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NC100668, A NEW TRACER TESTED FOR IMAGING OF VENOUS THROMBOEMBOLISM: PHARMACOKINETICS AND METABOLISM IN MAN

KIM G. TOFT, JUDITH A. JOHNSON, INGER OULIE, TORE SKOTLAND

Research and Development, GE Healthcare, Oslo, Norway (KGT, IO, TS) and Princeton, New Jersey (JAJ)

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Running title: Pharmacokinetics of NC100668 in man

Corresponding author: Dr. Kim Toft,

Research and Development, GE Healthcare,

Nycoveien 2, N-0401 Oslo, Norway

Telephone: +47 23185616

Fax: +47 23186008

E-mail: Kim.Toft@ge.com

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List of non-standard abbreviations: AUC, area under the curve; C_{max}, maximum observed concentration; LC-MS, liquid chromatography mass spectrometry; %ID, percent of injected dose; RSD, relative standard deviation; SD, standard deviation

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ABSTRACT

The ^{99m}Tc-complex of NC100668 is a new tracer tested for nuclear medical imaging of venous thromboembolism. NC100668 is a 13 amino acid peptide with a Tc-binding chelator (NC100194) linked to the C-terminal end. The present study was performed following injection of ^{99m}Tc-NC100668 in human healthy volunteers with five dose levels of NC100668 (20-2000 μg) and a constant radioactivity dose. The rate at which the radioactivity was cleared from blood was independent of gender and dose of NC100668; more than half of the 82% urinary clearance of radioactivity was obtained 2 h post-injection. The radioactivity in blood was reduced to 50% of initial values within 12 min; this was followed by a more gradual decline with half-life of 1.2 h and a terminal elimination half-life of 10.5 h. The plasma concentration of NC100668 declined rapidly with an initial half-life of 5-10 min. The half-life after this initial phase could be estimated for only two of the subjects in the highest dose-group as the NC100668 concentration in the other samples at these time points was below the limit of detection of the LC-MS method. LC-MS analyses of urine samples revealed the identity of two metabolites generated from the C-terminal end of the molecule; Gly-NC100194 was identified as the major metabolite and NC100194 as a minor metabolite. The estimated sum of these two metabolites is in the same magnitude as the recoveries of ^{99m}Tc in these samples indicating that most of the ^{99m}Tc excreted in urine is bound to one of these metabolites.

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(INTRODUCTION)

^{99m}Tc-NC100668 is a new diagnostic radiopharmaceutical evaluated for imaging of venous thromboembolism. NC100668 consists of a 13 amino acid peptide coupled to a Tc-chelator (NC100194) via the C-terminal glycine. Using the common three letter abbreviations for amino acids the structure of NC100668 is Acetyl-Asn-Gln-Glu-Gln-Val-Ser-Pro-Tyr(3-iodo)-Thr-Leu-Leu-Lys-Gly-NC100194 where NC100194 is represented by the chemical formula – NH-CH_2-CH_2-N(CH_2-CH_2-NH-C(CH_3)$_2$-C(CH_3)=N-OH)$_2$. The mechanism of action and the complete molecular structure of this compound can be found at Skotland et al. (2006).

Diagnostic radiopharmaceuticals are radioactive substances utilised for medical imaging of a variety of diseases. ^{99m}Tc ($t_{½}$ of 6.02 h) is the γ-emitter most often used in these agents (Liu et al., 1997). The ^{99m}Tc-based agents are distributed to hospitals in a Tc-free form as a freeze-dried product ready for labeling with technetium, which is eluted from a ^{99}Mo/^{99m}Tc generator and added to the product on the day of imaging. These radiopharmaceuticals contain a vast excess of the chelating agent compared to the added Tc; typically less than 1% of the injected agent is in the form of the Tc-complex (sum of the γ-emitter ^{99m}Tc and the non- γ-emitter ^{99}Tc, formed from ^{99m}Tc). Hence, the unlabelled chelating agent makes up almost the entire amount of the chemical entity injected, whereas it is the very small amount of the ^{99m}Tc-labelled agent which is responsible for the imaging effect.

We have earlier described a rapid clearance from blood of NC100668 following injection in rats, and that two metabolites and no parent compound were identified in rat urine (Skotland et al., 2006). The present data were obtained using samples collected in a Phase I study performed as a placebo-controlled, observer-blinded, randomized, single ascending-dose study with five intravenous dose levels of ^{99m}Tc-NC100668 injected into healthy volunteers. The injected radioactivity was kept constant at 190 MBq per subject whereas five dose levels of NC100668 (20-2000 µg) were injected (imaging dose expected to be 100 µg).

Materials and Methods

Reagents and Substances. All chemicals and substances used were as previously described in the rat metabolism study (Skotland et al., 2006).

In Life Part of the Study and Sample Collection. ^{99m}Tc-NC100668 was prepared using sterile conditions and aseptic techniques by reconstitution of the NC100668 Kits with Sodium (^{99m}Tc) Pertechnetate Injection USP/Ph.Eur. followed by heating at 60±5 °C for 10 min. The reconstituted ^{99m}Tc-NC100668 was used within 2 h and was not to be administered if an HPLC method showed the ^{99m}Tc-NC100668 peak to be less than 85% of the total radioactivity or any other peak to be more than 7% of total, or if an ITLC (Instant Thin Layer Chromatography) method showed reduced-hydrolyzed technetium of more than 5% of total.

The study included 8 subjects receiving ^{99m}Tc-NC100668 for each of the five dose levels (a total of 40 subjects). The nominal dose of NC100668 was 20, 100, 500, 1000 and 2000 µg with a constant radioactivity of 187±12 MBq (mean±SD). The molar ratio of ^{99m}Tc-NC100668 to NC100668 is approximately 5% at the lowest dose, 0.05% at the highest dose and close to 1% at the expected clinical dose of 100 µg. The age of the subjects ranged from 19 to 64 years (mean age at each dose level 31-47 years). There were more males than females; no females in the lowest dose group, 3 females in the highest dose group, and 2 females in the other dose groups. The study was conducted in full accordance with the current version of the Declaration of Helsinki.

Venous blood samples (anticoagulated with citrate) for gamma counting were collected 30 min prior to injection, at 5, 10, 15 and 30 min, and at 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after injection. Citrated plasma samples for LC-MS analyses were collected 30 min prior to injection, at 6, 12, and 30 min, and at 1, 1.5, 3, 6, 12, and 24 h after injection. Urine was collected 30 min prior to injection and at all urinary voids up to 24 h after injection. The samples were stored below -15 °C until being analyzed.

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Quantification of NC100668 in Blood Samples. The concentration of NC100668 was analyzed using a validated LC-MS method (Toft et al., 2005).

Metabolite Identification in Urine Samples using LC-MS Analysis. Just prior to LC-MS analysis the samples were thawed, centrifuged at 3000 x g for 10 min and cleaned by a solid phase extraction procedure using C18-SPE columns (100 mg, 1 ml from Varian Inc., Palo Alto, CA, USA). These columns were conditioned with 2 x 1 ml of methanol and equilibrated with 2 x 1 ml of water. The urine samples (100 µl) were then applied onto the SPE cartridges, which thereafter were washed with 2 x 1 ml of water. The cartridges were then eluted with 2 x 0.5 ml of 40% methanol in HCl into plastic cryotubes. Finally, the samples were evaporated under nitrogen and dissolved in 100 µl sample solvent, mixed and transferred to plastic micro HPLC vials. Metabolite identification was performed using the LC-MS system previously described in the rat metabolism study (Skotland et al., 2006).

Estimation of Gly-NC100194 and NC100194 in Human Urine. The amounts of NC100194 and Gly-NC100194 in the urine samples were determined using the same LC-MS system as used for metabolite identification by correlating the peak areas of the urine samples with the areas found for calibration standards. Calibration curves were made by plotting the peak area of NC100194 (*m/z* 345.3±1.0) or Gly-NC100194 (*m/z* 402.3±1.0) against the theoretical concentration of NC100194 or Gly-NC100194 in the calibration standards; the calibration curves were fitted to a non-linear equation; $y = a + bx + cx^2$ and weighted with a weighing factor of $1/y^2$.

Gly-NC100194 was estimated by comparing the signals with that obtained using calibration curves in the range 3.2-2000 ng/ml. The signal to noise ratio was approximately 4 for the lowest calibration sample. Although the goodness of fit values for the calibration points were within ±15% (back calculated values), the intermediate precision was rather poor; in the range of 30-60% for three control samples containing approximately 80, 280 and 1400 ng Gly-

NC100194/ml. It is believed that leakage from the SPE column made a major contribution to this poor precision.

NC100194 was estimated using calibration curves in the range 8-5000 ng/ml. The signal to noise ratio was approximately 4 for the lowest calibration sample. The validation data for this analysis were in the same range as that described above for Gly-NC100194.

Radiochemical Quantification of Blood and Urine Samples. The total radioactivity was determined using a Packard Cobra Model 5003 gamma counter (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Duplicate aliquots were assayed of each sample. The limit of quantification was defined as twice the background. All gamma counting data were decay-corrected to the time of injection and are presented in units of kBq/ml.

Analysis of Data and Pharmacokinetic Calculations. Analysis of chromatographic and mass spectrometry data were performed as previously described in the rat metabolism study (Skotland et al., 2006). Model-independent pharmacokinetic analyses were performed using PK Solutions 2.0 software (Summit Research Services, Montrose, CO, USA). For pharmacokinetic calculations, dose was taken to be the nominal NC100668 dose administered.

Results and Discussion

Pharmacokinetics of ^{99m}Tc in Plasma. The mean plasma concentrations of radioactivity for all five doses of NC100668 are shown in Fig. 1A. The rate of clearance of radioactivity from plasma was independent of gender (data not shown). The C_{max} (range 14.8-18.6 kBq/ml for the five dose levels) and AUC (range 30.9-39.9 kBq·h/ml) for plasma blood activity were independent of the NC100668 dose over the 100-fold range studied. Of the 39 subjects evaluable for pharmacokinetic analysis, the individual concentration versus time profiles of seven subjects (18%) exhibited a bi-exponential decline while 32 (82%) exhibited a tri-exponential decline. The initial decline in radioactivity (generally estimated over the interval from 5 min to 30 or 60 minutes) was rapid and reduced to 50% of the observed initial values within approximately 12 min. The decline from ~1 to 6 hours post-dose was more gradual with a half-life of approximately 1.2 h (range 1.18-1.31 h). Lastly, the observed elimination half-life estimated over the interval from 8 to 24 hours was approximately 10.5 h (range 9.2-11.9 h). Clearance was estimated to 68 ml/h/kg (range 61.7-74.8 ml/h/kg) and the volume of distribution to 1040 ml/kg (range 1005-1084 ml/kg). Since the majority of the radioactivity (~82%) was rapidly eliminated from the body via renal excretion during the distribution phase, the terminal elimination half-life should be interpreted with caution since this represents the disposition of a relatively minor fraction of the total administered dose. Therefore, the half-life for the second phase which characterizes the elimination of the majority of the administered dose, is likely to be more clinically relevant and is consistent with removal via glomerular filtration.

Pharmacokinetics of ^{99m}Tc in Urine. The renal clearance of radioactivity appeared to be independent of the dose of NC100668 and was estimated to be 57 ml/h/kg, which is approximately 83% of the estimated value of 68 ml/h/kg for the systemic clearance.The urinary recoveries were estimated to be 76±17%, 81±7%, 91±15%, 89±13%, and 72±14% (% of

injected dose; mean±SD; from the lowest to the highest dose group). Thus, approximately 82% of the injected radioactivity cleared through the kidneys, and more than half of this within 2 h post-injection.

Pharmacokinetics of NC100668 in Plasma. The mean plasma concentration data for all five dose levels are shown in Fig. 1B. Although the calculation of the pharmacokinetic parameters involved only the concentrations above the limit of quantification (LOQ) of 2 ng/ml, we have in Figure 1 never the less chosen to show all data above 1 ng/ml, i.e. half the LOQ. These data clearly show that valuable supportive information was obtained when including data below (but very close to) the LOQ.

The NC100668 plasma concentrations declined rapidly with an initial half-life of 5 to 10 min. The elimination half-life could be estimated only for 2 subjects in the highest dose group because the concentrations at later time points were below the limit of quantification. Consequently the AUC values as well as clearance and volume of distribution could not be accurately assessed from these data.

When comparing data in Fig. 1A and Fig. 1B, it should be noted that counting of radioactivity means counting the total radioactivity, not only ^{99m}Tc-NC100668, but also any ^{99m}Tc-containing impurities (such as the pertechnetate ion, $^{99m}TcO_4^-$) and ^{99m}Tc-NC100668 metabolites, and that only a very small fraction of NC100668 has ^{99m}Tc bound to its C-terminal chelate, even at the lowest dose of NC100668. A more rapid elimination of NC100668 than the radioactivity indicated that metabolites of NC100668 were formed in blood, similar to that observed in rats (Skotland et al., 2006). However, too few data are available in the present study to conclude if such metabolites were formed also in human blood.

Identification of Metabolites in Urine. The first part of the metabolite identification focused on analyzing the urine sample collected during the first hour after injection of NC100668 to subject #44, as this sample contained the highest metabolite concentration.

Using the LC-MS method developed for analysis of urine metabolites following injection of high doses (up to 5 mg NC100668/kg) in rats (Skotland et al., 2006), no difference was observed in the total ion chromatogram of the post-dose urine sample when comparing with the pre-dose sample (Figure 2A and 2B; inserts), even in the region with retention time of 11.60-12.00 min where differences were observed in the rat study. However, as also shown in Fig. 2A and 2B, differences in signal intensities were observed in the MS spectrum in this region of the chromatogram in the post-dose sample; new signals were observed at *m/z* 201.6 and 402.2 and new, but much weaker, signals were observed at *m/z* 303.3 and 345.2.

The substance in the human post-dose urine sample giving rise to the signal at m/z 402.2 had a retention time similar to that of Gly-NC100194, and MS-MS spectra of this substance showed exactly the same pattern as obtained with Gly-NC100194 (Fig. 3A and B). Thus, Gly-NC100194 was identified as a metabolite in this human urine sample similar to that reported for rats (Skotland et al., 2006). Furthermore, the MS-MS data in Fig. 3A and B indicated that the weak signal at *m/z* 303.3 in the post-dose urine (Fig. 2B) was due to a fragment of Gly-NC100194 formed in the MS. The fragmentation pattern for Gly-NC100194 is detailed in the rat metabolism report (Skotland et al., 2006).

The substance in the human post-dose urine sample giving rise to the weak signal at m/z 345.2 had a retention time similar to that of NC100194, and MS-MS spectra of this substance showed exactly the same pattern as obtained with NC100194 (Fig. 3C and D). Thus, NC100194 was identified as a metabolite in this human urine sample similar to that reported for rats (Skotland et al., 2006). As the NC100194 metabolite in urine eluted close to other substances, the MS-MS spectra showed additional signals compared with the standard. It should be noted that the main MS-MS fragments of NC100194 (*m/z* 187, 246 and 286; Fig. 3D) were all 57 units below those formed from Gly-NC100194 (*m/z* 244, 303 and 343; Fig. 3B). As Gly contributes with a mass of 57, this means that similar fragments were formed

from NC100194 as those described for Gly-NC100194.

Gly-NC100194 and NC100194 were observed as metabolites in urine samples from all subjects receiving the highest dose (Table 1). No other metabolite signal was identified in any of these subjects (data not shown), which was as expected based on the data obtained in the rat study.

Estimation of Gly-NC100194 and NC100194 in Urine Samples. The urine samples analyzed with their respective collection periods (up to approximately 13 h) and volumes for all subjects of the highest-dose group is listed in Table 1. As shown in this table the highest concentrations of both metabolites were observed in the urine samples collected during the first hour after injection. Moreover, in the samples collected during the first hour after injection, the amount of NC100194 was only 4 to 8% of that of Gly-NC100194 for 6 of the 8 subjects. This ratio increased with time after injection for all 8 subjects. Considerably higher ratios of NC100194 to Gly-NC100194 were obtained in the samples from subjects #42 and #45 than from the other 6 subjects. It should be noted that the extreme high ratios obtained for subject #45 were in part due to interferences from an endogenous substance (also observed in the pre-dose sample) resulting in an overestimation of the concentration of NC100194 in these samples. As the concentration of the interfering substance may vary between different urine samples, there is no good way of correcting for this interference and the data for subject #45 are therefore reported as they were obtained, i.e. without any correction.

The data in Table 1 were used to calculate the total amount of each metabolite excreted in the urine samples. The accumulated recovery of Gly-NC100194 during the first 13 hours after injection was in the range of 43 to 63% of the injected dose for 7 of the 8 subjects. In the samples from subject #45, the recovery of Gly-NC100194 was only 19% of the injected dose. This subject, who obviously metabolized NC100668 very fast, was the same subject that showed the endogenous urine substance which resulted in an overestimation of the amount of

NC100194. The estimated accumulated amount of Gly-NC100194 for all 8 subjects corresponds to 52±18% (mean±SD) of the injected dose of this part of NC100668. As mentioned above, 2 of the subjects showed a much higher recovery of NC100194 than for the 6 other subjects. Thus, a recovery of 33% and 56% of NC100194 was estimated for subject #42 and #45, respectively (although this number is certainly overestimated for #45), whereas the 6 others showed a recovery of 3 to 10%. The estimated accumulated amount of NC100194 corresponds to 15±19% (mean±SD) of the injected dose when all 8 subjects are included and 10±10% if the data for subject #45 are not included in the calculations.

It should be noted that quantification of these metabolites was not possible in the rat urine samples (Skotland et al., 2006), although these samples contained much higher concentrations of the metabolites than the human urine samples. The difficulty in quantifying these metabolites in rat urine is probably due to the much higher (and varying) concentration of both high- and low-molecular-weight substances in rat urine (Alt et al., 1985), and illustrates the differences in rat and human kidneys in handling proteins and peptides.

The recoveries for the two metabolites are listed in Table 1 together with the recovery of ^{99m}Tc in the same samples. As shown in Table 1 the estimated sums of the two metabolites were in the range 46.2 to 86.4% of the injected dose, whereas the accumulated recoveries of ^{99m}Tc in the same samples were in the range 50.6 to 91.4% of the injected dose. By looking at the data in Table 1 in more detail, it is clear that the estimated recoveries of the metabolites fit quite well with the ^{99m}Tc data for many of the samples, e.g. all samples analyzed for subject #42. On the other hand, there are rather large differences for some samples, e.g. the samples obtained during the first hour after injection for subjects #44, #49 and #50. The Gly-NC100194 levels estimated in these three samples were higher than expected from the radioactivity measurements. In addition, when comparing with the radioactivity measurements, there is a clear trend towards underestimation of the metabolite levels at very low metabolite

concentrations. As the radioactivity measurements are expected to be very accurate, the most likely explanation for this is the technical difficulty encountered in the estimation of the two metabolites as described in Materials and Methods. In summary, these data indicate that most of the ^{99m}Tc excreted in urine is bound to either Gly-NC100194 or NC100194. Furthermore, our conclusion is supported by the published results on the *in vivo* (rat) and *in vitro* metabolic profile of ^{99m}Tc-NC100668 (Edwards et al. 2006). Here it is concluded that the metabolites of ^{99m}Tc-NC100668 is ^{99m}Tc-Gly-NC100194, ^{99m}Tc-NC100194 and $^{99m}TcO_4^-$.

All data reported above for analyses of metabolites in the urine samples were obtained from the subjects receiving the highest dose. Some analyses were also performed on urine samples collected from subjects in the next highest dose-group. Although Gly-NC100194 could be detected also in these urine samples (data not shown), the concentration of the metabolite was not estimated due to the high degree of uncertainty in these data.

The present data indicate that the metabolism in man is very similar to that observed in rats as the two same metabolites were observed in a similar ratio in urine of both species. There are many proteases in the body that theoretically should be able to cleave peptides at the C-terminal side of Lys (and Arg) residues and thus be able to cleave Gly-NC100194 from NC100668. As discussed in rat metabolism study (Skotland et al. 2006), the “trypsin-like protease” present in large amounts in the brush-border membrane (Guder and Ross, 1984) is an obvious candidate for cleaving Gly-NC100194 from NC100668 and the NC100194 observed in urine is most likely formed from Gly-NC100194 by an attack of membrane alanyl aminopeptidase (EC 3.4.11.2), which is present in large amounts on the kidney brush border membrane (Turner, 1998).

In conclusion, analysis of blood and urine samples obtained following injection of ^{99m}Tc-NC100668 in healthy volunteers revealed a rapid elimination from blood and mainly urinary excretion fitting with a mechanism of glomerular filtration. The substance seems to be

metabolized similarly in rat and man as the two same C-terminal metabolites were detected in urine samples from both species.

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Footnote

Send reprint requests to:

Dr. Kim Toft,

Research and Development, GE Healthcare,

Nycoveien 2, N-0401 Oslo,

Norway.

Legends for figures

FIG. 1. *Mean plasma concentration of radioactivity (A), and mean plasma concentration with standard deviation of NC100668 (B), following administration of ^{99m}Tc-NC100668 Injection. The nominal dose of radioactivity was 190 MBq for all subjects, whereas the nominal doses of NC100668 were 2000 µg (●), 1000 µg (○), 500 µg (■), 100 µg (□) and 20 µg (▲).*

FIG. 2. *MS spectra (mass range = 150 to 800) of human urine samples from subject #44; pre-dose (A) and following injection of 1.6 mg NC100668 (B). MS spectra were collected at the retention time of 11.60 to 12.00 min from the total ion current chromatograms (mass range 150-2000) shown in the inserts.*

FIG. 3. *MS/MS spectra (mass range = 150 to 500) of the main peaks observed in the inserted chromatograms, i.e. following fragmentation in the MS of the ions giving rise to the signal at m/z = 402.3 ± 1.0 of the post-dose urine sample shown in Fig. 2B (A) and synthesized standard Gly-NC100194 (B) and at m/z = 345.5 ± 1.0 of the same post-dose urine sample (C) and synthesized standard NC100194 (D).*

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TABLE 1. Amount *of Gly-NC100194 and NC100194 excreted in urine samples from the highest-dose group, including accumulated recovery of the metabolites and radioactivity*

Sample ID and collection period (h)		Volume (ml)	Gly-NC100194 (ng/ml)	NC100194 (ng/ml)	Ratio of NC100194 to Gly-NC100194 (%)	Accumulated recovery (% of injected dose)	
						Gly-NC100194 + NC100194	^{99m}Tc
#41	1	150	823	61	8	47.5	48.2
	2	52	166	24	14	51.0	62.2
	4	80	109	32	30	55.0	73.8
	8	200	90	37	41	63.8	84.3
	13	250	26	<LOQ	NA	66.1	91.4
#42	1	23	699	213	30	7.6	8.3
	2	320	236	209	89	57.2	52.9
	4	90	186	148	80	67.7	64.9
	7	140	48	92	190	74.3	70.5
	8	70	70	50	71	77.3	73.7
	13	500	<LOQ	<LOQ	NA	77.3	76.6
#43	1	290	403	22	6	44.3	50.5
	2	62	187	16	9	48.8	66.0
	4	390	24	<LOQ	NA	52.2	82.7
	8	68	5.9	10	166	52.6	83.4
	13	260	<LOQ	<LOQ	NA	52.6	85.1
#44	1	88	1559	61	4	51.3	33.6
	2	44	871	64	7	66.1	45.0
	3	240	43	<LOQ	NA	69.8	55.2
	4	80	140	17	12	74.3	59.5
	7	260	49	16	33	80.2	67.7
	8	80	60	25	41	82.6	70.1
	9	26	130	108	83	84.7	70.8
	13	88	56	<LOQ	NA	86.4	72.8
#45	1	45	277	438[a]	158[a]	10.6[a]	7.4
	2	85	132	449[a]	340[a]	26.6[a]	22.0
	4	38	73	436[a]	598[a]	32.7[a]	27.7
	8	450	40	190[a]	475[a]	65.7[a]	50.2
	13	290	29	72[a]	252[a]	75.3[a]	55.4
#48	1	90	860	39	5	29.1	40.2
	2	37	411	45	11	35.1	51.1
	4	41	304	48	16	40.3	60.2
	8	135	62	29	47	44.5	69.3
	13	170	28	<LOQ	NA	46.2	72.0
#49	1	124	761	41	5	35.7	24.7
	2	78	263	15	6	43.5	36.3
	3	230	92	18	19	52.4	46.0
	4	143	24	<LOQ	NA	53.6	50.5
	5	345	26	<LOQ	NA	56.9	54.6
	8	350	10	<LOQ	NA	58.1	58.8
	10	150	<LOQ	<LOQ	NA	58.1	60.0
	16	200	<LOQ	45	NA	60.9	61.6
#50	1	93	1394	84	6	49.3	16.2
	2	140	104	29	28	55.9	26.6
	4	350	48	52	107	67.5	39.8
	7	300	23	11	47	71.0	45.7
	8	230	24	<LOQ	NA	73.0	48.2
	13	350	<LOQ	<LOQ	NA	73.0	50.6

[a] Overestimated due to presence of endogenous substance; NA: Not applicable

Figure 1

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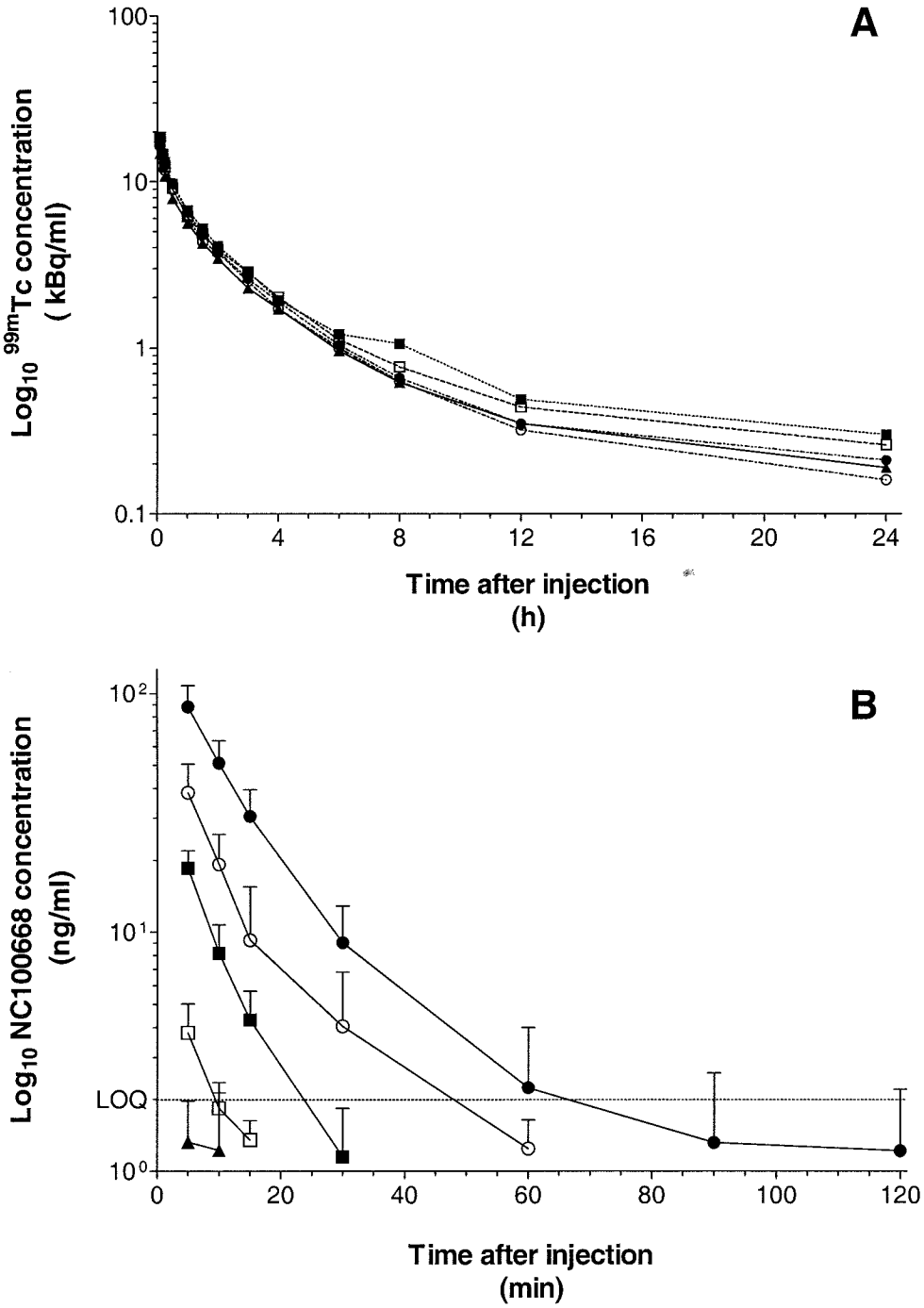

Figure 2

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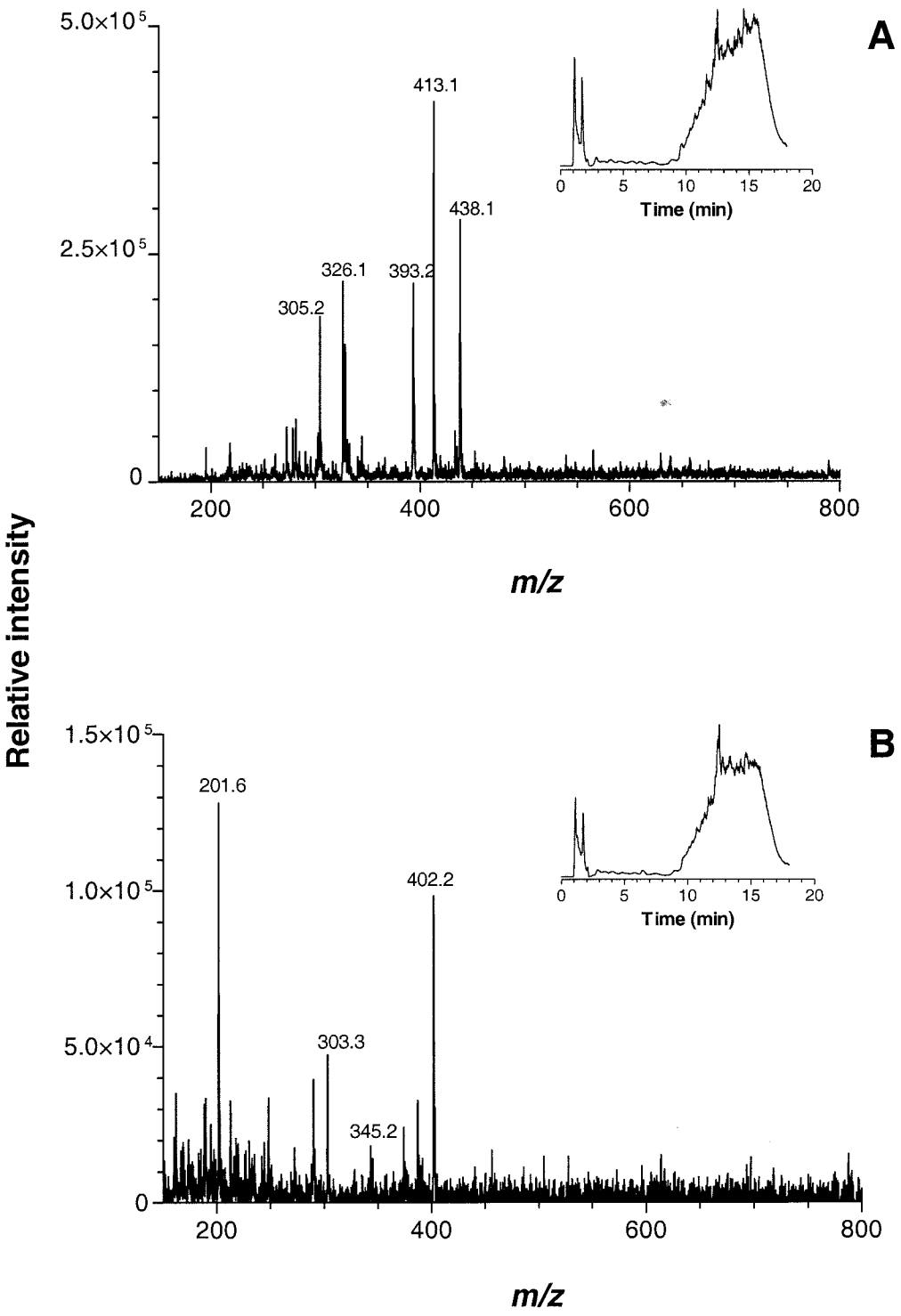

Figure 3

DMD Fast Forward. Published on August 6, 2007 as DOI: 10.1124/dmd.106.014126
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