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Short communication

Improved RP-HPLC method to determine neferine in dog plasma and its application to pharmacokinetics

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Abstract

Existing methods to determine neferine, a bisbenzylisoquinline alkaloid, either have no internal standard or lack selectivity, or take longer time. Here an improved reverse-phase high-performance liquid chromatographic (RP-HPLC) method was established in biological samples. The extraction recovery was 90.9% for neferine at concentration level of 0.2 μg/ml and 77.7% for dauricine (the internal standard) at 5 μg/ml in dog plasma, respectively. The linear quantification range of the method was 25–2 000 ng/ml in dog plasma, with linear correlation coefficients greater than 0.999. The intra-day and inter-day relative standard deviations (R.S.D.s) for nerferine at 50, 200 and 1 000 ng/ml levels in dog plasma fell in the range of 3.0–5.4% and 4.3–9.5%, respectively. The RP-HPLC method was successfully applied to a pharmacokinetics study, in which experimental dogs received a single dose of neferine (5 mg/kg i.v. or 10 mg/kg p.o.). The pharmacokinetic result was presented.

Keywords: Improved; RP-HPLC; Neferine; Dog plasma; Pharmacokinetics

1. Introduction

Nelumbo nucifera GAERTN., a perennial aquatic crop, is utilized all over the world. It is not only as an ornamental plant, but also for a dietary staple. Almost all its parts, i.e., leaves, flowers, seeds and rhizomes, are utilized but rhizomes hold the largest market share. The seed of *Nelumbo nucifera* GAERTN. is named "Lian Zi Xin" in China. Within it, three types of bisbenzylisoquinoline alkaloids were found, including neferine (structure shown in Fig. 1), liensinine and isoliensinine and the content of neferine is the highest of the three [9]. As a Traditional Chinese Medicine, "Lian Zi Xin" has been used for "clearing away heart-fire" [1] and treating high fevers and hyposomnia [2–8] in clinical practice for over 1000 years. In recent years, it was reported that neferine has extensive pharmacological effects in cardiovascular system, e.g., anti-arrhythmia [10–12], anti-platelet aggregation and anti-thrombosis formation [13,14]. Neferine also possesses a reversal effect of multidrug resistance (MDR) mediated by glutathione (GSH) detoxification system in K562/A02 cell line [15,16], and promotes vincristine-induced apoptosis in the human mammary MCF-7 multidrug-resistant cells [17]. Our laboratory demonstrated that neferine had effect on bleomycin induced pulmonary fibrosis in mice [18].

Hu et al. [19] reported a HPLC method to determine neferine in rabbit plasma. Our laboratory also developed a HPLC method to determine neferine in rat plasma and applied it to pharmacokinetic study in rats [20]. However, all these methods either had no internal standard as a control, or lacked selectivity (the lowest limit of quantitation was 50 ng/ml in plasma and if the concentration of neferine is below that level it cannot be quantitated), or took longer time for sample preparation (two or three times for extraction of the samples and evaporation of the solvent). Recently Chen et al. [21] developed a method to characterize, prepare and quantitatively analyze neferine based on liquid chromatography electrospray ionization tandem mass spectrometry. Nevertheless, they did not use the method to determine neferine in biological samples. Thus the development of an improved analytical method by HPLC for the measurement of neferine in biological samples is necessary. This present investigation was therefore designed to establish a rapid, accurate, sensitive, selective and reliable HPLC-UV method and to eval-

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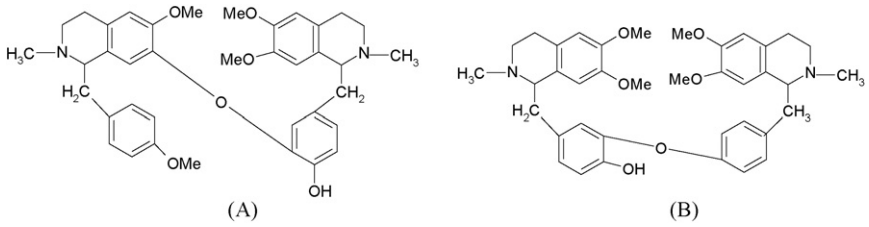

Fig. 1. Chemical structures of neferine (A) and internal standard dauricine (B).

uate the pharmacokinetics of neferine after oral and intravenous administration in dogs.

2. Experimental

2.1. Chemicals and reagents

Neferine (≥97% purity, measured by HPLC) was provided by Department of Pharmacology's Phytochemistry Laboratory, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Dauricine, another bisbenzylisoquinline alkaloid [11] (purity ≥ 99%, measured by HPLC) was obtained from the Center for Standard Compounds of the National Food and Drug Administration (Beijing, China). Analytical grade triethylamine, HPLC-grade methanol, acetonitrile, dichloromethane were purchased from Kermel Chemical Reagents Development Center (Tianjin, China). Distilled water was purified by a Milli-Q system (Millipore®). Mobile phase used in HPLC was filtered using a 0.2 μm membrane filter (Eilite Analytical Instruments Co., Dalian, China).

2.2. Instrumentation

The chromatographic system consisted of a Shimadzu LC-10ADVP System (a LC-10A isocratic pump, a manual injector with 20 μl loop, a SPD-10AVP variable wavelength UV detector). The above system was controlled by system Controller and HW chromatography software (Qian Pu Software Co., Shanghai, China). A TGL-16C centrifuge (Anting Instruments Factory, Shanghai, China) was utilized to centrifuge plasma samples and a homemade sample concentrator for evaporation of samples.

2.3. Plasma samples

Six-male dogs (11.42 ± 0.60 kg) were purchased from the Experimental Animal Science Co., Guangdong Institute of Pharmaceutical Industry (Certification: SCXK 2003-0006). The dogs were hosted at an animal facility that operated in the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, according to China Union regulations for the maintenance and experimentation on animals (document no. 55, 2001, Health Ministry of the People's Republic of China). Prior to administration of neferine, the plasma from the dogs was collected as blank. All blood samples were drawn into test tubes and centrifuged at 4 000 rpm (800 × *g*) for 10 min. The plasma was transferred into test tubes and frozen at −70 °C until analysis.

2.4. Chromatographic conditions

A reversed-phase Hypersil ODS-C_{18} column (4.6 mm × 200 mm, 5 μm particle size, Dalian Elite Analytical Instruments Co. Ltd.) equipped with a precolumn Hypersil ODS-C_{18} (4 mm × 10 mm, 10 μm particle size) was used. Mobile phase was composed of methanol–acetonitrile–water–triethylamine (20/40/40/0.12, v/v) and it was degassed for 10 min by ultrasonic treatment before use. The HPLC system was operated isocratically at a flow rate of 1.0 ml/min in ambient temperature and the detector was set at 282 nm. The injection volume was 20 μl and the retention times of neferine and the internal standard (I.S.) in plasma samples were approximately 11 and 8 min, respectively.

2.5. Solutions preparation

2.5.1. Stock solutions and standard solutions

As the stock solutions, neferine (100 μg/ml) and dauricine (250 μg/ml) were dissolved in distilled water with pH value 4.5 regulated with hydrochloric acid solution. Neferine standard solutions were prepared in the concentration range of 250–20 000 ng/ml for preparation of calibration curves, evaluation of the precision and accuracy of the analytical method and estimation of limits of detection and quantification. They were stable for at least 1 month at 4 °C.

2.5.2. Spiked plasma samples

Calibration standards were prepared freshly by spiking 900 μl of drug-free dog plasma with 100 μl concentrated standards in order to achieve concentration of 25, 50, 100, 200, 500, 1 000 and 2 000 ng/ml. Quality control samples were prepared in 45 ml of drug-free dog plasma by spiking with 5 ml of concentrated standards to 50, 200 and 1 000 ng/ml, which were low quality control, medium quality control and high quality control (LQC, MQC and HQC) concentrations, respectively. The quality control samples were used for the analytical method validation. These quality control samples were divided into aliquots of 1 ml into vials capped tightly, stored at −70 °C until analysis.

2.6. Analysis of plasma samples

Calibration curve standards, quality control and dog plasma samples were thawed and allowed to equilibrate at room temperature. The thawed samples were vortexed to ensure complete mixing of contents. Next, 100 μl of working solution of I.S. (50 μg/ml) was added to 1 ml of plasma sample and vor-

texed for 30 s. Sequently 0.5 ml of acetonitrile and 5 ml of dichloromethane were added and the new mixture was vortexed for 10 min. Then, the samples were centrifuged for 10 min in 4 000 rpm (800 × *g*). The clear organic phase was evaporated to dryness with a gentle stream of air in a water bath at 40 °C. Subsequently, the residue was redissolved in 100 μl of mobile phase and vortexed for 1 min. Then the solution was transferred into a small clean eppendorf tube and centrifuged for 5 min at 10 000 rpm (5 500 × *g*). Finally, 20 μl of the supernatant was directly injected into the HPLC system.

2.7. Method validation

The method was validated for selectivity, calibration curves, LLOQ, recovery, precision, accuracy and stability based on the guidelines of the state food and drug administration (SFDA) of China.

Specificity was studied by applying the extraction procedure to blank plasma samples to test the interference at the retention time areas of neferine and I.S. Calibration curves for neferine were constructed in the concentration range of 25–2 000 ng/ml ($n = 7$, each point was the mean of three experimental measurements) and calculated by plotting peak area ratio (y) of neferine and I.S. versus neferine concentrations (x, ng/ml). Regression equations were obtained through weighted least-square linear regression analysis ($w = 1/x^2$). LLOQ was experimentally chosen as the lowest concentration at which both precision and accuracy should be within the tolerable maximum of 20% and 80–120%. For absolute recovery, the peak areas of neferine or I.S. in plasma were compared with the corresponding ones in mobile phase, as follows: Recovery of extraction (%) = $A_x/A_s \times 100\%$. Here A_x represented the area of neferine or I.S. in plasma samples, analogously, A_s represented the area of neferine or I.S. in mobile phase. The precision of the method was studied by estimating intra- and inter-day relative standard deviations (R.S.D.s) of values using LQC, MQC and HQC plasma samples. Intra-day precision was determined by analysis of each sample in 1 day ($n = 5$). Inter-day precision was determined by repeated analysis in five consecutive days ($n = 5$). The stability of plasma samples, which were kept at room temperature, frozen at −70 °C and experienced through three cycles of freeze–thaw, were estimated. The stability of extracted samples (i.e. ready for HPLC analysis) at 4 °C was also evaluated.

2.8. Pharmacokinetic study

The pharmacokinetic study was based on a single-dose, randomized, two period crossover design at intervals of 1 week. The animals fasted 10 h were intravenously administrated 5 mg/kg or orally 10 mg/kg neferine solution to obtain absolute bioavailability. No food was allowed until 4 h after dosing while water intake was free. About 3 ml of blood samples was collected from the foreleg vein into heparinized test tubes at 0.017 h (for i.v. only), 0.083, 0.167, 0.333, 0.667, 1, 2, 3, 4, 6, 8, 12, and 24 h (for p.o. only) after administration. After a washout period of 7 days, the study was repeated once again.

3. Results and discussions

3.1. Mobile phase

In order to optimize the elution of neferine, several eluent mixtures were tested. A mixture of methanol–acetonitrile–water–triethylamine (20/40/40/0.12, v/v) was the simplest and also the most appropriate mobile phase for the elution of neferine from plasma. The elution time of neferine was approximately 11 min, i.e. quite reasonable for analysis of biological samples. By increasing the amount of methanol or acetonitrile, the total elution time was decreasing but I.S. peak was moving closer to the plasma background peaks. It was noticed that the use of buffers was not necessary because it did not improve any analytical parameter (e.g. resolution or elution time). However, triethylamine is essential. The appropriate content of triethylamine (within 1–2‰) was used to achieve satisfactory peak symmetry and peak resolution simultaneously. Since triethylamine is a kind of alkaloid and the amount of triethylamine was very small, the stability of the silica column was not affected under high pH condition.

3.2. Choice of I.S.

Several substances including tetrandrine, daurisoline and dauricine were tested as internal standards. Among these, dauricine (Fig. 1B) met all the typical requirements of a compound to be used as I.S., i.e. it was stable during the analysis, easily available, not an endogenous substance, its elution time was shorter than that of neferine, its peak did not interfere with the matrix of plasma samples, and its extraction recovery value was greater than 75%.

3.3. Treatment of samples

Liquid–liquid extraction and protein precipitation are the commonly used techniques for sample preparation. Liquid–liquid extraction usually offers much cleaner sample that in turn makes the method more robust and scalable. In this study for the isolation of neferine from plasma samples liquid–liquid extraction was used. Extraction conditions were optimized by using several solvents such as dichloromethane, diethyl ether, acetoacetate, methanol, acetonitrile and combinations of them. Among these, dichloromethane gave the highest recovery. The addition of 0.5 ml of acetonitrile to dichloromethane decreased the number of interfering endogenous peaks. Therefore, dichloromethane accompanied with acetonitrile was proved to be a simple, rapid and efficient way for extracting neferine from plasma. Also, it improved selectivity and sensitivity of the chromatographic assay, compared with other tested solvents. And it practically did not affect the lifetime of column. Optimization of the volume of dichlormethane was also performed. Five milliliters of dichloromethane containing 0.5 ml of acetonitrile was the optimum volume for neferine to be extracted from 1 ml of plasma. Higher dichloromethane volumes increased the time of evaporation and lower volumes decreased the recovery of samples. The reconstitution step of

the evaporated samples was also optimized. One hundred microliters of mobile phase was the appropriate volume for dissolving the amount of neferine after the evaporation. Further, the addition of mobile phase ensured the compatibility of the injected samples with the "chromatographic system". If the evaporated samples were reconstituted in water or methanol, the width and symmetry of the peak would be affected. The extraction and reconstitution steps were very important because not only isolation and quantitation of neferine was accomplished but also preconcentration of injected solutions (by 10 times) was achieved.

3.4. Method validation

3.4.1. Selectivity

Typical chromatograms of blank plasma and of plasma spiked with neferine (1 000 ng/ml) and I.S. (5 000 ng/ml) are shown in Fig. 2A and B, respectively. No interfering peak appeared in the chromatograms at the elution time of neferine and I.S., which indicated no endogenous components interfered. Good resolution for the two peaks was assured by the *R*s values, which were greater than 2. Also, both neferine and I.S. peaks were symmetrical (symmetry factors were between 1.00 and 1.04). Zhou et al. [22] studied the metabolites of neferine and its analogues in vitro microsomal and claimed there are at least six metabolites identified following the incubation of neferine with dog hepatocytes. It is possible that the extra peak at about 4.5 min (Fig. 2C) is one of these metabolites.

3.4.2. Calibration curves and LLOQ

Typical calibration curve parameters and back-calculated concentrations of neferine in dog plasma are listed in Table 1.

By analyzing plasma spiked with decreasing concentrations of neferine, the LLOQ was experimentally found to be 25 ng/ml. For this concentration, the precision and accuracy were 10.0% and 110.1%, respectively, which were consistent with SFDA guidelines.

3.4.3. Absolute recovery

The extraction recoveries of neferine ($n = 3$) from spiked dog plasma were satisfactory at low, middle and high concentrations, which varied from 85% to 92%. Recovery of the I.S. of dauricine was very consistent with a mean of 77.7% and a R.S.D. of 5.0% ($n = 3$).

3.4.4. Precision, accuracy and stability

Intra- and Inter-day relative standard deviations of neferine were less than 5.4% and 8.5%, respectively, while the corresponding intra- and inter-day accuracy values fell in the range of 96.4–104.3% and 96.9–110.5%, respectively.

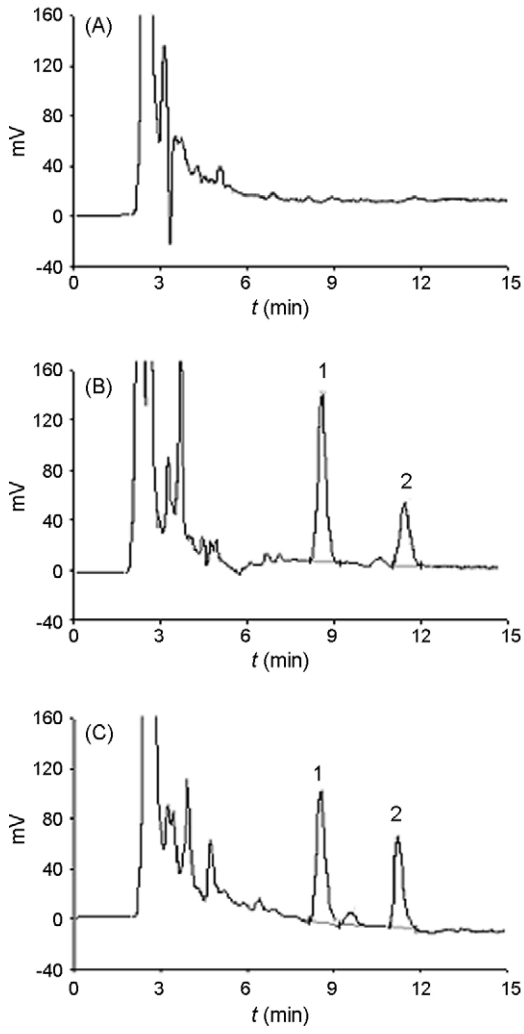

Fig. 2. Typical chromatograms of blank plasma from a dog (neferine free plasma) (A), of plasma spiked with neferine (1 000 ng/ml) and the internal standard dauricine (5 000 ng/ml) (B), and of plasma of a dog after oral administration neferine (10 mg/kg) (C). Retention times for dauricine and neferine were approximately 8 and 11 min, respectively. The chromatographic conditions were: ODS RP-C_{18} column (4.6 mm × 200 mm), mobile phase methanol–acetonitrile–water–triethylamine (20/40/40/0.12, v/v), flow rate 1 ml/min, detection wavelength 282 nm and room temperature: 1, dauricine (I.S.); 2, neferine.

Plasma samples left at room temperature for 12 h or frozen at −70 °C for 1 month, samples after extraction (i.e. reconstituted in the mobile phase, stored at 4 °C for 12 h and ready for HPLC analysis), and samples via three freeze–thaw cycles, were determined to evaluate the stability of neferine. The results indicate

Table 1
Typical calibration curve parameters and back-calculated concentrations of neferine in dog plasma

	Calibration curve (mean $n = 5$)							Curve parameters		
Added	25	50	100	200	500	1 000	2 000	*A*	*B*	*R*
Back-calculated	25.6	47.6	93.9	220.1	530.0	966.8	1869.2	0.00572	0.00196	0.9992
RE (%)	2.2	4.8	6.1	10.06	6.0	3.3	6.5			

RE: relative error; *A*: intercept of the calibration curve; *B*: slope rate of the calibration curve; *R*: correlation coefficient.

Table 2
Precision, accuracy and stability of HPLC analysis of neferine in dog plasma samples ($n = 5$)

Concentration (ng/ml)	Precision R.S.D.s (%)		Accuracy (%)		Stability (%)			
	Intra-day	Inter-day	Intra-day	Inter-day	Room temperature for 1 month	Extracted samples at 4 °C for 12 h	Frozen for 1 month	Three Freeze–thaw cycles
50	4.5	8.7	99.3	102.2	96.8	97.0	96.5	92.1
200	5.4	9.5	104.3	110.5	95.7	98.1	94.1	94.6
1 000	3.0	4.3	96.4	96.9	101.6	102.4	95.8	90.5

S.D.: standard deviation; R.S.D.: relative standard deviations.

that, taking account of the analytical variability, the processed sample stability was acceptable.

All the data of precision, accuracy and stability are shown in Table 2.

3.5. Pharmacokinetic application

The validated HPLC-UV method has been successfully used to determine the concentrations of neferine in dog plasma samples. The obtained plasma concentration versus time profiles of neferine are shown in Fig. 3. For intravenous administration the concentration of neferine in plasma decreased quickly in the first hour after giving the drug to dogs. While for oral administration, the concentration–time curve showed unusual two peaks and at 0.667 h the concentration was higher than that at 0.333 h, which was consistent with literature [20]. It looks as if enterohepatic cycling may be a reason behind the shoulder, since that neferine is a large molecule and it could be bile acid conjugated via the –OH groups.

The pharmacokinetic parameters were estimated by the DAS (Drug and Statistics for Windows) version 1.0 program. Pharmacokinetic analysis of neferine was performed by a three-compartment model and non-compartmental analysis for two different process of administrating the drug, respectively. The maximum plasma concentration (C_{max}) and corresponding peak time (T_{max}) of neferine was determined by the inspection of the individual drug plasma concentration–time profiles. The elimination rate constant (K_e) was obtained from the least-square fitted terminal log-linear portion of the plasma concentration–time profile. The elimination half-life ($t_{1/2}$) was calculated through $0.693/K_e$. The area under the plasma concentration–time curve of neferine, from time zero to infinity ($AUC_{0-\infty}$) was determined by the trapezoidal rule to the last measurable concentration (C_t) plus the additional area from time t to infinity, calculated as C_t/K_e. The absolute bioavailability, $F(\%)$, was calculated as follows: $F(\%) = (AUC_{p.o.} \times D_{i.v.})/(AUC_{i.v.} \times D_{p.o.}) \times 100\%$, here D denoted the dose of administration to the dogs. The obtained pharmacokinetic parameters of neferine are shown in Table 3.

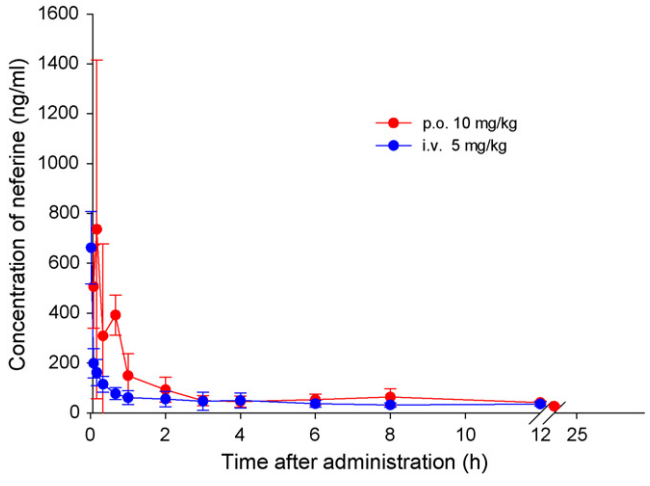

Fig. 3. Plasma concentration vs time profiles of neferine in dogs ($n = 6$) following intravenous administration of neferine 5 mg/kg or oral administration of neferine 10 mg/kg at a single dose.

Table 3
Pharmacokinetic parameters of neferine in dog plasma ($n = 6$)

Parameter	i.v.	p.o.
$AUC_{0-24\,h}$ (ng/ml × h)	835.1 ± 227.1	1090.7 ± 304.0
$AUC_{0-\infty}$ (ng/ml × h)	844.3 ± 228.2	1103.6 ± 302.3
MRT (h)		4.62 ± 1.18
T_{max} (h)		0.33 ± 0.14
C_{max} (ng/ml)		856.16 ± 251.49
$t_{1/2\gamma}$ (h)	8.95 ± 4.12	
$t_{1/2}$ (h)		7.45 ± 3.64
V_d (l)	0.079 ± 0.024	
CL (l/h)	0.0091 ± 0.0035	
F (%)		65.36

AUC: the area under the plasma concentration–time curve; MRT: mean residence time; T_{max}: time to reach maximum (peak) plasma concentration following drug administration; C_{max}: maximum plasma concentration following drug administration; $t_{1/2\gamma}$: terminal or elimination half-life (to be used in three-compartment model); $t_{1/2}$: elimination half-life (to be used in one or non-compartmental model); V_d: distribution volume; CL: clearance; F: bioavailability; i.v.: intravenous injection; p.o.: peros.

4. Conclusion

An improved reversed-phase isocratic HPLC method with UV spectrometric detection was established, optimized and validated for the determination of neferine in dog plasma. Compared to previously developed HPLC methods, the advantages of the proposed method include simplified single step liquid–liquid extraction procedure (i.e. better recovery and shorter sample preparation periods), use of I.S. (i.e. improved precision and accuracy) and low quantification limits (due to the preconcentration of injected solutions). This method was successfully applied to pharmacokinetic study. The results demonstrated that there

was a double-peak absorption in dogs after an oral administration of the drug; the absolute bioavailability of neferine was about 65%.

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