

# Preparation and quality control of radiolabeled streptokinase for Thrombosis imaging

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**Background:** In order to diagnose the site of thrombi, radiolabeled streptokinase can be prepared. The radiolabeled compound can be used in imaging of thrombi in many cardiovascular diseases. **Materials and Methods:** Streptokinase was successively labeled with [<sup>67</sup>Ga]-gallium chloride using cyclic DTPA-dianhydride. The conjugation with DTPA was optimized for concentration, time and temperature followed by size exclusion chromatography using G-50 Sephadex. The radiochemical purity of the tracer was checked using HPLC and ITLC methods. The biodistribution studies were performed in normal rats up to 167 h using tissue counting and preliminary SPECT studies up to 2 h. **Results:** The radiolabeled enzyme was prepared in 60 minutes after incubation at room temperature, with the radiochemical purity of >95% (HPLC) and >99% (ITLC) methods. The radioactivity was accumulated in lung, intestine and liver as shown by scarification and SPECT (Single Photon Emission Computed Tomography) methods. **Conclusion:** Radiolabeled Streptokinase was prepared in suitable radiochemical purity and its biodistribution is comparable to other radiolabeled proteins. Further studies are required to investigate the imaging properties of the tracer in appropriate animal model. *Iran. J. Radiat. Res., 2009; 6 (4): 195-200*

**Keywords:** Streptokinase, gallium-67, thrombosis, SPECT, radiopharmaceuticals.

## INTRODUCTION

Streptokinase (STP) is a highly purified substance derived from the culture filtrate of cocci of Lancefield Group C (molecular weight 47,000 daltons) with disappearance rate of 83 minutes in human serum<sup>(1)</sup> which finally leads to the hydrolysis of fibrinogen and other plasma proteins. The efficacy of STP in the analysis of venous thrombi and massive pulmonary emboli has been estab-

lished in clinical studies by angiographic evaluations, before and after treatment<sup>(2)</sup>. Since plasminogen is present in the thrombus/embolus, it can be a suitable macromolecular target to be detected by STP. The detection of magnitude and sites of thrombi in cardiovascular diseases is critical in the management of the patients. Thus, the preparation of a STP-based radio-tracer can be interesting in the diagnosis of thrombi.

A number of reports have described the radioactive labeling of STP as a radiopharmaceutical in the detection of blood clots<sup>(3-5)</sup>. Although STP has a strong affinity for thrombi, the radiolabeled enzymes reported have not been as successful in the location of clots as might be expected from theoretical considerations, so far. This relative lack of success may in part have resulted from unsatisfactory used labeling methods<sup>(6)</sup>.

For instance, Tc-99m STP has been prepared for detection of the clot via a direct labeling of the protein. However, due to many reasons the compound was not successful in biological evaluation<sup>(7)</sup>. Other studies focused on the I-131 labeling of STP for biodistribution and pharmacokinetic studies, but deiodination activity of serum caused a high free iodine background<sup>(8,9)</sup>.

The catabolic pathways for STP was studied using <sup>125</sup>I-STP in one study, and biodistribution was checked in rats, but no SPECT studies

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were performed<sup>(10)</sup>.

In order to obtain a STP conjugate for possible use in diagnostic studies using metallic positron emission tomography (PET), or single photon emission tomography (SPECT) radioisotopes, <sup>67</sup>Ga-labeled STP was prepared for preliminary biodistribution studies, based on our recent experiences on the preparation of gallium-labeled proteins<sup>(11)</sup>. Radiolabeled enzyme was injected intravenously to normal and thrombotic rats and biodistribution of the tracer was checked among the tissues using post-mortem studies and SPECT.

## MATERIALS AND METHODS

Production of <sup>67</sup>Ga was performed at AMIRS (Agricultural, Medical and Industrial Research School) 30 MeV cyclotron (Cyclone-30, IBA). Enriched zinc-68 chloride with enrichment of >95% was obtained from Isotopes Research Group at AMIRS. Sephadex G-50, sodium acetate, phosphate buffer components methanol and ammonium acetate were purchased from commercial vendors. Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N<sub>2</sub>. STP was a pharmaceutical sample (STREPTASE®), purchased from Aventis Co., and was used without further purification. Radiochromatography was performed by a AR-2000 Bioscan instrument, Paris, France, on polymer backed silica gel using 10 mM DTPA solution as eluent. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, flow scintillation analyzer (Packard-150 TR) and UV-visible (Shimadzu) using Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman, NJ, USA. Calculations were carried out based on the 184 keV peak for <sup>67</sup>Ga. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using student *t*-test.

Tissue counts were obtained using high purity germanium (HPGe) detector coupled with a Canberra (model GC1020-7500SL)

multichannel analyzer. Statistical significance was defined as P<0.05. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn (<http://asab.nottingham.ac.uk/downloads/guidelines2005.pdf>).

### Production of <sup>67</sup>Ga

<sup>68</sup>Zn (p,2n)<sup>67</sup>Ga was used as the best nuclear reaction for the production of <sup>67</sup>Ga. Radionuclidic and chemical impurities could be removed in the radiochemical separation process. After the target bombardment process, chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 M HCl (15 ml) and the solution was passed through a cation exchange resin (AG 50W, H<sup>+</sup> form, mesh 200-400, h:10 cm, Ø:1.3 cm) which had been preconditioned by passing 25 ml of 9 M HCl. The column was then washed by 25 ml of 9M HCl at a rate of 1 ml/min to remove copper and zinc ions. Finally, <sup>67</sup>Ga cation was washed out by 20 ml of 4 M HCl. Then, 10 M HCl (20 ml) was added to the 20 ml of 4 M eluent in order to obtain a 7M mixture to extract <sup>67</sup>Ga ions. Diisopropyl ether was used to extract <sup>67</sup>Ga from the aqueous phase (2 times). The mixed organic layers were back-extracted using 12.5 ml of 0.05 M HCl. The resulting high-purity <sup>67</sup>Ga chloride solution with 7.13 GBq/ml activity concentration was used for labeling after quality control.

### Quality control of the product

Control of Radionuclide purity: Gamma spectroscopy of the final sample was carried out using an HPGe detector coupled to a Canberra multi-channel analyzer for 1000 seconds. Chemical purity control: The presence of zinc and copper cations were checked by polarographic methods. Even at 1 ppm of standard zinc and copper concentrations, the area under the curve of the polarogram of the experimental samples were lower than the standards.

### **Conjugation of cyclic DTPA di-anhydride with STP**

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated with STP using a small modification of the well-known cyclic anhydride method<sup>(12)</sup>. Conjugation was performed at a 1:1 molar ratio. In brief, 20 µl of a 1 mg ml<sup>-1</sup> suspension of DTPA anhydride in dry chloroform (Merck, Darmstadt, Germany) was pipetted under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Commercially available STREPTASE (1 mg, 1 ml, pH 6.5) was subsequently added and gently mixed at room temperature for 60 min. Conjugation mixture was then passed through a Sephadex G-50 column (2 × 15 cm, 2 g in 50 ml of Milli-Q® water) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. The fractions containing the highest concentration of conjugated protein were chosen and kept at 4°C and for radiolabeling.

### **Radiolabeling of the DTPA-conjugated enzyme with <sup>67</sup>Ga**

The protein DTPA-conjugate was labeled using an optimization protocol according to literature<sup>(13)</sup>. Typically, 37-40 MBq of <sup>67</sup>Ga-chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. To the Ga containing vial, conjugated fraction was added in 1 ml of phosphate buffer (0.1 M, pH=7.4) and mixed gently for 30 seconds. The resulting solution was incubated at room temperature for 30 minutes. Following incubation, the radiolabeled protein conjugate was checked using ITLC/HPLC methods for the chemical and radiochemical purity. If percentage of impurities was more than 10%, the sample was purified using gel filtration as follows. Briefly, the radiolabeled mixture was passed through a Sephadex G-50 column (2 ×

15 cm, 2 g in 50 ml of Milli-Q® water) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. Control labeling experiments were also performed using <sup>67</sup>GaCl<sub>3</sub>, and DTPA with <sup>67</sup>GaCl<sub>3</sub>. Radio thin layer chromatography of the radiolabeled samples was performed on polymer backed silica gel using 10 mM DTPA solution. HPLC was performed on the final preparation using acetate buffer solution (50 mM pH. 5.5) as eluent (flow rate: 1ml/min pressure: 130 KgF/cm<sup>2</sup>) for 20 min in order to elute low molecular weight components. Radiolabeled protein was eluted using a gradient of the latter solution (100 to 0%) and citrate buffer solution (50mM, pH.4, 0 to 100%) using reverse stationary phase.

### **Stability testing of <sup>67</sup>Ga-DTPA-STP in final solution**

The stability of final radiolabeled product was checked at room temperature for 2 hours and at 5°C for 24 hours, while ITLC was performed using 5µL samples transferred on Whatman no. 2 paper and eluting by 10 mM DTPA solution at specific time intervals.

### **Stability testing of the <sup>67</sup>Ga-DTPA-STP in presence of serum**

Labeled compound stability in serum, was assessed by gel filtration 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; 0.5 mL fractions were collected.

### **Biodistribution of <sup>67</sup>Ga-DTPA-STP in normal rats**

To determine its biodistribution, <sup>67</sup>Ga-DTPA-STP was administered to normal rats. A volume (50 ml) of final <sup>67</sup>Ga-DTPA-STP solution containing 50±2 mCi radioactivity was injected intravenously to rats through their tail vein. The animals

were sacrificed at exact time intervals (2-168 h), and the ID/gr % of different organs was calculated as percentage of urea under the curve of 184 keV peak per gram using an HPGe detector.

### SPECT imaging of $^{67}\text{Ga}$ -DTPA-STP in rats

Images were taken at 24 and 48 hours after administration of the radiopharmaceutical by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. The useful field of view (UFOV) was 540 mm $\times$ 400 mm. The spatial resolution in the coincidence mode was 10 mm FWHM at the CFOV. Sixty four projections were acquired for 30 seconds per view with a 64 $\times$ 64 matrix.

## RESULTS

Conjugation of STP with DTPA cyclic di-anhydride and radiolabeling of STP with  $^{67}\text{Ga}$ : The labeling yield of  $^{67}\text{Ga}$ -DTPA-STP has been studied in the wide range of STP/DTPA ratios optimize the process and to improve  $^{67}\text{Ga}$ -DTPA-STP performance *in-vitro*. ITLC studies using whatman paper in 10 mM DTPA solution as eluting phase demonstrated the retention of the radiolabeled STP at the origin ( $R_f=0$ ), while free  $^{67}\text{Ga}$  migrates to a higher  $R_f$  (0.7) (figure 1).

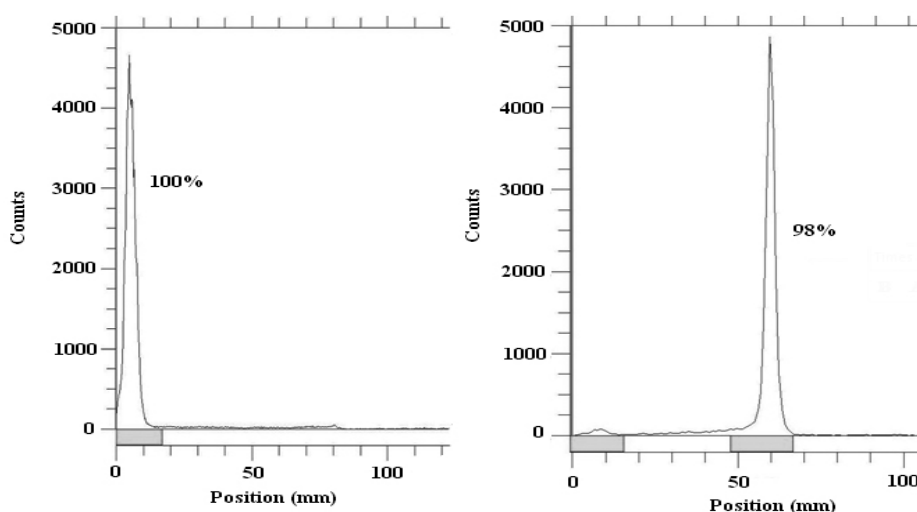


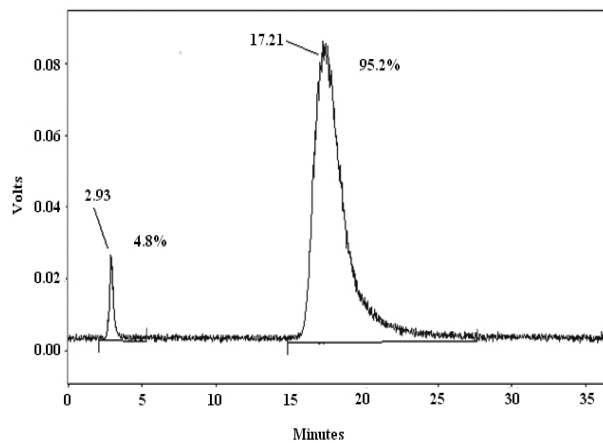
Figure 1. ITLC chromatograms for  $^{67}\text{Ga}$ -DTPA-STP (left) and starting  $^{67}\text{GaCl}_3$  used in the labeling reaction on polymer backed silica gel using 10 mM DTPA solution as eluent.

At this stage the mixture was tested by HPLC in order to determine the radiochemical purity before administration to rodent models for biodistribution studies. Figure 2 shows the HPLC chromatogram of the final solution. The fast eluting component (2.93 min) was shown to be a mixture of free  $^{67}\text{Ga}$  and  $^{67}\text{Ga}$ DTPA which were washed out on reverse phase stationary phase. Both compounds are ionic, so they are eluted at the same retention time.

### Stability of radiolabeled protein

The stability of the radiolabeled protein was determined after challenge with phosphate-buffered saline and serum. ITLC analysis showed that the proteins retained the radiolabel over a period of several hours, indicating that the  $^{67}\text{Ga}$ -protein chelate was of high affinity.

The results were confirmed by gel filtration chromatography. After incubation of [ $^{67}\text{Ga}$ ]-DTPA-STP with PBS for 2 h, almost all of the radioactivity eluted in the same position as [ $^{67}\text{Ga}$ ]-DTPA-STP; there was no evidence for large-scale release of free  $^{67}\text{Ga}$ . Similarly, gel filtration chromatography of  $^{67}\text{Ga}$ -DTPA-STP after a 2 h incubation with human serum showed that the radioactivity still eluted in the same position. Thus, there was no evidence for either degradation or



**Figure 2.** HPLC chromatogram of final radiolabeled solution on a reversed phase column using a gradient of acetate/citrate buffer.

transchelation of  $^{67}\text{Ga}$  to other serum proteins over a time period consistent with the normal blood clearance time of STP.

#### **Biodistribution studies in normal rats**

The distribution of [ $^{67}\text{Ga}$ ]-DTPA-STP among tissues were determined for untreated rats. A volume (0.1 ml) of final [ $^{67}\text{Ga}$ ]-DTPA-STP solution containing 4.4-5.2 MBq radioactivity (6 mg protein in 100 mL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a dose calibrator with a fixed geometry.

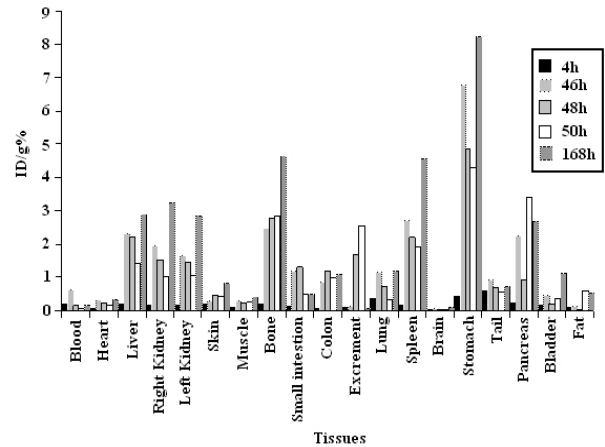
The animals were sacrificed by ether asphyxiation at selected times after injection (2, 4, 27, 48, 50 and 168 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, muscle, bone, colon, brain, stomach, lung, skin, pancreas, bladder, fat) and increments were weighed and their accumulated activities were determined with a  $\gamma$ -ray scintillation as a percent of the injected dose per gram of tissue (figure 3).

#### **Imaging of [ $^{67}\text{Ga}$ ]-STP in normal rats**

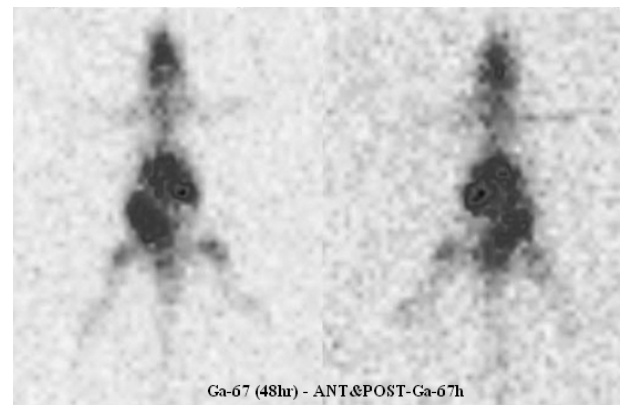
In order to compare the biodistribution pattern with thrombotic animals, the normal animals were administered with the tracer and the distribution was studied.

As shown in figure 4, the tracer is mostly accumulated in liver, kidney and GI

system and a pattern of bone accumulation is observed in the thigh bone, skull and slightly in vertebrae, as already demonstrated in the post-mortem studies.



**Figure 3.** Percentage of injected dose per gram (ID/g %) of  $^{67}\text{Ga}$ -DTPA-STP in normal rat tissues 2-168 h p.i.



**Figure 4.** SPECT images of  $^{67}\text{Ga}$ -DTPA-STP in normal rat 48 hours post injection.

## **DISCUSSION**

STP contains 35 lysine moieties that are suitable for conjugation using ccDTPA, so the least possible ccDTPA must be used. STP is a high positively charged protein with an isoelectric pH of 5.0. Total labeling and formulation of [ $^{67}\text{Ga}$ ]-DTPA-STP took about 60 minutes, with a radiochemical purity of more than 95% checked by ITLC and HPLC. A suitable specific activity product was formed via insertion of  $^{67}\text{Ga}$  cation. The radio-labeled complex was stable in human serum for at least 24 hours and no significant amount of free  $^{67}\text{Ga}$  as well as

$^{67}\text{Ga}$ -DTPA was observed. The final preparation was administered to normal rats and biodistribution of the radiopharmaceutical was checked 2 to 168 hours later. The tracer is washed out from blood and accumulated in liver in 4 hours, a percent of releases  $^{67}\text{Ga}$  is excreted from kidneys especially days after the injection due to metabolism of the tracer, the activities of muscle, skin, heart, fat are negligible, while significant bone uptake is observed. However one assumption is the interaction of positively charged of STP and hydroxyl apatite of bone tissue with negative charge. Although there are reports of STP biodistribution in the literature, but there is no reports for bone uptake.

In other radiolabeled STP compounds the biodistribution was affected by the dissociation of the tracers thus a real tracer was not reported, for instance  $^{99\text{m}}\text{Tc}$  compound was shown to be unstable and was not successful in biological evaluation (7). Other studies focused on the I-131 labeling of STP for biodistribution and pharmacokinetic studies, but deiodination activity of serum caused a high free iodine background (8, 9). The radiochemical purity of this tracer would allow appropriate molecular imaging of thrombi sites in cardiac diseases. [ $^{67}\text{Ga}$ ]-DTPA-STP can be a suitable probe for imaging of thrombosis in cardiovascular diseases. Use of Ga-68 labeled STP can also result in a superior labeled compound due to positron emission properties for PET studies as well as appropriate physical half live (68 minutes) compared to STP biological half life (80 minutes).

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