Biodistribution and kinetic studies of technetium-99m labeled *Naja naja karachiensis* venom via gamma scintigraphic and SPECT images

Muhammad Hassham Hassan Bin Asad[1], Durr-e-Sabih[2], Israr Ahmad[2], Bashir Ahmad Choudhry[3], Ghulam Murtaza[1]* and Izhar Hussain[1]

[1]Department of Pharmaceutical Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan
[2]Department of Nuclear medicine (MINAR), Nishtar Hospital, Multan, Pakistan
[3]Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan

Abstract: *Naja naja karachiensis* have been responsible for plentiful deaths in Pakistan. To investigate bio distribution and blood kinetics, venom was labeled with the radiotracer (technetium-99m) by following the method of direct labeling technique. Its maximum labeling percentage was 97.7% (pH 6, 100µg stannous chloride dihydrate) which was higher than some other reported venom. Radio labeled venom was stable for more than 4 hours both *in vivo* (96%) and *in vitro* (serum 94.1%, saline 94.3%) experimentations. Intravenous doses of venom (250µg, 0.5mCi) were found to be evenly distributed (having R/L ratio=1.0) in all parts of sacrificed rabbits. Kidneys (53.75% activity/g) and urinary bladder (23.70% activity/g) were found with the copious quantity of injected dose of venom. Rest of all other organs was found with subsequent remaining dose of venom. Among them, lungs (14.2% activity/g), liver (4.32% activity/g), bones (1.38% activity/g), heart (0.8% activity/g), blood (0.56% activity/g), skin (0.45% activity/g), intestines (0.35% activity/g), skeleton muscles (0.3% activity/g), brain (0.14% activity/g) and stomach (0.05% activity/g) are included. After 24 hours of injection, poisoned blood of rabbits was almost cleared from venom. Gamma scintigraphic images (up to 2 hours) along with bio distribution suggest that kidneys are main organs of excretion in rabbits. Elimination started immediately after administration of venom however, possible sites for metabolism of venom are liver and lungs. More accumulation of venom in heart compared to brain suggests its higher affinity (thus possible higher toxicity) to cardiac muscles as compared to brain tissues.

Keywords: *Naja naja karachiensis*, Technetium-99m, biodistribution, gamma scintigraphy.

INTRODUCTION

Incidences of snakebite are very common especially in tropical and subtropical areas of the world. Due to this, it is always remained a topic of discussion among various scientists to discover the underline phenomenon of snake poisoning (Zouari-Kessentini *et al*., 2013). All the snakes present on the earth are not poisonous rather their venoms are used for therapeutic purposes as antihypertensive, inhibitors of cancerous cell prolongation and activators of complement system (Pujatti *et al*., 2005). There are more than two hundred species of the snakes that are toxic. They can be categorized into Cortalidae, Viperidae, Elapidae and Hydrophidae families (Matsui *et al*., 2000; Warrell, 2010). In Asiatic countries particularly Pakistan lethal snakes of family Elapidae (belonging to genus Naja) are very common hencetheir bites are very frequent. According to literature survey 20,000 deaths annually reported in Pakistan and majority of them are due to *Naja naja karachiensis* (pattern-less black Pakistani cobra) bites. Victimize of these snakes bite suffer from different complications like hemorrhage, necrosis, pain, local inflammation, neurotoxicity as well as cardio toxicity (Razi *et al*., 2011). Equine animal's anti-sera are the effective therapy for snakebite patients but recovery of suffers depends on doses, neutralization and way of administration of anti-sera (Rocha *et al*., 2008).

For complete treatment of snakebite, standard protocols (for serum) are still not available this has resulted in deficiency of data about pharmacokinetic parameters of different venoms. Pharmacokinetic studies of venoms can help us to develop standard serum therapy protocols along with their systemic information. Many toxins from various sources have been studied previously for their biodistribution (Shirmardi *et al*., 2010(a); Pujatti *et al*., 2005) however; pattern-less black Pakistani cobra, which is found in southern Punjab province of Pakistan has not been studied previously for their pharmacokinetic parameters.

Pharmacokinetic study, particularly biodistribution, of venom can be easily performed by binding it with some radionuclide. Radionuclides have been used extensively in the field of nuclear medicine for their tagging on to some desired compound. Among them short lived technetium-99m (^{99m}Tc) is being used frequently due to their short half life (6 hours) and low energy photons (140 keV) exposure to the subjects. Technetium-99m binds with sulfahydril groups of various proteins of venoms in reducing environment to generate technetium labeled venom as represented in fig. 1. Labeling of ^{99m}Tc with

**Corresponding author:* e-mail: gmdogar356@gmail.com

different radio pharmaceuticals is a powerful tool to observemorphological imagesand their biodistribution to various organs (Rocha *et al*., 2008; Pujatti *et al*., 2005).

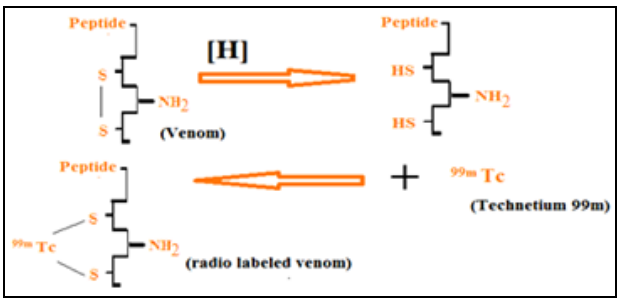

Fig. 1: Representation of binding of *Naja naja karachiensis* venom with ^{99m}Tc (Shirmardi *et al*., 2010)

In this present article snake venom (*Naja naja karachiensis*) is labeled with ^{99m}Tc to study its biodistribution and localization in different organs of healthy rabbits which might be useful in future for preparation of effective anti-venom for clinical use.

MATERIALS AND METHODS

Reagents

Unless and otherwise specified all the chemicals were purchased from Sigma, USA. However, ^{99m}Mo/ ^{99m}Tc generator was supplied by Isotope Production Division, PINSTECH, Islamabad, Pakistan. Black southern pattern-less form of Pakistani cobra snakes (*Naja naja karachiensis*) were collected with local charmers from Cholistan desert, Southern Punjab province of Pakistan (Snakebite, 2008). Venom was collected in low light environament at ambient temperature by squeezing the glands below their eyes and stored for further study after lyophilization (Razi *et al*., 2011).

Animals

Male rabbits having weight of 1.5±0.5kg were housed in metabolism cages, which facilitated the gathering of their waste. In addition they were provided with water and chow. All the experiments were carried out under the guidelines and after getting permission from institutional animal ethical committee (Ref. letter no: Administration /432/27/04/13/MINAR/Multan).

Radio labeling of Naja naja karachiensis venom

Venom was labeled with freshly eluted ^{99m}Tc (Na $^{99m}TcO_4$) from^{99}Mo/^{99m}Tc generator. Briefly, acidic solution of stannous chloride dihydrate was mixed with 125µL of venom solution (2mg/mL). Radioactivity (^{99m}Tc) equivalent to 18.5M Bq (0.5mCi) was added to the above mixture and incubated for 5-10 minutes. Percentage labeling was determined by the help of chromatographic method (Priyadarshani *et al*., 2010; Sajid and Mahmood, 2012; Yonamine *et al*., 2005).

Radiolabeling yield

Small aliquot (2µl) of radiolabeled venom was spotted at the end of a paper strip (1.5 cm × 10 cm) using acetone as mobile phase in small vial fitted with screw cap. After development of chromatogram, strip was removed and divided into 10 segments and activity in each segment was determined in a NaI(TI) well type gamma counter (Cap-Ria 16 gamma counter). Histogram was obtained by plotting radioactivity for each segment (1cm) of paper strip (Saha, 1984).

Effect of pH and stannous chloride on radiolabeling

Effect of different concentrations of stannous chloride dihydrate ($SnCl_2.2H_2O$) on the yield of percentage labeling of *Naja naja karachiensis* venom was determined. They were used in various concentrations from 10 to 200µg/mL by keeping the pH constant at 6. In another set of experiments, stannous chloride was used in the concentration of 100µg/mL but pH was changed from 5 to 7 and again percentage labeling was determined (Priyadarshani *et al*., 2010).

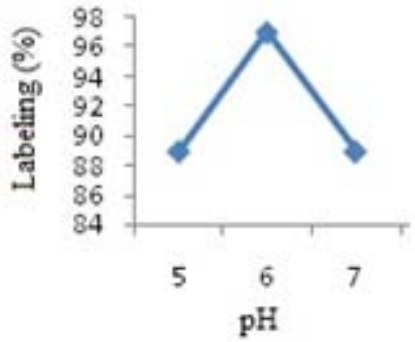

Fig. 2: Effect of pH on percentage labelling of venom *Naja naja karachiensis* with ^{99m}Tc

^{99m}Tc labeled venom and its stability

For *in vitro* experiments fresh human serum (1000µL) was mixed with 50µL of ^{99m}Tc labeled venom and incubated at 37ºC. Stability was determined up to 24 hours after application of 10µL of mixture by thin layer chromatographic method. Same procedure was repeated with saline instead of serum and observations were recorded (Shirmardi, 2010). Forstability determination (*in vivo*) rabbits were injected total volumes of 300µl (250µg venom) of ^{99m}Tc labeled venom (Priyadarshani *et al*., 2010).

Biodistribution of radiolabeled venom

^{99m}Tc labeled *Naja naja karachiensis* venom was injected intravenously into dorsal ear vein of the rabbit. For *ex vivo* studies rabbits were humanly scarified exactly after three hour. Various organs (blood, brain, bones, heart, Intestines, kidneys, liver, lungs, muscles, skin, stomach and urinary bladder) were separated, weighed and counted for radioactivity in a gamma counter and expressed as percentage of venom dose per gram of whole organ, %

ID/g. The injected dose of venom was corrected by subtracting the activity deposited in the ear (Shirmardi *et al*., 2010b; Sajid and Mahmood, 2012).

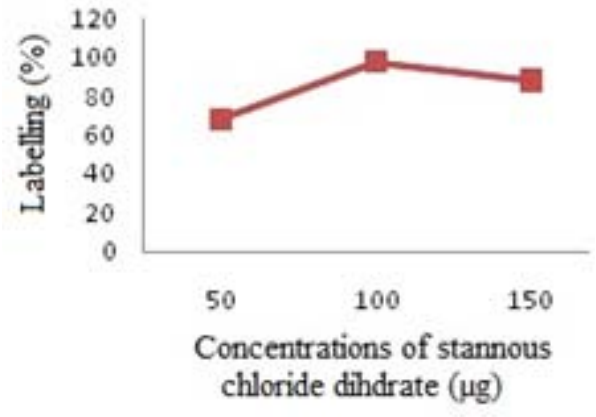

Fig. 3: Effect of various concentrations of stannous chloride dihydrate on labelling efficiecny of venom *Naja naja karachiensis* with ^{99m}Tc

Blood kinetics

Radiolabeled venom (*Naja naja karachiensis*) was injected intravenously having 18.5M Bq (0.5mCi) radio activity for monitoring of blood clearance. Blood was taken at different intervals and radioactivity was recorded. Total blood volume of rabbits was considered by taking 7% of total body weight (Priyadarshani *et al*., 2010).

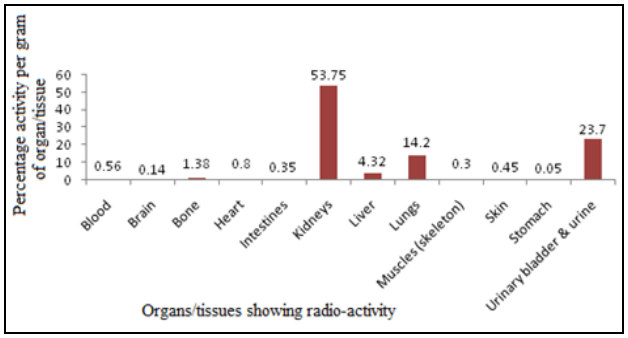

Fig. 4: Biodistribution of 99mTc labeled *Naja naja karachiensis* venom in healthy male rabbits after intravenous injections

Gamma scintigraphy and single positron emission computed tomographic (SPECT) images

After administration of radiolabeled venom (250µg), scintigraphy was carried out and various images were analyzed by gamma camera (Siemens, Digitrac 75). Rabbits were anaesthetized previously by preanaesthetic medication (buprenorphine, 0.05mg/kg) followed by induction of anaesthesia (propofol, 8mg/kg, i.v.). They were maintained with 0.6mg/kg/mindoses of propofol. After initial 2 hours of gamma scintillation whole body images, rabbits were subjected to take SPECT images at 360^{o} for transverse, coronal and sagittal sections (Priyadarshani *et al*., 2010; Martín-Cancho *et al*., 2006).

RESULTS

Complexation of Naja naja karachiensis with ^{99m}Tc

Venom was labeled sufficiently (97%) with ^{99m}Tc and its tagging was determined by chromatographic methods as shown in table 1. The optimum labeling percentage was recorded with 100 µg of stannous chloride at pH 6 overall results are expressed in figs. 2 and 3.

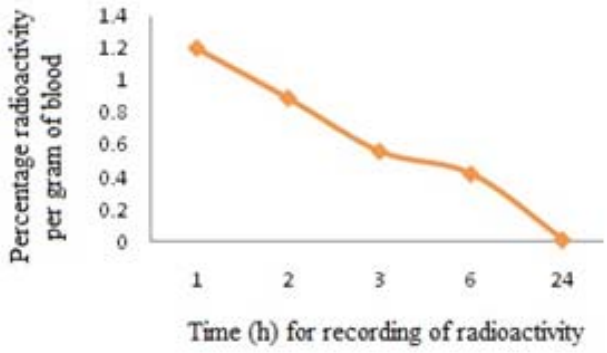

Fig. 5: Blood clearance of ^{99m}Tc labeled *Naja naja karachiensis* venom injected through ear vein in rabbit

Stability of ^{99m}Tc labeled Naja naja karachiensis

Stability of radio labeled venom *Naja naja karachiensis* was determined separately *in vitro* and *in vivo* experiments. Results have shown that complex of venom with ^{99m}Tc was stable both *in vivo* and *in vitro* experiments up to 4 hours. Complex of venom was stable *in vitro* up to 4 hours with 94 percent binding efficiency whereas *in vivo* it was 96 percent radio labeled. Summary of overall results is shown in table 2.

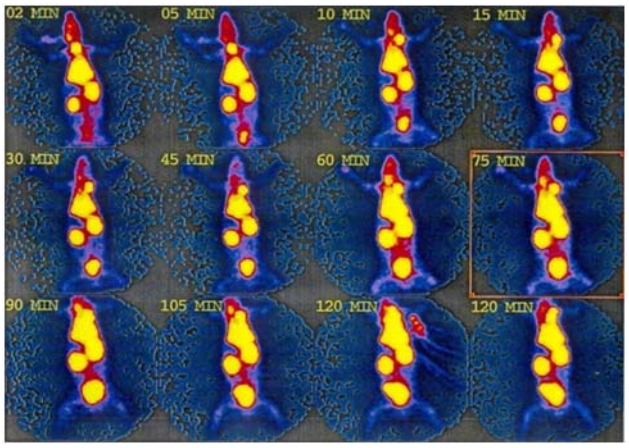

Fig. 6: The whole body gamma scintigraphic images of 99mTc labeled venom up to 2 h

Biodistribution of ^{99m}Tc labeled Naja naja karachiensis venom in rabbits

The least biodistribution of technetium labeled venom was found in brain. However the highest activity was found in kidneys and urinary bladder. Lungs and liver are also found with sufficient radio labeled venom. Little

quantity of venom was deposited in bone, heart and connective tissues (blood). The overall results with percentage activity per gram of rabbit's tissues are expressed in fig. 4.

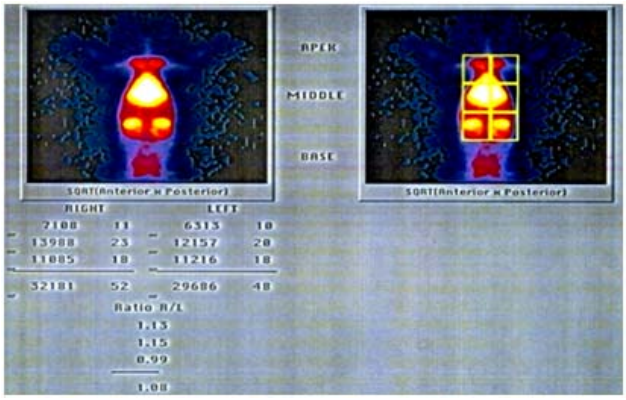

Fig. 7: Venom distribution in middle compartment of rabbit body interms of R / L ratio

Blood clearance (kinetics)

In vivo there was rapid clearance of venom from the blood of rabbits up to1 hour (1.3% /gram) after injection. There was slow elimination of venom after an initial hour and approximately all the venom eliminated within 24 hours. The 0.01% activity was maintained even after 24 hours. Complete detail is expressed in fig. 5.

Gamma scintigraphic and SPECT images

Localization of ^{99m}Tc labeled venom in rabbits as determined by gamma camera images is shown in fig. 6. After 2 minutes of intravenous injection, venom has been reached in lungs, liver and both kidneys via heartbeat. However venom reached in urinary bladder after 5 minutes. At the end of first hour urinary bladder has been filled with activity. Radio labeled venom cannot be visualized in brain via gamma scintigraphic images. Moreover, right over left (R/L) ratio of radio labeled venom in middle compartment of the rabbits is approximately equal. The ratio was 1.08 as shown in fig. 7.

SPECT images were acquired at 360º and venom's distribution and accumulation from head to feet were assessed by single photon emission. The overall images of transverse, sagittal and coronal sections are shown in fig. 8.

DISCUSSION

Biodistribution and kinetics studies have prime importance for clinical assessment of venom's toxicity. In this study, technetium 99m has been used successfully (by direct labeling technique) to tag it with snake venom *Naja naja karachiensis*. Stannous chloride was used to reduce disulfide bridges (i.e., a bond between sulfahydril groups of venom and ^{99m} Tc) in venom to develop labeling (Shirmardi *et al*., 2010a). Venom was found with maximum labeling yield of 97.7% as it is higher when compared with other reported venoms like *Mesobuthus eupeus* venom, Crotalus venom and *Scorpaena plumier* venom (Pujatti *et al*., 2005; Shirmardi *et al*., 2010**a**; Soprani *et al*., 2007).

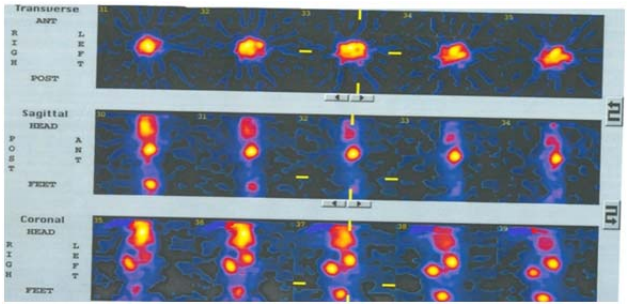

Fig. 8: Single photon emission computed tomography (SPECT) of rabbit body at 360º

Table 1: Labeling percentage of *Naja naja Karachiensis* venom with ^{99m}Tc

S. No	Strip Number	Counts/30 sec	Percentage Activity
1.	01	448	0.23
2.	02	185290	97.7
3.	03	501	0.26
4.	04	481	0.25
5.	05	472	0.24
6.	06	510	0.26
7.	07	1440	0.75
8.	08	205	0.10
9.	09	192	0.10
10.	10	100	0.05
11.	Total	189639	99.9

Stability studies of our venom extracted from *in vivo and in vitro* experimentations revealed that labeling is stable (94%) up to 4 hours as shown in table 2. Satisfactory stability profile of technetium labeled *Naja naja karachiensis* venom has enabled us to extend our study for biodistribution and kinetics parameters. For this purpose, rabbits have been reported previously in various procedures for assessment of venom's toxicities (Murugesan, 1999). Intravenous dose of Pakistani cobra venom was found the highest (77.45% activity/g) in the kidneys and urinary bladder. Lungs and liver were declared second (14.2% activity/g) and third (4.32% activity/g) the most effective venom accumulated organs. Present data has revealed that excretion of cobra venom occurred mainly through kidneys however participation of liver and lungs in metabolism is also important. Heart and brain of rabbits were found less accumulated with venom. Blood clearance of snake venom immediately started after i.v. injection (initially rapid) however; it was seen in urinary bladder after 5 minutes as shown in gamma

Table 2: Stability studies of ^{99m}Tc labeled *Naja naja karachiensis*venom

Time for incubation (hour)	Labeling %age (*in vitro*)		Labeling %age (*in vivo*) (Mean ±SEM)
	Saline (Mean ±SEM)	Human serum (Mean ±SEM)	
0	97.7±0.649	97.2±0.635	97.6±0.643
1	97.1±0.578	97.0±0.578	97.3±0.624
2	96.5±0.777	96.1±0.851	97.0±0.867
3	95.7±1.027	95.3±0.821	96.2±0.696
4	94.3±1.011	94.1±0.317	96±0.882

scintigraphic images (up to 2 hours with 15 minutes intervals) in fig. 6. It was observed that after 24 hours of envenomation there was negligible concentration of venom in blood pool of rabbits. Complete and summarized details about biodistribution and kinetics of Pakistani cobra venom are shown in fig. 4 and 5.

Another important parameter to assess even distribution of venom is right over left ratio (R/L). Venom distribution in terms of R/L ratio was almost equal to 1 which clearly indicates its evenly distribution in middle compartment (lungs, liver and kidneys) of animals. Additionally various SPECT images (transverse, sagittal and coronal sections) were also observed for possible illustration of venom distribution in various tissues of healthy male rabbits for their discrimination from one tissue to another. The overall SPECT images are shown in fig. 8 for possible explanation about accumulation of *Naja naja karachiensis* venom.

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