

The *in vivo* and *in vitro* metabolic profile of  $^{99m}\text{Tc}$ -NC100668, a new tracer for imaging venous thromboembolism: Identification and biodistribution of the principal radiolabeled metabolite

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List of non-standard abbreviations:  $\beta$ -NADPH,  $\beta$ -nicotinamide adenine nucleotide phosphate (reduced form); b.wt, body weight; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; FXIIIa, Factor XIIIa; GBq, Gigabecquerel; hepatic S9, a liver homogenate rich cytochrome P450; HPLC, high performance chromatography; pi, post injection; ITLC, instant thin layer chromatography; ip, intraperitoneal; iv, intravenous; MDP, methylene diphosphonic acid; MS, mass spectrometry; %id, percent of injected dose; RCP, radiochemical purity; RHT, reduced hydrolysed technetium; SD, standard deviation;  $\text{TcO}_4^-$ , pertechnetate ion; TFA, trifluoroacetic acid

## ABSTRACT

$^{99m}\text{Tc}$ -NC100668 is a radiopharmaceutical imaging agent being developed to aid the diagnosis of thromboembolism. The stability profile of  $^{99m}\text{Tc}$ -NC100668 was investigated by HPLC after *in vitro* exposure to blood and plasma obtained from rat and man as well as urine and bile obtained from rat. The metabolic profile of  $^{99m}\text{Tc}$ -NC100668 exposed to human and rat hepatic S9 was also studied. The profile of  $^{99m}\text{Tc}$ -labeled species in plasma, urine and bile was investigated following intravenous administration of  $^{99m}\text{Tc}$ -NC100668 to rat. The major species observed *in vitro* and *in vivo* consisted of the  $^{99m}\text{Tc}$ -chelator (NC100194) attached to the C-terminal amino acid residue and referred to as  $^{99m}\text{Tc}$ -complex of Gly-NC100194. The identity of the major metabolite was confirmed by co-chromatography with an authentic standard and the genuine metabolite using a second HPLC method. The minor metabolites were sodium pertechnetate ( $^{99m}\text{Tc}$ ) and  $^{99m}\text{Tc}$ -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 towards plasma clot was established using a plasma clot forming assay. A minor uptake of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 in the plasma clot and a rapid removal from the body were noted. In conclusion the metabolites of  $^{99m}\text{Tc}$ -NC100668 are not anticipated to have a negative impact on the ability of the test substance to image blood clots.

(INTRODUCTION)

$^{99m}\text{Tc}$ -NC100668 is currently being evaluated as an *in vivo* diagnostic marker of active thromboembolic disease (Edwards et al., 2006). Rapid diagnosis is essential as 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'Neil et al., 2004). NC100668 (Figure 1a) is based on a linear peptide with homology to the N-terminal sequence of  $\alpha_2$ -antiplasmin, a known FXIIIa substrate (Lijnen et al., 1987). FXIIIa catalyses the formation  $\epsilon$ -( $\gamma$ -glutamyl)lysyl bonds that hold the fibrin clot together (Gorman et al., 1984).

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

Due to the short radioactive half-life of  $^{99m}\text{Tc}$ -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  $^{99m}\text{TcO}_4^-$  obtained from a  $^{99}\text{Mo}/^{99m}\text{Tc}$  generator. In order to ensure that the radiolabeling process achieves the highest RCP possible; the ligand is present in vast excess in relation to the amount of  $^{99m}\text{TcO}_4^-$  added. Typically less than 1% of the ligand present is actually bound to  $^{99m}\text{Tc}$  following reconstitution. The NC100668 kits are all formulated with 100  $\mu\text{g}$  ligand, therefore in a reconstituted vial less than 1  $\mu\text{g}$  is actually chelated to  $^{99m}\text{Tc}$ .

Changes in the physicochemical properties (net charge, lipophilicity etc) of a molecule occur following chelation of  $^{99m}\text{Tc}$ . These changes can affect the biological behavior of the molecule relative to its unchelated analogue. For example, the dopamine transporter tracer, TRODAT, can not cross the blood brain barrier whereas  $^{99m}\text{Tc}$ -chelated TRODAT can (Kung et al., 1997). These

changes may also alter the affinity and selectivity of a  $^{99m}\text{Tc}$ -chelated ligand for metabolizing enzymes relative to its unchelated counterpart. Therefore, the radiolabeled metabolites present must be characterized independently of the unlabeled metabolites in order 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 can not be addressed by conventional LC-MS techniques alone.

The purpose of this study was to investigate the *in vivo* metabolism of  $^{99m}\text{Tc}$ -NC100668 following iv injection to rats and to identify the major radioactive metabolites present by co-chromatography with authentic potential metabolites. The *in vitro* stability of  $^{99m}\text{Tc}$ -NC100668 was studied in whole blood and plasma as well as hepatic S9 of rat origin and compared with that obtained from samples of human origin. The identity of the major metabolite was confirmed by co-chromatography with an authentic standard using a separate HPLC method. Finally, the impact of the presence of the major  $^{99m}\text{Tc}$ -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 (Acetyl-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr(3-iodo)-Thr-Leu-Leu-Lys-Gly-NC100194), NC100194 (N,N-Bis(N-(1,1-dimethyl-2-(hydroxylimino)propyl)aminoethyl)aminoethylamine) and Gly-NC100194 were supplied by GE Healthcare (Little Chalfont, UK). Each kit contained approximately 50 nmoles of ligand plus buffer, reductants and radiostabilisers. These excipients facilitated the radiolabeling of the test substance following reconstitution with 2-5 ml sodium pertechnetate ( $^{99m}\text{Tc}$ ) solution, containing approximately 1-2.5 GBq  $^{99m}\text{Tc}$  obtained from a Drytec<sup>TM</sup> or Amertec<sup>TM</sup> II  $^{99m}\text{Tc}$  Sterile Generator (GE Healthcare). 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.

After cooling, the RCP of each preparation was determined by reverse phase HPLC and silica gel ITLC. HPLC (Gilson, Luton, UK) was performed by applying aliquots of each preparation to a 250 mm x 4.6 mm (5 $\mu\text{m}$ ) Luna C<sub>18(2)</sub> column (Phenomenex, Macclesfield, UK) which was eluted with 0.05% 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 minutes at a flow rate of 1 ml/min.  $^{99m}\text{Tc}$ -labeled species were detected post-column 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, Poole, UK) and 1 M ammonium acetate (VWR International). The strips were allowed to dry and were scanned on a Packard Instantimager (Perkin Elmer, Beaconsfield, UK). The image produced was integrated around the origin to give the amount of RHT ( $^{99m}\text{Tc}$ ) in the preparation. All other radiolabeled species moved with the solvent front.

All  $^{99m}\text{Tc}$ -NC100668 preparations had a RCP of greater than 85%. The major impurity of

$^{99m}\text{Tc}$ -NC100668 was the kinetic  $^{99m}\text{Tc}$ -complex of NC100668 which accounted for at least 6% of the radioactivity present. Under radiolabeling conditions the kinetic  $^{99m}\text{Tc}$ -complex of NC100668 was rapidly formed. Heat was used to convert most of this through to the thermodynamic complex,  $^{99m}\text{Tc}$ -NC100668. The kinetic  $^{99m}\text{Tc}$ -complex of NC100668 is believed to be a different arrangement of the technetium amine-oxime complex. The remaining radiochemical impurities were RHT ( $^{99m}\text{Tc}$ ) (approximately 1%),  $^{99m}\text{Tc}$ -MDP and hydrolytic decomposition products of  $^{99m}\text{Tc}$ -NC100668. The RCP of the  $^{99m}\text{Tc}$ -NC100194 preparations used was at least 45% and 85% for the  $^{99m}\text{Tc}$ -complex of Gly-NC100194 preparations used. The remaining radioactivity in these preparations was associated with  $^{99m}\text{Tc}$ -MDP and  $^{99m}\text{TcO}_4^-$ .

$^{99m}\text{Tc}$ -DTPA was prepared by addition of 5 ml sodium pertechnetate ( $^{99m}\text{Tc}$ ) solution, containing 1 GBq of  $^{99m}\text{Tc}$ , to a commercially available labeling kit containing calcium trisodium diethylenetriaminepentaacetate (GE Healthcare). Each preparation was allowed to stand for 30 minutes before RCP analysis by silica gel ITLC according to the manufacturer's instructions. The  $^{99m}\text{Tc}$ -DTPA preparations used in this study had an RCP of at least 94% before use. All other chemicals were of analytical grade quality.

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

$^{99m}\text{Tc}$ -NC100668 (25 MBq) was dispensed into pre-warmed 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 minutes of incubation. All incubations were performed in duplicate.

***In Vitro* Metabolism of  $^{99m}\text{Tc}$ -NC100668.** A total 20  $\mu\text{l}$  of a 10 mg/ml  $\beta$ -NADPH (Sigma)

solution and 10 µl of a 20 mg/ml protein S9 fraction (Xenotech, Lenexa, Ka) obtained from either rat or human liver was added to 1.69 ml 50 mM tris(hydroxymethyl)hydrogen chloride buffer (Sigma) pH adjusted to 7.4 and pre-warmed to 37°C. <sup>99m</sup>Tc-NC100668 (10 MBq) was then added. The hepatic S9 mixture was placed in a shaking 37°C water bath. Samples were removed immediately following the addition of <sup>99m</sup>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.

Cytochrome P450 independent metabolism was investigated by incubating <sup>99m</sup>Tc-NC100668 in rat and human hepatic S9 fractions deficient in β-NADPH over 90 minutes at 37°C.

**Hydrolysis <sup>99m</sup>Tc-NC100668 by Trypsin.** Immobilised trypsin beads (200 units/ml) (Perbio Science, Cramlington, UK) were prepared for use by washing 3 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 in a bench top centrifuge for 10 minutes. After the third wash the trypsin bead pellet was re-suspended in 1 ml of 20 mM calcium chloride. A 50 µl 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 <sup>99m</sup>Tc-NC100668 (50 MBq). The mixture was incubated for 60 minutes at 37°C before analysis.

***In vivo* Metabolism of <sup>99m</sup>Tc-NC100668.** Six male Wistar outbred rats (CrI: (WI) BR) (Charles River, Margate, UK) weighing between 180 and 260 g at the time of study, were injected with <sup>99m</sup>Tc-NC100668 (50 MBq) via the lateral tail vein, under light anaesthesia with halothane (Merial, Harlow, UK) (6% in O<sub>2</sub>). The animals were then deeply anaesthetised following the administration of urethane (150 mg/kg) (Sigma) by ip injection. This level of anaesthesia was maintained by further ip injection of urethane as required. Three animals were killed by cervical dislocation 5 minutes pi and three animals 60 minutes pi.

At the time of sacrifice, the abdomen of each animal was opened and 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 a 25 gauge hypodermic needle, containing sodium citrate solution. After the blood sample had been collected a urine sample was taken by aspiration of the bladder using a separate needle and syringe.

A third group of 3 adult male Wistar rats was lightly sedated with halothane (6% in O<sub>2</sub>) and then deeply anaesthetised by an ip injection of urethane. The right femoral vein was exposed and cannulated with a 25 gauge needle attached to a 5 cm length of tubing, with an internal diameter of 0.4 mm and external diameter of 1.0 mm (Portex, Hythe, UK).

A portion of the bile duct approximately 0.5 cm from the liver was exposed after laparotomy. This was then cannulated using tubing with an internal diameter of 0.5 mm and an external diameter of 1.0 mm (Portex). The viscera of each animal were laid to one side and wrapped in damp gauze. For the duration of the study the gauze was kept moist by the regular application of 0.9% saline (Fresenius, Warrington, UK). Bile was allowed to flow for 5 to 10 minutes in order to ensure the patency of the cannula.

Following the preparation period, <sup>99m</sup>Tc-NC100668 (50 MBq) was administered via the femoral cannula. Bile samples were collected through the bile duct cannula over 3 collection periods: 0 to 15 minutes, 16 to 45 minutes, and 46 to 90 minutes pi.

**Preparation of <sup>99m</sup>Tc-Labeled Samples for HPLC Analysis.** Samples of blood were centrifuged for 20 minutes to remove the blood cells. Once the red blood cells had been removed, the blood samples were treated as plasma samples. The plasma and hepatic S9 samples were added to ice cold acetonitrile. A total of 200 µl sample was added to 800 µl acetonitrile. This was followed by centrifugation at 10 000 rpm with a fixed angle bench top centrifuge for 10 minutes to sediment precipitated protein. The samples of bile and urine were collected and centrifuged in a bench top centrifuge for 5 minutes at 10 000 rpm prior to analysis by HPLC. The supernatants were separated from the precipitated material, 100 µl of which was diluted with an appropriate

volume of de-ionized water such that approximately 50 KBq of radioactivity could immediately be analyzed by HPLC with an on-line radiochemical detector (Perkin Elmer).

**HPLC Analysis of  $^{99m}\text{Tc-NC100668}$  Metabolites.** HPLC was used to analyse the radiochemical profile of the metabolites of  $^{99m}\text{Tc-NC100668}$ . In the initial studies radiolabeled species were separated on a VYDAC C<sub>18</sub> (250 mm x 4.6 mm, 5  $\mu\text{m}$ ) HPLC column (Hichrom, Reading, UK). A C<sub>18</sub> 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/minute. 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.

Co-chromatography studies were performed in order to identify the principal metabolite present. In these experiments processed urine samples containing the major metabolite were combined with either  $^{99m}\text{Tc-NC100194}$  or  $^{99m}\text{Tc-complex}$  of Gly-NC100194 (produced by the trypsin mediated proteolysis of  $^{99m}\text{Tc-NC100668}$ ). 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 if peaks due to the genuine metabolite and the peaks due to 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 co-elute with the authentic standard of  $^{99m}\text{Tc-complex}$  of Gly-NC100194. This method used a 250 mm x 4.6 mm (5 $\mu\text{m}$ ) Luna C<sub>18(2)</sub> 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 minutes at a flow rate of 1 ml/min.

$^{99m}\text{Tc}$ -labeled species were detected post-column using a Bioscan radiodetector.

**Biogenesis of  $^{99m}\text{Tc}$ -Complex of Gly-NC100194.** Eight male Wistar rats were lightly anaesthetised with halothane (6% in  $\text{O}_2$ ) and injected ip 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  $^{99m}\text{Tc}$ -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 following 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 8 animals was pooled. The radioactive content of the pooled urine was determined using an Ion Chamber (Capintec, Ramsey, NJ) and the radiochemical composition 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 (CrI: (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 anaesthesia with halothane (6% in  $\text{O}_2$ ). After injection, animals were individually housed in metabolism cages to allow separate collection of voided urine and faeces. Three males were sacrificed at 2, 10 and 20 minutes, and 1, 4 as well as 7 hours 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 was determined by assay for radioactivity in a twin crystal automatic gamma counter. Blood, bone, muscle, skin and fat samples were collected into pre-weighed 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 in order 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 calculated as a percentage of the total radioactive dose administered.

***In Vitro Plasma Clot Assay.***  $^{99m}\text{Tc}$ -complex of Gly-NC100194,  $^{99m}\text{Tc}$ -NC100668 and  $^{99m}\text{Tc}$ -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 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 (Sigma) (the plasma clot-forming buffer) or 0.84 ml 50 mM tris(hydroxymethyl)aminomethane buffer only (the non-clot-forming buffer). Equivalent volumes of plasma, each spiked with an identical amount of each test substance, were also dispensed into P7 vials to act as counting standards.

After incubation of both the clot forming and non-clot forming mixtures at ambient temperature for 1 hour, the clotting reaction was terminated by the addition of 1 ml of 0.4 M EDTA (VWR International). Each vial was washed with 50 mM tris(hydroxymethyl)aminomethane buffer containing 0.1% (v/v) Tween 20 detergent (Sigma) and the vial contents dried on nitrocellulose filters with a pore diameter of 0.45  $\mu\text{m}$  (Whatman, Maidstone, UK) over a vacuum manifold (Millipore, Watford, UK). Before use these filters were pre-treated, by incubating them with 1.5% (w/v) bovine serum albumin (Sigma) overnight in a refrigerator at 2 to 8°C, in order to minimize non-specific binding.

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

The plasma clot uptake (due to either specific or non-specific incorporation) was expressed as a percentage of the total amount of each test substance added to each vial, after subtraction of

non-specific 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 Chromeleon version 6 software.

## Results

***In Vitro* Stability of  $^{99m}\text{Tc-NC100668}$ .** Three  $^{99m}\text{Tc}$ -labeled species in addition to the test substance were produced by the incubation of  $^{99m}\text{Tc-NC100668}$  in rat blood, rat plasma and saline (Figure 2). The major peak present, with a retention time of approximately 21 minutes corresponded to  $^{99m}\text{Tc-NC100668}$ . The test substance was more stable in saline and rat plasma than in whole rat blood. After 90 minutes 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  $^{99m}\text{TcO}_4^-$ , with a retention time of approximately 3 minutes. Its identity was confirmed by a retention time identical to that of authentic sodium pertechnetate ( $^{99m}\text{Tc}$ ). There was also evidence of the presence of the  $^{99m}\text{TcO}_4^-$  ion in rat blood. The identity of the peak at approximately 10 minutes in whole blood corresponded to the  $^{99m}\text{Tc}$ -complex of Gly-NC100194. The remaining  $^{99m}\text{Tc}$ -labeled species observed in whole blood, with a retention time of 19 minutes, has not been identified.

In contrast, there was much less evidence of any metabolism occurring in human blood or plasma. Following 90 minutes incubation the amount of radioactivity associated with  $^{99m}\text{Tc-NC100668}$  dropped by less than 3 and 1% respectively (data not shown).

***In Vitro* Metabolism of  $^{99m}\text{Tc-NC100668}$ .** The rate of turnover of  $^{99m}\text{Tc-NC100668}$  was faster in rat than in man (Figure 3), after 90 minutes 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 minutes was identified as  $^{99m}\text{Tc}$ -complex of Gly-NC100194 (retention time approximately 10 minutes). Three additional metabolites were observed in both human and rat hepatic S9, with retention times of between 11 and 19 minutes. With the exception of the metabolite that eluted after approximately 15 minutes the remaining species were all 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 due to the

partial proteolytic cleavage of the test substance. The metabolic profile produced when  $^{99m}\text{Tc-NC100668}$  was incubated with hepatic S9 in the absence of  $\beta\text{-NADPH}$  was identical to that seen in the presence of  $\beta\text{-NADPH}$ .

**Hydrolysis of  $^{99m}\text{Tc-NC100668}$  by Trypsin and Co-Chromatography with  $^{99m}\text{Tc-NC100194}$ .** The major species present following the exposure of  $^{99m}\text{Tc-NC100668}$  to trypsin had a retention time similar to the metabolites found in urine (Figure 4). Furthermore, when authentic metabolites from rat urine were co-chromatographed with the trypsin cleaved material, the major urinary metabolite was observed to co-elute with the major species produced as a consequence of the trypsin cleavage of  $^{99m}\text{Tc-NC100668}$ .

When authentic  $^{99m}\text{Tc-NC100194}$  was co-chromatographed with  $^{99m}\text{Tc-NC100668}$  which had been treated with trypsin, it was observed that the trypsin metabolite and  $^{99m}\text{Tc-NC100194}$  species could still be distinguished from each other.

**Confirmation of the identity of the major metabolites of  $^{99m}\text{Tc-NC100668}$  using the short HPLC method.** It was possible to resolve the authentic standards ( $^{99m}\text{Tc}$ -complex of Gly-NC100194 and  $^{99m}\text{Tc-NC100194}$ ) when a mixture of the two were analyzed using the short HPLC method (Figure 7).  $^{99m}\text{Tc-NC100194}$  had a retention time of approximately 7 minutes compared with 7.5 minutes for  $^{99m}\text{Tc}$ -complex of Gly-NC100194. The peaks that eluted before the two standards corresponded to  $^{99m}\text{TcO}_4^-$  and  $^{99m}\text{Tc-MDP}$  left over from the radiolabeling reaction.

Using this HPLC method it was possible to confirm that the major metabolite present following the hydrolysis of  $^{99m}\text{Tc-NC100668}$  in the presence of trypsin co-resolved with authentic  $^{99m}\text{Tc}$ -complex of Gly-NC100194 standard and not  $^{99m}\text{Tc-NC100194}$  (Figure 8 A to C). Similarly the presence of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 and  $^{99m}\text{Tc-NC100194}$  as the two metabolites present in urine was confirmed by co-chromatography using authentic standards on the same system (Figure 8 D to F).

**In Vivo Metabolism of  $^{99m}\text{Tc-NC100668}$ .** The predominant  $^{99m}\text{Tc}$ -labeled metabolite in

plasma, urine and bile was  $^{99m}\text{Tc}$ -complex of Gly-NC100194 (Figures 5 and 6).  $^{99m}\text{Tc}$ -NC100668 was present in plasma at all time points up to 90 minutes pi, but only in bile up to 30 minutes pi.

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

$^{99m}\text{Tc}$ -NC100668 was stable in bile and urine. During 90 minutes of incubation very little degradation of the test substance was observed. The only species observed to grow over time was  $^{99m}\text{TcO}_4^-$ .

**Biodistribution of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 in rats.** The clearance of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 from blood was rapid. Only 18% id was retained in the blood by 2 minutes and after 7 hours less than 0.1% id was present (Table 2). There was some uptake of radioactivity into lung (2% id) and heart (1% id) 2 minutes pi. Radioactivity was rapidly lost from these two tissues. Twenty minutes pi the % id retained in these tissues had more than halved.

The distribution of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 to other organs and tissues was rapid with a high % id found in muscle (26% id), skin (21% id) and kidneys (12% id) 2 minutes pi. By 1 hour pi the % id retained within muscle (2% id), skin (3% id), and kidneys (4% id) had significantly declined. By 7 hours pi, only the kidneys (3% id) and liver (4% id) still retained more than 1% id. After 2 minutes, 4% id was detected in the liver. This increased to 6% id by 1 hour, before returning to 4% by 7 hours pi.



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

## Discussion

This study has demonstrated that the metabolites of  $^{99m}\text{Tc}$ -NC100668 were  $^{99m}\text{Tc}$ -complex of Gly-NC100194,  $^{99m}\text{Tc}$ -NC100194 and  $\text{TcO}_4^-$ . The similar metabolic profile of  $^{99m}\text{Tc}$ -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 fact that the metabolism of  $^{99m}\text{Tc}$ -NC100668 in hepatic S9 is not affected by the absence of  $\beta$ -NADPH suggests that the test substance is not metabolized by cytochrome P450.

The identity of the major metabolite, the  $^{99m}\text{Tc}$ -complex of Gly-NC100194 was determined from the findings that this metabolite had a retention time similar, but not identical, to that of  $^{99m}\text{Tc}$ -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  $\text{NH}_2$  groups (e.g. dansylcadaverine) have shown themselves to be effective FXIIIa substrates (Hantagan., 1982). The protease trypsin, cleaves to the C-terminal of basic amino acids, in the case of  $^{99m}\text{Tc}$ -NC100668, a potential cleavage sites exist after the lysine residue at position 12. Following hydrolysis of  $^{99m}\text{Tc}$ -NC100668 with trypsin *in vitro* only a single radiolabeled species was observed, which did not co-resolve with  $^{99m}\text{Tc}$ -NC100194. By implication the most likely identity of this metabolite is the  $^{99m}\text{Tc}$ -complex of Gly-NC100194. Finally, it has been demonstrated 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 on the basis of co-chromatography with an authentic  $^{99m}\text{Tc}$ -complex of Gly-NC100194 standard using a second HPLC method (the short method).

Given the difference in charge and lipophilicity between NC100668 and  $^{99m}\text{Tc}$ -NC100668, which would occur as a result of  $^{99m}\text{Tc}$  chelation, it was possible that the metabolism of

$^{99m}\text{Tc-NC100668}$  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  $^{99m}\text{Tc-NC100668}$  and NC100668 that both entities are metabolized by the same trypsin like proteases that are abundant in blood and kidney brush boarder (Skotland et al., 2006).

In addition small amounts of  $^{99m}\text{Tc-NC100194}$  were observed in urine and bile after the administration of  $^{99m}\text{Tc-NC100668}$  to rat. Following intravenous 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 alanylaminopeptidase (EC.3.4.11.2) in the kidneys. The presence of  $^{99m}\text{Tc-NC100194}$  would suggest that  $^{99m}\text{Tc-complex}$  of Gly-NC100194 is also a substrate for this enzyme.

The biodistribution of the  $^{99m}\text{Tc-complex}$  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 is 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  $^{99m}\text{TcO}_4^-$  has been studied previously (McElvany et al., 1981); following administration,  $^{99m}\text{TcO}_4^-$  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  $^{99m}\text{Tc-NC100668}$  is rapid (Edwards et al., 2006). Following iv administration to rat,  $^{99m}\text{Tc-NC100668}$  is rapidly removed from all tissues apart from liver and kidneys (3% id and 17% id at 1 hour pi respectively), with elimination primarily into the urine (66% id at 1 hour pi).

The biodistribution data indicates that the presence of either the  $^{99m}\text{Tc}$ -complex of Gly-NC100194 or  $\text{TcO}_4^-$  *in vivo* is unlikely to adversely affect the ability of  $^{99m}\text{Tc}$ -NC100668 to image blood clots. The rapid clearance of  $^{99m}\text{Tc}$ -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 adversely impact upon 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  $^{99m}\text{Tc}$ -NC100668. These species were only observed following prolonged incubation and are not physiologically relevant given the rapid elimination of  $^{99m}\text{Tc}$ -NC100668 *in vivo*. *In vitro*, in bile, urine and saline the only species observed to grow over time was  $^{99m}\text{TcO}_4^-$ , 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 figure 2 suggests that RCP was in fact higher. The major impurities of the  $^{99m}\text{Tc}$ -NC100668 labeling reaction, the kinetic  $^{99m}\text{Tc}$ -complex of NC100668 and  $^{99m}\text{Tc}$ -MDP, are in fact intermediates of the final  $^{99m}\text{Tc}$ -labeled product. The kinetic  $^{99m}\text{Tc}$ -complex of NC100668 is rapidly converted to the final thermodynamic product on heating. The kinetic  $^{99m}\text{Tc}$ -complex of NC100668 and the final product are believed to be different arrangements of the technetium-oxime complex.  $^{99m}\text{Tc}$ -MDP is present because MDP is incorporated into the kit as  $\text{Sn}^{2+}$  solubiliser ( $\text{Sn}^{2+}$  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 pre-warmed incubation mixture drives the labeling reaction to completion. As a result initially, the RCP appears higher than was originally stated. The only significant remaining impurity, RHT ( $^{99m}\text{Tc}$ ), is a colloid and as a result any of this material present will be trapped at the head of the HPLC column and will not show up on a HPLC trace.

There are reports in the literature of mass spectrometry being used to elucidate the structures of radiopharmaceuticals (Vanbilloen et al., 2003; Vanderghinste et al., 2003). Despite this it has yet to be demonstrated that it is possible to identify the structures of the radiolabeled metabolites using such technology. This is due to the low chemical concentration of radiopharmaceutical present after administration to 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 co-chromatographic identification with authentic standards, must be used to identify the radioactive metabolites associated with novel radiotracers like  $^{99m}\text{Tc-NC100668}$ .

In conclusion, this study has identified that the major radiolabeled metabolite of  $^{99m}\text{Tc-NC100668}$  *in vivo* in rat is the  $^{99m}\text{Tc}$ -complex of Gly-NC100194. This metabolite is also present in human hepatic S9 following incubation with  $^{99m}\text{Tc-NC100668}$ , suggesting that this metabolite would also be present in man after administration of  $^{99m}\text{Tc-NC100668}$ .

Biodistribution and plasma clot uptake studies have shown that the  $^{99m}\text{Tc}$ -complex of Gly-NC100194 will not have a negative impact upon the clinical utility of  $^{99m}\text{Tc-NC100668}$ .

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## References

- Edwards D, Lewis J, Battle M, Lear R, Farrar G, Barnett DJ, Godden V, Oliveira A, Coombes C  
Ahlström H (2006)  $^{99m}\text{Tc}$ -NC100668, a new tracer for imaging venous thromboemboli:  
Pre-clinical biodistribution and incorporation into plasma clots *in vivo* and *in vitro*. *Eur J  
Nucl Med*. In press.
- Gorman JJ, Folk JE (1984) Structural features of glutamine substrates for transglutaminases.  
Role of extended interactions in the specificity of human plasma factor XIIIa and of the  
guinea pig liver enzyme. *J Biol Chem* 259:9007-9010.
- Hantagan R (1982) Steady-state fluorescence of dansylcadaverine-fibrinogen: evidence for  
flexibility. *Biochemistry* 21:1821-1829.
- Kung MP, Stevenson DA, Plössl K, Meegalla S, Beckwith A, Essman WD, Mu M, Lucki, Kung  
HF (1997) [ $^{99m}\text{Tc}$ ]TRODAT-1: a novel technetium-99m complex as a dopamine transporter  
imaging agent. *Eur J Nucl Med* 24:372-380.
- Lijnen HR, Holmes WE, van Hoef B, Wiman B, Rodriguez H, Collen D (1987) Amino-acid  
sequence of human alpha 2-antiplasmin. *Eur J Biochem* 166:565-574.
- McElvany KD, Wagner SJ, Welch MJ (1981) Radiochemistry and radiopharmacology, in  
Nuclear Medicine: Technology and Techniques (Bernier DR, Langan JK, Wells DL eds)  
pp162-176, Mosby-Year Book Inc Missouri
- O'Neill JM, Wright L, Murchison JT (2004). Helical CTPA in the investigation of pulmonary  
embolism: a 6-year. *Clin Radiol* 59:819-825.
- Skotland T, Hustvedt SO, Oulie I, Jacobsen PB, Friisk GA, Langøy AS, Uran S, Sandosham J,  
Cuthbertson A and Toft KG (2006) NC100668, a new tracer for imaging of venous

thromboembolism: disposition and metabolism in rats. *Drug Metab Dispos* 34:111-120.

Vanbilloen HP, Cleynhens BJ, de Groot TJ, Maes A, Bormans GM, Verbruggen AM (2003) RP-HPLC separation of the diastereomers of technetium-99m labeled tropanes and identity confirmation using radio-LC-MS. *J Pharm Biomed Anal* 32:663-668.

Vanderghinste D, Van Eeckhoudt M, Terwinghe C, Mortelmans L, Bormans GM, Verbruggen AM, VanbilloenHP (2003) An efficient HPLC method for the analysis of isomeric purity of technetium-99m-exametazime and identity confirmation using LC-MS. *J Pharm Biomed Anal* 32:679-685.



**Footnotes**

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### Legend for Figures

FIG.1. *The molecular structures of NC100668 (A), NC100194 (B) and Gly-NC100194 (C).*

*The precise structures of the  $^{99m}\text{Tc}$  complexes have not been elucidated. Therefore, in the interests of accuracy the  $^{99m}\text{Tc}$  atom is not shown.*

FIG. 2. *HPLC chromatograms of  $^{99m}\text{Tc}$ -NC100668 following incubation in saline after 5 (A) and 60 (B) minutes incubation; rat plasma after 5 (C) and 60 (D) minutes incubation as well as rat blood after 5 (E) and 60 (F) minutes incubation. All incubations were performed at 37°C and the plasma and blood samples were anti-coagulated with sodium citrate. The saline samples (A and B) were analysed on a separate system to the plasma (C and D) or blood (E and F) samples, hence the slight discrepancy in retention times.*

FIG. 3. *HPLC chromatograms of  $^{99m}\text{Tc}$ -NC100668 following incubation in rat hepatic S9 after 5 (A), 45 (B) and 90 (C) minutes incubation as well as human hepatic S9 after 5 (D), 45 (E) and 90 (F) minutes 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 ( $^{99m}\text{Tc}$ ) standard (A), HPLC profiles of  $^{99m}\text{Tc}$ -complex of Gly-NC100194 from rat urine (B),  $^{99m}\text{Tc}$ -NC100668 after exposure to trypsin (C). Authentic  $^{99m}\text{Tc}$ -NC100194 standard (D),  $^{99m}\text{Tc}$ -NC100668 after exposure to trypsin co-chromatographed with a similar amount of the authentic metabolite obtained from rat urine (E) and  $^{99m}\text{Tc}$ -NC100668 after exposure to trypsin co-chromatographed with a similar amount of  $^{99m}\text{Tc}$ -NC100194 (F).*

FIG. 5. HPLC chromatograms of blood (A, B, C) and urine samples (D, E, F) obtained following injection of  $^{99m}\text{Tc-NC100668}$  in male Wistar rats. The chromatograms were obtained using blood and urine samples collected 5, 60 and 90 minutes after injection, respectively.

FIG. 6. HPLC chromatograms of bile (A, B, C) obtained following injection of  $^{99m}\text{Tc-NC100668}$  in male Wistar rats. The chromatograms were obtained using bile samples collected 0-15, 16-45 and 46-90 minutes after injection, respectively. HPLC chromatograms of  $^{99m}\text{Tc-NC100668}$  following incubation in rat bile after 0 (D) and 60 (E) minutes incubation. The reduced signal to noise ratio observed in figures 6A, B and C was due to the limited amount of radioactivity collected in bile.

FIG 7. Authentic  $^{99m}\text{Tc-complex of Gly-NC100194}$  and  $^{99m}\text{Tc-NC100194}$  standards co-chromatographed using the short HPLC method.

FIG 8. HPLC chromatography and co-chromatography of the major urinary metabolites present in rat urine or following trypsin mediated hydrolysis of  $^{99m}\text{Tc-NC100668}$  using the short HPLC method. Trypsin cleavage product only (A). Trypsin cleavage and authentic  $^{99m}\text{Tc-complex of Gly-NC100194}$  (B). Trypsin cleavage and authentic  $^{99m}\text{Tc-complex of NC100194}$  (C). Urine metabolites only (D). Rat urine and authentic  $^{99m}\text{Tc-complex of Gly-NC100194}$  (E). Rat urine and authentic  $^{99m}\text{Tc-complex of NC100194}$  (F).

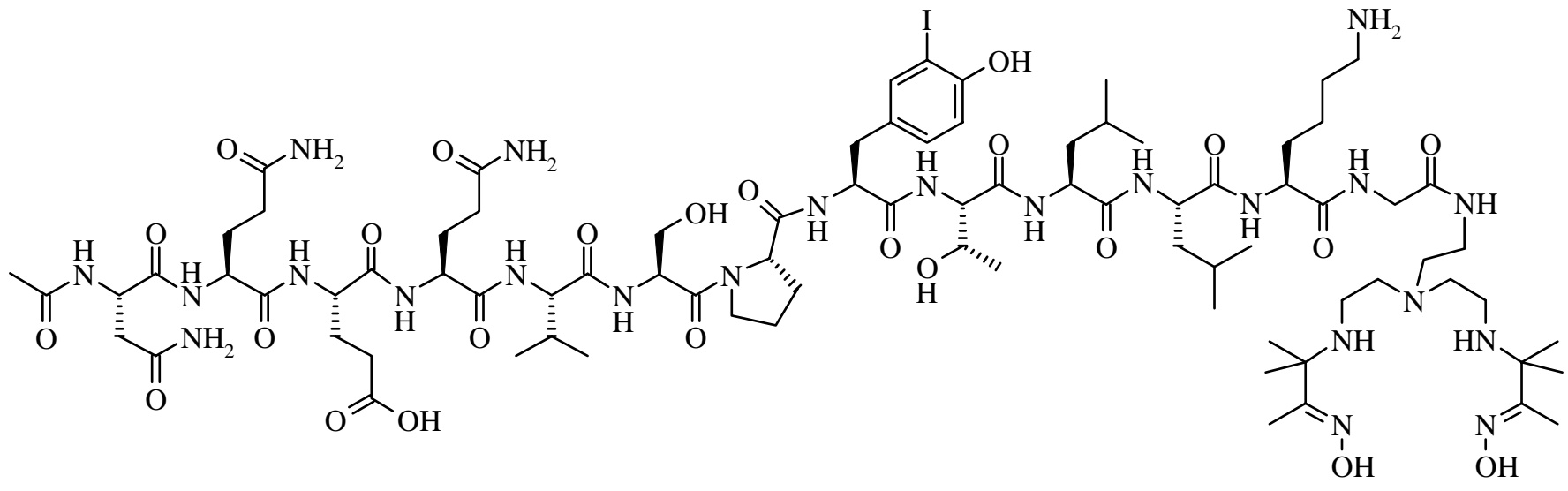
Table 1. *HPLC gradient conditions*

Time (minutes)	% Solvent A	% Solvent B
0	100	0
3	100	0
20	60	40
22	60	40
22.1	50	50
25	50	50
30.1	100	0
35	100	0

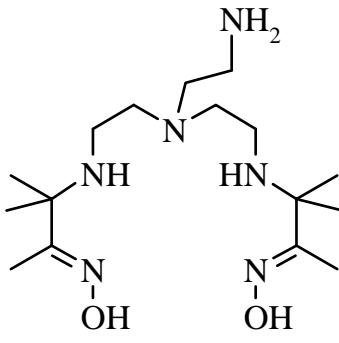
Table 2. *The biodistribution of radioactivity after injection of <sup>99m</sup>Tc-complex of Gly-NC100194 in male Wistar rats. The data are presented as % injected dose in tissues/organs (mean ± SD; n=3).*

Tissue/organ	2 minutes	10 minutes	20 minutes	1 hour	4 hours	7 hours
Bone	3.91 ± 0.36	4.01 ± 0.18	2.03 ± 0.28	0.56 ± 0.07	0.27 ± 0.12	0.23 ± 0.03
Muscle	25.82 ± 1.33	25.84 ± 3.19	10.52 ± 1.78	2.03 ± 1.05	0.41 ± 0.09	0.37 ± 0.05
Blood	17.83 ± 0.87	16.11 ± 0.61	6.24 ± 0.38	1.56 ± 0.10	0.06 ± 0.01	0.04 ± 0.00
Kidneys	11.85 ± 2.36	9.43 ± 7.10	10.04 ± 0.70	4.44 ± 0.50	4.23 ± 0.34	3.44 ± 0.42
Bladder and urine	0.48 ± 0.68	0.55 ± 0.23	43.26 ± 5.21	75.50 ± 1.70	79.54 ± 4.97	80.22 ± 2.14
Lung	1.49 ± 0.27	1.25 ± 0.15	0.49 ± 0.04	0.17 ± 0.03	0.04 ± 0.00	0.03 ± 0.01
Liver	4.36 ± 0.18	4.64 ± 0.45	5.74 ± 0.11	6.25 ± 0.70	4.99 ± 0.87	4.31 ± 0.44
Spleen	0.27 ± 0.02	0.23 ± 0.04	0.10 ± 0.02	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
Stomach	0.83 ± 0.07	0.92 ± 0.08	0.36 ± 0.04	0.08 ± 0.02	0.03 ± 0.01	0.02 ± 0.00
Stomach contents	0.08 ± 0.01	0.12 ± 0.03	0.43 ± 0.44	0.04 ± 0.02	0.09 ± 0.09	0.03 ± 0.02
Small intestine	2.85 ± 0.60	2.46 ± 0.40	1.63 ± 0.27	1.00 ± 0.21	0.34 ± 0.02	0.23 ± 0.03
Small intestine contents	0.54 ± 0.16	0.45 ± 0.07	0.87 ± 0.16	2.59 ± 0.30	1.26 ± 0.43	0.34 ± 0.04
Large intestine	1.49 ± 0.27	0.96 ± 0.59	0.67 ± 0.12	0.16 ± 0.03	0.19 ± 0.03	0.17 ± 0.04
Large intestine contents	0.26 ± 0.10	0.52 ± 0.44	0.18 ± 0.03	0.06 ± 0.02	5.97 ± 3.04	5.23 ± 1.23
Heart	0.48 ± 0.08	0.43 ± 0.05	0.17 ± 0.01	0.05 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Thyroid	0.15 ± 0.04	0.16 ± 0.07	0.08 ± 0.01	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Brain	0.11 ± 0.06	0.08 ± 0.03	0.04 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eyes	0.06 ± 0.01	0.06 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Salivaries	0.43 ± 0.04	0.34 ± 0.01	0.14 ± 0.04	0.03 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
Pancreas	0.49 ± 0.39	0.63 ± 0.36	0.22 ± 0.20	0.07 ± 0.06	0.02 ± 0.02	0.02 ± 0.01
Adrenals	0.05 ± 0.02	0.04 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Testes	0.69 ± 0.37	0.64 ± 0.01	0.39 ± 0.05	0.10 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
Skin	20.62 ± 1.33	23.42 ± 1.81	10.63 ± 0.87	2.95 ± 0.14	0.92 ± 0.25	0.81 ± 0.12
Fat	4.63 ± 0.28	3.75 ± 2.55	2.96 ± 0.22	0.63 ± 0.07	0.14 ± 0.09	0.11 ± 0.02
Faeces	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.11	0.05 ± 0.08	1.03 ± 0.99	3.75 ± 2.59

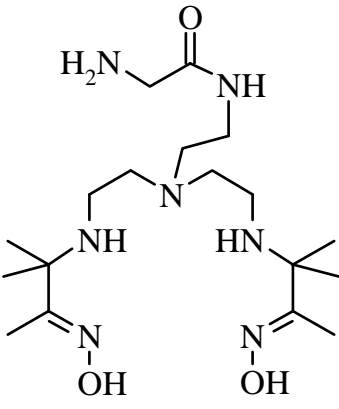
Fig 1  
A



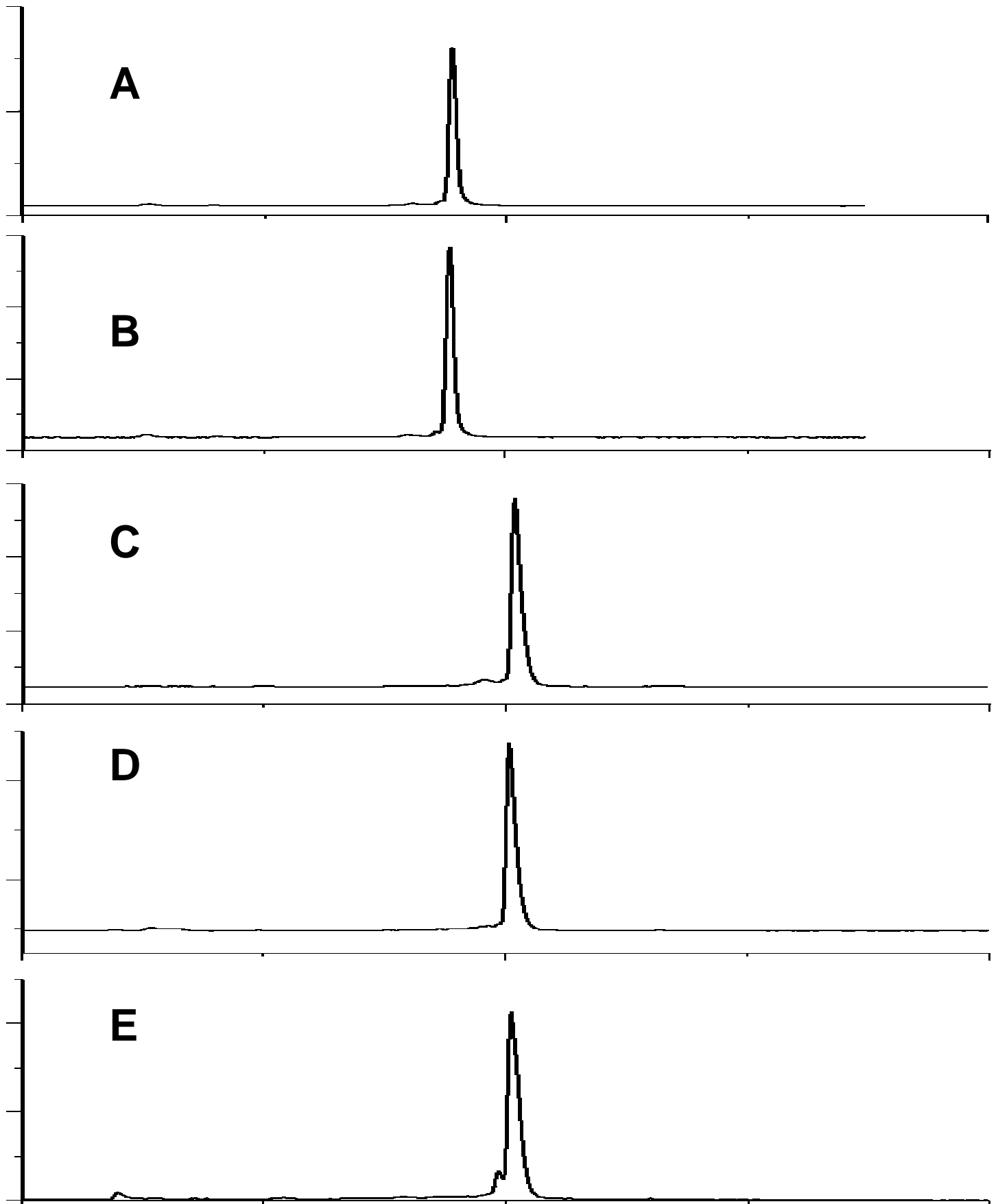
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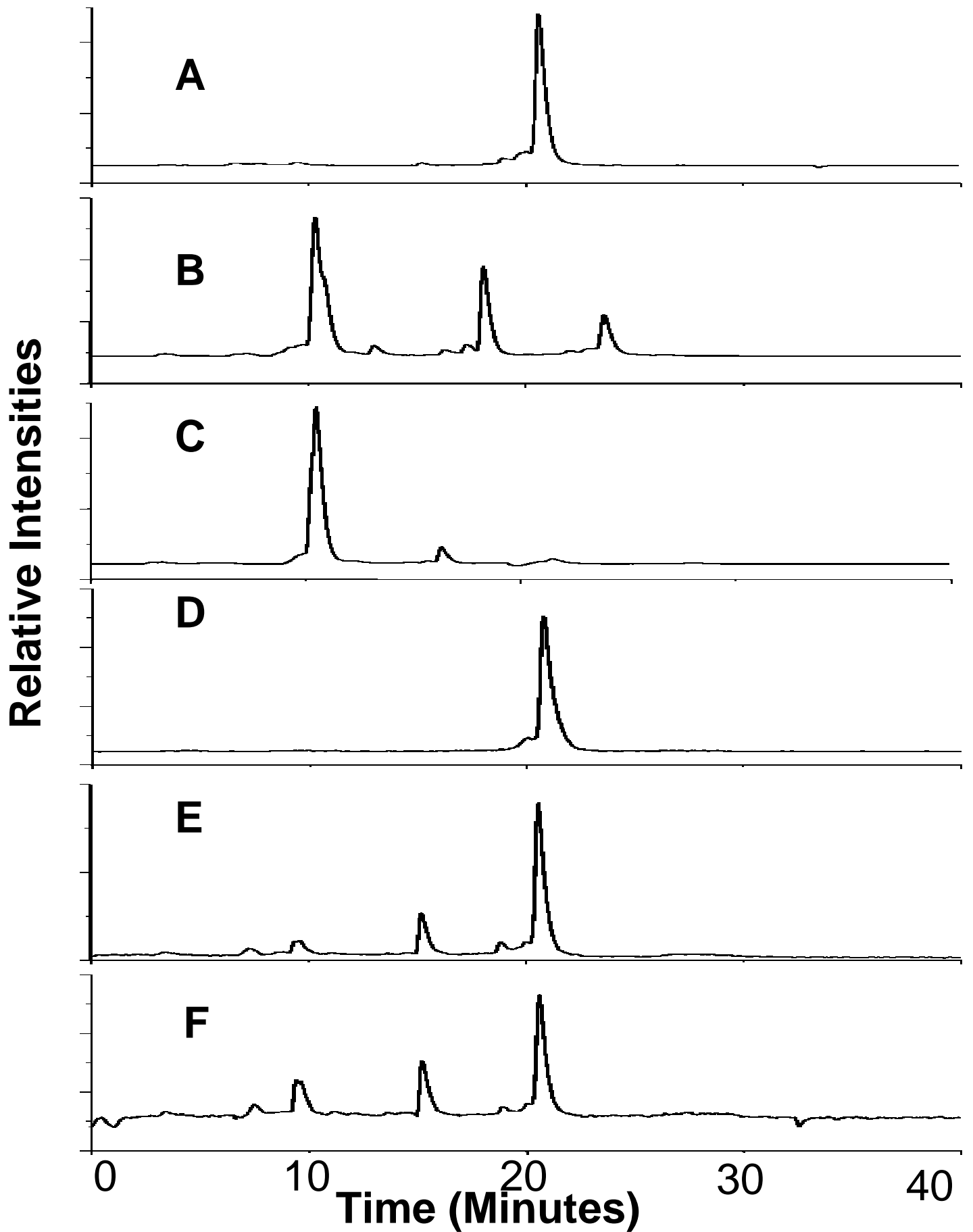
C



**Fig 2**

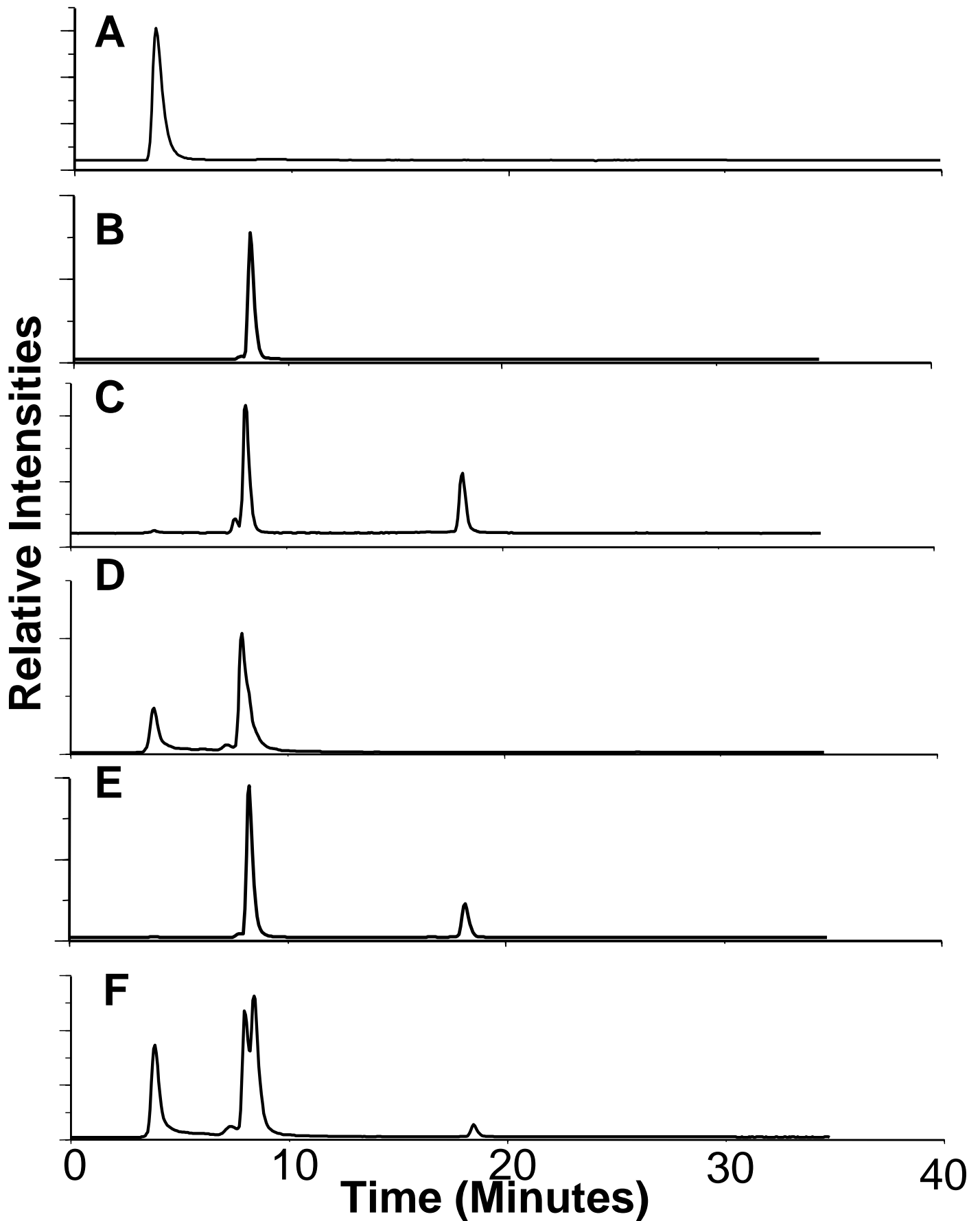


**Fig 3**

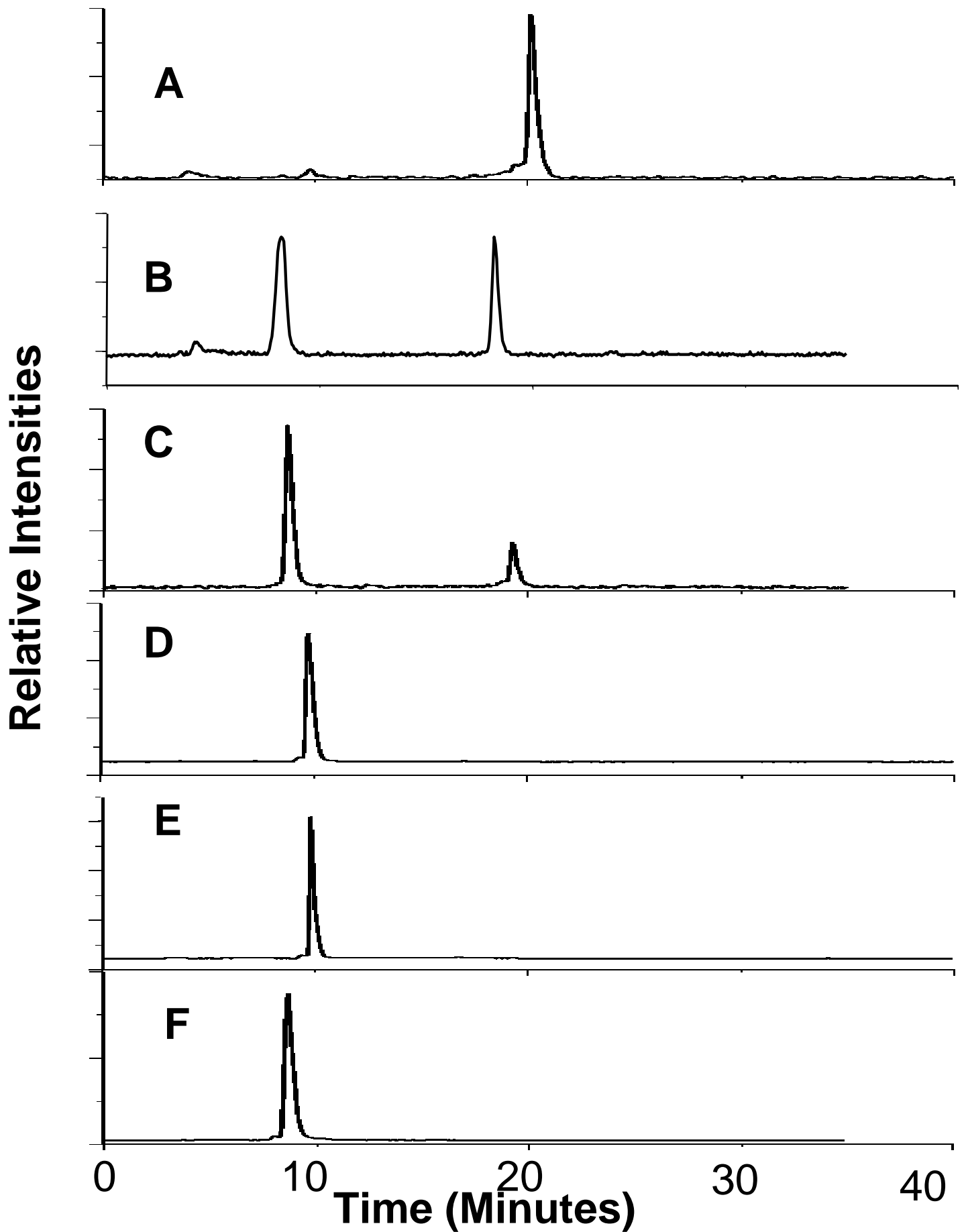




**Fig 4**



**Fig 5**



**Fig 6**

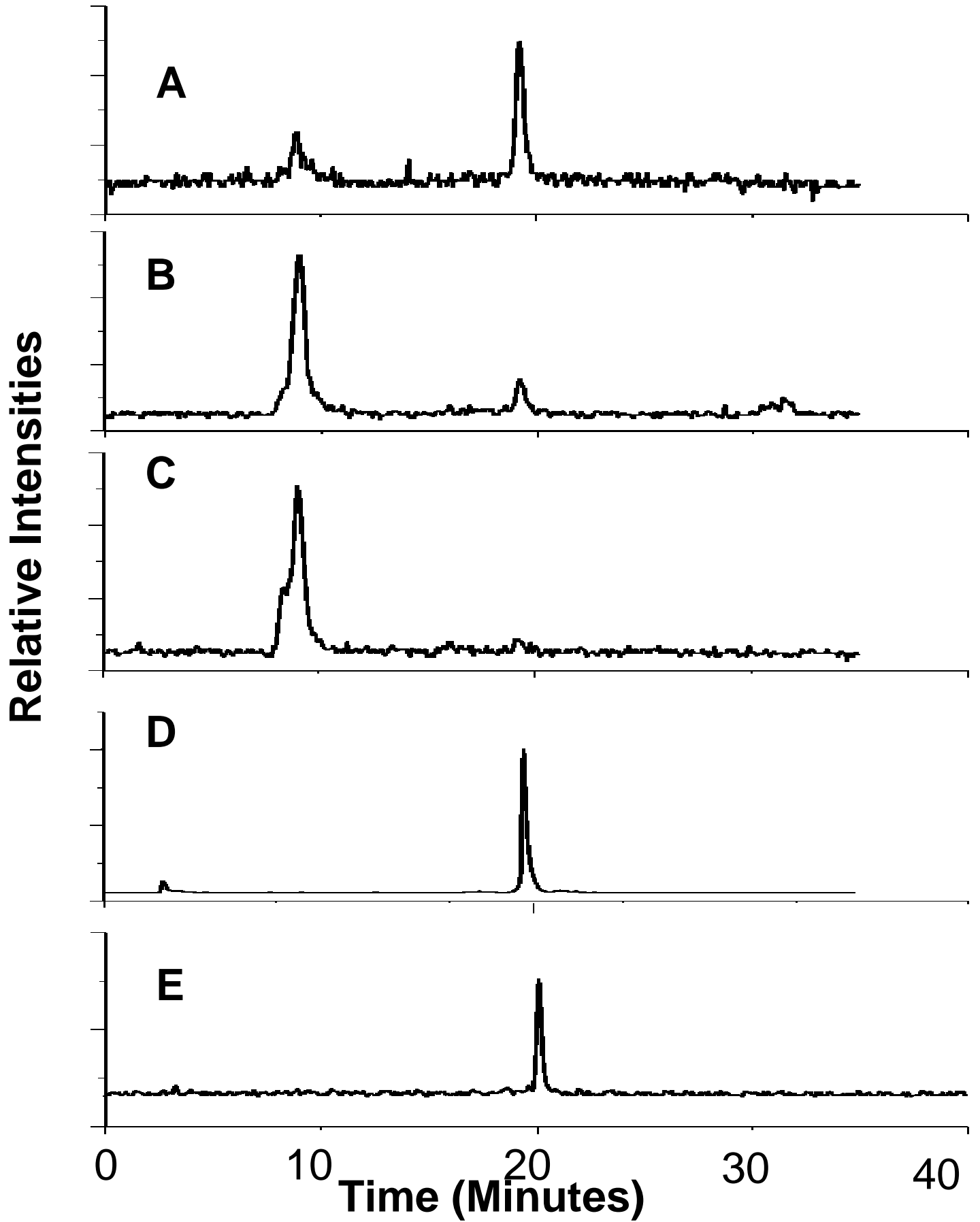
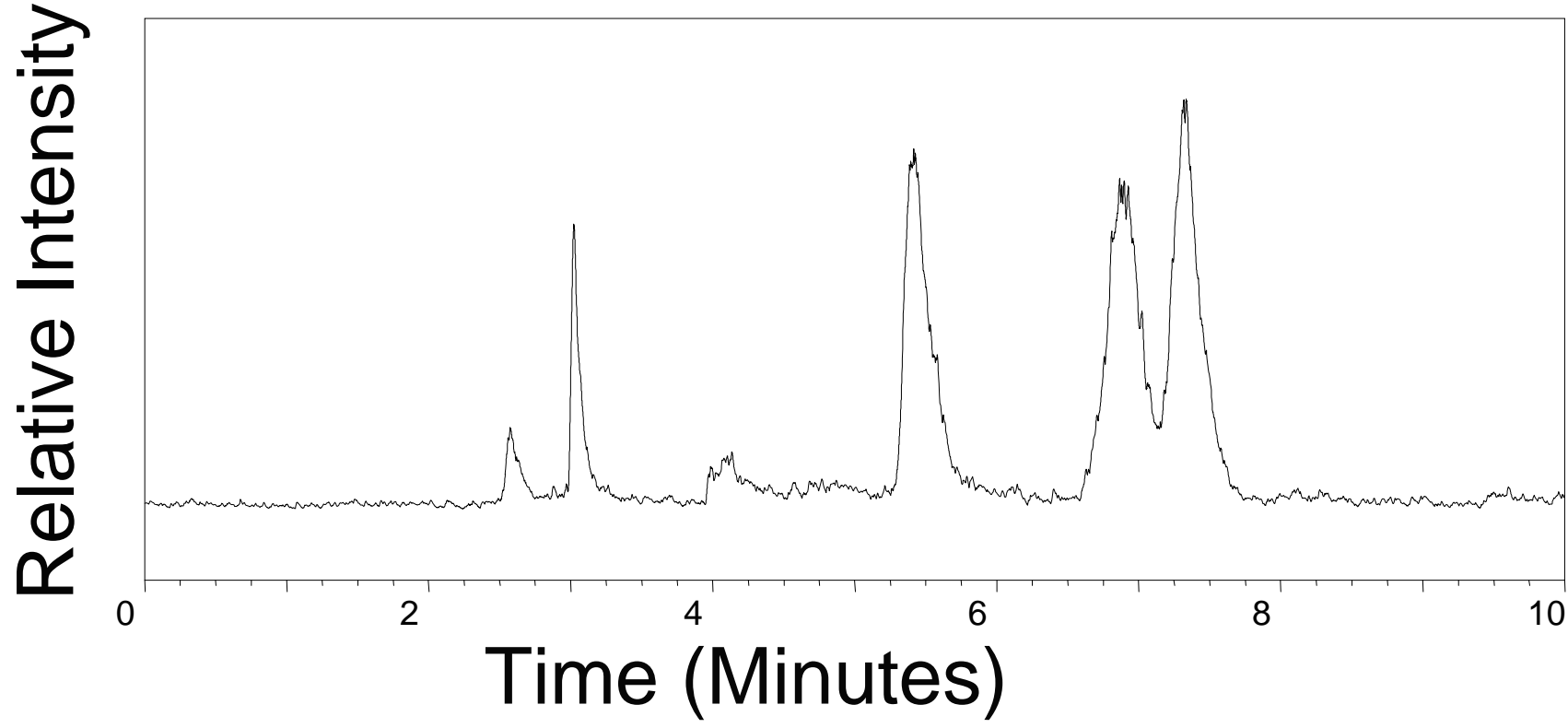


Fig 7



# Fig 8

