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The ultra-performance liquid chromatography tandem mass spectrometry method for detection and quantification of C4NP in rat plasma and its application to pharmacokinetic studies

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ABSTRACT

Introduction Combretastatins, which are excellent anticancer agents, are isolated from *Combretum*. A sensitive ultra-performance liquid chromatography tandem mass spectrometry method was developed and validated for the pharmacokinetic study of a combretastatin analog (C4NP) in rats.

Methods Sample pretreatment was finished by simple protein precipitation in which methanol was added to plasma containing an internal standard (buspirone hydrochloride). Liquid chromatograph separation was accomplished on a reverse-phase Kinetex XB-C18 column [50×4.6 mm; internal diameter: 2.6 μm (Phenomenex, Torrance, CA, U.S.A.)] with a gradient mobile phase of acetonitrile (0.05% formic acid, volume for volume) and water (0.05% formic acid) at a flow rate of 0.3 mL/min. The analytes were analyzed in the positive ion by electrospray ionization and quantified in the selective reaction monitoring mode. The entire procedure was validated following the U.S. Food and Drug Administration guidelines for bioanalytical methods validation.

Results Our study investigated, for the first time, the detection and pharmacokinetic characteristics of C4NP in Sprague–Dawley rat plasma. The pharmacokinetic results suggest that C4NP is predominantly restricted to blood or extracellular fluid and is not extensively distributed to most organ tissues. In addition, C4NP can be cleared by renal filtration and active tubular secretion in Sprague–Dawley rats. Toxicokinetics of C4NP in these rats indicate that no saturation of the metabolic or excretion process occurs for C4NP, and metabolic induction and accumulation of toxic injury from multiple dosing are both absent.

Conclusions For 100 μL of analyte, recovery plus high accuracy and reproducibility indicate that our new ultra-performance liquid chromatography tandem mass spectrometry method is a reliable and high-throughput analytical tool for the pharmacokinetic study of C4NP in rats. Those results should be useful for risk assessment.

Key Words Ultra-performance liquid chromatography tandem mass spectrometry, C4NP, method validation, pharmacokinetics, safety

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INTRODUCTION

Combretastatins are heterocyclic plant alkaloids derived originally from the bark of the African bush willow *Combretum caffrum*[1–4]. They have been investigated for use as anticancer agents because of their inhibitive activity against tumour blood flow. Combretastatin A-4 phosphate (CA4P) is a synthetic phosphorylated prodrug of the natural product combretastatin A-4 (CA4). As a novel antitumour vascular-disrupting agent, CA4 is structurally similar to colchicine and can discriminate between normal vessels and tumour vessels, selectively disrupting the abnormal

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tumour vasculature, leading to vascular collapse[5]. Combretastatin A-4 can bind strongly to the colchicine-binding site on tubulin, inhibiting microtubule assembly[6–8].

Histologic studies show that several tubulin-binding agents (colchicine, podophyllotoxin, vincristine, and vinblastine, for instance) and other antineoplastic agents (for example, tumour necrosis factor, flavone acetic acid, and related compounds) can induce vascular damage in tumours. However, that induction is achieved only at approximately the maximum tolerated dose, which limits the applicability of those agents[9–13]. In contrast, in murine models, CA4P can induce vascular shutdown in tumours at less than one tenth the maximum tolerated dose[14,15].

Combretastatin A-4 phosphate is the lead product candidate as a clinical vascular disrupting agent, and it is the first combretastatin analog to enter the clinic. The U.S. Food and Drug Administration (FDA) has approved CA4P for the treatment of thyroid cancer. Phase III clinical trials are currently evaluating whether CA4P in combination with paclitaxel or carboplatin can improve the safety and efficacy of those drugs against anaplastic thyroid carcinoma[16–21].

Combretastatin 4-N phosphate (C4NP), a modified version of CA4P, is a new combretastatin analog that is stable in aqueous solution. It is expected to become a novel anticancer drug. Pharmacokinetic study of CA4P in rats is therefore very important before the compound can enter into clinical application. Traditional methods for molecule quantification such as high-performance liquid chromatography do not meet the requirements of a shorter run time and high sensitivity. Recently, ultra-performance liquid chromatography (UPLC) has significantly improved peak resolution and expedited analyses. With its relatively short analysis time and increased sensitivity, UPLC tandem mass spectrometry (MS/MS) has been used as a novel tool in pharmacokinetic studies.

The aim of the present study was to develop a rapid and sensitive UPLC MS/MS method for the detection of C4NP in Sprague–Dawley rat plasma and to understand the *in vivo* disposition of C4NP. The entire procedure was validated following the FDA guidelines for bioanalytic methods validation[22]. We also intended to study the long-term toxicity and toxicokinetics of C4NP as a clinical drug, providing toxicologic evidence for its safety as a clinical medication. To the best of our knowledge, no publication has reported a UPLC MS/MS method for the determination of C4NP or the application of such a method for pharmacokinetic or toxicokinetic studies in Sprague–Dawley rats.

METHODS

The Second Military Medical University (Shanghai, P.R.C.) provided C4NP (purity: ≥98%). Buspirone hydrochloride (internal standard) was obtained from Sigma–Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile and methanol of mass spectrometry grade were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Ultrapure water was produced using a Milli-Q Plus apparatus (Millipore, Bedford, MA, U.S.A.). All other chemicals and solvents were of analytical or chromatographic grade (the highest quality available) and obtained from commercial sources.

UPLC MS/MS

The UPLC MS/MS system consisted of a TSQ Quantum triple-stage quadrupole mass spectrometry meter (Thermo Finnigan, San Jose, CA, U.S.A.) interfaced by an ESI probe with an Acquity UPLC meter (Waters, Milford, MA, U.S.A.). The liquid chromatography MS/MS system control, data acquisition, and data processing were finished using the Acquity UPLC Console and Thermo LCquan software packages (Waters).

For method validation and sample analysis, chromatographic separation was conducted on a reversed-phase Kinetex C18 XB column [50×4.6 mm; internal diameter: 2.6 μm (Phenomenex, Torrance, CA, U.S.A.)]. The liquid chromatography mobile phases were 0.05% formic acid in water (phase A) and 0.05% formic acid in acetonitrile (phase B). This gradient elution scheme was used: The organic phase was increased linearly from 10% to 30% in 2 minutes and was then maintained for another 1 minute. Finally, after 1 minute of 90% B, the column was led to the original ratio of 10% B and 90% A within 0.5 minutes, followed by re-equilibration at 10% B for a further 0.5 minutes, which enabled equilibration of the column. A timed switch valve drove the effluent to the source from minutes 1.5 to 3.5 only. The resulting total runtime was 5 minutes.

Infusion experiments were used to optimize the parameters of the positive-ion ESI MS/MS instrument and thereby to maximize the generation of protonated molecules and the efficient production of characteristic fragment ions in the analytes. All analytes were detected in positive ionization using an ion spray voltage of 3500 V, sheath gas pressure of 45 bar, auxiliary gas pressure of 5 bar, and capillary temperature of 300°C. The precursor-to-product ion pair was monitored at m/z 407.08–327.07 for C4NP and at m/z 386.00–122.09 for the internal standard in the select reaction monitoring mode. The mass spectrometer was operated at unit mass resolution for both the first and third quadrupoles.

Sample Preparation

Plasma samples were stored at –80°C before analysis. Before the experiments, samples were allowed to thaw at room temperature and were vortexed for 30 s. Rat plasma (90 μL) was mixed with 10 μL of the internal standard (100 ng/mL) and then vortexed. After methanol 400 μL was added to each sample, the sample was vortexed again for 10 minutes and then centrifuged at 12,000 rpm and 4°C for 10 minutes, after which the precipitated proteins were removed. The upper supernatant (400 μL) was transferred to a new tube and evaporated at 35°C under a nitrogen stream until dry. That residue was reconstituted in 100 μL of a 9:1 (volume-to-volume) water–acetonitrile mixture, vortexed for 10 minutes, centrifuged at 12,000 rpm and 4°C for 10 minutes, and transferred to amber glass vials. The resulting supernatant (10 μL) was analyzed by liquid chromatography MS/MS.

Construction of Standard Solutions and Quality Control Samples

The primary stock solution of C4NP was prepared by dissolving accurately weighed analytes in water to a

concentration of 1.026 mg/mL. The stock standard solution of the internal standard was prepared by dissolving an appropriate amount of the standard in CH_3OH:H_2O (50:50, volume-to-volume) to a concentration of 1 mg/mL.

During the validation process and the study, duplicate 8-point calibration curves were plotted routinely for blank rat plasma spiked at C4NP concentrations of 5.70, 11.40, 22.80, 57.00, 114.00, 228.00, 456.00, and 912.00 ng/mL. Quality control samples were prepared at three concentrations—10.68, 106.78, and 854.22 ng/mL—and were used in the validation and during the toxicokinetic study. A standard curve was then plotted using weighted ($1/x$) linear regression of the peak area of C4NP, expressed as a ratio to the internal standard peak area, against the corresponding nominal plasma concentration of C4NP.

Validation

Assays were validated according to the FDA guidance on bioanalytic method validation, *FDA Guidance for Industry on Bioanalytical Method Validation*[22]. The method was documented and validated on separate days with respect to these parameters: specificity, linearity, lowest limit of quantitation (LLOQ), intraday precision and accuracy, interday precision and accuracy, effects of matrix, extraction recovery, effects of dilution, and the stability of the analyte during the sample storage and treatment procedures.

Assay selectivity was assessed by monitoring ion pairs of the analytes when peaks in the plasma samples obtained from 5 untreated rats appeared. The LLOQ was defined as the lowest concentration on the standard calibration curve from 6 batches in which both precision and accuracy were 20% or less, with a signal-to-noise ratio greater than 10. The linearity of the calibration curve was evaluated over the range 5.70–912.00 ng/mL. The resulting correlation coefficient ($r > 0.99$) was considered satisfactory. Precision and accuracy were assessed using the concentrations of the analytes covering the range of the calibration curve. The criteria for acceptability were defined as an accuracy of ±15% standard deviation and a precision of ±15% relative standard deviation from the nominal values. The intraday accuracy and precision were evaluated by analyzing the quality control samples (10.68, 106.78, and 854.22 ng/mL) with 6 duplicated levels per concentration on the same day. The interday accuracy and precision were assessed over 3 days. The effects of matrix and the extraction efficiency were examined by comparing the peak areas of analytes between 3 sample sets. The stability of C4NP was evaluated by comparison under mimicked situations likely to be encountered, including post-preparative stability test, freeze–thaw cycle test, and long-term stability test. Quality control samples at low, medium, and high serum concentrations were determined at 6 duplicated levels. The stability of the extracts was evaluated by holding them at room temperature and at 4°C for 24 hours. Samples of C4NP maintained at –80°C for 60 days was evaluated by comparing the post-freeze measured concentration with the initial concentration added to the sample. The freeze–thaw stability of the samples was assessed over 3 freeze–thaw cycles by thawing the samples at room temperature and refreezing them at –80°C for 24 hours.

Toxicokinetic Experiments

Animal experiments were conducted according to the protocols approved by the Review Committee of Animal Care and Use at the Second Military Medical University (Shanghai, P.R.C.). Sprague–Dawley rats ($n = 18$; both sexes; weight range: 210–220 g; Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, P.R.C.) were randomly divided into three groups ($n = 6$ per group). The groups were treated with 2, 6, and 18 mg/kg C4NP once daily for 28 consecutive days. Serial blood samples were collected from the orbital sinus into EDTA-2Na–anticoagulated tubes under light ether anesthesia. The collection time points were set at 0 minutes (before the first dose), 1 minute (immediately after the first dose), and at 5, 10, 20, 30, 45, 60, and 120 minutes after the first dose. The blood samples were centrifuged (12,000*g* for 10 minutes at 4°C) and kept frozen at –80°C until analysis.

All pharmacokinetic parameters were analyzed by a non-compartmental method using the Kinetica 2000 software package (version 3.0: InnaPhase Corporation, Philadelphia, PA, U.S.A.), including time to peak (T_{max}), peak concentration (C_{max}), half-life ($t_{1/2}$), and area under the curve ($AUC_{0\text{-}t}$). Experimental data are expressed as mean ± standard deviation ($n \geq 3$) or simple mean ($n = 2$). Data processing and statistical analyses were performed using Microsoft Excel (2003 version: Microsoft, Redmond, WA, U.S.A.).

RESULTS AND DISCUSSION

Liquid Chromatography MS/MS method

Given the high hydrophilicity of the analytes, plasma sample preparation used a simple protein precipitation method instead of serum protein electrophoresis, which is relatively tedious and expensive. Because of its simplicity and low cost, protein precipitation would be favourable for analyzing a large number of plasma samples obtained from toxicokinetic experiments. To achieve efficient extraction, two precipitating agents (methanol and acetonitrile) were tested. Methanol was finally chosen as the precipitating agent because the precision and accuracy after processing by methanol are superior to those after processing by acetonitrile.

In the initial experiments, we developed an efficient ionization method for detection of C4NP. The C4NP showed high level of protonated molecule peak in the positive-ion ESI source. In the MS/MS spectra, the highest signal intensity peaks of the product ions were at m/z 327.07 (optimal collision energy: 23 V). The main parameters—that is, sheath gas pressure, auxiliary gas pressure, spray voltage, and capillary temperature—were set at 45 bar, 5 bar, 3500 V, and 300°C respectively. The internal standard was decided at m/z 386.00→122.09. Figure 1 shows two mass spectra of C4NP.

Acetonitrile was chosen as the organic phase because it can improve the peak shape. Gradient elution is typically used to improve the peak shape and to eliminate higher matrix effect resulting from co-elution between the analyte and the endogenous plasma components. When the organic phase was ramped from 10% to 30% organic in 2 minutes and maintained at 30% to 3 minutes in the gradient method, the peaks of analyte and internal standard appeared at the retention time of 2.35 minutes and

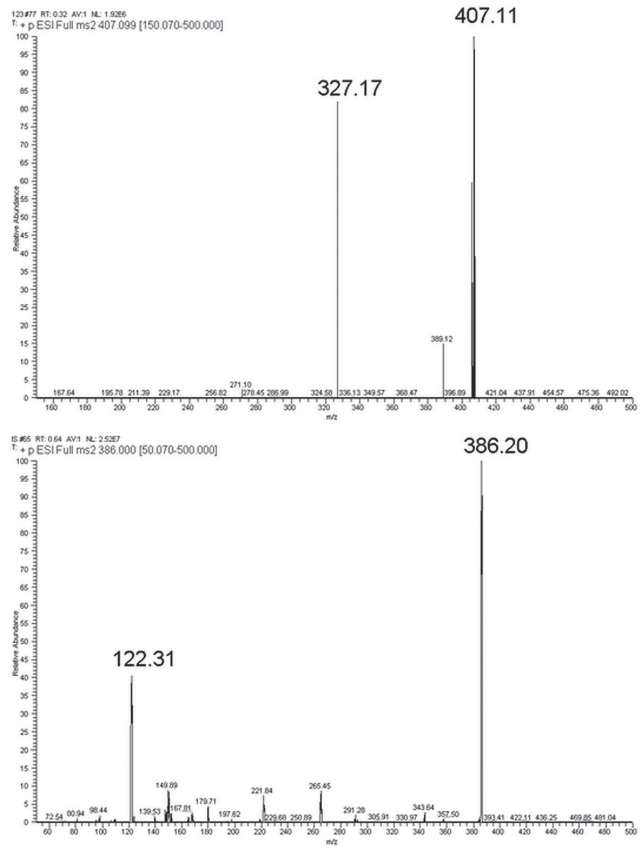

FIGURE 1 Secondary mass spectra of combretastatin analog and buspirone hydrochloride.

2.41 minutes respectively. The total chromatographic run time was 5.0 minutes. Figure 2 shows the typical liquid chromatography MS/MS profiles of the standard solution spiked in rat plasma, the blank plasma, and the actual samples.

Method Validation

Specificity

Selectivity was assessed by analyzing the peak corresponding to C4NP to check the appearance of peaks in plasma from 5 untreated rats. No peaks of endogenous compounds were detected at the retention time of the analyte in the blank plasma (Figure 2), indicating that our method had high selectivity.

Linearity and Sensitivity

The calibration curve had a linear range of 5.70–912.00 ng/mL and the typical regression equation was $y = -0.0161409 + 0.0264429x$ ($r = 0.9996$). The LLOQ was 5.70 ng/mL determined as the lowest concentration of the calibration curve with a signal-to-noise ratio greater than 10 (relative standard deviation: 3.74%; accuracy: 111.87%). The acceptance criterion for each point of back-calculated standard concentration was ±15% or less deviation from the nominal value, except at LLOQ, which was set at ±20%[22].

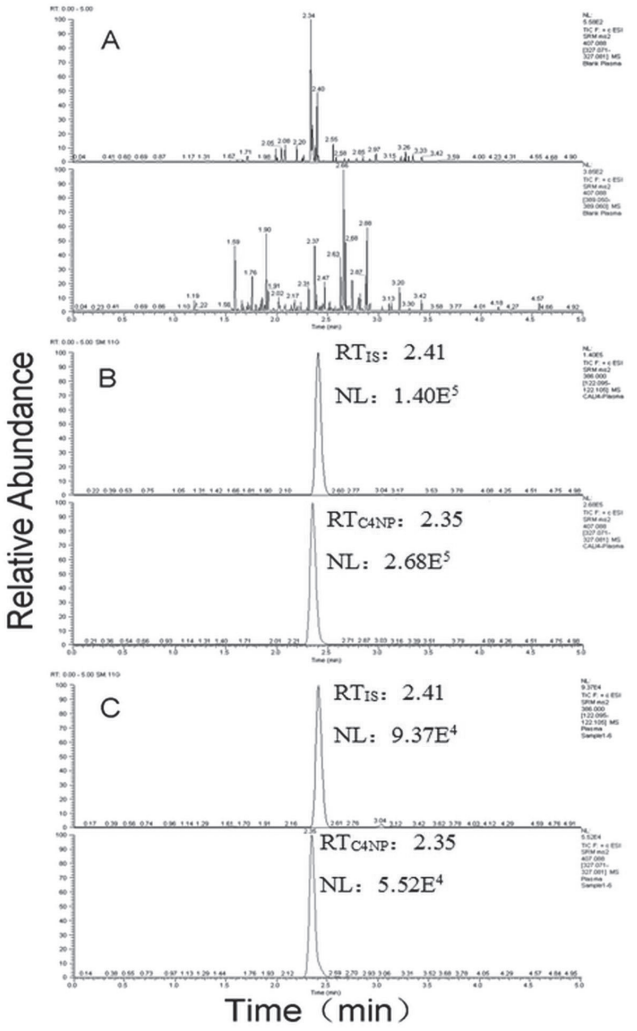

FIGURE 2 Liquid chromatography tandem mass spectrometry chromatograms of a combretastatin analog (C4NP) in (A) a blank sample of rat plasma; (B) the same blank sample spiked with known concentrations of C4NP (51.33 ng/mL) and buspirone hydrochloride [internal standard (10 ng/mL)]; and (C) a plasma sample obtained from rat 1 45 minutes after the first intravenous administration of C4NP. RT = retention time; NL = nominal level.

Precision and Accuracy

The precision and accuracy were evaluated by determining C4NP quality control samples at 3 concentration levels (10.68, 106.78, and 854.22 ng/mL). The intraday precisions and accuracies were assessed at 6 duplicated levels for each concentration during a single analytical run; interday precisions and accuracies were measured in replicate for each concentration on 3 successive days. Table I presents the results of the precision and accuracy evaulations. The relative standard deviations of the precision did not exceed 15%, and the accuracies were within 85%–115%. The foregoing results indicate that the new method is acceptable for determination of C4NP in plasma.

Extraction Recovery and Matrix Effects

The extraction recovery and matrix effects were examined at 6 duplicated levels by comparing the peak areas of the analytes between the 3 sample sets. In set 1, the analytes were dissolved in a matrix component–free solution of acetonitrile and water (10:90, volume-to-volume). In set 2, the analytes were added into post-extraction plasma obtained from 5 untreated rats. In set 3, the analytes were added to blank plasma before extraction and were then extracted. The extraction efficiency and absolute matrix effect were calculated as follows:

$$\text{Extraction efficiency} = (\text{Mean peak area})_{\text{set 3}} / (\text{Mean peak area})_{\text{set 2}}$$

$$\text{Absolute matrix effect} = (\text{Mean peak area})_{\text{set 2}} / (\text{Mean peak area})_{\text{set 1}}$$

Table II summarizes the results of extraction recovery and matrix effects.

Stability

The stability of C4NP was determined at 3 concentrations (10.68, 106.78, and 854.22 ng/mL) under conditions covering the entire storage and assay process, including short-term stability (storage at 20°C and 4°C for 24 hours), freeze–thaw stability (3 cycles), and long-term stability (storage at –80°C for 2 months). The results showed that all samples were stable during the assay process under the tested conditions (Table III).

Dilution Effect

Dilution effects were performed in 6 replicates by a 500-times dilution with blank plasma. Plasma samples were spiked with C4NP at 427.11 μg/mL, diluted at a dilution factor of 500 (854.22 ng/mL), and then analyzed. The results (relative standard deviation: 5.45%; accuracy: 104.63%) showed that samples could be diluted with blank plasma without affecting the final concentration.

Toxicokinetics of C4NP in Sprague–Dawley Rats

The described method was successfully applied in a toxicokinetic study of C4NP administration in Sprague–Dawley rats at 3 doses (2, 6, and 18 mg/kg). The mean plasma concentration–time profiles of C4NP plotted in Figure 3 suggest that the C4NP concentrations increased as the dose increased from 2 mg/kg, to 6 mg/kg, and to 18 mg/kg.

Pharmacokinetics of C4NP in Sprague–Dawley Rats

Table IV presents the major pharmacokinetic parameters of C4NP. The C_{max} of C4NP was reached immediately for all 3 doses. The $AUC_{0-\infty}$ values of the 3 doses were 62,138 ± 10,757 ng·min^{-1}·mL^{-1}, 212,186±26,517 ng·min^{-1}·mL^{-1}, and 725,088±154,230 ng·min^{-1}·mL^{-1} respectively; the C_{max} values were 27,244 ± 3,407 ng/mL, 93,587 ± 12,353 ng/mL, and 292,392 ± 40,795 ng/mL respectively. The mean steady-state distribution volumes for C4NP were 148 mL/kg, 191 mL/kg, and 185 mL/kg respectively. Those results were smaller than the rat total body water (668 mL/kg) and a little larger than the rat blood volume (54 mL/kg)[23], which suggests

TABLE I Intra- and interday precision and accuracy of the measured combretastatin analog (C4NP) concentration

Nominal concentration (ng/mL)	Intraday (n=6)			Interday (n=3)		
	Mean measured concentration (ng/mL)	Precision (% RSD)[a]	Accuracy (%)[b]	Mean measured concentration (ng/mL)	Precision (% RSD)[a]	Accuracy (%)[b]
10.681	10.386±0.323	3.12	97.07	10.881±0.493	4.53	101.88
106.811	112.304±2.426	2.16	105.14	104.154±7.301	7.01	97.51
854.489	933.426±16.853	1.81	109.24	885.239±47.879	5.41	103.60

[a] (Standard deviation / mean) × 100.
[b] (Mean measured concentration / nominal concentration) × 100.
RSD = relative standard deviation.

TABLE II Extraction recovery and matrix effects (n = 6)

Nominal concentration (ng/mL)	Peak area			Extraction recovery		Matrix effect	
	$Mean_A$	$Mean_B$	$Mean_C$	(%)[a]	RSD (%)	(%)[b]	RSD (%)
10.681	142125.0	103555.6	114108.8	72.86	9.25	124.55	8.80
106.811	1486816	1157646	1196244	77.86	3.95	124.29	7.80
854.489	11602116	8599668	11101098	74.12	2.60	104.51	4.76

[a] ($Mean_B$ / $Mean_A$) × 100.
[b] ($Mean_A$ / $Mean_C$) × 100.
$Mean_A$ = standards spiked before extraction; $Mean_B$ = standards spiked after extraction; $Mean_C$ = standards spiked in 90% acetonitrile; RSD = relative standard deviation.

TABLE III Stability of quality control sample under conditions mimicking the entire assay process (n = 6)

Sample condition	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Precision (% RSD)[a]	Accuracy (%)[b]
Bench-top stability				
4°C, 24 hours	10.681	11.451±0.314	2.75	107.21
	106.811	108.729±4.917	4.52	101.80
	854.489	902.024±84.698	9.39	105.56
25°C, 24 hours	10.681	9.650±0.221	2.29	90.35
	106.811	109.643±3.949	3.60	102.65
	854.489	942.366±32.573	3.46	110.28
Freeze–thaw stability				
3 Freeze–thaw cycles[c]	10.681	10.078±0.493	4.89	94.35
	106.811	107.083±12.016	11.22	100.25
	854.489	827.187±39.177	4.74	96.80
Long-term stability				
–80°C for 2 months	10.681	9.582±0.482	5.03	89.71
	106.811	97.914±6.054	6.18	91.67
	854.489	929.041±19.864	2.14	108.72

[a] (Standard deviation / mean) × 100.
[b] (Mean measured concentration / nominal concentration) × 100.
[c] –80°C to 25°C.
RSD = relative standard deviation.

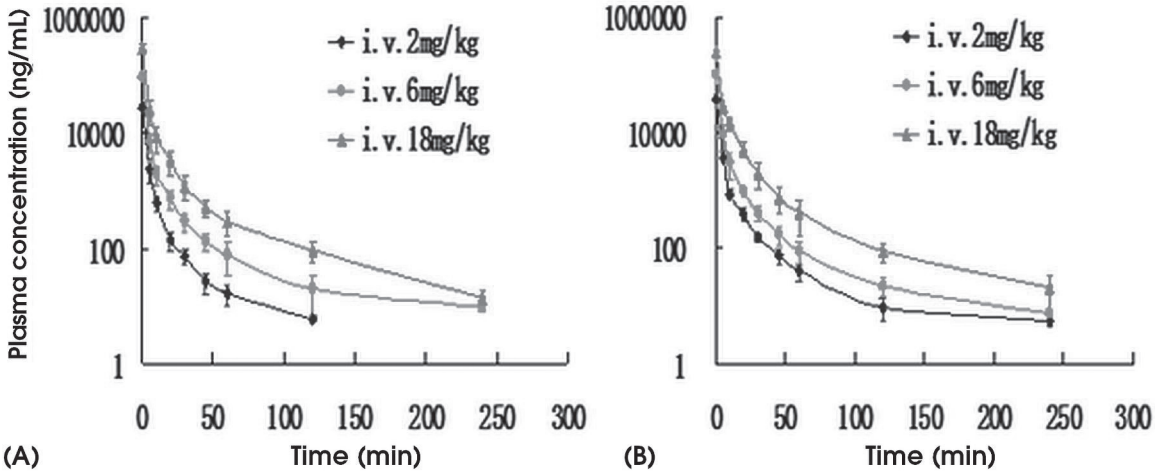

FIGURE 3 Mean plasma concentrations of a combretastatin analog (C4NP) over time after (A) first and (B) last administration of C4NP to rats at 3 doses: 2 mg/kg, 6 mg/kg, and 18 mg/kg.

that C4NP is restricted predominantly to blood and is not extensively distributed to most organ tissues.

In our results, clearance values were 33.03 $mL \cdot min^{-1} \cdot kg^{-1}$, 28.66 $mL \cdot min^{-1} \cdot kg^{-1}$, and 25.77 $mL \cdot min^{-1} \cdot kg^{-1}$ respectively. The observed physicochemical properties (small polar molecules), clearance values, and glomerular filtration rate (5.24 $mL \cdot min^{-1} \cdot kg^{-1}$)[23] in the rats suggest that C4NP can be cleared by renal filtration and active tubular secretion in Sprague–Dawley rats.

Linear Correlation Study of the Level of System Exposure After Long-Term Intravenous Administration of C4NP in Rats

Linear regression analysis was carried out using the AUC_{0-24} with dose and the C_{max} with dose. Significant positive linear correlations were observed between the systemic exposure levels to C4NP in Sprague–Dawley rats and the dose used for intravenous administration of C4NP (2, 6, or 18 mg/kg) for 28 consecutive days (Figure 4). Those

TABLE IV Pharmacokinetic parameters[a] of a combretastatin analog (C4NP) after first and last administration to 18 Sprague–Dawley rats at 3 doses

Parameter	Administration	Dose of C4NP (mg/kg)		
		2 mg/kg	6 mg/kg	18 mg/kg
C_{max} (ng/mL)	First	27,243.5±3,407.0	93,586.5±12,353.0	292,392.2±40,794.8
	Last	37,192.3±2,729.9	99,619.9±9,206.3	251,753.8±30,907.8
T_{max} (min)	First	0	0	0
	Last	0	0	0
$t_{1/2}$ (min)	First	21.57±10.32	39.56±17.70	42.94±4.92
	Last	44.29±31.90	51.49±11.65	43.80±9.97
Clearance ($mL \cdot min^{-1} \cdot kg^{-1}$)	First	33.03±5.90	28.66±3.71	25.77±5.39
	Last	21.27±1.73	24.63±3.25	23.34±1.68
V_{SS} (mL/kg)	First	147.97±28.31	191.37±84.91	185.41±38.77
	Last	153.45±32.89	176.91±29.13	210.01±39.80
MRT (min)	First	4.60±1.15	6.65±2.29	7.29±1.37
	Last	7.28±1.73	7.18±0.78	9.09±2.22
AUC_{0-t} ($ng \cdot min^{-1} \cdot mL^{-1}$)	First	61,909±10,677	211,666±26,493	724,261±154,063
	Last	94,150±8,233	246,617±34,639	773,508±61,190
$AUC_{0-\infty}$ ($ng \cdot min^{-1} \cdot mL^{-1}$)	First	62,138±10,757	212,186±26,517	725,088±154,230
	Last	94,584±8,253	247,331±34,529	774,762±61,484

[a] All values presented as mean ± standard deviation.
C_{max} = peak concentration; T_{max} = time to peak concentration; $t_{1/2}$ = half-life; V_{SS} = steady-state distribution volume; MRT = mean residence time; AUC_{0-t} = area under the curve for time 0 to time t; $AUC_{0-\infty}$ = area under the curve for time 0 to ∞.

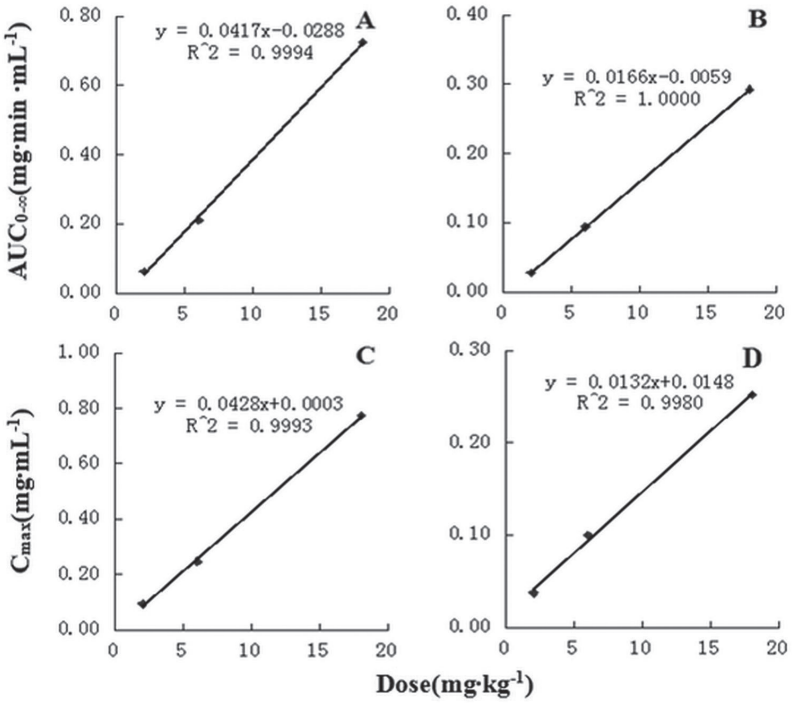

FIGURE 4 Linear correlations for the level of systemic exposure after long-term intravenous administration of a combretastatin analog (C4NP) in rats. Correlations of area under the curve (AUC) with (A) first C4NP administration and (B) last C4NP administration; and correlations of peak concentration (C_{max}) with (A) dose of first C4NP administration and (B) dose of last C4NP administration.

results indicate that the time course of C4NP is linear in Sprague–Dawley rats and that no saturation of metabolic or excretion processes occurred.

Accumulation of C4NP in Sprague–Dawley Rats After Long-Term Intravenous Injection

The histogram of AUC_{0-24} with dose and C_{max} with dose (Figure 5) indicates that the systemic exposure levels to C4NP in Sprague–Dawley rats after administration of C4NP for 28 consecutive days at 3 doses (2, 6, or 18 mg/kg) were similar to those seen with the first dose, suggesting an absence of metabolic induction or accumulation of toxic injury from multiple doses.

CONCLUSIONS

We developed a rapid, sensitive, and reliable liquid chromatography MS/MS method for the determination of C4NP. The method demonstrates high linearity, precision, accuracy, recovery, and stability, together with a sufficient lower detection limit and a shorter run time. This validated method is suitable to detect C4NP in Sprague–Dawley rat plasma and was also successfully applied in a pharmacokinetic study after intravenous administration. To the best of our knowledge, this liquid chromatography MS/MS method is the first to be published for the determination of C4NP in Sprague–Dawley rat plasma. The method has been fully validated and was successfully applied in a pharmacokinetic study. The resulting data should be useful for use in risk assessment.

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CONFLICT OF INTEREST DISCLOSURES

We have read and understood *Current Oncology*'s policy on disclosing conflicts of interest, and we declare that we have none.

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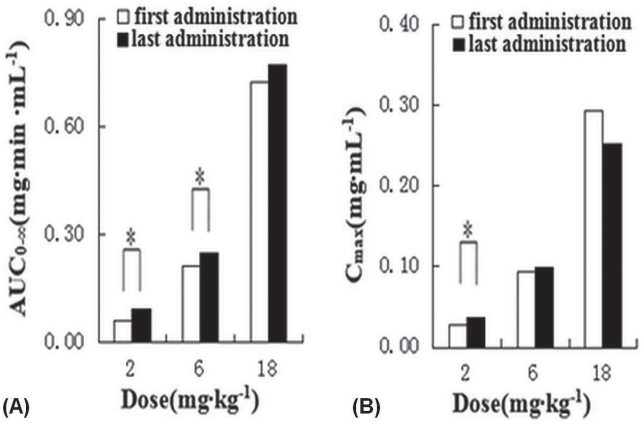

FIGURE 5 Histograms of (A) 24-hour area under the curve (AUC_{0-24}) against dose, and (B) peak concentration (C_{max}) against dose. * $p < 0.05$.

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