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Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 164–169
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Metabolism in rats of NC100692, an RGD-peptide for imaging of angiogenesis

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Received 3 July 2007; received in revised form 13 December 2007; accepted 14 December 2007
Available online 23 December 2007

Abstract

The ^{99m}Tc complex of NC100692 is being evaluated as a diagnostic agent for imaging of angiogenesis. We here report *in vivo* studies performed with NC100692 and ^{14}C-labelled NC100692 using liquid chromatography coupled to either an ion-trap mass spectrometer or a radiochemical detector. Following injection of ^{14}C-labelled NC100692, only the parent compound and no metabolites was observed in rat blood, whereas no parent compound and only 1 metabolite was observed in urine. Analysis of rat urine samples with liquid chromatography with mass spectrometric detection following administration of NC100692 verified the absence of the parent compound and showed the presence of 2 metabolites. The structures of the 2 metabolites were identified based on mass spectra and accurate mass determinations. The major metabolite was identified as the molecule obtained following hydrolytic cleavage at the end of the C-terminal amino acid of NC100692. The minor metabolite was identified as that obtained following removal of phenylalanine within the cyclic structure of the major metabolite.
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Keywords: NC100692; Rat; LC–MS; Metabolism; Angiogenesis

1. Introduction

Diagnostic radiopharmaceuticals are radioactive or radiolabelled imaging agents. For radiolabelling purposes ^{99m}Tc is most frequently used due to a short physical half-life (6.02 h) and a γ photon emission of 140 keV suitable for high-efficiency detection resulting in low radiation exposure to the patient [1]. Due to the short half-life, the ^{99m}Tc-based radiopharmaceuticals are commercially distributed in a Tc-free form (i.e. the ligand). These products are normally supplied as a freeze-dried product which is added ^{99m}Tc at the hospital prior to imaging by reducing $^{99m}TcO_4^-$ eluted from a ^{99}Mo/^{99m}Tc generator [1]. The added ^{99m}Tc binds to a chelator part of the ligand. There is a vast excess of the ligand compared with the added ^{99m}Tc in such products as normally less than 1% of the ligand in the injected solution is in the form of a ^{99m}Tc-complex. Hence, the unlabelled ligand constitutes almost the entire amount of the injected drug, despite the fact that it is the very small amount of the ^{99m}Tc-labelled agent that is responsible for the diagnostic imaging information obtained.

Angiogenesis is a process present in several pathologies for which diagnostic imaging may be useful, e.g. disease screening, staging, monitoring, and follow-up purposes. The role of angiogenesis in tumors is well known [2]. Other diseases in which angiogenesis may be involved include endometriosis [3], coronary arterial disease [4], atherosclerosis [5], and arthritis [6]. Targeting receptors expressed selectively or up-regulated on endothelial cells in areas undergoing neo-vascularisation is an approach to both the diagnosis and treatment of angiogenesis-dependent diseases. Integrin $\alpha_v\beta_3$ is one such receptor [7]. Substances binding specifically and with a high affinity to this receptor are well known and include small peptides containing an RGD (arginine–glycine–aspartic acid) motif [7,8]. The NC100692 ligand contains an RGD sequence which binds selectively with high affinity to the vitronection class of integrin receptors, such as the $\alpha_v\beta_3$ receptor [9].

The ^{99m}Tc complex of NC100692 is being evaluated as a diagnostic imaging agent for the detection of angiogenesis [10], and was recently used for detection of malignant lesions in patients with breast cancer [11]. NC100692 consists of a peptide-based pharmacophore containing a cyclic RGD

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doi:10.1016/j.jpba.2007.12.026

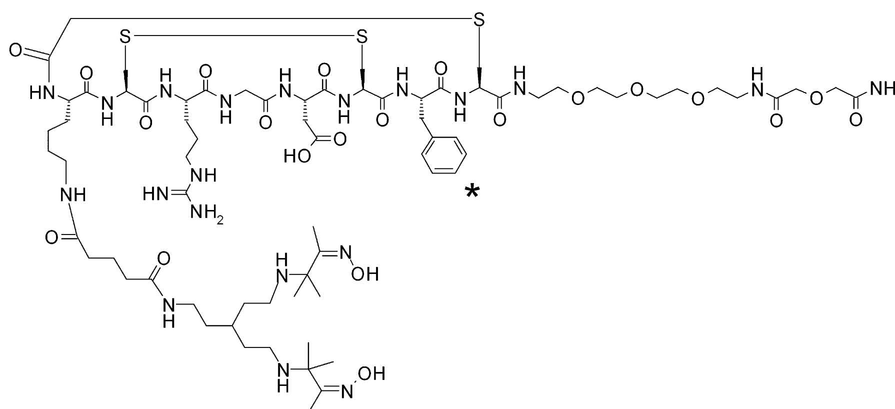

Fig. 1. Structure of NC100692. The asterisk (*) denotes the site of uniformly ^{14}C labelling of [Phe-U-^{14}C]NC100692.

sequence coupled to a biomodifier on the C-terminal part of the peptide and a Tc chelator on the N-terminal part. The structure of NC100692 is shown in Fig. 1; the synthesis of NC100692 has been described by Indrevoll et al. [9]. We have previously developed and validated a quantitative method for the determination of NC100692 in plasma [12]. The aim of the present study was to elucidate the metabolism of NC100692 following intravenous injection in rats.

2. Materials and methods

2.1. Chemicals

NC100692 was delivered by GE Healthcare (Oslo, Norway); radiolabelled NC100692 ([Phe-U-^{14}C]NC100692) was supplied by GE Healthcare (Amersham, UK). All other chemicals were of analytical grade quality. Water was purified by reversed osmosis, ion exchanged and filtered through 0.45 μm filter on a Milli-Q Reagent Water System (Millipore, Molsheim, France).

2.2. Animals and husbandry

Three male Bkl:SD (Sprague-Dawley) rats from Scanbur-BK (Sollentuna, Sweden) and 3 male HanTac:WH (Wistar Hannover) rats from Møllegaard (Lille Skensved, Denmark), each 260–300 g at termination, were injected intravenously with NC100692. Four male Hsd:SD (300–350 g) from Harlan (Horst, the Netherlands) were given an intravenous injection with [Phe-U-^{14}C]NC100692. The animals of each breed were allocated to a common cage after assignment to study in the NC100692 biotransformation experiments. The animals were acclimatized for at least 5 days prior to study start and were housed in a polycarbonate cage type IV and placed on racks in the assigned room. Bedding was Beekay lab bedding from B & K Universal A/S (Nittedal, Norway) and was changed every other day. On the day of dosing the animals were placed in fresh cages. The temperature was kept at 21 ± 2 °C, and the humidity maintained at 55 ± 10%. Lighting was controlled to give 12 h light and 12 h dark per 24 h in phase with natural daylight. Ventilation provided approximately 20 air changes per hour. The rats were provided municipal tap water and diet (Rat & Mouse No. 1 Maintenance Diet, Special Diets Service, Northwith, UK) *ad libitum.*

2.3. Dosing and sampling

A brief summary of the study design is presented in Table 1. All rats from which urine was sampled were trained for confinement to the metabolism cages during the acclimatization period before study start. Pre-dose urine samples (0.5 ml) were collected from all animals in the metabolism cages during the training period. On the day of urine sampling, the animals were placed individually in metabolism cages for urine sampling for 5 h after dosing. All 3 Bkl:SD rats were administered a single intravenous injection of 750 μg NC100692/kg body weight (bw), while all 3 HanTac:WH rats were administered a single intravenous injection of 75 μg NC100692/kg bw. The 2 Hsd:SD rats were administered 1.76 MBq (833 μg [Phe-U-^{14}C]NC100692/kg bw) in 0.5 ml injection solution as a bolus injection in the tail vein. The urine sampled was kept on ice during the collection period, and subsequently frozen below

Table 1
Study design overview

Substance injected	Amount (μg/kg)	Blood collected (min)	Urine collected	Use of samples
[Phe-U-^{14}C]NC100692	833 (1.76 MBq)	5, 15, 30, 60	–	
[Phe-U-^{14}C]NC100692	1036 (2.85 MBq)	2, 5, 10, 15	–	Metabolic screening[a]
[Phe-U-^{14}C]NC100692	833 (1.76 MBq)	–	Up to 5 h	
NC100692	75	–	Up to 5 h	Metabolite identification[b]
NC100692	750	–	Up to 5 h	

[a] Screening by use of liquid chromatography with radiochemical detection.
[b] Identification by use of liquid chromatography with mass spectrometric detection.

−15 °C and stored for maximum 11 weeks prior to analysis. The urine samples were diluted (urine + saline, 1 + 2 (v/v)) prior to analysis using liquid chromatography with radiochemical detection.

The 4 Hsd:SD rats to be blood sampled were administered 1.76 MBq (833 μg [Phe-U-^{14}C]NC100692/kg bw) (2 rats) or 2.85 MBq (1036 μg [Phe-U-^{14}C]NC100692/kg bw) (2 rats) in 0.5 ml injection solution as a bolus injection in the tail vein after being anaesthetised with subcutaneous injections of Dormicum in combination with Hypnorm. Dormicum (midazolam 5 mg/ml) and Hypnorm (fentanyl 0.315 mg/ml with fluanison 10 mg/ml) were both mixed 1 + 1 (v/v) with sterile water, and an injection solution was prepared by mixing diluted Hypnorm and Dormicum solutions 1 + 1 (v/v) and injecting 0.2–0.3 ml/100 g bw. Blood samples from the tail vein (1 ml) were collected in a tube containing citrate as an anticoagulant (1.76 MBq: 5 and 30 min post-injection from 1 rat and 15 and 60 min from the other rat; 2.85 MBq: 2 and 10 min post-injection from 1 rat and 5 and 15 min post-injection from the other rat). Plasma was prepared after collection by centrifugation at 1500 × *g* for 10 min at room temperature, and frozen below −15 °C if not analyzed immediately. Prior to analysis the plasma samples were protein precipitated (sample + 5% (v/v) trifluoroacetic acid in acetonitrile, 1 + 1 (v/v)). The protein precipitated sample was incubated at 4 °C for 30 min prior to the centrifugation (13,000 RPM) for 10 min at 4 °C before removal of the supernatant which after dilution (supernatant + saline, 1 + 2 (v/v)) was analyzed using liquid chromatography with radiochemical detection.

All rats used for urine sampling were killed by an intravenous overdose of Pentobarbital (100 mg/kg bw) immediately after urine sampling was completed, while the animals used for blood collection were killed by exsanguination from the *vena cava* while under deep surgical anaesthesia with Hypnorm and Dormicum. During intravenous administrations (both dosing and termination) the rats were immobilised by a designated restraining device and the injections were administered through the tail vein using a 1 or 2 ml syringe with 25 G butterfly needle.

2.4. Liquid chromatography with radiochemical detection

A Spectra Physics SP 8800 ternary pump (Mountain View, CA) coupled to a Gilson 231 autoinjector (Middleton, WI) was used for LC on a 4.6 mm × 100 mm Waters Symmetry 3.5 μm C_{18} analytical column (Waters, Milford, MA) using a binary mobile phase gradient at 1 ml/min at ambient temperature. The mobile phase consisted of Mobile phase A (0.1% (v/v) trifluoroacetic acid in water) and Mobile phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) according to the following: Initially 15% B, 15%–20% B in 15 min, 20%–35% B in 5 min, 35% B for 5 min, 35%–15% B in 0.1 min and subsequently re-equilibration for 20 min. The radiochemical detector was a Packard Radiomatic Flo-One\Beta A 140 A (PerkinElmer) at standard ^{14}C setting with a 250 μl flow-cell and 3 ml/min scintillation liquid (Packard Ultima-Flo M; PerkinElmer) flow rate. An injection volume of 10 μl was used for the *in vitro* stability study and the metabolism profiling of rat urine while 50–100 μl was injected for the metabolism profiling of rat plasma.

2.5. Liquid chromatography with mass spectrometric detection

A Hewlett Packard series 1100 LC (Agilent Technologies, Palo Alto, CA) hyphenated with an ion-trap MS (Finnigan LCQ; Thermo, Waltham, MA) was used for LC–MS analysis. The LC was controlled by the Hewlett Packard ChemStation software, version A.06.01 and Xcalibur (Thermo) was used to control the MS detector and for data sampling. The chromatographic method used with radiochemical detection was not compatible with mass spectrometric detection with regard to the use of trifluoroacetic acid in the mobile phase. Furthermore it was of interest to reduce the volumetric flow in addition to improve the peak performance by decreasing the column dimensions. Therefore, a new chromatographic method was developed for the use of mass spectrometric detection.

A 2 mm × 4 mm Phenomenex C18 column was used as the guard column (Torrance, CA) and a 2.1 mm × 75 mm Supelco Discovery HS C18 (3 μm particle diameter) delivered by Supelco (Bellefonte, PA) was used as the analytical column at a flow rate of 0.3 ml/min at ambient temperatures. An injection volume of 10 μl was used with autosampler cooling to 4 °C. Mobile phase A (0.1% (v/v) formic acid in water) and Mobile phase B (0.1% (v/v) formic acid in acetonitrile) were used for gradient elution according to the following: Initially 5% B, 5%–50% B in 8 min, 50% B for 1 min, 50%–5% B in 1 min and subsequently re-equilibration for 9 min. The ion-trap MS was used with positive electrospray mode. Full scan was performed from *m/z* 150 to 2000, while selective ion monitoring (SIM) of NC100692, Metabolite A and Metabolite B was performed using $[M+2H]^{2+}$ (*m/z* 849.4 ± 0.5) and $[M+3H]^{3+}$ (*m/z* 566.6 ± 0.5), $[M+H]^{+}$ (*m/z* 1408.6.6 ± 0.5) and $[M+2H]^{2+}$ (*m/z* 704.8 ± 0.5), and $[M+H]^{+}$ (*m/z* 1279.6 ± 0.5) and $[M+2H]^{2+}$ (*m/z* 640.3 ± 0.5), respectively. Exact mass determination was performed using a 2695 Alliance LC system (Waters) coupled to a QTOF Micromass spectrometer (Waters). Five microlitres of rat urine were injected.

The amount of the metabolites in the urine samples was estimated by using the calibration curve for NC100692 covering the ranges 5–5000 ng NC100692/ml or 50–10000 ng NC100692/ml. The calibration standards were prepared by diluting a stock solution (1 mg NC100692/ml in water) with human urine obtained from 2 healthy volunteers. Fresh calibration standards were made for each analytical sequence. The calibration curve was made by plotting the peak area of the total ion current of NC100692 (sum of *m/z* 566.6 ± 0.5 and *m/z* 849.4 ± 0.5) against the nominal concentration of NC100692 in the calibration standards. The calibration curve was fitted to a non-linear equation, $y = a + bx + cx^2$, and weighted with a weighing factor of $1/y$. Due to the lack of pure standards the amounts of Metabolites A and B were estimated by assuming similar response as the parent compound NC100692. Hence, the estimated amount of each metabolite was obtained by correlating the peak area of the total ion current of the metabolite in the urine samples with

the area found in the NC100692 calibration standards using the calibration curve.

3. Results

3.1. Studies performed with [Phe-U-^{14}C]NC100692

Only the chromatographic peak corresponding to [Phe-U-^{14}C]NC100692 was observed in blood following injection of [Phe-U-^{14}C]NC100692 in male rats, i.e. no biotransformation was observed in blood. In the urine samples, only 1 metabolite and no parent compound was observed (data not shown). The radioactive peak in the urine chromatogram was shown to be different from the parent compound by spiking experiments (the metabolite and the parent compound eluted at 13.6 and 15.3 min, respectively).

3.2. Qualitative analysis of rat urine samples following injection of NC100692

NC100692 was not present in the post-dose rat urine samples at detectable levels using LC–MS, in agreement with the results obtained following injection of [Phe-U-^{14}C]NC100692. To evaluate all metabolites of NC100692 potentially present in rat urine, pre-dose and post-dose samples were analyzed using LC with full scan MS detection in the range *m/z* 150–2000 (Fig. 2). Two metabolites, Metabolites A and B, were observed in post-dose urine from animals receiving 75 or 750 μg NC100692/kg bw. Metabolite A was the major and least hydrophilic metabolite and Metabolite B was the minor and most hydrophilic metabolite as determined from the elution order. Spiking experiments revealed co-elution of NC100692 reference standard and Metabolite A in the chromatographic system used for LC–MS.

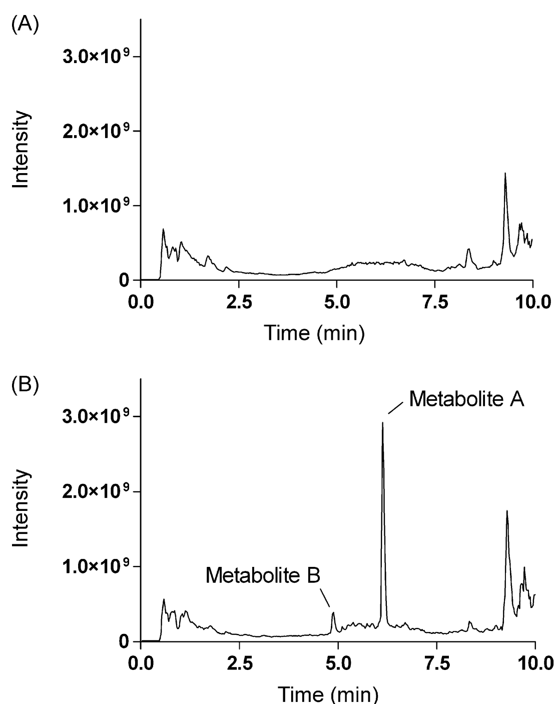

Fig. 2. Total ion current chromatograms (*m/z* 150–2000) of the (A) pre-dose and (B) post-dose urine samples.

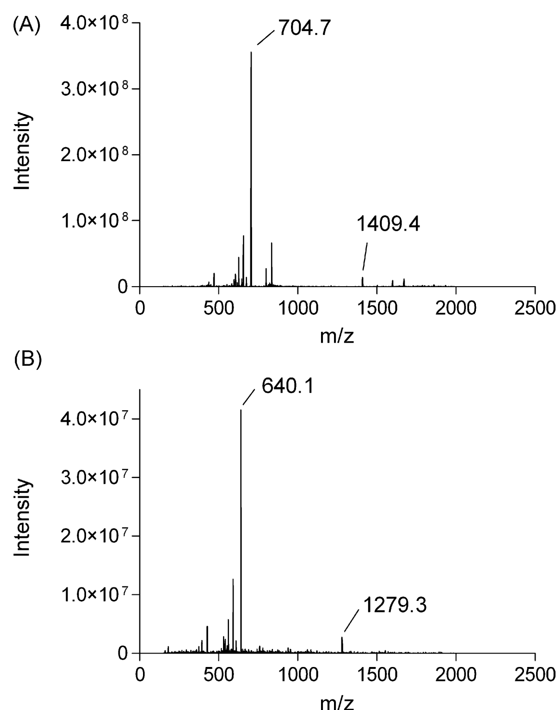

Fig. 3. Mass spectra of (A) Metabolite A obtained at 6.1 min in the chromatogram of the post-dose urine sample in Fig. 2B and (B) Metabolite B obtained at 4.9 min in the chromatogram of the post-dose urine sample in Fig. 2B.

A mass spectrum of Metabolite A is shown in Fig. 3a. For all animals both $[M+H]^+$ (only at high dose level) and $[M+2H]^{2+}$ (at both dose levels) were detected at *m/z* 1408.7 ± 0.6 and 704.7 ± 0.1, respectively. The possibility of metabolism in the chelate part of NC100692 was investigated. MS–MS of the $[M+H]^+$ ions of NC100692, Metabolite A, and Metabolite B demonstrated a loss of 99 mass units for all chemical species (*m/z* 1697.8 → *m/z* 1598.4, *m/z* 1408.3 → *m/z* 1309.3, and *m/z* 1279.3 → *m/z* 1180.3, respectively), similarly to that described for another agent containing the same chelate [13]. The same mass loss was proposed by Mass Frontier^{TM} software version 3.0 (Thermo) for the fragmentation of the chelate part, thereby showing the chelate part to be intact in both Metabolites A and B. Metabolite A was further investigated using exact mass determination as $[M+H]^+$ resulting in an *m/z* of 1408.6556. This result fit very well with a metabolite formed upon hydrolysis on the C-terminal end of the cysteine (Cys) residue corresponding to a theoretical *m/z* of 1408.6540 for $[M+H]^+$. Thus, Metabolite A was identified as the structure shown in Fig. 4A.

A mass spectrum of Metabolite B is shown in Fig. 3b. For all rats both $[M+H]^+$ (only at high dose level) and $[M+2H]^{2+}$ (at both dose levels) were detected at an *m/z* of 1279.6 ± 0.6 and 640.1 ± 0.1, respectively. Metabolite B was also further investigated using exact mass determination as $[M+H]^+$ resulting in an *m/z* of 1279.5976. This result fits very well with the mass of Metabolite A following the removal of the phenylalanine (Phe) residue corresponding to a theoretical *m/z* of 1279.5961 for $[M+H]^+$. The exact mass difference observed between Metabo-

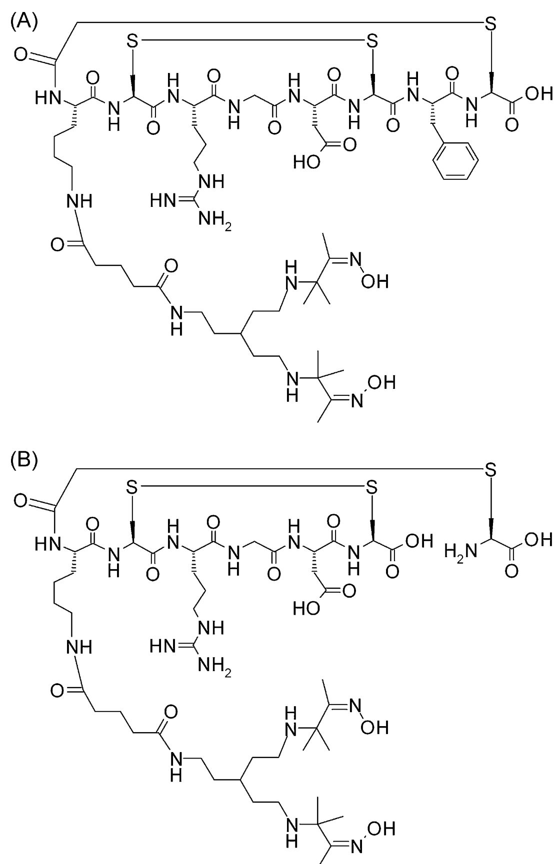

Fig. 4. Structures of (A) Metabolite A and (B) Metabolite B.

lites A and B is 129.0580 which correspond to the residue mass of Phe minus H_2O (theoretically 129.0579). Thus, Metabolite B was identified as the structure shown in Fig. 4b. Interestingly; the theoretical intermediate between Metabolites A and B (hydrolysed Metabolite A with an *m/z* of A + 18) was not present at detectable levels.

3.3. Estimation of metabolite amounts

Due to the lack of pure standards the amounts of Metabolites A and B could not be accurately quantified. Instead, the amounts of the metabolites were estimated by assuming similar response as the parent compound NC100692. A urinary excretion in male rats of 3.5–7.3% of injected dose was estimated for Metabolite A, whereas a recovery of 0.2–0.8% of the injected dose was estimated for Metabolite B. These experiments were performed at a high dose level (750 μg/kg bw) using Sprague-Dawley strain and at a reduced dose level (75 μg/kg bw) using Wistar strain (Wistar Hannover) without observing any differences.

4. Discussion

Following injection of [Phe-U-^{14}C]NC100692 in rats, only the parent compound and no metabolites were observed in blood, whereas only 1 metabolite and no parent compound was observed in urine. The absence of NC100692 in urine was verified with LC–MS analyses, which also clearly demonstrated the presence of 2 metabolites in urine. The structures of these metabolites were revealed using exact mass determination. The major and least hydrophilic metabolite (Metabolite A) was identified as the molecule following hydrolytic cleavage at the end of the C-terminal amino acid of NC100692. The minor metabolite (Metabolite B) was identified as that obtained following removal of Phe from Metabolite A. These results thus explain why only 1 metabolite was observed in urine following injection of [Phe-U-^{14}C]NC100692, as Metabolite B lacks the ^{14}C labelled Phe, precluding detection of Metabolite B with the radiochemical detector.

The different chromatographic systems used in the ^{14}C work and in the LC–MS study explain why the radiolabelled species observed in urine did not co-elute with the parent compound, while Metabolite A was found to co-elute with the parent compound in the LC–MS system. In the ^{14}C study, a significantly less steep gradient was employed in addition to the use of different chromatographic columns.

In a quantitative whole body autoradiography study, the results following intravenous injection of [Phe-U-^{14}C]NC100692 in rats were very similar to those obtained using [^{14}C]Phe only (data not shown). This further supports the observation in the present study, as Metabolite B is generated following the removal of Phe from Metabolite A; thus the released Phe may explain the similarity of the autoradiography results between injection of [Phe-U-^{14}C]NC100692 and [^{14}C]Phe. In conclusion, all data obtained in studies using the ^{14}C-labelled NC100692 fit with the identified structures for Metabolites A and B. It should be noted that the enzymatic cleavage reactions leading to the 2 metabolites do not take part in the chelating part of the molecule or close to the chelating part and thus probably do not affect the chelating ability of the metabolites compared with NC100692. Moreover, the present data are in agreement with data obtained following injection of ^{99m}Tc-NC100692 in rats, where only the parent compound and no metabolites were observed in blood, whereas no parent compound and 2 radioactive metabolites were observed in urine (Dave Edwards, personal communication; data generated within GE Healthcare).

Our estimates of the amounts of Metabolites A and B in rat urine are highly uncertain, mainly for 2 reasons. Firstly, the assumption of similar responses for these metabolites and NC100692 may not be valid. Both underestimation and overestimation may result from such estimations. Secondly, the effect of ion suppression may result in underestimation of the signal responses and should therefore also be considered [14]. Ion suppression may be expected both due to the complex and variable nature of the matrix but also due to the limited sample preparation performed (dilution only). In our previous experience with a similar substance quantification of metabolites in rat urine has proven difficult, probably due to the much higher (and varying) concentration of both high- and low-molecular weight substances in rat urine [15]. In light of that more than 50% of the radioactivity is excreted in urine 5 h after injection of ^{99m}Tc-NC100692 in rats (Dave Edwards, personal communication), we believe that the low recovery of the 2 metabolites in urine in the present study is due to a large underestimation of the metabolites due to reasons discussed above.

It is not known which enzymes are involved in forming the 2 metabolites observed, but some speculations regarding this issue are presented in the following. The bond within NC100692 which must be hydrolyzed to generate Metabolite A is quite unusual and probably non-existent in endogenous substances. It is very likely that this metabolite is generated by one of the many kidney brush border enzymes. Theoretically there exist at least 4 possibilities for obtaining Metabolite B from Metabolite A. The first possibility is to have a protease splitting on the C-terminal of the Phe residue, followed by removal of Phe by a carboxypeptidase. A chymotrypsin-like protease has been reported to be present in kidney brush border [16] and should theoretically be able to split C-terminal to Phe. Although this pathway should not be excluded, we are not aware of any carboxypeptidase in kidney brush border that has been described to have a "carboxypeptidase A-like" activity and thus be able to split off the new C-terminal Phe. The second possibility is to split Metabolite A at the N-terminal side of the Phe residue. This could be obtained by neprilysin, also called kidney brush border neutral proteinase, neutral endopeptidase or NEP (EC 3.4.24.11), which constitutes approximately 4% of the protein of the renal brush border membrane proteins [17]. Following splitting at the N-terminal side of Phe, the Phe residue may be removed by membrane alanyl aminopeptidase (EC 3.4.11.2) which represent as much as 8% of the kidney brush border membrane proteins and has broad substrate specificity with a high activity concerning removal of Phe [18]. The third possibility is that the first split of Metabolite A may be performed by peptidyl dipeptidase (also called angiotensin I converting enzyme; EC 3.4.15.1) present in kidney brush border followed by removal of Phe by the membrane alanyl aminopeptidase. Although this dipeptidase has been described to be able to cleave off different C-terminal dipeptides, it would be somewhat surprising if it is able to perform this task with the very special C-terminal structure of Metabolite A. Finally, there is a theoretical possibility to obtain Metabolite B from Metabolite A by having a two-step attack from a carboxypeptidase. As mentioned above, we are not aware of any carboxypeptidase described to have this kind of activity in kidney brush border. It is therefore unlikely that there exists an enzyme able to produce Metabolite B without a significant amount of intermediate between Metabolites A and B, i.e. having the mass of Metabolite A plus 18. Interestingly such an intermediate was not detected.

In conclusion, our data indicate a rapid formation of Metabolite A from NC100692 in kidney brush border. Metabolite B has to be formed by 2 enzymatic steps from Metabolite A; the absence of an intermediate in this reaction indicates the second step to be much faster than the first step. Based upon theoretical considerations Metabolite B is most likely formed from Metabolite A by the sequential attack of neprilysin and membrane alanyl aminopeptidase, which both are very abundant in kidney brush border membrane. It should be noted that the urinary excretion of peptides/proteins may differ between different species and between rat strains and sexes [19]; no difference was observed in the metabolites in urine of the 3 rat strains used in the present study.

Acknowledgment

We thank Åsmund Larsen for performing the exact mass determinations.

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