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Quantification of NC100668, a new tracer for imaging of venous thromboembolism, in human plasma using reversed-phase liquid chromatography coupled with electrospray ionization ion-trap mass spectrometry

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Abstract

NC100668 is being developed as a new tracer for radiopharmaceutical imaging of venous thromboembolism. NC100668 consists of a targeting peptide of 13 amino acids with a ^{99m}Tc-binding chelator linked to the C-terminal amino acid. The present report describes a method for quantification of NC100668 in human citrated plasma. The method is based on solid-phase extraction followed by reversed-phase liquid chromatography using a gradient of water and acetonitrile with 0.1% formic acid. The chromatographic system was coupled on-line with an electrospray mass spectrometer. The analyses were performed by selective ion monitoring of the $[M+2H]^{2+}$ and the $[M+3H]^{3+}$ ions of NC100668 and an internal standard which was identical to NC100668 except for not being iodinated in the tyrosine residue. The limit of quantification of the method was 2 ng NC100668/ml plasma. The calibration curve ranged from 2 to 250 ng NC100668/ml plasma and was fitted to a linear equation with a weighing factor of $1/y^2$ and found to be highly reproducible. The total precision of the method, expressed as the relative standard error of the mean, was 23.2, 8.8 and 14.7% for the low, medium and high control samples, respectively. The accuracy of the method was 108.5, 100.0 and 105.0% for the low, medium and high control samples, respectively. NC100668 was stable in human plasma during at least three freeze/thaw cycles, during 30 h on dry ice and up to 3 months when stored in a −20 °C freezer.
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Keywords: NC100668; Human plasma; LC–MS; Ion-trap; Imaging; Venous thromboembolism

1. Introduction

^{99m}Tc-NC100668 is being developed as a diagnostic radiopharmaceutical for imaging of venous thromboembolism, which is a major health problem with an estimated average annual incidence in the US exceeding 1/1000 [1]. NC100668 consists of a 13 amino acid peptide, N-terminally blocked with an acetyl group, containing an iodinated tyrosine and 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–iodoTyr–Thr–Leu–Leu–Lys–Gly–NC100194 where NC100194 is; $-NH-CH_2-CH_2-N[CH_2-CH_2-NH-C(CH_3)_2-C(CH_3)=N-OH]_2$ (Fig. 1).

The peptide moiety of NC100668 is similar to the N-terminal sequence in human α_2-antiplasmin [2,3], which during the coagulation process is covalently linked to fibrin by the enzymatic action of blood coagulation factor XIIIa. It has been shown that a covalent bond is formed between a glutamine residue in α_2-antiplasmin (corresponding to Gln-2 in NC100668) and a lysine residue in fibrin [4]. It is believed that a similar reaction mediated by factor XIIIa is the mechanism of action for binding of NC100668 to fibrin in blood clots [5,6], thus making it possible to obtain a scintigraphic image of the clot.

^{99m}Tc is one of the principal radionuclides used in nuclear medicine due to its short half-life (6.02 h) and favourable γ-emitting energy. The ^{99m}Tc-based radiopharmaceuticals are distributed to the pharmacy as a lyophilised kit in a Tc-free form.

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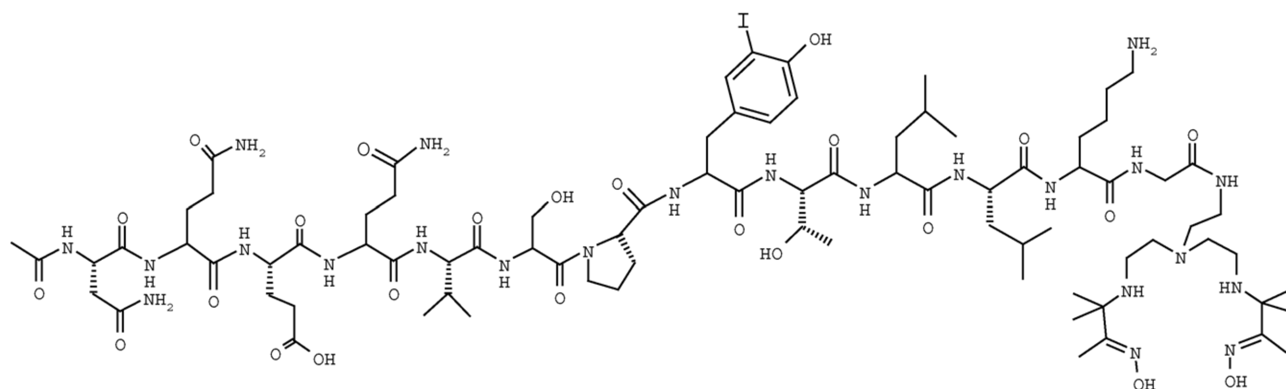

Fig. 1. The structure of NC100668.

Prior to use, ^{99m}Tc (in the form of the pertechnetate anion, i.e. TcO_4^-, eluted from a $^{99}Mo/^{99m}Tc$ generator with saline) is added to the freeze-dried active substance (called the ligand; NC100668 in the present study) and the added ^{99m}Tc binds to the chelator part of the ligand. There is a vast excess of the ligand compared with the added ^{99m}Tc. Less than 1% of the ligand in the injected solution is in the form of a ^{99m}Tc-complex. Hence, the unlabelled ligand makes up 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 images obtained. During the last decade, several highly specific synthetic peptides or small molecular markers have been efficiently labelled with ^{99m}Tc in order to image the acute pathology of venous thromboembolism [7].

As part of the development of NC100668 it was necessary to establish a method to describe the kinetics of this agent in animals receiving high doses (toxicokinetics) and in humans receiving doses close to the clinical imaging dose (pharmacokinetics). As one clinical imaging dose consists of less than 100 μg NC100668, a method with low limit of quantification was needed for this purpose. We here report validation data obtained with a method based on SPE of human citrated plasma followed by reversed-phase liquid chromatography coupled with electrospray ion-trap mass spectrometry (LC-ESI-MS). This method was found suitable for toxicokinetic purposes and it has also been used to describe the pharmacokinetics of NC100668 in humans (to be published elsewhere).

2. Experimental

2.1. Materials

Acetonitrile and methanol were LiChrosolve grade and hydrochloric acid (37%) was pro-analysis grade, all from Merck (Darmstadt, Germany). Formic acid was pro-analysis grade from Ratburn Chemicals Ltd. (Walkerburn, Scotland). NC100668 and TT106 (internal standard; structure identical to NC100668 except for not being iodinated in the tyrosine residue) were from GE Healthcare (Oslo, Norway). Water was purified by reversed osmosis, ion exchanged and filtrated through a 0.45 μm filter using a Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were of analytical grade. Citrated human blood for standard and control samples was obtained from two healthy volunteers.

2.2. Standard and control samples

The calibration standards and the control samples were made by dilutions in citrated human plasma. The calibration standards contained 2–250 ng NC100668/ml in addition to the internal standard (100 ng TT106/ml) and were made fresh for every analytical sequence. Control samples were made at three concentration levels, i.e. 10 ng (low), 100 ng (medium) and 200 ng (high) NC100668/ml and stored at −20 °C. Before analyses, the control samples were added internal standard (100 ng TT106/ml).

2.3. Sample preparation

Plasma was prepared by centrifugation at $3000 \times g$ for 10 min. The plasma samples (250 μl) were extracted by a SPE procedure using a VacMaster® Sample Processing Station from IST (Hengoed, UK). The plasma samples were applied onto Bond Elut-C18 cartridges (100 mg) obtained from Varian Associates (Harbor City, CA, USA), which were preconditioned with 2 ml of methanol and 2 ml of water. TT106, the internal standard, and plasma were added to the cartridge which were washed with 2 ml of water and 2 ml of methanol + 0.1 M HCl + water (40 + 20 + 40, v/v/v). Finally, NC100668 and TT106 were eluted with 2 ml of 80% methanol in 20% 0.1 M HCl (80 + 20, v/v). The samples were evaporated under a stream of nitrogen at room temperature and the residues were dissolved in 150 μl of 20% (v/v) acetonitrile in water; 10 μl were injected onto the LC-column.

2.4. Chromatographic conditions

A Hewlett Packard Series HP1100 system (Agilent Technologies, Palo Alto, CA, USA) was used with a Waters Symmetry Shield RP8, 100 mm × 2.1 mm I.D. (3.5 μm particle diameter) column (Waters Corporation, MA, USA) with a Waters Symme-

try Shield RP8 10 mm × 2.1 mm I.D. (3.5 μm particle diameter) guard column (Waters Corporation, MA, USA). Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B 0.1% (v/v) formic acid in acetonitrile. To separate NC100668 from endogenous peptide and protein material, a gradient was run starting at 95% mobile phase A, decreasing to 50% A in 10 min and then back to 95% A in 1 min. Including 11 min equilibration time, the total chromatographic run time was 22 min. The flow rate was 0.2 ml/min and the analysis was performed at ambient temperature (approximately 22 °C). The samples were kept in the autosampler at 4 °C during the entire analytical sequence and 10 μl was injected for each analysis.

2.5. *Mass spectrometry*

The LC system was coupled on-line to a LCQ Classic ion-trap quadrupole mass spectrometer (Thermo Finnigan, San Jose, FL, USA); the interface between the LC and the MS was electrospray ionization (positive mode). The ion-source and ion-optic parameters were optimised with respect to the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of NC100668, i.e. $m/z = 986.5 \pm 1.0$ and $m/z = 658.0 \pm 1.0$, respectively. Quantification of NC100668 in the calibration samples and control samples were conducted by single ion monitoring (SIM) of four selected ions, the two ions mentioned above for NC100668 and two ions for the internal standard TT106, i.e. the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions at $m/z = 923.5 \pm 1.0$ and $m/z = 616.0 \pm 1.0$, respectively.

2.6. *Sample analysis and validation parameters*

The samples were analysed by LC–MS (SIM of *m/z* 658.0, 986.5, 616.0 and 923.5) in sequence together with calibration standards, control samples and injection blanks. The calibration standards were positioned at the beginning of each sequence, while the control samples and the blanks were randomly placed in the sequence together with the samples. The standard curve was evaluated from six calibration curves analysed on six different days. The precision was measured by analysing the three control samples (9.87, 98.7 and 197 ng NC100668/ml) in triplicate in a total of six analytical sequences. The standard deviations were determined from the mean square values of an ANOVA single factor calculations of the results where $\text{S.D.}_{w(p)}{}^2$ = within-group mean square and $n\text{S.D.}_x{}^2$ = between-group mean square (where n is the number of replicates per analysis). Accuracy of estimating NC100668 in human plasma was calculated from the mean value of the precision data.

2.7. *Data handling*

Thermo Finnigan Xcalibur version 1.0 SR1 was used for sampling and integration of the chromatograms and LCquan version 1.0 SR1 for quantification of NC100668 in the plasma samples. GraphPad Prism, version 2.0 was used for statistical evaluation of the regression parameters. Microsoft Excel, version 5.0 was used for the ANOVA calculations and the other statistical calculations.

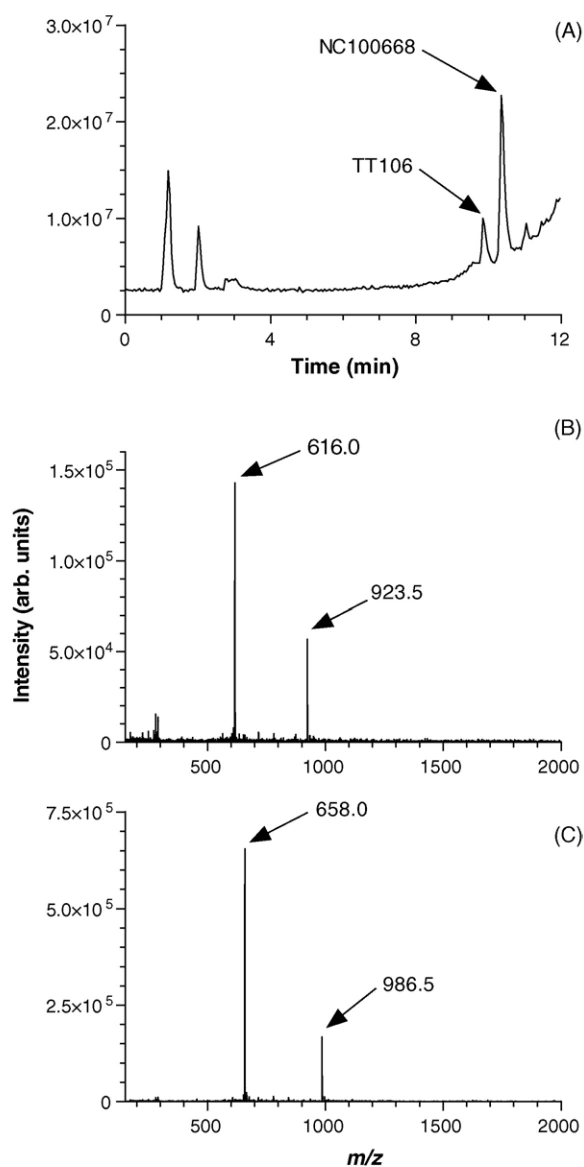

Fig. 2. Typical total ion current full scan (*m/z* 150–2000) chromatogram and mass spectrum of a standard containing 200 ng NC100668/ml and 200 ng TT106/ml; (A) LC–MS chromatogram, (B) mass spectrum of the TT106 peak from (A) with retention time of 9.90 min and (C) mass spectrum of the NC100668 peak from (A) with retention time of 10.40 min.

3. Results and discussion

3.1. *Specificity of the method*

Chromatographic resolution of NC100668 and the internal standard was achieved by using the LC–MS method as shown in Fig. 2A. The mass spectrum of NC100668 (Fig. 2C) shows that the most abundant peaks are *m/z* 658.0 which is the $[M+3H]^{3+}$ ion and *m/z* 986.5 which is the $[M+2H]^{2+}$ ion, respectively. The molecular ion of NC100668 with an *m/z* 1972.0 $[M+H]^{+}$ was not observed in this mass spectrum. The mass spectrum of the internal standard TT106 (Fig. 2B) shows that the most abundant peaks are *m/z* 616.0 which is the $[M+3H]^{3+}$ ion and *m/z* 923.5

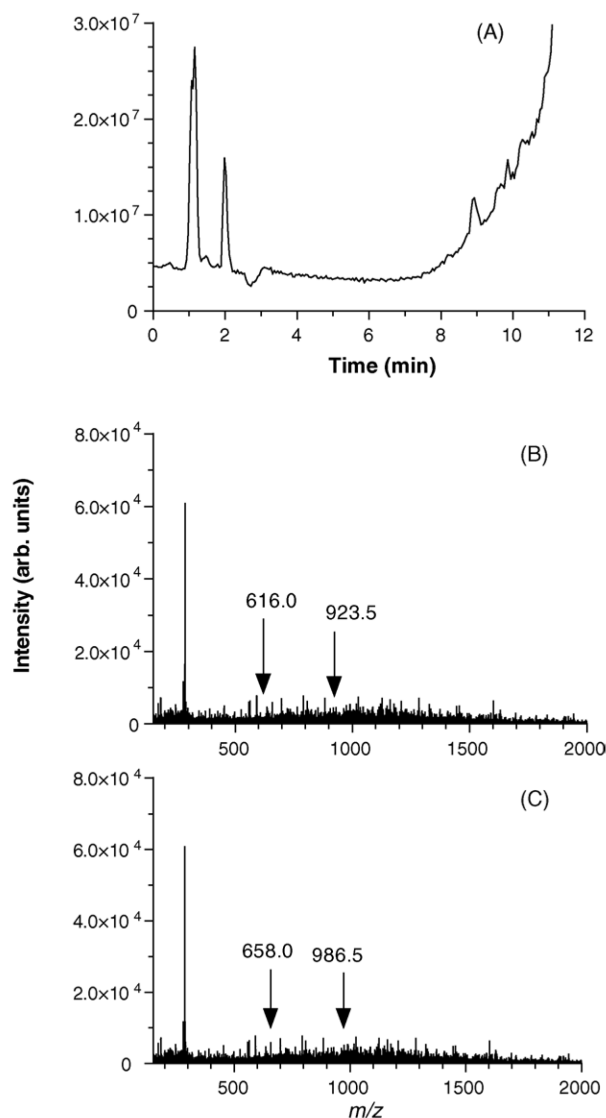

Fig. 3. Typical total ion current full scan (m/z 150–2000) LC–MS chromatogram and mass spectrum of a plasma sample from a healthy volunteer; (A) LC–MS chromatogram, (B) mass spectrum at the retention time of TT106 peak at 9.90 min from (A) and (C) mass spectrum at the retention time of NC100668 peak (10.40 min) from (A).

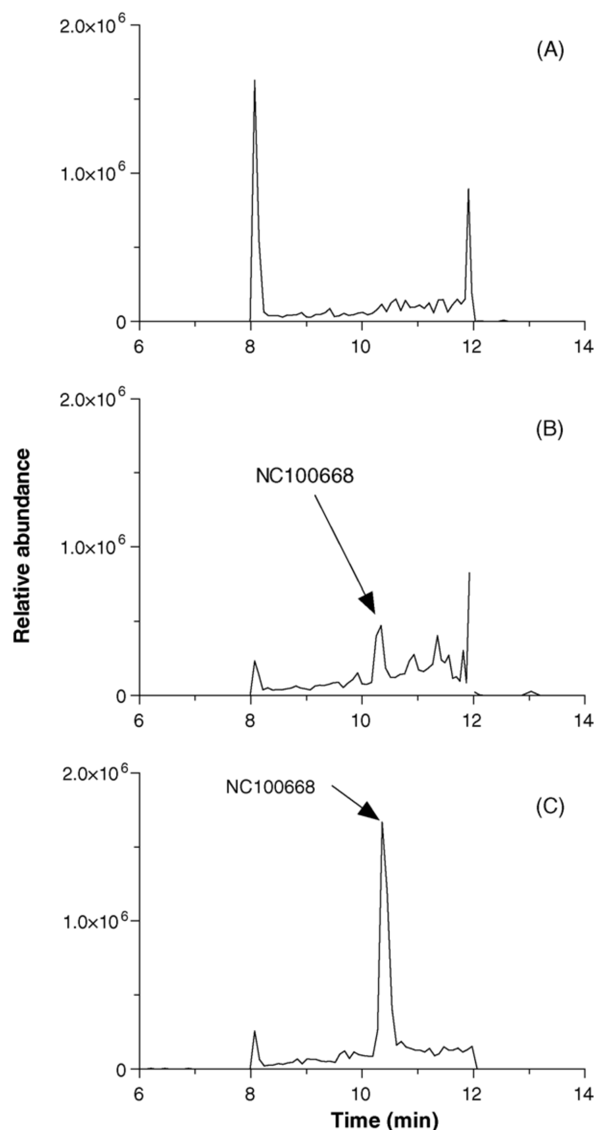

Fig. 4. Typical chromatograms in the SIM mode of; (A) a blank sample, (B) an LOQ sample, and (C) a real plasma sample from the clinical trial. Note that only the time window between 8 and 12 min in the chromatographic run time was analysed by the MS for the clinical samples.

which is the $[M+2H]^{2+}$ ion, respectively. The molecular ion of TT106 with an m/z 1845.0 $[M+H]^{+}$ was not observed in this mass spectrum.

The selectivity and specificity of the method was measured by analysing extracted plasma samples from two donors in the validation study. In addition, extracted plasma samples from nine healthy volunteers that received saline injections in stead of NC100668 in the clinical phase I study were also tested for selectivity and specificity. As shown in Fig. 3 there were no major interfering peaks in the chromatogram and the mass spectra at the retention time of NC100668 or TT106. The late eluting peaks in Fig. 3A originated from peptide and protein material that were not removed during the sample preparation step. The selectivity of the method is further demonstrated in Fig. 4 showing chromatograms in the SIM mode of a blank sample (A), an LOQ sample (B), and a real plasma sample from the clinical trial (C).

The retention times of TT106 and NC100668 in human plasma samples showed very little variation throughout the analytical sequences (i.e. results from one typical analytical sequence were 9.85 ± 0.03 min (mean ± S.D., $n = 25$) and 10.35 ± 0.03 min (mean ± S.D., $n = 25$), for TT106 and NC100668, respectively).

3.2. Solid phase extraction

In order to avoid clogging of the LC column and MS capillary, the plasma samples were extracted via solid phase extraction. It was not possible to obtain percentage extraction recoveries of NC100668 and TT106 by comparing water and plasma as

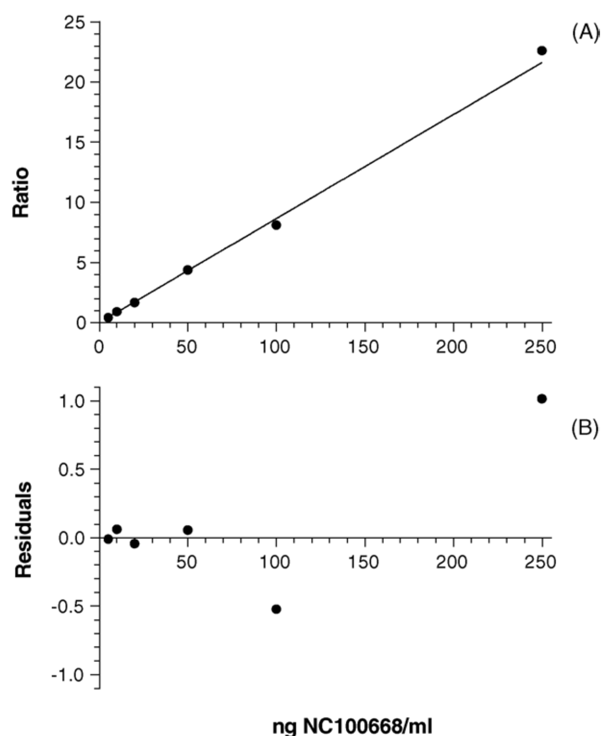

Fig. 5. Typical calibration curve (A) and residual plot (B) of the calibration standards.

matrices, as these peptides in water solution adhere to surfaces whether it is glass or plastic.

3.3. Quantification

Carry-over effects were minimized by regular washing of the LC and the heated MS capillary with solutions of different polarity. In addition, the guard column was back flushed with mobile phase for 1–2 h between every sequence. The carry over was normally less than 1 ng NC100668/ml when analysing a blank plasma sample after the highest calibration standard or the highest control sample, i.e. less than 0.5%.

The calibration curve was made by plotting the peak area ratio of NC100668 to TT106 against the theoretical concentration of NC100668. The curvature was evaluated by using an *F*-test, comparing linear ($y = a + bx$) and quadratic regression ($y = a + bx + cx^2$), with the linear regression giving the best fit (data not shown). The calibration curve was weighted with a weighing factor of $1/y^2$ to get the best fit of the lower calibration standards. An example of a calibration curve and residual plot are shown in Figs. 5 and 6. The calculated regression parameters together with the 95% confidence interval of the calibration curves from six analytical sequences are listed in Table 1. The results show that the slope is statistically different form 0, the intercept is not statistically different from 0, and the regression coefficient is not statistically different from 1.00. The goodness of fit of the calibration point to the calibration curve was also calculated and the largest deviation from the theoretical concentration was 8% (Table 2).

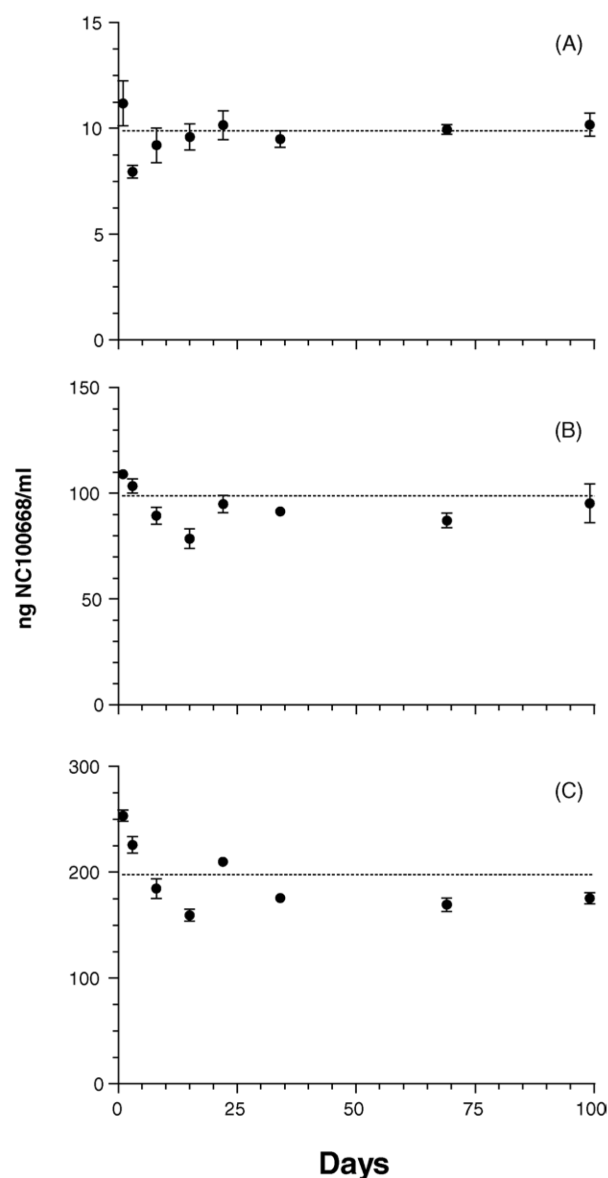

Fig. 6. Stability of NC100668 in human plasma stored at −20 °C. Three concentration levels were tested; (A) 10 ng/ml, (B) 100 ng/ml, and (C) 200 ng/ml. The dotted lines indicate the theoretical concentration levels.

3.4. Limit of quantification

Based on pre-validation work, 2 ng NC100668/ml (corresponding to 40 pg injected) was chosen as the lowest standard of the calibration curve. The repeatability of this calibration stan-

Table 1
Regression parameters of the calibration curve fitted to the equation $y = a + bx$ and weighted with $1/y^2$. The mean values are calculated based on six individual calibration curves

Regression parameters	Mean ($n = 6$)	95% confidence interval of the mean
Intercept, *a*	0.102	±0.114
Slope, *b*	0.070	±0.010
Regression coefficient, r^2	0.990	±0.008

Table 2
The goodness of fit of the calibration points

Target concentration (ng NC100668/ml)	Recovery (% of theoretical value)
2.0	106 ± 4
5.0	93 ± 6
10	104 ± 6
20	96 ± 7
50	104 ± 9
100	98 ± 5
250	108 ± 6

The values were estimated from six analytical series for each calibration standard and are given as mean ± S.D.

Table 3
The analytical precision of the method calculated by ANOVA single factor calculations

Control sample	Mean value (ng/ml)	$S.D._{w(p)}$ (ng/ml)	$S.D._t$ (ng/ml)	$R.S.D._w(p)$ (%)	$R.S.D._t$ (%)
High	209	13	31	6.4	14.7
Medium	99	6	9	6.5	8.8
Low	10.7	1.1	2	10.5	23.2

Table 4
The accuracy of the method

Control sample	Concentration (ng NC100668/ml)		Accuracy (%)
	Theoretical	Found (mean ± S.D.)	
High	197	209	106.0
Medium	98.7	98.7	100.0
Low	9.87	10.7	108.5

dard was during the validation study, found to be 28.8% R.S.D. ($n = 6$). Although this value is higher than stated in the recommendations for estimation of LOQ (i.e. 20%) [8,9], this value was chosen as the LOQ of the method. Since each calibration curve was made new for every analytical sequence, the concentration of the calibration standards varied to a small extent. Thus, the lowest calibration standard was either slightly below or slightly above 2 ng/ml. Calculations of the S/N ratio for five different LOQ calibration standards gave very similar results (mean 7.4; range 6.3–8.3).

3.5. Precision and accuracy

The precision and accuracy of the method was calculated using the three quality control samples as described in Section 2.6. The repeatability R.S.D. of the control samples was found to be 10.5, 6.5 and 6.4% for the low, medium and high control samples, respectively (Table 3). The intermediate precision R.S.D. was found to be 23.2, 8.8 and 14.7% for the low, medium and high quality control samples, respectively (Table 3). These data show a good precision, except for the lowest control sample, where the between-run variation was slightly higher than 20%. The accuracy was found to be 108.5, 100.0 and 106.0% for the low, medium and high control samples, respectively (Table 4), i.e. these data show a good accuracy of the method.

3.6. Sample stability

The stability of NC100668 in human plasma stored at −20 °C was tested by analysis of the three control samples for a period of 99 days (Fig. 5). The data show that NC100668 is stable in human plasma for at least 3 months when stored at −20 °C. Moreover, analyses of the three control samples showed NC100668 to be stable in human plasma during at least three freeze/thaw cycles and for at least 30 h on dry ice (data not shown). NC100668 extracted from plasma was found to be stable in the autosampler for at least 32 h at 4 °C (data not shown).

4. Conclusion

An LC–MS method for quantification of NC100668 in human plasma was developed and validated. The method was found to be suitable for quantification of NC100668 in animal plasma for toxicokinetic calculations and in human plasma for calculations of pharmacokinetic parameters. The pharmacokinetic data will be published separately as part of a clinical phase I study.

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