

Evaluation of Radiolabeled Streptokinase for Thrombosis Imaging

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ABSTRACT

Introduction: Cardiovascular disease is the major cause of morbidity and mortality in developing and developed countries. Rapid diagnosis of the thrombosis can be an essential step in management of the stroke.

Methods: In this work a recently developed radiolabeled streptokinase (STP) tracer was evaluated in an animal thrombotic model using SPECT imaging and biodistribution studies. Locally labeled [⁶⁷Ga]-Streptokinase was checked by ITLC, HPLC and SDS-PAGE experiments to check the tracer integrity and purity. The biodistribution studies were performed in thrombotic femoral vein of rats using tissue counting and preliminary SPECT studies, respectively (up to 2 h).

Results: [⁶⁷Ga]-Streptokinase prepared with suitable radiochemical purity (HPLC >95%, ITLC >99%) was administered to FeCl₃ induced thrombotic rats and the percentage of injected dose per gram of tissue (ID/g%) as well as the SPECT images demonstrated the good specific binding of the tracer in thrombotic clots located in heart and aorta 2 hours post injection.

Conclusion: [⁶⁷Ga]-DTPA-STP can be a suitable probe for imaging of thrombosis in cardiovascular diseases. Ga-68 labeled STP has the potential to be an alternative superior labeled compound due to positron emission properties for PET studies as well as appropriate physical half life.

Key words: Streptokinase, Gallium-67, Thrombosis, SPECT, Radiopharmaceuticals

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INTRODUCTION

Since plasminogen is present in the thrombus/embolus, it can be a suitable macromolecular target to be detected by STP. Detection of the magnitude and sites of thrombi in cardiovascular diseases is critical in the management of patients (1). Thus the preparation of a streptokinase (STP)-based radiotracer can be interesting in the diagnosis of thrombi.

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 (2) which finally leads to the hydrolysis of fibrinogen and other plasma proteins.

In molecular imaging where a molecular target is considered, a radiotracer could be designed, compounds such as ¹⁸F-FDG targeting hexokinase enzyme, ¹⁸F-FLT targeting DNA polymerase are very interesting examples (3).

A number of reports have described the radioactive labeling of STP to be used as a radiopharmaceutical in the detection of blood clots (4). Although STP has a strong affinity for thrombi, the radiolabeled enzymes yet reported have not been as successful in the localization of clots (2) as might be expected from theoretical considerations. This relative lack of success may in part result from unsatisfactory labeling methods used (5).

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 this compound was not successful in biological evaluation (6). 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 (7). The catabolic pathways for STP was studied using ¹²⁵I-STP in one study with evaluation of biodistribution in rats but no SPECT imaging was performed (8). The research for early detection of thrombosis is continuing using radiolabeled compounds

as, MRI targeting contrast agents (9) and also various ultrasound modalities (10) however none of them have focused on the use of trivalent radionuclides especially Ga radionuclides. Ga is so important since the Ga-68 isotope is a splendid radioisotope in today's nuclear medicine already used in the production of receptor specific imaging agents well-known to nuclear medicine society (11).

In the last 37 years, no Tc-99m labeled tracer based on streptokinase is produced however, Tc-99m is used in radiolabeling of antibodies and fragments for the targeting of other thrombosis factors (12).

Based on our recent study in production of ⁶⁷Ga-labeled STP (13), radiolabeled enzyme was injected intravenously to thrombotic rats and tissue distribution of the tracer was checked using post-mortem studies and SPECT method.

METHODS

Production of ⁶⁷Ga was performed at Agricultural, Medical and Industrial Research School (AMIRS) 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 N₂. STP was a pharmaceutical sample (STREPTASE®) purchased from Aventis Co. According to the drug in-let and manufacturer website, none of the excipients used in the pharmaceutical sample contained interfering agents such as amines and metals. Thus we used the pharmaceutical sample directly 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 x 4.6 mm, Whatman, NJ, USA.

Calculations were based on the 184 keV peak for ⁶⁷Ga. 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.

Preparation of ⁶⁷Ga-DTPA-STP

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated with STP using a small modification of the well-known cyclic anhydride method (14) at a 1:1 molar ratio of ccDTPA and commercially available Streptase for 60 min. Conjugation mixture was then passed through a Sephadex G-50 column and eluted fractions checked for the presence of protein using visible folin-phenol colorimetric assay. The fraction containing the highest protein concentration was added to a conical vial containing 37-40 MBq of ⁶⁷Ga-chloride (in 0.2M HCl) and the mixture was incubated at room temperature for 30 minutes followed by ITLC/HPLC checks for radiochemical purity. The stability of the tracer in final product and presence of human serum at 37°C were also studied (Jalilian et al, 2008, unpublished data).

SDS-Polyacrylamide Gel Electrophoresis

The starting commercial material, conjugated and radiolabeled protein samples were analyzed for integrity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled protein was evaluated with and without reduction by 2-mercaptoethanol. Approximately 200,000

cpm of each preparation was applied per lane and the 4–20% polyacrylamide were run according to the method of Laemmli (15).

Induction of left femoral vein thrombosis in experimental rats

This procedure was performed according to reported method using ferric chloride solution with modifications (16). Briefly, a normal healthy male rat was anesthetized by IP injection of a ketamine/xylazine (3:7, v/v, 1 ml) mixture. The animal was ready for surgery 25 minutes post injection. Left femoral skin was cut using a razor and left femoral vein was isolated and fixed for the treatment. A 5×5 mm Whatman paper soaked in 60% FeCl₃ solution (5 ml), was wrapped around the isolated vein. The paper was treated with FeCl₃ solution every 10 min using a dropper. After 30 minutes the brown stain on the Whatman paper demonstrated the formation of thrombosis at the paper wrapped area of the vein and the surgery was terminated by stitching the cut. The whole process was performed in a sterile HEPA cabinet unit.

Biodistribution of ⁶⁷Ga-DTPA-STP in thrombosis-bearing rats

A volume (50 µl) of final ⁶⁷Ga-DTPA-STP solution containing 50±2 µCi radioactivity was injected intravenously to rats through the 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 injected dose (calculated from urea under the curve of 184 keV peak, efficacy and branching ratio) per gram using an HPGe detector.

SPECT imaging of ⁶⁷Ga-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×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 64×64 matrix.

RESULTS AND DISCUSSION

Preparation of ⁶⁷Ga-DTPA-STP

The labeling yield of ⁶⁷Ga-DTPA-STP has been studied in the wide range of STP/DTPA ratios in order to optimize the process and to improve ⁶⁷Ga-DTPA-STP performance in vitro.

At this stage the mixture was tested by HPLC in order to determine the radiochemical purity before administration to rodent models for biodistribution studies. The fast eluting component (2.93 min) was shown to be a mixture of free ⁶⁷Ga and ⁶⁷Ga-DTPA which were washed out on reverse phase stationary phase. Both compounds are ionic, so they are eluted at the same retention time (curves not shown).

Protein integrity test using SDS-Polyacrylamide Gel Electrophoresis

In order to demonstrate the integrity of the protein after conjugation and radiolabeling gel electrophoresis was performed on the SDS PAGE gels using 16% bisacrylamide gel.

The loaded samples were STP pharmaceutical sample, DTPA-STP and radiolabeled protein samples an hour and also a week after the experiment while kept in the fridge. The 3 samples were showed to have similar pattern of migration in the gel electrophoresis as shown in Figure 1.

Biodistribution studies in thrombosis-bearing rats

Tracer administration and tissue biodistribution studies in treated rats were performed (as mentioned above). Biodistribution data was obtained (not shown), however with respect to biological half life of STP a comparative demonstration has been presented in normal and thrombosis-bearing rats 2 hours post

injection of tracer in 18 different tissues (Figure 2).

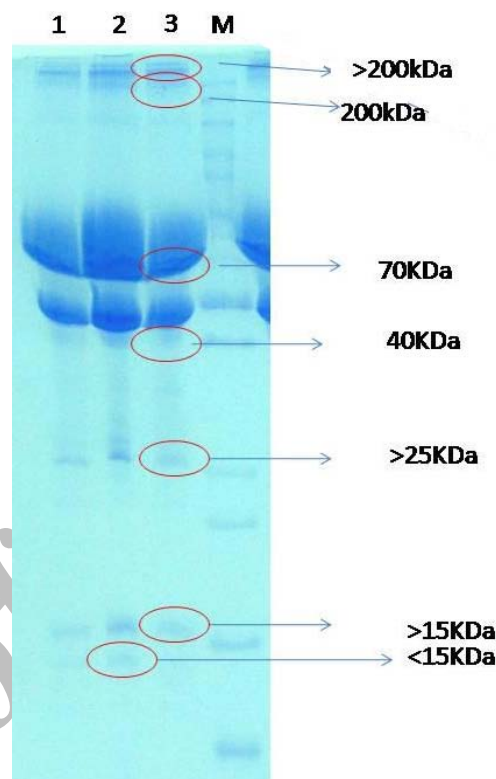


Figure 1. SDS-PAGE lane patterns for STP pharmaceutical sample (1), DTPA-STP conjugate (2) and radiolabeled protein (3) and a protein standard ladder (M) samples.

Due to the formation of vein thrombotic clots in blood stream in FeCl₃ induced thrombosis, much higher amounts of tracer remains in blood than normal rats (10-15 times), this retention in circulation causes a decrease in radioactivity content in many other organs compared to normal animal distribution such as liver (2 times), bone (8 times), spleen (3 times) and pancreas (5-6 times).

It has been shown that most of the femur fractures and thrombi production in lower limbs lead to cardiac arrest and lung embolism. The biodistribution studies performed 2 hours post injection, however it is very possible that femoral accumulation

could be detected much earlier at first few minutes. On the other hand the formation of clots results in the increased accumulation of the radioactivity in blood (8-10 times), kidneys (2-2.5 times), lung (6-7 times). A logical explanation for these observations would be more availability of the tracer in blood resulting in more urinary excretion of the tracer and its low molecular weight metabolites. The alveolar retention of the clots is significant as well due to the migration of the clots into lung circulation similar to thromboemolism in lungs. The change in activity distribution pattern is not significant in other tissues such as skin, muscle, brain, intestine, colon and fat, which are not major organs of accumulation.

Imaging of [⁶⁷Ga]-STP in thrombotic rats

As shown in Figure 3, the best time period for scanning showed to be one hour to 2 hours post injection, since as mentioned in the text the circulation time for the enzyme would be 80 minutes and after that the enzymatic degradation of the protein takes place in the circulation. Thirty minutes post injection of the tracer no significant activity was observed while an activity increase in chest area is observed. After an hour heart and chest area demonstrates significant accumulation which reaches to its maximum after 1.5 hour, after 90 minutes the enzymatic degradation starts and the chest activity decreases, but still is significant.

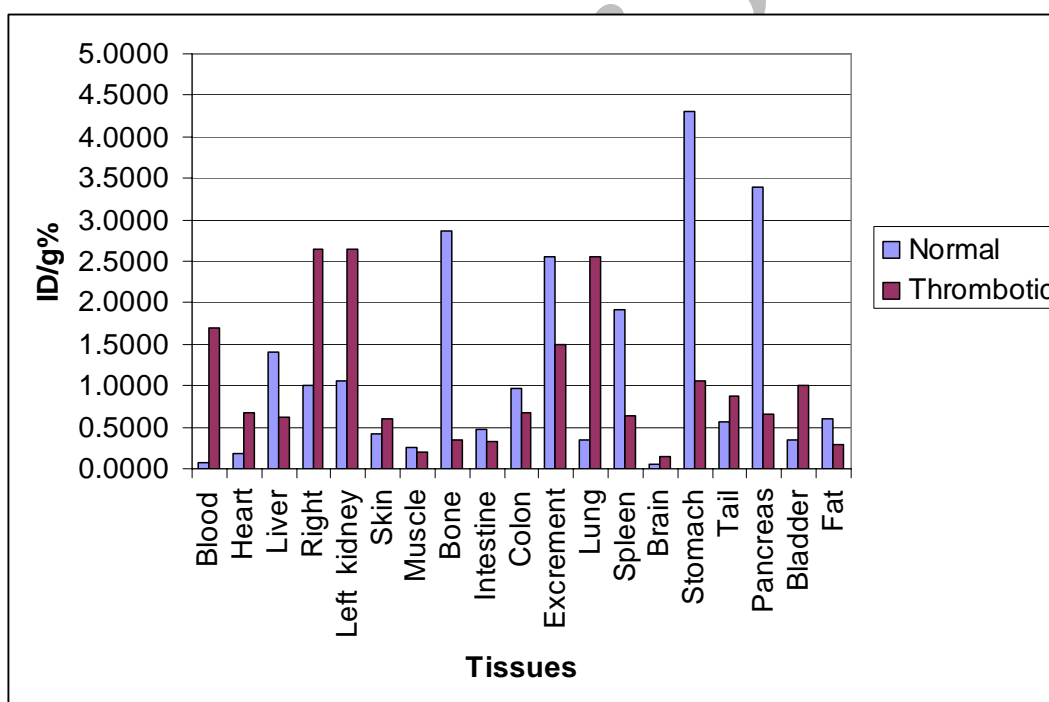


Figure 2. The biodistribution of the tracer among the treated and untreated animals 2 hours post injection.

At the first glance one may observe that 30 minutes after injection, bladder is a major accumulation site, while not much activity is present in the kidneys which usually must have activity in connection to bladder. Kidneys must be the first organs before the bladder to be filled with the tracer. A major

reason for the activity accumulation in that region is possibly the movement of radiolabeled clot along the ascending veins, since in 1-2 hour, activity is located in heart. The biopsy showed the accumulation of a big clot in the heart (1.5h post injection).

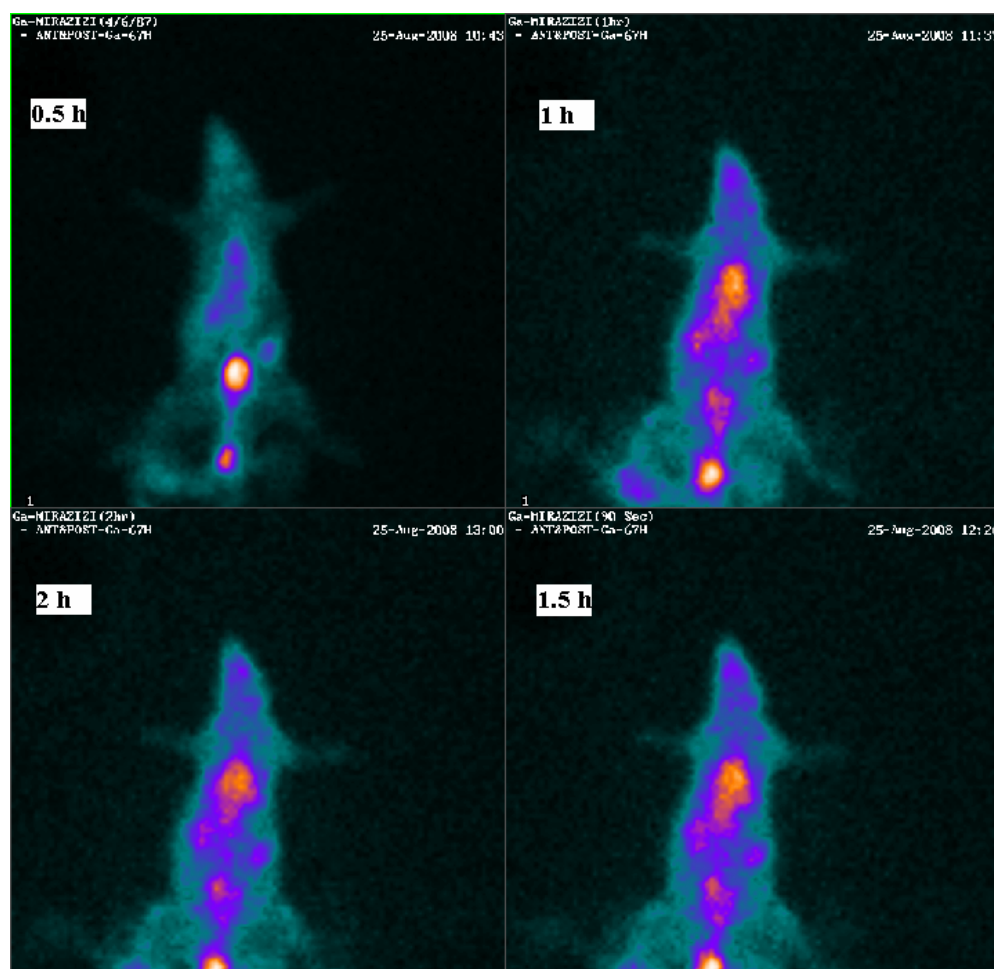


Figure 3. SPECT images of ⁶⁷Ga-DTPA-STP in thrombotic rat 0.5-2 hours post injection

CONCLUSION

Total labeling and formulation of [⁶⁷Ga]-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 ⁶⁷Ga cation. The radio-labeled complex was stable in human serum for at least 24 hours and no significant amount of free ⁶⁷Ga as well as ⁶⁷Ga-DTPA was observed. The final preparation was administrated to normal rats and biodistribution of the radiopharmaceutical

was checked 2 to 168 hours later. The tracer administration in FeCl₃-induced thrombotic animals demonstrated increased blood retention of the tracer (10-15 times), due to the initiation of thrombolytic activity of the tracer on the clot particles, as well as kidneys (2-2.5 times), lung (6-7 times) and decrease in accumulation of liver (2 times), bone (8 times), spleen (3 times) and pancreas (5-6 times) compared to the tracer biodistribution in normal animals. According to our knowledge this study presents the first successful streptokinase-based macromolecular imaging of

thrombosis in a living experimental animal using preliminary SPECT studies. [⁶⁷Ga]-DTPA-STP can be a suitable probe for imaging of thrombosis in cardiovascular diseases. Use of Ga-68 labeled STP can result in a superior labeled compound due to positron emission properties for PET studies as well as appropriate physical half life (68 minutes) compared to STP biological half life (80 minutes).

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