



## Applying radiolabeled novel recombinant insulin via $^{99m}\text{Tc}$ for ensuring native like action by *in vivo* biodistribution in mice.

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In order to study functionality and pharmacodynamics of any important biological molecule such hormones and enzymes, it takes a lot of time and complexed strategies to do that. Here we study the incidence of new recombinant human insulin prevalence in body tissues in real time with aid of radiotracing technique. A new designed model of recombinant human insulin and produced in *E.coli* suggested to be functional without post translational modification, has been radiolabeled with Technetium-99m, then tracing its biodistribution with  $^{99m}\text{Tc}$  in mice. The new construct of human recombinant Insulin protein subjected to the formation of  $^{99m}\text{Tc}$ -complex using sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) as a reducing agent, whole reaction conditions subject to optimization of the pH, temperature, substrate amount and a reducing agent amount. Then  $^{99m}\text{Tc}$ -Insulin complex has been intravenously administered for biodistribution study *in vivo* in Albino Swiss mice. The optimized conditions for preparing the  $^{99m}\text{Tc}$  –insulin complex with the highest radiochemical yield (93.3%) disclosed that using 100  $\mu\text{g}$  of insulin in the presence of 20  $\mu\text{g}$  of a reducing agent sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) at pH 8 and within 30 minutes reaction time. The radioactive complex of  $^{99m}\text{Tc}$ -Insulin gives a much better result after IV injection, where no accumulation in distinct organ. And due to aqueous nature of Insulin, it show clearance from both renal and hepatic route, Also prevalence of Insulin in body according every organs mass or size, have a great impact of functionality of recombinant insulin molecule. Consequently, this method seems to be much rapid and effective for evaluation of biological molecule *in vivo* via radioactive tracing technique.

**Key words:** Recombinant human Insulin,  $^{99m}\text{Tc}$  Radiolabeling, Biodistribution.

### Introduction

The world's population is increasing steadily at a rate of 1–2 percent, while reports at the same time point to a significant increase in insulin-dependent diabetes, which may be as high as 5–6 percent [1], considering Insulin to be the most important therapeutic drug produced by recombinant DNA technology, with huge economic importance. Genentech researchers have deproduced recombinant insulin as the first recombinant

human insulin model for therapeutic use in K12 E. Many trials from other companies such as Elli Lilly and Novo Nordisk [3] have been studied. Functional human insulin hormone composed of two polypeptide chains A and B with a 51 a.a, with a [7]. molecular weight of 5.8 kDa, chains are linked by two inter-molecular disulfide bonds between the residues CysA7-CysB7 and CysA20-CysB19, whereas an intra-molecular disulfide bond joins the residues CysA6-CysA11 [4].

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All trials for characterizing insulin biodistribution pattern and metabolism depend on plasma clearance of unlabeled insulin [5,6] or radioiodoinsulin [7]. External imaging using a  $\gamma$ -camera after intravenous administration of insulin labeled with a suitable radionuclide offers a better, simple and non-invasive substitute to understand its biokinetics.  $^{123}\text{I}$ -insulin metabolism investigated by external detection using scintillation camera in ordinary rats and humans [8]. Observation shows dimensions of biological activity and breakdown of insulin after radioiodination, is denatured and metabolized differently from native insulin due to iodinated insulin [9]. Depending on the number of iodine atoms per insulin molecule, immunological characteristics, electrophoretic properties and hormonal activity of  $^{131}\text{I}$ -insulin have changed to different levels [10]. Furthermore, insulin residue positions of the iodine atom influence the biological conduct of the labeled molecule [11]. Literature study revealed that technetium-99m is the most appropriate radionuclide for use in nuclear medicine due to its ideal physical properties ( $T_{1/2} = 6.02\text{h}$  and  $I = 140\text{ keV}$ ), low cost and great availability [12,13]. The strategy of using radiolabeled insulin with technetium as a tumor imaging agent showed insulin receptor over expression on surfaces of cell tumor cells [14]. This study aims to maximum exploitation of radioactive tracing technique. Particularly, with available radionuclide Technetium-99m in studying the real time functionality and activity of newly recombinant insulin molecule that has been produced in *E.coli* [15].

## Experimental Method

### Materials

A 6.92 kDa insulin molecule was produced using a novel simple method, created by special PCR program to build and amplify a new human insulin gene construct. Followed by insertion in a suitable cloning/expression vector pTriEx-4 plasmid, then transformed in *Origami 2(DE3) pLacI* Competent *E.coli* that used as an expression host. Insulin is then purified and characterized through normal laboratory testing for purity such as SD-PAGE and affinity western blot [15]. Egyptian Atomic Energy Authority (RPF), supplied the  $^{99\text{m}}\text{Tc}$  ( $T_{1/2} = 6\text{h}$ ,  $E_c = 140\text{ keV}$ ) for labeling. All reagents were of the grade of analytical and molecular biology. Radioactivity measurements were

performed in a NaI (TI) well-type gamma counter (Scaler Ratemeter SR7 model: Nuclear enterprises LTD, Edinburg, TX, USA). Whatman No.1 paper chromatography (Whatman International Ltd, Maidstone, Kent, UK) identified the radiochemical yield.

### Preparation of $^{99\text{m}}\text{Tc}$ -Insulin complex

A reducing agent of 20 mg of sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) was added to 100 $\mu\text{g}$  of human insulin dissolved in PBS (1X,  $\text{pH} = 7.4$ ). After adjusting the pH to 8 sodium [ $^{99\text{m}}\text{Tc}$ ]-pertechnetate was added to the reaction mixture, with mild shaking and retained for 30 min at room temperature, then the radiolabeling yield was measured using paper chromatographic technique.

The various parameters were studied in order to optimize the radiolabeling reaction such as pH (6,7,8, 9 and 10), temperature (25 and 37 °C), substrate quantity (50, 100, 250 and 500  $\mu\text{g}$  / rxn), reduction of the quantity of the agent (5, 10, 20 and 40 mg / rxn) and reaction time from 5 to 360 min to obtain the highest radiochemical yield.

### Paper chromatography

Ascending paper chromatography technique was used to determine the radiochemical yield using paper strips (13 cm long and 1 cm wide). It was marked from the lower end at a distance of 2 cm and lined into segments up to 10 cm. By using a hypodermic syringe, two drops of the  $^{99\text{m}}\text{Tc}$ -Insulin complex solution were placed to the strips at stage 0 and the strips were then put ascending in two various developer as mobile phases (acetone and saline) to determine the yield of radiolabeling. Ascending paper chromatography was used to evaluate the percentage of colloid species  $^{99\text{m}}\text{Tc}$ -Insulin, free  $^{99\text{m}}\text{TcO}_4$ , and reduced hydrolyzed- $^{99\text{m}}\text{Tc}$ . Acetone used to verify the percentage of free  $^{99\text{m}}\text{TcO}_4$  in ascending paper chromatography, where it moved up with the solvent ( $R_f = 0.9$ ), while other species stayed at the spotting point ( $R_f = 0.0$ ). The other chromatographic solvent was saline, which used to determine the percentage of reduced colloid- $^{99\text{m}}\text{Tc}$  hydrolyzed species that remained at the starting point ( $R_f = 0.0$ ), while  $^{99\text{m}}\text{Tc}$ -insulin complex and free  $^{99\text{m}}\text{TcO}_4$  went to the top of the paper ( $R_f = 0.9$ ).

### Biodistribution studies

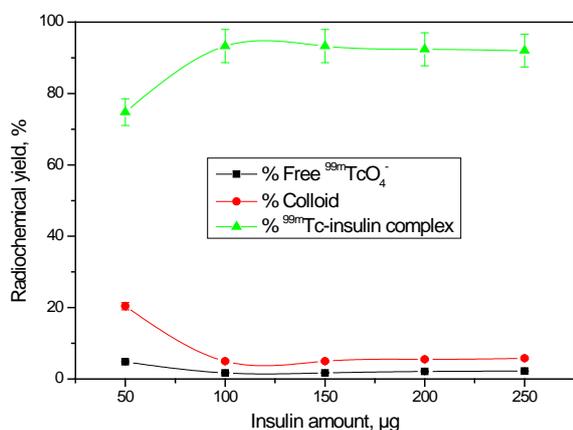
This study follows animal ethics committee guidelines of the Egyptian Atomic Energy

Authority. Swiss Albino mice (20–25gm) purchased from Cairo, Egypt, National Cancer Institute. Biodistribution was determined for the  $^{99m}\text{Tc}$ -insulin complex. Exactly 100  $\mu\text{l}$  of the optimized  $^{99m}\text{Tc}$ -insulin solution containing about  $1.85 \pm 0.5$  MBq ( $\sim 50$   $\mu\text{Ci}$ ) was injected intravenously into the mice tail vein. After anesthetizing using chloroform, the animals sacrificed at exact time intervals (5, 15, 30, 60, 120 and 180 min). With 0.9 percent saline solution, all body organs and tissues were washed, then weighed separately. Muscle, bone, and blood samples were gathered and weighted at 40, 10, and 7% respectively of total body weight [16]. The radioactivities of the organs as well as the background were measured by  $\gamma$ -counter. The percentage of ID/g was calculated for the different organs as the percentage of the injected dose.

## Results and Discussion

### Effect of insulin amount

Insulin quantity per reaction was studied in the 50–250  $\mu\text{g}$  range in **Fig 1**. The highest radiochemical yield obtained for the studied insulin was 92.2% using 100  $\mu\text{g}$  Insulin and remaining roughly stable though increasing the Insulin to 250  $\mu\text{g}$ . Where the lowest yield was observed at 50  $\mu\text{g}$  insulin was (74.8 %) which may be due to the insufficient Insulin to complex all  $^{99m}\text{TcO}_4^-$ .

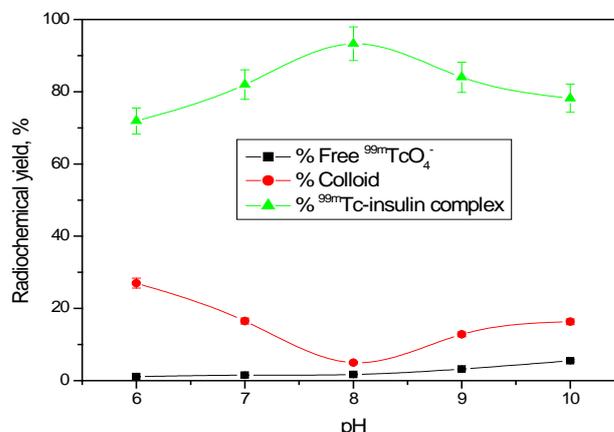


**Fig. 1.** Effect of insulin amount on the radiochemical yield of  $^{99m}\text{Tc}$ -insulin complex. Reaction conditions: 20 mg sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), pH 8, 0.3 mL of  $\text{Na}^{99m}\text{TcO}_4^-$  solution at room temperature after 20 min. Error bars indicate the standard error of the mean (SEM) for N=3 independent experiments.

### Effect of pH

The effect of pH on the  $^{99m}\text{Tc}$ -insulin complex radiochemical yield was studied in **Fig. 2** of the pH range from 6 to 10. The radiochemical yield was

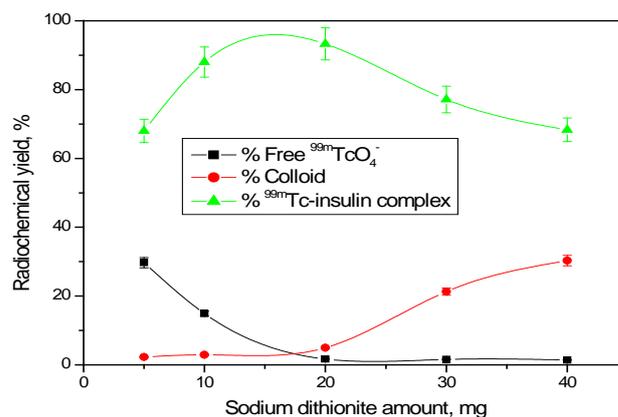
moderately small at a lower and greater pH value (6 and 10), respectively  $\sim 71.9$  % and 78.2 %, where the best pH value was at pH 8, at which the highest radiochemical yield of 92.2 % was achieved.



**Fig. 2.** Effect of pH on the radiochemical yield of  $^{99m}\text{Tc}$ -insulin complex. Reaction conditions: 20 mg sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), 100  $\mu\text{g}$  substrate (Insulin) amount, 0.3 mL of  $\text{Na}^{99m}\text{TcO}_4^-$  solution at room temperature after 20 min. Error bars indicate the standard error of the mean (SEM) for N=3 independent experiments.

### Effect of Sodium Dithionite $\text{Na}_2\text{S}_2\text{O}_4$ amount

By using the sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) as a reducing agent, the radiochemical yield of  $^{99m}\text{Tc}$ -insulin was highly affected by sodium dithionite content in the range 5–40 mg. The highest radiochemical yield of 93.3% was obtained at 20 mg of reducing agent, where the yield was lower and higher than 20mg as shown in **Fig 3**.



**Fig. 3.** Effect of reducing agent sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) on the radiochemical yield of  $^{99m}\text{Tc}$ -insulin complex. Reaction conditions: 100  $\mu\text{g}$  substrate (insulin) amount, pH 8, 0.3 mL of  $\text{Na}^{99m}\text{TcO}_4^-$  solution at room temperature after 20 min. Error bars indicate the

standard error of the mean (SEM) for N=3 independent experiments.

#### Effect of reaction time and stability

Reaction time effect on the formation of the  $^{99m}\text{Tc}$ -insulin complex was studied to determine the minimum time at which the maximum radiochemical yield of  $^{99m}\text{Tc}$ -Insulin complex was determined. Eight intervals varying from 5 to 360 min were studied. The radiochemical yield gives 93.3% after 30 min of labeling and remains stable until 360 min without altering Fig 4, which gives a reasonable indication of the stability of the reaction over time

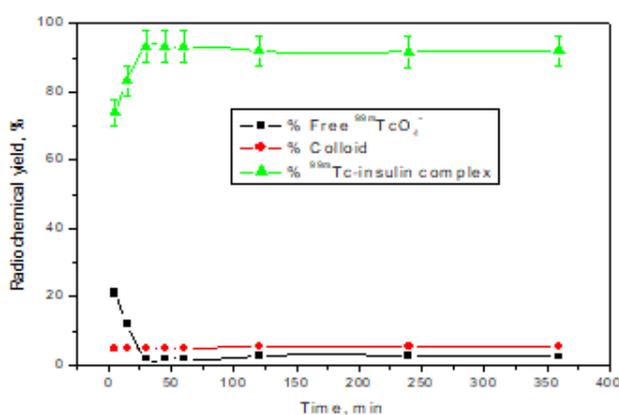


Fig. 4. Effect of reaction time on the radiochemical yield of  $^{99m}\text{Tc}$ -insulin complex. Reaction conditions: 100  $\mu\text{g}$  substrate (Insulin) amount, 20 mg sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), pH 8, 0.3 mL of  $\text{Na}^{99m}\text{TcO}_4^-$  solution at room temperature after 20 min. Error bars indicate the standard error of the mean (SEM) for N=3 independent experiments.

#### Biodistribution study of $^{99m}\text{Tc}$ -insulin complex

In vivo biodistribution study of the  $^{99m}\text{Tc}$ -insulin complex was performed at 5, 15, 30, 60, 120 and 180 min with different time intervals of the biodistribution assay Fig 5. As a percentage of injected dose per gram of organ or fluid (% ID / g organ) were the results demonstrated.  $^{99m}\text{Tc}$ -insulin complex shows normal uptake by all body organs and tissue as compared to body density and weight.  $^{99m}\text{Tc}$ -insulin complex showed relatively low to the initial kidney uptake (7.185 percent) then increased over time to 32.32% after 3 hours of injection, indicating clearance through the urinary pathway. Also decreasing the concentration in blood by the time indicate its transport from blood to tissues normally, however its complete uptake

from blood (13.9 % at 5 min and 6.25% at 180 min) due to body homeostasis.

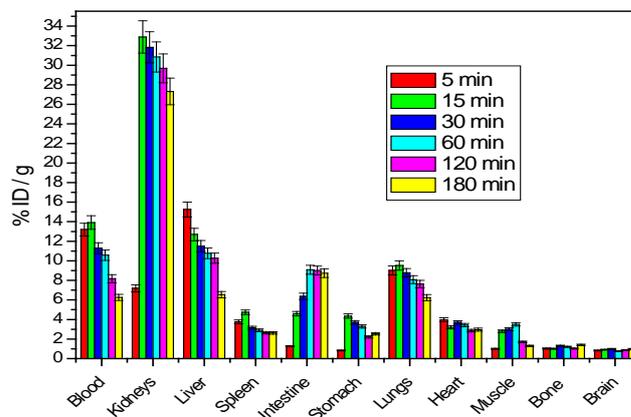


Fig. 5. Biological distribution of  $^{99m}\text{Tc}$ -insulin complex in normal Albino Swiss mice. Error bars indicate the standard error of the mean (SEM) for N=5 independent experiments

## DISCUSSION

### Paper chromatography separation

According to [17] acetone used to determine the percentage of free  $^{99m}\text{TcO}_4^-$ , which migrate to top of the chromatography paper, leaving the  $^{99m}\text{Tc}$ -Insulin and reduced hydrolyzed- $^{99m}\text{Tc}$  colloid species remains at the bottom. While the  $^{99m}\text{Tc}$ -insulin complex and free  $^{99m}\text{TcO}_4^-$  moved to the top of the paper when using saline as another mobile phase, the reduced hydrolyzed- $^{99m}\text{Tc}$  colloid species stayed at starting point in the bottom. The percentage of  $^{99m}\text{Tc}$ -insulin complex can be easily determined. Very small amount of colloid formed which is a major limitation in direct labeling of proteins [18].

### Radiolabeling of Insulin

Technetium radiolabeled complexation must employ a reducing agent to reduce technetium from 7 to the desired oxidation state, with the most commonly used reducing agent being stannous chloride [19] and sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) [20]. Sodium dithionite was used for reduction, as it has low precaution for the reaction condition. The yield of the  $^{99m}\text{Tc}$ -Insulin complex was decreased by the use of small and high quantities of sodium dithionite ( $10 \text{ mg} < x > 20 \text{ mg}$ ) may be due to an incomplete reduction of  $^{99m}\text{TcO}_4^-$  species and increased  $^{99m}\text{Tc}$ -colloid formation, respectively [21].

The amount of insulin also has a major impact on the percentage of radiolabeling yield, where small amounts of substrates have low labeling yield because this amount was insufficient to chelate all the reduced  $^{99m}\text{Tc}$  species [22]. The maximum radiolabeling yield was 93.3% at 100  $\mu\text{g}$ ; there is no change in the labeling yield to further increase the substrate quantity. The pH is more or less than pH 8, the radiochemical yield tends to be low due to the formation of reduced  $^{99m}\text{Tc}$ -colloid hydrolyzed as the main radiochemical impurity [24].

#### Biodistribution

Depending on the results of the biodistribution, there is no specific accumulation of  $^{99m}\text{Tc}$ -insulin complex in any body organs. As can be seen from figure 5, the  $^{99m}\text{Tc}$ -insulin complex was excreted mainly through the urinary pathway and used before [18] to follow the same requirements of indigenous insulin. Since the insulin receptor (IR) found in all body cells, this explains the high muscle uptake of the  $^{99m}\text{Tc}$ -insulin complex [23], because the muscle to body volume represents about 40% of the complete body weight, the same values also shown in the bone representing about 12% of the body weight. Partial low accumulation of radioactive material in the stomach and intestine above normal ratios, indicating a very small free  $^{99m}\text{TcO}_4^-$  (pertechnetate) percentage that was less than 3% in optimal condition. Homogenous  $^{99m}\text{Tc}$ -insulin complicated distribution confirms the correct folding and native action of recombinant human insulin produced in *E. coli* and tested by radiolabeling.

#### Conclusion

Low cost and effective technique used in *E. coli* to determine the biological activity of newly modeled recombinant human insulin. Radiolabeling takes place with  $^{99m}\text{Tc}$ , giving a good yield of about 93.3% for radiolabeling and studying the iodistribution of  $^{99m}\text{Tc}$ -Insulin complex in mice. Results showed that our novel insulin protein is well absorbed by all the mice body organs and tissues, indicating that our protein construction considers a promising candidate for diabetes treatment, radiolabeling method is a low-cost approach for in vivo examination of recombinant therapeutic proteins to demonstrate its real-time insulin pharmacokinetics. Also labeled insulin can be used as a probe for cancer tracing via single photon emission computed tomography (SPECT).

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