RESEARCH ARTICLE

IMAGING OF DEEP VENOUS THROMBOSIS USING RADIOACTIVE LABELED TIROFIBAN: ANIMAL MODEL EVALUATION

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Abstract:
Imaging of acute thrombus, especially the very prevalent condition of acute deep vein thrombosis is usually relied on conventional imaging techniques utilizing either ultrasonography or contrast venography. The former procedure is limited by accuracy and the latter by technical considerations. Recent advances in the understanding of the pathogenesis of acute clot at the molecular level have suggested new opportunities for detection of the acute thrombotic process based on the biomolecular behavior of components of the clotting process including the formed element of the blood, the platelet.
Thus, development of radionlabelled small peptide or peptidomimetic ligands that can bind platelets and their specific expressed receptor have been suggested as a new approach to detect clot location and, more essentially, determine the age and morphology of the evolving thrombus. This new approach has focused on the use of aseries of radionlabelled platelet GPIIb/IIIa receptor antagonists. Tirofiban N-(butylsulfonyl)-4-O-(4-(4-piperidyl)-L-tyrosine is a non-peptide tyrosine derivate. The aim of thestudy was to introduce radioactive labelled tirofiban as a specific imaging agent foracute DVT. The labeling was performed with Technetium-99 in the presence of a stannousreducing agent. The labelled preparation showed a fast blood clearance in the normal rat model (without induced thrombosis). More than 80% of the injected dose was eliminated from the circulation in the first hour after injection. Biodistribution and visualization of the labelled molecule was carried out using anexperimental model of thrombosis in the male Wistar rat. Planar images were obtained 30 min and 60 min after application of \(2 \times 10^5\) imp/min 99mTechnetium-tirofiban, in the rat’s tail vein. Sensitivity and specificity were determined using the ratio ‘left leg positive for DVT’ and ‘right leg negative for DVT’. The obtained ratio was 1.54 after 30 min and 5.04 after 60 min. These values were considered as positive in the detection of acute DVT. The high DVT uptake show that radiolabelled tirofiban in the introduced rat model can be the promising agent for imaging of deep venous thrombosis.

Key Words: Radionuclide labeling, Imaging, Deep Venous Thrombosis; 99mTc-Tirofiban, Rat model

Introduction
Venous thromboembolism is a complex vascular disease with a multifactorial pathogenesis that results in significant morbidity and mortality. The first and more common manifestation is deep venous thrombosis (DVT), which usually arises in the deep veins of the calf and spreads upwards. Pulmonary embolism, the second and more serious manifestation, occurs as a complication of DVT proximal to the deep calf veins [1].
Because the incidence of deep venous thrombosis (DVT) and pulmonary embolism (PE), in high-risk populations, in hospitalized patients, as well as in the general population remains is still remarkably high, yet an accurate diagnosis of DVT and PE continues to be unreliable [2,3].
All available imaging procedures (duplex ultrasound, magnetic resonance and contrast venography as a standard test for validating new diagnostic procedures) do not reflect the metabolic activity of the clot and therefore they may overestimate the presence of active clots [4]. Only nuclear medical examinations can provide an image that includes information on thrombus formation [25]. The development of radioactive agents for scintigraphic imaging of DVT and PE is centered on the use of 99mTc-labeled peptides or peptidomimetics specific for resting or activated platelets [4-9]. They are smaller in size, easier to produce than monoclonal antibodies, expected to clear more rapidly from circulation than radiolabeled proteins, are less likely to induce any immunologic reaction, and yet, in most cases, they enjoy receptor specificity and binding constants as high as those of the monoclonal antibodies.
99mTechnetium is radionuclide inexpensive, easy to obtain worldwide, and decays with emission of \( \gamma \)-rays (140 keV, 90\%) that can be efficiently detected by \( \gamma \)-cameras. Its half-life (6 h) is long enough to perform examinations before excessive radioactive decay has occurred, yet not too long to persist in the body long after the examinations have been performed.

Our approach was focused on the use of a series of radiolabelled platelet GPIIb/IIIa receptor antagonists [6, 10]. The GPIIb/IIIa receptors are expressed on the membrane surface of activated platelets and play an integral role in platelet aggregation and thrombus formation [10]. Initial actions in thrombus formation frequently involve the activation of platelets by thrombogenic conditions and their subsequent aggregation. Platelet aggregation is mediated by fibrinogen, which binds via the Arg-Gly-Asp (RGD) sequence through GPIIb/IIIa receptor expressed on activated platelets. Since the GPIIb/IIIa receptors are expressed only on the membrane surface of activated platelets, with 50 000–90 000 GPIIb/IIIa binding sites per platelet [2], the GPIIb/IIIa receptor makes an excellent target for the development of an imaging agent that binds with high specificity to activated rather than to unactivated platelets. They would be differentially incorporated in the thrombus (activated platelets) and the circulating platelets (resting or relatively less activated) [11, 12]. These molecules represent glycoprotein (GPIIb/IIIa) receptor antagonists and they act as true biochemical markers of active thrombosis [10, 11].

One peptide, 99mTc-P280 (5), is approved by the Food and Drug Administration under the tradename AcuTect (99mTc-P280; Diatide, Inc.) (8), which can image acute thrombi but not old clots or PE.

The goal of our work was to use the small non-peptide derivate or peptidomimetic ligands with high specificity for the GPIIb/IIIa receptor and incorporate a convenient radionuclide for imaging purposes was initiated from the already existing data presented using labelled peptides and GPIIb/IIIa receptor antagonists in the diagnosis of acute DVT. One promising GPIIb/IIIa receptor antagonist is tirofiban (Aggrastat\textsuperscript{TM}, Merck, Inc.), a non-peptideteryosine derivate that inhibits fibrinogen binding [14] Tirofiban hydrochloride monohydrate is chemically described as N-(butylsulfonyl)-O-(4-[4-piperidinyl]butyl)-L-tyrosine monohydrochloride monohydrate Fig. 1. The empirical formula of Tirofiban is C\textsubscript{22}H\textsubscript{36}N\textsubscript{2}O\textsubscript{5}S·HCl·H\textsubscript{2}O and molecular weight of 495.08 [2,3].

It is useful in combination with heparin and aspirin in the management of patients with unstable angina or non-Q-wave myocardial infarction, including patients who may subsequently undergo percutaneous transluminal coronary angioplasty (PTCA) [13, 14].

Adjunctive therapy with a GP IIb/IIIa-receptor inhibitor can reduce the incidence of cardiac ischemic events, including subsequent myocardial infarction (MI) and death, in patients with non-ST-segment-elevation acute coronary syndromes [5-6].

When Tirofiban is prescribed and administered to the patient according to the recommended treatment, >90\% inhibition is attained by the end of the 30-minute infusion. Platelet aggregation inhibition is reversible following cessation of the infusion of Tirofiban. Tirofiban with a half-life of approximately 2 hours [15] is not strongly bound to plasma protein, and protein binding is concentration-independent in the range of 0.01–25 µg mL\textsuperscript{-1}. The unbound fraction in human plasma is 35\%. The distribution volume of Tirofiban in the steady state is about 30 liters.

To obtain the concentration of tirofiban appropriate for labeling as potential imaging radiopharmaceuticals and in the same time not receive therapeutical effect, we have labeled tirofiban with 99mTcTechnetium and evaluated it in vitro and in rats, with experimental venous thrombosis established and modified afterwards, according to the needs of a particular line of research [16, 17].

**Material and Methods**

**Materials**

**Tirofiban**

The structure of tirofiban (tirofiban hydrochloride monohydrate) N-(butylsulfonyl)-O-[4-(4-piperidinyl)butyl]-L-tyrosine monohydrochloride monohydrate is shown in Fig. 1. Tirofiban hydrochloride monohydrate is a white to off-white, non-hygroscopic, free flowing powder, with a molecular weight of 495.08. It is very slightly soluble in water [2,3].

Isotope- 99mTechnetium
99mTechnetium-pertechnetate was obtained from a commercial 99Mo/99mTc generator (10 GBq) (Schering /CIS-biointernational).

**Radiolabelling**

Labelling of tirofiban with 99mTechnetium

Tirofiban was labelled using the method of direct labelling under nitrogen. The kit freeze dried formulation contains a mixture of tirofiban(20nmol) dissolved in buffer (ethanol 96%/PBS 0.01M, pH7.3, 1:3 vol./vol.) and stannous chloride (10nmol) as a reducing agent. Sodium (99mTc) perthechnetate(specific activity 740–4500 MBq/mL) containing 100 MBq/mL was added and the reaction mixture incubated for 15 min at room temperature. The quality control was done by paper chromatography and instant thin layer chromatography (ITLC) using two solvents — 95% acetone and saline. The percentage labelled yield was recorded by gamma scanner (VeenstraInstrumenten B.V. VCS-103 V1.06).

**Blood clearance**

99mTechnetium-tirofiban was injected intravenously into rats. Blood samples were drawn from the previously prepared carotid vein using a sterile syringe at 5, 15, 30, 45 min and 1, 2, 4, 6 and 24 h after injection. All samples were of the same volume and their radioactivity measured in a gamma counter and compared with a standard.

In vitro platelet binding

The binding study was performed with rat and human platelets isolated and treated according to protocol for platelet labelling (TROMBO-SCINT). Platelets were incubated for 30 min with tirofiban radiolabelled with 99mTechnetium. The percentage of binding was measured after one and two washing steps.

**Animal studies**

Male Wistar rats weighing 220–250 g were used throughout the study, anaesthetized by intraperitoneal injection of water solution of Nesdonal(concentration 20 mg/kg body weight).

Experimental animal model [18, 19]

Venous thrombosis was induced by ligation of the femoral vein in rats whose blood was made hypercoagulable by intravenous administration of tissue thrombin [17]. For the thrombosis model, the body temperature of the rats was maintained at 37°C. In brief, a short incision was made in the skin and subcutaneous tissue in the left groin region and the femoral neurovascular sheath was gently exposed [20]. An approximately 10 mm long portion of the left femoral vein, distal to the inguinal ligament was isolated by rubbing it against the blade of a pair of forceps and this segment was collapsed. The collapsed segment of femoral vein between the clamps was traumatized by striking and the twenty units of thrombin (in 0.2 mL saline) were injected into the segment with a needle. A semiconstricting ligature was placed down stream to prevent the clot from slipping away.

Imaging

The thrombi developed in the rats were visualized using tirofiban radiolabelled with 99mTechnetium. Planar images were obtained 30 min and 60 min after application of 2 × 10⁶ counts/min in 50–100 μL 99mTechnetium-tirofiban in the rat’s tail vein. Syringes should be measured before and after injection in order to determine accurately the radioactivity of material injected. The sensitivity and specificity of the radiopharmaceuticals were determined using the ratio ‘left leg positive for DVT’ and ‘right leg negative for DVT’ using the ROI technique.

**Biodistribution**

The biodistribution studies were carried out using the same experimental model of thrombosis in male Wistar rat and by injection of the radiolabelled products. After the desired time period had elapsed, the animals were sacrificed and the samples of organ of interest (heart, lung, liver, spleen, kidney, trombotic and normal tissue) were collected and placed in pre-weighed counting tubes. These were then counted in a gamma counter together with a standard prepared from a known dilution of the injected material (preferably prepared at the time of injection). The total activity injected into each rat was determined and the activity remaining in the tail subtracted. The uptake of the labelled products in each tissue was calculated and the specific uptake of the products quantified using ratio ‘left leg positive for DVT’ and ‘right leg negative for DVT’.

**Results**

This study used a rat model of DVT to evaluate radiolabelled tirofiban, platelets GPIIb/IIIa antagonist for its potential use in the detection of rapidly growing venous thrombi. Two different methods of labelling were selected using 99mTechnetium as radioisotope.

Labelling of tirofiban with 99mTechnetium

The specific activity of the radiolabelled product was 9.2 × 1018 to 1.0 × 1019 counts/min/mol. The percentage of obtained complex after labelling was more than 95% (in acetone), more than 85% (in saline), with the free pertechnetate (99mTcO4-) less than 5% (Fig. 2).

The labelled product was stable without changing the percentage of labelling after 2 h at room temperature.
After determination of radiochemical purity, it was concluded that the product could be tested for in vitro binding using normal platelets from humans and rats.

In vitro platelet binding
The binding study made for in vitro stability of platelets showed that $^{99m}$Tc labelled tirofiban has high percent of labeling for both, normal platelets from humans and rats 69.30% and 68.79% (Fig. 3). The stability of binding did not change after two washing steps, 58.63% and 57.58%.

Blood clearance
The obtained results showed that more than 80% of injected dose from the labelled preparation of tirofiban was eliminated from the circulation in the first hour after injection (Fig. 4).

Imaging studies
Whole body images of distribution of $^{99m}$Technetium-tirofiban in normal and experimental induced thrombosis in rats 30 and 60 min after administration are presented in Fig. 5. At 30 min and 60 min, post-injection, experimental induced thromboses are visualized compared with the normal rat distribution of the tracer. Biological data demonstrated $^{99m}$Technetium-tirofiban accumulation in the thyroid and in the liver. This accumulation could be related to the normal distribution of circulated platelets in the liver and spleen.

Biodistribution studies
Biological distribution data for the labelled $^{99m}$Technetium-tirofiban at different times after IV administration are presented in Fig. 6. These biodistribution studies demonstrated some different behavior. Results obtained from the radioactivity distribution of $^{99m}$Technetium-tirofiban at 30 min and 60 min after IV administration clearly present an accumulation in the critical organs. This is in accordance with the presence of destroyed platelets carrying radioactivity tirofiban. However, the radiolabelled product was rapidly cleared from the circulation as represented by a low percentage of radioactivity in the heart and lung. The obtained ratio ‘left leg positive for DVT’ and ‘right leg negative for DVT’ was 1.54 after 30 min and 5.04 after 60 min for the tirofiban labelled with technetium (Fig. 7). These values were considered as positive in the detection of acute DVT.

FIGURES

![Chemical structure of Tirofiban hydrochloride.](image)
a.

![Graph showing Tc-99m isotope and acquisition data](chart.png)

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b. ITLC radiochromatograms of 99mTc-tirofiban in (a) 95% acetone (b) saline.
Figure 3. In vitro binding of rat and human platelets isolated and treated with radiolabelled tirofiban.

Figure 4. Blood clearance of radiolabelled tirofiban - 99mTc-tirofiban in normal rat
Figure 5. Gamma camera images after IV injection of 99mTc-tirofiban in rats
DISCUSSION

Blood coagulation has long been considered an important factor in the pathogenesis of venous thrombosis. The relative contribution of stasis, altered coagulability of the blood, vessel wall damage and circulation of leucocytes or platelets to the pathogenesis of venous thrombosis remains in dispute. On the other hand, it is known that a hypercoagulable state contributes significantly to the thrombotic process. Animal models based on these activation mechanisms have been designed as a means of studying potential imaging agents for diagnosis of acute DVT. The venous stasis thrombosis model consists of inducing blood stasis in the femoral vein of rats after prior injection of a procoagulant. A radionuclide imaging agent that binds to platelets being incorporated into an active thrombus but one which, if not bound, clears rapidly from the blood would have great potential for acute DVT detection. Platelets expressed from the cell surface of GPIIb/IIIa receptors undergo the conformational change that makes them available for binding fibrinogen. Cross-linkage of activated platelets by the bivalent fibrinogen molecule to form a hemostatic plug is the primary episode of thrombosis. An imaging agent, ideally labelled with 99mTc and capable of binding actively and specifically to the GPIIb/IIIa receptor on activated platelets, would give images of active or acute venous thrombosis.
The aim of the study was to evaluate tirofiban as a specific imaging agent to GPIIb/IIIa receptors in the case of experimentally induced acute DVT in the rat experimental model. Radionuclide imaging offers considerable potential as a successful diagnostic agent which would address a few important criteria, i.e., rapid, non-invasive, cost effective and accurate. The published data [7, 13, 19] indicate that radiolabelled tirofiban binds preferentially to GPIIb/IIIa receptors on activated platelets and can differentiate acute DVT from chronic venous thrombosis. Radiolabelled tirofiban is a functional rather than anatomical imaging modality. The use of radiotracers allows an understanding of the bioavailability process, biodistribution and kinetics of any molecule labelled with an isotope, a procedure which does not alter the molecule’s biological properties. In the current work, technetium (to evaluate tirofiban as a specific imaging agent to GPIIb/IIIa receptor in the case of experimental induced acute DVT in the rat experimental model) was chosen as a radiotracer for biodistribution and imaging studies in the experimental thrombosis induced in rats for its favourable radiation and physical characteristics, ready availability, possibility of labeling and cost.

Commercially available technetium–apcitide (AcuTect), previously known as 99mTc-P280, which binds to the GPIIb/IIIa receptor, is the first imaging agent used in the clinical studies to detect acute DVT [21, 22]. In this study, although the 99mTc–apcitide images obtained at 2 h after tracer injection show the greatest overall accuracy in comparison with earlier images, combined analysis of image sets from at least two time points (30 min and 60 min) provides greater accuracy in the detection of acute DVT in the patients. These results correlate with data obtained from the animal model. 99mTc Technetium-tirofiban is accurate in the detection of acute DVT, especially 60 min after application. The results obtained from the animal design experimental studies showed that the ratios ‘left leg positive for DVT’ and ‘right leg negative for DVT’ for the radiolabelled preparation of tirofiban are well within the range of that expected for a successful imaging agent.

Conclusion
Animal models provide convenient screening tools for radiolabelled products before a new radiopharmaceutical is further developed in clinical trials [23]. The tirofiban labelled with 99mTc Technetium was found that have good thrombus uptake in vivo experiments. Obtained data indicate that radiolabelled tirofiban binds preferentially to GPIIb/IIIa receptors on activated platelets and can differentiate acute DVT from chronic venous thrombosis. These results can be helpful in the further clinical investigation of patients with acute DVT.

References