Original Article

Preparation and Biodistribution Study of a ^{99m}Tc-Labeled Toxic Fraction of Iranian Mesobuthus Eupeus Scorpion Venom

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ABSTRACT

Introduction: Iranian scorpion species are classified in Buthidae and Scorpionidae with 16 genera and 25 species. In Iran, similar to other parts of the world, there are a few known species of scorpions responsible for severe envenoming. Mesobuthus eupeus is the most common species in Iran. Its venom contains several toxin fractions which can affect the ion channel. In this study purification, labeling and biological evaluation of Mesobuthus eupeus scorpion venom are described.

Methods: To separate different venom fractions, soluble venom was loaded on a chromatography column packed with sephadex G50 gel then the fractions were collected according to UV absorption at 280 nm wavelength. Toxic fraction (F3) was loaded on anionic ion exchanger resin (DEAE) and then on a cationic resins (CM). Finally toxic fraction F319 was labeled with ^{99m}Tc and radiochemical analysis was determined by paper chromatography. The biodistribution was studied after injection into normal mice.

Results: Toxic fraction of venom was successfully obtained in purified form. Radiolabeling of venom was performed at high specific activity with radiochemical purity more than 95% which was stable for more than 4 h. Biodistribution studies in normal mice showed rapid clearance of compound from blood (2.64% ID at 4 h) and tissues except the kidneys (27% ID at 4 h).

Conclusion: As tissue distribution studies are very important for clinical use, results of this study suggest that ^{99m}Tc labeling of venom can be a useful tool for in vivo studies and is an excellent approach to follow the process of biodistribution and kinetics of toxins.

Keywords: Mesobuthus eupeus, Venom, Purification, Radiolabeling, Chromatography

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INTRODUCTION

Scorpions are widespread in all populated and rural areas in Iran. Iranian scorpion species are classified in buthidae and scorpionidae with 16 genera and 25 species. The most common species are: Mesobuthus eupeus, Odontobuthus doriae, Scorpio maurus, and Hemiscorpius lepturus. Mesobuthus eupeus from the family of buthidae is the most common species in Iran (1-5). Scorpion venom can cause various effects ranging from pain, inflammation, muscle paralysis and occasionally death in children. Pharmacological characterizations of this venom, as well as its' sub-fractions has not yet been reported.

Scorpion venom is mainly composed of basic peptides and several toxin fractions which can affect the exposed fibers and the muscles directly or through motor nerves causing neuromuscular intoxication. Moreover, Scorpion venoms are particularly rich source of small, mainly neurotoxic proteins or peptides interacting specifically with various ionic channels in excitable membrane sizes. These short-chain toxins are composed of 30–40 amino acid residues and are mainly cross-linked by two or four disulfide bridges which are mostly active on K^+ or Cl^- and Ca^{2+} channels (6-8). An example of above toxins is chlorotoxin which is consisted of 4 disulfide bonds and can block Cl^- channels (9-11).

By now, about 400 toxic peptides have been identified in scorpion venoms, mainly among those are from buthidae family. The main focus has been placed upon the buthidae family due to some species within this family possess particularly potent venoms that could be harmful to humans (7). Understanding the biodistribution and localization of these venoms in different organs is helpful for preparation of good antivenom for clinical use.

A good approach to see the biodistribution of an unknown compound in different organs is labeling with radionuclide. ^{99m}Tc continues to be at the forefront of these tracers due to its ideal nuclear characteristics (6-hour half life and gamma energy of 140 keV), its availability from a ^{99}Mo/^{99m}Tc generator, and well-established labeling chemistries.

In this study preparation, purification, labeling of Iranian Mesobuthus eupeus (IMe) and biological behavior of labeled compound through biodistribution studies in normal mice is evaluated.

METHODS

All chemical materials were prepared from commercial sources Sigma and Fluka. Sodium pertechnetate ($Na^{99m}TcO_4$) obtained from commercial ^{99}Mo/^{99m}Tc generator (Radioisotope Division, AEOI).

Venom preparation

Crude Venom of IMe was obtained from Iranian Mesobuthus eupeus scorpion. The scorpions were collected with UV light at night from different parts of Iran and they were milked by electric shock at the end of tail. The venom was water dialyzed and freeze dried at -20°C. The freeze dried venom (180 mg) was dissolved in distilled water and placed in dialysis bag and dialyzed against distilled water at 4°C for 48 h. After dialysis the venom solution was centrifuged at 14000 RPM for 17 min and the supernatant was collected.

Gel filtration on Sephadex G-50

Lyophilized crude venom (180 mg) was dissolved in ammonium acetate Buffer (pH 8.6) and the insoluble material was removed by centrifugation and filtration (for example: Mucoproteins). Supernatant was applied to a column of Sephadex G-50 (2.5 × 125 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 8.6). The elution was carried out with the same buffer at a flow rate of 60 ml/h. Volumes of 10 ml were collected and

all those volumes corresponding to each fraction were identified by UV spectrophotometer then were mixed. Toxicity of each fraction was determined in mice via killing effect on them.

Anion- exchange chromatography

A DEAE column (1.5 × 25.0 cm) was equilibrated with 0.02 M Tris base buffer (pH 8.3). Toxic fraction from the previous stage was dialyzed and condensed with PEG and applied to the column. The elution was carried out with a linear gradient from 0.0 M to 0.5 M sodium chloride in buffer at a flow rate of 20 ml/h. Volumes of 5 ml were collected and all those volumes corresponding to each fraction which identified by UV then were mixed. Toxicity for each fraction also was determined.

Cation-exchange chromatography

A CM column (1.5 × 25.0 cm) was equilibrated with 0.02 M sodium acetate buffer (pH 4.8). Toxic fraction separated in anion-exchange chromatography was dialyzed and condensed with PEG and applied to the column. The elution was carried out with a linear gradient from 0.0 M to 0.5 M sodium chloride in buffer at a flow rate of 20 ml/h. Volumes of 5 ml were collected and all volumes corresponding to each fraction were mixed and toxicity determined. For toxic fraction, protein concentration was measured by the formula which is related to any protein with unknown extinction coefficient: protein concentration = 1.55 × Absorbance at 280 nm – 0.77 × Absorbance at 260 nm (12).

Labeling with ^{99m}Tc

The toxic fraction obtained from the Cation-exchange step was labeled with ^{99m}Tc by direct method using stannous chloride and sodium borohydride as a reducing agents (13, 14). Preliminary studies were done to establish the optimum conditions for obtaining the highest yield of labeled venom. Briefly, stannous chloride (4 µg) and sodium borohydride (20 µg) were transferred to a vial containing lyophilized F319 (67 µg) for reduction of pertechnetate anions. The pH was adjusted to 7.5 with sodium hydroxide. $Na^{99m}TcO_4$ (1.85 MBq) freshly eluted from a $^{99}Mo/^{99m}Tc$ generator was added to the reaction vial. The mixture was incubated for 20 min at room temperature under vacuum condition. Then a volume 0.5 ml saline solution was added to reaction vial in order to interrupt the labeling reaction (15, 16).

Radiochemical analysis

^{99m}Tc-labeled venom was characterized by ITLC on silica gel 60 (Merck) and Whatman paper No. 1 previously saturated with 1.0% bovine serum albumin solution. As a mobile phase acetone was used for silica gel system ($^{99m}TcO_4^-$ Rf = 1) and saline for Whatman paper No. 1 (^{99m}Tc-colloid Rf = 0). The radioactivity was quantified by cutting the strip (1.5 × 10 cm^2) into 1 cm pieces and counting in a well type gamma counter.

^{99m}Tc-Venom stability

A volume of 50 µl of the labeled venom solution was incubated at 37°C with 1 mL of fresh human serum. Radiochemical stability was determined by taking samples of 10 µl at different times up to 24 h for analysis by ITLC. Stability in PBS also was determined by incubation of 50 µl of the labeled venom with 1 mL PBS solution storing in room temperature for 24 h. For stability analysis TLC method was performed.

Toxicity and Biodistribution

Animal experiments were performed in compliance with the regulations of nuclear science and technology research institute (NSTRI), and with generally accepted guidelines governing such work. For determination of toxicity, each fraction was injected to groups (2 each) mice and a dead mouse was an indication of toxicity. In

animal biodistribution study, male mice weighing 25-30 g were injected with 0.02 MBq of radiolabeled peptide in saline into the tail vein. For exvivo counting mice were sacrificed after 10 min, 1 and 4 h and various organs were dissected, weighed and counted for radioactivity. Data were expressed as the percentage of injected dose per gram of tissue (%ID/g).

Statistical analyses

The calculations of means and standard deviations for internalization and biodistribution were performed on Microsoft Excel. Student's T test was used to determine statistical significance.

RESULTS

Four fractions were obtained by gel filtration chromatography process (F1, F2, F3 and F4) (Fig. 1). From these F3 was recording as a toxic one. In second step of purification by anion- exchange chromatography process 3 fractions (F31, F32, and F33) were collected which F31 was identified as a toxic fraction (Fig. 2). In final purification step by cation-exchange chromatography process 10 fractions (F311 to F3110) were collected and from these No. F319 was identified as a toxic one (Fig. 3).

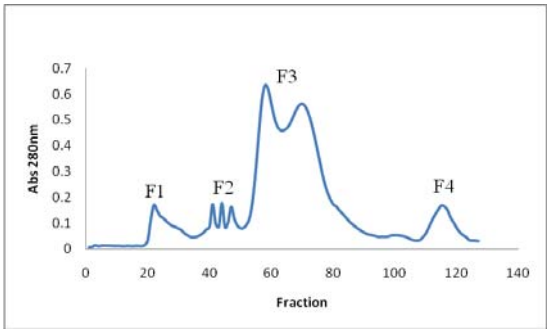

Figure 1. Output of Gel chromatography in sephadex G 50 for venom

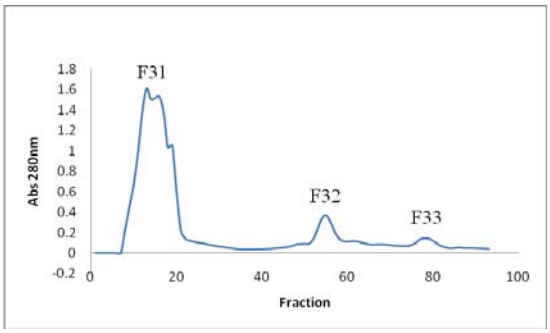

Figure 2. Output of Anion- exchange chromatography on DEAE resin for F3 fraction.

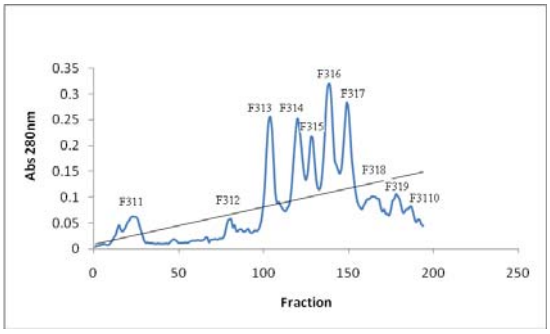

Figure 3. Output of cation- exchange chromatography on CM resin for F31 fraction.

The Radiolabeling of fraction F319 was performed and a labeling yield of >95% with specific activity of 27.6 kBq/µg was obtained. After 4 h radiochemical purity in human serum and PBS remained >40% and >43% respectively. The scheme for direct labeling of compound with ^{99m}Tc has been shown in figure 4.

The result of biodistribution in mice is shown in figure 5. The tissue distribution of radioactivity showed a rapid clearance of compound from the blood and most tissues except the kidneys. The initial uptake in the blood after 10 min was 22.16% decreasing to 2.64%, 4 h post-injection. The liver uptake after 10 min was 9.28% decreasing to 7.48%, 4 h post-injection.

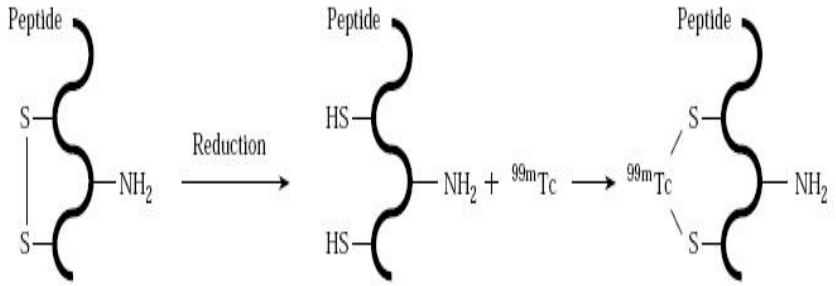

Figure 4. The schematic presentation of venom labeling with ^{99m}Tc by direct method.

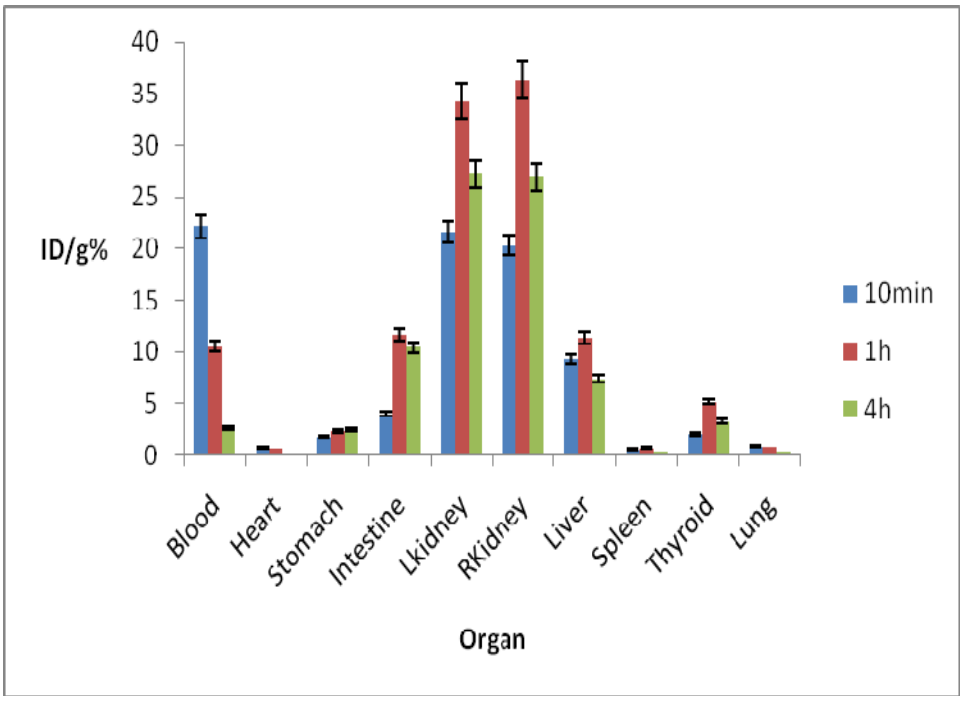

Figure 5. Biodistribution of F319 fraction in mice at 10 min, 1 and 4 h. Data are presented as %ID/g ± SD and results are the means of groups of three animals.

The uptake of F319 by the kidneys was about 21% at 10 min and increased to 27% after 4 h post injection. The concentration of complex in the stomach was insignificant. The maximum uptake in the intestine was 10.46% 4 h post injection. The whole body scintigraphic image of the mouse at 1 h post injection is shown in figure 6.

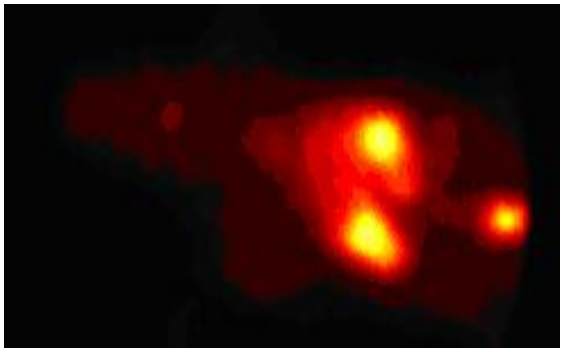

Figure 6. Whole body scintigraphic image of the mouse at 1 hr post injection of F319 fraction.

DISCUSSION

The present study examined the toxic fractions and subfractions present in the venom of Iranian Mesobuthus eupeus (IMe) which has been find in different places in Iran. The crude venom was dialyzed against deionized water for 24 hours and centrifuged for separating soluble peptides and proteins from the insoluble mucoproteins. The soluble venom was applied to a sephadex G-50 gel filtration. The toxicity of each fraction was determined by intravenous injection to mice and each toxic fraction was further purified by two steps ion-exchange chromatography. Finally F319 as a main proteic component of Iranian Mesobuthus eupeus venom with cytotoxic activity was obtained for further study. These finding shows that our purification method is very suitable in obtaining the toxic fraction from venoms.

In order to study biodistribution, final purified toxic fraction (F319) was labeled with sodium pertechnetate. ^{99m}Tc is the most important radionuclide in nuclear medicine applications. About 80% of the radiopharmaceuticals used in clinic are ^{99m}Tc based, the reason for this being optimal nuclear physical characteristics and the ready availability. The half-life is short enough to enable the administration of reasonably high doses allowing good quality images. Moreover ^{99m}Tc is readily available from a ^{99}Mo/^{99m}Tc generator in high quality and at low cost (17).

For ^{99m}Tc labeling of different compounds a large number of radiolabeling techniques have been developed and extensively reviewed (18-21). They can be classified into three main categories: direct labeling, chelate approach, and the indirect labeling approach (22, 23). The direct labeling approach usually uses a reducing agent to convert a number of disulfide linkages into free thiols, which are able to bind the ^{99m}Tc very efficiently. It has been reported that both thiolate sulfur and imidazole nitrogen are involved in bonding to the ^{99m}Tc (24). This method applies mostly to proteins or their fragments because of their disulfide bonds. Direct labeling method is simple and easy to perform and does not require synthetic modification or blocking and deblocking of functional groups. We have used $SnCl_2$ to reduce the disulfide bridges to provide sulfahydril groups for ^{99m}Tc binding. In our method we produced ^{99m}Tc labeled F319 with high efficiency and acceptable stability.

Pharmacokinetic studies are very important for clinical trials and radioisotope-based compounds are very convenient for these approaches. The present study demonstrates that ^{99m}Tc-F319 can be readily prepared by direct reduction techniques and is an excellent approach to follow the process of biodistribution and kinetics of toxins.

CONCLUSION

In this study, we have shown an approach toward preparation and purification of a toxic fraction from venom of Mesobuthus eupeus. Radiolabeling of venom was performed in order to assess the most optimum conditions for labeling and potential usage in biological evaluation. Furthermore, the conjugates showed relatively good specific activity and demonstrated considerable radiochemical stability. These properties suggest that labeling with ^{99m}Tc can be a useful tool for in vivo studies of venoms.

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