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Original Article

Quantification of neomangiferin in rat plasma by liquid chromatography–tandem mass spectrometry and its application to bioavailability study ☆

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ABSTRACT

Neomangiferin, a natural C-glucosyl xanthone, has recently received a great deal of attention due to its multiple biological activities. In this study, a rapid and sensitive ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method for the quantification of neomangiferin in rat plasma was developed. Using chloramphenicol as an internal standard (IS), plasma samples were subjected to a direct protein precipitation process using methanol (containing 0.05% formic acid). Quantification was performed by multiple reactions monitoring (MRM) method, with the transitions of the parent ions to the product ions of m/z 583.1→330.9 for NG and m/z 321.1→151.9 for IS. The assay was shown to be linear over the range of 0.2–400 ng/mL, with a lower limit of quantification of 0.2 ng/mL. Mean recovery of neomangiferin in plasma was in the range of 97.76%–101.94%. Relative standard deviations (RSDs) of intra-day and inter-day precision were both $<10\%$. The accuracy of the method ranged from 94.20% to 108.72%. This method was successfully applied to pharmacokinetic study of neomangiferin after intravenous (2 mg/kg) and intragastric (10 mg/kg) administration for the first time. The oral absolute bioavailability of neomangiferin was estimated to be $0.53\% \pm 0.08\%$ with an elimination half-life ($t_{1/2}$) value of 2.74 ± 0.92 h, indicating its poor absorption and/or strong metabolism in vivo.

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1. Introduction

Neomangiferin (NG) was first extracted from the dried rhizome of *Anemarrhena asphodeloides*, and its structure was confirmed as a new C-glucosyl xanthone in comprehensive spectral analysis [1]. There have been previous studies reporting the extraction of NG from the rhizome of *Belamcanda chinensis* [2] and *Gentiana rhodantha* [3]. Also, the plant extracts which contain NG have already been widely used for the treatment of diabetes [4,5] and osteoporosis [6]. However, the pharmacological effects of NG have not been clearly defined yet. Zhou et al. [7] have demonstrated that NG has significant therapeutic effects on high-fat diet-induced nonalcoholic fatty liver disease (NAFLD) in rats, suggesting that NG could play an important role in modulating the mRNA and protein expression involved in free fatty acid uptake, lipogenesis and lipid oxidation.

Despite the potential medical benefits of NG, the possible mechanism is still complex and not fully understood yet. The concentration of NG in vivo is critical to its bioavailability, and the pharmacokinetic profile is essential for a better understanding of pharmacological and clinical effects of this active compound.

To the best of our knowledge, the information about the pharmacokinetic properties of NG in raw materials and related traditional Chinese prescriptions is already available [8,9].

There were several liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) methods reported to quantify NG in plasma. For example, Sun et al. [8] developed an LC–MS/MS method for the quantification of NG in rat plasma after oral administration of *A. asphodeloides* extract and Er-Mu preparation with totally more than 20 min of running time. Li et al. [9] reported a method to detect NG in rat plasma after oral administration of Baihe Zhimu Tang by LC–MS/MS. But the method had some limitations, including long running time (10 min), large volume of plasma sample (200 μL), inadequate sensitivity (lower limit of quantification (LLOQ): 10.3 ng/mL) and narrow linear concentration range (10.3–1660 ng/mL). Obviously, the pharmacokinetic properties of the constituent contained in crude plant materials are quite different from those of respective pure compounds, owing to the presence of multiple components [10]. However, until now, the pharmacokinetic characteristics of the pure NG have not been investigated yet. Moreover, little information is given on the quantification and pharmacokinetic research of NG after intravenous administration, which could not provide more essential

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data for clinical trials of this compound.

Therefore, in this paper we were dedicated to developing and validating a rapid, simple and sensitive ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) method for the determination of NG in rat plasma after intravenous (2 mg/kg) and intragastric (10 mg/kg) administration. The method has the advantages of small volume of plasma sample (50 μL), simple sample preparation (one-step protein precipitation), good chromatographic resolution, specific and sensitive mass spectrometric conditions and short runtime (4 min), with the concentration range of 0.2–400 ng/mL.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards of NG and chloramphenicol (internal standard, IS) were purchased from Dingrui Pharmaceutical Co., Ltd (Shanghai, China). Their purities were determined to be over 98% by normalization of the peak areas detected by UHPLC–MS/MS. Methanol, acetonitrile, ammonium acetate and formic acid of MS-grade were purchased from Fisher Scientific (Tustin, CA, USA). Ultra-pure water was prepared by Milli-Q System (Millipore, Bedford, MA, USA). Other reagents used here were of analytical grade.

2.2. Instrumentation and conditions

Chromatographic separations were performed on a Shimadzu LC system (Kyoto, Japan) equipped with system controller (CBM-20A), pump (LC-20ADXR), auto-injector (SIL-20AC), online degasser (DGU-20A3), and column heater (CTO-20AC). Then separations were achieved on a Poroshell 120 EC-C_{18} column (100 mm × 3.0 mm, 2.7 μm; Agilent, USA) with an EC-C_{18} guard column (5 mm × 2.1 mm, 2.7 μm; Agilent, USA). Isocratic analysis was made through a mobile phase of solvent A (water containing 5 mM ammonium acetate)-solvent B (methanol containing 0.1% formic acid) (10:90, v/v). The flow rate was 0.6 mL/min and the injection volume was 5 μL. The column and sample temperature were maintained at 25 °C and 4 °C, respectively.

An AB SCIEX QTRAP® 5500 tandem mass spectrometer (AB, USA) was connected to the LC system through an electrospray ionization (ESI) interface. The ESI source was operated in negative ionization mode. Quantification was performed by multiple reactions monitoring (MRM) method, with the transitions of the parent ions to the product ions of m/z 583.1 → 330.9 for NG and m/z 321.1 → 151.9 for IS. The conditions of ionization source were as follows: curtain gas, 25 psi; ion spray voltage, 4000 V; temperature, 500 °C, nebulizer gas, 35 psi; and heater gas, 60 psi. Compound dependent parameters for NG included declustering potential (DP): −134.2 V, entrance potential (EP): −10.4 V, collision energy (CE): −51.6 V and collision exit potential (CXP): −36.3 V; for IS DP: −117.2 V, EP: −11.3 V, CE: −21.2 V and CXP: −15.4 V. System control and data analysis were performed by AB Sciex Analyst software (version 1.6.1).

2.3. Standard solutions, calibration standards and quality control

Stock solutions of NG and IS were prepared in methanol at a concentration of 1 mg/mL. Then the stock solutions were diluted with methanol to obtain fresh standard working solutions. The plasma calibration standard solutions were prepared at concentrations of 0.2, 0.4, 1, 4, 10, 40, 100 and 400 ng/mL for NG and 40 ng/mL for IS by spiking the moderate amounts of working solutions into 50 μL of blank plasma.

Low-, mid- and high-level quality control (QC) samples containing 0.5, 10 and 320 ng/mL of NG were prepared in a manner similar to that used for the preparation of calibration samples. All stock solutions, working solutions, calibration standards and QC samples were stored at −20 °C and were brought to room temperature before analysis.

2.4. Sample preparation

Before analysis, the plasma sample was thawed to room temperature. An aliquot of 50 μL of plasma was mixed with 50 μL of IS working solution (40 ng/mL) and 400 μL of methanol (containing 0.05% formic acid). After vortexing for 2 min, the mixture was centrifuged at 4 °C for 10 min at 13,000 *g*. The supernatant (5 μL) was injected into the UHPLC–MS/MS system for analysis.

2.5. Method validation

The method was validated for selectivity, linearity, precision, accuracy, matrix effect, recovery and stability according to the guidelines set by the United States Food and Drug Administration (USFDA) [11] and European Medicines Agency (EMA) [12] for the validation of bioanalytical method. Validation runs were conducted on three consecutive days. Each validation run consisted of one set of calibration standards and six replicates of QC plasma samples.

The selectivity of the method was evaluated by analyzing six blank plasma samples from different rats, blank plasma spiked NG and IS, as well as a rat plasma sample. The "cross-talk" between MRM transitions was evaluated by analyzing these different blank samples.

Calibration curves were constructed by analyzing spiked calibration samples on three separate days. Peak area ratios of NG to IS were plotted against analyte concentrations, and standard curves were well fitted to the equations by linear regression with a weighting factor of the reciprocal of the concentration ($1/x$) in the concentration range of 0.2–400 ng/mL. The limit of detection (LOD) was determined as the plasma concentration giving a signal-to-noise ratio of 3 and the LLOQ was defined as the lowest concentration on the calibration curves; NG peak should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80%–120%.

To evaluate the matrix effect, six blank plasma samples from different rats were extracted and then spiked with the analyte at 0.5, 10 and 320 ng/mL. The corresponding peak areas were then compared to those of neat standard solutions at equivalent concentrations, and this peak area ratio is defined as the matrix effect. The matrix effect of IS was evaluated at the concentration of 40 ng/mL in the same manner.

Accuracy and precision were assessed by the determination of QC samples at three concentration levels (0.5, 10 and 320 ng/mL) in six replicates on three validation days. The precision was expressed by relative standard deviation (RSD).

The recovery of NG was evaluated by comparing peak areas of extracted QC samples with those of reference QC solutions reconstituted in blank plasma extracts ($n = 6$). The recovery of IS was determined in a similar way.

Carry-over was assessed following injection of two processed blank matrix samples immediately after three repeats of the upper limit of quantification (ULOQ) and the response was checked [13].

Stability of NG in rat plasma was evaluated by analyzing three replicates of plasma samples at the concentrations of 0.5, 10 and 320 ng/mL, which were exposed to different conditions. These results were compared with those of freshly prepared plasma samples. The short-term stability was determined after the exposure of the spiked samples to room temperature for 4 h, and the

ready-to-inject samples (after protein precipitation) in the LC autosampler at room temperature for 24 h. The freeze/thaw stability was evaluated after three complete freeze/thaw cycles (−20 to 25 °C) on consecutive days. The long-term stability was assessed after storage of the standard spiked plasma samples at −20 °C for 30 days.

If the concentration in a real sample was above the ULOQ, the sample was analyzed after dilution with blank plasma. The dilution integrity experiment was carried out at 5 times of the ULOQ concentration (2000 ng/mL) and also at ULOQ level (400 ng/mL). Six replicate samples, each of which has the concentration of 1/10 of 5 × ULOQ (200 ng/mL) and ULOQ concentration (40 ng/mL), were prepared and their concentrations were calculated, by applying the dilution factor against the freshly prepared calibration curve for NG. The percentage change from the comparison sample should be within ±15%.

2.6. Pharmacokinetic study

Male Sprague-Dawley (SD) rats (body weight: 200 ± 20 g) were obtained from Third Military Medical University (Chongqing, China). All animals were bred in a breeding room at 25 ± 2 °C with 60% ± 5% humidity, and a 12 h dark-light cycle. The animals were acclimatized to the facilities for 5 days, and then fasted with free access to water for 12 h prior to each experiment. The dosing solution of NG was prepared by dissolving appropriate amount of NG in 0.5% carboxymethyl cellulose sodium (CMC-Na) aqueous solution. All rats were randomized into two groups (six in each group): NG 2 mg/kg body weight by intravenous administration and 10 mg/kg body weight by intragastric administration, respectively. The intravenous solution injection rate was controlled within 1 min. After a single dose was administered, about 300 μL of blood samples were collected in heparinized tubes via the orbital vein at 0 (predose), 0.033, 0.083, 0.167, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h for intravenous administration and 0 (predose), 0.083, 0.167, 0.33, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10 and 16 h for intragastric administration, respectively. Following centrifugation at 2200 *g* at 4 °C for 3 min, plasma samples were obtained and frozen at −20 °C until analysis.

Pharmacokinetic parameters were determined by non compartmental methods using the Drug and Statistics software (DAS, version 2.0, Clinical Drug Evaluation Center, Wannan Medical College, Anhui, China). The pharmacokinetic parameters, such as maximum plasma concentration (C_{max}) and time of maximum concentration (T_{max}), were obtained directly from the plasma concentration-time plots. The elimination rate constants (k) were determined by linear regression analysis of the logarithmic transformation of the last four data points of the curve. The elimination half-life ($t_{1/2}$) was calculated using the following equation: $t_{1/2}=0.693/K$. The area under the plasma concentration-time curve up to the last time (t) ($AUC_{0–t}$) was determined using the trapezoidal rule. The $AUC_{0-\infty}$ values were calculated by adding the value of $C_t \times k^{-1}$ to $AUC_{0-\infty}$. Bioavailability (F) was calculated by $(AUC_{ig}/AUC_{iv}) \times (dose_{iv}/dose_{ig}) \times 100\%$.

3. Results and discussion

3.1. Method development and optimization

The ion intensities of both NG and IS were high in the negative ionization mode. $[M–H]^-$ was selected as the precursor ion, and the most specific fragment ions were selected as the product ions in MRM acquisition to avoid any interference from the matrix. As a result, the MRM transitions on the negative ionization of NG and IS were selected at m/z 583.1/330.9 and m/z 321.1/151.9, respectively (Fig. 1).

The mobile phase played a critical role in achieving good chromatographic behavior and appropriate ionization [14]. Various combinations of organic diluents (methanol/acetonitrile) together with ammonium acetate or formic acid were investigated. Methanol (containing 0.1% formic acid) and water (containing 5 mM ammonium acetate) were chosen as the mobile phase because they could provide symmetrical and sharp peak shape. As isocratic elution is simple and provides relatively short running time without column equilibration/re-equilibration time, isocratic elution was used in the present assay and it did not encounter any issues associated with possible buildup of contaminants or endogenous components during each run. Because one analytical run could be finished within 4 min, our method enabled a sample throughput of at least 250 samples per day.

3.2. Method validation

3.2.1. Selectivity and matrix effect

Fig. 2 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with NG and IS, and a plasma sample. No interfering endogenous substance was observed at the retention time of the analyte and IS.

To investigate the matrix effect, three concentration levels of NG (0.5, 10 and 320 ng/mL) in six replicates were analyzed, and the values are shown in Table 1. The matrix effects for NG and IS ranged from 89.44% to 113.03%, and the corresponding RSDs were less than 6.35%. Besides, the matrix effects for the dosing formulation CMC-Na and the anticoagulant heparin sodium that might exist in plasma were investigated as well, and no significant

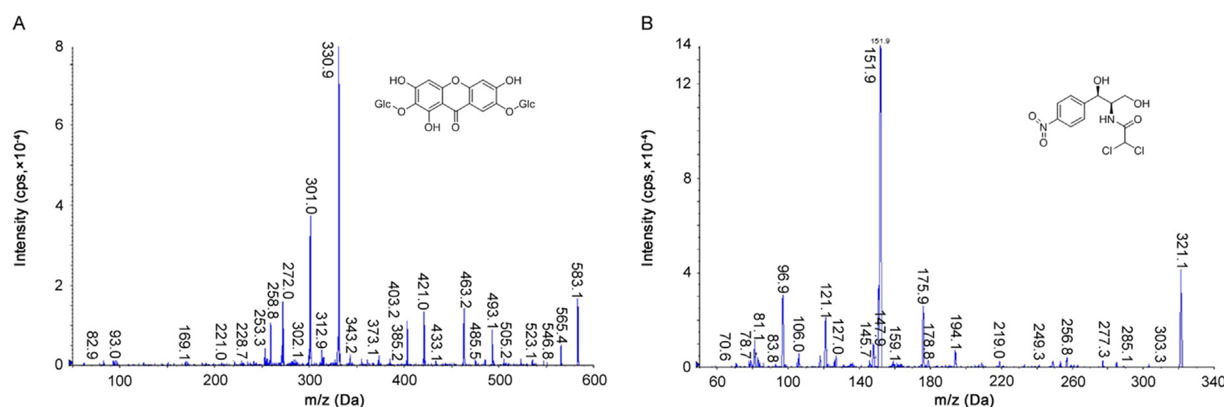

Fig. 1. Full-scan product ion spectra of $[M–H]^-$ ions and fragmentation schemes for (A) NG and (B) chloramphenicol (IS).

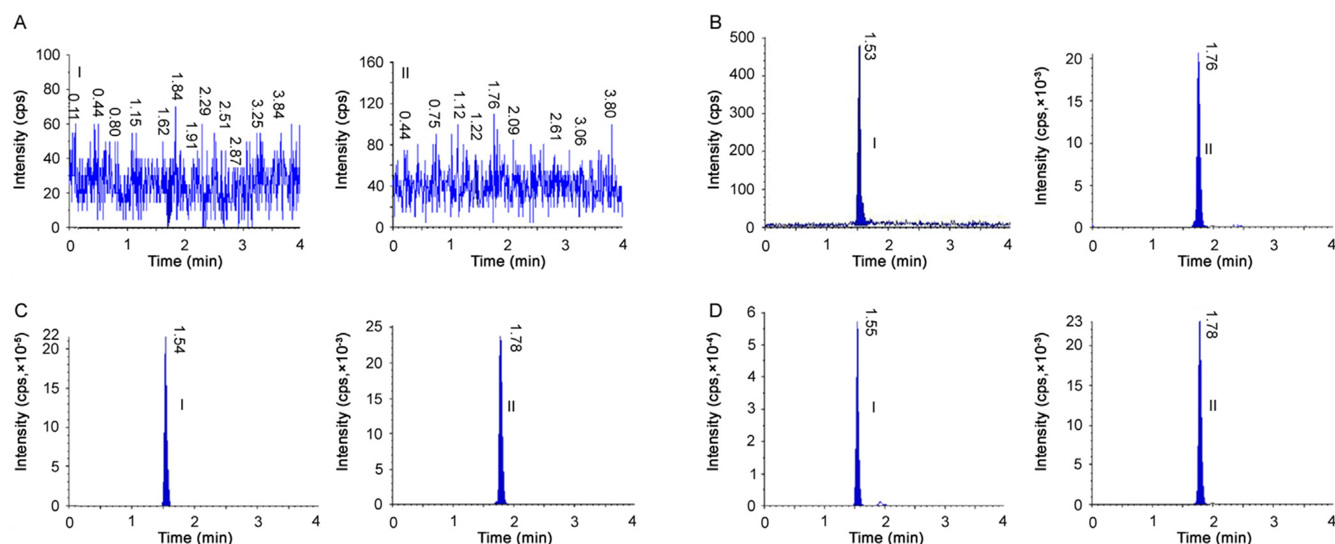

Fig. 2. Representative MRM chromatograms of (A) a blank rat plasma sample; (B) a blank rat plasma spiked with NG at LLOQ (0.2 ng/mL) and IS (40 ng/mL); (C) a rat plasma sample at 0.5 h after an intravenous dose of NG (2 mg/kg) to rats and (D) a rat plasma sample at 0.5 h after an intravenous dose of NG (10 mg/kg) to rats (I: retention time, 1.54 min; m/z 583.1→330.9 for NG and II: retention time, 1.78 min; m/z 321.1→151.9 for IS).

Table 1
Matrix effect of NG and IS.

Compound	Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RSD (%)	Matrix effect (%)
Neomangiferin	0.5	0.45 ± 0.01	2.77	89.44 ± 2.48
	10	10.93 ± 0.18	1.67	109.30 ± 1.82
	320	307.68 ± 9.37	3.05	96.15 ± 2.93
IS	40	45.21 ± 2.87	6.35	113.03 ± 7.18

interference of these reagents was found. These results demonstrated that the processing procedure of plasma samples was highly acceptable with no significant ion suppression or enhancement.

3.2.2. Linearity and sensitivity

The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range 0.2–400 ng/mL for NG in rat plasma. A typical equation of the calibration curve was obtained as follows: $y=0.0393x+0.00911$ ($r=0.9993$), where y is the peak-area ratio of NG to IS and x is the plasma concentration of NG, respectively.

The LLOQ for NG was 0.2 ng/mL, which was sensitive enough for the pharmacokinetic study of NG in rats. The precision and accuracy at this concentration level were acceptable, with the RSD of 11.5% and the accuracy of 98.2%, respectively. The LOD, defined as a signal/noise ratio of 3, was 0.1 ng/mL for NG in rat plasma.

3.2.3. Precision and accuracy

The intra- and inter-day precision and accuracy of the method were investigated by analyzing QC samples (0.5, 10 and 320 ng/mL). All the values are shown in Table 2. Intra-day RSD was below 8.13% and inter-day RSD was below 4.79%. The accuracy was within 94.20%–108.72%. The method was proved to be highly accurate and precise.

Table 2
Accuracy and precision for the analysis of NG in QC samples.

Spiked concentration (ng/mL)	Found concentration (ng/mL)	Accuracy (%)	Precision (%RSD)	
			Intra-day ($n=6$)	Inter-day ($n=3$)
0.5	0.47 ± 0.04	94.20	8.13	3.10
10	10.87 ± 0.21	108.72	1.93	4.20
320	345.92 ± 8.59	108.10	2.48	4.79

3.2.4. Extraction recovery and stability

The extraction recoveries of NG were 97.76% ± 2.81%, 101.67% ± 2.15% and 101.94% ± 3.84% at three concentrations of 0.5, 10 and 320 ng/mL, respectively, while the recovery of IS was 103.49% ± 6.49%. These results suggested that the recoveries of NG and IS were consistent and were not concentration-dependent. All the values are shown in Table 3.

The autosampler, room temperature, freeze-thaw and long-term (30 days) stability results indicated that the analyte was stable under the storage conditions described above since the bias in concentration was within ± 15% of nominal values (Table 4).

3.2.5. Carry-over

Carry-over was tested by injecting two processed blank matrix samples subsequently after injection of a ULOQ sample in three independent runs. The response in the first blank matrix at the retention time of NG should be less than 20% of the mean response of an LLOQ sample for NG. The response in the first blank matrix at the retention time of the analyte was less than 4.7% of the mean response at the LLOQ for NG. Therefore, the carry-over test was found to be acceptable.

3.2.6. Cross-talk

The phenomenon of cross-talk may have occurred if the analytes had similar retention time, which will lead to greater deviation of the results. Therefore, it is essential to monitor two or

Table 3
Recoveries of NG and IS ($n=6$).

Compounds	Concentration (ng/mL)	RSD (%)	Recovery (%, mean ± SD)
Neomangiferin	0.5	2.87	97.76 ± 2.81
	10	2.11	101.67 ± 2.15
	320	3.77	101.94 ± 3.84
IS	40	6.27	103.49 ± 6.49

Table 4
Stability of quality control samples of NG (n=6).

Condition	Concentration (ng/mL)		CV (%)	Accuracy (%)
	Nominal	Found		
Short-term stability (4 h at room temperature)	0.5	0.48 ± 0.01	1.71	95.52
	10	10.74 ± 0.37	3.43	107.36
	320	347.84 ± 5.21	1.50	108.70
Autosampler stability (24 h at room temperature)	0.5	0.47 ± 0.02	4.85	93.00
	10	10.62 ± 0.23	2.15	106.16
	320	341.36 ± 4.67	1.37	106.68
Freeze-thaw stability (three cycles)	0.5	0.46 ± 0.02	3.77	91.96
	10	10.88 ± 0.20	1.79	108.79
	320	352.35 ± 7.60	2.16	110.10
Long-term stability (30 days at −20 °C)	0.5	0.47 ± 0.02	5.07	93.80
	10	10.34 ± 0.13	1.25	103.44
	320	318.48 ± 7.26	2.28	99.53

more channels for MRM analysis so as to avoid cross-talk. Two methods are usually used to avoid cross-talk: selecting different quantitated ions to improve the selectivity; and optimizing the mobile phase to completely separate the analytes [15]. In the present study, NG and IS had different quantitated ions and retention time (t_R=1.54 min for NG, and t_R=1.78 min for IS). The results indicated that there was no interference between the four monitoring channels, and no relevant cross-talk was observed.

3.2.7. Dilution integrity

The mean back-calculated concentrations for 1/10 dilution samples of 5 × ULOQ and ULOQ were 96.1% and 97.2%, respectively. The RSDs were 9.5% and 7.4%, respectively.

3.3. Application of the method to pharmacokinetic study

The method was successfully applied to the quantification of NG in rat plasma after intravenous (2 mg/kg) and intragastric dosings (10 mg/kg). Intravenous dose was limited by NG solubility in a vehicule compatible with this administration route. Anticipating a possibly low bioavailability, a higher dose was chosen for oral administration in order to make quantitation possible. These doses were based on the tolerance observed in previous experiments (unpublished results). Indeed, no sign of acute toxicity was observed at these doses. The mean plasma concentration-time curves are shown in Fig. 3. An important inter-individual variability was observed for both intravenous and intragastric administrations.

The main pharmacokinetic parameters from non-compartment model analysis are summarized in Table 5. The absolute bioavailability of NG was calculated to be 0.53% ± 0.08%. Many factors that could be responsible for the low bioavailability of this compound include the potential hydrolysis in the gastrointestinal tract, poor permeability through the intestinal epithelial membrane and first-pass effect in the liver [16]. Thus, the aglycone of NG or its other metabolites that might exist in plasma may be paid more attention to in our further studies. In addition, our results further support efficacy results following intravenous administration.

Table 5
Pharmacokinetic parameters of NG after intravenous (2 mg/kg) and intragastric (10 mg/kg) administrations in rats (mean ± SD, n=6).

Parameters	Intravenous	Intragastric
$t_{1/2}$ (h)	1.70 ± 0.14	2.74 ± 0.92
C_{2min} (ng/mL)	6330.11 ± 854.68	–
T_{max} (h)	–	0.58 ± 0.29
C_{max} (ng/mL)	–	72.17 ± 7.97
AUC_{0-t} (ng h/mL)	3484.31 ± 324.58	92.26 ± 17.53
$AUC_{0-\infty}$ (ng h/mL)	3487.24 ± 325.28	93.31 ± 17.53
Vz (L/kg)	1.41 ± 0.06	–
MRT (h)	2.54 ± 0.41	3.76 ± 0.92
F (%)		0.53 ± 0.08

$t_{1/2}$: elimination half-time; C_{2min}: the observed plasma concentration at 2 min; T_{max}: time to peak value; C_{max}: peak concentration; AUC_{0-t}: area under the plasma concentration-time curve from time zero to t; $AUC_{0-\infty}$: area under the curve from time zero to infinity; Vz:apparent volume of distribution.; MRT: mean residence time; F: bioavailability.

There have been some references on determination of NG after intragastric administration of *A. asphodeloides* extract and related traditional Chinese prescriptions in rat plasma [8,9]. But no method about determination of pure NG in plasma is available in the literatures. The pharmacokinetic profiles of NG in raw materials or prescriptions were slightly different from that observed in pure form, and the possible reasons might be the complexity of Traditional Chinese Medicine (TCM) for their interaction between multitudinous compounds in formulas in vivo.

4. Conclusion

This developed method was shown to be specific, sensitive, precise and accurate for the quantification of NG in rat plasma. This method represented an improvement over the previous published procedures [8,9] in the following aspects: (i) sample preparation by fast and cheap protein precipitation with methanol (containing 0.05% formic acid) had no influence on the recovery of the analyte and therefore caused no detectable matrix effects;

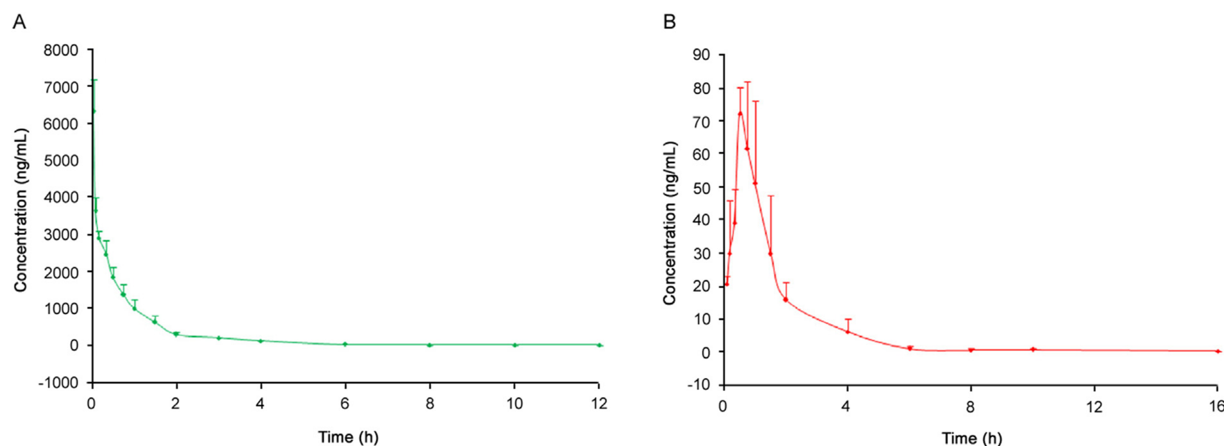

Fig. 3. Mean plasma concentration-time curves of NG in rats following (A) intravenous (2 mg/kg) and (B) intragastric (10 mg/kg) administrations (mean ± SD, n=6).

(ii) this method featured the highest sensitivity with an LLOQ of 0.2 ng/mL so far; (iii) run time of only 4 min enabled a high sample throughput of at least 250 samples per day; and (iv) in contrast to all other published methods, our method was comprehensively validated according to the current USFDA and EMA guidelines for bioanalytical method validation. The established method has already been successfully applied to a pharmacokinetic study of NG after intravenous (2 mg/kg) and intragastric (10 mg/kg) administrations to rats. The pharmacokinetic profile of NG in rat was characterized and the bioavailability of NG was reported to be 0.53% ± 0.08% for the first time. Due to the poor bioavailability of NG, more attention should be paid to the metabolites of NG that might exist in plasma in our further studies.

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