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Recombinant human insulin as a solid tumor potential imaging agent: Radio-synthesis and biological evaluation

Gamal Abdelaziz [1,2*]; Motaleb, M.A.[1]; Farouk, N.[1]; Adli A. Selim[1*]

[1]Labeled Compounds Department, Hot Laboratories Center, Egyptian Atomic Energy Authority, P.O. Box 13759, Cairo, Egypt; [2]laboratoire structure et Activité des biomolécules normales et pathologiques" U1204 – INSERM - Université Evry-Val d'Essonne (UEVE).

[*]Corresponding authors E-mail: gamal_abdelazeez@yahoo.com; adli_a_selim@yahoo.com

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Abstract

The aim of this study was to synthesis an imaging agent for tumor targeting. New recombinant insulin analogue was successfully produced from *E. coli* by recombinant DNA technique, and was well labeled with Technetium-99m with a high radiochemical yield of 93.3 ± 2.1 %. Moreover, it showed good *in-vitro* stability in both saline and human serum. Preclinical evaluation of Technetium-99m [^{99m}Tc] Tc-insulin in solid tumor-bearing mice showed high accumulation in tumor tissues. The T/NT (target to non-target ratio) was of 5.4, after 60 min. of post injection (p.i). The direct intra-tumoral (I.T) injection of [^{99m}Tc] Tc-insulin showed good retention in tumor tissues with a ratio more than 50 % after 15 min. As a result of the promising bio-distribution studies; the newly recombinant insulin showed good uptake in tumor site, which assured high concentration of insulin receptor on tumor cell surface, accompanied with high cell density of tumor cells as well. This work affords a potential radio-carrier that could be used as a good tumor marker and imaging probe via SPECT (Single Photon Emission Computed Tomography) technique, after further preclinical studies.

Keywords: Human recombinant insulin, Labeling, Technetium-99m, Imaging, Tumor

1. Introduction

Cancer is the main cause of mortality, and the number of diseased patients with cancer is increasing at an alarming rate. Tumor was developed when cells got mutated and multiplied in an uncontrolled manner (Motaleb and Selim, 2019). The most effective way for cancer treatment is its early detection (Sakr *et al*., 2013; Badenhorst *et al*., 2015; Ma *et al*., 2015). Nuclear medicine technique was one of the most popular techniques used for cancer detection (Ibrahim *et al*., 2014; Sakr *et al*., 2014). Technetium-99m is the most radionuclides which emit gamma rays used in nuclear medicine; due to its perfect characteristics (6.02 h physical half-life and 140 keV γ-ray energy),

low cost and good availability (Dilworth and Parrott, 1998; Sakr *et al*., 2012; Motaleb *et al*., 2018b).

As previously reported by Wang *et al*., (2013), insulin has a good affinity towards tumor site. Insulin receptors pathway was mainly focused in anti-diabetic therapies; but nowadays it has rapidly gained attention as a novel target in cancer. According to Malaguarnera and Belfiore, (2011), these new concepts must be exploited for cancer prevention and therapy. Papa *et al*., (1990); Belfiore, (2007) pointed that malignant cells have over-expression insulin receptors more than expression levels observed in classical insulin target organs, such as the liver. Insulin receptor isoform A (IR-A) expression has been detected in cancer cells of the breast; lung, colon (Frasca *et al*.; 1999), ovaries (Kalli *et al*., 2002), thyroid gland (Vella *et al*., 2002), smooth and striated muscles (Sciacca *et al*., 2002).

The new human recombinant insulin was labeled with Technetium-99m, and factors affecting the percent of labeling yield were studied in details. Moreover, *in-vivo* studies were carried out on solid tumor bearing mice.

2. Material and methods

2.1. Chemicals

Sodium dithionite were purchased from Merck, Darmstadt, Germany. ^{99m}Tc was obtained from a 99Mo-99mTc generator (RPF, Egyptian Atomic Energy Authority, Egypt). Radioactivity measurements were done in a NaI (TI) well-type gamma counter (Scaler Ratemeter SR7 model, Nuclear enterprises LTD, Edinburg, TX, USA). Labeling yield was detected using Whatman No.1 paper chromatography (PC) (Whatman International Ltd, Maidstone, Kent, UK). All chemicals and reagents used were of high purity grade, double distilled water was used.

2.2. Synthesis of human recombinant insulin

De novo strategy has been developed for producing recombinant human insulin protein (6.92 kDa) in *E. coli*, via direct assembly of full-length gene contacting chain A and B connected by only five residues of amino acids. This was followed by PCR amplification, and then inserting in pTriEx-4 plasmid as a cloning/expression vector. Chemical transformation process has been undergone in Origami 2(DE3) pLacI competent E. coli. When the gene was expressed and insulin protein was produced intracellularly; insulin was then purified by chromatography via Ni-NTA beads. After that the purity and affinity were checked by Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE), and western blot according to Aziz *et al*., (2016).

2.3. Radiochemical yield of [^{99m}Tc] Tc-insulin complex

According to Essa *et al*., (2015), [^{99m}Tc] Tc-insulin complex radiochemical yield was evaluated by ascending paper chromatography (Whatman No.1, Whatman International Ltd, Maidstone, Kent, UK) using two different mobile phases; acetone (for detection of free $^{99m}TcO_4^-$), and water: ethanol: ammonia mixture (5:2:1, v/v/v) for detection of RH-^{99m}Tc. Papers chromatography of 1 cm wide and 12 cm long were marked from lower end at a distance of 2 cm, and then lined into parts (1 cm each) up to 10 cm. The % of free $^{99m}TcO_4$ was determined at Rf = 1 by using acetone as a developing solvent, whereas the mixture was used to determine the % of hydrolysed ^{99m}Tc colloid at Rf = 0. After completing the developing process; the strips were dried, cut into 1 cm pieces, and then counted using the γ-ray scintillation counter. The % of [^{99m}Tc] Tc-insulin complex was calculated as follows:

Free $^{99m}TcO_4^-$, (%) = (Activity at R_f = 1.0)/ (Total activity) ×100

RH-^{99m}Tc, (%) = (Activity at R_f = 0.0)/ (Total activity) ×100

[^{99m}Tc]Tc-insulin complex, (%)= 100- (Free $^{99m}TcO_4^-$ + %"RH^{-99m}Tc)

2.4. Labeling of recombinant insulin with Technetium [^{99m}Tc]

Labeling studies of insulin were carried out using sodium dithionite ($Na_2S_2O_4$) as a reducing agent in reference to Essa *et al*., (2015). Desired amount of insulin was added to appropriate amount of sodium dithionite dissolved in sodium pertechnetate at optimum pH value, and allowed to stand at room temperature. Reaction conditions such as; quantity of insulin (50-250 µg), pH (6-10), sodium dithionite amount (5-40 mg), reaction time, were studied to obtain a maximum radiochemical yield, and to determine the best time for bio-evaluation studies.

2.5. Physiological in-vitro stability study

Physiological *in-vitro* stability was performed in saline and in human serum. 0.2 ml of the [^{99m}Tc] Tc-insulin was completed to 2 ml with 0.9 % saline or human serum, and then incubated at 37ºC for 24 h. The radiochemical yields were checked at 0, 3, 6, 12 and 24 h of incubation period (Motaleb *et al*., 2018a).

2.6. Bio-distribution studies

Assays were carried out according to the protocol of this study approved by the Egyptian Atomic Energy Authority (EAEA), animal ethics committee (EAEA/2017/167).

2.6.1. Solid tumor induction in mice

The parent tumor line used was Ehrlich Ascites Carcinoma. This tumor line was withdrawn from a donor Albino mouse, and then diluted with physiological saline. About 0.1 ml of this solution was intramuscularly injected in the right thigh of a mouse; the solid tumor however was produced after 5-7 days (Essa *et al*., 2015).

2.6.2. Bio-distribution

The Bio-distribution studies of the [^{99m}Tc] Tc-insulin were performed in two groups of mice "A", and "B" (solid tumor bearing mice). The radioactive solution was injected intravenously (I.V.) into group A; but group "B" was injected intra tumor (I.T.). After that; the animals were sacrificed via spinal cord dislocation at 5, 15, 30, 60, 120 and 180 min. (p.i.), for each group. Blood, bone and muscle weight were taken as 7, 10 and 40 % of the total body weight, respectively (Motaleb *et al*., 2018b). The obtained results were expressed as % dose per gram of organ.

2.7. Statistical analysis

Results of each experiment was analysed statistically by one way ANOVA test, and were expressed as mean ± SEM. Each experiment was repeated twice.

3. Results and Discussion

3.1. Radiolabeling of human recombinant insulin

3.1.1. Effect of pH of the reaction medium

The effect of different pH values of the reaction mixture on the radiochemical yield of [^{99m}Tc] Tc-insulin was studied as shown in Fig. 1. Maximum radiochemical yield of 93.3 ± 2.1 % was obtained at optimum pH of 8. However; at pH 6 and 10, the radiochemical yield was decreased and reached to 71.9 ± 1.9 % and 78.2 ± 2.0 %, respectively. The radiochemical yield was low at pH below or above the optimum pH of the reaction medium. This was attributed to the formation of reduced hydrolyzed Technetium (RH-99mTc) colloid, which was the main radiochemical impurity in reference to Essa *et al*., (2015).

3.1.2. Effect of amount of reducing agent

Labeling studies of insulin were carried out using sodium dithionite ($Na_2S_2O_4$) as a reducing agent. Fig. 2 showed the radiochemical yield of [^{99mTc}] Tc-insulin which was highly affected by the content of the

reducing agent. Maximum radiochemical yield of 93.3 ± 2.1 % was obtained by using the optimum amount of sodium dithionite (20 mg). The radiochemical yield was decreased and become 68 ± 1.4 % at 5 mg of sodium dithionite, due to incomplete reduction of 99mTcO4- as reported by Essa et al., (2015).

On increasing the sodium dithionite content over the optimum amount (40 mg); the radiochemical yield was decreased to 68.3 ± 1.4 %, whereas the RH-^{99m}Tc colloid formation was increased in accordance with Motaleb *et al*., (2018a).

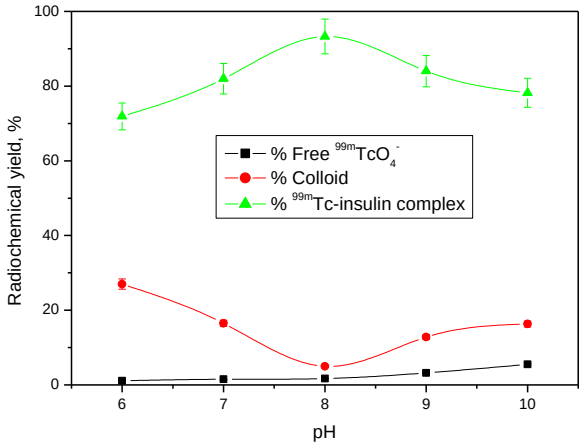

Fig.1. Radiochemical yield of [^{99m}Tc] Tc-insulin as a function of pH

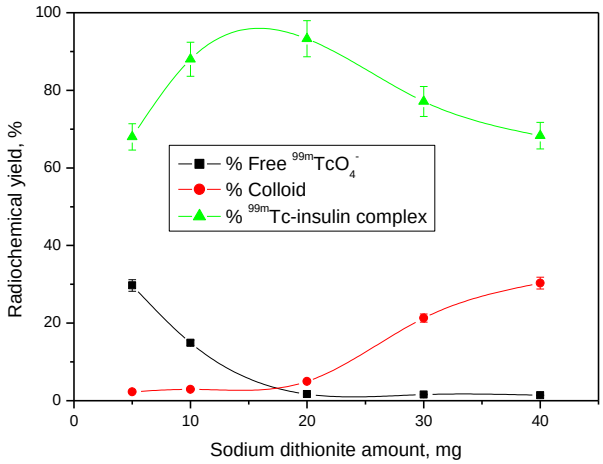

Fig. 2. Radiochemical yield of [^{99m}Tc] Tc-insulin as a function of $Na_2S_2O_4$ amount

3.1.3. Effect of the amount of insulin

Fig. 3 shows the effect of insulin amount on the radiochemical yield. At low amount of insulin (50 µg); the radiochemical yield was low (74.8 ± 1.6 %), because the amount of insulin required to chelate all the reduced 99mTc was insufficient (Al-Wabli *et al*., 2016). Maximum radiochemical yield of (93.3 % ± 2.1 %) was obtained at 100 µg of insulin. However, further increase in the insulin amount had nearly no impact on the radiochemical yield.

3.1.4. Effect of reaction time and *in-vitro* stability

As clear in Fig. 4, the rate of formation of [^{99m}Tc] Tc-insulin started with a yield of 74 ± 1.5 % after 5 min. The highest radiochemical yield of 93.3 ± 2.1 % was achieved at 30 min. of reaction time. The effect of time on the in vitro stability of the [^{99m}Tc] Tc-insulin complex was studied up to 6 h, where the radiochemical yield remained around maximum value.

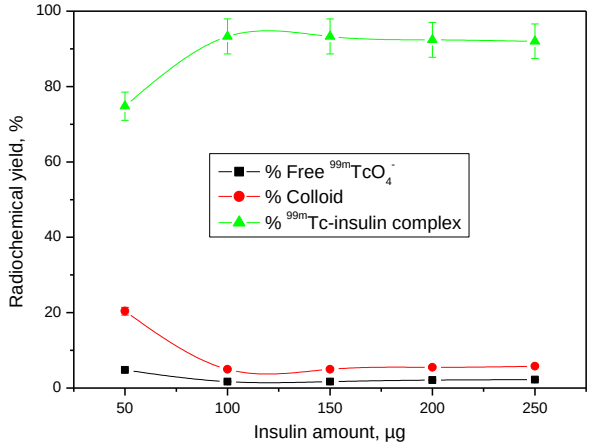

Fig. 3. Radiochemical yield of [^{99m}Tc] Tc-insulin as a function of insulin amount

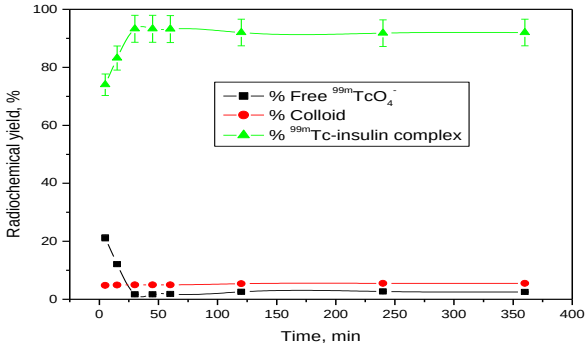

Fig. 4. Radiochemical yield of [^{99m}Tc] Tc-insulin as a function of time

3.2. Physiological *in-vitro* stability

Study of *in-vitro* stability of the [^{99m}Tc] Tc-insulin under physiological conditions in both saline and human serum revealed negligible changes in radiochemical yield. Only 1.2 % of the complex was lost after 6 h, and about 3 % decrease in the radiochemical yield was recorded after 24 h of incubation period. This indicated that [^{99m}Tc] Tc-insulin had prolonged stability and was suitable for *in-vivo* studies.

3.3. Bio-distribution studies

In group "A", [^{99m}Tc] Tc-insulin was accumulated in tumor cells with a good yield (Fig. 5). This was observed by calculating Target/non target (T/NT) (tumor muscles to normal muscles) ratio which reached 5.4 after 60 min. as clear in (Fig. 6).

This higher uptake in tumor cells was better than many of recently developed agents based on T/NT ratio such as; [^{99m}Tc] Tc-N-MAG-AMCPP (1.83 at 1 h) (Ding *et al*., 2012), [^{99m}Tc] Tc-DMSAme (2.49 at 2 h) (Zhang *et al*., 2010), [^{99m}Tc] Tc-sunitinib (3 at 1 h) (Sakr *et al*., 2013), [^{99m}Tc] Tc-(CO)3-labeled chlorambucil analoge (3.2 at 3 h) (Satpati *et al*., 2009), ((2E,2'E,3E,3'E)-3,3'-(cyclohexane-1,2-diylbis (azanylylidene)) bis (butan-2-one) dioxime) (3.4 ± 0.2 at 30 min.) (Motaleb *et al*., 2018b), [^{99m}Tc] Tc-BnAO-NI (3.96 at 2 h) (Hsia *et al*., 2011), [^{99m}Tc] Tc-gemcitabine (4.9 at 120 min.) (Ibrahim *et al*., 2014), and ((2E,2'E,3E,3'E)-3,30-(pyrimidine-4,5-diylbis(azanylylidene) bis-(butan-2-one) dioxime) (5.14 ± 0.25 at 30 min.) (Motaleb *et al*., 2017).

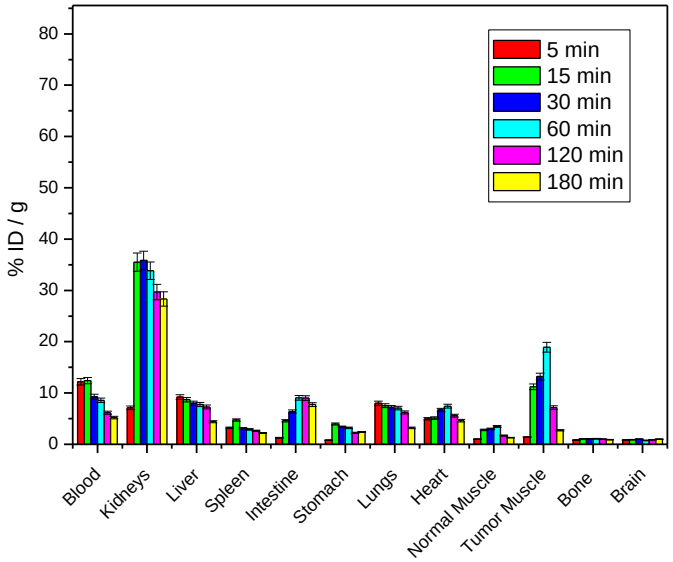

Fig. 5. Bio-distribution studies of [^{99m}Tc] Tc-insulin at different time intervals post I.V. injection in solid tumor bearing Albino mice

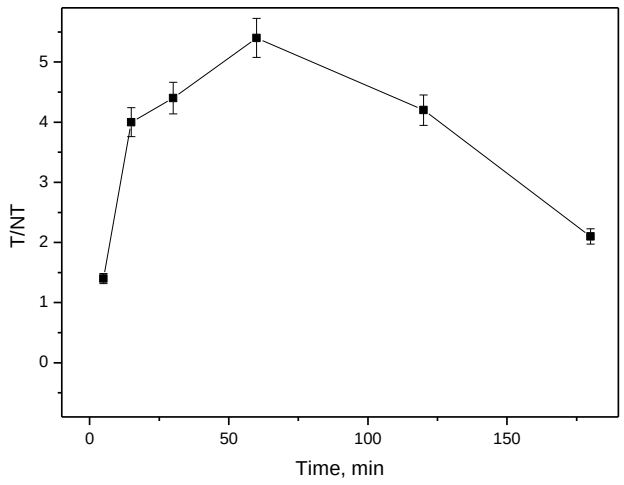

Fig. 6. T/NT ratio of [^{99m}Tc] Tc-insulin at different time intervals post I.V. injection in solid tumor bearing Albino mice

In comparison with intravenous injection; [^{99m}Tc] Tc-insulin can be directly injected in large doses to tumor site, without toxicity effect on other organs (intra-tumoral injection IT). On the other hand; in the second group "B", [^{99m}Tc] Tc-insulin was retained in tumor muscles with a ratio more than 50% after 15 min. (Fig. 7).

These promising results in both "A" and "B" groups were encouraging to conclude that insulin can be used as a good and safe carrier for Technetium-99m to tumor sites; in addition, it can be used as a potential candidate for tumor imaging.

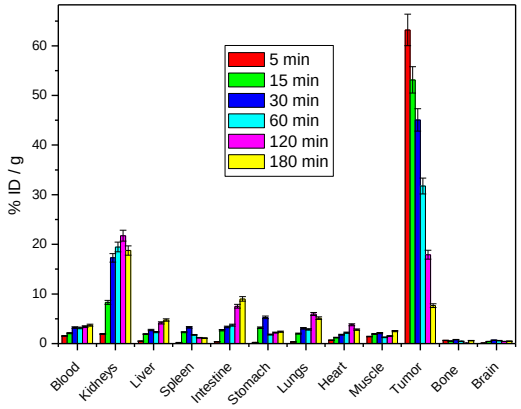

Fig. 7. Bio-distribution studies of [^{99m}Tc] Tc-insulin at different time intervals post I.T. injection in solid tumor bearing Albino mice

Conclusion

A new approach was explained for preparing [^{99m}Tc] Tc-insulin as a new candidate for tumor imaging with high radio-stability. The accumulation in tumor cells was investigated via *in-vivo* studies in tumor bearing Albino mice models, which showed high accumulation in tumor site with T/NT of 5.4 after 60 min. through IV injection. Moreover, there was an attractive retention in tumor muscles after IT injection with a ratio of more than 50 % after 15 min. This newly created agent ([^{99m}Tc] Tc-insulin) was in good stability, and showed high uptake in tumor site compared with the recently developed radiopharmaceuticals. Thus it affords a great promise as a radiopharmaceutical, to be employed as an effective candidate for tumor imaging after further preclinical studies.

Conflict of interests

The authors declare that there was no conflict of interests.

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