ABSTRACT:

$^{99m}$Tc-NC100668  [Acetyl-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr(3-iodo)-Thr-Leu-Leu-Lys-Gly-NC100194] is a radiopharmaceutical imaging agent being developed to aid the diagnosis of thromboembolism. The stability profile of $^{99m}$Tc-NC100668 was investigated by high-performance liquid chromatography (HPLC) after in vitro exposure to blood and plasma obtained from rat and human, as well as to urine and bile obtained from rat. The metabolic profile of $^{99m}$Tc-NC100668 exposed to human and rat hepatic S9 (a liver homogenate-rich cytochrome P450) was also studied. The profile of $^{99m}$Tc-labeled species in plasma, urine, and bile was investigated following i.v. administration of $^{99m}$Tc-NC100668 to rat. The major species observed in vitro and in vivo consisted of the $^{99m}$Tc-chelator (NC100194) [N,N-Bis(N-(1,1-dimethyl-2-(hydroxylimino)propyl)]aminoethyl]aminoethylamine] attached to the C-terminal amino acid residue and referred to as $^{99m}$Tc-complex of Gly-NC100194. The identity of the major metabolite was confirmed by chromatography with an authentic standard and the genuine metabolite using a second HPLC method. The minor metabolites were sodium pertechnetate ($^{99m}$Tc) and $^{99m}$Tc-NC100194. In addition, a small number of other species were transiently observed in vitro; they were not investigated further. The biodistribution of the major metabolite was studied in male Wistar rats. The affinity of the major metabolite toward plasma clot was established using a plasma clot-forming assay. A minor uptake of $^{99m}$Tc-complex of Gly-NC100194 in the plasma clot and a rapid removal from the body were noted. In conclusion, the metabolites of $^{99m}$Tc-NC100668 are not anticipated to have a negative impact on the ability of the test substance to image blood clots.

$^{99m}$Tc-NC100668 is currently being evaluated as an in vivo diagnostic marker of active thromboembolic disease (Edwards et al., 2006). Rapid diagnosis is essential because the mortality rate associated with untreated pulmonary embolism can be up to 30%. Timely intervention can reduce this rate to as low as 2% (O’Neill et al., 2004). NC100194 (Fig. 1A) is based on a linear peptide with homology to the N-terminal sequence of α2-antiplasmin, a known factor XIIIa (FXIIIa) substrate (Lijnen et al., 1987). FXIIIa catalyzes the formation of γ-glutamyllysyl bonds that hold the fibrin clot together (Gorman and Folk, 1984).

$^{99m}$Tc is a γ-emitting radioisotope used in diagnostic nuclear medicine. Usually, $^{99m}$Tc is complexed to a ligand before administration. The precise distribution of $^{99m}$Tc is then determined by the physicochemical properties of the $^{99m}$Tc-ligand complex. The distribution of radioactivity within the body can be detected externally using a γ-camera. The pattern of radioactive uptake and retention in the acquired image is then used to help make a diagnosis.

Because of the short radioactive half-life of $^{99m}$Tc-labeled diagnostic radiopharmaceuticals, they are usually distributed to the point of use as freeze-dried kits ready for radiolabeling. Before use, kits are reconstituted with a saline solution containing $^{99m}$TcO$_4^-$ (pertechnetate ion) obtained from a $^{99m}$Mo/$^{99m}$Tc generator. To ensure that the radiolabeling process achieves the highest radiochemical purity (RCP) possible, the ligand is present in vast excess in relation to the amount of $^{99m}$TcO$_4^-$ added. Typically, less than 1% of the ligand present is bound to $^{99m}$Tc following reconstitution. The NC100668 kits are all formulated with 100 μg of ligand; therefore, in a reconstituted vial less than 1 μg is chelated to $^{99m}$Tc.

Changes in the physicochemical properties (e.g., net charge, lipophilicity) of a molecule occur following chelation of $^{99m}$Tc. These changes can affect the biological behavior of the molecule relative to its unchelated analog. For example, the dopamine transporter tracer TRODAT cannot cross the blood-brain barrier, whereas $^{99m}$Tc-chelated TRODAT can (Kung et al., 1997). These changes may also alter
the affinity and selectivity of a $^{99m}$Tc-chelated ligand for metabolizing enzymes relative to its unchelated counterpart. Therefore, the radiolabeled metabolites present must be characterized independently of the unlabeled metabolites to ensure that any potential for accumulation and retention of radioactivity in an unrelated organ or pathology is understood. In the event that this information is not available, it may be possible that on some occasions a false-positive diagnosis might be made. Given the low chemical concentration of radiolabeled material involved, characterization of any radiolabeled metabolites present is not a trivial challenge and cannot be addressed by conventional liquid chromatography/mass spectrometry techniques alone.

The purpose of this study was to investigate the in vivo metabolism of $^{99m}$Tc-NC100668 following i.v. injection to rats and to identify the major radioactive metabolites present by cochromatography with authentic potential metabolites. The in vitro stability of $^{99m}$Tc-NC100668 was studied in whole blood and plasma, as well as in hepatic S9 (a liver homogenate-rich cytochrome P450) of rat origin, and compared with that obtained from samples of human origin. The identity of the major metabolite was confirmed by cochromatography with an authentic standard using a separate high-performance liquid chromatography (HPLC) method. Finally, the impact of the presence of the major $^{99m}$Tc-metabolite on the clinical usefulness was studied by a combination of in vivo biodistribution and in vitro plasma clot uptake experiments.

**Materials and Methods**

*Reagents and Substances.* Freeze-dried kits containing NC100668, NC100194, and Gly-NC100194 were supplied by GE Healthcare (Little Chalfont, UK). Each kit contained approximately 50 nmol of ligand plus buffer, reductants, and radiostabilizers. These excipients facilitated the radiolabeling of the test substance following reconstitution with 2 to 5 ml of sodium pertechnetate ($^{99m}$Tc) solution, containing 1 GBq of $^{99m}$Tc, obtained from a Drytec or Amertec II $^{99m}$Tc Sterile Generator (GE Healthcare).

After cooling, the RCP of each preparation was determined by reverse-phase HPLC and silica gel instant thin layer chromatography (ITLC). HPLC (Gilson, Luton, UK) was performed by applying aliquots of each preparation to a 250 mm × 4.6 mm (5 μm) Luna C18(2) column (Phenomenex, Macclesfield, UK) that was eluted with 0.05% trifluoroacetic acid (TFA) (Sigma, Gillingham, UK) in water (solvent A) (VWR International, Poole, UK) and 0.04% TFA in acetonitrile (solvent B) (Rathburn, Walkerburn, UK). A linear gradient from 16% solvent B to 40% solvent B was applied over 20 min at a flow rate of 1 ml/min. $^{99m}$Tc-labeled species were detected postcolumn using a Bioscan radiodetector (Lablogic, Sheffield, UK).

ITLC was carried out using ITLC-silica gel strips (Pall, Portsmouth, UK) run in a cylinder containing a 50:50 mixture (v/v) of methanol (VWR International) and 1 M ammonium acetate (VWR International). The strips were allowed to dry and were scanned on a Packard InstantImager (PerkinElmer, Beaconsfield, UK). The image produced was integrated around the origin to give the amount of reduced hydrolyzed technetium (RHT) ($^{99m}$Tc) in the preparation. All the other radiolabeled species moved with the solvent front.

All the $^{99m}$Tc-NC100668 preparations had an RCP of greater than 85%. The major impurity of $^{99m}$Tc-NC100668 was the kinetic $^{99m}$Tc-complex of NC100668, which accounted for at least 6% of the radioactivity present. Under radiolabeling conditions, the kinetic $^{99m}$Tc-complex of NC100668 was rapidly formed. Heat was used to convert most of this through to the thermodynamic complex, $^{99m}$Tc-NC100668. The kinetic $^{99m}$Tc-complex of NC100668 is believed to be a different arrangement of the technetium amine-oxime complex. The remaining radiochemical impurities were RHT ($^{99m}$Tc) (approximately 1%), $^{99m}$Tc-methylene diphosphonic acid (MDP), and hydrolytic decomposition products of $^{99m}$Tc-NC100668. The RCP of the $^{99m}$Tc-NC100194 preparations used was at least 45 and 85% for the $^{99m}$Tc-complex of Gly-NC100194 preparations used. The remaining radioactivity in these preparations was associated with $^{99m}$Tc-MDP and $^{99m}$Tc$_2$O$_4^-$. $^{99m}$Tc-diethylenetriaminepentaacetic acid (DTPA) was prepared by addition of 5 ml of sodium pertechnetate ($^{99m}$Tc) solution, containing 1 GBq of $^{99m}$Tc, to a commercially available labeling kit containing calcium trisodium diethylenetriaminepentaacetic (GE Healthcare). Each preparation was allowed to stand for 30 min before RCP analysis by silica gel ITLC according to the manufacturer’s instructions. The $^{99m}$Tc-DTPA preparations used in this study had an RCP of at least 94% before use. All the other chemicals were of analytical grade quality.

*In Vitro Stability of $^{99m}$Tc-NC100668.* An aliquot of $^{99m}$Tc-NC100668 (50 MBq) was added to glass scintillation vials containing 1.9 ml of prewarmed saline or plasma (obtained from male Wistar rats and human volunteers, anticoagulated with sodium citrate). $^{99m}$Tc-NC100668 (75 MBq) was added to glass scintillation vials containing 3 ml of prewarmed citrate antico-
agulated rat or human blood. Samples of plasma or blood were removed after 5, 45 (60 min for the saline samples), and 90 min of incubation at 37°C in a shaking water bath. 

\(^{99m}\text{Tc-NC100668}\) (25 MBq) was dispensed into prewarmed scintillation vials containing 3 ml of rat bile or urine. The vials were returned to the 37°C shaking water bath. Samples were removed after 5, 60, and 120 min of incubation. All the incubations were performed in duplicate.

**In Vitro Metabolism of \(^{99m}\text{Tc-NC100668}\).** Twenty microliters of a 10- mg/ml \(\beta\)-NADPH (Sigma) solution and 10 \(\mu\)l of a 20-mg/ml protein S9 fraction (Xenotech, Lenexa, KS) obtained from either rat or human liver was added to 1.69 ml of 50 mM tris(hydroxymethyl)hydrogen chloride buffer (Sigma), pH adjusted to 7.4, and prewarmed to 37°C. \(^{99m}\text{Tc-NC100668}\) (10 MBq) was then added. The hepatic S9 mixture was placed in a shaking 37°C water bath. Samples were removed immediately after the addition of \(^{99m}\text{Tc-NC100668}\) (defined as 0 min for the purposes of this experiment) and after 45 and 90 min of incubation. All the incubations were performed in duplicate and analyzed separately.

Cytochrome P450-independent metabolism was investigated by incubating \(^{99m}\text{Tc-NC100668}\) in rat and human hepatic S9 fractions deficient in \(\beta\)-NADPH over 90 min at 37°C.

**Hydrolysis \(^{99m}\text{Tc-NC100668}\) by Trypsin.** Immobilized trypsin beads (200 units/ml) (Perbio Science, Cramlington, UK) were prepared for use by washing three times in 1 ml of 20 mM calcium chloride solution (Sigma). After each wash, the trypsin bead pellet was collected by centrifugation at 10,000 rpm with a fixed angle bench-top centrifuge for 10 min. After the third wash, the trypsin bead pellet was resuspended in 1 ml of 20 mM calcium chloride. A 50-\(\mu\)l aliquot (containing approximately 10 units of enzyme activity) was removed and added to 0.85 ml of 20 mM calcium chloride, to which was added \(^{99m}\text{Tc-NC100668}\) (50 MBq). The mixture was incubated for 60 min at 37°C before analysis.

**In Vivo Metabolism of \(^{99m}\text{Tc-NC100668}\).** Six male Wistar outbred rats [Crl: (WI) BR] (Charles River, Margate, UK) weighing between 180 and 260 g were anesthetized with halothane (6% in O\(_2\)) and then deeply anesthetized by an i.p. injection of urethane. The right femoral vein was exposed and cannulated with tubing with an internal diameter of 0.5 mm and an external diameter of 1.0 mm (Portex). The viscera moved to one side to expose the bladder and vena cava. A venous blood sample of 3 ml was taken from the vena cava using a 10-ml syringe fitted with sodium citrate solution. After the blood sample had been collected, a urine sample was taken by aspiration of the bladder using a 25-gauge hypodermic needle containing sodium citrate solution. After each sample had been collected, the blood samples were treated as plasma samples. The plasma and hepatic S9 samples were added to ice-cold acetonitrile. A 200-\(\mu\)l sample was added to 800 \(\mu\)l of acetonitrile. This was followed by centrifugation at 10,000 rpm with a fixed angle bench-top centrifuge for 10 min to sediment precipitated protein. The samples of bile and urine were collected and centrifuged in a bench-top centrifuge for 5 min at 10,000 rpm before analysis by HPLC. The supernatants were separated from the precipitated material, 100 \(\mu\)l of which was diluted with an appropriate volume of deionized water such that approximately 50 KBq of radioactivity could immediately be analyzed by HPLC with an on-line radiochemical detector (PerkinElmer).

**HPLC Analysis of \(^{99m}\text{Tc-NC100668}\) Metabolites.** HPLC was used to analyze the radiochemical profile of the metabolites of \(^{99m}\text{Tc-NC100668}\). In the initial studies, radiolabeled species were separated on a VYDAC C\(_{18}\) (250 mm \(\times\) 4.6 mm, 5 \(\mu\)m) HPLC column (Hichrom, Reading, UK). A C\(_{18}\) Nova-Pak guard column (Waters, Watford, UK) was fitted in front of the HPLC column. The HPLC column was eluted at a constant flow rate of 1 ml/min. The separation was performed at ambient temperature. The aqueous mobile phase (solvent A) was 0.1% (v/v) TFA in analytical grade water, and the organic mobile phase (solvent B) was 0.1% (v/v) TFA in acetonitrile. The gradient used to elute the radiolabeled metabolites is described in Table 1.

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<th>Time</th>
<th>Percentage Solvent A</th>
<th>Percentage Solvent B</th>
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<td>35 min</td>
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A second HPLC method (referred to as the short HPLC method) was developed to confirm that the major metabolite present in urine and as a consequence of the trypsin-mediated cleavage of \(^{99m}\text{Tc-NC100668}\) would still coelute with the authentic standard of \(^{99m}\text{Tc-complex of Gly-NC100194}\) prepared in this way. The amounts of radioactivity in the \(^{99m}\text{Tc-NC100194}\) and \(^{99m}\text{Tc-complex of Gly-NC100194}\) samples were diluted with analytical grade water until they were approximately equivalent to that in the processed urine sample. The resulting chromatograms were inspected to see whether peaks caused by the genuine metabolite and peaks caused by the presence of either \(^{99m}\text{Tc-NC100194}\) or \(^{99m}\text{Tc-complex of Gly-NC100194}\) could be resolved from one another.

A second HPLC method (referred to as the short HPLC method) was developed to confirm that the major metabolite present in urine and as a consequence of the trypsin-mediated cleavage of \(^{99m}\text{Tc-NC100668}\) would still coelute with the authentic standard of \(^{99m}\text{Tc-complex of Gly-NC100194}\). This method used a 250 \(\times\) 4.6 mm (5 \(\mu\)m) Luna C\(_{18}\) (2) column, which was eluted with 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). A linear gradient from 5% solvent B to 10% solvent B was applied over 10 min at a flow rate of 1 ml/min. \(^{99m}\text{Tc-NC100668}\)-labeled species were detected post-column using a Bioscan radiodetector.

**Biodegeneration of \(^{99m}\text{Tc-Complex of Gly-NC100194}\.** Eight male Wistar rats were lightly anesthetized with halothane (6% in O\(_2\)) and injected i.p. with 1 part ketamine (100 mg/ml) (Pharmacia Animal Health, Corby, UK) and 1 part xylazine (2% w/v) (Bayer, Newbury, UK) at a dose of 1.0 ml/kg body weight. The right femoral vein of each animal was exposed. A cannula was inserted into the vein, and 0.5 ml of \(^{99m}\text{Tc-NC100668}\) (approximately 500 MBq) was injected. Once the \(^{99m}\text{Tc-NC100668}\) had been administered, the urethra of these animals was clamped to prevent loss of urine.

Sixty minutes after the administration of \(^{99m}\text{Tc-NC100668}\), each animal was sacrificed by cervical dislocation. The abdomen was opened, and the urine removed from the bladder. The urine from the eight animals was pooled. The radioactive content of the pooled urine was determined using an Ion Chamber (Capintec, Ramsey, NJ), and the radiochemical composition was determined by HPLC. The RCP of \(^{99m}\text{Tc-complex of Gly-NC100194}\) prepared in this way was determined to be more than 95% by HPLC.

**Biodistribution of \(^{99m}\text{Tc-Complex of Gly-NC100194}\.** Male Wistar outbred rats [Crl: (WI) BR] weighing between 180 and 260 g were injected with a bolus of the biogenerated \(^{99m}\text{Tc-complex of Gly-NC100194}\) (50 MBq) via a lateral tail vein under light anesthesia with halothane (6% in O\(_2\)). After

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**TABLE 1**

**HPLC gradient conditions**

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<th>Time</th>
<th>Percentage Solvent A</th>
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injection, animals were individually housed in metabolism cages to allow separate collection of voided urine and feces. Three males were sacrificed at 2, 10, and 20 min, and three were killed 1, 4, and 7 h after injection.

Animals were killed by cervical dislocation under halothane anesthesia. Each carcass was weighed, and after dissection the percentage injected dose (% id) in the tissues and organs was determined by assay for radioactivity in a twin crystal automatic gamma counter. Blood, bone, muscle, skin, and fat samples were collected into preweighed containers and assayed for radioactivity; appropriate body composition factors (blood 5.8% b.wt., bone 5% b.wt., muscle 43% b.wt., skin 18% b.wt., and fat 7% b.wt.) were applied to data for these tissue samples to calculate the % id in the entire tissue. All the other tissues, organs, and excreta as listed in Table 3 were collected intact at dissection, and the % id was calculated as a percentage of the total radioactive dose administered.

In Vitro Plasma Clot Assay. 99mTc-complex of Gly-NC100194, 99mTc-NC100668, and 99mTc-DTPA were each added to separate plasma samples obtained from rat, which were anticoagulated with 100 mM sodium citrate (10% v/v) (Sigma). Aliquots of 0.4 ml of plasma were then added to P7 vials (Patterson Scientific, Luton, UK) containing either 0.84 ml of 110 mM calcium chloride (Sigma) with 40 units of bovine thrombin (Sigma) in a 50 mM tris(hydroxymethyl)aminomethane buffer containing 0.1% (v/v) Tween 20 detergent (Sigma), and the vial contents were dried on nitrocellulose filters with a pore diameter of 0.45 μm (Whatman, Maidstone, UK) over a vacuum manifold (Millipore, Watford, UK). Before use, these filters were pretreated by incubating them with 1.5% (w/v) bovine serum albumin (Sigma) overnight in a refrigerator at 2 to 8°C to minimize nonspecific binding.

The radioactivity present on these dried filters and in plasma counting standards was then measured using a sodium iodide gamma scintillation counter (PerkinElmer).

The plasma clot uptake (resulting from either specific or nonspecific incorporation) was expressed as a percentage of the total amount of each test substance added to each vial after subtraction of nonspecific binding to the filter observed in the non-clot-forming buffer.

Analysis of Chromatographic Data. The chromatographic data obtained using the radiochemical detector was processed using either Gilson Unipoint version 2.1 or Dionex Chromelone version 6 software.

Results

In Vitro Stability of 99mTc-NC100668. Three 99mTc-labeled species in addition to the test substance were produced by the incubation of 99mTc-NC100668 in rat blood, rat plasma, and saline (Fig. 2). The major peak present, with a retention time of approximately 21 min, corresponded to 99mTc-NC100668. The test substance was more stable in saline than in whole rat blood. After 90-min incubation, less than 10% of the radioactivity present in saline and plasma had been metabolized compared with 30% in rat blood.

The major species produced in plasma and saline was 99mTcO4–, with a retention time of approximately 3 min. Its identity was confirmed by a retention time identical to that of authentic sodium pertechnetate (99mTcO4–). There was also evidence of the presence of the 99mTcO4– ion in rat blood. The identity of the peak at approximately 10 min in whole blood corresponded to the 99mTc-complex of Gly-NC100194. The remaining 99mTc-labeled species observed in whole blood, with a retention time of 19 min, has not been identified.

In contrast, there was much less evidence of any metabolism occurring in human blood or plasma. After 90-min incubation, the amount of radioactivity associated with 99mTc-NC100668 decreased by less than 3 and 1%, respectively (data not shown).

FIG. 2. HPLC chromatograms of 99mTc-NC100668 after incubation in saline after 5 (A) and 60 (B) min of incubation; rat plasma after 5 (C) and 90 (D) min of incubation; rat blood after 5 (E) and 90 (F) min of incubation. All the incubations were performed at 37°C, and the plasma and blood samples were anticoagulated with sodium citrate. The saline samples (A and B) were analyzed on a separate system to the plasma (C and D) or blood (E and F) samples, hence the slight discrepancy in retention times.

In Vitro Metabolism of 99mTc-NC100668. The rate of turnover of 99mTc-NC100668 was faster in rat than in humans (Fig. 3); after 90-min incubation, less than 4% of the test substance remained in rat hepatic S9 compared with 52% in human hepatic S9. The major metabolite present in rat hepatic S9 fraction after 90 min was identified as 99mTc-complex of Gly-NC100194 (retention time, approximately 10 min). Three additional metabolites were observed in human and rat hepatic S9, with retention times of between 11 and 19 min. With the exception of the metabolite that eluted after approximately 15 min, the remaining species were all the minor components of the matrix; individually, each species represented no more than 2% of the radioactivity present. None of these species were observed in vivo; they most likely arise because of the partial proteolytic cleavage of the test substance. The metabolic profile produced when 99mTc-NC100668 was incubated with hepatic S9 in the absence of β-NADPH was identical to that seen in the presence of β-NADPH.

Hydrolysis of 99mTc-NC100668 by Trypsin and Chromatography with 99mTc-NC100194. The major species present after the exposure of 99mTc-NC100668 to trypsin had a retention time similar to the metabolites found in urine (Fig. 4). Furthermore, when authentic metabolites from rat urine were cochromatographed with the trypsin-cleaved material, the major urinary metabolite was observed to coelute with the major species produced as a consequence of the trypsin cleavage of 99mTc-NC100668.
When authentic $^{99m}$Tc-NC100194 was cochromatographed with $^{99m}$Tc-NC100668 that had been treated with trypsin, it was observed that the trypsin metabolite and $^{99m}$Tc-NC100194 species could still be distinguished from each other.

Confirmation of the Identity of the Major Metabolites of $^{99m}$Tc-NC100668 Using the Short HPLC Method. It was possible to resolve the authentic standards (99mTc-complex of Gly-NC100194 and $^{99m}$Tc-NC100194) when a mixture of the two was analyzed using the short HPLC method (Fig. 5). $^{99m}$Tc-NC100194 had a retention time of approximately 7 min compared with 7.5 min for $^{99m}$Tc-complex of Gly-NC100194. The peaks that eluted before the two standards corresponded to $^{99m}$TcO$_4^-$ and $^{99m}$Tc-MDP left over from the radiolabeling reaction.

Using this HPLC method, it was possible to confirm that the major metabolite present after the hydrolysis of $^{99m}$Tc-NC100668 in the presence of trypsin coresolved with authentic $^{99m}$Tc-complex of Gly-NC100194 and not $^{99m}$Tc-NC100194 as the two metabolites present in urine was confirmed by cochromatography using authentic standards on the same system (Fig. 6, D–F).

In Vivo Metabolism of $^{99m}$Tc-NC100668. The predominant $^{99m}$Tc-labeled metabolite in plasma, urine, and bile was $^{99m}$Tc-complex of Gly-NC100194 (Figs. 7 and 8). $^{99m}$Tc-NC100668 was present in plasma at all the time points up to 90 min after injection, but only in bile up to 30 min after injection.

In the urine and bile samples, a second $^{99m}$Tc-labeled species, identified as $^{99m}$Tc-complex of Gly-NC100194 by cochromatography with authentic standard (Fig. 4F), was observed to elute immediately adjacent to $^{99m}$Tc-complex of Gly-NC100194. The major species in bile and urine was the $^{99m}$Tc-complex of Gly-NC100194, accounting for more than 95% of the radioactivity in urine at all the time points and 90% of the radioactivity in bile, apart from the 0- to 15-min postinjection collection period.

$^{99m}$Tc-NC100668 was stable in bile and urine. During 90 min of incubation, very little degradation of the test substance was observed. The only species observed to grow over time was $^{99m}$TcO$_4^-$. Time (Minutes) Relative Intensities

FIG. 3. HPLC chromatograms of $^{99m}$Tc-NC100668 after incubation in rat hepatic S9 after 5 (A), 45 (B), and 90 (C) min of incubation, as well as human hepatic S9 after 5 (D), 45 (E), and 90 (F) min of incubation. The HPLC system was poorly primed for run B, hence the slight discrepancy in retention time compared with runs A and B through to F inclusive.

FIG. 4. Authentic sodium pertechnetate (99mTc) standard (A), HPLC profiles of $^{99m}$Tc-complex of Gly-NC100194 from rat urine (B), and $^{99m}$Tc-NC100668 after exposure to trypsin (C). Authentic $^{99m}$Tc-NC100194 standard (D), $^{99m}$Tc-NC100668 after exposure to trypsin cochromatographed with a similar amount of the authentic metabolite obtained from rat urine (E), and $^{99m}$Tc-NC100668 after exposure to trypsin cochromatographed with a similar amount of $^{99m}$Tc-NC100194 (F).

In Vitro Plasma Clot Assay. The percentage uptake of $^{99m}$Tc-complex of Gly-NC100194 (3 ± 1%) into the rat-forming clot assay was far less than that for $^{99m}$Tc-NC100668 (43 ± 1%) and comparable with the value obtained for $^{99m}$Tc-DTPA, the negative control (2 ± 1%).
Discussion

This study has shown that the metabolites of $^{99m}$Tc-NC100668 were $^{99m}$Tc-complex of Gly-NC100194, $^{99m}$Tc-NC100194, and TcO$_4^-$\textsuperscript{−}. 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 humans is likely to be similar to that observed in rat in vivo. The fact that the metabolism of $^{99m}$Tc-NC100668 in hepatic S9 is not affected by the absence of β-NADPH suggests that the test substance is not metabolized by cytochrome P450.

The identity of the major metabolite, the $^{99m}$Tc-complex of Gly-NC100194, was determined from the findings that this metabolite had a retention time similar, but not identical, to that of $^{99m}$Tc-NC100194, suggesting that the two molecules have similar structures. The plasma clot binding assay indicated that there were no groups available to this metabolite to permit FXIIIa-mediated incorporation into the forming plasma clot. This fact suggested that the lysine residue at position 12 was absent, as other small molecules with free NH$_2$ groups (e.g., dansylcadaverine) have shown themselves to be effective FXIIIa substrates (Hantagan, 1982). The protease trypsin cleaves to the C terminus of basic amino acids; in the case of $^{99m}$Tc-NC100668, a potential cleavage site exists after the lysine residue at position 12. After hydrolysis of $^{99m}$Tc-NC100668 with trypsin in vitro, only a single radiolabeled species was observed, which did not coresolve with $^{99m}$Tc-NC100194. By implication, the most likely identity of this metabolite is the $^{99m}$Tc-complex of Gly-NC100194. Finally, it has been shown that the major urinary metabolite of the NC100668 ligand is Gly-NC100194 (Skotland et al., 2006). The identity of the major metabolite was then confirmed based on the cochromatography with an authentic $^{99m}$Tc-complex of Gly-NC100194 standard using a second HPLC method (the short method).

Given the difference in charge and lipophilicity between NC100668 and $^{99m}$Tc-NC100668, which would occur as a result of $^{99m}$Tc chelation, it was possible that the metabolism of $^{99m}$Tc-NC100668 might not be identical to that of NC100668. If the radiolabeled metabolites were not characterized, then there was always the possibility that they could be retained in an unrelated pathology, with the consequence for
a false-positive diagnosis being made. In this case, it appears from the similarity of the metabolic profile of 99mTc-complex of Gly-NC100194 and NC100668 that both entities are metabolized by the same trypsin-like proteases that are abundant in blood and kidney brush border (Skotland et al., 2006).

In addition, small amounts of 99mTc-NC100194 were observed in urine and bile after the administration of 99mTc-NC100668 to rat. After i.v. administration of NC100668, small amounts of NC100194 were also observed in urine (Skotland et al., 2006). The occurrence of NC100194 was ascribed to the metabolism of Gly-NC100194 by alanyl aminopeptidase (EC.3.4.11.2) in the kidneys. The presence of 99mTc-NC100194 would suggest that 99mTc-complex of Gly-NC100194 is a substrate for this enzyme.

The biodistribution of the 99mTc-complex of Gly-NC100194 in rat indicates that it was rapidly removed from the body, principally by urinary excretion. The values quoted in this study are in fact slightly exaggerated because the biodistribution data are not corrected for the residual radioactivity in the blood contained within the tissues. Therefore, the actual amount of radioactivity associated with each of these organs will be slightly lower than stated here.

The disposition of 99mTcO4− has been studied previously (McElvany et al., 1981); 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-NC100668 are rapid (Edwards et al., 2006). Following i.v. administration to rat, 99mTc-NC100668 is rapidly removed from all the tissues apart from liver and kidneys (3% id and 17% id at 1 h after injection, respectively), with elimination primarily into the urine (66% id at 1 h after injection).

The biodistribution data indicate that the presence of either the 99mTc-complex of Gly-NC100194 or TcO4− in vivo is unlikely to adversely affect the ability of 99mTc-NC100668 to image blood clots. The rapid clearance of 99mTc-complex of Gly-NC100194 with no obvious sites of retention indicates that its presence is unlikely to give rise to a false-positive diagnosis or an adverse impact on the radiation dosimetry.

A number of other minor radiolabeled species were observed in vitro; most likely, they are the product of protease activity and chemical degradation of 99mTc-NC100668. These species were only observed after prolonged incubation and are not physiologically relevant given the rapid elimination of 99mTc-NC100668 in vivo. In vitro in bile, urine, and saline, the only species observed to grow over time was 99mTcO4−, this being most likely a consequence of radiochemical degradation.

The RCP of the preparations used in this study were at least 85%. However, inspection of Fig. 2 suggests that RCP was in fact higher. The major impurities of the 99mTc-NC100668 labeling reaction, the kinetic 99mTc-complex of NC100668 and 99mTc-MDP, are in fact intermediates of the final 99mTc-labeled product. The kinetic 99mTc-complex of NC100668 is rapidly converted to the final thermodynamic product on heating. The kinetic 99mTc-complex of NC100668 and the final product are believed to be different arrangements of the technetium-oxime complex. 99mTc-MDP is present because MDP is incorporated into the kit as Sn2+ solubilizer (Sn2+ is present to reduce the Tc ion to the correct oxidation state to permit chelation to NC100668). MDP also stabilizes the reduced Tc atom long enough to allow incorporation into the NC100668 molecule. Most likely, the act of adding the test compound to prewarmed incubation mixture drives allowing incorporation into the NC100668 molecule. Most likely, the act of adding the test compound to prewarmed incubation mixture drives allowing incorporation into the NC100668 molecule.
the test system. In addition, there are potential problems associated with the extraction and concentration of short-lived gamma-emitting isotopes (radioactive decay to other elements and radiolytic decomposition). Until these issues can be satisfactorily resolved, other methods, such as cochromatographic identification with authentic standards, must be used to identify the radioactive metabolites associated with novel radiotracers like $^{99m}$Tc-NC100668.

In conclusion, this study has identified that the major radiolabeled metabolite of $^{99m}$Tc-NC100668 in vivo in rat is the $^{99m}$Tc-complex of Gly-NC100194. This metabolite is also present in human hepatic S9 after incubation with $^{99m}$Tc-NC100668, suggesting that this metabolite would also be present in humans after administration of $^{99m}$Tc-NC100668. Biodistribution and plasma clot uptake studies have shown that the $^{99m}$Tc-complex of Gly-NC100194 will not have a negative impact on the clinical utility of $^{99m}$Tc-NC100668.

Acknowledgments. We thank Drs. B. Higley, R. Pickett, S. McCully, T. Skotland, and K. Toft for their help and advice in preparing this article.

References