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Quantification of NC100692, a new tracer for ^{99m}Tc-imaging of angiogenesis, in human plasma using reversed-phase liquid chromatography coupled with electrospray ionization ion-trap mass spectrometry

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Abstract

NC100692 is under development as a diagnostic radiopharmaceutical for targeting angiogenesis associated with diseases, such as cancer and endometriosis. NC100692 consists of a cyclic RGD-containing peptide with an ethylene glycol chain linked to the C-terminal amino acid and a ^{99m}Tc-binding chelator linked to the N-terminal amino acid. The present report describes a method for quantification of NC100692 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 NC100692 and the internal standard, which was identical to NC100692 except for containing twice the length of the ethyleneglycol chain. The limit of quantification of the method was 0.5 ng NC100692/ml plasma. The calibration curve ranged from 0.5 to 250 ng NC100692/ml plasma and was fitted to a quadratic equation with a weighing factor of $1/y$ and found to be highly reproducible. The total precision of the method, expressed as the relative standard error of the mean, was 11.1, 10.8 and 9.7% for the low, medium and high control samples, respectively. The accuracy of the method was 103.4, 111.1 and 107.5% for the low, medium and high control samples, respectively. NC100692 was stable in human plasma during at least 3 freeze/thaw cycles, during 48 h on dry ice and at least 8 weeks when stored in a −20 °C freezer.

Keywords: NC100692; Human plasma; LC–MS; Ion-trap; Imaging; Angiogenesis

1. Introduction

The ^{99m}Tc complex of NC100692 is being evaluated as a diagnostic imaging agent for detection of angiogenesis, and was recently used for detection of malignant lesions in patients with breast cancer [1]. NC100692 consists of a peptide based pharmacophore containing a cyclic RGD sequence coupled to an ethylene glycol biomodifier in the C-terminal end of the peptide and a ^{99m}Tc-binding chelator linked to the N-terminal end. The structure of this substance is shown in Fig. 1; the synthesis was recently described by Indrevoll et al. [2].

^{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 resulting in low radiation exposure to the patients [3]. ^{99m}Tc-based radiopharmaceuticals are distributed to the pharmacy as lyophilised kits in a Tc-free form. 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) [3] is added to the freeze-dried ligand (NC100692 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. Accordingly, it is the

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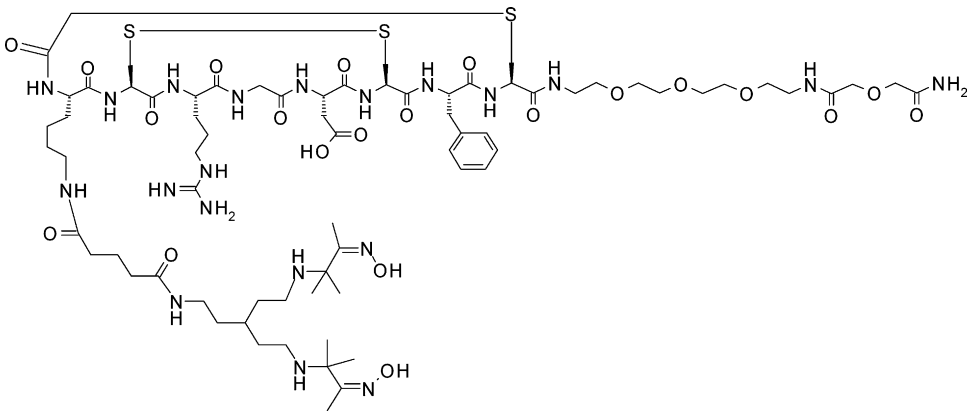

Fig. 1. The structure of NC100692.

unlabelled ligand that is used to describe the kinetic parameters for such products.

As part of the development of NC100692 it was necessary to establish a method to describe the kinetics of this agent in humans receiving doses close to the clinical imaging dose (pharmacokinetics). As one clinical imaging dose consists of not more than 75 μg NC100692 [1], a method with low limit of quantification was needed for this purpose. We here report validation data obtained following normal guidelines for such work [4,5], with a method based on solid phase extraction (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 the intended use and has been used to describe the pharmacokinetics of NC100692 in humans (to be published elsewhere).

2. Experimental

2.1. Materials

Acetonitrile and methanol were LiChrosolve grade and acetic acid was pro-analysis grade, all from Merck (Darmstadt, Germany). Formic acid was pro-analysis grade from Rathburn Chemicals LTD (Walkerburn, Scotland) and ammoniumhydroxid was from Sigma (Sigma–Aldrich Co., St.Louis, MO, USA). NC100692 and the internal standard (NC100682) were from GE Healthcare (Oslo, Norway); the internal standard was identical to NC100692 except for having twice the length of the ethylene glycol chain. 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 and specificity and selectivity experiments were obtained from 8 healthy volunteers.

2.2. Standard and control samples

The calibration standards and the control samples were made by dilutions in human citrated plasma (Blood Bank, Ullevål University Hospital, Oslo, Norway) and stored at −20 °C. The calibration standards contained 0.5 to 250 ng NC100692/ml, and the control samples were made at 3 concentration levels, i.e. 1.5 ng NC100692/ml (low), 75 ng NC100692/ml (medium) and 200 ng NC100692/ml (high).

2.3. Sample preparation

Plasma was prepared by centrifugation at 3000 × *g* for 10 min at room temperature. The internal standard (50 μl) was added to the plasma samples (250 μl) and extracted by a SPE procedure using Gilson ASPEC XL4 from Gilson Inc. (Middleton, WI, USA). The SPE columns (30 mg Waters Oasis HLB) obtained from Waters (Milford, MA, USA) were preconditioned with 1 ml of methanol and 1 ml of water. The cartridges were washed with 2 ml of water and 2 ml of methanol + ammoniumhydroxid + water (35 + 2 + 63, v/v/v). Finally, NC100692 and the internal standard were eluted with 0.75 ml of methanol + acetic acid + water (80 + 2 + 18, v/v/v). The samples were evaporated under a stream of nitrogen at room temperature and the residues were dissolved in 125 μl of 20% (v/v) acetonitrile in water; 30 μl were injected onto the LC-column.

2.4. Chromatographic conditions

A TSP Surveyor LC-system (ThermoFinnigan, San Jose, FL, USA) was used with a Supelco Discovery HS C18, 100 × 2.1 mm i.d. (3 μm particle diameter) column (Supelco, Bellafonte, PA, USA) fitted with a Phenomenex C8 4 × 2.0 mm i.d. guard column (Phenomenex, Torrance, CA, 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 NC100692 from endogenous peptide and protein material, a gradient was run starting at 95% mobile phase A, decreasing to 50% A in 8 min, holding for 1 min, and then returning to 95% A in 1 min. Including 9 min equilibration time, the total chromatographic run time was 19 min. The flow rate was 0.3 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.

2.5. Mass spectrometry

The LC system was coupled on-line to a LCQ DECA XP^{Plus} LCQ ion-trap quadrupole mass spectrometer (Thermo Finnigan); 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 NC100692, i.e. $m/z = 566.6 \pm 0.5$ and $m/z = 849.4 \pm 0.5$, respectively. Quantification of NC100692 in the calibration samples and control samples were conducted by single ion monitoring (SIM) of 4 selected ions, the 2 ions mentioned above for NC100692 and similarly 2 ions for the internal standard, i.e. the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions at $m/z = 663.3 \pm 0.5$ and $m/z = 994.5 \pm 0.5$, respectively.

2.6. Sample analysis and validation parameters

The samples were analysed by LC–MS (SIM of *m/z* 566.6, 849.4, 663.3 and 994.5) 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 6 calibration curves analysed on 6 different days by 2 different operators. The precision was measured by analysing the 3 control samples in triplicate in a total of 6 analytical sequences. The standard deviations were determined from the mean square values of an ANOVA single factor calculations of the results where $S.D.w^2_{(p)}$ = within-group mean square and $nS.D._x^2$ = between-group mean square (where n is the number of replicates per analysis). Accuracy of estimating NC100692 in human plasma was calculated from the mean value of the precision data.

2.7. Data handling

Thermo Finnigan Xcalibur Version 2.0 SR1 was used for sampling and integration of the chromatograms and LCquan Version 1.0 SR1 for quantification of NC100692 in the plasma samples. GraphPad Prism, Version 4.0 was used for statistical evalua-

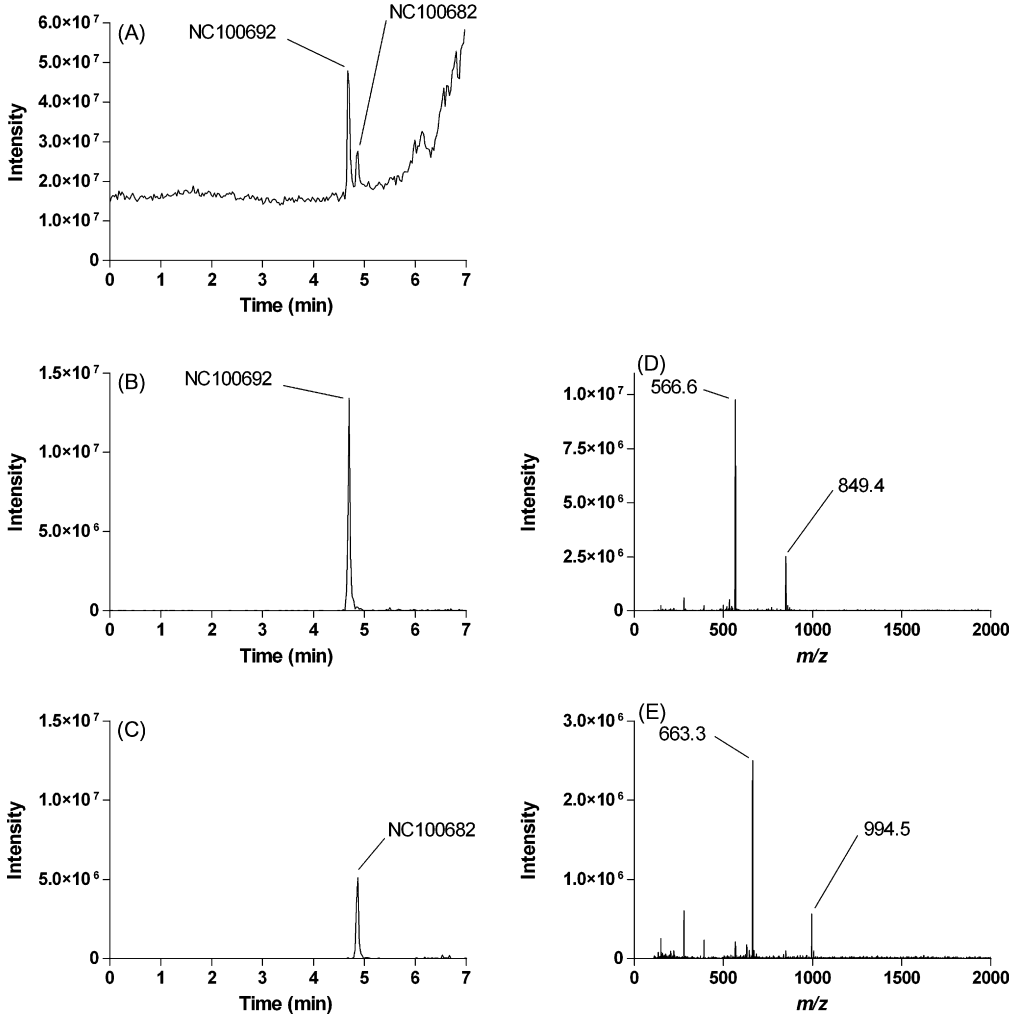

Fig. 2. Typical chromatograms and mass spectra of a standard containing 100 ng NC100692/ml and 100 ng internal standard (NC100682)/ml: (A) Total ion current full scan (*m/z* 150–2000) chromatogram of NC100692 and the internal standard, (B) SIM ($m/z = 566.6 \pm 0.5$ and $m/z = 849.4 \pm 0.5$) chromatogram of NC100692, (C) SIM ($m/z = 663.3 \pm 0.5$ and $m/z = 994.5 \pm 0.5$) chromatogram of the internal standard, (D) mass spectrum of NC100692 from (A) with retention time 4.7 min and (E) mass spectrum of the internal standard peak from (A) with retention time 4.9 min.

tion of the regression parameters. Microsoft Excel, Version 5.0 was used for the ANOVA calculations and the other statistical calculations.

3. Results and discussion

3.1. Specificity of the method

NC100692 and the internal standard were chromatographically resolved as shown for the full scan mode in Fig. 2A. The representative chromatograms using the SIM mode are shown in Fig. 2B and Fig. 2C for NC100692 and the internal standard, respectively. The mass spectrum of NC100692 (Fig. 2D) shows that the most abundant peaks are *m/z* 566.6.0 which is the $[M+3H]^{3+}$ ion and *m/z* 849.4 which is the $[M+2H]^{2+}$ ion, respectively. The molecular ion of NC100692 with an *m/z* 1697.80 $[M+H]^{+}$ was not observed. The mass spectrum of the internal standard (Fig. 2E) shows that the most abundant peaks are *m/z* 663.3 which is the $[M+3H]^{3+}$ ion and *m/z* 994.5 which is the $[M+2H]^{2+}$ ion, respectively. The molecular ion of the internal standard with an *m/z* 1987.0 $[M+H]^{+}$ was not observed.

The selectivity and specificity of the method were investigated by analysing extracted plasma samples from 6 donors. No major interfering peaks were observed in the chromatogram and the mass spectra at the retention time of NC100692 or the internal standard (Fig. 3). 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 NC100692 and the internal standard in human plasma samples showed very little variation. Based on the results from 1 typical analytical sequence, the retention times were 4.66 ± 0.01 min (mean ± S.D., $n = 21$) and 4.83 ± 0.01 min (mean ± S.D., $n = 21$), for NC100692 and the internal standard, respectively.

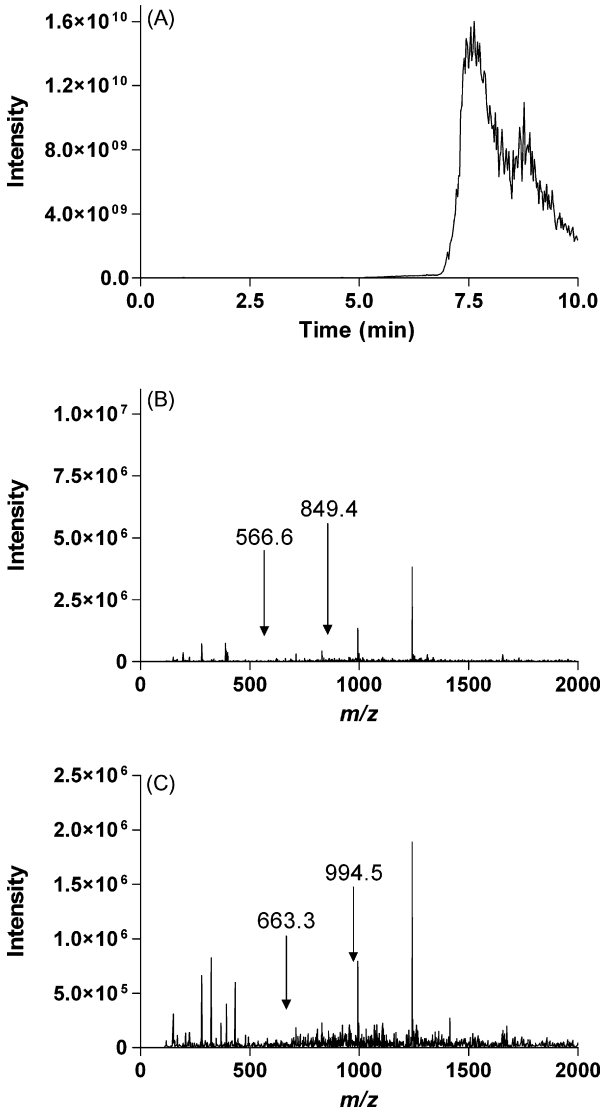

Fig. 3. Typical total ion current full scan (*m/z* 150–2000) LC–MS chromatogram and mass spectrum of a plasma sample from a healthy volunteer: (A) LC–MS chromatogram, (B) mass spectrum at the retention time of the NC100692 peak (4.7 min) from (A) and (C) mass spectrum at the retention time of the internal standard (NC100682) peak (4.9 min) from (A).

3.2. Quantification

Regular washing of the LC and the MS instruments with solutions of different polarity was performed in order to minimize carry-over effects. In addition, the guard column was changed between every analytical sequence. The carry over was normally <0.1% when analysing a blank plasma sample after the highest calibration standard.

The calibration curve was made by plotting the peak area ratio of NC100692 to the internal standard against the theoretical concentration of NC100692. The simplest model that adequately described the concentration–response relationship was $y = a + bx + cx^2$ weighted with a weighing factor of $1/y$ to get the best fit of the lower calibration standards. An example of a calibration curve and residual plot are shown in Fig. 5. The calculated regression parameters together with the 95% confidence interval of the calibration curves from 6 analytical sequences are listed in Table 1. The results show that both the slope and the curvature are statistically different from 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 12% (Table 2).

Table 1
Regression parameters of the calibration curve fitted to the equation $y = a + bx + cx^2$ and weighted with $1/y$

Regression parameters	Mean ($n = 6$)	95% Confidence interval of the mean
Intercept, *a*	−0.01	0.01
Slope, *b*	0.08	0.01
Curvature, *c*	4.0×10^{-5}	1.8×10^{-5}
Regression coefficient, r^2	0.9988	0.0008

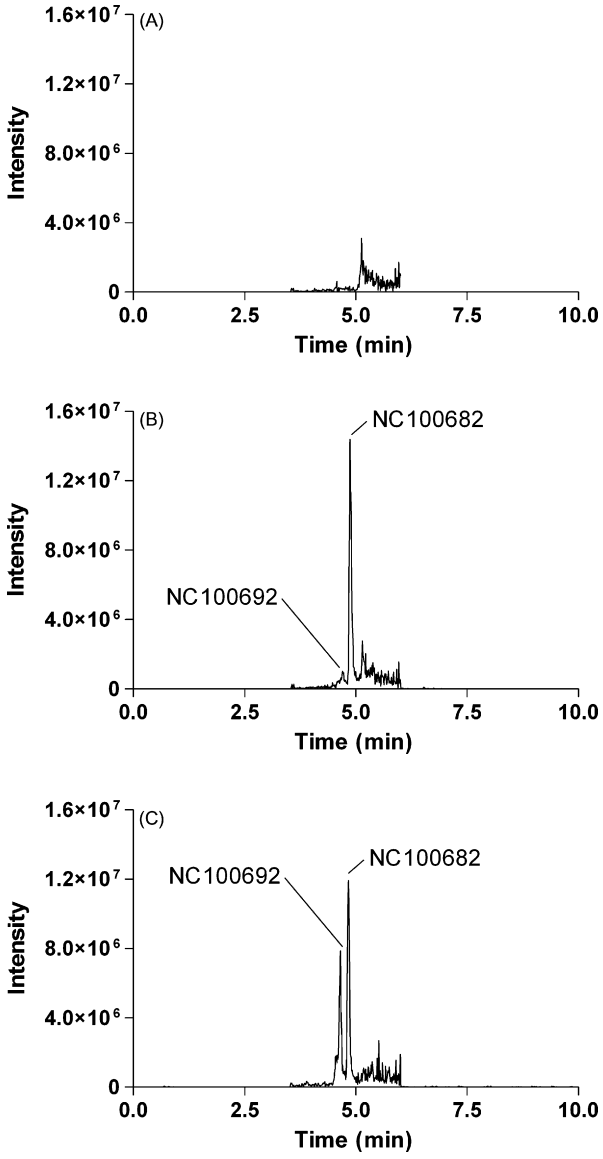

Fig. 4. Typical chromatograms in the SIM mode of: (A) a blank sample, (B) an LOQ sample and (C) a real plasma sample from the clinical trial.

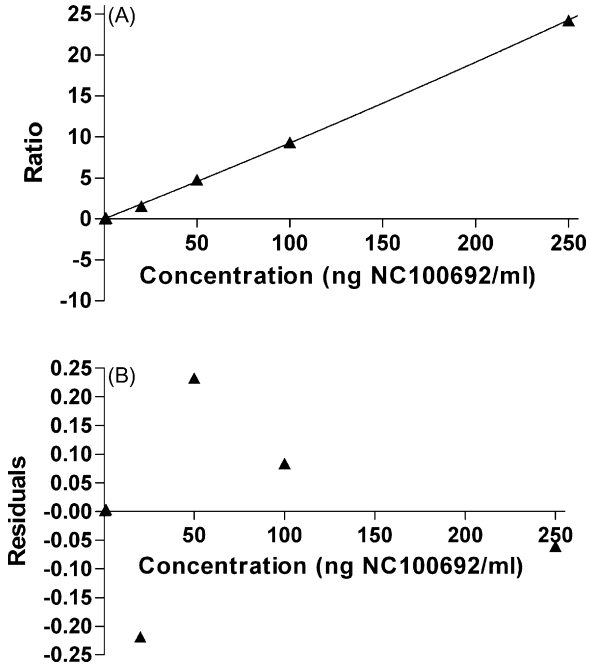

Fig. 5. Typical calibration curve (A) and residual plot (B) of the calibration standards.

Table 2
The goodness of fit of the calibration points

Target concentration (ng NC100692/ml)	Recovery (% of theoretical value)
0.5	111 ± 6
1.0	92 ± 10
5.0	88 ± 9
20	95 ± 4
50	102 ± 6
100	101 ± 5
250	100 ± 1

The values were estimated from 6 analytical series for each calibration standard and are given as mean ± S.D.

3.3. Limit of quantification

Based on pre-validation work, 0.5 ng NC100692/ml (corresponding to 15 pg injected) was chosen as the lowest standard of the calibration curve. The repeatability and accuracy of this calibration standard were found to be 9.0% R.S.D. ($n = 5$) and 90.4% ($n = 5$), respectively. These data show a precision and accuracy well within that required [5], and 0.50 ng NC100692/ml was therefore accepted as the limit of quantification (LOQ) for the method.

3.4. Precision and accuracy

Three quality control samples were used to calculate the precision and accuracy of the method as described in Section 2.6. The repeatability R.S.D. of the control samples was found to be 9.3, 9.1 and 6.5% for the low, medium and high control samples, respectively (Table 3). The intermediate precision R.S.D. was

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	215	14	21	6.5	9.7
Medium	81.5	7.4	8.8	9.1	10.8
Low	1.55	0.15	0.17	9.3	11.1

Table 4
The accuracy of the method

Control sample	Concentration (ng NC100692/ml)		Accuracy (%)
	Theoretical	Found (mean ± S.D.)	
High	200	215 ± 20	107.5
Medium	73.3	81.5 ± 8.7	111.1
Low	1.50	1.55 ± 0.17	103.4

found to be 11.1, 10.8 and 9.7% for the low, medium and high quality control samples, respectively (Table 3). The accuracy was found to be 103.4, 111.1 and 107.5% for the low, medium and high control samples, respectively (Table 4). The precision and accuracy of the method were within acceptance criteria for bioanalytical methods [5].

3.5. Sample stability

NC100692 was shown to be stable in human plasma when stored at −20 °C for 8 weeks (analysis performed using 3 control samples; data not shown). Moreover, analyses of the control samples at 3 concentration levels showed NC100692 to be stable in human plasma during at least 3 freeze/thaw cycles and for at least 48 h on dry ice (data not shown). Finally, NC100692 extracted from plasma was found to be stable in the autosampler for at least 47 h at 4 °C (data not shown).

3.6. Solid phase extraction recovery

The plasma samples were extracted using SPE in order to avoid clogging of the LC column and MS capillary. The percent extraction recovery of NC100692 and the internal standard was >80% in samples with plasma as matrices compared to non-extracted standards prepared in 20% acetonitrile in water (data not shown).

4. Conclusion

An LC–MS method for quantification of NC100692 in human plasma was developed and validated. The method was found to be suitable for quantification of NC100692 in human plasma for calculations of pharmacokinetic parameters. These studies will be published separately.

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