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RESEARCH ARTICLE

Preparation and Biological Evaluation of ^{67}Gallium- Labeled Iranian *Hemiscorpius Lepturus* Scorpion Venom

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Abstract: ***Background*:** The *Hemiscorpius lepturus* (*H. lepturus*) is a deadly scorpion species living in the southern Iran.

***Objective*:** *H. lepturus* induces delayed toxicity symptoms and understanding the long term biodistribution/biokinetic of the venom is of great interest in toxicology.

Methods: A Ga-67 labeled venom was prepared using a DOTA -conjugated venom followed by radiolabeling using $^{67}GaCl_3$ at 40°C for 90 min. The purification of the radiolabeled venom was performed using size exclusion-chromatography (radiochemical purity 71%). The radiolabeled venom was stable in the final solution in the presence of human serum at 37°C for 72 hours. The tissue distribution was studied in blood, heart, liver, spleen, muscle, brain, kidney, intestine and skin tissues at the intervals of 1, 4, 24, 48 and 72 hours using tissue counting and SPECT imaging.

Results: The radiolabeled venom mixture obtained with an estimated molar activity of 0.52 MBq/µg. The main accumulation tissues during the first 72 hours were kidneys, blood, liver, intestines, stomach and skin, respectively. Therefore, it is likely that *H. lepturus'* clinical effects and renal toxicity are primary and caused by direct effects of the *H. lepturus* venom.

Conclusion: The results have largely shown the direct clinical effects on the studied tissues during the 72-hour period and antivenom administration can strongly alleviate the toxicity effects as early as 72 hours in the management of the patients.

Keywords: *Hemiscorpius lepturus,* scorpion venom, gallium-67, radiolabeling, SPECT, ^{67}Ga-DOTA-HLV.

1. INTRODUCTION

Hemiscorpius lepturus scorpion, (*H. lepturus*, *Gadim* in local language), is one of the most dangerous scorpions in the world. Its sting often does not cause pain, but cause severe clinical complications in humans [1]. These signs include severe hemolytic symptoms, renal failure, physiological disturbances of the central nervous system, cardiovascular disorders, and severe cellular toxicity. Death may occur due to acute renal failure or cardio-respiratory arrest accompanied by severe symptoms of the central nervous system [2].

H. lepturus sting has severe and delayed complications, especially renal complications, even after 3-7 days. Therefore, to obtain the tissue distribution pattern, the gallium binding to *H. lepturus* venom was performed.

Biodistribution studies of long-term circulating molecules in the body (including, antibodies, polypeptides, toxins *etc*.) can be performed using radiolabeled probes followed by com monly used molecular imaging methods such as single photon emission computed tomography (SPECT) *etc*. Gallium-67 (half-life: 78.25 h) has been widely used in the biodistribution of such molecules [3, 4] due to its availability in cyclotrons, rather long half life, acceptable photon emission characteristics. Ga-67 citrate has been long used as a radiopharmaceutical approved by FDA and EU pharmacopeia since the 1970's and has been considered a safe radionuclide for human studies.

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To provide a stable Ga-67 protein molecule, a (DOTA: 1,4,7,10 –tetra azacyclododecne-1,4,7,10-tetra acetic acid) based venom conjugate preparation was developed and was then used in Ga-67 labeling using known methodologies. DOTA, a universal chelator for many metallic cations of various valences has been widely used in the development of radiopharmaceutical kits such as DOTATATE, DOTATOC, DOTANOC, and is approved worldwide [5].

In this study, Gallium-67 labeled *H. lepturus* venom (^{67}Ga-HLV) was prepared and administrated to the wild type Balb/c mice to determine the long-term tissue distribution of *H. lepturus* venom, relationship evaluation of long-term tissue concentrations of the venom with delayed clinical manifestations and determine the main venom target tissues in the long-term to justify clinical treatment.

2. MATERIALS AND METHODS

$^{67}GaCl_3$ obtained from 30 MeV cyclotron (Cyclone-30, IBA), Pars Isotope, Karaj, Iran. NHS (N-hydroxysuccinimide)-DOTA was purchased from Macrocycles (NJ, USA). Radio-chromatography was performed by using a Bioscan AR-2000 radio TLC scanner instrument (Bioscan, Paris, France). A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for counting distributed activity in rat organs. Calculations were based on the 184 keV peak for ^{67}Ga. All values were expressed as Mean ± Standard Deviation (Mean ± SD) and the data were compared using student's t-test. Statistical significance was defined as $p < 0.05$. All animal studies were approved by Research and Ethical Committees of Ahvaz Jundishapur University of Medical Sciences. All animal studies follow international accepted ethical guidelines such as the Animal Welfare Act of 1966 (AWA). The project was found to be in accordance with the ethical principles and the national norms and standards for conducting medical research in Iran.

2.1. Preparation and Validation of the Venom

Lyophilized *H. lepturus* venom was provided by Dr Babak Vazirian, Ahvaz Jundishapur University of Medical Sciences. Briefly, *H. lepturus* scorpions were trapped and their venoms were collected by electrical stimulation on their telsons (15 V). The collected venom was pooled, lyophilized and stored at –20°C before use. For the experiment, an aliquot of the lyophilized venom was reconstituted by the addition of phosphate buffered saline solution (PBS).

2.2. Conjugation of NHS-DOTA with the HLV Mixture

Radiolabeling of the venom mixture was performed according to the developed protocols for monoclonal antibody labeling at NSTRI with some modifications [3]. Briefly, HLV mixture was diluted with PBS (pH = 7.8) buffer solution. The venom concentration was measured using a biophotometer (Eppendorf) at OD = 280 nm. 20±5 µg of venom was used for labeling. This amount of HLV contains about 70±10.5 microcouri activity in the 100 microliter solution. The solution was passed through a Vivaspin 2 (20 min, 2.684 g) two times in order to remove the impurities. The venom can then be removed from the upper part of the filter using bicarbonate buffer (0.2 M Na_2CO_3, pH = 9.2). The final concentration was re-measured using biophotometric assay as well as a structure integrity test using SDS-PAGE. Then, DOTA-NHS (1.3 mg, excess 120 times) dissolved in bicarbonate buffer (400µL, 0.2 M, pH=9.2) was added to the purified venom solution (3.3 mg/mL) in a borosilicate vial and mixed gently 20 times by pipetting. The mixture was gently shaken and incubated at room temperature for 24 h. The mixture was then transferred on a Vivaspin 2 cut-off filter (30KD) and centrifuged at 2.684 g for 15 min. To terminate the conjugation step and provide the suitable radiolabeling pH, the upper filter fraction is washed using ammonium acetate buffer (0.2M, pH = 5.5) three times in order to remove excess of DOTA-NHS. At this stage, acetate buffer is added to the upper fraction (1 mL) and the mixture is pipetted 10-20 times for immunoconjugate dissolution. The filter is then centrifuged upside-down at 2.684 g for 5 min. The venom concentration was measured using a biophotometer (Eppendorf) at OD = 280 nm.

2.3. Radiolabeling of the Venom conjugate with ^{67} Ga

Typically, 370 MBq of [^{67}Ga] $GaCl_3$ (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. Acetate buffer (700 µL, pH = 5.5) was added to the gallium-containing vial and the vial was vortexed for 10 min. The conjugate containing fraction (500 µg) in acetate buffer with the measured protein content was added to the vial and mixed gently for 5 min using pipetting (10-20 ×). The mixture is then incubated at 40ºC for 90 min followed by testing the radiochemical purity by ITLC (Instant Thin Layer Chromatography) using a radio TLC scanner (Whatman No.1, 1 mM DTPA). Finally, ETDA solution (10 µL, 10 mM) is added to the labeling mixture and incubated for 10 min to scavenge the unlabeled Ga cation. The mixture is then passed through the disposable PD10 desalting column (Amersham) to further increase the radiochemical purity of the mixture. The final solution is then passed through a 0.22-micron biological filter for animal studies.

2.4. Stability Testing of the Radiolabeled Venom in Final Formulation

Stability of [^{67}Ga]-DOTA-HLV mixture in PBS was determined by storing the final solution at 4°C for 72 h and performing frequent ITLC analysis to determine radiochemical purity. ITLC analysis of the conjugated product was performed to monitor the degradation of products or other impurities. ITLC was performed by sampling the radiolabeled complex on a Whatman paper followed by developing in 1 mM DTPA aqueous solution [6].

2.5. Stability Testing of the Radiolabeled Compound in the Presence of Human Serum

Radiolabeled venom stability in serum was assessed by size exclusion chromatography on a Sepharose column (1 × 30 cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5 mL/min at room temperature; 1 mL of fractions was collected.

2.6. Biodistribution of ^{67} Ga-DOTA-HLV Mixture in Balb/c Mice

To determine the biodistribution, the ^{67}Ga-DOTA-HLV mixture was administered to Balb/c mice. Fifteen male mice (23-28 g) were randomly divided into five groups with three animals each. A volume 100 µL of the final radioactive solution containing 4.4 MBq radioactivity was injected intravenously to mice through their tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1 mL syringe before and after the injection in a dose calibrator with a fixed geometry. At the predetermined time point, 1, 4, 24, 48 and 72h after injection, all mice were sacrificed. After an intraperitoneal injection of ketamine/xylazine (Sigma, USA) solution (20 µl of solution per gram body weight; ketamine: 10 mg/ml; xylazine: 1 mg/ml), the mice were euthanized with heart puncture using a 1 ml syringe, prewashed with diluted heparin (5000 IE/ml). Blood and other organ samples (heart, liver, spleen, lung, muscle, brain, kidney, intestine, stomach, skin, feces) were collected and weighed and their specific activities were determined with an HPGe detector counting the area under the curve of the 184 keV peak. The tissue uptakes were calculated as the percent of the area under the curve of the related photo peak per gram of tissue (%ID/g).

2.7. Imaging Studies

For gamma camera imaging twelve healthy male mice (four groups with three animals each) were intravenously injected through the tail vein with 4.4 MBq (100 µL) of 67Ga-DOTA-HLV. Immediately before imaging, the animals were sacrificed by overdosing ketamine/xylazine. Imaging was obtained at 4,24,48,72 h after injection by a dual-head single-photon emission computed tomography (SPECT) system (SMV-GE, DST-XL). The distance of animal to high energy septa was 12 cm. The useful field of view was 3.5 mm.

3. RESULTS

3.1. Radiolabeling of the Venom Conjugate with ^{67} Ga

The venom mixture was labeled with ^{67}Ga using NHS-DOTA as a chelator. Labeling time was 90 min at 40°C. On the other hand, the DTPA solution (1mM) was used as a mobile phase to determine R_f (retention factor) of $^{67}Gacl_3$ on ITLC (Fig. **1**). A radiochemical purity of 56% was observed, as determined by ITLC analysis using a radio TLC scanner (Whatman No.1, 1 mM DTPA) without further purification (Fig. **2**). To achieve higher radiochemical purity of mixture and scavenging the unlabeled Ga cation, the ETDA solution (10 µL, 10 mM) is added to the labeling mixture and incubated for 10 min and then the mixture passed through the disposable PD10 desalting column (Amersham). As well as, ITLC confirmed that the final radiochemical purity was 71% (Fig. **3**).

3.2. *In vitro* Stability of ^{67}Ga-DOTA-HLV

The radiolabeled venom showed a stability of more than 85% up to 72 h after dilution in PBS at 4°C (Fig. **4**). The result of the serum stability test showed that the radiolabeled venom was stable more than 90% up to 4 h after dilution in human serum (Fig. **4**). The labeled venom did not cause any physiological disturbance or toxic effect.

3.3. Biodistribution of ^{67}Ga-DOTA- HLV in Balb/c Mice

The mass of injected radiolabeled venom could not directly be determined, however the protein content was measured just before labeling. The biodistribution data for ^{67}Ga-DOTA-venom in male Balb/c mice at 1, 4, 24, 48 and 72 h after injection is shown in Fig. (**5**). The radiolabeled venom showed high kidney uptake as 26.55 ± 0.62%, 20.66 ± 0.11%, 12.56 ± 0.21%, 5.52 ± 0.33%, 1.99 ± 0.35% in 1, 4, 24, 48 and 72 h after injection, respectively. Slow blood clearance and as well as low radioactivity uptakes were observed in the majority of other organs. It should be noted that along with the kidney, liver uptake of radiolabeled venom was also higher than other organs as 12.23 ± 0.235, 11.55 ± 0.11% at 1 and 4 h post injection (Fig. **5**).

3.4. Gamma Camera Imaging

The image acquired 4, 24, 48 and 72 h after the intravenous injection of ^{67}Ga-DOTA- HLV into Balb/c mice confirmed biodistribution pattern at these times. As predicted from the biodistribution studies, the high kidney and liver uptake was apparent in the images as well. Other organ and tissues were less visualized than kidneys (Fig. **6**).

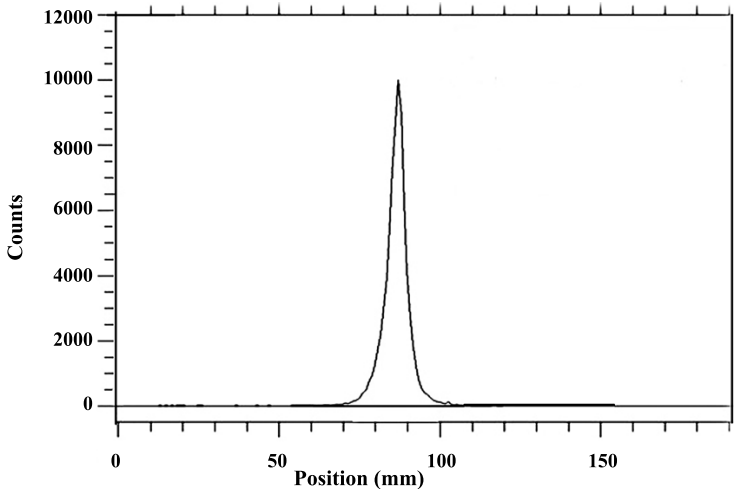

Fig (1). RTLC (Radio Thin Layer Chromatography) of ^{67}Ga in 10 mM DTPA in H_20 as mobile phase and Si stationary phase.

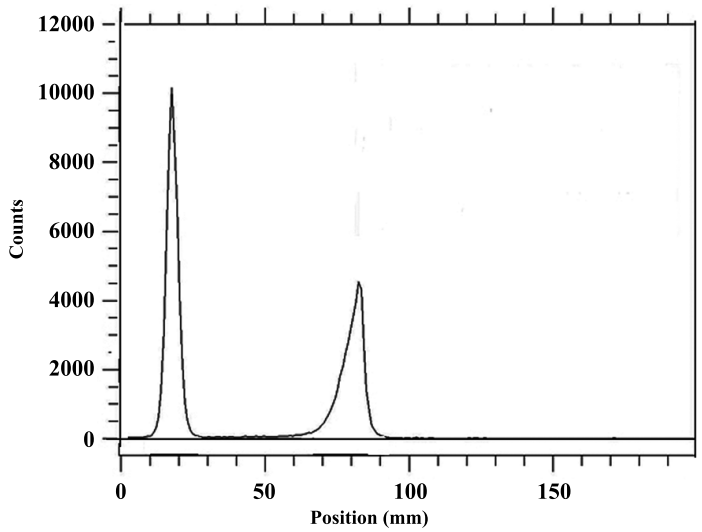

Fig (2). . RTLC of ^{67}Ga-DOTA-HLV in 1mM DTPA as mobile phase at 90 min after reaction. Radiochemical purity was 56% without further purification.

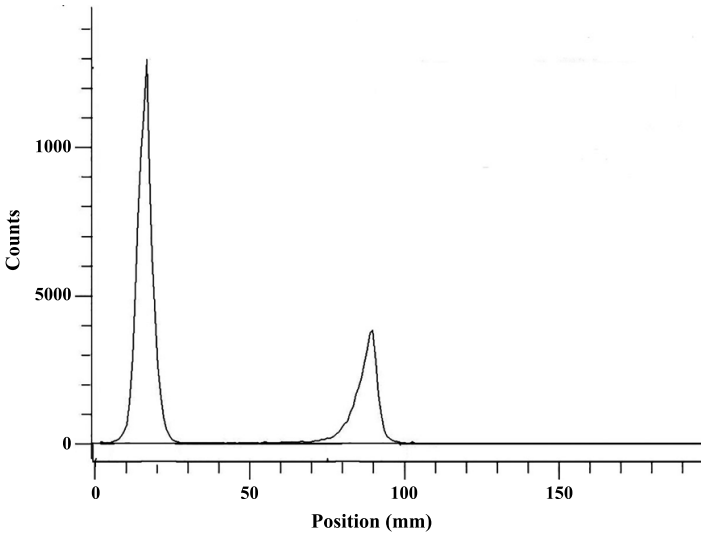

Fig (3). RTLC of ^{67}Ga-DOTA-HLV in 10 mM DTPA (10 µl) as mobile phase after purification by PD10 column. Radiochemical purity reached to 71%.

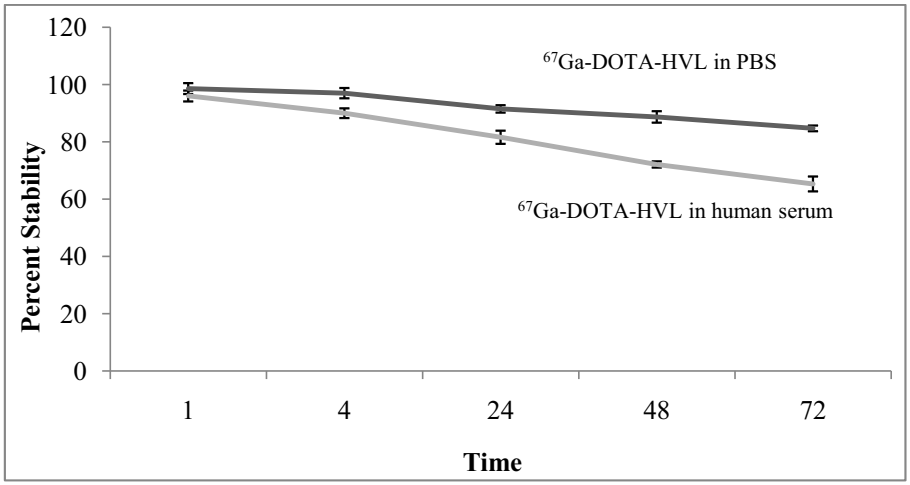

Fig (4). Stability of ^{67}Ga-DOTA-HLV in PBS and human serum up to 72 h after dilution. Data are presented as mean ± standard deviation (n = 3).

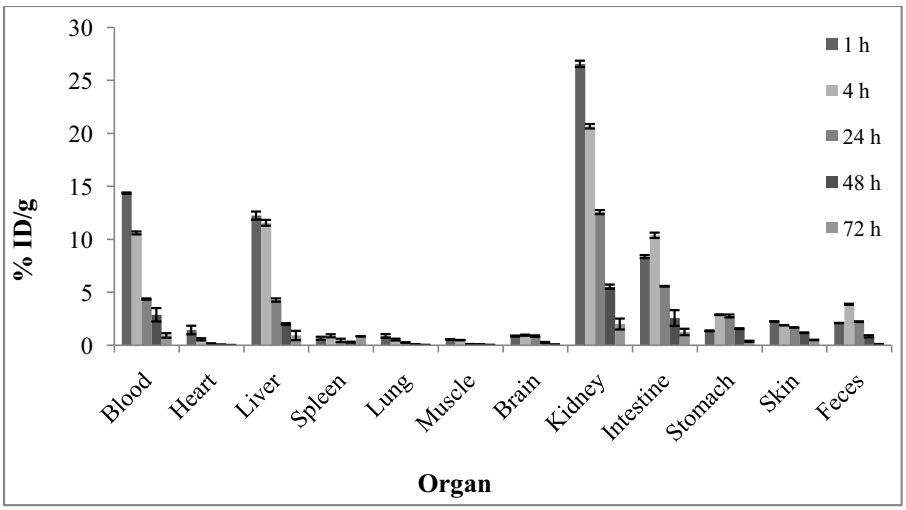

Fig (5). Biodistributions of ^{67}Ga-DOTA-HLV in male Balb/c mice at 1, 4 and 24 h after injection. Data are expressed as % ID/g for tissues. Data are presented as mean ± SD for 3 animals.

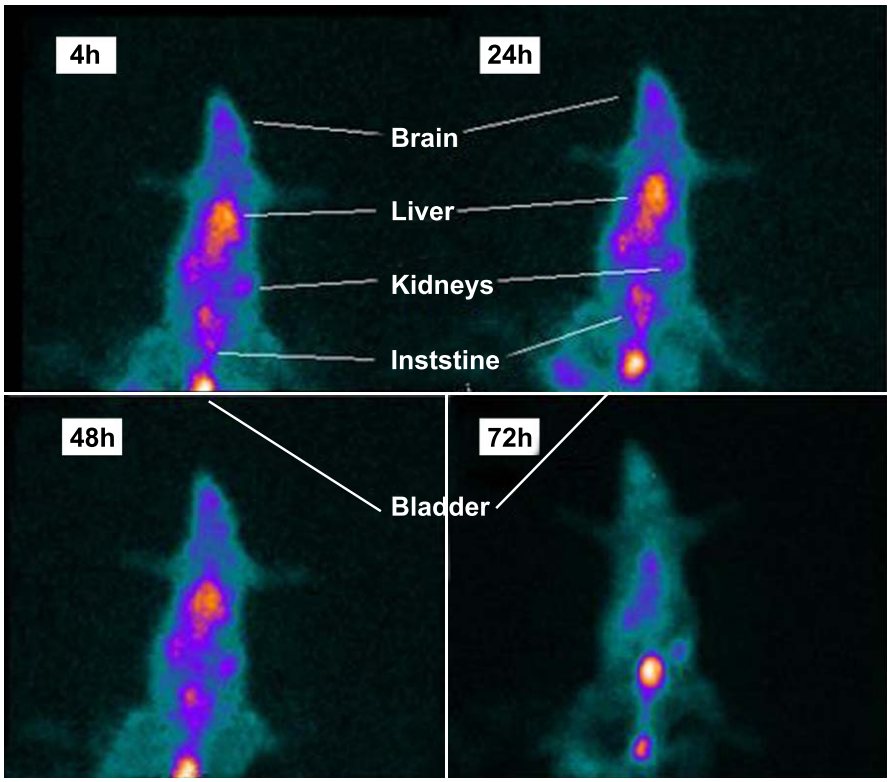

Fig (6). Imaging in Balb/c male mice using ^{67}Ga-DOTA-HLV at 4, 24, 48 and 72 h after injection. Mice were intravenously injected through the tail with 4.4 MBq of radiolabeled venom. Immediately before imaging, the animals were sacrificed by overdosing ketamine/axylazine.

4. DISCUSSION

The deaths caused by the scorpion sting in Iran are mostly from Scorpion *Hemiscorpius lepturus* that it is called Gadim in the southwest of Iran. For this reason, Gadim is known as the most dangerous scorpion in Iran [2]. The venom of this scorpion does not cause pain in the place of sting, but it can lead to severe clinical symptoms. The most important clinical symptoms of sting with *H.lepturus* scorpion include severe skin complications such as ulcers, edema, cellulitis, inflammation, necrosis at the site of the sting, which indicates the cytotoxicity of scorpion venom [7] and blood disorders such as lethal hemolysis, red blood cell wall destruction, hematuria and lymphocyte destruction and suppression of the lymphopoietic system. Another most important complication of this scorpion is kidney complications in the form of severe damage to the kidney cells and tissue [8, 9].

The pharmacokinetics parameters and the biodistribution profile of venom during a 400-minute period for the venom

have already been published. This study was performed using the chloramine-T method [10]. In the present study, the biodistribution of *H.lepturus* scorpion venom was investigated by the longest available period of lasting radiolabeled venom. For this reason, the venom was labeled by ^{67}Ga using DOTA as a chelator and then the level of ^{67}Ga-DOTA- HLV radioactivity in blood and other tissues was measured. The use of ^{67}Ga as radionuclides with a long half-life ($t_{1/2}$ =78.25 h) helps to track the venom uptake for more than 24 h. Various similar pharmacokinetic studies were carried out and the pharmacokinetic parameters of scorpion venoms were measured by radioactive materials [11-14]. In our previous studies, tissue distribution and the pharmacokinetic parameters of *H.lepturus* scorpion venom were investigated by radioactive matter of iodine 125 within 400 minutes after administration [15, 16].

Although radiolabeling and purification of Ga-67 proteins and antibodies have been performed frequently in high radiochemical purity and stability in our lab with success [3, 4, 6] in the last few decades. For unknown reasons the stability of the labelled toxin mixture, was low and possible decomposition of the HVL to smaller parts and chelates release led to low radiochemical purity of the compounds. Various working conditions (a fried room, darkroom, addition of stabilizers) were as used formerly for mAb fragments and did not improve the results. More or less this was attributed to the toxin stability itself than radiolysis.

Due to low stability of the mixture, it was difficult to calculate molar ratio of the complexes, however considering the radiochemical purity (71%) of the cocktail an estimated molar ratio of 0.52 MBq/μg was obtained. According to the results, it can be said that the main tissues with high remaining of radiolabeled venom up to 72 hours after injection include kidney, liver, blood, and intestines. It should be noted that the concentration of venom decreased rapidly during the 4 hours after injection, but after this time, the amount of venom in all tissues slowly decreased. These findings indicate that the venom is persistent in tissues and justifies the causes of its delayed and severe complications. It has also been reported in the previous study that the venom has slow blood clearance and a long half-life elimination period [15]. Considering the results of the previous studies and the results of this study, it seems that the venom of this scorpion has the ability of accumulation in certain tissues. This can be explained by the fact that venom may have quick access to specific tissue compartments, including liver, blood and kidneys, and a slow access to other compartments. Accumulation of venom in the brain was very low compared with organs such as kidney. Therefore, it is likely that venom concentration is not directly responsible for neurologic manifestations of envenomation like seizure.

Gadim's scorpion clearance is 0.20 ml/min, which is lower than the Iranian scorpion *Odonthubuthus doriae* venom, with a clearance of 12.2 ml / min [17]. The gradual absorption and slow distribution of Gadim scorpion venom, compared to *O.doriae* scorpion venom emphasize that Gadim has a slower distribution. This observation may be related to the delayed clinical symptoms of Gadim's scorpion sting.

According to the result of biodistribution data, high liver and intestine uptake of ^{67}Ga-DOTA-venom was seen. Based on the effects of scorpion venom on the gastrointestinal system, such as necrosis of the gut mucus, gastric spasm, decreased H^+ concentration in gastric secretions, abdominal pain and nausea and vomiting and severe reduction in LDH, SGPT, SGOT and changes in liver metabolic enzymes can be used to justify the effect of Gadim's scorpion venom in these organs. Considering the high values of the labeled venom in 72 hours, and previous studies suggesting a significant change in liver enzymes, it is reasonable to say that venom compounds have deleterious effects on hepatocytes [9, 18].

It is noteworthy that kidneys have the highest uptake of radiolabelled venom, so, 60 minutes after injection, the most uptake was seen in the kidneys. The serious presence of venom in the kidney system during the study suggests that the kidneys are a special target for venom action. *H. lepturus* venom itself causes direct kidney toxicity, which is commonly reported in patients.

According to the low uptake of the brain tissue, it is possible that small amounts of venom pass through the blood-brain barrier and cause severe brain toxicity. The low uptake of the brain tissue can be reflected by the integrity of proteins. However, serious neurological complications due to sting cannot be justified by a negligible distribution in the brain tissue, and it can be concluded that these complications are due to the secondary effects of venom on the nervous system and the brain, not its tissue concentration. Therefore, it is logical to deduce that some of the observed clinical effects, such as seizure, restlessness, and loss of consciousness, are not due to the presence of toxic compounds in the brain. It can also be concluded that compounds in the venom are not able to pass through the blood-brain barrier due to their physical-chemical properties, lipophilicity and molecular weight. The effect of venom in the cardiovascular system, such as postural hypertension, tachycardia, arrhythmias in the cardiogram electrode (a specific decrease in ST segment) and the effect of venom on the muscles and joints can be due to the secondary effects of venom on these tissues and organs [7-9]. There is a low level of radioactivity in heart and muscle. Therefore, complications cannot be justified by the tissue concentration of the venom in the heart, nervous system, muscle and joints.

The radioactivity decreases between 4-24 hours after injection of venom. During this period, the radioactivity in the blood is less than that of the kidneys. Therefore, we can conclude that the kidneys have the highest radioactivity within that time interval. According to the biodistribution profile, the maximum activity is observed in the kidneys during the first hours as well as in the rest of the study hours. This amount is greater than blood, and the average amount of blood is higher than the gastrointestinal tract. It is simply determined that the accumulation of the labeled compound in the kidney is clear. This finding is broadly consistent with the recent findings of the collaborated studies of Iran and Tunisia, which indicate the intrinsic affinity of some of the compounds from the venom to the kidneys [19]. Despite the 72 hours of injection, more than 35% of the labeled values are present in the kidneys. Therefore, it is reasonable to say that the prevalence of the destructive effects of venom occurs from the early hours of the sting.

An important point is the presence of 14.3% of the total labeled compound in the blood, which may indicate the binding or storage of the compound in blood vessels, including blood cells. The gradual increase in the amount of venom in the plasma and its maximum value at 60 minutes after the time of injection and the gradual decrease in the concentration, points out that venom has a relatively long contact with blood. Clinical outcomes have confirmed the blood-related complications and the hemolytic effects of this venom. The kidney toxicity appears to be due to several factors, including the red blood cell lysis, and this problem persists for as long as there are toxins in the kidneys. One of the most important points is that about 71.5% of the entire labeled compounds were found in the first hour of the study (based on the biodistribution profile). This shows the correctness of the selected tissues studied.

CONCLUSION

Given the available information and the continued existence of the labelled compound after 72 hours in different tissues, in particular, the kidneys, liver and blood, antivenom administration is recommended for up to 72 hours after the sting, especially to prevent damage to these tissues. This finding is the main conclusion of this study, which should be taken into consideration by medical groups.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocol of this study was approved by the ethics committee affiliated with Ahvaz Jundishapur University of Medical Sciences (Approval code: U 94140).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All animal research procedures were performed in accordance with the standards of Guide for the US National Research Council's "Guide for the Care and Use of Laboratory Animals".

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during the current study are included in this published article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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