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A HPLC method for determination of nicousamide in dog plasma and its application to pharmacokinetic studies

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Abstract

A sensitive and reproducible high performance liquid chromatography (HPLC)-UV method for determination of nicousamide, an inhibitor of rennin and transforming growth factor-beta1 (TGF-β1) type II receptors, has been developed and validated. Following acetonitrile deproteiniation, samples were separated by isocratic reversed-phase HPLC on an Aichrom Bond-AQ C_{18} column and quantified using UV detection at 320 nm. The mobile phase was acetonitrile/water (ratio 62:38 containing 0.1% H_3PO_4), with a flow-rate of 1.0 ml/min. A linear curve over the concentration range 5–200 ng/ml ($r^2 = 0.9978$) was obtained. The coefficients of the variation for the intra- and inter-day precisions ranged from 1.4–10.7% and 1.8–7.1%, respectively. The percentage of relative recovery was 91.56–105.45%. The method was used to determine the plasma concentration–time profiles for nicousamide after oral doses of 30, 100 and 300 mg/kg in dogs. A nonlinear pharmacokinetics was found in dogs at doses from 30 to 300 mg/kg. Following 30 mg/kg oral dose, the C_{max} and AUC in females were lower than that in male. There is a potential for accumulation in dogs following multiple doses.

Keywords: Nicousamide; HPLC; Pharmacokinetic; Dog; Diabetic nephropathy

1. Introduction

Tubulointerstitial fibrosis is a typical finding in virtually all progressive renal diseases. The molecular mechanism that progress renal disease has begun to be elucidated recently [1]. Renin and TGF-β1 have been found to play a pivotal role in the tubulointerstitial pathology [2–5]. The clinical study showed that the inhibition of the RAS is an effective way to intervene in the pathogenesis of cardiovascular and renal disorders [6,7]. The inhibition of TGF system might be a critical mechanism for the development of diabetic renal hyperfiltration [8,9].

Nicousamide (3-(3′-carboxy-4′-hydroxy-anilino-carbo-)-6-nitro-7-hydroxy-8-methyl-coumarin, Fig. 1) is an inhibitor of rennin and transforming growth factor-beta1 (TGF-β1) receptor for the treatment of diabetic nephropathy. Recent studies have shown that nicousamide can attenuate albuminuria, glomerular sclerosis, tubulointerstitial fibrosis and renal tubule vacuolar degeneration in streptozotocin-induced diabetic rats. Interestingly, nicousamide also can reduce the incidence of diabetic cataract. To study the pharmacokinetics of nicousamide in plasma samples, a sensitive, accurate and reproducible method which is a reverse-phase HPLC system, with UV detection for the analysis was established. In order to elevate the sensitivity of nicousamide determination in plasma, we acidified the aqueous layer after the plasma was deproteinized. The method was successfully applied for a pharmacokinetic study of nicousamide in dogs.

2. Experimental

2.1. Materials

Nicousamide was synthesized at the Laboratory of Chemical Synthesis (Chinese Academy of Medical Sciences). The chemical structure of nicousamide was characterized by X-crystal diffraction, NMR, MS, infrared spectra, and HPLC-UV. The purity of nicousamide was 99.7%. Acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany) and all other reagents were of analytical grade.

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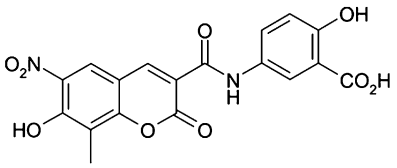

Fig. 1. Chemical structures of nicousamide.

Drug free plasma was obtained from untreated male beagle dogs weighing 6–8 kg purchased from Beijing Marshall Inc (Beijing, China).

2.2. Preparation of stocks, calibration standards and quality control samples

Nicousamide was dissolved in dimethyl sulphoxide (1 mg/ml) as stock solution and small volume aliquots for single-time use were stored at −20 °C until required. Working standards were prepared freshly by diluting the stock solution with water.

Calibrations standards samples were prepared by adding different concentrations of the working standards in drug-free plasma. The final concentrations of nicousamide were 5, 10, 20, 40, 80, 160 and 200 ng/ml.

High-, mid- and low-level quality control samples contained 150, 50 and 10 ng/ml each of the nicousamide analytes. These samples were prepared in a manner similar to that used for preparation of the calibrator samples.

2.3. Preparation of samples

A 300 μl nicousamide sample was spiked with 300 μl acetonitrile, the mixtures were vortexed for 10 s followed by centrifugation at 14,000 rpm for 5 min at room temperature. The aqueous layer (300 μl) was acidificated by adding phosphoric acid (3 μl). A 100 μl of the supernatant sample was injected into the HPLC system.

2.4. Chromatography

The analysis of calibrator, quality control and experimental samples were performed on an Agilent 1100 LC system (Palo Alto, CA, USA), equipped with a quaternary pump and degasser, an autosampler and column compartment (35 °C), a photodiode array detector, and ChemStation software. The analyte was eluted with acetonitrile/water (ratio 62:38 containing 0.1% H_3PO_4) at a flow rate of 1.0 ml/min on a reverse phase column (Aichrom Bond-AQ C_{18}, 5 μm, 4.6 mm × 250 mm; USA) with a C18 (5 μm, 8 mm × 4 mm; Dikma) guard column, followed by specific measurement at 320 nm. Nicousamide was eluted at 6.6 min under above conditions.

2.5. Bioanalytical method validation

2.5.1. Linearity, precision and accuracy

The linearity of the HPLC method for the determination of nicousamide was evaluated by a calibration curve in the range of 5–200 ng/ml. The calibration curve was obtained by plotting the peak area of each analyte versus nicousamide concentration. Least squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The calibration curve requires a correlation coefficient (r^2) of 0.99 or better. To evaluate the precision, at least five QC samples of three different concentrations of nicousamide were processed and injected on a single day (intra-day) and at different days (inter-day). The variability of nicousamide determination was expressed as the coefficient of variation (%CV) which should be ≤15% at all the concentrations. Accuracy is expressed as % bias which should be within limits of ±15% at all concentrations of nicousamide.

2.5.2. Recovery

The recoveries of nicousamide from plasma were determined at different standard concentrations by spiking the drug into the corresponding blank plasma. The percentage of recovery was calculated by comparing the peak area of deproteinized samples with samples in which the compound was spiked directly in acetonitrile. The recoveries at three QC concentration levels of nicousamide in plasma were examined at least five times.

2.5.3. Stability studies

Three freeze-thaws, long-term, short-term and post-preparative stabilities of nicousamide in plasma was tested using high-, mid- and low-quality control samples. The freeze-thaw stability of the analyte was determined over three freeze-thaw cycles. In each freeze-thaw cycle, the samples were frozen and stored at −20 °C for 24 h, then thawed at room temperature. To evaluate long-term stability of nicousamide, the plasma samples were stored at −20 °C for 10 days. For the short-term stability, fresh plasma samples were kept at room temperature for 12 h before sample preparation. The stability of the prepared plasma samples was tested after keeping the samples at room temperature for 12 h.

2.6. Pharmacokinetic experiments in dogs

The Institute Animal Care and Welfare Committee approved all animal protocols. Male and female beagle dogs weighing 6–8 kg purchased from Beijing Marshall Inc. (Beijing, China) were housed in individual cages during these studies. Dogs were fasted 12 h before receiving nicousamide and fed 4 h after administration. The dosing solutions used for all animal studies were prepared by suspending the required amounts of nicousamide in 0.5% sodium carboxymethyl cellulose. The plasma pharmacokinetics of nicousamide were studied in male and female beagles (3/sex) after a single oral 30, 100, 300 mg/kg dose and after a multiple dose (daily oral 100 mg/kg for 7 days) of nicousamide. Blood samples were collected via the foreleg vein into heparinized syringes at 0.05, 0.1, 0.17, 0.33, 0.5, 0.67, 1, 2, 3, 4, 6, 8, 12 and 24 h after oral dosing, immediately centrifuged and the resulting plasma prepared according to the procedure given for the calibrators. Pharmacokinetic analysis of nicousamide concentrations in plasma was performed using noncompartmental methods via the proprietary DAS (Drug and statistics) computer software package (Chinese Pharmacology Society).

3. Results and discussion

3.1. Method development

As nicousamide has an extremely low solubility in both water and organic solvents, therefore, a deproteinized method to detect plasma nicousamide has been developed. To diminish the dilution of the sample during deproteinize, diverse proportional solvents (methanol, acetonitrile, trichloracetic acid, perchloric acid) were selected in the deproteinize process. Although a 500 μl sample spiked with 100 μl 30% trichloracetic acid can produce the minimal dilution, optimum peak shape and an increase of detector sensitivity accordingly, the recovery was unsuitable. However, the plasma deproteinized with equal volume of acetonitrile followed acidification by adding phosphoric acid produced a similar optimum peak shape and the satisfactory recovery. Additionally, acidification the samples instead of strongly acidic the mobile phase can avoid a shorter column life caused by the latter. The chromatograms of a blank plasma sample, a nicousamide quality control sample (20 ng/ml) and a dog plasma sample after a 30 mg/kg dosing with nicousamide was summarized in Fig. 2. Endogenous plasma components did not interfere with the elution of the compounds of interest.

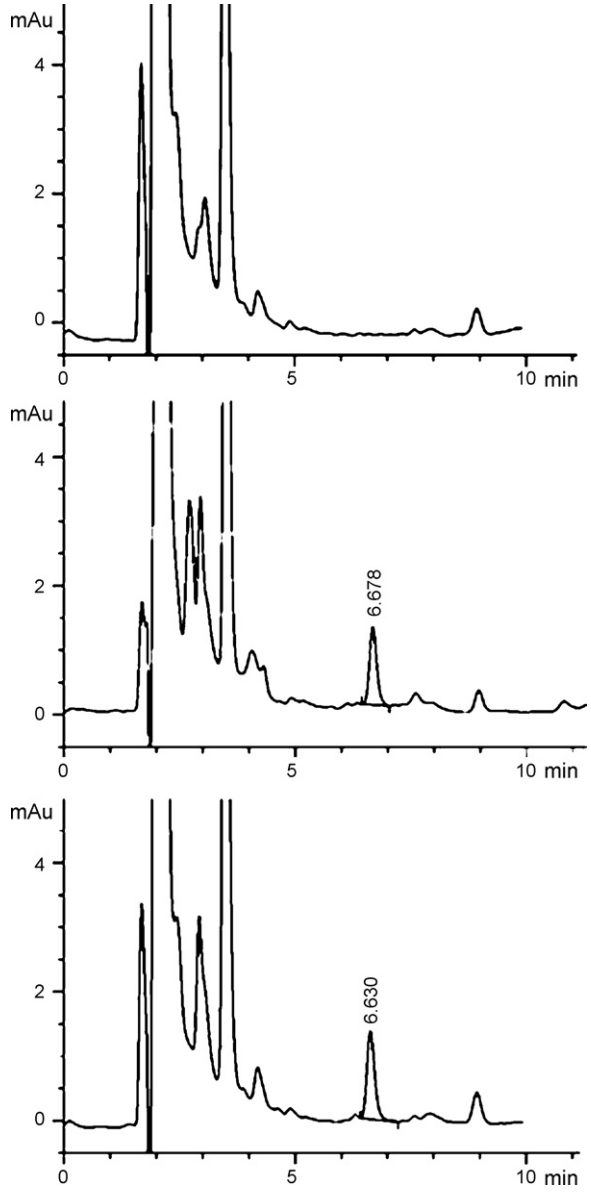

Fig. 2. Chromatograms of nicousamide in dog plasma: blank plasma sample (top trace); a 20 ng/ml nicousamide quality control sample (middle trace); a dog plasma sample after a 30 mg/kg dose of nicousamide (bottom trace).

3.2. Method validation

A linear relationship was found between plasma peak areas and nicousamide concentrations within the range of 5–200 ng/ml. The mean (±S.D.) regression equation for calibration curves in plasma was $(0.5515 \pm 0.0142)C + (2.5101 \pm 0.6386)$, $r^2 = 0.9978 \pm 0.0024$ ($n = 5$). The coefficient of variations of slope for nicousamide were found to be <15%, which indicates a high precision of the present assay. The average recoveries of nicousamide after deproteinized procedure ranged from 91.6 to 105.8% (Table 1).

The intra- and inter-day variability of plasma assay is listed in Table 2. It was demonstrate that the HPLC method for the determination of nicousamide was reliable and reproducible since both %CV and % bias were below 15% for all estimated concentrations of nicousamide. The LLOQ was evaluated by analyzing the plasma sample spiked with the analyte at a final concentration of 5 ng/ml at which the signal-to-noise ratio (S/N) was 10.

3.3. Stability studies

Stability of nicousamide during the determination was assessed under a variety of conditions and the maximum period was presented in Table 3. The deviation of the mean test responses were within ±15% of appropriate controls in all stability tests of nicousamide in dog plasma. Three freeze-thaw cycles of the QC samples appeared to have no effect on quantification of the analyte. QC samples stored in a freezer at −20 °C remained stable for at least 10 days. No effect on quantification was observed for the short-term stability of the samples kept at room temperature for 12 h. The deproteinized samples can be analyzed after at least 12 h at room temperature. These results suggested that dog plasma samples containing nicousamide can be handled under normal laboratory conditions without significant loss of compound.

Table 1
Absolute recovery of the method for determining the concentration of nicousamide in plasma samples ($n = 5$)

Concentration (ng/ml)	Absolute recovery (mean ± S.D.; %)	%CV
10	105.8 ± 7.6	7.2
50	102.4 ± 5.1	5.0
150	91.6 ± 4.0	4.4

Table 2
Intra- and inter-day precision and accuracy of nicousamide measurements in dog plasma

Concentrations (ng/ml)	Intra-day			Inter-day		
	Mean ± S.D. (n = 5)	Precision (%CV)	Accuracy (% bias)	Mean ± S.D. (n = 5)	Precision (%CV)	Accuracy (% bias)
10	11.5 ± 1.2	10.7	14.9	10.4 ± 0.7	7.1	3.5
50	54.4 ± 4.3	7.9	8.8	54.6 ± 1.9	3.5	9.2
150	164.7 ± 2.4	1.4	9.8	166.2 ± 2.9	1.8	10.8

Table 3
Stability of nicousamide in dog plasma

Stability (n = 5)	Concentration (mean ± S.D.; ng/ml)		
	10	50	150
Freeze-thaw stability			
Initial	11.5 ± 1.6	57.3 ± 4.0	170.9 ± 3.0
Measured	11.4 ± 1.3	57.1 ± 1.3	172.1 ± 2.9
Deviation (%)	−1.0	−0.2	0.7
Long-term stability			
Initial	9.9 ± 1.3	57.5 ± 1.9	171.1 ± 1.7
Measured	10.4 ± 1.5	56.6 ± 2.3	172.4 ± 2.4
Deviation (%)	4.6	−1.6	0.8
Short-term stability			
Initial	9.9 ± 1.3	57.5 ± 1.9	171.1 ± 1.7
Measured	10.0 ± 1.1	57.0 ± 1.9	171.3 ± 5.0
Deviation (%)	1.1	−0.8	0.1
Post-preparative stability			
Initial	11.5 ± 1.6	57.3 ± 4.0	170.9 ± 3.0
Measured	11.2 ± 1.5	56.4 ± 2.0	171.6 ± 7.7
Deviation (%)	−3.0	−1.6	0.4

3.4. Pharmacokinetic study of nicousamide in dogs

The validated assay was used to determine the plasma concentration profiles of nicousamide in beagle dogs after a single oral dose of 30, 100, 300 mg/kg and after a multiple dose (daily oral 100 mg/kg for 7 days). The mean plasma concentration versus time profiles of nicousamide after single oral doses were shown in Fig. 3, and the mean pharmacokinetic parameters were summarized in Table 4. After three oral dosages, the peak of nicousamide in blood occurred rapidly, which was approximately 0.3, 1.8 and 1.6 h in males, and 0.6, 0.7 and 0.6 h in females, respectively. Nicousamide was eliminated from plasma with a MRT of 1.4, 4.3 and 12.1 h in males, and 1.6, 3.2 and 12.8 h in females at dose of 30, 100 and 300 mg/kg. The female dogs showed lower C_{max} (33.5 μg/l versus 123.5 μg/l) and $AUC_{0-\infty}$ (46.7 μg h/l versus 107.6 μg h/l) than the males after a dose of nicousamide at 30 mg/kg, no significant differences in C_{max} and $AUC_{0-\infty}$ between male and female dogs at dose of 100 and 300 mg/kg.

The lack of linearity in C_{max} value and the significant extension of MRT with the increase of nicousamide dosage suggested the saturation in the absorption and elimination of nicousamide, and the nonlinear pharmacokinetics in dogs from 30 to 300 mg/kg.

Table 4
Pharmacokinetic parameters of nicousamide in dogs following single oral doses of 30, 100, or 300 mg/kg (n = 3)

Gender	$AUC_{0-\infty}$ (μg h/l)	C_{max} (μg/l)	T_{max} (h)	MRT (h)
Dose: 30 mg/kg				
Male	107.6	123.5	0.3	1.4
Female	46.7	33.5	0.6	1.6
Dose: 100 mg/kg				
Male	448.4	151.6	1.8	4.3
Female	468.6	158.3	0.7	3.2
Dose: 300 mg/kg				
Male	1846.8	136.7	1.6	12.1
Female	1907.8	110.2	0.6	12.8

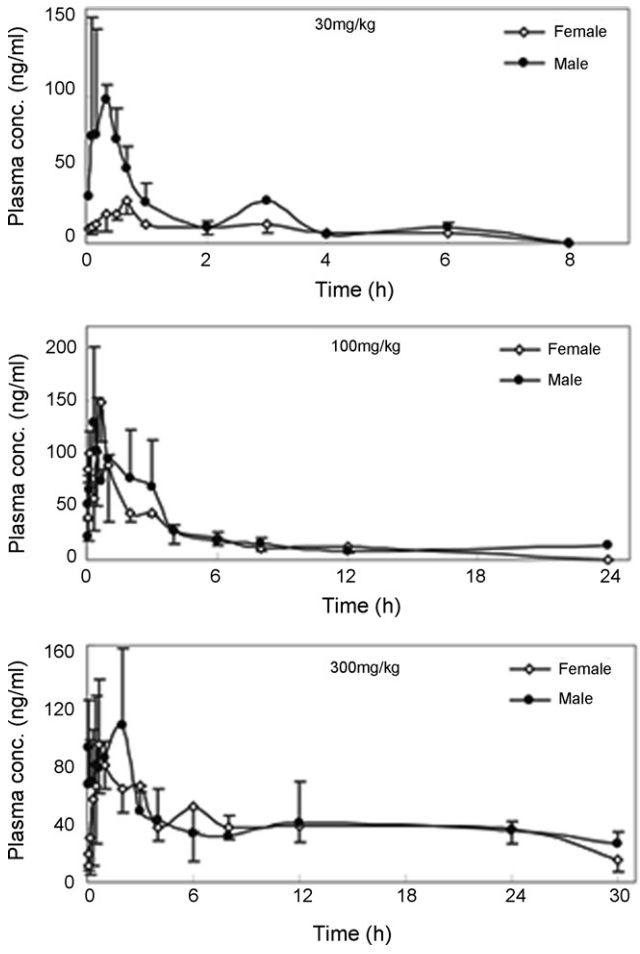

Fig. 3. Plasma concentration–time profiles of nicousamide after single oral doses of 30, 100 and 300 mg/kg in dogs (3/sex).

Table 5
Mean plasma pharmacokinetic parameters of nicousamide in dogs following multiple oral doses of 100 mg/kg ($n = 3$)

Gender	$AUC_{0-\infty}$ (μg h/l)	C_{max} (μg/l)	T_{max} (h)	MRT (h)
Dose (mg/kg): single dose				
Male	448.4	151.6	1.8	4.3
Female	468.6	158.3	0.7	3.2
Dose (mg/kg): multiple doses				
Male	5235.3	184.0	0.7	10.2
Female	4133.7	205.4	0.5	10.2

The mean pharmacokinetic parameters for nicousamide from the multiple dosing studies were compared with single dosing in Table 5. After seven daily doses of nicousamide (100 mg/kg), the C_{max} of male and female dogs increased 30% and 21%, $AUC_{0-\infty}$ values raised 10.7 and 7.8 times, and MRT extended 3.2 and 2.4 times compared with single dosing, suggesting there was a potential for accumulation in dogs following multiple doses.

In conclusion, the newly developed HPLC method provided a new simple, sensitive, reproducible and validated assay for the determination of nicousamide in plasma. This HPLC method will help in further studies in characterizing the tissue distribution and excretion of nicousamide.

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