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RESEARCH ARTICLE

IMAGINGOF DEEP VENOUS THROMBOSISUSINGRADIOACTIVE 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 radiolabelled small peptide or peptidomimetic ligandsthat 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 andmorphology of the evolving thrombus. This new approach has focused on the use of aseries of radiolabelled 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 obtained30 min and 60 min after application of 2×10^6 imp/min 99mTechnetium-tirofiban, in the rat's tail vein. Sensitivityand specificity were determined using the ratio 'left leg positive for DVT' and 'right legnegative 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 acuteDVT. The highDVT 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 multifactorialpathogenesis that results in significant morbidity and mortality.Thefirst and more common manifestation is deep venous thrombosis (DVT), whichusually 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 metabolicactivity of the clot and therefore they may overestimate the presence of activeclots [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 centeredon 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 rapidlyfrom 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 thoseof the monoclonal antibodies.

99mTechnetium is radionuclide inexpensive, easy to obtain worldwide, and decays withemission of γ-rays (140 keV, 90%) that can be efficiently detected by γ-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 radiolabelledplatelet GPIIb/IIIa receptor antagonists [6, 10]. The GPIIb/IIIa receptors areexpressed on the membrane surface of activated platelets and play an integral role in platelet aggregation and thrombus formation [10]. Initial actions inthrombus formation frequently involve the activation of platelets by thrombogenicconditions and their subsequent aggregation. Platelet aggregation is mediated by fibrinogen, which binds via the Arg-Gly-Asp (RGD) sequence tothe GPIIb/IIIa receptor expressed on activated platelets. Since the GPIIb/IIIareceptors are expressed only on the membrane surface of activated platelets, with 50 000–90 000 GPIIb/IIIa binding sites per platelet [2], the GPIIb/IIIareceptor makes an excellent target for the development of an imaging agent than bound with high specificity to activated rather than to unactivatedplatelets. They would be differentially incorporated in the thrombus (activatedplatelets) and the circulating platelets (resting or relatively less activated) [11, 12].

These molecules represent glycoprotein (GPIIb/IIIa) receptorantagonists and they act as true biochemical markers of active thrombosis[10, 11].

Onepeptide, 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 oldclots or PE.

The goal of our work was to use the small non-peptide derivate or peptidomimeticligands with high specificity for the GPIIb/IIIa receptor and incorporatea convenient radionuclide for imaging purposes was initiated from the alreadyexisting data presented using labelled peptides and GPIIb/IIIa receptorantagonists in the diagnosis of acute DVT. One promising GPIIb/IIIa receptorantagonist is tirofiban (AggrastatTM, Merck, Inc.,), non-peptidetyrosine 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 $C_{22}H_{36}N_2O_5S{\cdot}HCl{\cdot}H_2O$ 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 (PTCA0 [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 administrated 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^{-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 99mTechnetium 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 monohydrochloridemonohydrate is shown in Fig. 1. Tirofiban hydrochloride monohydrate is a white to off-white, non-hygroscopic, free flowing powder, with a molecularweight 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 intravenouslyinto rats. Blood samples were drawn from the previously prepared carotid veinusing 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 agamma counter and compared with a standard.

In vitro platelet binding

The binding study was performed with rat and human platelets isolatedand treated according 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 ligature of the femoral vein in rats whose blood was made hypercoagulable by intravenous administration oftissue thrombin [17]. For the thrombosis model, the body temperature of the rats was maintained at 37^0C. In brief, a short incision was made in the skinand subcutaneous tissue in the left groin region and the femoral neurovascularsheath was gently exposed [20]. An approximately 10 mm long portion of the left femoral vein, distal to the inguinal ligament was isolated by rubbing itagainst 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 radiolabelledwith 99mTechnetium. Planar images were obtained 30 min and 60 min afterapplication of 2×10^6 counts/min in 50–100 μL 99mTechnetium-tirofiban in the rat'stail vein. Syringes should be measured before and after injection in order todetermine accurately the radioactivity of material injected. The sensitivity andspecificity of the radiopharmaceuticals were determined using the ratio 'left legpositive for DVT' and 'right leg negative for DVT' using the ROI technique.

Biodistribution

The biodistribution studies were carried out using the same experimentalmodel of thrombosis in male Wistar rat and by injection of the radiolabelledproducts. After the desired time period had elapsed, the animals were sacrified and the samples of organ of interest (heart, lung, liver, spleen, kidney,trombotic and normal tissue) were collected and placed in pre-weighedcounting tubes. These were then counted in a gamma counter together with astandard prepared from a known dilution of the injected material (preferablyprepared at the time of injection). The total activity injected into each rat wasdetermined and the activity remaining in the tail substracted. The uptake of the labelled products in each tissue was calculated and the specific uptake of theproducts quantified using ratio 'left leg positive for DVT' and 'right legnegative 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 oflabelling after 2 h at room temperature.

After determination of radiochemical purity, it was concluded that theproduct 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 that99mTc 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 thelabelled preparation of tirofiban was eliminated from the circulation in the first hour after injection (Fig. 4).

Imaging studies

Whole body images of distribution of 99mTechnetium-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 visualizedcompared with the normal rat distribution of the tracer. Biological data demonstrated 99mTechnetium-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 thespleen.

Biodistribution studies

Biological distribution data for the labelled 99mTechnetium-tirofiban at differenttimes after IV administration are presented in Fig. 6. These biodistribution studies demonstrated somedifferent behavior. Results obtained from the radioactivity distribution of 99mTechnetium-tirofiban at 30 min and 60 min after IV administration clearly present a the accumulation in the critical organs. This isin accordance with the presence of destroyed platelets carrying radioactivetirofiban. 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 forDVT’ was 1.54 after 30 min and 5.04 after 60 min for thetirofiban labelled with technetium (Fig. 7). These values were considered as positive in the detection of acute DVT.

FIGURES

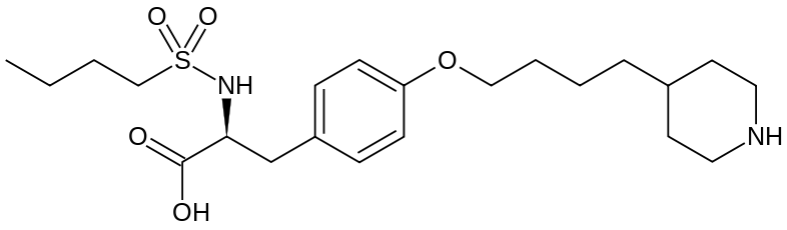
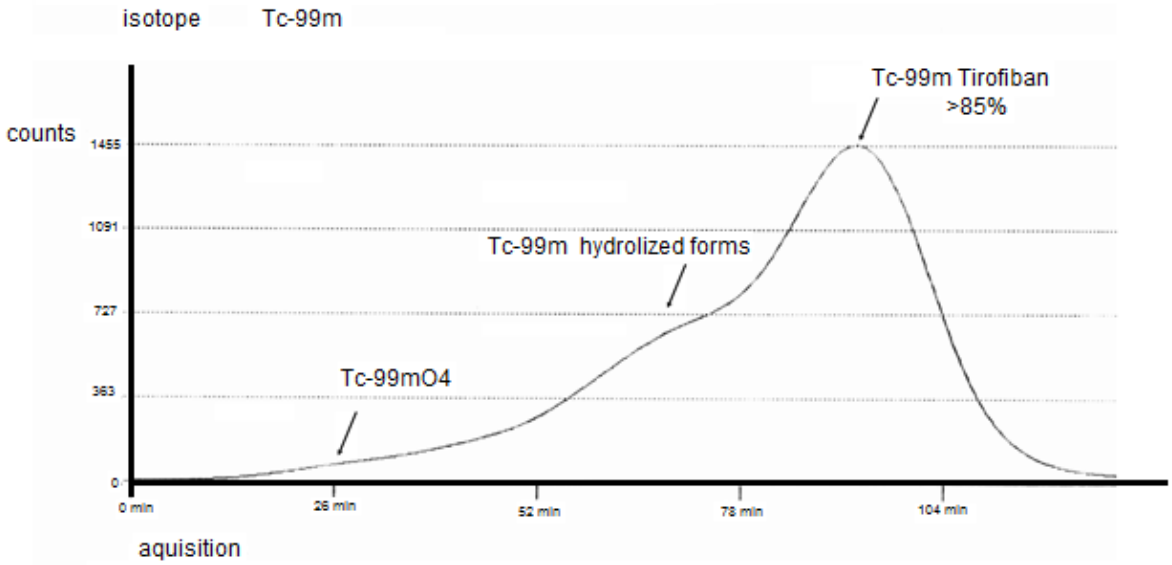


Figure 1. Chemical structure of Tirofiban hydrochloride.

a.



Real time:	65.00s	Live time:	61.29s (5.7 % Dead time)
Length:	130.0 mm		
Scan Window:	135.0 - 145.0 keV	Background Sub:	0 counts per channel
Counts:	49706	Peak:	1455 counts @ 93.2 mm
Detector			
LLDT:	3.0 %	High Tension:	600 V
LLD:	10.0 %	Gain:	50.119 x
ULD:	100.0 %		
Zero:	0.0 %	Conversion Gain:	2048

b.

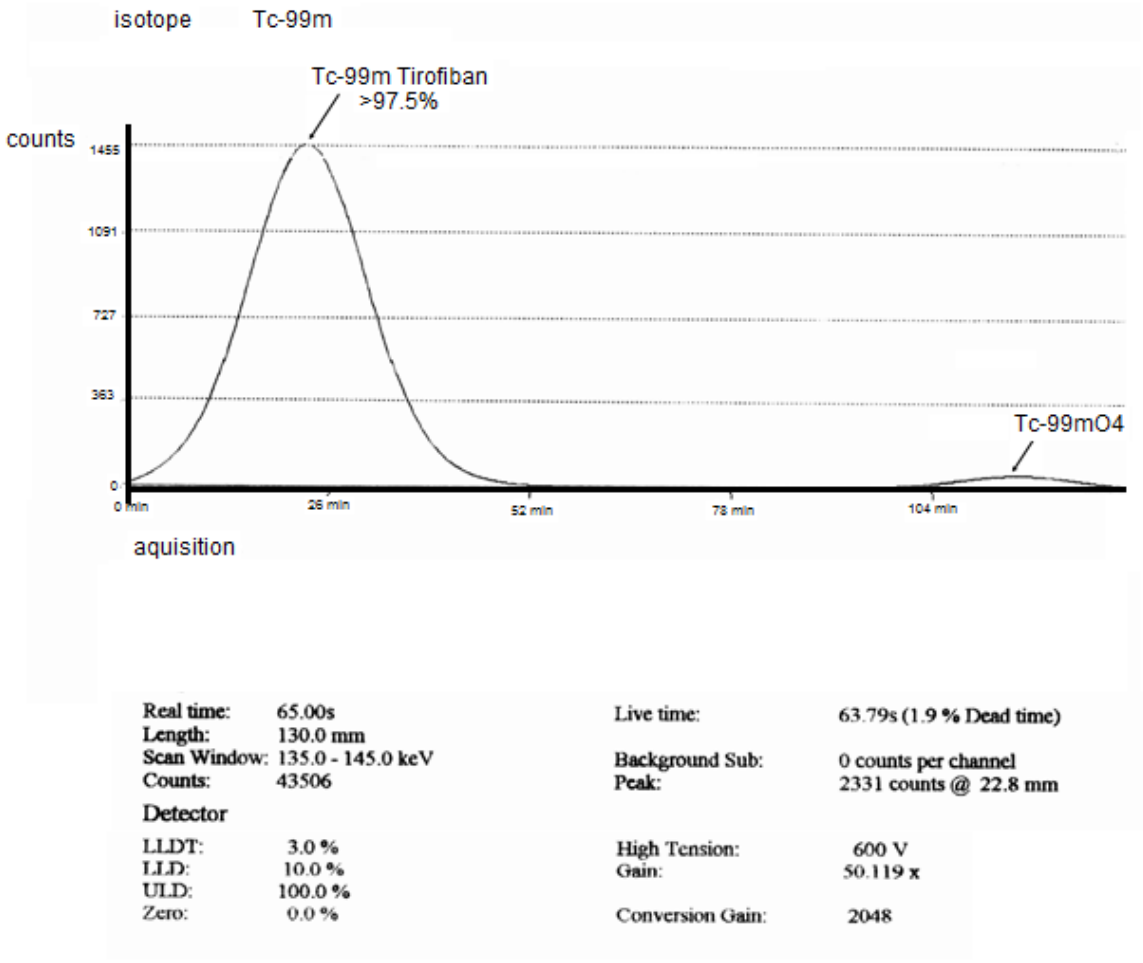


Figure 2.ITLC radiochromatograms of 99mTc-tirofiban in (a) 95% acetone (b) saline.

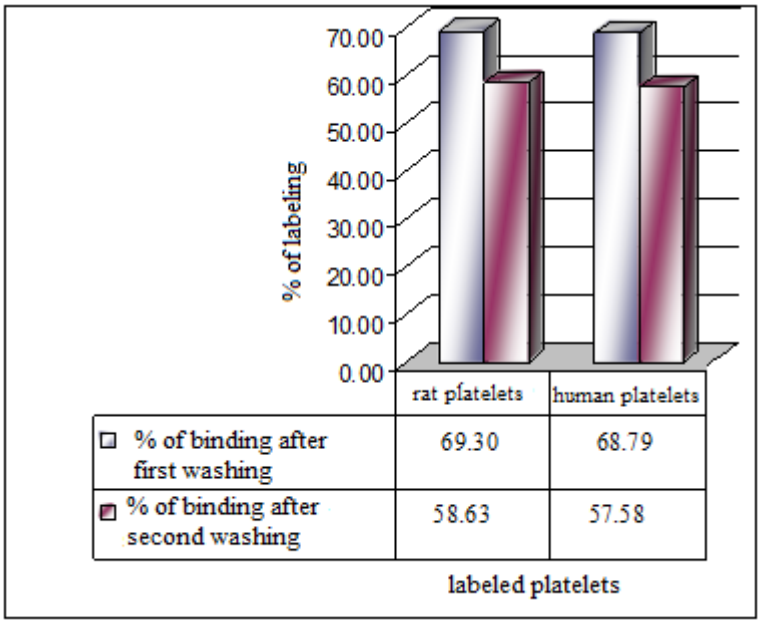


Figure 3.In vitro binding of rat and human platelets isolated and treated with radiolabelledtirofiban.

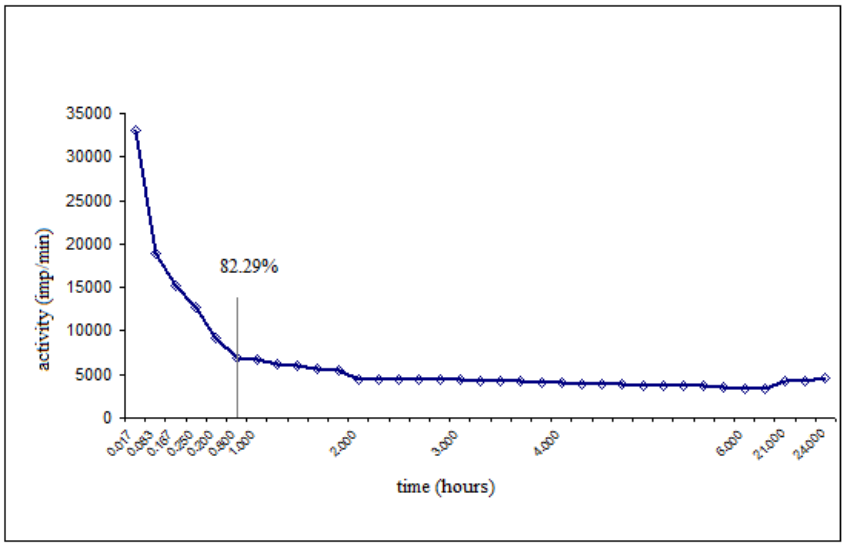
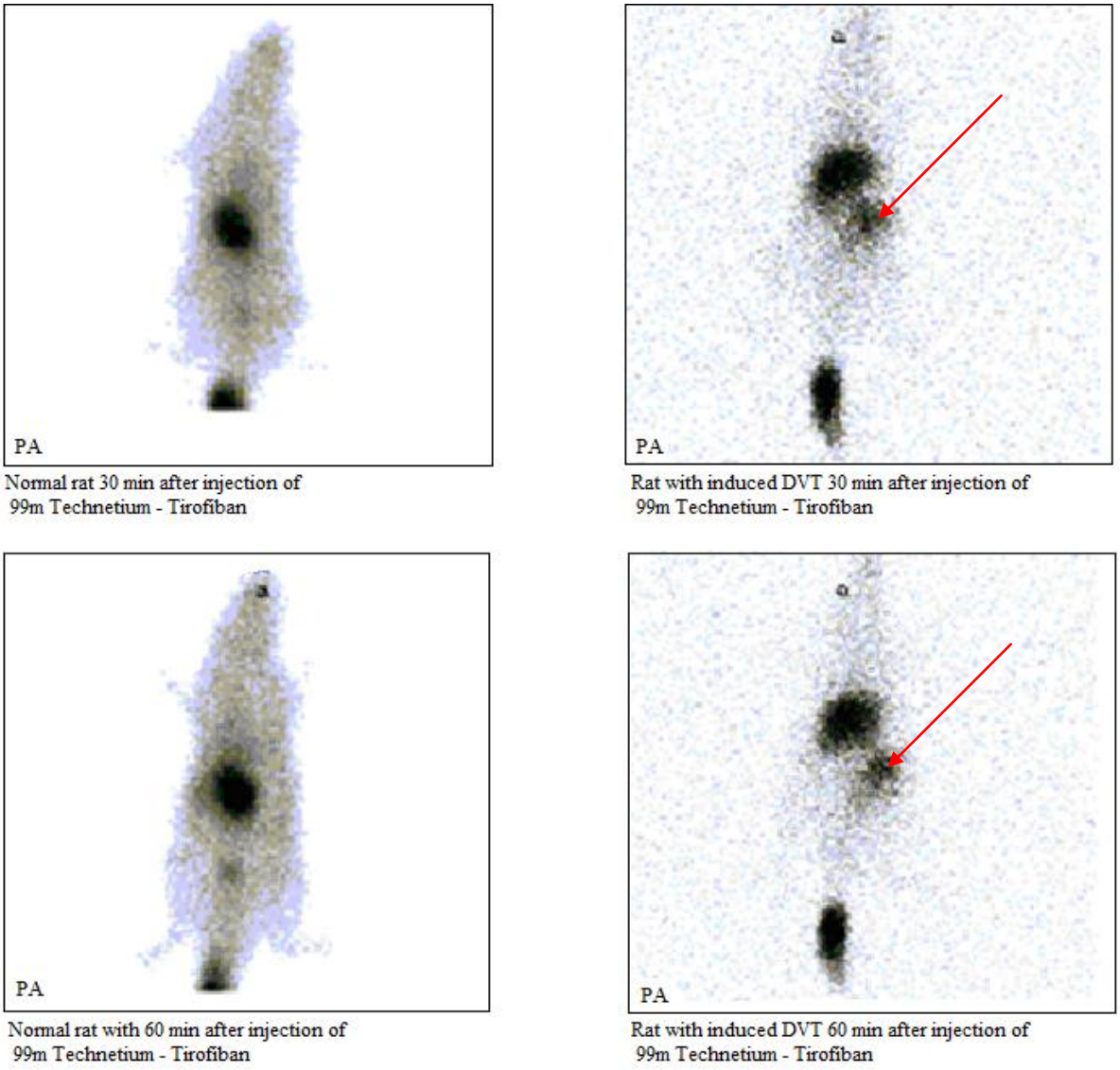


Figure 4. Blood clearance of radiolabelled tirofiban - 99mTc-tirofiban in normal rat



Normal rat 30 min after injection of 99m Technetium - Tirofiban

Rat with induced DVT 30 min after injection of 99m Technetium - Tirofiban

Normal rat with 60 min after injection of 99m Technetium - Tirofiban

Rat with induced DVT 60 min after injection of 99m Technetium - Tirofiban

Figure 5.Gamma camera images after IV injection of 99mTc-tirofiban in rats

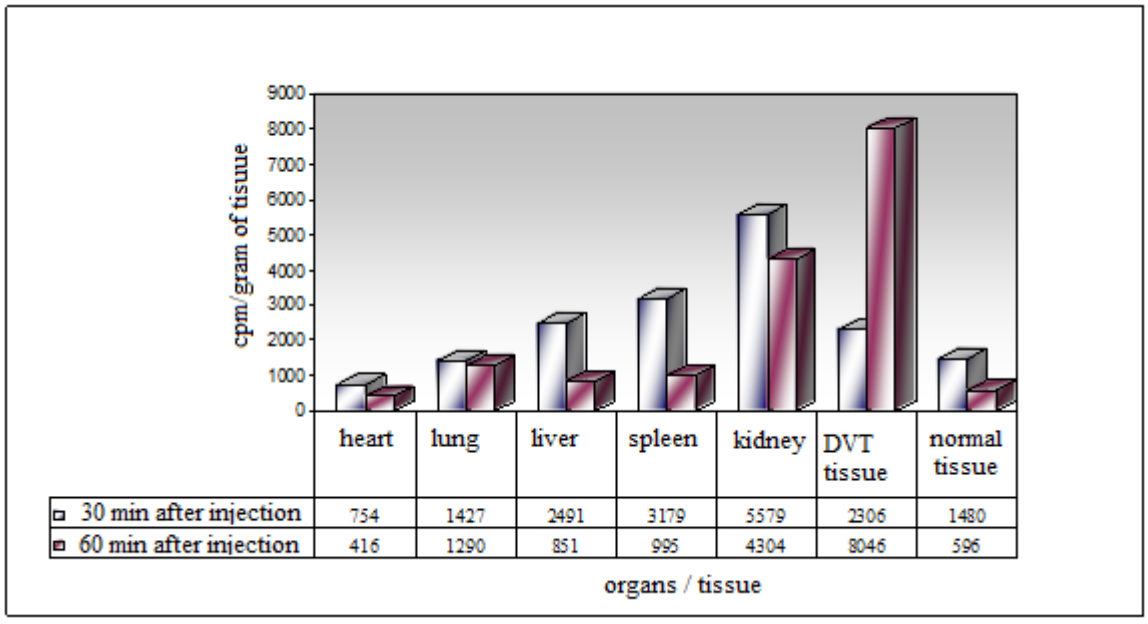


Figure 6. Biodistribution studies in rat with induced DVT and after injection of 99m Technetioum labeled tirofiban

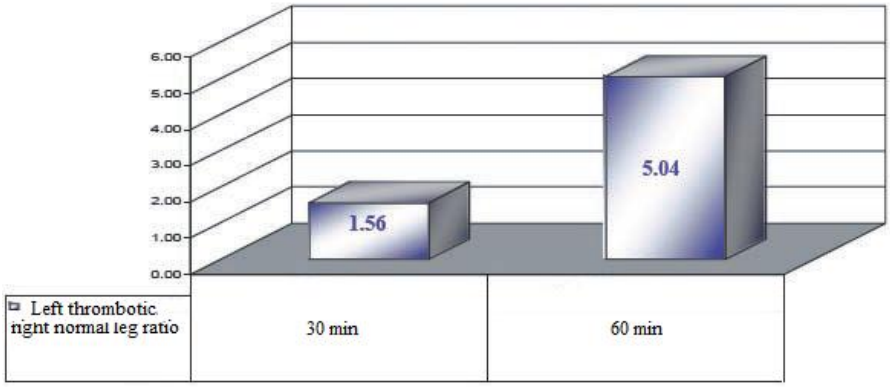


Figure 7. Left thrombotic/right normal leg ratio using ROI technique from gamma cameraimages after injection of 99mTechnetium-tirofiban.

DISCUSSION

Blood coagulation has long been considered an important factor in the pathogenesis of venous thrombosis. The relative contribution of stasis, alteredcoagulability of the blood, vessel wall damage and circulation of leucocytes orplatelets 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 mechanismshave been designed as a means of studying potential imaging agents fordiagnosis 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 undergothe conformational change that makes them available for binding fibrinogen. Cross-linkage of activated platelets by the bivalent fibrinogen molecule to forma 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 agentto GPIIb/IIIa receptors in the case of experimentally induced acute DVT in the rat experimental model. Radionuclide imaging offers considerable potential asa 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 bindspreferentially to GPIIb/IIIa receptors on activated platelets and can differentiateacute 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, aprocedure which does not alter the molecule's biological properties. In the current work, technetium (to evaluate tirofiban as a specific imaging agent toGPIIb/IIIa receptor in the case of experimental induced acute DVT in the ratexperimental model) was chosen asradiotracer for biodistribution and imaging studies in the experimental thrombosis induced in rats for its favourableradiation and physical characteristics, ready availability, possibility of labeling and low cost.

Commercially available technetium–apcitide (AcuTect), previouslyknown as 99mTc-P280, which binds to the GPIIb/IIIa receptor, is the firstimaging agent used in the clinical studies to detect acute DVT [21, 22]. In thisstudy, although the 99mTc–apcitide images obtained at 2 h after tracer injectionshow the greatest overall accuracy in comparison with earlier images, combinedanalysis 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. Theseresults correlate with data obtained from the animal model. 99mTechnetium-tirofiban is accurate in the detection of acute DVT, especially 60 min afterapplication. The results obtained from the animal design experimental studiesshowed that the ratios 'left leg positive for DVT' and 'right leg negative forDVT' for the radiolabelled preparation of tirofiban are well within the range ofthat expected for a successful imaging agent.

Conclusion

Animal models provide convenient screening tools for radiolabelledproducts before a new radiopharmaceutical is further developed in clinicaltrials [23].

The tirofiban labelled with 99mTechnetium wasfound that have good thrombus uptake in vivo experiments. Obtained data indicate that radiolabelled tirofiban binds preferentially toGPIIb/IIIa receptors on activated platelets and can differentiate acute DVTfrom chronic venous thrombosis.

These results can be helpful in the further clinical investigation of patientswith acute DVT.

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