

## Full-length article

# Pharmacokinetics of CPU0213, a novel endothelin receptor antagonist, after intravenous administration in mice<sup>1</sup>

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## Key words

CPU0213; high pressure liquid chromatography; mice; pharmacokinetics; acute toxicity

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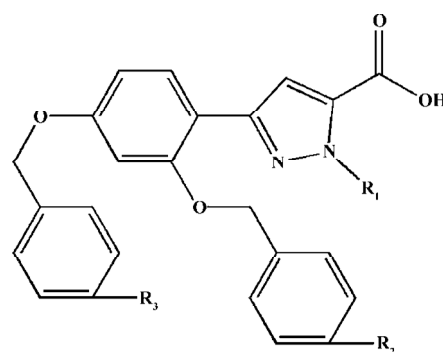
## Abstract

**Aim:** To determine the pharmacokinetics associated with acute toxic doses of CPU0213, a novel endothelin receptor antagonist in mice after a single intravenous administration. **Methods:** Concentrations in serum and the pharmacokinetic parameters of CPU0213 were assayed by high pressure liquid chromatography (HPLC) following a single intravenous bolus of CPU0213 at concentrations of 25, 50, and 100 mg/kg in mice. The intravenous acute toxicity of CPU0213 was also assessed in mice. **Results:** A simple, sensitive and selective HPLC method was developed for quantitative determination of CPU0213 in mouse serum. The concentration-time data conform to a 2-compartment model after *iv* administration of CPU0213 at concentrations of 25, 50, 100 mg/kg. The corresponding distribution half-lives ( $T_{1/2\alpha}$ ) were 3.6, 4.2, 1.1 min and the elimination half-lives ( $T_{1/2\beta}$ ) were 39.4, 70.3, 61.9 min. There was a linear increase in  $C_0$  proportional to dose, and the same as  $AUC_{0-t}$  and  $AUC_{0-\infty}$ .  $AUC_{0-t}$  and  $AUC_{0-\infty}$  were 4.511, 13.070, 23.666  $g \cdot min \cdot L^{-1}$  and 4.596, 13.679, 24.115  $g \cdot min \cdot L^{-1}$ , respectively. The intravenous  $LD_{50}$  was 315.5 mg/kg. **Conclusion:** First order rate pharmacokinetics were observed for CPU0213 within the range of doses used, and the acute toxicity of CPU0213 is mild.

## Introduction

Endothelin (ET), a 21 amino acid peptide, is the most potent known constrictor of human resistance and capacitance vessels. It is produced in many different tissues from vascular endothelium<sup>[1]</sup>, and in higher amounts from diseased myocardium, and vascular smooth muscle cells. ET-1 appears to play an important role in pathological processes underlying several cardiovascular diseases, including heart failure<sup>[2]</sup>, systemic hypertension<sup>[3]</sup>, myocardial infarction, atherosclerosis, pulmonary hypertension, renal failure<sup>[4]</sup>, and cardiovascular complications of diabetes, *etc* ET levels are elevated in both tissue and plasma<sup>[5]</sup> in various disease states. Endothelin receptor antagonists (ETRA) are currently being investigated and developed for a wide spectrum of indications<sup>[6]</sup>. Bosen-tan, the first endothelin receptor antagonist, was launched in November 2001 in the US for the treatment of pulmonary hypertension.

CPU0213 (Figure 1), synthesized by the Center of New



**Figure 1.** Chemical structures of CPU0213 and CPU0204.

CPU0213 R<sub>1</sub>: -(CH<sub>2</sub>)<sub>3</sub>CH<sub>3</sub> R<sub>2</sub>: -Cl R<sub>3</sub>: -Cl

CPU0204 R<sub>1</sub>: -(CH<sub>2</sub>)<sub>2</sub>CH<sub>3</sub> R<sub>2</sub>: -H R<sub>3</sub>: -Cl

Drug Research and Development, China Pharmaceutical University, is a new non-selective endothelin receptor antagonist that has been shown to be an effective suppressor of vasoconstriction and ET-1 binding to rat myocardial mem-

brane preparations<sup>[7]</sup>. The  $pA_2$  values for the suppression of ET-1-induced contraction of rat thoracic aorta (mediated by  $ET_A$ ) and sarafotoxin (S6c)-induced contraction of guinea pig bronchial smooth muscles ( $ET_B$  mediated) are  $8.52 \pm 0.26$  and  $7.28 \pm 0.04$ , respectively<sup>[7]</sup>, approximately 20 times the potency of bosentan<sup>[8]</sup>. CPU0213 attenuates cardiac hypertrophy<sup>[9]</sup> and chronic heart failure<sup>[10]</sup> in rats. It inhibits oxidative stress, the overexpression of matrix metalloproteinases (MMPs) and modulates the collagen content in hypertrophic myocardium induced by levothyroxine<sup>[11,12]</sup>. In hypoxia and monocrotaline (MCT)-induced pulmonary artery hypertension (PAH) model, CPU0213 markedly relieves pulmonary hypertension and right ventricular hypertrophy. The therapeutic effects of CPU0213 on vascular endothelium and kidney injury in streptozotocin (STZ)-induced diabetic rats are also notable. Therefore, it is important to investigate the pharmacokinetic behavior and obtain basic information about the pharmacokinetic characteristics of the compound. The aim of the present study is to investigate the pharmacokinetics of CPU0213 after a single iv bolus of 3 different doses of CPU0213 in relation to the acute toxic doses by iv in mice.

## Materials and methods

**Chemicals and reagents** CPU0213 (batch No: 20040501) and CPU0204 (batch No: 20040501) (Figure 1) were provided by the Center of New Drug Research and Development, China Pharmaceutical University, Nanjing, China. CPU0213 was prepared in normal saline (at concentrations of 2.5, 5.0, 10.0 g/L for the pharmacokinetics study and 27.54, 30.60, 34.00, 37.78 g/L for the acute toxicity study) containing 1% (w/w) poloxamer. Methanol was high performance liquid chromatography (HPLC) grade and all other chemicals were of analytical reagent (AR) grade.

**Animals** Kunming mice (equal numbers of males and females), weighing 18–22 g, were supplied by the Nanjing Jiangpu Animal Center (certificate No: SCXK2002-0016). The investigation conformed to the Guidelines for the Care and Use of Laboratory Animals of Jiangsu Province.

**Sample preparation** After 5 mL of dichloromethane was added to 0.1 mL serum mixed with 0.1 mL of the internal standard (10 mg/L CPU0204), the samples were vortexed for 1 min and centrifuged at  $3000 \times g$  for 10 min. The organic layer was transferred into clean tubes and evaporated at 55 °C under a gentle stream of nitrogen. Residues were dissolved in 100  $\mu$ L of the mobile phase and 20  $\mu$ L was injected into HPLC column.

**Chromatography** The HPLC system consisted of a LC-10AT VP liquid chromatograph (Shimadzu, Japan), an SPD-

10A Detector (Shimadzu, Japan). The column was a Hypersil C<sub>18</sub>, with 5  $\mu$ m particle size, and 15 cm  $\times$  4.6 mm internal diameter. The mobile phase comprised methanol and water (68:32, v/v). The chromatography assays were performed at 25 °C with a flow rate of 1 mL/min and UV detection at a wavelength of 254 nm.

Blank serum was assessed to identify any endogenous materials in the serum interfered with peaks of CPU0213 and an internal reference compound CPU0204.

Calibration curves for the assay of CPU0213 in serum were prepared by adding various amounts of CPU0213 to blank serum over the range of 2–560 mg/L (at 2, 4, 10, 20, 40, 80, 160, 320, 560 mg/L). All samples for the calibration curves were analyzed in the same manner as the plasma samples from dosed animals. The peak-area ratios of CPU0213 to the internal standard were calculated. Linear regression was carried out based on the peak-area ratios of CPU0213 to the internal standard plotted against the concentrations of CPU0213 spiked in serum.

Samples of CPU0213 (2, 40, 560 mg/L) were added to blank serum and assessed to determine the accuracy of the method according to the formula:

Recovery (%) = concentration prepared / concentration measured  $\times$  100%.

Various concentrations of CPU0213 (2, 40, 560 mg/L) were added to blank serum and the concentration of each sample was determined 5 times to arrive at an intra-day coefficient of variation (CV) during the same day and an inter-day coefficient of variation on 5 separate days [CV (%) = SD/mean  $\times$  100%].

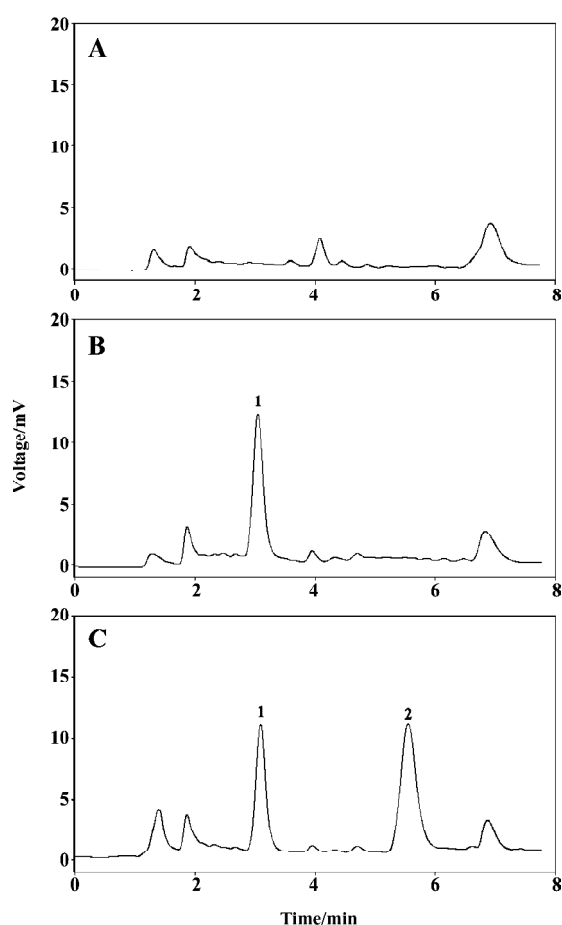
**Pharmacokinetics assay** Mice were assigned to receive CPU0213 at concentrations of 25, 50, or 100 mg/kg iv via the tail vein. At different time points (1, 5, 10, 20, 40, 60, 90, 120, 180, 240, 300, 420, and 600 min after intravenous administration), blood samples were collected from the postorbital vein plexus and centrifuged. Three male and 3 female mice were used per sampling time point in each dose group. The separated serum samples were stored at -20 °C until assay. The concentrations vs time were analyzed by using the 3P87 program (version 1.0; Chinese Society of Mathematical Pharmacology, Beijing, China) to calculate the pharmacokinetic parameters.

**Acute toxicity** The mice were divided into 4 groups of 5 males and 5 females at random, and CPU0213 was administered iv via the tail vein at doses of 275.4, 306.0, 340.0, and 377.8 mg/kg, which were carefully determined after a series of preliminary trials. Changes in the appearance and behavior, and mortality in mice were monitored for 14 d after administration. LD<sub>50</sub> values and the corresponding confidence limits

were determined by the Bliss method<sup>[13]</sup> using NDST software (version 21; Chinese Society of Mathematical Pharmacology, Beijing, China).

## Results

**Chromatography validation** In the present study, a sensitive and selective HPLC method was developed for the quantitative determination of CPU0213 in mouse serum. The retention times of CPU0213 and CPU0204 were 5.6 min and 3.1 min, respectively. There was no interference at these peaks, so these were free from endogenous interference (Figure 2).



**Figure 2.** Typical HPLC chromatograms for CPU0213 and the internal standard in mouse serum. (A) Blank serum sample; (B) blank serum sample spiked with internal standard; (C) serum sample 90 min after iv administration of 25 mg/kg CPU0213. Peaks 1 and 2 refer to the internal standard and CPU0213, respectively.

The calibration curve of the peak-area ratio (y) against the respective sample concentrations (x) was constructed.

The equation of mean linear regression ( $n=6$ ) was  $y=0.0496x+0.0925$  ( $r=0.998$ ) for the concentration range from 2 to 560 mg/L.

The range of recovery in serum at concentrations of 2, 40, and 560 mg/L was 94.2%–103.0% (Table 1).

**Table 1.** Recovery for HPLC assay of CPU0213 in mouse serum. Mean±SD.  $n=6$ .

Concentration prepared (mg/L)	Concentration measured (mg/L)	Recovery (%)
2	2.06±0.13	103.0±3.1
40	37.7±1.7	94.2±4.0
560	539±25	96.4±4.5

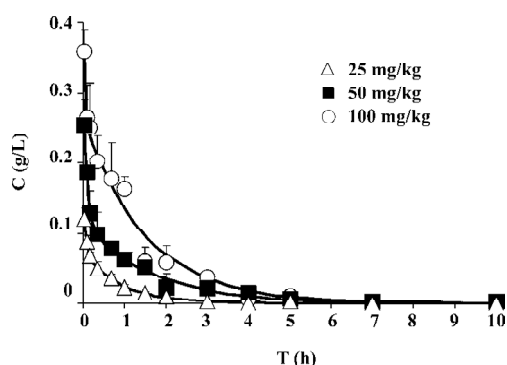
Precision was determined by 5 assays of samplings at 2, 40, and 560 mg/L. Satisfactory results were obtained for both intra-day and inter-day assays. The intra- and inter-day CV were in the range of 5.1%–5.4% and 4.9%–6.8%, respectively (Table 2).

**Table 2.** Coefficient of variation (CV) of intra- and inter-day for HPLC assay of CPU0213 in mouse serum. Mean±SD.  $n=5$ .

Concentration prepared (mg/L)	Intra-day		Inter-day	
	Concentration measured (mg/L)	CV (%)	Concentration measured (mg/L)	CV (%)
2	1.97±0.10	5.1	2.05±0.14	6.8
40	37.2±1.9	5.1	38.5±2.4	6.2
560	540±29	5.4	546±27	4.9

**Pharmacokinetics** The serum CPU0213 concentration-time data were well characterized by a 2-compartment model (Figure 3). The initial concentrations ( $C_0$ ) for the 3 doses were 0.133, 0.283, and 0.581 g/L respectively. The distribution half-lives ( $T_{1/2\alpha}$ ) and elimination half-lives ( $T_{1/2\beta}$ ) ranged from 1.1 to 4.2 min, and from 39.4 to 70.3 min, respectively. Parameters calculated according to statistical moment were also in the table.  $AUC_{0-t}$  is the area under the curve from time zero to the last time point of measurable concentration.  $AUC_{0-\infty}$  is the area under the curve from time zero to infinity. Mean residence time (MRT) was also calculated. The system serum clearance (CL) ranged from 0.00366 to 0.00544  $L \cdot min^{-1} \cdot kg^{-1}$  (Table 3).

**Acute toxicity by iv** After iv administration of the high



**Figure 3.** Serum concentration of CPU0213 versus time after iv administration of CPU0213 at concentrations of 25, 50, and 100 mg/kg in mice.  $n=6$ .

**Table 3.** Pharmacokinetic parameters of CPU0213 after intravenous administration of 25, 50, 100 mg/kg in mice ( $n=6$ ).

Parameter	Dose (mg/kg)		
	25	50	100
$C_0$ (g/L)	0.133	0.283	0.581
$T_{1/2\alpha}$ (min)	3.6	4.2	1.1
$T_{1/2\beta}$ (min)	39.4	70.3	61.9
$AUC_{0-t}$ (g·min·L <sup>-1</sup> )	4.511	13.070	23.666
$AUC_{0-\infty}$ (g·min·L <sup>-1</sup> )	4.596	13.679	24.115
MRT (min)	61.8	105.7	90.9
CL (L·min <sup>-1</sup> ·kg <sup>-1</sup> )	0.00544	0.00366	0.00414

doses of CPU0213, the mice became immediately eclamptic and their pupillae were enlarged. After 2 or 3 h, the spontaneous movements decreased and the activated muscular activity subsided. Most deaths occurred 5 min to 2 h after iv administration, and no death occurred 48h after iv administration. The mortality of mice in the 14 d after intravenous administration of CPU0213 at concentrations of 275.4, 306.0, 340.0, and 377.8 mg/kg was 0%, 50%, 70%, and 100%, respectively. The intravenous LD<sub>50</sub> was 315.5 mg/kg and the 95% confidence limits were 301.5–330.2 mg/kg.

## Discussion

Only one blood sample was obtained from each mouse. Therefore, the pharmacokinetic parameters in mice are based on mean concentration-time data. Compartment models were chosen on the basis of the Akaike Information Criterion (AIC)<sup>[14]</sup>. The R<sup>2</sup> values of the 3 dose groups for the 2-compartment model are more than 0.999, which indicates that the concentration-time data are well fitted to a 2-compartment model. Parameters calculated by statistical moment are

in agreement with those calculated by using the compartment model, providing further evidence that the 2-compartment model of the pharmacokinetic behavior is reliable.

After intravenous administration, there was a linear increase in  $C_0$  proportional to dose ( $r>0.9999$ ), the same as for the  $AUC_{0-t}$  ( $r=0.992$ ) and  $AUC_{0-\infty}$  ( $r=0.989$ ). There was no prolongation of the  $T_{1/2\beta}$  found with the large dose, and the CL of the 3 doses were similar. It is reasonable to surmise that CPU0213 adheres to first order rate pharmacokinetics, and no saturation was found at concentrations from 25 to 100 mg/kg.

CPU0213 is well tolerated in mice after iv bolus administration, and the acute toxicity is mild according to the LD<sub>50</sub>. The iv doses used in this pharmacokinetic study were 25, 50, and 100 mg/kg, which covers approximately one third of the LD<sub>50</sub>.

In the present study, we found that the  $T_{1/2\beta}$  was not sufficiently long, being approximately 1 h following iv bolus administration. It is doubtful that CPU0213 administration once per day could offer therapeutic effects for the treatment of certain disorders. However, CPU0213 has been administered orally in other experiments, including treating animal models of pulmonary hypertension and cardiovascular complications of diabetes and hyperthyroidism<sup>[9–12]</sup>. The outcomes of these experiments show that daily oral administration at doses of 25, 50, and 100 mg/kg is sufficient to prevent pathological progression of these conditions. These data on CPU0213 provide convincing evidence that oral administration once per day provides satisfactory therapeutic efficacy, despite the short  $T_{1/2\beta}$ .

The initiation of a pharmacological effect is dependent on the existence of a sufficient concentration of a drug in the plasma, and occurs when the moiety of drug is bound to the receptor site. The disappearance of a drug's effect results from the dissociation of the drug from its receptor. In general, the effective plasma level is the dominant factor controlling a drug's effect. The duration of drug effects, however, are not plasma level-dependent. As shown by a well-known example of a "hit and run" drug<sup>[15]</sup>, even if plasma levels are negligible for a long period of time, the drug effect remains unless new enzymes are synthesized. Anticancer drugs are another group of drugs for which initiation is dependent on the effective concentration, but the duration of drug effect is not correlated with the plasma concentration of the drugs. We previously discovered that the duration of the pharmacological effect of bepridil is longer than that of the plasma concentrations of the drug<sup>[16]</sup>. This suggests that despite the  $T_{1/2\beta}$  of CPU0213 being short, effective therapy can still be achieved with a single daily oral dose. It is possible that

a counter-clockwise hysteresis loop<sup>[17,18]</sup> is involved in the effect–plasma level relationship, which would explain the prolonged effect against a relatively short half-life.

In conclusion, the first order rate pharmacokinetics and mild acute toxicity were observed for CPU0213. The drug effect is likely to be sustained after disappearance of the drug in the plasma, and this property should be investigated further.

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