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An HPLC–ultraviolet detection method for the determination of Z24 in mouse whole blood and its application to pharmacokinetic studies

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ABSTRACT

A sensitive and reproducible high-performance liquid chromatography (HPLC)–UV method for the determination of Z24, a tumorigenesis and angiogenesis inhibitor, has been developed and validated in mouse whole blood. Blood samples were extracted with ether, evaporated, and the residue was reconstituted in mobile phase. An aliquot was separated by isocratic reversed-phase HPLC on a Hypersil ODS-2 column and quantified using UV detection at 390 nm. The mobile phase was 50% (v/v) acetonitrile/water with a flow rate of 0.8 ml/min. A linear curve over the concentration range of 0.05–6 μg/ml (r^2 = 0.9976) was obtained. The coefficient of the variation for the intra- and inter-day precision ranged from 3.0 to 10.9% and 5.7 to 10.3%, respectively. The absolute recovery of Z24 was 89.2–108.5%. The method is simple, economical and sufficient for in vivo pharmacokinetic studies on Z24. Nonlinear pharmacokinetics was found in mice at doses from 20 to 80 mg/kg.

1. Introduction

The developments in understanding the biochemical mechanisms in the multistep process of carcinogenesis have disclosed numerous molecular targets for anticancer drug therapy. Apoptosis plays an important role in controlling cell proliferation in both physiological and pathological conditions [1,2]. It was well documented that upregulation of genetic pathways controlling apoptosis can inhibit growth and development of a tumor [3]. The bcl-2 family are major regulators of apoptosis [4], which can not only prevent apoptosis induced by a large variety of stimuli in many cell types [5,6] but also delay tumor progression by induction of angiogenesis [7].

Z24 [3*Z*-3-[(1*H*-pyrrol-2-yl)-methylidene]-1-(1-piperidinylmethyl)-1,3-2*H*-indol-2-one hydrochloride salt], a novel synthetic indolin-2-ketone small-molecule compound was designed by computer screening based on the 3D structure of bcl-2 protein. Studies have shown that Z24 can suppress angiogenesis in chicken chorioallantoic membrane [8]. Furthermore, Z24 was shown to induce tumor cell apoptosis and inhibit tumor angiogenesis dose-dependently in mice [9]. To study the pharmacokinetics of Z24 in blood samples, a sensitive, accurate and reproducible method using reverse-phase high-performance liquid chromatography (HPLC) and UV detection was developed and successfully applied to the pharmacokinetic study of Z24 in mice.

2. Experimental

2.1. Materials

Z24 was synthesized at the Laboratory of Drug Synthesis (Beijing Institute of Pharmacology and Toxicology), as described previously [10]. The chemical structure of Z24 was identified by nuclear magnetic resonance, mass spectrometry, and elemental analysis. Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany) and all other reagents were of an analytical grade (Fig. 1).

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

In order to avoid the isomerization of Z24, the preparations were carried out under light-protected conditions. Z24 was dissolved in methanol (0.25 mg/ml) as stock solution and aliquots for single-time use were stored at −20 °C until required. Working standards were prepared freshly by diluting the stock solution with distilled water.

Calibration standards were prepared by adding 0.1 ml of working standards at different concentrations in 0.4 ml of fresh blank blood. The final concentrations of Z24 were 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 μg/ml.

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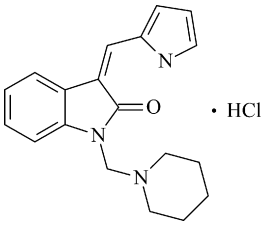

Fig. 1. Chemical structure of Z24.

The quality control samples (QCs) were prepared in duplicate from separate stock solutions. High-, mid- and low-level quality control samples contained 2, 0.5 and 0.1 μg/ml of Z24 were prepared in a manner similar to that used for the preparation of the calibration standards.

2.3. Preparation of samples

A 500-μl aliquot of whole blood was spiked with ether (2 ml × 2 ml), and the mixtures were vortexed for 30 s followed by centrifugation at 4000 rpm for 5 min at room temperature. The organic phase was removed and evaporated to dryness in a water bath at 40 °C. The residue obtained was reconstituted with 100 μl of mobile phase and 50 μl of the reconstituted sample was injected into the HPLC system.

2.4. Chromatography

The analysis of calibration standards, QCs, and samples were performed on an HPLC system, equipped with a P1000 pump, an UV2000 variable wavelength UV–vis detector (Spectra Physics, Mountain View, CA, USA). The analyte was eluted with 50% (v/v) acetonitrile/water at a flow rate of 0.8 ml/min on a reverse phase column (Hypersil ODS-2, 5 μm, 150 mm × 4.6 mm) with a C_{18} (5 μm, 10 mm × 4.6 mm) guard column, followed by specific measurement at 390 nm. Z24 was eluted at 8 min under the above conditions.

2.5. Bioanalytical method validation

2.5.1. Linearity, precision and accuracy

The linearity of the HPLC method for the determination of Z24 was evaluated by a calibration curve in the range of 0.05–6 μg/ml. The calibration curve was obtained by plotting the peak area of each analyte versus Z24 theoretical concentration. Least squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. The calibration curve required a correlation coefficient (r^2) of 0.99 or better, which was considered appropriate for a validated method. To evaluate precision, at least five QCs at each of the three different concentrations were processed and injected on a single day (intra-day) and on different days (inter-day). The variability of Z24 determination was expressed as coefficient of variation (%CV), which should be ≤15% for all concentrations. Accuracy was expressed as % bias of theoretical versus calculated concentrations, and it should be within limits of ±15% for all concentrations of Z24.

2.5.2. Recovery

The absolute recoveries of Z24 from blood were determined at different standard concentrations by spiking the drug into the corresponding fresh blank blood. The percentage of recovery was calculated by comparing the peak area of extracted samples with samples in which the same amount of compounds were diluted with mobile phase and injected directly. The recoveries at three QC concentration levels of Z24 in blood were examined at least five times.

2.6. Pharmacokinetic experiments in mice

All experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of the Beijing Institute of Pharmacology and Toxicology. Male Kunming mice weighing 18–22 g were obtained from Academy of Military Medical Sciences Animals Center (Beijing, China) and fasted 12 h before receiving Z24 and fed 4 h after administration. For oral pharmacokinetic studies, Z24 was suspended in 0.5% sodium carboxymethyl cellulose and administered by gavage (20, 40, 80 mg/10 ml/kg). Blood samples (five animals/time point) were collected via the orbital plexus into heparinized tubes at 0, 5, 10, 20, 40 min, 1, 1.5, 2–7, and 9 h after dosing. A 0.5-ml aliquot was taken from each sample and prepared immediately according to the procedure given for the calibration standards. Pharmacokinetic analysis of Z24 concentrations in blood was performed using noncompartmental methods via the proprietary drug and statistics (DAS) computer software package (Anhui Provincial Center of Drug Clinical Evaluation).

3. Results and discussion

3.1. Method development

The solid substance of Z24 is stable. Z24 can be isomerized when the solution is exposed to light. After knowing the light sensitive character of Z24, experiments were successfully performed under light-protected conditions. Initially, diverse combinations of organic phase (ether, chloroform, ethyl acetate) had been tested in the processing of Z24 samples. It was found that the ether-extraction method produced satisfactory recovery and clean chromatogram of Z24. The mobile phase containing 50% acetonitrile for the separation of Z24 showed optimum peak shape and the detector sensitivity was increased, compared with that of 70% methanol. The chromatograms of a blank blood sample, a Z24 QC sample (0.5 μg/ml) and a mouse blood sample after a 40-mg/kg dosing with Z24 are summarized in Fig. 2. Blank blood showed no peaks of interfering endogenous compounds around the retention time of Z24. In addition, the retention time of Z24 was short enough so that it is suitable for routine analysis.

3.2. Stability of Z24

In order to assure that there was no isomerization of Z24 during sample handling and preparation, the stability of Z24 was studied. Five groups of Z24 were determined using a standard Z24 solution of 1.5 μg/ml in methanol contained in ampules. Group 1: the solution was exposed to light for 7 h. Group 2: the solution was kept under light protection for 7 h. Group 3: the solution was in a water bath at 50 °C under light protection. Group 4: ampules were kept under light protection at 4 °C for 10 days. Group 5: ampules were kept under light protection at −20 °C for 10 days. The results are shown in Fig. 3, which indicates that the concentration of Z24 decreased rapidly when exposed to light during the first 10 min, and then diminished slowly. Z24 was stable for 7 h under light-protected conditions both at room temperature and in a 50 °C water bath. In a freezer at −20 °C, Z24 remained stable for at least 10 days. The stability of Z24 in blood samples was also studied under light-protected conditions. The results suggested that Z24 in blood samples was stable for at least 7 h at room temperature.

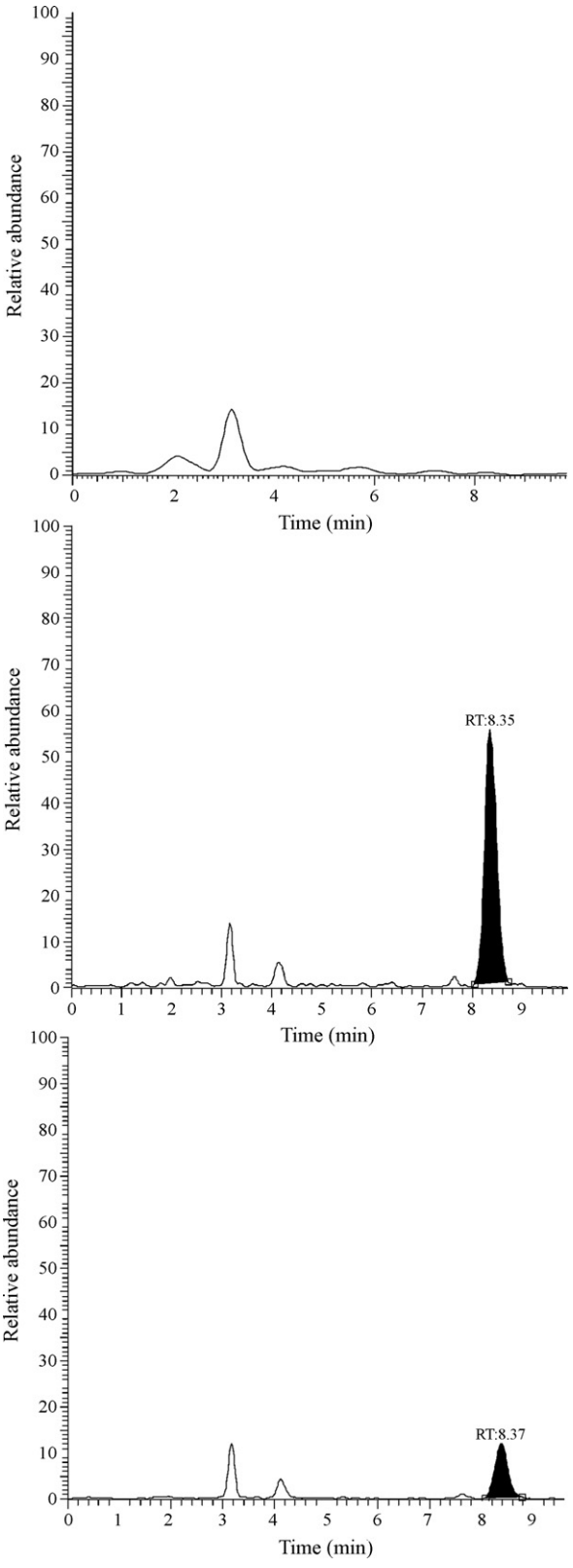

Fig. 2. Representative HPLC chromatograms: (A) blank blood sample, (B) a 0.5-μg/ml Z24 quality control sample, and (C) a mouse blood sample drawn at 4 h after a 40-mg/kg dose of Z24.

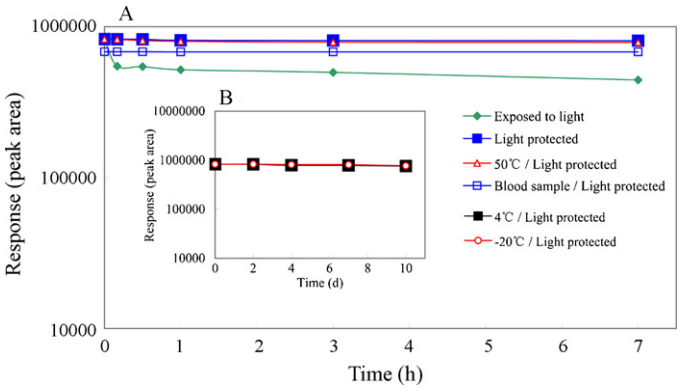

Fig. 3. The stability of Z24 in methanol and blood ((A) a 7-h experiment and (B) a 10-day experiment).

Therefore, the samples were prepared under light-protected conditions immediately after blood collection and determination of Z24 should be completed within 7 h.

3.3. Method validation

A linear relationship was found between peak areas of Z24 in blood and theoretical concentrations within the range of 0.05–6 μg/ml. The mean (±S.D.) regression equation for calibration curves in blood was $(479049.3 \pm 40928.0)C + (7235.7 \pm 66.3)$, $r^2 = 0.9976 \pm 0.0018$ ($n = 5$). The coefficient of variations of slope for Z24 were found to be <15%, which indicated a high precision of the present assay. The absolute recovery of Z24 after liquid–liquid extraction ranged from 89.2 to 108.5% and %CV of recovery was below 15% for each concentration (Table 1).

The intra- and inter-day variabilities of blood assay are listed in Table 2. It was demonstrated that the HPLC method for the determination of Z24 was reliable and reproducible since both %CV and % bias were below 15% for all estimated concentrations of Z24. The LLOQ was evaluated by analyzing blood sample spiked with the analyte at a final concentration of 0.05 μg/ml at which the signal-to-noise (S/N) ratio was 10 (Fig. 4). These results suggested that the reproducibility and recovery were acceptable over the studied concentration range.

3.4. Pharmacokinetic study of Z24 in mice

The validated assay was used to determine the blood concentration profiles of Z24 in mice after a single oral dose of 20, 40 and 80 mg/kg. The mean blood concentration versus time profiles of Z24 after single oral doses are shown in Fig. 5, and the mean pharmacokinetic parameters are summarized in Table 3. After oral administration, Z24 was absorbed rapidly and it can be detected in mice blood at 5 min postdose. The peak blood concentrations of Z24 (20, 40, 80 mg/kg) appeared at 10 min postdose, while the $t_{1/2z}$ was 1.15, 1.14 and 2.68 h, respectively. A dose proportionality study indicated that there was good correlation between AUC and dose ($AUC_{0-\infty}$ versus dose; $r^2 = 0.982$), however, the lack of linearity in C_{max} value and the significant extension of $t_{1/2z}$ with the increase

Table 1
Absolute recovery of the method for determining the concentration of Z24 in blood samples ($n = 5$)

Concentration (μg/ml)	Absolute recovery (mean ± S.D., %)	%CV
0.1	108.5 ± 0.1	5.9
0.5	103.3 ± 0.1	13.9
2.0	89.2 ± 0.1	12.2

Table 2
Intra- and Inter-day precision and accuracy of Z24 measurements in mice blood

Concentrations (μg/ml)	Intra-day			Inter-day		
	Mean ± S.D. (n = 5)	Precision (%CV)	Accuracy (% bias)	Mean ± S.D. (n = 5)	Precision (%CV)	Accuracy (% bias)
0.1	0.10 ± 0.01	10.9	−2.6	0.09 ± 0.01	10.3	−8.0
0.5	0.46 ± 0.02	3.4	−8.8	0.52 ± 0.06	12.3	4.7
2.0	2.24 ± 0.07	3.0	12.1	2.18 ± 0.12	5.7	9.0

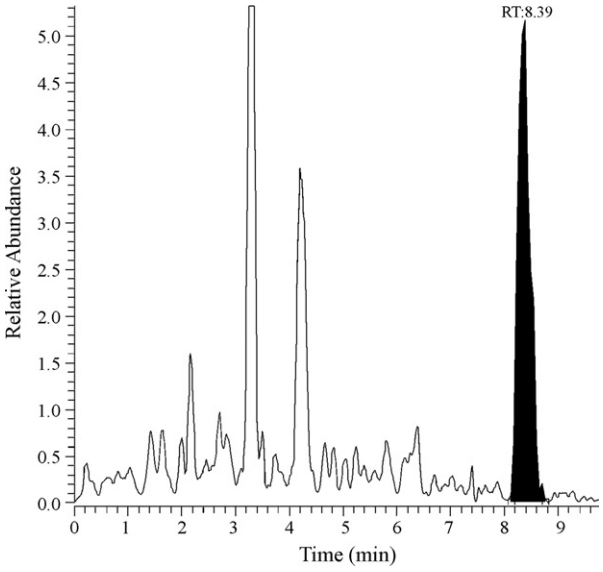

Fig. 4. The LLOQ chromatogram of Z24 (0.05 μg/ml).

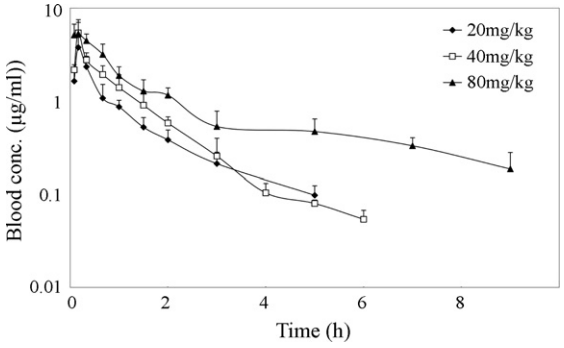

Fig. 5. Blood concentration–time profiles of Z24 after single oral doses of 20, 40 and 80 mg/kg in mice (n = 5).

Table 3
Pharmacokinetic parameters of Z24 in mice following single oral doses of 20, 40, or 80 mg/kg

Dose (mg/kg)	$AUC_{0-\infty}$ (mg h/l)	C_{max} (mg/l)	T_{max} (h)	MRT (h)	$t_{1/2z}$ (h)
N = 5					
20	2.98	3.78	0.17	1.34	1.15
40	4.21	5.42	0.17	1.25	1.14
80	8.88	5.32	0.17	3.10	2.68

of Z24 dosage suggested saturation in absorption and elimination of Z24. The results indicated a nonlinear pharmacokinetics in mice from 20 to 80 mg/kg.

4. Conclusion

In conclusion, the newly developed HPLC method provided a new, sensitive, reproducible and validated assay for the determination of Z24 in blood. This HPLC method will be used in further studies to characterize the tissue distribution of Z24.

Acknowledgements

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