Original Article:

Radiolabeling and Bio-distribution study of ICD-85 with Technetium-99m as a cancer treatment agent in mice

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ABSTRACT

ICD-85 is a combination of three poly-peptides, ranging from 10,000 to 30,000 Dalton, derived from the venoms of an Iranian brown snake *(Agkistrodon halys)* and a yellow scorpion *(Hemiscorpius lepturus)*. Labeling of this ICD-85 was successfully achieved with ^{99m}Tc, through direct method using SnF_2 as reducing agent. Labeled ICD-85 was injected into mice to determine the excretion pathway. The results show that the maximum labeling yield (>75%) was obtained by using 30 µg of ICD-85 in phosphate buffer (60 µl, pH 7.1) at room temperature. Bio-distribution studies with radiolabeled ICD-85 shows moderate clearance of the complex from blood. The improvement of the immunotherapeutic treatment of cancer requires a better knowledge of the biological actions of the ICD-85 since tissue distribution studies are very important for clinical purpose.

Keywords: Radiolabeling; Tc-99m; Bio-distribution; Venom; ICD-85

INTRODUCTION

Snakes and scorpions are widespread in all urban and rural areas. Hemiscorpius lepturus is the most common scorpion's species [1-5]. Venom is a mixture of proteins, peptides (poly-peptides) and toxin fractions which can affect the exposed fibers and the muscles directly or through motor nerves, causing neuromuscular intoxication. Most of these proteins and polypeptides, existing as monomers, but some of them form complexes in the venom. Venom can cause various effects such as pain, inflammation, muscle paralysis or death in people. Moreover, scorpion venoms are mainly rich sources of short-chain toxins composed of 30–40 amino acid residues and are mainly cross-linked by two or four disulfide bridges. These are mostly active on ion channels (K^+or $Cl^-$and Ca^{2+}) [2, 6-9].

ICD-85 is a combination of three peptides that range from 10 to 30 kDa and are derived from the venom of an Iranian snake *(Agkistrodon halys)* and a scorpion *(Hemiscorpius lepturus)* [10-14]. Previous studies revealed an inhibitory effect of ICD-85 (venom derived peptides) on breast cancer cell line MDA-MB231 [10]. ICD-85 was also confirmed by in vivo studies to suppress the breast tumor in mice [11]. Finding the bio-distribution and localization of these venoms in different body tissues is useful for preparation of good agent for clinical use. A common method to see the bio-distribution of an unknown compound in different tissues is labeling with radioisotopes. For therapy by venom, there are reports that present the cytotoxic activity of various venoms through employing some cancer cells [15-17]. It was indicated that a protein from Cobra venom was selectively cytotoxic to cancer cells [15].

Labeling of a compound with radioisotopes is a proper approach for observing the bio-distribution in vital organs. Technetium-99m (^{99m}Tc) has numerous applications as a tracer, and remains at the forefront of such investigations due to its ideal nuclear characteristics (6-hour half-life and

gamma energy of 140 keV), ready availability from a ^{99}Mo /^{99m}Tc generator, and well-established labeling chemistries [1, 18, 19].
The aim of this study is the labeling of ICD-85, its stability study and its bio-distribution in mice, so that the biological behavior of the labeled compound is evaluated in animal through bio-distribution assay.

MATERIALS AND METHODS

All chemical materials were purchased from Sigma and Fluka (Germany) company. Technetium-99m was extracted from a ^{99}Mo/^{99m}Tc generator. Venom (ICD-85) was provided by Razi Vaccine and Serum Research Institute of Iran [10-14]. ICD-85 derived from scorpion and snake venom (Iranian brown snake (Agkistrodon halys) and yellow scorpion (Hemiscorpius lepturus)) consisted of three poly peptides, ranging from 10,000 to 30,000 Dalton. The ICD-85 polypeptides were selected based on a study of crude venom cytotoxicity. Similar to previous researches by Zare et al. [10-14], the venom indicated antigrowth activity on some cell lines. Then, the venoms were fractionated; the active peptides were isolated and tested on the cell lines.
The ICD-85 was labeled with ^{99m}Tc through direct method using stannous fluoride as reducing agents. The direct method of radiolabeling usually uses a reducing agent to convert a disulfide bonds into free thiols that are able to bind the Technetium-99m quite efficiently. This procedure often applies to peptides, proteins and antibodies or their fragments because of their disulfide bonds.
Preliminary studies were done to establish the optimum conditions for obtaining the highest yield of labeled venom. In this study, labeling process was divided into two steps. As for first step (Figure 1), 10 microgram of (in distilled water 10 µg/10µl) SnF_2 was added to vial containing ICD-85 (in phosphate buffer, pH=7.1, 1M) for the reduction of S-S bonds (15min). In the second step (Figure 2), $Na^{99m}TcO_4$ (2mCi) freshly eluted from a ^{99}Mo/^{99m}Tc generator was added to the reaction vial. The pH was adjusted to 7.1. The mixture was incubated for 15 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 [1, 18]. Gel chromatography separated peptides and proteins on the basis of size. In this system, Small molecules diffuse into the pores of the gel beads and therefore move through the bed more slowly, while large molecules move through the bed quickly. Chromatography set-up (Sephadex) for labeled ICD-85 separation is shown figure 3.

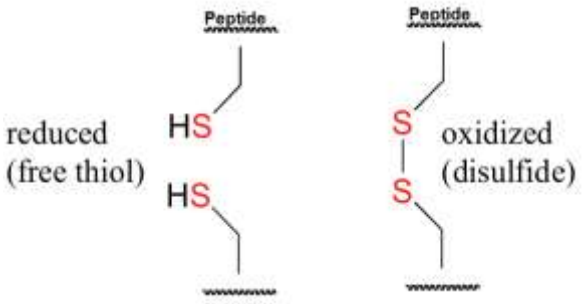

Figure 1. Reduced and Oxidized disulfide

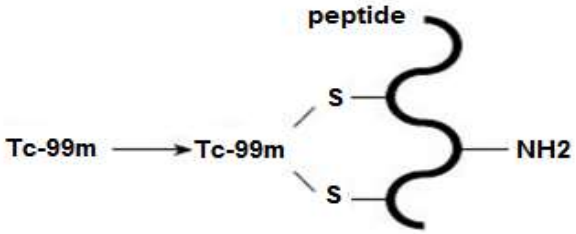

Figure 2. labeling of peptide (ICD-85) with Tc-99m

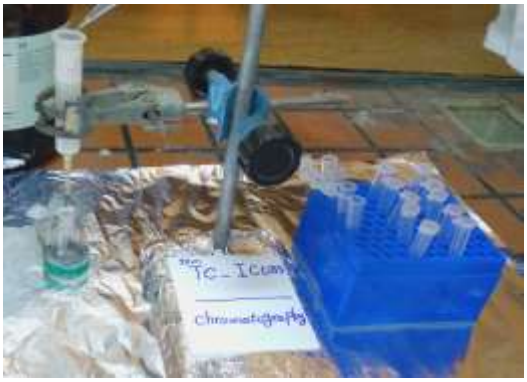

Figure 3. Chromatography set-up (Sephadex) for labeled ICD-85 separation

Experimental Animals

Animal studies were performed in compliance with the regulations of the Nuclear Sciences and Technology Research Institute (NSTRI), and with generally accepted guidelines governing such works. To pursue that aim, cancerous male mice, weighing between 25 and 30 g were injected with venom and investigated. Melanoma cancer was created by B16F10 cell line.

Bio-distribution

Bio-distribution study by killing the mice is an important step for preclinical investigations. The data obtained by dissecting and counting the organs is more accurate than other methods, while other methods such as SPECT imaging can also be used. Mice were injected with a saline solution containing radiolabeled ICD-85 into the tail vein. For ex vivo counting, the animals were killed 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).

RESULTS

In our method, ^{99m}Tc was labeled ICD-85 with high efficiency and good stability. After direct labeling of ICD-85 with Tc-99m, the complex was loaded on sephadex chromatography column (1.5*5.5cm) to separate the labeled ICD-85. The column was eluted by phosphate buffer (pH=7.1, 0.1 M) and collected into 1ml tubes. Finally, these tubes were counted by well type counter (Quantitative gamma counting was performed on an EG&G/ORTEC (Model 4001M, Jackson, USA) Mini Bin and Power Supply counter.). According to the results, the efficiency of radiolabeling was >75%. The results of radiolabelling are shown in Figure 4.

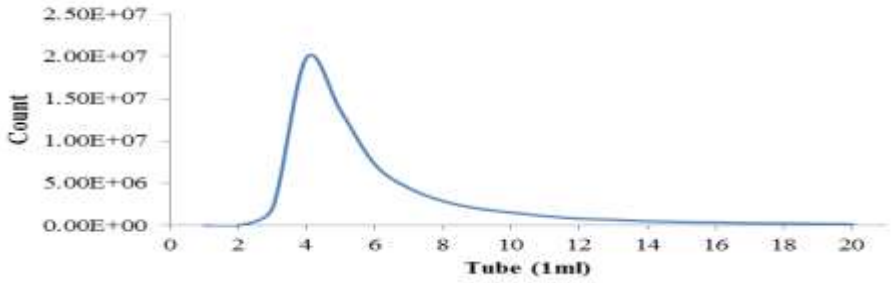

Figure 4. Chromatogram shows counts of 1ml tubes after radiolabeling and counting in NaI(Tl) well type counter

When the mice were injected with the labeled ICD-85, they did not show any clinical symptoms. After 30 min, they were sacrificed and their organs dissected and counted by well type counter. Bio-distribution study in normal mice presented moderate clearance of the compound from blood. The Bio-distribution results for labeled ICD-85 and ^{99m}TcO4 are shown in Tables 1, 2 and Figures 5 and 6.

Table 1. The Bio-distribution of labeled ICD-85 and ^{99m}TcO4 in mice after 30 min post injection

Organs	^{99m}Tc-ICD85 (ID/g%)	^{99m}TcO4 (ID/g%)
Blood	3.72±0.2	2.2±0.15
Heart	2.12±0.15	3.1±0.2
Lung	3.01±0.16	3.83±0.22
Tumor	1.87±0.11	1.79±0.12
Stomach	1.36±0.08	10.04±1.16
Intestine	0.84±0.06	1.97±0.12
Thyroid	1.43±0.07	14.33±1.8
Liver	6.1±0.4	4.15±0.3
Spleen	1.79±0.08	2.2±0.13
Kidney	8.2±0.5	3.61±0.17
Tissue	0.82±0.04	1.65±0.1
Brain	0.3±0.02	0.4±0.04

Table 2. Target to non–Target ratio in 5 organs for labeled ICD-85

Organs	Percentage
Tumor / Thyroid	130%
Tumor / Kidney	22.8%
Tumor / Liver	30%
Tumor / Heart	88%
Tumor / Lung	62%

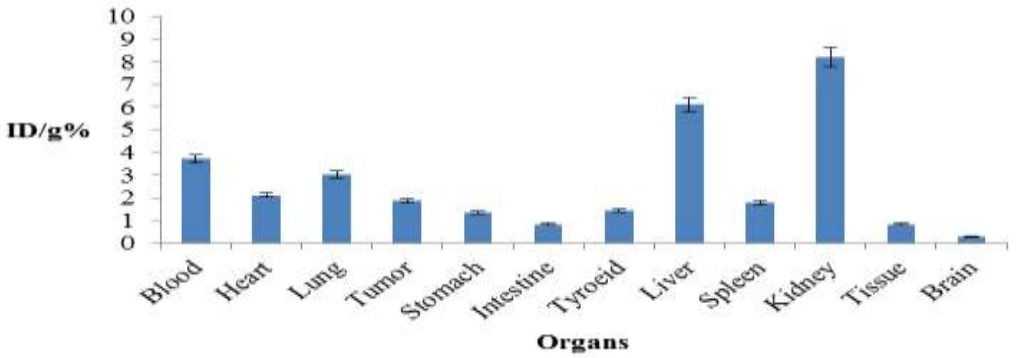

Figure 5. The bio-distribution of labeled ICD-85 in mice

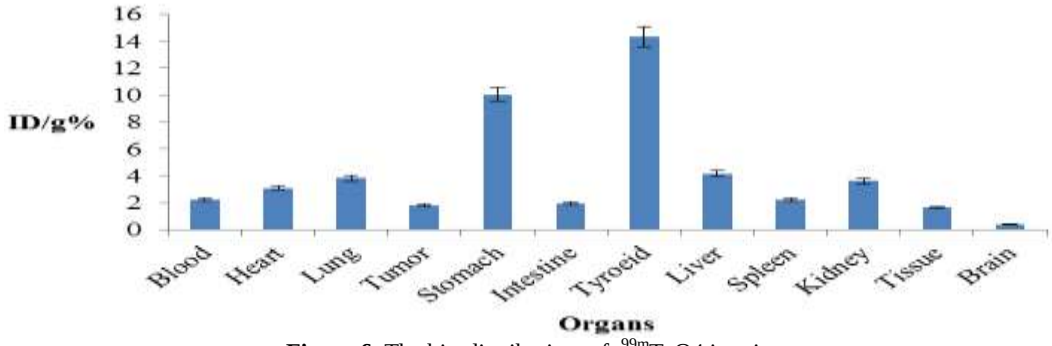

Figure 6. The bio-distribution of ^{99m}TcO4 in mice

DISCUSSION

In order to study ICD-85 bio-distribution, this material was labeled with sodium pertechnetate ($Na^{99m}TcO4$). ^{99m}Tc is the most important radionuclide in nuclear medicine applications; about 85% of the radiopharmaceuticals used in diagnostic applications are based in this substance. The reasons for this central position include favorable nuclear physical characteristics

and the availability. The half-life is short enough to enable the administration of reasonably high doses which, in turn, allow for good quality images. Moreover, ^{99m}Tc is available from ^{99}Mo/^{99m}Tc generators in high quality and at low cost [1, 18]. For ^{99m}Tc labeling, a large number of techniques have been developed and extensively reviewed [1, 20-23]. They may be classified into three main categories: direct labeling, preformed chelate approach, and indirect labeling approach [22]. The direct labeling approach usually uses a reducing agent to convert a number of disulfide linkages into free thiols, which are able to bind to ^{99m}Tc efficiently. It has been reported that both thiolate sulfur and imidazole nitrogen are involved in bonding with ^{99m}Tc [24]. This method applies mostly to proteins or their fragments because of their disulfide bonds. The direct labeling method is simple and easy to perform and does not require synthetic modification nor blocking and deblocking of functional groups. SnF_2 was used to reduce the disulfide bridges to provide sulfahydril groups for ^{99m}Tc binding.

In this study, the labeling of ICD-85 is successfully achieved with Tc-99m using direct SnF_2 reduction procedures. Tc-99m radionuclide is a pure gamma emitter which is ideal for medical scanning (gamma energy 140 keV). Also the dose delivered to the organs of interest may be extremely negligible compared to many other radionuclides. Bio-distribution study in normal mice presented moderate clearance of the compound from blood (The uptake of ICD-85 in blood after 30 minutes was 3.72±0.2 (ID/g%), which shows the moderate clearance of labeled ICD-85 from the body). Results showed that both liver and kidneys comprise secretion pathway of the labeled ICD-85. No significant accumulation in the stomach and thyroid was observed, indicating that there was low free ^{99m}Tc in the labeled ICD-85. Liver and kidney uptake after 30 minutes were 6.1±0.4and 8.2±0.5 (ID/g%) respectively. The concentration of labeled ICD-85 in the stomach and thyroid (target organs for free pertechnetate) was almost insignificant.

CONCLUSION

Radiolabeling of the ICD-85 was accomplished in order to assess the most optimum conditions for labeling and potential usage in biological evaluation. Furthermore, the conjugate showed acceptable specific activity and demonstrated considerable radiochemical stability. These properties suggest that ^{99m}Tc labeling of ICD-85 may be a useful tool for in vivo studies and is an excellent approach to follow the process of bio-distribution and kinetics of toxins.

"The authors declare no conflict of interest"

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