Pre-Clinical Evaluation of a Novel Radiotracer for the Diagnosis of DVT and Pulmonary Embolism.

DAVID EDWARDS
Dissertation presented at Uppsala University to be publicly examined in Föreläsningssalen, Röntgen, Ing. 70, 1 tr, Akademiska sjukhuset, UPPSALA, Friday, December 8, 2006 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract


Deep vein thrombosis (DVT) and pulmonary embolism (PE) are different aspects of a single condition, venous thrombo-embolic disease (VTE), a major cause of morbidity and mortality in the western world. Rapid diagnosis is critical, as timely medical intervention can have a substantial beneficial effect on the mortality rate.

Irrespective of its presentation, VTE is a difficult disease to diagnose. Pathologies unrelated to VTE can give rise to a clinical presentation similar to DVT or PE, resulting in a false positive diagnosis. This raises the risk of a patient being treated inappropriately. Therefore, there is a need for an agent that has high specificity and sensitivity for the detection of active blood clots, which are amenable to treatment by anticoagulant and/or thrombolytic therapy.

This work describes the pre-clinical efficacy studies performed on one such agent, $^{99m}$Tc-NC100668. $^{99m}$Tc-NC100668 is a substrate for factor XIIIa and as a potential physiological, rather than anatomical, marker of VTE it is hoped it will not give rise to the false negative and positive diagnoses that are inherent in the currently available diagnostic techniques, such as the ventilation perfusion (V/Q) scan, multidetector computer tomography or ultrasound.

It is reported in this work that $^{99m}$Tc-NC100668 uptake and retention in blood clot was rapid and maintained over at least a 4 hour period in a rat model of DVT. Anticoagulant and thrombolytic therapies commonly used to treat thrombosis did not seriously impair the ability of $^{99m}$Tc-NC100668 to detect thrombi. No significant tissue retention, which could interfere with the ability to image thrombi in vivo, was observed. Biodistribution and plasma clot uptake studies showed that $^{99m}$Tc complex of gly-NC100194, the major metabolite of $^{99m}$Tc-NC100668, would be unlikely to affect adversely the clinical utility of the test substance.

The in vitro uptake of $^{99m}$Tc-NC100668 into forming plasma clots indicated that retention into human blood clots would be comparable with the observations made in the rat preclinical models.

The uptake of $^{99m}$Tc-NC100668 in vitro and in vivo was much greater than could be accounted for by physical entrapment into the forming blood clots. The reduced uptake of a biologically inactive analogue of $^{99m}$Tc-NC100668 both in vitro and in vivo indicated that the blood clot uptake and retention of $^{99m}$Tc-NC100668 was mediated by factor XIIIa.

In conclusion, $^{99m}$Tc-NC100668 might be useful in the detection of thromboembolism.

David Edwards, Department of Oncology, Radiology and Clinical Immunology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

© David Edwards 2006

ISSN 1651-6206
ISBN 91-554-6726-1
urn:nbn:se:uu:diva-7321 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7321)
Study I
99mTc-NC100668, a new tracer for imaging venous thrombo-emboli: Pre-clinical biodistribution and incorporation into plasma clots in vivo and in vitro.

Reprinted with kind permission of Springer Science and Business Media.

Study II
The in vivo and in vitro metabolic profile of 99mTc-NC100668, a new tracer for imaging venous thrombo-embolism: Identification and biodistribution of the principal radiolabelled metabolite.

Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.

Study III
99mTc-NC100668, an agent for imaging venous thrombo-embolism. The effect of anticoagulant or thrombolytic therapy on the uptake and retention of radioactivity.

Accepted Nucl Med Commun. 2006.
Reprinted with permission of Lippincott Williams & Wilkins. Accepted for publication Nuclear Medicine Communications, Copyright © (2006).

Study IV
The biodistribution of NC100668 and the effect of excess NC100668 on the biodistribution of 99mTc-NC100668 in rat.

Submitted Nucl Med and Biol.
# CONTENTS

1 ABBREVIATIONS ........................................................................7

2 INTRODUCTION .......................................................................11

2.1 Platelet activation .................................................................11

2.2 Blood coagulation .................................................................11

2.3 Regulation of haemostasis ....................................................14

2.4 Protection of the blood clot from lysis and eventual resolution 15

2.5 Thrombosis and pulmonary embolism ...............................15

2.6 Incidence and outcomes .......................................................18

2.7 Diagnosis of VTE .................................................................18

2.8 Why target factor XIIIa? .......................................................21

2.9 Why develop a radiotracer targeted to factor XIIIa? ............23

2.10 Production of $^{99m}$Tc .........................................................23

2.11 Radiolabelling with $^{99m}$Tc .................................................24

2.12 Planar and SPECT imaging using $^{99m}$Tc .........................25

3 STUDY AIMS ...........................................................................28

Main aim ..................................................................................28

Specific aims .............................................................................28

4 METHODS ...............................................................................29

Test substances (Studies I to IV inclusive) ...............................29

Rat biodistribution model (Studies I, II and IV) .......................29

Rat bile duct cannulation model (Study II) .............................29

Rat model of DVT (Studies I, III and IV) ..................................30
1 ABBREVIATIONS

AH110563 Ac-Asn-Ala-Glu-Ala-Val-Ser-Pro-Tyr-Thr-Leu-Leu-Ala-Gly-NC100194.

The biologically inactive analogue of NC100668

aPTT Activated partial thromboplastin time. The time needed for plasma to form a fibrin clot following the addition of calcium and a phospholipid reagent; used to evaluate the intrinsic clotting system.

AT-III Antithrombin III is a protein, which stimulates the removal of blood clots in the bloodstream.


DTPA Diethylene triamine pentaacetic acid.

DVT Deep vein thrombosis.
Final intermediate  The radiotracer in the formulated kit prior to radiola-
belling with 99mTc.

FoV  Field of view.

GpIIb IIIa  A member of the vitronectin class of integrin receptors expressed on platelets.

hCT  Helical computerised tomography.

HMWK  High molecular weight kinogen.

HPLC  High performance liquid chromatography.

MDP  Methylene diphosphonic acid.

NC100194  3,3,11,11-tetramethyl-7-aminoethyl-4,7,10-triazatridec-
ane-2,12-dionedioxime.

\[
\begin{array}{c}
\text{N} \\
\text{NH} \\
\text{NH} \\
\text{N} \\
\text{N} \\
\text{NH} \\
\text{OH} \\
\text{OH}
\end{array}
\]

NC100668  Ac-Asn-Gln-Glu-Gln-Val-Ser-Pro-(I-Tyr)-Thr-Leu-Leu-
Lys-Gly-NC100194.

\[
\begin{array}{c}
\text{N} \\
\text{NH} \\
\text{NH} \\
\text{N} \\
\text{N} \\
\text{NH} \\
\text{OH} \\
\text{OH}
\end{array}
\]

PE  Pulmonary embolism.

PF  Platelet factor. Platelet released protein that promotes blood clotting by neutralising heparin.

pi  Post injection.

PIOPED  Prospective investigation on pulmonary embolism diagnosis.

PMT  Photomultiplier tube.

RHT  Reduced Hydrolysed Technetium-99m.

Sensitivity  The probability that a test result will be positive given that the subject has the disease.

SPECT  Single photon emission computerised tomography.
Specific activity  The number of atoms of a radioactive substance that disintegrate per unit time per unit mass of a radioisotope.

Specificity  The probability that a test result will be negative given that the subject does not have the disease.

TAFI  Thrombin-activatable fibrinolysis inhibitor.

TF  Tissue factor. A membrane glycoprotein of around 250 residues which initiates blood clotting after binding factors VII or VIIa.

$^{99m}$Tc-NC100668  The NC100668 ligand radiolabelled with $^{99m}$Tc.

TFPI  Tissue factor pathway inhibitor.

t-PA  Tissue plasminogen activator. The vascular form of a serine protease that acts on plasminogen to generate plasmin.

vWF  Von Willibrand factor. Plasma factor involved in platelet adhesion through an interaction with Factor VIII.

VTE  Venous thrombo-embolism.

V/Q  Ventilation perfusion scan. A V/Q scan is an imaging technique used in nuclear medicine for the diagnosis of PE. The ventilation (V) component is performed by imaging the lung when the patient breathes in air that contains $^{81m}$Kr, $^{133}$Xe or occasionally $^{99m}$Tc as Technegas™. The image generated reveals the extent to which the lung is ventilated. The perfusion (Q) component is performed by giving the patient a bolus injection of $^{99m}$Tc-labelled macroaggregated albumin ($^{99m}$Tc-MAA). The $^{99m}$Tc-MAA lodges in the terminal pulmonary capillaries and when the patient is imaged gives an indication of the extent of blood perfusion in the lungs. By assessing the degree of mismatch between the two images it is possible for a physician to decide if a diagnosis of PE is likely or not.
2 INTRODUCTION

Haemostasis is a protective mechanism that has evolved to protect the body from blood loss following a traumatic injury. Haemostasis is a complex process and subject to biochemical, cellular and physiological control. Dysregulation of any of these processes due to disease, trauma or any number of other environmental or genetic factors can result in the inappropriate deposition of blood clots, a phenomenon that will have a serious negative impact on a person’s well-being.

2.1 Platelet activation

Following damage to endothelial tissue, collagen and von Willibrand factor (vWF) in the underlying basement membrane are exposed to circulating platelets. Damaged endothelial cells adjacent to the tissue trauma express P and E-selectins [1]. These proteins trap platelets from the circulating blood to the area around the damaged tissue.

The binding of collagen, vWF and selectins to platelets causes them to undergo a conformational change leading to their activation. As part of the activation process, many more receptors (αβ, GpIb-IX-V and GpIIb/IIIa) are externalised to facilitate further platelets-substratum, cell and platelet interactions [2].

Activated platelets release numerous compounds including fibrinogen, vWF factor XIII, factor V, growth factors (TGF-β, PDGF) and platelet factor four (PF-4) as well as ADP, ATP and serotonin [3]. These compounds all facilitate blood clotting, platelet activation or vasoconstriction.

Negatively charged phosphatidylserine is transferred to the external surface of the activated platelet where it provides a reactive surface on which clotting factors can aggregate, accelerating the blood clotting process [4].

2.2 Blood coagulation

According to the classical theory of blood coagulation, the blood clotting cascade is composed of two separate arms, the intrinsic and the extrinsic arm. The two pathways meet at the level of factor Xa which catalyses the activation of thrombin (Figure 1). Thrombin then cleaves fibrin from fibrinogen. Fibrin, thrombin and calcium released from activated platelets activate factor XIII, which stabilises the fibrin clot by covalently cross-linking the fibrin monomers [5, 6].

The intrinsic arm is initiated when factor XII and pre-kallikrein are activated in response to contact with a negatively charged surface such as collagen. Factor XIIa activates pre-kallikrein and the kallikrein generated responds by activating further factor XII. Gradually the concentration of factor XIIa and kallikrein present increases until there is sufficient factor XIIa present to activate factor XI. Factor XIa activates factor IX. Factor IXa complexes with factor VIIIa and this complex activates factor Xa.

The extrinsic arm is initiated when tissue factor (TF) is expressed on the surface of damaged endothelial cells. TF binds to circulating factor VII and cal-
Calcium. This complex associates with a negatively charged phospholipid on the surface of activated platelets, resulting in activation of factor X.

Recently the theory has been modified to take into account the fact that the extrinsic arm appears to dominate the intrinsic arm. The two arms may be able to “talk” to one another above the level of factor X (Figure 2) [7]. In this model, the intrinsic clotting cascade is believed to play only a minor role in the initiation of coagulation. The revised theory places TF at the centre of the blood clotting mechanism.

TF externalised by damaged endothelium binds to and activates circulating factor VII [8]. TF-factor VIIa complex forms on the endothelial cell surface adjacent to negatively-charged phospholipids that provide anchorage points for factors IX and X. The TF-factor VIIa complex generates small amounts of factors IXa and Xa (Figure 2). Factor Xa can then convert small amounts of prothrombin to thrombin.

Figure 1.
Classic theory of blood clotting.
However, in the absence of factor Va, the factor Xa formed by the TF-factor VIIa complex is rapidly inhibited by tissue factor pathway inhibitor (TFPI). If insufficient TF is produced to sustain the blood clotting process, the factor Xa generated is degraded by TFPI [5].

Factor VIIIa, delivered to the site of injury by vWF, complexes with phospholipid, calcium and factor IXa, to form a stable complex that is capable of generating large amounts of factor Xa. However, this complex can only form if there is sufficient TF-factor VIIa complex present to generate factor IXa.

The activation of factor XIII is a complex multi-step process. The inactive form of the enzyme exists as a heterodimer, composed of two A units and two B units [9]. The A units are the catalytic units responsible for the cross linkage of fibrin monomers. The B units serve as regulatory domains of factor XIII. They dissociate from the A subunits in the presence of fibrin and calcium, thereby unmasking the catalytic activity of the A subunits.
2.3 Regulation of haemostasis

Endothelial cells secrete prostacyclin and nitrous oxide, which suppress the activation of circulating platelets as well as causing vasodilation of the blood vessels facilitating blood flow. In contrast, platelets release thromboxane, a potent vasoconstrictor. Both thromboxane and prostacyclin have very short plasma half-lives. Therefore, any changes to either endothelium or platelet activity quickly affect the balance of these paracrine factors which in turn alters the blood flow to the surrounding vasculature.

In addition, enzymes such as adenosine diphosphate (ADP) phosphatase expressed on the surface of endothelial cells remove the circulating ADP that might activate platelets [10]. Finally, the endothelial cells act as a blanket, preventing platelets from accessing extracellular matrix components such as collagen and vWF.

When blood clotting occurs in response to a localised injury, mechanisms exist to prevent it from growing into and then occluding healthy veins.

The thrombomodulin receptor expressed on the surface of endothelial cells, monocytes and platelets acts as a thrombin sink, removing free thrombin from the blood [11]. This helps to limit the extent to which a blood clot can grow away from the site of injury.

Thrombin bound to thrombomodulin complexes and then activates protein C. The protein Ca/S complex binds to phosphatidylserine on predominantly activated platelets (the γ-carboxyglutamic acid-rich domain on protein S facilitates this process). From this position the protein Ca/S complex is able to inhibit factor Va and factor VIIIa [12].

The endothelial protein C receptor (EPCR) is found adjacent to the thrombomodulin receptor. Protein Ca bound to this receptor down-regulates the synthesis of procoagulatory factors such as PDGF and P and E-selectin [13, 14].

TFPI is released by the endothelium in response to elevated concentration of thrombin [15]. TFPI sequesters factor Xa and the TF/factor VIIa complex, thus rendering them unavailable to participate in further thrombin generation [7, 10]. The TFPI-factor Xa-TF/factor VIIa complex is then internalised by monocytes or degraded in the liver [8].

Antithrombin III (AT-III) regulates activity within the blood clot itself, rather than mopping up the over-spill activity. AT-III contains a number of arginine-rich centres that react irreversibly with the active sites of thrombin, factor IXa, factor Xa and factor XIIa, inactivating these enzymes. Heparin binding to AT-III induces a conformational change in AT-III which increases the affinity of the arginine residue for the active site of these enzymes [16].
2.4 Protection of the blood clot from lysis and eventual resolution

The incorporation of $\alpha_2$-antiplasmin into a blood clot by factor XIIIa renders it resistant to proteolysis by plasmin. $\alpha_2$-antiplasmin binds irreversibly to the active site of plasmin, rendering it inactive. Once the $\alpha_2$-antiplasmin incorporated into the blood clot has been exhausted, it will become susceptible to the activity of plasmin. Plasmin is activated by tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA) secreted by endothelial cells. In addition, factors XIIa and XIa, as well as kallikrein, up-regulate plasmin activity [17].

Thrombin-activatable fibrinolysis inhibitor (TAFI) circulates in the blood as an inactive zymogen associated with both plasmin and platelets [18, 19]. TAFI is activated by plasmin or thrombomodulin complexed to thrombin. Activated TAFI cleaves the carboxy terminal arginine and lysine from fibrin, which prevents plasminogen binding to fibrin [20]. At low thrombin/thrombomodulin complex concentrations protein C activity dominates over TAFI activation and coagulation is suppressed. As the concentration of thrombin rises, as would be expected following haemorrhage or tissue damage, TAFI activity predominates, resulting in blood clot stabilisation [21].

Gradually under the control of factor XIIIa the composition of the blood clot changes. Fibrin is replaced with fibronectin and vitronectin secreted by fibroblasts. These proteins are constituents of the extra-cellular matrix and form adhesion points for fibroblasts and other cells. Eventually, the blood clot is digested by the action of plasmin and other proteases such as elastase and cathepsin G that are released from the neutrophils that infiltrate the blood clot [22].

2.5 Thrombosis and pulmonary embolism

DVT and PE are different presentations of a single condition, venous thromboembolic disease. Venous thrombi may form anywhere in the body but DVT that arise in the legs, in particular in the iliac, femoral or popliteal veins are the most common (Figure 3) [23]. However, venous thrombi created in other anatomical regions, such as the arms, are becoming more common due to the increasing use of indwelling catheters in certain patient groups, such as cancer patients [24].

DVT are triggered by the convergence of 3 separate factors (Virchow’s triad): hypercoagulability, vascular injury and venous stasis. Vascular injury exposes the blood to tissue factor on damaged endothelium as well as the negatively charged basement membrane, both of which can activate platelets. Venous stasis impairs the clearance of activated coagulation factors and/or the influx of inhibitory thrombomodulin. Finally, hypercoagulability occurs when the inhibitory elements of coagulation are overwhelmed by the pro-coagulatory factors, usually because of illness or genetic factors [25]. Typically, the genesis of these DVT is behind the valves in the veins of the leg where the valve wall promotes the formation of eddy currents that favour venous stasis (Figure 4) [26].
If the venous blood clot (or a fragment of a large blood clot) detaches from the wall of the vein, it eventually lodges in the lung. The critical period for this phenomenon appears to be 5 to 10 days after the formation of the DVT [27]. Typically, human thrombi are between 4 and 6 mm wide. These dimensions correspond to the vasculature of the sub-segmental pulmonary vessels, where many pulmonary emboli are found [28].

Figure 3.
Location of the major sites of venous thromboembolism.
As a result of a vascular stasis, trauma or other cause of hypercoagulability a blood clot forms behind a valve in a leg vein (1 & 2), the blood clot grows proximally (3), eventually the blood clot will start to fragment (4), and fragments will eventually reach the lungs where they will form pulmonary emboli (4a). Alternatively, the surrounding endothelium might encapsulate the blood clot, stabilising it (5). Finally, the blood clot might be totally resorbed (not shown).

Figure 4.
Genesis and potential fate of a deep vein thrombus.
2.6 Incidence and outcomes

VTE is a major cause of morbidity and mortality in the western world. The annual incidence of this disease is estimated to be somewhere between 100 and 200 per 100,000 general population based on estimates made in Europe and the USA [29]. Post-mortem studies have indicated that the true incidence of VTE may be even higher [30] and with timely intervention the mortality rate can drop from 30% to 2% [31]. Therefore, rapid diagnosis is critical.

There are numerous pre-disposing factors to VTE. These include (but are not restricted to) age, obesity, recent surgery, a previous history of VTE, malignancy, congestive heart failure and oral contraceptive therapy [29].

Genetic variability is a significant contributing factor in the incidence of VTE. Factor V Leiden (an autosomal dominant mutation of factor V that renders the gene product more resistant to degradation by protein Ca) is estimated to be present at a frequency of 3% to 7% in the general Caucasian population, but in one study has been found in 27% of patients diagnosed with PE [32]. Similarly, the prothrombin 20210A mutation occurs at a frequency of 1% in the general population and 18% in patients diagnosed with PE. Finally, patients being treated for VTE are five times as likely to have an inherited protein C or protein S deficiency and are twice as likely to have an inherited AT-III deficiency as that of the general population [32, 33].

There is also a relationship between ethnicity and pre-disposition to idiopathic VTE. A study performed in the USA demonstrated that people of Afro-Caribbean descent run a relative risk of idiopathic VTE of 1.3, Hispanics 0.6 and Asians 0.3 compared with 1.0 in the white European population [34]. These differences are thought to be related to the genetic polymorphisms described above.

There is evidence of seasonal variation in the incidence of DVT and PE, even after allowing for confounding factors such as increased rates of hospital admission during the colder winter months [35]. The increase in DVT and PE incidence in winter may be due to increased viscosity in blood that precipitates abnormal blood coagulation. Finally, the incidence of PE has been shown to peak in the early part of the day, from 08:00 to 12:00. It has been hypothesised that the surge in PE at this time is a consequence of unstable DVT released by the increased vascular tone and muscular activity that occurs in the morning [36].

2.7 Diagnosis of VTE

The typical clinical presentation of DVT is usually a swollen leg, often accompanied by inflammation and some redness which is tender to the touch. The symptoms of PE include dyspnea, pleuritic chest pain, haemoptysis and syncope [30]. Unfortunately, the signs of DVT are often dismissed as trivial. In contrast, the symptoms of PE may be confused with a number of other life-threatening conditions (for example myocardial infarction, pneumothorax or aortic dissection).
The diagnosis of DVT and/or PE is often made based on a patient's medical history, as well as a thorough clinical examination plus numerous supplementary tests. A number of diagnostic decision trees are available to assist the physician in making a diagnosis of PE in particular; Wells et al (Table 1) have developed one of the best-known decision trees [37].

The initial tests performed to confirm diagnosis of PE include electrocardiography, arterial blood gas analysis and chest X-ray. These tests are relatively non-specific, but they are useful to rule out an alternative diagnosis such as myocardial infarction [37].

If the preliminary findings warrant further examination, the patient is referred for a diagnostic scan to confirm the presence of a thrombus (DVT) or embolism (PE). Pulmonary angiography is the gold standard for the diagnosis of central and segmental PE [38] whilst ascending vein venography is the gold standard for the diagnosis of DVT [39].

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous history PE or DVT</td>
<td>+1.5</td>
</tr>
<tr>
<td>Heart rate &gt; 100 beats per minute</td>
<td>+1.5</td>
</tr>
<tr>
<td>Recent surgery or immobilisation</td>
<td>+1.5</td>
</tr>
<tr>
<td>Clinical signs of DVT</td>
<td>+3.0</td>
</tr>
<tr>
<td>Alternate diagnosis less likely than PE</td>
<td>+3.0</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>+1.0</td>
</tr>
<tr>
<td>Diagnosed with cancer</td>
<td>+1.0</td>
</tr>
</tbody>
</table>

Clinical probability of PE

<table>
<thead>
<tr>
<th>Level</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0-1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2-6</td>
</tr>
<tr>
<td>High</td>
<td>&gt;7</td>
</tr>
</tbody>
</table>

Table 1. The Well's algorithm.
Cannulation of the pulmonary artery is invasive and associated with a significant risk of morbidity and mortality, approximately 6% and 0.5% respectively [38]. Therefore, the application of pulmonary angiography has tended to be restricted to a few specialist centres. In addition, ascending vein venography is falling out of use because it is uncomfortable for the patient. In addition, it is impossible to evaluate whole body embolic burden with this technique.

Until recently, the most common in vivo diagnostic technique for the diagnosis of PE was the V/Q scan. A V/Q scan may have three possible diagnostic outcomes; high probability for PE (there is a strong mis-match between the ventilation and perfusion scan), intermediate probability for PE (there is some mismatch between the ventilation and perfusion scan) and low probability for PE (there is no mis-match between the ventilation and perfusion scan).

Between 50% and 70% of all V/Q scans performed are diagnosed as intermediate whereas only 30% fall into the high probability category [40]. As a result, a significant proportion of patients who have an intermediate V/Q scan will still require further diagnostic work before a definitive diagnosis can be made. The PIOPED (Prospective Investigation of Pulmonary Embolism Diagnosis) study found that only 46% of patients with PE confirmed by angiography actually had a high probability V/Q scan [41]. According to the PIOPED investigators, significant numbers of patients with PE could remain untreated because they have an intermediate (40% of confirmed PE patients) or even a low (14% of confirmed PE patients) probability scan.

Due to the deficiencies in the V/Q scan it has been replaced in many institutions by the hCT scan. Unlike pulmonary angiography, hCT is minimally invasive and widely available. Compared with the V/Q scan it can discriminate between thrombi and other pathologies similar to PE, for example pneumothorax or aortic dissection [38]. Acute PE is usually identified by hCT as a filling defect that forms at an acute angle to the blood vessel [42].

The sensitivity and specificity for PE diagnosed by hCT vary from 53% to 100% and 67% to 100% respectively. Sensitivity and specificity are dependent on many factors including the experience of the reader, the size and location of the thrombus and the collimation used (smaller collimation size increases spatial resolution). When used to detect DVT the sensitivity and specificity ranged from 89% to 100% and 94% to 100% respectively [42]. PE lodged in the lobar or segmental arteries are readily detected by hCT. However, between 6% and 30% of patients with documented PE present with sub-segmental thrombi which are less readily detected by hCT [38].

Compression ultrasound (also known as B-mode ultrasound) has now replaced ascending vein venography as the standard technique for the diagnosis of proximal-vein DVT. The sensitivity and specificity of ultrasound for the detection of DVT are well in excess of 80% for proximally located thrombi and 30% for thrombi in the calf muscle. Thrombi located in the pelvis are difficult to detect due to the issues with the local anatomy. Ultrasound examinations can be confounded by numerous other factors including calcification and atherosclerosis,
previous history of DVT, obesity and haemotoma overlying the blood vessels of interest. It is thought these factors may reduce the diagnostic accuracy in up to 16% of all ultrasound procedures performed [43].

All of these imaging modalities look for anatomical obstruction. When a patient has a history of thromboembolism (which is very often the case), it is difficult to differentiate between new active thrombi and older inactive thrombi. Furthermore, image interpretation in these patients may be complicated by the development of collateral veins around old blood clots. Such a scenario could result in an incorrect diagnosis and the potential for inappropriate therapeutic intervention [44]. Attempts to overcome this problem with the use of diagnostic probes targeted towards active blood clots have been evaluated.

Active blood clots have been targeted using many different radiotracers. One of the earliest approaches was to label fibrin with $^{125}$I. Radioactive accumulation was measured using a scintillation meter. This technique is no longer used. The low energy of $^{125}$I meant that thrombi forming in the illeal and popliteal veins could not be accurately detected. In addition, there was the risk of infection to the patient as the fibrinogen used was derived from human donors. A $^{99m}$Tc-labelled fragment of the fibrin alpha-chain N-terminal peptide ($^{99m}$Tc-TP 850) has also been pre-clinically evaluated. Studies in rabbits have shown that experimental blood clots up to 24 hours old could be visualised [45]. Another approach is to use fibronectin radiolabelled with $^{99m}$Tc or $^{111}$In, however the potential for these radiotracers to detect older blood clots has been questioned [46]. A $^{99m}$Tc-radiolabelled antibody (Thrombo View®) raised against a fibrin epitope has been demonstrated to detect DVT in man [47].

Activated platelets have been used to target blood clots. The simplest approach has been to label platelets with $^{111}$In and follow their distribution following injection [48]. More recently, radiotracers targeted towards the GPIIbIIIa receptor, which is specific to activated platelet, have been evaluated [49]. However, as platelets are incorporated into a blood clot they rapidly become exhausted; as a consequence the uptake of these agents declines as the blood clot ages.

### 2.8 Why target factor XIIIa?

Factor XIIIa is responsible for the covalent cross-linking of fibrin monomers as well as the incorporation of $\alpha_2$-antiplasmin, fibronectin and vitronectin into the blood clot.

The activation of factor XIII is a complex multi-step process. The inactive form of the enzyme exists as a heterodimer, composed of two catalytic “A” units and two regulatory “B” units [50]. In the presence of calcium the “B” units dissociate from the “A” units to produce factor XIIIa. The concentration of calcium required to break the hydrophobic and electrostatic bonds between the “A” and “B” units is reduced in the presence of fibrin. The concentration of fibrin present is in turn dependent on the presence of thrombin. This multi-step process towards the activation of factor XIII appears to serve as a physiological brake ensuring that factor XIII activity occurs only in the correct environment,
i.e. regions of tissue damage where one would expect to find elevated levels of thrombin, fibrin and calcium.

A radiotracer that is a factor XIIIa substrate, would provide a highly specific agent that would target active over inactive blood clots (Figure 5). Potentially, as an enzyme substrate, it would be capable of giving rise to a stronger signal than a radiotracer directed at a receptor. Finally, as an active blood clot is by definition one where fibrin deposition is occurring (i.e. factor XIIIa must be present), it should be possible to detect even aged blood clots using a factor XIIIa substrate due to the local elevated increase in calcium and fibrin.

![Covalent cross-linking of fibrin monomers by factor XIIIa](image1)

![Incorporation of $^{99m}$Tc-NC100668 into clots](image2)

**Figure 5.**
Proposed mechanism of action of $^{99m}$Tc-NC100668.
2.9 Why develop a radiotracer targeted to factor XIIIa?

Nuclear medicine is a mature branch of radiology. Most hospitals have access to a nuclear medicine facility. Consequently, it would be relatively straightforward to introduce an imaging agent labelled with a suitable radioisotope into a hospital without any significant additional capital or training costs.

The most commonly used radioisotope for *in vivo* diagnostic imaging is $^{99m}$Tc. $^{99m}$Tc has a half-life of 6.02 hours and releases a 140 KeV photon when it undergoes internal conversion to $^{99}$Tc.

It is possible to convert the $^{99m}$Tc atom to a range of different oxidation states. As a result it can complex with many different chelating groups (the chelator is that part of the radiotracer that is responsible for binding the $^{99m}$Tc atom). Therefore, the chelating groups can be designed to suit the chemistry of very many different pharmacophores with diverse biological properties. For example, $^{99m}$Tc-complexed to Exametazine or TRODAT produces very lipophilic molecules that can cross the blood brain barrier; in contrast $^{99m}$Tc-complexed to DTPA produces a very hydrophilic agent that is used as a marker of kidney function.

2.10 Production of $^{99m}$Tc

$^{99m}$Tc is produced from a molybdenum-99/technetium-99m generator. At its heart is an alumina column, upon which the radioisotope $^{99}$Mo (t$_{1/2}$ 67 hours) is impregnated. $^{99}$Mo decays, by electron capture, to $^{99m}$Tc (87% of all transitions) and $^{99}$Tc (13% of all transitions).

$^{99m}$Tc is eluted from the generator by drawing a saline solution through it. The amount of $^{99m}$Tc obtained is dependent on the amount of $^{99}$Mo originally added to the alumina cartridge, the number of times the cartridge has previously been eluted and the time between each elution as well as the age of the generator. Most commercially supplied generators have a shelf life of approximately 14 days [51].

The “m” in $^{99m}$Tc indicates that this particular isotope is metastable. This means that it is still in a high energy state. The excess energy is removed from the nucleus with an emission of a photon of γ-radiation with energy of 140 KeV. The conversion of $^{99m}$Tc to $^{99}$Tc is known as internal conversion and has a half-life of 6.02 hours. The 140 KeV photons emitted are sufficiently high in energy to ensure that minimal energy attenuation will occur as the photon leaves the body, whilst at the same time being low enough in energy that a sodium iodide scintillation crystal can efficiently absorb them. Thus the efficiency of energy transfer from patient to detector is optimal [51]. An additional attraction of using $^{99m}$Tc in nuclear medicine is that as $^{99m}$Tc decays it does not emit any β radiation, thus being a distinct advantage in terms of limiting the radiation dose to the patient [52].

Eventually $^{99}$Tc decays by β− emission to $^{99}$Ru ($^{99}$Tc→$^{99}$Ru+β−). The half-life of this final decay is 2.1 x 10$^4$ years, a period so long that it is not normally considered significant in any biological process or medical procedure.
2.11 Radiolabelling with $^{99m}$Tc

$^{99m}$Tc exists in a range of oxidation states from -1 to +7. The +7 oxidation state, $^{99m}$TcO$_4^{-}$, is usually associated with freshly collected generator eluate. However, the lower oxidation states of -1, +1, +2 and +3 are usually required for $^{99m}$Tc radiolabelling [51, 52].

$^{99m}$Tc must be reduced to successfully radiolabel a molecule. This reduction process is usually accomplished by the presence of a small amount of a reducing agent. Typically, Sn$^{2+}$ salts provide this reducing power although sodium borohydride has also been used.

$^{99m}$Tc has a very high specific activity. Typically less than 0.1 μM $^{99}$Tc and $^{99m}$Tc is present in the generator eluate. Once the generator eluate has been diluted to provide the patient with a suitable radioactive dose, the chemical dose of $^{99m}$Tc will be reduced even further.

$^{99m}$Tc is chemically reactive and will readily complex with groups that can donate electrons. Given the small chemical concentration present, it is feasible that significant amounts of $^{99m}$Tc present could also complex with any impurities present as well as the final intermediate (the un-complexed radiopharmaceutical).

Methylene diphosphonate (MDP), gluconate or tartrate are incorporated into the kit as a Sn$^{2+}$ solubiliser (Sn$^{2+}$ is present to reduce the $^{99m}$Tc ion to the correct oxidation state to permit chelation to the final intermediate). However, these molecules are also weak $^{99m}$Tc-chelators. They stabilise the reduced Tc atom in the correct oxidation state long enough to permit their incorporation into the un-complexed final intermediate.

Tc that does not complex with the final intermediate will exist either as the pertechnetate ion ($^{99m}$TcO$_4^{-}$) or as reduced hydrolysed technetium ($^{99m}$Tc) (RHT). These radiolabelled impurities can be retained in the body, adversely affecting radiation dosimetry (a measure of the amount of energy imparted by an administered radioisotope to the body) and potentially interfering with the images acquired. Consequently, it is important to minimise the amount of these species produced by the radiolabelling process.

The amount of $^{99m}$TcO$_4^{-}$ present is controlled by limiting the amount of oxygen and $^{99m}$Tc present. Controlling these two species limits the potential for the formation of oxygen radicals which can compete with the $^{99m}$Tc-chelates generating $^{99m}$TcO$_4^{-}$.

RHT is a colloid formed when reduced $^{99m}$Tc complexes with other inorganic components that may be present in the kit (e.g. SnO, Al$_2$O$_3$ etc). The process that forms RHT is in direct competition with the ligand chelating process. Therefore, the amount of RHT can be controlled by ensuring that sufficient excess ligand is present to compete with RHT formation.

Commercially available $^{99m}$Tc-based radiopharmaceuticals are supplied to the user as freeze-dried kits. These kits contain the ligand (e.g. NC100668) as well as the correct amount of stannous salts, trans-chelating agents and buffers to control pH etc. The presentation of kits in this format means that, provided
the correct amount of $^{99m}\text{Tc}$ is added, it is very easy to manufacture the radiopharmaceutical. This reduces the skill and equipment required to produce these compounds. As a result radiopharmaceuticals derived from these kits are relatively cheap to make and widely available.

2.12 Planar and SPECT imaging using $^{99m}\text{Tc}$

A gamma camera is used to acquire clinical data. The camera is composed of at least one detector (although some machines have up to three detectors) mounted on a gantry (Figure 6) [53, 54].

Each detector is composed of a lead collimator, scintillation crystal and an array of photomultiplier tubes (PMT). The collimator acts as a focusing device that directs the radiation towards the scintillation crystal. The scintillation crystal converts the radiation into photons of light and the PMT detect these photons and convert them to electrical signals. The electrical signals are then sent to a computer for further processing.

The scintillation crystal is sandwiched between the collimator and a bank of PMTs. Radioactivity impinging upon the crystal is converted into light, which is captured by the PMT. The PMTs convert the light signal to an electrical signal which is amplified and fed to an analogue to digital converter (ADC). The digital signal is passed to an imaging centre where the image is recreated.

![Figure 6. Overview of a gamma camera imaging system.](image-url)
Figure 7 demonstrates the principle behind the collimator. Only those photons that are emitted from the source and are perpendicular to the plane of the collimator will pass through it and interact with the sodium iodide crystal. Photons A and B are emitted at 90º to the plane of the collimator and give rise to images C and D on the crystal. Photon E hits the collimator at an oblique angle and is absorbed before it can interact with any of the PMT.

Figure 7 also illustrates the inherent inefficiency of any gamma camera because the detector can only cover a small fraction of the emission sphere from the source (patient). In this example photon F is emitted from the source, but is not detected.
The effective atomic mass (z) of the crystal is sufficiently high that it is able to attenuate the radiation. The scintillation crystal is composed of a crystal lattice, usually sodium iodide that has been doped with thallium. Sodium iodide is not particularly efficient at converting the photons to light energy. Typically, approximately 10% of the radiation falling on the detector is converted to detectable light. The energy from each photon is converted to a quantum of light. The light emitted by individual incident photons of radiation will be detected by several adjacent PMT.

The signal processing software is designed to calculate the spatial location of an incident photon in an artesian (x,y) framework, on the basis of which PMTs are activated. The energy of each photon is calculated and applied to a peak height analyser. Photons above a certain threshold will be accepted and the data point used to contribute to the image being formed. Those photons that fall below the threshold are thought to be the result of scatter and will not be allowed to contribute to image formation.

By collecting photons emitted in more than one plane it is possible to make more effective use of the administered radioactivity. These multi-head detectors are used in Single Photon Emission Computer Tomography (SPECT). SPECT scintigraphy can create sophisticated transaxial images of the body. These SPECT images can be far more informative than the conventional 1-dimensional planar images, but they require more computer processing power to recreate the images and more time is required to prepare the patient. The patient needs to be placed as near as possible to the centre of rotation of the detector(s) if significant image distortion is to be avoided, thus adding to the time taken for the examination.

Given the constraints described above, careful consideration has to be given to how much radioactivity must be administered to a patient in order to produce a diagnostically useful scan. The constraints imposed by the dosimetric considerations of the radiopharmaceutical, the efficiency of the camera, the size of the lesion and the compliance of the patient will all have to be considered before a patient is given a radiopharmaceutical for any diagnostic investigation. Typically, the radioactive dose associated with a single scan might be between 500 and 750 MBq of $^{99m}$Tc. However, with some scans, for example myocardial infarction imaging, doses in excess of 1 GBq may be administered.
3 STUDY AIMS

Main aim
The principal aims of this work were to ascertain the suitability of $^{99m}$Tc-NC100668 as an \textit{in vivo} diagnostic marker of DVT and PE.

Specific aims
The specific aims of this work were to:

1. Confirm the mechanism of uptake of $^{99m}$Tc-NC100688 using appropriate \textit{in vitro} and \textit{in vivo} models.
2. Evaluate the biodistribution of $^{99m}$Tc-NC100688 in healthy and lesion-bearing animals.
3. Investigate the metabolism of $^{99m}$Tc-NC100688 and ascertain whether the principal metabolites had the potential to interfere with the uptake of $^{99m}$Tc-NC100688.
4. Demonstrate that the presence of excess NC100668 in the formulation of the NC100668 kit would not adversely affect the biodistribution of $^{99m}$Tc-NC100688 or its retention in thrombi.
4 METHODS

Test substances (Studies I to IV inclusive)

$^{99m}{\text{Tc}}$-NC100668, $^{99m}{\text{Tc}}$-AH110563 and $^{99m}{\text{Tc}}$-NC100194, as well as authentic $^{99m}{\text{Tc}}$-complex of Gly-NC100194, were all prepared from lyophilised kits containing approximately 50 nmoles ligand. Excipients including buffer, reductants and radiostabilisers were included to facilitate radiolabelling with 2.5 GBq Sodium Pertechnetate ($^{99m}{\text{Tc}}$) solution, obtained from an Amertec™ II Technetium-99m Sterile Generator. Reconstituted kits were heated at 60-65°C for 10-12 minutes in a water bath and then removed and left for a further 10 minutes to cool to room temperature. Following this cooling step the radiochemical purity was determined by high performance liquid chromatography and instant thin layer chromatography.

$^{99m}{\text{Tc}}$-labelled Nanocolloid and $^{99m}{\text{Tc}}$-DTPA were prepared following manufacturers’ instructions from commercially available labelling kits obtained from GE Healthcare. $[^{14}\text{C}]$Dansyl cadaverine, $[^{14}\text{C}]$dansyl hexylamine hydrochloride and $[^{14}\text{C}]$NC100668 were all obtained from GE Healthcare.

Rat biodistribution model (Studies I, II and IV)

Wistar outbred rats were injected with a bolus of test substance via a lateral tail vein whilst under light anaesthesia with halothane (6% in O$_2$). Three different test substances were studied: $^{99m}{\text{Tc}}$-NC100668, $^{99m}{\text{Tc}}$-AH110563 and biologically generated $^{99m}{\text{Tc}}$-complex of Gly-NC100194 obtained from the urine of rats that had been dosed with $^{99m}{\text{Tc}}$-NC100668. After injection, animals were housed individually in metabolism cages to allow separate collection of voided urine and faeces. Animals were sacrificed at 2 and 20 minutes, and 1, 4, 7 hours pi and, for $^{99m}{\text{Tc}}$-NC100668 only, 24 hours post injection (pi).

Animals were killed by cervical dislocation under halothane anaesthesia. Each carcass was weighed and, after dissection, the percentage injected dose (% id) in the tissues and organs dissected was determined by assay for radioactivity in a twin crystal automatic gamma counter.

Rat bile duct cannulation model (Study II)

Adult male Wistar rats were lightly sedated with halothane (6% in O$_2$) and then deeply anaesthetised by an intra peritoneal injection of urethane. The right femoral vein was exposed and cannulated. The bile duct was exposed by laparotomy and then cannulated. The viscera was laid to one side and wrapped in damp gauze.

$^{99m}{\text{Tc}}$-NC100668 was administered via the femoral cannula. Bile samples were collected through the bile duct cannula up to 90 minutes pi. Samples were analysed by HPLC.
Rat model of DVT (Studies I, III and IV)

DVT were induced in the inferior vena cava of Wistar rats using the method of MacIomhair and Lavelle [55]. Briefly, animals were deeply anaesthetised by intra peritoneal injection of 1 part ketamine (100 mg/ml) and 1 part xylazine hydrochloride (2% w/v) at a dose of 0.1 ml per 100 g of bodyweight. The right femoral vein was exposed and cannulated. Following a laparotomy the inferior vena cava was exposed and an L shaped sharp platinum wire, approximately 1 cm in length, was inserted into the vena cava. Five minutes after the insertion of the wire, a 0.4 ml solution of 0.12 mM ellagic acid (Sigma) was administered via the femoral cannula. After allowing a period of time (1 hour in most experiments) for the blood clot to mature, the test substance was administered via the indwelling femoral cannula. Animals were sacrificed at different times pi, depending on the study, by the administration of 3 ml 1% (v/v) glutaraldehyde directly into the vena cava.

The inferior vena cava was then clamped on either side of the wire and the entire section of vein removed. The blood clot was removed from the blood vessel and platinum wire, washed gently with saline, blotted dry and weighed. Each carcass was weighed and, after dissection, the % id in the tissues and organs was determined by assay for radioactivity in a twin crystal automatic gamma counter.

Hepatic S9 incubations (Study II)

A total of 10 MBq $^{99m}$Tc-NC100668 was added to hepatic S9 (0.2 mg β-NADPH, 0.2 mg protein S9 fraction) in a total of 2 ml 50 mM tris(hydroxymethyl)hydrogen chloride buffer (pH 7.4) and placed in a shaking 37°C incubator. Aliquots of the S9 mixture were removed immediately following the addition of $^{99m}$Tc-NC100668 (defined as 0 minutes for the purposes of this experiment) and after 45 and 90 minutes of incubation. All incubations were performed in duplicate and analysed separately. Samples were analysed by HPLC after organic solvent sedimentation of the protein fraction.

Stability assays in rat blood, plasma, bile and urine (Study II)

A total of 50 MBq $^{99m}$Tc-NC100668 was added to aliquots of pre-warmed saline, as well as human and rat plasma, serum and anti-coagulated whole blood. Samples of plasma or whole blood were removed after 5, 45 (60 minutes for the saline samples) and 90 minutes of incubation at 37°C in a shaking incubator. $^{99m}$Tc-NC100668 was added to 3 ml of rat bile or urine. The vials were placed in a 37°C shaking incubator. Samples were removed after 5, 60 and 120 minutes of incubation. All incubations were performed in duplicate.
HPLC analysis (Study II)
HPLC was used to analyse the radiochemical profile of the metabolites of $^{99m}$Tc-NC100668. Two methods were employed. The first method was used to profile the radiolabelled species present in hepatic S9, plasma, urine and bile and to assign a tentative identity of the metabolites by co-chromatography with authentic or biogenerated standards. A second HPLC method was used to confirm the identity of the metabolites, again by co-chromatography with authentic standards only.

In vitro plasma clot model (Studies I, II and III)
A plasma clot-forming buffer was produced by the addition of 110 mM calcium in a 50 mM tris(hydroxymethyl)aminoethane buffer containing 40 units of bovine thrombin solution to glass vials. The non-clot-forming buffer used to determine non-specific binding was produced by adding a tris(hydroxymethyl)-aminoethane buffer to glass vials.

Appropriate test substances were added to plasma samples, aliquots of which were then added to the clot-forming and non-clot-forming buffers. Equivalent volumes of plasma, each spiked with an identical amount of each test substance, were also dispensed into glass vials to act as counting standards.

After incubation at room ambient temperature for 1 hour, the clotting reaction was terminated by the addition of 1 ml of 0.4 M EDTA. Each vial was washed with 50 mM tris(hydroxymethyl)aminoethane buffer containing 0.1% (v/v) Tween 20 detergent and the contents dried on protein-blocked (1.5% (w/v) bovine serum albumin nitrocellulose filters over a vacuum manifold.

The radioactivity present on these dried filters and in plasma counting standards was then measured.
5 RESULTS

Study I

\(^{99m}\text{Tc-NC100668}\) was incorporated into forming plasma clots \textit{in vitro}. In contrast the negative controls (\(^{99m}\text{Tc-DTPA,}\) \(^{99m}\text{Tc-AH110563,}\) \(^{99m}\text{Tc-Nanocolloid}\) and \([^{14}\text{C}]\text{dansyl hexylamine hydrochloride}\)) which did not possess any functional groups that may serve as pharmacophores for factor XIIIa were not incorporated into forming plasma clots \textit{in vitro} (Figure 8).

In order to obtain an adequate radiometric response approximately 0.12 pmoles of NC100668 was added to each P7 vial compared with approximately 37 nmoles of \([^{14}\text{C}]\text{dansyl cadaverine}\). Therefore, the difference observed between the \textit{in vitro} plasma uptake of \([^{14}\text{C}]\text{dansyl cadaverine}\) and \(^{99m}\text{Tc-NC100668}\) may be due to the fact that the amount of FXIIIa present in the plasma clot was insufficient to incorporate all of the \([^{14}\text{C}]\text{dansyl cadaverine}\) present in the time available. \(^{99m}\text{Tc-Nanocolloid}\) is a particulate species that has no affinity towards factor XIIIa. It is most likely that the retention into forming plasma clot observed in Figure 8 is due to physical entrapment of this particular test substance in the forming plasma clot.

Some minor differences between the biological fate of intravenously injected \(^{99m}\text{Tc-NC100668}\) in male and female Wistar rats were observed. However, these differences were evident only at the earliest time points pi and were reflected in

![Figure 8.](image_url)

\textbf{Figure 8.}

Uptake of \(^{99m}\text{Tc-NC100668}\) as well as positive and negative controls into rat plasma clots \textit{in vitro}.

32
the relative rates of urinary excretion, which was faster in the female animals. There was evidence to demonstrate slower elimination of radioactivity from kidneys and, to a lesser extent, liver than other tissues, but otherwise the elimination of radioactivity was rapid (Figure 9). These results suggest that the background clearance of radioactivity following administration of $^{99m}$Tc-NC100668 would be suitable to permit the visualisation of blood clot *in situ*.

There were some additional differences observed in the biodistribution of $^{99m}$Tc-NC100668 in normal Wistar rats compared with those bearing a surgically induced blood clot. Principally, reduced elimination of radioactivity from the blood and lower urinary accumulation was observed in the lesioned animals compared with the healthy animals. These differences were most likely to be due to the effect of surgery on the lesioned animals.

The ratio of the radioactive concentration in the blood clot to blood of those animals dosed with $^{99m}$Tc-NC100668 was 5 to 10 fold greater than that of those dosed with either $^{99m}$Tc-Nanocolloid or $^{99m}$Tc-AH110563. The retention of $^{99m}$Tc-Nanocolloid observed in the blood clots of animals dosed with these test substances was thought to be the result of physical entrapment in the forming blood clot due to the physical size of the colloidal $^{99m}$Tc-Nanocolloid species. This finding was consistent with the *in vitro* data. Given that $^{99m}$Tc-AH110563 has no affinity towards factor XIIIa, it must be assumed that the small amount of uptake observed into blood clot is due to the presence of low levels of radiolabelled colloidal material that are associated with all $^{99m}$Tc-labelled radiopharmaceuticals.

![Biodistribution of $^{99m}$Tc-NC100668 in male and female Wistar rats.](image)

**Figure 9.** Biodistribution of $^{99m}$Tc-NC100668 in male and female Wistar rats.
Study II

This study identified the metabolites of $^{99m}$Tc-NC100668 as the $^{99m}$Tc-complex of Gly-NC100194 (major metabolite in blood, bile and urine) (Figure 10, Figure 11 and Figure 12), $^{99m}$Tc-NC100194 (minor metabolite in urine) (Figure 11) and $^{99m}$TcO$_4^-$ (present in bile) (Figure 12). The identity of these metabolites was determined by co-chromatography with authentic standards (Figure 10). The similar metabolic profile of $^{99m}$Tc-NC100668 following incubation in rat and human hepatic S9 as well as in blood and plasma suggested that the metabolism of this compound in man is likely to be similar to that observed in rat in vivo.

The biodistribution of the $^{99m}$Tc-complex of Gly-NC100194 in rat indicates that it was rapidly removed from the body, principally by urinary excretion (Figure 13). The in vitro plasma clot assay demonstrated that the major metabolite observed in blood ($^{99m}$Tc-complex of Gly-NC100194) was unlikely to have any affinity for blood clot in vivo (Figure 14).

Figure 10.
HPLC trace of radioactivity in plasma 5, 60 and 90 minutes pi of $^{99m}$Tc-NC100668.
Figure 11.
HPLC trace of radioactivity in urine 5, 60 and 90 minutes pi of $^{99m}$Tc-NC100668.

Figure 12.
HPLC trace of radioactivity in bile 0-15, 16-45 and 46-90 minutes pi of $^{99m}$Tc-NC100668.
Figure 13. Biodistribution of $^{99m}$Tc-complex of Gly-NC100194 in male Wistar rats.

Figure 14. Uptake of $^{99m}$Tc-complex of Gly-NC100194, $^{99m}$Tc-DTPA and $^{99m}$Tc-NC100668 in rat plasma clot in vitro.
Study III

The rat model of DVT demonstrated that the uptake of $^{99m}$Tc-NC100668 was unrelated to the age of the blood clot. Irrespective of the age of the blood clot (2 minutes to 4 hours at the time the test substance was administered) between 2 and 4% id/g was retained within the clot at 60 minutes pi, despite the fact that the weight of the recovered blood clots decreased with age (Figure 15 and Figure 16).

In the rat DVT model, there was a marked increase in the radioactive uptake ratio in the blood clot to selected neighbouring organs (blood, heart, lung and liver) with increasing time pi (Figure 17). This was due to a substantial reduction in the background tissue radioactivity because of elimination of radioactivity via the bladder and urine.

The uptake of radioactivity into the blood clot of animals bearing a one hour old experimentally induced DVT in the presence of anticoagulant was broadly comparable with the saline control. In contrast, reduced uptake of $^{99m}$Tc-NC100668 into blood clot was observed in the presence of the tissue plasminogen activator (Table 2).
Figure 15.
The effect of age on the uptake of $^{99mTc}\text{-NC100668}$ into blood clot ratio in a rat model of DVT following the administration of $^{99mTc}\text{-NC100668}$ to animals with 1 hour old blood clots.

* 80 & 300 minute data statistically significant (p<0.05), ANOVA followed by Tukey’s test for significance.

Figure 16.
The effect of age on the weight of recovered blood clots.
Figure 17.
The effect of increasing time pi on the blood clot to selected background tissue ratio in a rat model of DVT following the administration of $^{99m}$Tc-NC100668 to animals with 1 hour old blood clots.

Table 2.
The effect of anticoagulant or thrombolytic therapy on the retention of $^{99m}$Tc-NC100668 in blood clot.

<table>
<thead>
<tr>
<th></th>
<th>Low molecular weight heparin</th>
<th>Unfractionated heparin</th>
<th>Thrombin inhibitor</th>
<th>Tissue plasminogen activator</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood clot (mg)</td>
<td>*20 ± 4</td>
<td>*28 ± 12</td>
<td>*24 ± 9</td>
<td>*22 ± 8</td>
<td>41 ± 12</td>
</tr>
<tr>
<td>Blood clot (% id/g)</td>
<td>2.90 ± 0.56</td>
<td>3.22 ± 1.56</td>
<td>3.36 ± 1.45</td>
<td>*0.03 ± 0.29</td>
<td>3.46 ± 1.91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Blood clot/blood</th>
<th>Blood clot/muscle</th>
<th>Blood clot/lung</th>
<th>Blood clot/liver</th>
<th>Blood clot/heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood clot (mg)</td>
<td>6.62 ± 1.95</td>
<td>6.15 ± 3.64</td>
<td>6.22 ± 1.59</td>
<td>*1.93 ± 0.69</td>
<td>6.27 ± 2.50</td>
</tr>
<tr>
<td>Blood clot (% id/g)</td>
<td>6.72 ± 1.95</td>
<td>6.31 ± 10.35</td>
<td>5.14 ± 4.31</td>
<td>5.55 ± 1.53</td>
<td>6.37 ± 7.48</td>
</tr>
<tr>
<td>Blood clot/blood</td>
<td>7.85 ± 2.23</td>
<td>8.66 ± 4.89</td>
<td>6.84 ± 2.05</td>
<td>*2.95 ± 1.47</td>
<td>6.37 ± 2.18</td>
</tr>
<tr>
<td>Blood clot/muscle</td>
<td>14.14 ± 2.37</td>
<td>13.91 ± 8.64</td>
<td>13.19 ± 4.74</td>
<td>4.64 ± 1.75</td>
<td>15.30 ± 7.47</td>
</tr>
<tr>
<td>Blood clot/lung</td>
<td>12.63 ± 3.35</td>
<td>13.29 ± 7.85</td>
<td>11.86 ± 4.46</td>
<td>*4.31 ± 1.53</td>
<td>12.82 ± 5.44</td>
</tr>
</tbody>
</table>

a Statistically significant difference with the corresponding saline control value as assessed by an unpaired t-test (*p<0.05)
Study IV

The in vitro uptake of $^{99m}$Tc-NC100668 and [Asn-U-$^{14}$C]NC100668 into rat plasma clots were comparable with each other, indicating that $^{99m}$Tc-chelation has no effect on the affinity of NC100668 for factor XIIIa (Table 3).

The biodistribution of [Asn-U-$^{14}$C]NC100668 and $^{99m}$Tc-NC100668 was similar. Less liver retention was observed following the administration of $^{99m}$Tc-NC100668 than [Asn-U-$^{14}$C]NC100668.

Overall, the biodistribution and blood clot uptake of $^{99m}$Tc-NC100668 was unaffected over a 5000 fold NC100668 dose range. An inverse correlation between NC100668 chemical dose and retention of radioactivity in the kidneys was observed (Figure 18). In addition, there was a trend for increasing radioactivity being voided into the urine at later time points pi as the NC100668 dose increased.

In this study 400 mg/kg (3 mmoles/kg) L-lysine reduced the kidney retention of $^{99m}$Tc-NC100668 by a third, relative to untreated control animals. In contrast, a dose of 889 μg/kg (450 nmoles/kg) NC100668 halved the radioactivity retained in the kidneys (Figure 19). A peptide with no substrate affinity for transglutaminase, $^{99m}$Tc-AH110563, had kidney retention of only 5% id at 1-hour pi (Figure 18). These data suggest that non-specific charge mediated endocytosis is not the primary mechanism responsible for the kidney retention of $^{99m}$Tc-NC100668.

### Table 3.
Comparison of the biodistribution of [Asn-U-$^{14}$C]NC100668 and $^{99m}$Tc-NC100668.

<table>
<thead>
<tr>
<th>Tissue/Organ</th>
<th>% id/g of $^{14}$C after injection of [Asn-U-$^{14}$C]NC100668</th>
<th>% id/g of $^{99m}$Tc after injection of $^{99m}$Tc-NC100668</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.36 ± 0.18</td>
<td>0.45 ± 0.31</td>
</tr>
<tr>
<td>Lung</td>
<td>0.22 ± 0.06</td>
<td>0.29 ± 0.09</td>
</tr>
<tr>
<td>Liver</td>
<td>0.52 ± 0.11</td>
<td>0.25 ± 0.66</td>
</tr>
<tr>
<td>Heart</td>
<td>0.16 ± 0.05</td>
<td>0.15 ± 0.11</td>
</tr>
<tr>
<td>Blood clot</td>
<td>2.42 ± 0.38</td>
<td>2.78 ± 1.04</td>
</tr>
</tbody>
</table>

| Blood clot to blood ratio | 7.69 ± 2.84 | 8.46 ± 4.22 |
| Blood clot to heart ratio | 15.65 ± 4.08 | 24.34 ± 12.08 |
| Blood clot to liver ratio | 4.78 ± 0.84 | 12.15 ± 5.62 |
| Blood clot to lung ratio  | 11.46 ± 3.84 | 11.07 ± 5.96 |

a Liver retention was statistically significant as assessed by an unpaired t-test (p<0.01).

b Blood clot to liver ratio was statistically significant as assessed by an unpaired t-test (p<0.05).
Figure 18.
The effect of NC100668 dose on the retention of $^{99m}$Tc-NC100668 in rat kidney, $^{99m}$Tc-AH110563 data included for comparison purposes.

Figure 19.
The effect of NC100668 and L-lysine on the retention of $^{99m}$Tc-NC100668 in rat kidney 1 hour post injection.
6 DISCUSSION

The difference in uptake between $^{99m}$Tc-NC100668 and the negative control compounds could not be accounted for by non-specific uptake or retention in the forming plasma clot. The uptake of $^{99m}$Tc-NC100668 was lower in rat than human plasma clots. These findings support a previous study that demonstrate that α2-antiplasmin is less efficiently incorporated into rat plasma clots than human plasma clots in vitro, possibly due to minor structural differences in human and rat fibrin and/or factor XIIIa [56]. However, these in vitro findings suggest that $^{99m}$Tc-NC100668 will be retained by human blood clots in vivo.

A recent publication by Jaffer et al reported the uptake of an optical imaging agent coupled to a peptide similar to NC100668 into human plasma clot is dependent on the glutamine at the equivalent position to glutamine 2 on NC100668 [57]. Simply replacing this residue with alanine abolished all biological activity. It may be that the biological activity of NC100668 is due to the glutamine residue at position 2 from the N-terminal of NC100668.

The biodistribution data collected in studies I and III suggest that rapid reduction in background radioactivity will facilitate early imaging but also imply that imaging at later time points may be improved due to reduced background noise from the surrounding tissues. Study IV has shown that the biodistribution of [$^{14}$C]NC100668 is equivalent to that of $^{99m}$Tc-NC100668 in DVT bearing animals.

Study III has demonstrated that anticoagulants which function by inhibiting the coagulation process do not affect the uptake of radioactivity into blood clot. $^{99m}$Tc-NC100668 has a very fast blood clearance profile; essentially the blood clot is labelled during the first pass of the radiotracer. Once it has been incorporated into the blood clot it cannot be removed by anticoagulant. Tissue plasminogen activator activates the fibrin lysing enzyme plasmin. In the presence of active plasmin, covalently bound $^{99m}$Tc-NC100668 released from the blood clot explains the reduced radioactivity in the blood clots of animals treated with tissue plasminogen activator.

It is likely that in the clinic older organised blood clots, which are resistant to the action of tissue plasminogen activator, will be encountered more often than younger blood clots [58]. Therefore, the ability of tissue plasminogen activator to affect $^{99m}$Tc-NC100668 blood clot retention may be more limited than suggested in this work.

Effective anticoagulant treatment requires the dose administered to achieve an aPTT value approximately 2 to 3 times that of the baseline control value [59]. In this study, the weights of the blood clots in the treated animals were approximately half the weight of the blood clots recovered from the untreated animals (Table 2). This demonstrates that sufficient anticoagulant or tPA was administered to elicit a biological effect.
The model of DVT used in these studies induced a temporary procoagulatory state using ellagic acid (a factor XIIa agonist) [55]. Once the procoagulatory phase had passed, anticoagulatory mechanisms dominated as the haemostatic balance reasserted itself. As a result the older blood clots underwent lysis, hence the reduction in weight of the blood clots recovered 5 hours post-formation.

Coagulation and blood clot lysis are not discrete processes, both occur simultaneously in a blood clot. Whether the blood clot grows or shrinks will depend upon which process dominates. The current results have shown that even under conditions of lysis it is still possible to detect the presence of blood clot.

The major metabolites of ⁹⁹mTc-NC100668 are the ⁹⁹mTc-complex of Gly-NC100194, and ⁹⁹mTcO₄⁻. ⁹⁹mTc-NC100194 is a minor metabolite observed in urine only. The similar metabolic profile of ⁹⁹mTc-NC100668 in rat and human hepatic S9, as well as blood and plasma, indicates that the metabolites observed in rat are likely to be identical to those encountered in man.

The disposition of ⁹⁹mTcO₄⁻ has been studied [60]. Following administration, ⁹⁹mTcO₄⁻ is widely distributed throughout the body with elimination predominantly via the kidneys (although a significant hepatobiliary route of elimination also exists). Retention of radioactivity is noted in the gastric mucosa, choroid plexus, thyroid and salivary glands. In comparison the distribution and elimination of ⁹⁹mTc-complex of Gly-NC100194 is rapid; there is no evidence of any accumulation and radioactivity is rapidly eliminated into the urine. The biodistribution of these metabolites indicate that they are unlikely to adversely affect the ability of ⁹⁹mTc-NC100668 to image blood clots.

The renal accumulation of radiopeptides can be minimised by saturating the negative charge on the basement surface of the proximal tubule with a positively charged amino acid co-administered with the radiolabelled peptide [61, 62]. However, study IV found that the kidney retention of ⁹⁹mTc-NC100668 was more effectively inhibited by an excess of NC100668 than L-Lysine.

QWBA studies using ¹⁴C-labelled NC100668 have demonstrated that it is concentrated in the kidney cortex [63]. The current work has shown that the biological behaviour of NC100668 is equivalent to ⁹⁹mTc-NC100668. Factor XIIIa and tissue transglutaminase are both expressed by cells in the proximal tubules [64, 65]. Therefore, it is quite plausible that the kidney retention observed in this study is due to factor XIIIa and/or tissue transglutaminase. As the amount of NC100668 present increases, the more likely it is that the active site of the transglutaminases in the proximal tubule will be occupied by NC100668 and not ⁹⁹mTc-NC100668. As a result there is less likelihood of the ⁹⁹mTc-NC100668 being trapped by reaction with transglutaminase. This is the first time that transglutaminase and/or factor XIIIa has been shown to be responsible for the renal retention of radioactivity. It suggests that the mechanism that underpins the renal retention of radioactivity may be more complicated than is currently thought [66].
In summary, the pre-clinical data reported here suggest that $^{99m}$Tc-NC100668 is retained in blood clot by a factor XIIIa specific mechanism and that it is rapidly cleared following administration, facilitating good signal to background ratios. The retention of $^{99m}$Tc-NC100668 into a blood clot is unlikely to be significantly affected by the presence of the anticoagulants and thrombolytic therapies commonly used to treat VTE. The metabolism of $^{99m}$Tc-NC100668 is unlikely to interfere with the uptake of the test substance into blood clot or give rise to any spurious false positive signal. The effect of excess NC100668 is unlikely to impede the uptake of $^{99m}$Tc-NC100668 into blood clot. However, it has demonstrated that the renal retention observed with this test substance is not due to charge mediated endocytosis which is normally associated with radiolabelled peptides.
7 CLINICAL RELEVANCE OF THE PRE-CLINICAL DATA

Pre-clinical data can only be successfully applied to the clinic if the investigator has a good knowledge of the limitations of the models used in terms of their relevance to man. In the studies described in this thesis, the Wistar rat has been used as the in vivo system to model the metabolism, kinetics and mechanism of action of $^{99m}$Tc-NC100668.

The rat is a well established model used to predict the biodistribution and radiation dosimetry of new radiotracers in man [67, 68]. This is despite the fact that there are numerous biochemical, physiological and anatomical differences between the two animal species. For example, the rat lacks a gall bladder and has a faster rate of metabolism than man. However, as these differences are well known it is possible to make allowance for them when extrapolating data to man.

Knowledge of the degree of homology between the human and rat haemostatic systems is critical if the pre-clinical data presented in this body of work can be extended to man. The affinity of a number of different platelet receptors for their respective ligands is higher in man than rat [69, 70]. However, as these cellular differences are remote from the site of action of $^{99m}$Tc-NC100668 they are unlikely to affect the integrity of the studies described in this work. At the biochemical level there is a great deal of similarity between rat and man. Blood clotting factors obtained from one animal species can be substituted into another without any appreciable effect on the blood clotting process [71]. The PT and aPTT times of plasma obtained from man and rat are broadly in line with one another according to the data recently presented in a review by Kurata et al [6].

It has been reported that less $\alpha_2$-antiplasmin is incorporated into the fibrin mesh of rat blood clots compared with human blood clots [56]. The concentration of $\alpha_2$-antiplasmin in human plasma is greater than those found in human serum. In contrast, there is no difference in the concentration of $\alpha_2$-antiplasmin in rat plasma and serum. However, rat $\alpha_2$-antiplasmin will combine with human fibrin to prolong clotting times, but not vice versa. These findings all suggest that rat fibrinogen is not identical to human fibrinogen.

Prior knowledge of these species differences has minimised their impact on the outcome of these studies as it was possible to understand their impact on the data.

The rat model of DVT selected in these experiments had some limitations from the point of view of developing a new in vivo marker for the diagnosis of thromboemboli. DVT and pulmonary emboli are not presented to the clinician until they are many hours or days old. As an acute model of thromboembolism, it was impractical to investigate the uptake and retention of agents into blood
clots more than 4 hours old. Although the experimental blood clots were only a few hours old they showed signs of being in a state of lysis (a consequence of the short-lived action of ellagic acid, the pharmacological agent used to induce hypercoagulability). Although lysis is only one characteristic of older, clinically relevant blood clots, the fact that $^{99m}$Tc-NC100668 was retained in these aged blood clots suggests that there will still be sufficient factor XIIIa present to enable these clinically relevant blood clots to be detected.

The experimental blood clot was situated in the inferior vena cava. This position is not amenable to imaging in a small rodent due to the proximity of the bladder and kidneys. The location of the blood clot in a blood vessel not normally associated with thromboembolism also meant that it was not possible to account for the potential effect of attenuation of photons as they pass through the body or the impact of overlying structures, such as the liver, which also retain radioactivity on the overall image quality.

Once again the potential impact of these factors was recognised and an attempt was made to minimise their influence on the interpretation of the data. This was performed by quoting relevant data as a ratio of radioactive concentration in the blood clot to that in key tissues that might be anticipated to overlie a DVT or pulmonary embolism.

Finally, the blood clot-bearing animals were an acute model of a chronic disease. The blood clots were created by temporarily disturbing one aspect of the haemostatic balance (administration of a factor XII agonist). In reality, venous thromboembolic disease is usually a symptom of another underlying condition such as cancer, traumatic tissue damage or surgery [72, 73]. These diseases can either activate the clotting cascade or up-regulate factor XIIIa directly [74, 75]. Obviously, it would be technically difficult and ethically suspect to create a number of different disease models in one animal in such a way as to give any meaningful data.

Therefore, images acquired in the clinic following the administration of $^{99m}$Tc-NC100668 must be considered along with the clinical history when being used to make a diagnostic decision.
8 CONCLUSION

- $^{99m}$Tc-NC100668 is retained by plasma clots (rat and man) in vitro and blood clots (rat) in vivo.

- $^{99m}$Tc-NC100668 uptake and retention is rapid and maintained over at least a 4 hour period in a rat model of deep vein thrombosis.

- Anticoagulant and thrombolytic therapies used to treat thrombosis do not seriously impair the ability of $^{99m}$Tc-NC100668 to detect thrombi.

- The major radiolabelled metabolite of $^{99m}$Tc-NC100668 in vivo in rat as well as in human and rat hepatic S9 is $^{99m}$Tc-complex of Gly-NC100194.

- The biodistribution of $^{99m}$Tc-NC100668 is not significantly affected (apart from the kidneys) by the presence of a large excess of unlabelled NC100668.

- These experiments have demonstrated that the kidney retention of $^{99m}$Tc-NC100668 is a result of the presence of factor XIIIa and/or transglutaminase in the proximal tubule.

The evidence presented in this work suggests that $^{99m}$Tc-NC100668 might be useful in the detection of thrombo-embolism.
9 ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all my co-authors whose help and support made this possible.

In addition, I owe a great deal to my colleagues at GE Healthcare; in particular Brian Higley, Roger Pickett, Stuart McCully, Tore Skotland, Kim Toft, Stephen Clegg and Paul Jones for sanity checking the manuscripts. Finally, in the UK, thanks to Jo Martins for organising all my travel arrangements and making sure I always had somewhere to stay whenever I was in Uppsala.

In Uppsala, I owe a particular debt to Prof. Ahlström, not only for accepting me as a PhD student, but also for having the patience to edit the papers and manuscripts as well as all the help and guidance he has provided me with. Thanks also to Christl Richter-Frohm for answering what must have seemed like a constant stream of questions from me as well as dealing with all the administration. I would also like to thank Håkan Pettersson for formatting the thesis.

I should say thanks as well to my former colleague Karen Briley-Sæbø for introducing me to Prof. Ahlström and giving me the idea that I could do this.

Most importantly thanks to my wife Louise for proofreading the thesis and my daughter Charlotte for putting up with all those weekends that I was hunched over the computer typing all this up.

Finally, thanks to GE Healthcare Ltd for the financial support.
10 REFERENCES


41 The PIOPED investigators. Value of the ventilation/perfusion scan in acute pulmonary embolism. Results of the Prospective Investigation of Pulmonary Embolism Diagnosis (PIOPED). *JAMA.* 1990; 263: 2753-2759.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 203

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)