Detection of antidiabetic activity by crude paratoid gland secretions from common Indian toad (bufomelano stictus)

Prasad Neerati

Department of Pharmacology, DMPK and Clinical Pharmacology Division, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India

Address for correspondence:
Dr. Prasad Neerati, Department of Pharmacology, DMPK and Clinical Pharmacology Division, University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Andhra Pradesh, India.
E-mail: prasadneerati@gmail.com

Abstract

Background: Amphibians have provided a remarkable array of biological active compounds, which are secreted from so-called granular skin glands which serve to protect the amphibians from predators due to its noxious effects on buccal tissue and at least in the case of some peptides, to protect from bacterial (or) protozoan infections. Given the respiratory and antimicrobial functions of amphibian skin, it is likely that some of the novel molecules found in amphibian granular gland secretions might be of use in the treatment of skin and respiratory infections. Secretions from common Indian toad (Bufo melanostictus) a member of Bufonidae family has the history of medicinal use however the anti-diabetic activity is not reported. The present study is aimed to determine whether paratoid gland extract have any influence on the diabetes and the pharmacokinetics and pharmacodynamics of glimepiride (GLM) in normal and diabetic rats.

Materials and Methods: An aqueous and methanolic extracts of paratoid glandular secretions were prepared, air dried and used to determine the antidiabetic activity in rats. The blood sampling was done at preset time intervals between 0, 0.5, 1, 2, 4, 6, 8 and 12 h, using heparinized capillaries. The blood glucose levels are estimated by glucose oxidase-peroxidase method, and reversed-phase high-performance liquid chromatography is used to determine the pharmacokinetic parameters of GLM using glibenclamide as an internal standard.

Results: Both the aqueous and methanolic extracts produced better glycemic control in diabetic rats, and methanolic extract is better than the aqueous extract. Serum concentrations of GLM increased at 2nd h, and the percentage glucose reduction is maximal at the 4th h with both aqueous and methanolic extracts of paratoid secretions of common Indian toad. Conclusions: Paratoid gland secretions of the common Indian toad is antidiabetic, in addition it has beneficial effects in combination with GLM. Further, it requires the systematic structure elucidation of the compounds and pharmacokinetic studies to explore the beneficial effects.

Key words: Common Indian Toad, glimepiride, paratoid gland secretions, reversed-phase high-performance liquid chromatography

INTRODUCTION

Toads have very large glands, and they secrete a large amount of venomous secretions with variable dose-dependent effects, this can affect other animal and human health care system. The primary aim is to find substances with completely novel structures leading directly or indirectly (via a new set of lead substances) to the next generation of Pharmaceuticals with new or improved activities. Such molecules may prove to be of general or academic, biochemical and biophysical, as well as of pharmacological interest since novel molecular structures may possess previously unknown and interesting pharmacological and biochemical properties. The major source of biochemical in amphibians is in their skin gland secretions for the possible potential as an anti-diabetic. Amphibian skin is highly permeable to water and fulfills many different physicochemical functions. Most amphibians show a marked preference for damp, if not aquatic habitats, which
are literally teeming with potentially pathogenic bacteria and fungi. They need to protect themselves against infection and indeed have developed a formidable array of integrated antimicrobial defense systems. Toads (order: Anura; family: Bufonidae; genus: Bufo) are distributed throughout the world, but are mainly in areas on tropical and humid temperate climates.[3] Each region is characterized by presence of some species of these amphibians found in India; the most commonly found species are Bufo melanostictus.

Toads do not possess the venom inoculation system, but they are venomous animals, as the glands covering the whole surface of their skin secrete highly toxic venom.[4] Amphibian skin glands basically consist of only two types - mucous glands and granular glands both of them are alveolar (oracinar); a third type, the tubulosoacicular or alveolar glands, is confined to a small groups of frogs and have limited, specialized functions. Other types have been described in certain anuran species.[5,6] Bufotenin is produced from a toad is a psychoactive compound.[7] Diabetes is a growing epidemic, which has been estimated to affect over 350 million people worldwide in 2011, and its prevalence is expected to increase to approximately 550 million by 2030.[8] It is considered as one of the five leading causes of death in the world. It is indeed to develop more compounds with antidiabetic potential to meet the needs of the treatment of diabetes and/or diabetic complications. Exenatide (Byetta, Bydureon) is taken by injection, similar to insulin, but they’re not insulin used to improve blood sugar control by mimicking the action of a hormone called glucagon-like peptide 1 and may also lead to weight loss. Byetta is a synthetic version of a protein found in the Gila monster, a poisonous lizard that lives in the Southwest and in Mexico, and this Lizard-Derived Diabetes Drug Is Approved by the Food Drug and Administration.[9] In our earlier study we proved that Indian toad (Bufo melanostrictus) parotoid gland crude secretion shown the dipeptidyl peptidase-IV (DPP-IV) inhibitory activity, these results triggered to do research on potential use of common toad parotoid gland crude extracts as antidiabetic.[10]

**MATERIALS AND METHODS**

**Animals and diet**
Male Wistar rats weighing 180–220 g were used in the study. They were maintained under standard laboratory condition at ambient temperature. They were fed with pellet diet and water ad libitum. The protocol was approved by institutional animal ethics committee (IAEC/15/UCPSC/KU/2013) Kakatiya University, Warangal.

**Drugs and chemicals**
Glimepiride (GLM) and Glibenclamide (internal standard [IS]) are gift samples from Chandra labs, Hyderabad, parotoid gland extract was freshly obtained by compressing the parotoid glands (it oozes out as white mass) with the help of forceps aseptically, and used in the study. Glucose estimating kits are supplied by Excel diagnostics Pvt. Ltd., Hyderabad and methanol high-performance liquid chromatography (HPLC) grade, Merck, Mumbai and diethyl ether, alloxan monohydrate is supplied by Sigma, Aldrich, and Bangalore.

**Induction of diabetes in rats**
Diabetes was induced in rats (fasting for 12 h) by the administration of Alloxan monohydrate in ice cold normal saline at a dose of 120 mg/kg intraperitoneally.[11] After 3 h 0.5 ml 10% glucose was given intraperitoneally and 5% oral glucose was given for following 24 h. After 10 days, the blood sample was collected from rats by orbital puncture of all surviving animals and the serum was analyzed for glucose levels. Rats with blood glucose levels of 250 mg/dl and above[12] were considered as diabetic and selected for the study.

**Pharmacokinetic and pharmacodynamic evaluation**

**Study in nondiabetic and diabetic rats**
Pharmacokinetic and pharmacodynamic interaction study in nondiabetic and diabetic rats. Two sets of the following animal groups (n = 6) are used in the study one set is for a non-diabetic group and the other set is for the diabetic group. All the parotoid gland extracts, and GLM are orally given to the animals and the parotoid extracts were nontoxic.

**In nondiabetic groups**
- Group 1 - GLM (orally 1 mg/kg)
- Group 2 - Parotoid gland extract in saline (Saline Parotoid Crude Extract [SP]-10 µg/kg) 7 days → on 8th day also SP
- Group 3 - Parotoid gland extract in saline (SP-10 µg/kg) 7 days → on 8th day (Followed by GLM)
- Group 4 - Parotoid gland extract in methanol (Methonolic Parotid Extract. [MP]-10 µg/kg) 7 days → on 8th day also MP
- Group 5 - Parotoid gland extract in methanol (MP-10 µg/kg) 7 days → on 8th day (followed by GLM).

**In diabetic groups**
- Group 1 - GLM (orally 1 mg/kg)
- Group 2 - Parotoid gland extract in saline (SP-10 µg/kg) 7 days → on 8th day also SP
- Group 3 - Parotoid gland extract in saline (SP-10 µg/kg) 7 days → on 8th day (Followed by GLM)
- Group 4 - Parotoid gland extract in methanol (MP-10 µg/kg) 7 days → on 8th day also MP
- Group 5 - Parotoid gland extract in methanol (MP-10 µg/kg) 7 days → on 8th day (followed by GLM).
Blood Samples were collected from orbital puncture at time intervals between 0, 0.5, 1, 2, 4, 6, 8 and 12 h using heparinized capillaries. Serum was separated by centrifugation. And blood glucose levels were determined using glucose oxidase-peroxidase method and remaining serum was stored in vials at ~70°C for pharmacokinetic parameters analysis.

**High-performance liquid chromatography description**
A Shimadzu Class VP series HPLC system with two LC-10AT pumps, a SPD-10A variable wavelength programmable ultraviolet/visible detector, a SCL-10A system controller and a RP C-18 column (Merck, Hiber; 250 mm × 4.6 mm; particle size 5 µm) was used. The system was equipped with N2000 software.

**Preparation of standard graph**
The stock solutions of GLM and glibenclamide were prepared in methanol at a concentration of 1 mg/ml each. Glibenclamide was used as an IS. By appropriately diluting the stock solution of GLM different concentrations (1.25, 2.5, 5, 12.5, 25, 50, 100, 125 µg/ml) and glibenclamide (100 µg/ml) were prepared. A volume of 100 µl of blank rat serum was mixed with 20 µl of 0.1N HCL, with different dilutions of 20 µl standard GLM solution and with 20 µl glibenclamide (100 µg/ml), vortex for 5 min. Then methanol (400 µl) was added and vortexed for 5 min, and it is centrifuged at 5000 rpm for 10 min. Supernatant 1 was collected; residue was added again with methanol (400 µl) by same procedure supernatant 2 was collected and added to supernatant 1. Supernatant (1 + 2) was evaporated, and methanol (200 µl) was added to the residue, then vortexed and centrifuged. Finally, supernatant was spiked into the column.

**Calculation of pharmacokinetic parameters**
Pharmacokinetic parameters were calculated using “KINETICA” software Adept Scientific, UK. All the data were expressed as mean ± standard deviation.

**Statistical analysis**
Difference in between concentration time profiles; in between pharmacokinetic parameters; in between serum glucose levels and difference between over the entire range tested were analyzed by one-way ANOVA (Bonferroni post-test). The differences were considered to be significant at \(P < 0.05\).

**RESULTS**
The HPLC detection of the glimepiride and glibenclamide is shown in [Figure 1]. Both the aqueous and methanolic extracts produced better glycemic control in diabetic rats, and methanolic extract is better than the aqueous extract. Serum concentrations of GLM increased at 2nd h from 8.24 ± 0.61 to 11.37 ± 1.15 and 12.5 ± 1.3 (\(P < 0.001\)) with saline parotoid and methanol parotoid extracts respectively [Table 1], and the percentage glucose reduction is maximal at the 4th h with both aqueous and methanolic extracts of parotoid secretions of common Indian toad [Table 2]. The saline and methanolic extracts (25%, 26% respectively) blood glucose percentage reduction activity is lesser than GLM (35%) alone, and greater than the combination with saline and methanolic extracts of parotoid gland secretions with glimepiride [Figure 2].

**Figure 1:** High-performance liquid chromatography chromatogram of glimepiride and glibenclamide in rat serum. Glibenclamide: Retention time-8.958 min, Glimepiride: Retention time-11.372 min
DISCUSSIONS

The crude toad secretions are very complex structure, and they had two major groups of molecules such as biogenic amines, bufotenins, bufotinins and steroid derivatives like bufodienolides and bufotoxins. Toads of genus Bufo produce a highly toxic substance, they cannot inoculate these substances but can be eliminated when the glands are compressed. So far the studies have been focused on the toxic effects of the toad venom extensively, but the beneficial effects of the toad venom are yet to be proved.

The chemical that is from a toad secretion is called 5-methoxy-N,N-dimethyltryptamine, and it is a derivative of bufotenine, and it is an extremely potent and causes vivid hallucinations. Bufotoxins and other secretions from toad venom also contain other chemicals including substances that can affect the heart and mimic hormones like adrenaline. This can lead to fatal arrhythmias, such as ventricular fibrillation, as well as vasoconstriction and death. The toads release the substances through their skins using specialized mucous glands and secrete mucus, whereas granular glands secrete toxins. The toxins are protecting the toad from predators like birds, mammals, snakes and crocodiles.

Diabetes is a chronic metabolic disorder and needs prolonged treatment for maintenance of normal blood glucose levels. Diabetes may precipitate cardiovascular, renal, neurological disorders in the long run leading to the existence of several disorders. Frog skin is considered as pharmacopeia having many potential compounds with pharmacological effects. The current study was

Table 1: Mean serum concentration of GLM in presence of saline extract and methanolic extract of paratoid gland

| Time (h) | GLM (µg/ml) | GLM+SP (µg/ml) | GLM+MP (µg/ml) |
|----------|-------------|----------------|----------------|
| 0        | 0           | 0              | 0              |
| 0.5      | 1.71±0.28   | 1.80±0.23      | 1.9±0.38       |
| 1        | 4.18±0.92*  | 4.2±0.83*      | 4.5±1.0*       |
| 2        | 8.24±0.61** | 11.37±1.15*** | 12.5±1.3**     |
| 4        | 7.69±1.12   | 8.12±1.35      | 9.3±1.1        |
| 6        | 4.20±0.41*  | 5.10±0.70*     | 5.13±1.1       |
| 8        | 1.68±0.40   | 1.6±0.55       | 1.8±0.71       |
| 12       | 0.96±0.3    | 1.1±0.34       | 1.65±0.28      |

Means±SD: ***Significant at P<0.001, **Significant at P<0.01, *Significant at P<0.05 compared to glimepiride control. SP: Saline extract of paratoid gland, MP: Methanolic extract of paratoid gland, SD: Standard deviation, GLM: Glimepiride

Table 2: Mean blood glucose changes and percent glucose reduction in nondiabetic group and diabetic group

| Time (h) | Mean blood glucose changes in nondiabetic group | Mean blood glucose changes in diabetic group |
|----------|-----------------------------------------------|---------------------------------------------|
|          | GLM (mg/dl) | GLM+SP (mg/dl) | GLM+MP (mg/dl) | GLM (mg/dl) | GLM+SP (mg/dl) | GLM+MP (mg/dl) |
| 0        | 108.66±6.5 | 108.66±11.7 | 108.66±13.5 | 310±18.02 | 310±11.53 | 310±24.54 |
| 0.5      | 102.25±4.0 | 94.92±9.6 | 93.52±15.14 | 279.17±3.96 | 283.65±10.4 | 276.52±13.78 |
| 1        | 84.55±8.02 | 80.46±8.23 | 75.55±10* | 246.11±13.48 | 258.54±11.03 | 243.04±7.8 |
| 2        | 63.55±4.20 | 62.98±6.8 | 58.89±5.1 | 191.54±2.5 | 251.72±20.00 | 222.58±12.58* |
| 4        | 76.39±12.4 | 71.5±7.14* | 65.93±13.2* | 156.92±31 | 231.88±5.03* | 196.23±12.38 |
| 6        | 89.77±7.9 | 83.97±9.8* | 80.74±7.1* | 222.58±9.10 | 218.86±19.63 | 200.26±7.65 |
| 8        | 101.29±12.5 | 97.05±12.5* | 95.33±10.8* | 252.78±4.21 | 239.63±8.5* | 216.38±14.63* |
| 12       | 105.5±0.8 | 101.71±11.5 | 97.04±8.66 | 281.53±5.06 | 264.74±13.22 | 253.58±18.61 |

Percent glucose reduction in nondiabetic group

| Time (h) | Percent glucose reduction in nondiabetic group |
|----------|-----------------------------------------------|
| 0        | 0±0                                           |
| 0.5      | 6.48±1.01 | 12.65±1.67 | 13.94±2.46 |
| 1        | 19.95±2.3* | 25.93±1.64* | 30.48±3.2* |
| 2        | 41.32±2.8*** | 42.04±2.81** | 45.8±14.2*** |
| 4        | 28.6±2.8 | 34.2±3.6* | 39.3±3.64* |
| 6        | 17.31±2.6 | 22.73±3.54* | 29.5±4.12* |
| 8        | 6.73±1.0 | 10.69±2.18* | 12.27±2.7* |
| 12       | 2.74±0.56 | 6.4±0.62 | 10.7±1.56 |

Percent glucose reduction in diabetic group

| Time (h) | Percent glucose reduction in diabetic group |
|----------|-----------------------------------------------|
| 0        | 0±0                                           |
| 0.5      | 6.89±1.85 | 9.26±1.25 | 10.9±2.15 |
| 1        | 20.5±2.02 | 18.6±2.75 | 22.7±3.3 |
| 2        | 27.2±3.86 | 22.5±4.36 | 29.3±2.6* |
| 4        | 35.6±4.3 | 40.2±3.7** | 43.7±6.4*** |
| 6        | 33.8±3.06 | 30.4±4.7 | 34.5±4.12 |
| 8        | 29.5±2.93 | 23.7±2.4* | 31.2±4.6* |
| 12       | 17.12±3.36 | 15.6±1.8 | 19.2±2.6 |

Mean±SD: ***Significant at P<0.001, **Significant at P<0.01, *Significant at P<0.05 compared to glimepiride control. SP: Saline extract of paratoid gland, MP: Methanolic extract of parotoid gland. Statistical analysis was performed using two-way ANOVA (Boneferroni post test). SD: Standard deviation, GLM: Glimepiride
undertaken to determine anti-diabetic potential of Bufo compounds derived from parotoid skin gland of toad. GLM is a known oral hypoglycemic of 3rd generation sulfonyl urea. It is primarily metabolized by CYP2C9.29 Thus, the present study was planned to investigate the influence of Bufo compounds on the pharmacokinetics and pharmacodynamics of GLM in rat’s in vivo, in both normal and alloxan induced diabetic conditions. In the study, it was evident that the parotoid gland extract has anti-diabetic potential on its own exhibiting percentage blood glucose reduction. The compound also affected pharmacodynamics of GLM relatively and pharmacokinetics of GLM after oral administration was also effected as exhibited by changes in AUMC, Cmax, t1/2. These results indicate that natural compound parotoid gland may inhibit the metabolism of GLM to some extent. This study leaves scope for further exploring the mechanism of action of parotoid gland extracts and also their interactions with other oral hypoglycemic agents.

CONCLUSIONS

Parotoid gland secretions of the common Indian toad is anti-diabetic, in addition it has beneficial effects in combination with GLM. These secretions may inhibit the metabolism of glimepiride and may also inhibit DPP-IV activity. Further, it requires the systematic structure elucidation of the compounds and pharmacokinetic studies to explore exact mechanism of action and the beneficial effects.

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