frogs like peptides, steroids alkaloids and many other uncharacterized toxins with a variety of biological activities [17, 18]. Toxins from toads and frogs secretions proved to possess cardiotonic, micotoxic and neurotoxic, vasoconstrictive, hypotensive, hallucinogenic effects along with their harmful effects to their predators. Upon systematic investigations of these secretions will be helpful to develop new chemical entities [19]. Permeability glycoprotein (P-g) is a protein that efflux out the drugs and chemicals to the outside of cell likely to be present on all cells acts as a defence mechanism against harmful substances and is *ABCB1* gene-encoded [20, 21].

Many of the drugs are P-gp substrates, because of its effect their concentrations inside the cell not maintained and if any P-gp inhibitor is given in combination, it will elevate the intracellular concentration. P-gp inhibiting drugs increase the concentration of the drugs those are substrates of P-gp and thus enhance the pharmacological and/or toxicological effects of the substrate drugs. Glyburide (GLY) is a P-gp substrate, and Indian toad parotid gland secretion (TPGS) if exhibits

P-gp inhibitory effect, there may be the possibility to alter the pharmacokinetics and pharmacodynamics and improve the oral bioavailability of GLY.

2. MATERIALS AND METHODS

Acetonitrile (Merck, Mumbai), methanol (Merck, Mumbai), Glyburide, (Sigma Aldrich, Bangalore), Potassium dihydrogen phosphate (Sigma Aldrich, Bangalore), Gliclazide, Fexofenadine (Aurobindo labs), Streptozotocin (Sigma Aldrich, Bangalore), Glucose kit (GOD-POD-KIT) (Viola, Mumbai). Types of equipment used are, HPLC (contains C18 column coated with 5micron particles), Biofuge (Heraeus instrument- Germany), Micropipettes (Torsons), Microcentrifuge tubes (Tarsons), Butterfly catheter, Ultra sonicator. LC HRMS (Instrument: Agilent Technologies, Modal: 0530).

2.1. **Animals**

Wistar rats procured from Mahaveer Enterprises, Hyderabad, acclimatized then used, with standard diet and water *ad libitum,* and 12 hours fasting was maintained before the start of the study.

2.2.**Sample collection and Preparation:**

Adult live Indian Toads (40-50 grams), Order– *Anura*, family – *Bufonidae*, genera – Bufo, subgenera–*melanostictus* were collected from the near vicinity of Kakatiya University, Warangal. *Bufonidae* includes more than 300 species the family includes 25 genera and the above-selected species was authenticated by Zoology professor YV from our university. TPGS was freshly obtained by compressing the parotid glands by milking process (it oozes out as white mass) with the help of forceps and hands aseptically from living individual's secretions were collected on the surface of glass Petri plates and it's containing dried extract was used in the study [22-24] and the Toads were released to live.

2.3.**Spectrometric analysis study by LC-HRMS:**

The aliquots of TPGS was dissolved in methanol and subjected for LCHRMS study mass spectra were recorded by Electron Spray Ionization -ESI [25] in the positive ion mode by direct infusion.

The Q-TOF MS used a quadrupole (four parallel rods arranged in a square formation), a collision cell, and a time of light unit to produce spectra. Lighter ions accelerate faster down the flight tube to the detector thus determining the ions mass-to-charge ratios.

2.4.**Toxicity assessment:**

TPGS suspended in water and toxicity findings were done under OECD guidelines in female Wistar rats. Animals separated to (4 groups, n=3) normal saline-treated group, TPGS 5mg/ kg/p.o treated group, TPGS 50mg/kg/p.o treated group and TPGS 300 mg/ kg/p.o the treated group, then observed for the possible toxicity up to 48 h. The mortality was noted to determine the lethal dose of TGPS [26-28].

2.5.***In Vitro*** **study**

Non-everted sac method:

Rats were grouped up to 4 (n=4) after overnight fasting intestines were isolated under anaesthesia by using thiopental sodium (50mg/kg/i.p), and ice-cold saline was used to flush. The rats were exsanguinated, the jejunum and ileum portions isolated and a segment of each was cut into a length of 10cm for preparation of the sac for the experiment. First group intestinal sacs were loaded with 500µg/mL of fexofenadine (FEX) a P-gp substrate alone and, second FEX+Verapamil (VER) as an inhibitor, third FEX+TPGS 1000 µg/ml and fourth FEX+TPGS1500 µg/ml was included in the sacs for the study protocol and apparent permeability coefficient (P_{app}) was calculated [29].

2.6.***In Vivo*** **Study:**

Rats were subjected for induction of diabetes and more than 200mg/dl glucose concentration was selected as diabetic [30].

Pharmacodynamics and Pharmacokinetic interaction study in diabetic rats:

Animals were grouped into four different sets like first as normal control, the second set as diabetic control, third set as glyburide treated and fourth set as GLY+ TPGS treated. The treatment is given for seven days; the interaction study was done with an initial day as a single dose and on the seventh day as a multiple-dose study. At the present, time points blood sampling

was done from tail vein (0, 0.5,1, 2, 4, 6, 8, 12 and 24h), at predetermined time intervals between by using butterfly catheter (23GB). Serum was separated by centrifugation. Blood glucose concentrations were estimated by GOD-POD method [31], and pharmacokinetic parameters estimated.

2.7.**Pharmacokinetics evaluation:**

HPLC description:

A Shimadzu Class VP series HPLC system with two LC-10AT pumps, an SPD-10A variable wavelength programmable UV/VIS detector, an SCL-10A system controller and an RP C-18 column (Merck, Hiber; 250 mm×4.6 mm; particle size 5 µm) was used. The system was equipped with N2000 software. The mobile phase consists of 60:40 v/v acetonitrile: monobasic potassium dihydrogen orthophosphate (pH 3.5 adjusted with phosphoric acid) at 60:40 v/v ratios was run by the isocratic system through the reverse-phase analytical column and gliclazide was used as an internal standard [32]. The flow rate was 1 ml/min and the effluent was monitored at 253nm. The total run time of the method was set at 15 min.

2.8.**Statistical Analysis:**

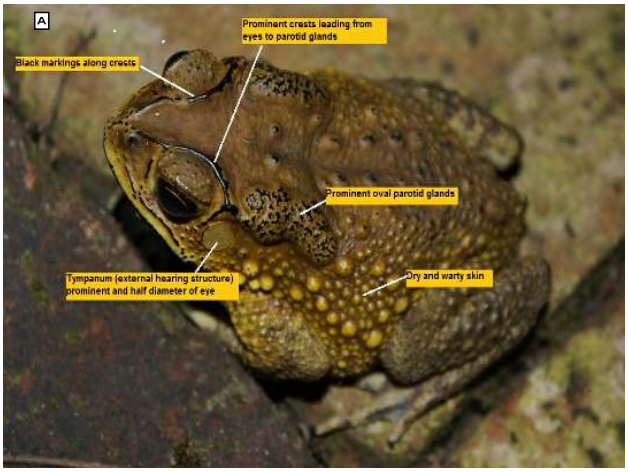
Analyzed the data help of WinNonlin 6.2 software and Graph Pad Prism software version 8.4.2, two way ANOVA (Bonferroni post-test) was used for statistical analysis and the values are explained as mean±SD.

3. RESULTS

3.1.**Spectrometric analysis study by LC HRMS:**

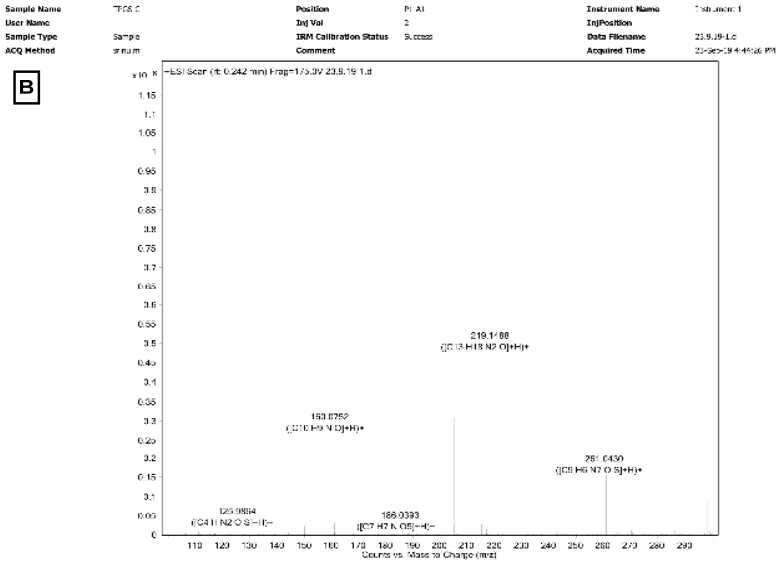
The presence of parotid gland is depicted in the Fg1A.

Fig 1. A). Courtesy. NUS Wiki.nus Web page.



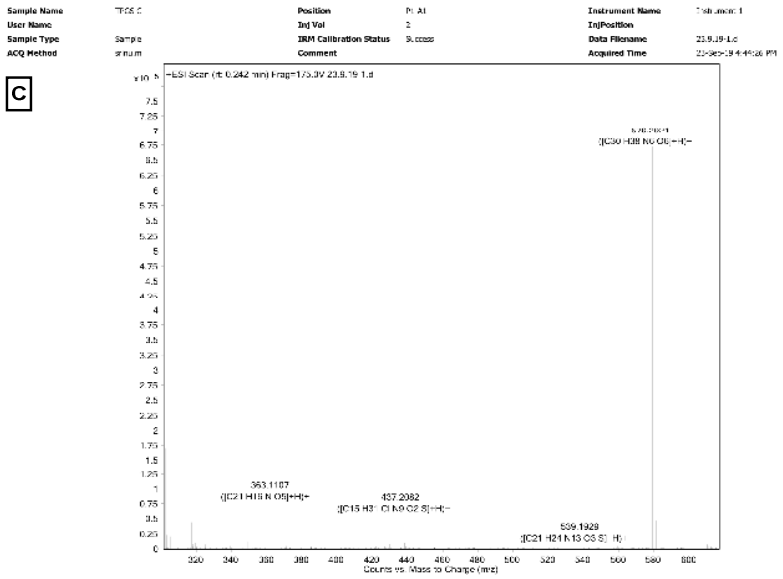
The following mass followed by the proposed molecular formula was obtained from LCHRMS studies with TPGS. They are 125.9864 ([C4 H N2 O S] +H) +, 160.0752 ([C10 H9 N O] +H) +, 219.1488 ([C13 H18 N2 O] +H) +, 261.0430 and are shown in Fig 1B.

Fig 1B. Identified Mass and probable Molecular formulas of TPGS range from 100-300.



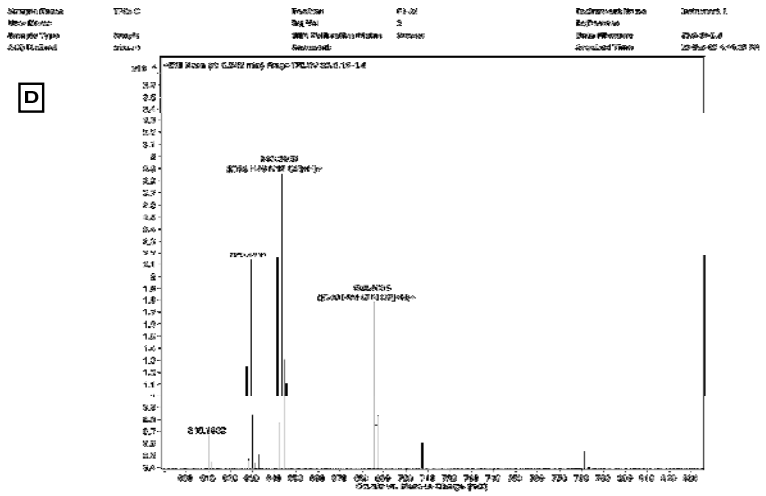
Whereas molecular formulas found from 300 to 600 range are ([C9 H6 N7 O S]+H)+, 363.1107 ([C21 H16 N O5]+H)+, 437.2082 ([C15 H31 Cl N9 O2 S]+H)+, 539.1929 ([C21 H24 N13 O3 S]+H)+, 579.2921 ([C30 H38 N6 O6]+H)+ and shown in Fig 1C.

Fig 1C. Identified Mass and probable Molecular formulas of TPGS range from 300-600.



Further the proposed molecules with molecular formula in the range of 600 to 800 are 643.3939 ([C33 H46 N12 O2]. +H) +, 685.4066 ([C40 H59 Cl N O6] +H) + and are shown in Fig 1D.

Fig 1D. Identified Mass and probable Molecular formula of TPGS ranges from 600-800.



3.2.Toxicity Assessment:

The mortality was observed with TPGS 300mg/kg and maximally tolerated dose (MTD) was decided as 50mg/kg/p.o. Table 1.

Table 1. Effect of TPGS of *Bufo melanostictus* for toxicity assessment.

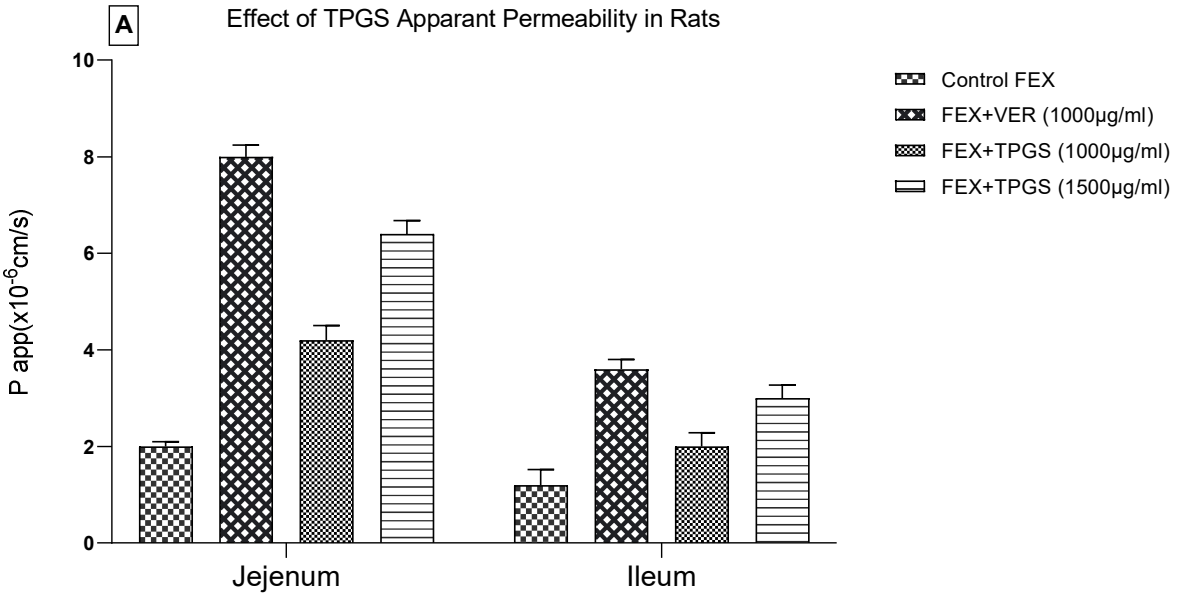
Groups	Treatment	Sign of toxicity (TS/NS)	Mortality (D/S)	% L/D
Normal control	Normal saline	0/3	0/3	100/0
TPGS	5mg/kg	0/3	0/3	100/0
TPGS	50mg/kg	0/3	0/3	100/0
TPGS	300mg/kg	2/3	2/3	34/66

TPGS= Toad Parotid Gland Secretion, TS= Toxicity Sign, NS=No sign of toxicity, D=Death, S= Survival, L=Live (n=3).

3.3. *In Vitro* Study:

TPGS P_{app}($X10^{-6}$ cm/s) in rat jejunum and ileum was significantly increased from 2.0±0.1 to 6.4±0.3 and 1.2±0.3 to 3.0±0.3 respectively Fig.2A.

Fig.2 A. Effect of TPGS influence on P_{app} of FEX in rats.

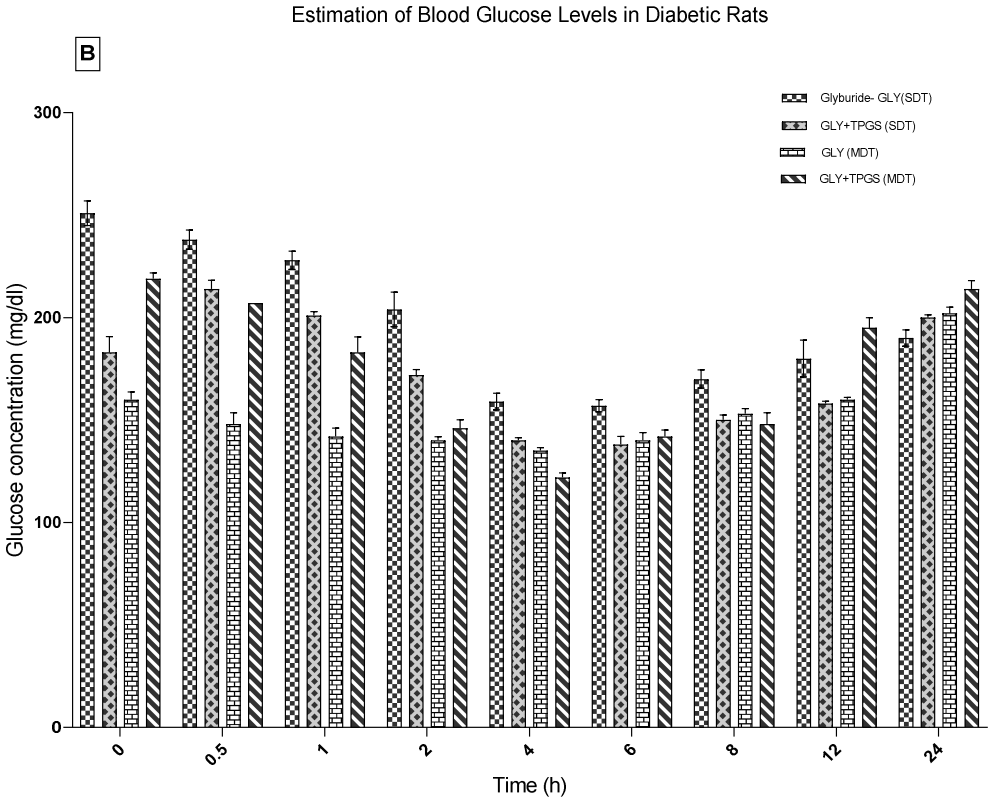


TPGS= Toad parotid Gland Secretion, FEX= Fexofenadine, VER= Verapamil, and data represents (n=6). Statistical analysis was performed using Two way ANOVA (Bonferroni post-test) with Mean±Standard deviation.

3.4.***In Vivo*** **Study:**

Blood glucose concentration in rats more than 250 mg/dl were considered as diabetic and in single, multiple-dose interaction studies (SDI, MDI) the concentrations decreased from 140.0±2.0 and 122.0±2.2 µg/dl respectively Fig 2 B.

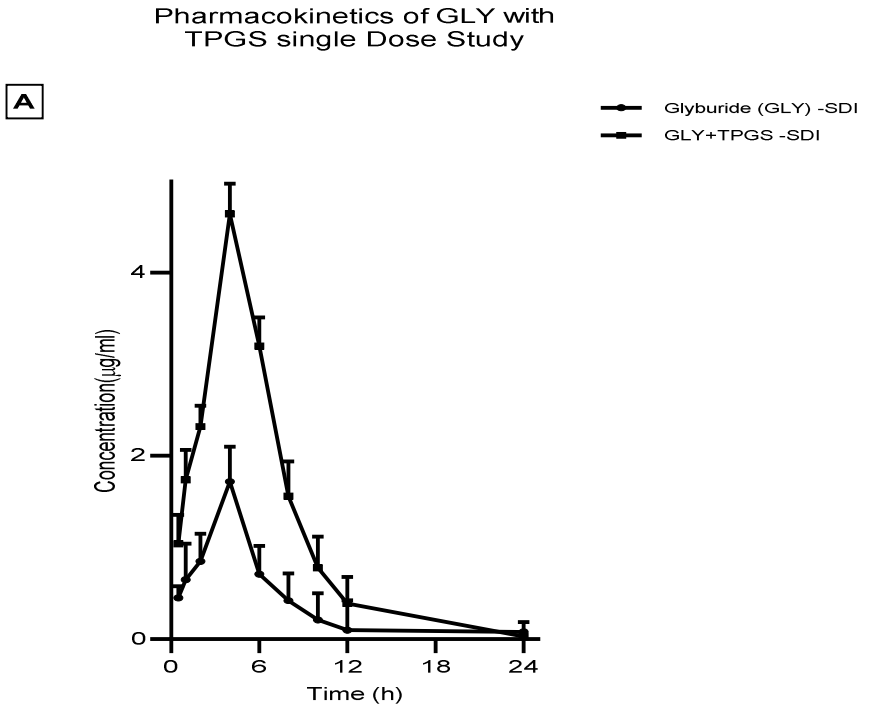
Fig 2B). Estimation of blood glucose levels in diabetic rats with TPGS and GLY.



SDT (single dose-treated); MDT (multi dose treated) Statistical analysis was performed using Two way ANOVA (Bonferroni post-test) with Mean±Standard deviation.

The pharmacokinetic parameters like C_{max}, Cl and in SDI, MDI significant increase of C_{max} and AUC_t and decrease of Cl was noticed. **C_{max}** of Gly was increased from 1.725±0.299 µg/mL to 4.300±1.160 µg/mL, 7.600±1.071 µg/mL with Gly +TPGS single dose Fig 3 A.

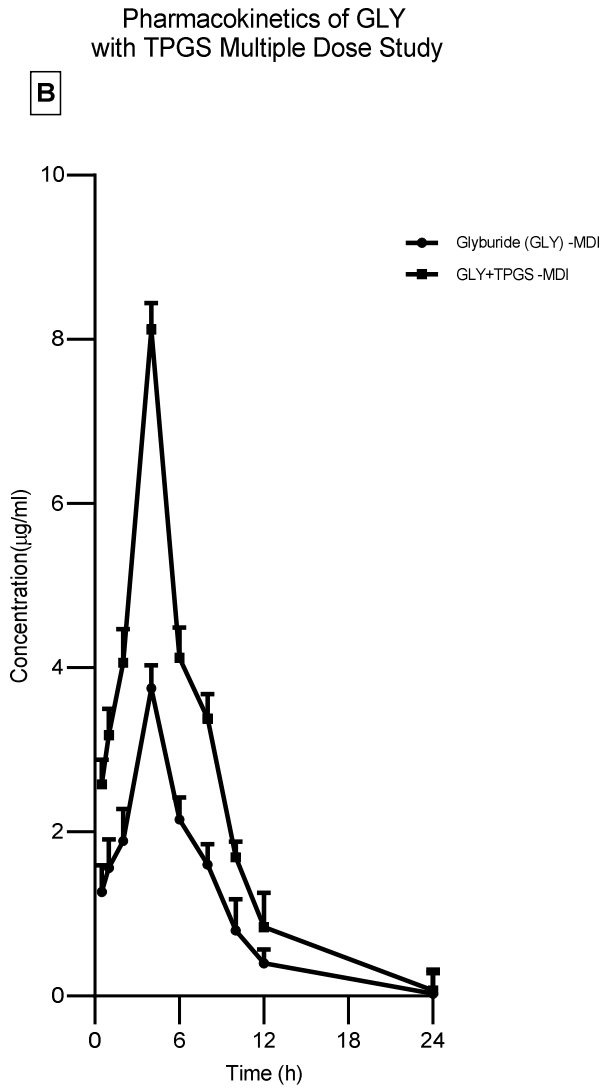
Fig. 3. A. Pharmacokinetic profiles of GLY and TPGS in diabetic rats for 7 days treatment with Single-Dose Study.



GLY= Glyburide, TPGS=Toad Parotid Glandular Secretion., Each symbol with a bar represents the mean ± S.D (n=6).

C_{max} of GLY was increased from 7.600±1.071 µg/mL with GLY+TPGS multiple-dose treatment Fig 3B.

Fig. 3B. Pharmacokinetic profiles of GLY and TPGS in diabetic rats for 7 days treatment with Multiple Dose Study.



GLY= Glyburide, TPGS=Toad Parotid Glandular Secretion**.,** Each symbol with a bar represents the mean ± S.D (n=6).

Clearance $_{of}$ GLY was decreased from 3536.9±277.72 mL/h/kg to 1130.409±311.1 mL/h/kg, 576.042±149.991 mL/h/kg with GLY +TPGS single-dose and multiple-dose treatment Table 2.

Table 2: GLY and GLY along with TPGS pharmacokinetic parameters with diabetes.

Pk parameter	GLY (SDT)	GLY (MDT)	GLY + TPGS (SDT)	GLY + TPGS (MDT)
C_{max} (µg/mL)	1.725±0.299	3.750±0.810**	4.300±1.160	7.600±1.071**
T_{max} (h)	4.000±0.000	4.000±0.000	4.000±0.000	4.000±0.000
$T_{1/2}$ (h)	2.028±0.327	3.951±2.420	2.953±1.270	3.612±1.316
AUC_t (h. µg /mL)	7.263±0.754	18.056±4.561	21.019±5.358**	36.044±3.549**
AUC_∞ (h. µg /mL)	8.519±0.626	28.081±7.665	27.850±6.421**	54.497±12.490**
CL (mL/h/kg)	3536.9±277.72	1146.5±387.9	1130.409±311.1***	576.042±149.991*
Vd (mL/kg)	10395.4±2158.065	5855.008±2245.70	4638.1±1692.73*	2804.3±612.884
Kel (h^{-1})	0.349±0.057	0.219±0.102	0.262±0.086	0.221±0.111
MRT (hr)	3.964±0.087	4.181±0.206	4.301±0.272	4.160±0.381

GLY= Glyburide, TPGS=Toad Parotid Glandular Secretion**.** Mean ±SD: *** p<0.001; ** p<0.01; * p<0.05 tested to GLY; SDT (Single dose treated); MDT (Multi dose treated). Two way ANOVA (*Bonferroni post-test*) was used for statistical analysis.

4. DISCUSSION:

Diabetes needs lifelong treatment for the optimal blood glucose concentration to maintain and if not maintained, it may result in other several disorders which affect the major organ systems in the body. Frogskin glands proved to have many potential compounds with pharmacological effects [33, 34]. LC HRMS study showed the presence of new chemical entities with a wide range of mass starting from 100 -900. In *an in-vitro* study, FEX P-gp substrate [35], and TPGS as testing inhibitor were used with jejunum and ileum, to confirm the P-gp inhibitory potential. This study evidenced that FEX, P_{app} was increased with VER as P-gp inhibitor [36] and

also with different concentrations of TPGS on jejunum and ileum indicates the P-gp inhibitory effect of TPGS these results are comparable with previous studies[37]. P-gp inhibitors can improve the oral bioavailability of P-gp substrate drugs [38-41] significantly similar to these results our results are correlated for the improvement of oral bioavailability of GLY a P-gp substrate drug and TPGS as P-gp inhibitor. The current study was undertaken to determine the antidiabetic potential of compounds derived from TPGS by using GLY as a P-gp substrate and to investigate the influence of TPGS on the absorption kinetics of GLY in STZ induced diabetic model. The enhanced absorption of GLY was due to P-gp inhibition by TPGS. Enhanced oral bioavailability by TPGS is related to the inhibition of P-pg substrate drugs by P-gp inhibitors [42, 43]. TPGS enhanced the oral pharmacokinetics of GLY suggesting that combined use may reduce the efflux of GLY. This indicates that natural compounds from Indian Toad may inhibit the P-gp driven GLY pumping outside the cell to some extent.

5. CONCLUSIONS

TPGS was found to be lethal at 300mg/kg. Shown to have P-gp inhibitory activity and enhanced the oral bioavailability of GLY. These results conclude that TPGS is a good source of the new chemical compounds of scientific interest to develop novel P-gp inhibitors. Further studies are warranted to isolate pure compounds as new chemical entities with potential pharmacological activities.

This study gives the scope for further exploring the systematic isolation of new chemical compounds to explore the mechanism of action of TPGS and also their interactions with other P-pg substrate drugs.

CONSENT

It is not applicable

ETHICAL APPROVAL

The protocol was approved by the Institutional Animal Ethical Committee, Kakatiya University, Warangal (IAEC/15/UCPSc/KU/2018).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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