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Pharmacokinetics of cyadox and its main metabolites in rats, pigs, chickens, and carps following oral administration at three dosage

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ABSTRACT

Cyadox (CYA), a 1,4-dioxide quinoxaline, is a safe and effective antibacterial agent with potential use in food-producing animals. The aim of this study was to compare the pharmacokinetics of CYA (Cy0) and its main metabolites [bisdeoxycyadox (Cy1), 4-desoxycyadox (Cy2), N-(quinoxaline-2-methyl)-cyanide acetyl hydrazine (Cy4), quinoxaline-2-carboxylic acid (Cy6), and 2-hydromethyl-3-hydroxy-quinoxaline (Cy12)] after oral administration at three dosages in pigs, chickens, carps, and rats. The concentration vs. time profile in plasma after single oral administration indicated that CYA was rapidly dissociated into its metabolites and showed the widest distribution in all animals, with the highest apparent volume of distribution. Cy0 and Cy6 persisted for the longest time at lower concentration. Cy1and Cy4 concentration was the highest in pig and rat plasma, while Cy1 was undetectable in chickens, and Cy4 was undetectable in carps following administration at three dosages. Different dosage of the CYX and its metabolites had no significant effect on wash-out period. This study revealed obvious species-specific differences in the kinetic behavior of CYA and its metabolites, which may be related to clinical efficacy and toxicity. Our results would facilitate the judicious use of CYA in different animals.

1. Introduction

Quinoxaline 1, 4-di-N-oxides (QdNOs) are known as excellent antibacterial agents. Olaquindox and carbadox are important members of the QdNOs and were widely used as veterinary medicines in animals for decades (Wang et al., 2015a). Mequindox, another member of this class, is an effective therapeutic drug for piglet diarrhea and has been used in pigs and chickens in China since the 1980s (Ihsan et al., 2010, 2013; Huang et al., 2015a). However, the use of these agents in food-producing animals had been banned or limited in many countries owing to their potential toxicity. Cyadox (CYA, Fig. 1), 2-formylquinoxaline-N1, N4-dioxide cyanoacetylhydrazone, is also a synthetic quinoxaline derivative, and previous studies have found that CYA is a safer and effective antibacterial agent of QdNOs with much lower toxicity in animals than other members of this class (Wang et al., 2015b; Liu et al., 2017; Fang et al., 2006; He et al., 2006). Therefore, it has been regarded as a potential replacement of olaquindox and carbadox, and many researchers predicted that CYA has the prospect of being used in livestock and poultry breeding (Ding et al., 2006a, 2006b; Cheng et al., 2016).

Knowledge on the pharmacokinetic properties of a parent drug and its important metabolites is necessary for comprehensive evaluation of the corresponding kinetic processes. During the drug development process, failures are often attributed to the adverse pharmacokinetic properties of the tested compounds. Comparative pharmacokinetic studies help to explain differences in absorption and disposition processes that may cause species variations in animal responses to fixed dosages of a drug (Baggot, 1992; Lin and Lu, 1997). Some studies (Zhao et al., 2013; Li et al., 2013; Sattar et al., 2016; Harnud et al., 2018;

Abbreviations: CYA, cyadox; HPLC, high performance liquid chromatography; DMSO, dimethyl sulfoxide; SPE, solid phase extraction column; HLB, hydrophilic-lipophilic-balanced column; LOD, limit of detection; LOQ, limit of quantification.

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Huang et al., 2018) have described the pharmacokinetics and elimination characteristics of CYA and its main metabolites in swine, chicken, and beagle dog following administration via different routes. However, these studies mainly focused on the pharmacokinetics and elimination characteristics of CYA and few of its known metabolites, namely 1,4-bis-desoxycyadox, quinoxaline-2-carboxylic acid, and 4-desoxycyadox, at same dosage. Moreover, a comprehensive evaluation of CYA and its important metabolites at different dosages in different animals has not been conducted. Therefore, further complete comparative pharmacokinetic studies are necessary to scientifically evaluate the rational use of CYA in food-producing animals.

In vitro and *in vivo* experiments have revealed that CYA could be extensively metabolized in rats, pigs, chicken, and carps (Liu et al., 2009; Zhao et al., 2013; Huang et al., 2015b). The metabolism process involves N–O group reduction, C═N cleavage, hydrogenation, and hydrolysis on the side chain, resulting in a total of 15 metabolites following a single oral gavage of [^{3}H]-CYA (Huang et al., 2015b). Zheng et al. showed that CYA can be metabolized through both enzymatic and non-enzymatic pathways in both the liver and gastrointestinal tract (Zheng et al., 2011). Among the 11 metabolites found in plasma, the most common metabolites were 1,4-bisdesoxycyadox (Cy1), 4-desoxycyadox (Cy2), *N′*-(quinoxaline-2-methyl)-cyanide acetyl hydrozine (Cy4), quinoxaline-2-carboxylic acid (Cy6), and 2-methylol-1-oxide (Cy12) (Fig. 1). Furthermore, different analytical techniques have been employed for the qualitative and quantitative determination of CYA and its main metabolites in various biological specimens (Huang et al., 2008, 2018; Wu et al., 2012; Zhao et al., 2013). However, few of these reported methods were optimized to simultaneously quantify a mixture of CYA and its metabolites in plasma. Some studies have reported the metabolism, pharmacological effects, and residue depletion of the metabolites of CYA (Qiu et al., 2012; Li et al., 2013; Yang et al., 2015; Wu et al., 2012; Huang et al., 2015a), revealing that certain metabolites have a potential toxic effects and should be considered together with the parent drug for food safety concerns. Pharmacokinetic parameters are used to predict drug residues in food-producing animals. Hence, the pharmacokinetic profile of the main metabolites of CYA should also be considered in determining the pharmacokinetic parameters of CYA in animals.

The present study investigated the pharmacokinetics of CYA and its metabolites (Cy1, Cy2, Cy4, Cy6, and Cy12) in rats, pigs, chickens, and carps after oral administration at three different dosages for thorough and comprehensive evaluation of the kinetic processes of CYA and its main metabolites. The findings of this study will provide knowledge on the effect of dose and species on the pharmacokinetics of CYA and its metabolites. Such data will be essential to support the judicious use of CYA as an antibacterial agent in food-producing animals.

2. Material and methods

2.1. Chemicals and reagents

CYA (Cy0; 99.8%, for plasma drug concentration analysis; ≧99.0%, for drug administration), 1,4-bisdesoxycyadox (Cy1, 99.5%), 4-desoxycyadox (Cy2, 99.3%), *N′*-(quinoxaline-2-methyl)-cyanide acetyl hydrozine (Cy4, 99.2%), quinoxaline-2-carboxylic acid (Cy6, 99.6%), and 2-methylol-1-oxide (Cy12, 99.5%) were synthesized by the Institute of Veterinary Pharmaceuticals (Wuhan, China). HPLC-grade methanol, acetonitrile, and formic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Purified water was produced using the Milli-Q water purification system from Millipore, Inc. (Bedford, MA, USA). All other reagents used in this study were of analytical grade and purchased in China.

Compounds were dissolved in DMSO to obtain standard solutions (1000 mg/L). Working solutions were prepared via dilution in methanol (all 10 mg/L, except for Cy4: 20 mg/L). All stock solutions were prepared in brown glass vials, stored in darkness at −20 °C, and tested weekly for investigation of stability. The stock solutions were shown to be stable for 3 months, with a slight change of 1.34% in content.

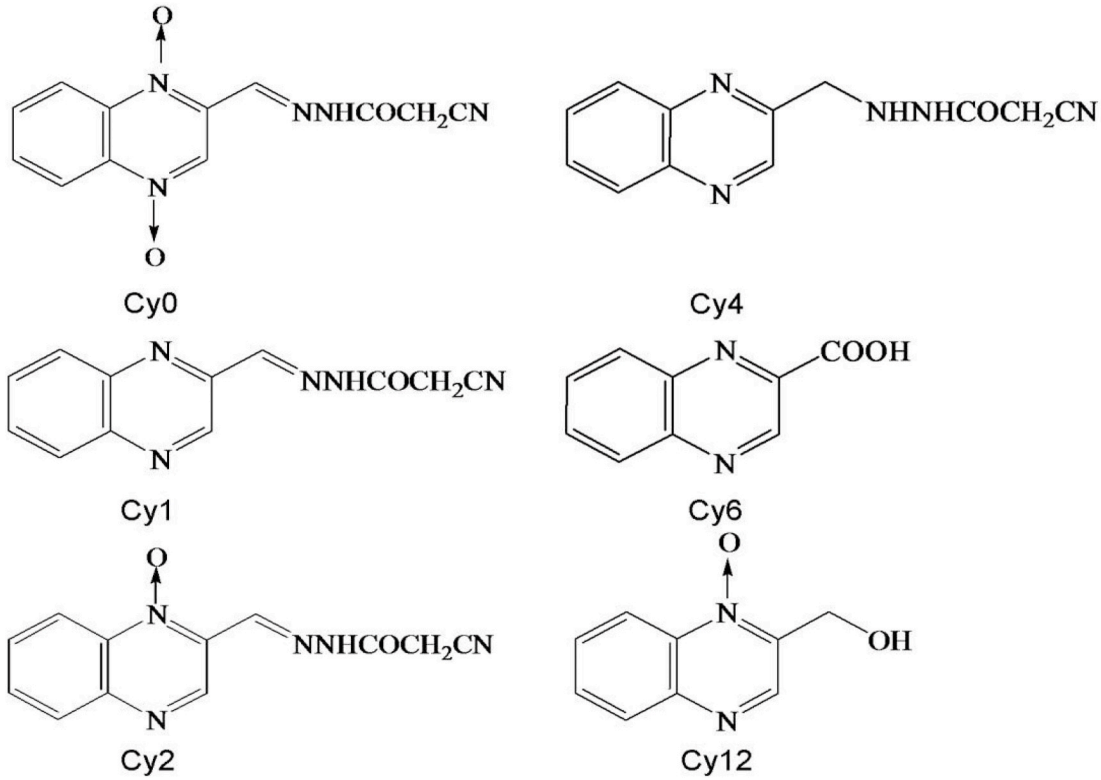

Fig. 1. Molecular structures of cyadox and its 5 main metabolites.

2.2. Animals

Male and female Wistar rats (weight 225 ± 18 g) were purchased from Hubei Experimental Animal Research Center (Wuhan, China). Healthy castrated cross-bread (Duroc × Landrace × Large White) pigs (weight 34.6 ± 5.5 kg) were purchased from the China Breeding Pigs Test Center (Wuhan, China). Male and female Avian chickens (weight 2.0 ± 0.2 kg) were purchased from Wuhan Chai Tai Co., Ltd. (Wuhan, China). Healthy carp (weight 450 ± 30 g) were purchased from Aquatic Base in Huazhong Agricultural University (Wuhan, China). The animals were fed a basal diet without antimicrobial agents/compounds and acclimatized for 1 week before the experiment, and were maintained under standard environmental conditions using the routine methods of animal husbandry and aquaculture. Rats, pigs, and chickens were housed in a temperature-controlled room (20 ± 2 °C) with a 12-h light/dark cycle, and maintained at a relative humidity of 40–70%. Carps were held in the same temperature-controlled room (20 ± 2 °C), and water-quality parameters were maintained at total ammonia nitrogen of no more than 0.8 mg/L, nitrite nitrogen of no more than 0.04 mg/L, pH 7.8–8.0, and 8–10 mL/L dissolved oxygen. Throughout the study period, feed was withheld approximately 12 h before to 4 h after drug administration, whereas water was available *ad libitum*. The experimental procedures involving animals in this study were approved by the Animal Care Center, Hubei Academy of Medical Sciences (Wuhan, China). All the *in vivo* experiments complied with the policy on the care and use of laboratory animals of the National Institutes of Health.

2.3. Dosing and sampling

A randomized parallel design was used for the study. A total of 90 rats, 24 pigs, 30 chickens, and 180 carps were divided into three groups on average. According to the recommended dosage, CYA was administered p. o. via gavage at dosages of 100, 300, and 500 mg/kg bw for rats; 10, 40, and 100 mg/kg bw for pigs; 50, 100, and 200 mg/kg bw for chickens; and 10, 20, and 40 mg/kg bw for carps. Next, 0.5 mL of blood samples were collected from the retinal vein of the rats via venipuncture into tubes containing heparin before drug administration and at 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 18 h, 24 h, and 36 h after p. o. administration. For pigs, 3–4 mL of blood samples were collected from the superior vena cava via venipuncture into tubes containing heparin before drug application and at 10 min, 30 min, 1 h, 2 h, 4 h, 5 h, 6 h, 7 h, 8 h, 10 h, 12 h, 16 h, 24 h, 36 h, and 48 h after p. o. administration. For chickens, 2 mL of blood samples were collected from the brachial vein into tubes containing heparin before drug administration and at 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, and 6 h after p. o. administration. For carps, 3 mL of blood samples were collected from the tail vein into tubes containing heparin before drug administration and at 15 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 16 h, and 24 h after p. o. administration. Six parallel data at different time points were obtained. All blood samples were centrifuged at 3000 r/min for 10 min at room temperature (25 °C). The separated plasma samples were kept at −20 °C until HPLC analysis.

2.4. Sample preparation

Briefly, 1.0 mL of pig, chicken, or carp plasma, or 0.2 mL of rat plasma was placed in a 10-mL polypropylene centrifuge tube, and 200 μL 2% (v/v) metaphosphoric acid and 2 mL methanol were added to the tube. The mixture was vortex-mixed for 2 min and centrifuged for 10 min at 15000×*g*. The supernatants were collected into another 50-mL polypropylene centrifuge tube and diluted five times. Next, the pH of the supernatant was adjusted to 4 or 5, and the mixture was vortex-mixed for 10 s. Final clean-up of the extracts was performed by offline SPE on an Oasis HLB cartridge. The SPE column [mixed mode anion-exchange columns–Oasis HLB sorbent (60 mg, 3 mL; Milford, MA, USA)] was pre-conditioned with methanol (3 mL) and water (3 mL). All flow rates for conditioning and washing were set at 3 mL/min. The entire extracts were loaded onto the SPE column at a flow rate of 1 mL/min. The column was washed with 5% (v/v) methanol (3 mL) and methanol (3 mL), and then dried by purging nitrogen at a rate of 10 mL/min. Next, 500 μL of the initial mobile phase was added to dissolve the residues, and the tube was vortex-mixed for 30 s. The solution was then transferred to a tapered micro-vial for analysis.

For HPLC analysis, 0.2 mL of rat plasma was added into a 1.5-mL polypropylene centrifuge tube and deproteinized by vortex-mixing each sample with MeOH (0.1 mL) and acetonitrile (0.1 mL) for at least 2 min. The samples were then centrifuged (15000×*g*, 4 °C, 10 min), and the supernatant was collected.

2.5. HPLC conditions

The HPLC system consisted of a Waters 2695 separations module and 2487 dual λ absorbance detector (Waters Co., Ltd, USA). A ZORBAX SB-C18 column (250 mm × 4.6 mm I.D., 5 μm; Agilent Technology, USA) was used for separating CYA and its metabolites. The operating temperature of the column was set at 30 °C. The injection volume was 40 μL. The mobile phase consisted of A (water/acetic acid, 100/0.5, v/v) and B (acetonitrile), and the flow rate was 1 mL/min. The UV detector was operated at a wavelength of 320 nm. The gradient elution program of the mobile phases for HPLC analysis is shown in Table 1.

2.6. Validation of the analytical method

Method validation followed the guidance for bioanalytical method validation from the US Food and Drug Administration (FDA, 2018). Selectivity was assessed by comparing the plasma samples spiked with Cy0 and internal standard with blank samples.

Standard calibration curves were obtained by plotting concentration (μg/L) against peak area. For calibration, 1.0 mL blank pig, chicken, or carp plasma, or 0.2 mL blank rat plasma, was spiked with 20 μL of a series of diluted working standard solutions of CYA and its metabolites and then analyzed as above. Linearity was evaluated over the range of 0.01, 0.02, 0.04, 0.16, 0.32, 0.64, and 1.28 μg mL^{-1} (except for Cy4: 0.02, 0.04, 0.16, 0.32, 0.64, 1.28, and 2.56 μg mL^{-1}). The limit of quantification (LOQ) was investigated by analyzing five replicates of the lowest concentration of the analyte with an acceptable accuracy and precision (signal-to-noise ratio >3), and the limit of detection (LOD) was determined as a concentration with a signal-to-noise ratio >10.

Precision and accuracy (intra- and inter-day) were assessed via quantification of five replicates prepared at low (2.0 μg mL^{-1}), medium (10.0 μg mL^{-1}), and high (50.0 μg mL^{-1}) concentrations of each analyte. Method precision and accuracy were both expressed as coefficient of variation (CV%). Recovery was determined by comparing the peak area ratio of extracted quality control samples with that of standard samples at the same concentrations. Stability study evaluated three replicates of

Table 1
The gradient elution program of the mobile phases for HPLC analysis.

Animals	Time (min)	mobile phase	
		A	B
Rat	0–4	86%	14%
	5–26	86%–70%	14%–30%
	26.01–30	86%	14%
Pig	0–5	86%	14%
	5–21	86%–70%,	14%–30%
	21.01–25	86%	14%
Chicken	0–5	85%	15%
	5–26	85%–75%,	15%–25%
	26.01–25	85%	15%
Carp	0–4	86%	14%
	4–26	86%–75%	14%–25%
	26.01–30	86%	14%

quality control samples (2.0, 10.0 and 50.0 μg mL^{-1}) at room temperature or in an autosampler, and after three successive freeze and thaw cycles. CYA and its metabolites were identified by matching their retention time and spectral characteristics examined by the Photodiode Array Detector (PDA) against those of standards.

2.7. Calculation of pharmacokinetic parameters and statistical analysis

Plasma concentration-time data of CYA and its metabolites were analyzed using a non-compartmental model based on statistical moment theory (Cutler, 1978; Yamaoka et al., 1978; Devane, 2010). A commercially available software program (Winnonlin 6.1; Pharsight Corporation, CA, USA) was used to estimate the pharmacokinetic parameters. The maximum plasma concentration (C_{max}) and the time to reach this concentration (T_{max}) were taken directly from the plasma concentration-time profiles. The area under the concentration-time curve ($AUC_{0-\infty}$) and the area under the first moment curve ($AUMC_{0-\infty}$) were calculated using the linear/logarithmic trapezoidal rule up to the last determined concentration, and were extrapolated to infinity (Devane, 2010). The first order rate constant associated with the terminal (log-linear) portion of the curve (lz) was estimated using linear regression of the terminal log-linear portion of the plasma concentration-time profile, and the terminal half-life ($T_{1/2}$) was calculated as ln2/lz. The mean residence time (MRT) was determined as the ratio of $AUMC_{0-\infty}$ to $AUC_{0-\infty}$, and the apparent total body clearance (CL) was determined by dividing the administered CYA dose by the $AUC_{0-\infty}$. All data are expressed as mean ± standard error of the mean (SE).

Statistical analysis was used to evaluate the differences in pharmacokinetics parameters between the same metabolite in the plasma of the same animal at different doses and between different metabolites in the plasma of the same animal at the same doses. The results were statistically analyzed using IBM SPSS Statistics 25. All tests were performed using Student's t-test, with $p < 0.05$ considered as statistically significant.

3. Results

3.1. Method validation

The method developed in this study was selective for the substance analyzed, and no endogenous interference was observed on the chromatograms (shown in Figs. 2 and 3). The calibration curve of CYA and its metabolites was in the range of 0.01–1.28 (except for Cy4: 0.02–2.56) μg mL^{-1} ($r \geq 0.9992$) in the plasma of four different animals, showing excellent linearity (Table 2). The LOQ was 0.02 μg mL^{-1} for CYA and its metabolites (except for Cy4: 0.04 μg mL^{-1}) (S/N > 10) in pig, chicken, and carp plasma, and 0.08 μg mL^{-1} in rat plasma. The inter-day precision expressed as the percentage of CV of the compounds was lower than 10% at all tested concentrations. It should be noted that variations of lower than 15% in inter-day precision are acceptable (Causon, 1997). The recoveries of CYA and its metabolites from different plasma samples were between 72% and 91% (Table 3).

3.2. Pharmacokinetics of CYA and its metabolites

3.2.1. Rat

Cy0, Cy1, Cy4, and Cy6 were detected in rat plasma. Cy2 and Cy12 were not detected or was detected at below the LOQ in rat plasma at any sampling time point after dosing. The concentrations of the compounds in rat plasma were below the LOQ within 36 h after oral dosing. The plasma concentration of the compounds reached the C_{max} within 8 h after administration on average and then progressively declined over time (Fig. 4). There was no dose-dependent increase in the $T_{1/2}$, T_{max} and MRT between same compounds at different dosages ($0.05 < p$) except for T_{max} of the Cy0 that significant difference at high dosage ($p < 0.05$). The $T_{1/2}$ of Cy6 was 8.50, 8.43, and 8.73 h after administration at 100, 300, and 500 mg/kg bw, respectively, and was longer than that of Cy1 and Cy4. There was a dose-dependent increase in $AUC_{(0\sim\infty)}$ between different dosages of the Cy1, and the $AUC_{(0\sim48h)}$ and $AUC_{(0\sim\infty)}$ of the Cy1 and Cy4 were much larger than that of their parent drug (Table 4). These results showed that most of Cy0 was rapidly transformed into its metabolites after p. o. administration, and that the metabolites may play an important role from a residue perspective.

3.2.2. Pig

Cy0, Cy1, Cy2, Cy4, and Cy6 were detected in pig plasma at dosages of 40 and 100 mg/kg bw, whereas Cy0, Cy1, and Cy2 were not detected at a dosage of 10 mg/kg bw. The peak plasma concentrations of the parent drug, 0.036 and 0.065 μg mL^{-1}, were observed at 2 h after dosing at 40 and 100 mg/kg bw, respectively. In contrast, the peak plasma concentrations of Cy1 were observed at 6, 7, and 6 h, and those of Cy4 were observed at 7, 10, and 10 h after dosing at 10, 40, and 100 mg/kg bw, respectively (Fig. 4). $T_{1/2}$ and MRT of the all five compounds was no dose-dependent characteristic after p. o. administration at 10, 40, and 100 mg kg-1 bw, and there were no significant differences between all five compounds at different dosages ($p < 0.05$). The $AUC_{(0\sim48h)}$ and $AUC_{(0\sim\infty)}$ of the Cy4 was larger than that of the parent drug (Cy0), Cy1 and Cy2 at same dosage ($p < 0.05$) (Table 5) indicate that Cy4 is widely distributed in pigs.

3.2.3. Chicken

Cy0 and Cy6 were detected in chicken plasma after dosing. After oral dosing at 50 and 100 mg/kg bw, Cy0 concentrations in chicken plasma were below the LOQ (0.02 μg mL^{-1}). Cy6 concentrations in plasma were below the LOQ (0.02 μg mL^{-1}) at 4 h after oral administration at all three level dosages (Fig. 4). In plasma, the C_{max} of Cy6 was calculated to

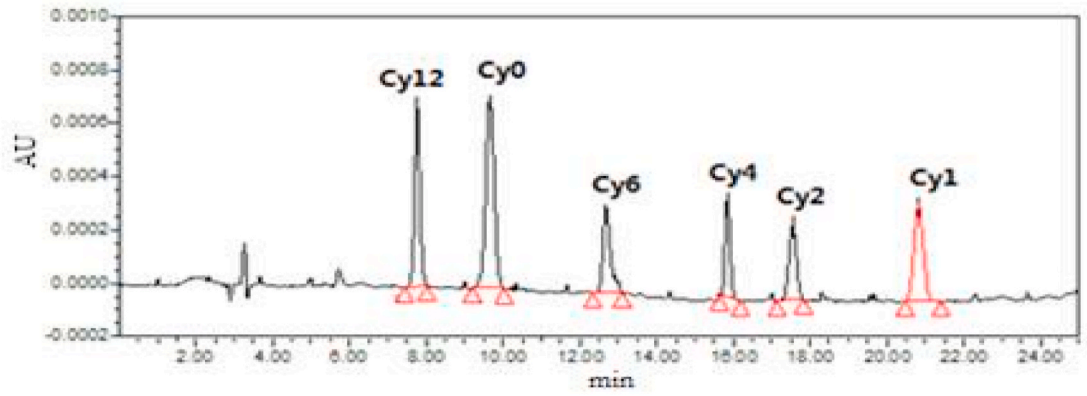

Fig. 2. HPLC chromatograms of standard solutions of 6 analytes at the concentration of 0.04 μg/mL.

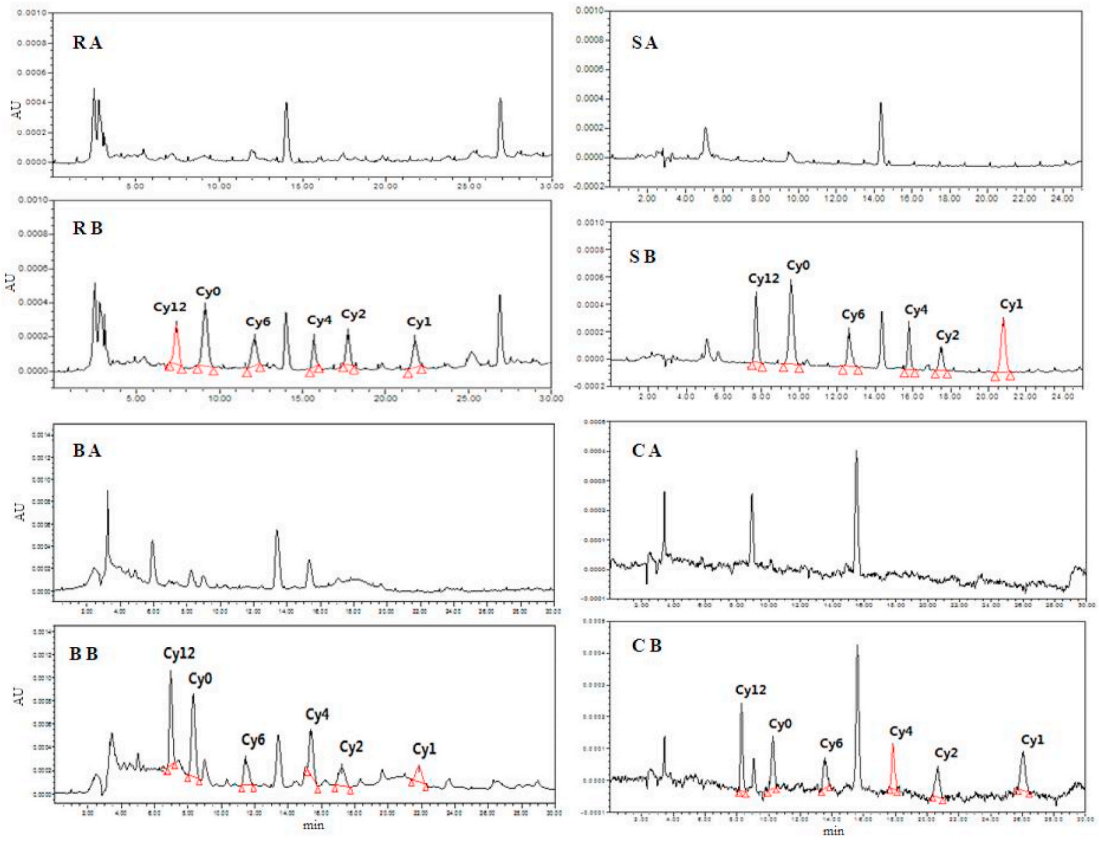

Fig. 3. HPLC chromatograms of blank plasma and spiked plasma of rat (RA, RB), pig (SA, SB), chicken (BA, BB) and carp (CA, CB) with CYA (Cy0) and its 5 major metabolites at the concentration of 0.02 μg mL^{-1} (rat 0.05 μg mL^{-1}).

Table 2
Standard curve of cyadox and its metabolites.

Compounds	linear equation	Correlation Coefficient (r)	Linearity (μg/mL)
Cy0	y = 304718x - 1575	1.0000	0.01–1.28
Cy1	y = 181532x - 738.11	0.9999	0.01–1.28
Cy2	y = 127464x - 156.01	0.9998	0.01–1.28
Cy4	y = 118964x - 1061.8	0.9998	0.02–2.56
Cy6	y = 85220x - 69.812	0.9992	0.01–1.28
Cy12	y = 105220x - 495.81	0.9994	0.01–1.28

Table 3
Recovery and inter-day RSD of Cyadox and its metabolites in pig plasma (%).

Compounds	20 μg/L		100 μg/L		500 μg/L	
	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD
Cy0	81.2 ± 4.4	5.47	80.9 ± 3.4	4.16	81.2 ± 3.5	4.33
Cy1	85.3 ± 4.8	5.64	84.3 ± 2.7	3.17	82.9 ± 3.5	4.23
Cy2	74.0 ± 2.9	3.95	73.8 ± 3.3	4.53	76.0 ± 4.7	6.20
Cy4	81.1 ± 5.6	6.95	78.3 ± 3.7	4.76	84.2 ± 4.5	5.34
Cy6	84.5 ± 5.7	6.78	87.2 ± 3.5	4.04	83.5 ± 4.6	5.54
Cy12	72.9 ± 4.7	5.64	81.6 ± 4.8	5.83	86.1 ± 4.7	5.47

be 0.031 ± 0.002 μg mL^{-1} at 1.25 ± 0.26 h and 0.037 ± 0.004 μg mL^{-1} at 1.25 ± 0.26 h after p. o. administration. At 4 h after oral administration at 200 mg/kg bw, Cy0 concentrations in plasma were all below the LOQ (0.02 μg mL^{-1}). In plasma, the C_{max} of Cy0 was calculated to be 0.034 ± 0.003 μg mL^{-1} at 0.78 ± 0.14 h after p. o. administration. No significant difference between the pharmacokinetic parameters of Cy0 and Cy6 was observed after p. o. administration at three level dosages except V_F and Cl_F of the Cy6 at high dosage. It was indicate that the elimination of CY6 is relatively slow at high dosage (Table 6).

3.2.4. Carp

Cy0 (at dosage of 40 mg/kg bw only), Cy1 (at dosages of 20 and 40 mg/kg bw), and Cy6 were detected in carp plasma (Fig. 4). After administration at 10 mg/kg bw, only one compound (Cy6) was detected in plasma. Cy6 concentrations in plasma were below the LOQ (0.02 μg mL^{-1}) at 16 h after oral dosing at all three level dosages. In plasma, the C_{max} of Cy6 was calculated to be 0.042 ± 0.003 μg mL^{-1} at 1.25 ± 0.26 h after p. o. administration. After administration at 20 mg/kg bw, two compounds (Cy1 and Cy6) were detected in plasma. The concentrations of Cy1 and Cy6 in plasma were all below the LOQ (0.02 μg mL^{-1}) at 16 h after oral dosing. In plasma, the C_{max} of Cy1 and Cy6 was calculated to be 0.051 ± 0.006 μg mL^{-1} at 5.20 ± 1.09 h and 0.050 ± 0.004 μg mL^{-1} at 4.80 ± 1.10 h after p. o. administration, respectively. At 16 h after oral dosing at 40 mg/kg bw, Cy0 concentrations in carp plasma were all below the LOQ (0.02 μg mL^{-1}). In plasma, the C_{max} of CYA was calculated to be 0.063 ± 0.006 μg mL^{-1} at 3.20 ± 1.10 h after p. o. administration. The concentrations of Cy1 and Cy6 in plasma were all below the LOQ (0.02 μg mL^{-1}) at 16 and 24 h after oral dosing. In plasma, the C_{max} of Cy1 and Cy6 was calculated to be 0.069 ± 0.004 μg mL-1 at 4.80 ± 1.10 h and 0.061 ± 0.007 μg mL-1 at 5.60 ± 0.89 h after p. o. administration, respectively. After administration at different dosages, T1/2 and Tmax were no significant differences between two compounds at different dosages ($p < 0.05$), it was indicate that the Cy1 and Cy6 were no dose-dependent characteristic after p. o. administration at 10, 40, and 100 mg/kg bw. There was significant difference in $T_{1/2}$ and MRT between Cy1 and Cy6, except T1/2 between Cy0 and Cy1 at high dosage

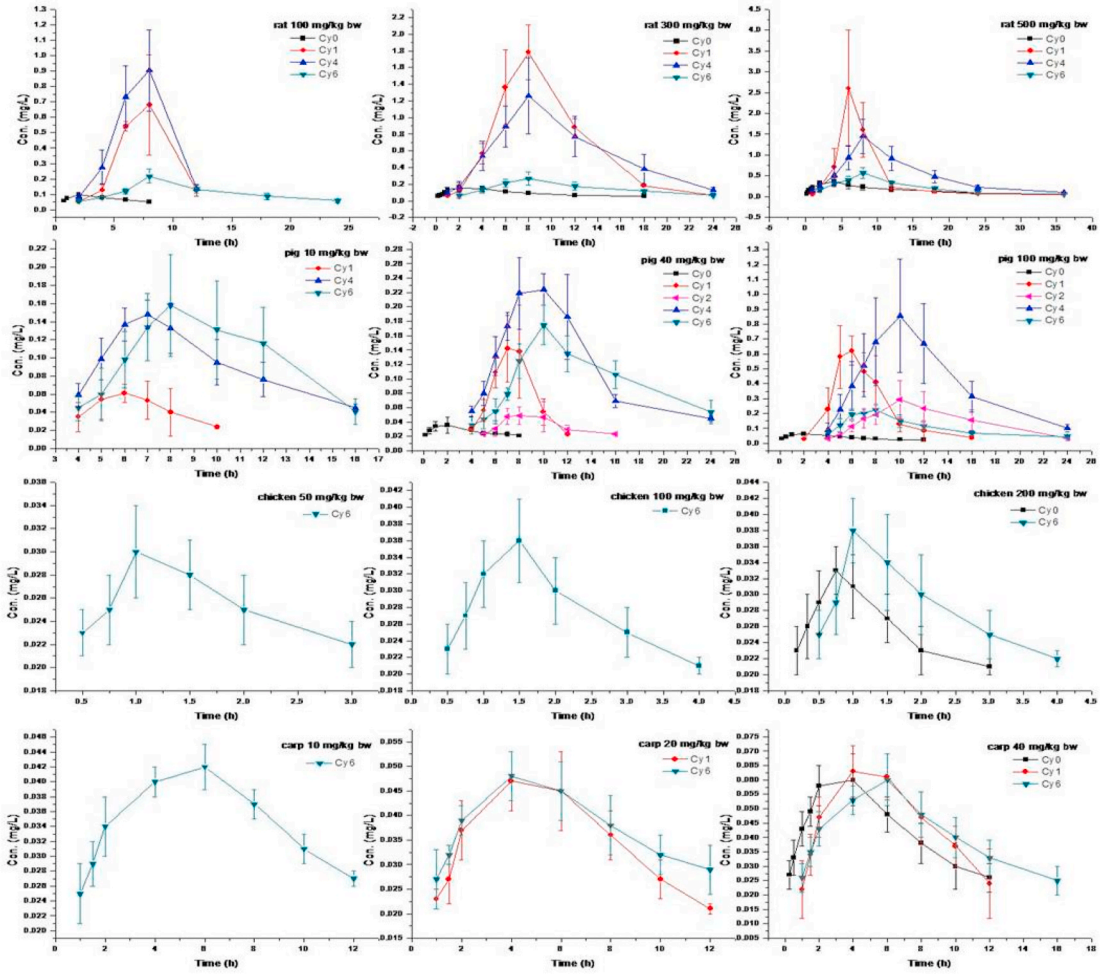

Fig. 4. Mean plasma concentration-time curves of cyadox and its metabolites after a single oral administration of cyadox in rat (100 mg/kg bw, 300 mg/kg bw and 500 mg/kg bw; n = 6), pig (10 mg/kg bw, 40 mg/kg bw and 100 mg/kg bw; n = 8), chicken (50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw; n = 10) and carp (10 mg/kg bw, 20 mg/kg bw and 40 mg/kg bw; n = 5).

(Table 7).

4. Discussion

In view of the potential to be used in food producing animals of CYA that due to its relatively low toxicity and strong antibacterial effects, the present study comprehensively investigated the pharmacokinetic characteristics of CYA through a comparative study of the pharmacokinetics of CYA and its metabolites in animals, which will offer useful scientific knowledge on the effect of CYA intake via diet. For this purpose, an HPLC method was developed and validated for simultaneous determination of CYA and its five metabolites (Cy1, Cy2, Cy4, Cy6, and Cy12) in plasma. This method achieved proper separation of analytes. The established method was subsequently employed in comparative pharmacokinetic study of CYA and its metabolites after p. o. administration in rats, pigs, chickens, and carps at different dosages.

A viable drug candidate should be absorbed into the blood stream, remain there for a sufficient time to exert its efficacy, and then be eliminated without producing any toxic effect. Each drug class has its own unique pharmacokinetic characteristics (Singh, 2006). In a previous study (Huang et al., 2015a), 5 (Cy1, Cy4, Cy5, Cy6, and Cy11), 11 (Cy1, Cy2, Cy3, Cy4, Cy5, Cy6, Cy9, Cy11, Cy12, Cy13, and Cy14), 4 (Cy2, Cy4, Cy5, and Cy6), and 3 (Cy1, Cy2, and Cy5) metabolites were observed in the plasma of rats, pigs, chickens, and carps, respectively, after a single oral dose of CYA. The result showed that no parent drug (Cy0) was detectable in the plasma of four animals at 6 h after administration. However, according to the present study, Cy0 was detectable in rat plasma for 0.17–8 h, 0.17–18 h, and 0.17–36 h following administration at three different dosages. Moreover, after administration at the high dosage, Cy0 persisted for 3–12 h in the plasma of the other species. In contrast, after CYA administration at the low dose, Cy1 and Cy4 were undetectable in rat plasma, Cy2 was undetectable in pig plasma, and Cy1 was undetectable in carp plasma (Fig. 4). These results suggested that CYA can be absorbed into the bloodstream, and that CYA dose and species may affect the formation, concentration, and elimination of its metabolites in plasma.

Pharmacokinetic parameters can provide information about the appropriate dosage regimen of antibacterial drugs (Theuretzbacher, 2012). In preclinical studies, toxicologists also face a difficult dilemma when attempting to extrapolate the observed toxicity of high doses to the safety of low doses. The underlying difficulty is that the kinetic behavior may be dose-dependent, resulting in a greater-than- or less-than-dose-proportional response in AUC, with unpredictable toxicologic consequences. Thus, an understanding of the effect of dose on drug pharmacokinetics is important in the evaluation of the efficacy and toxicity of new drugs (Lin, 1994). According to the recommended clinical dose of CYA in our laboratory, the 300, 40, 100, and 20 mg/kg bw doses were selected as the medium dose for rats, pigs, chickens, and carps, respectively. The low and high doses used in this study were within two- or four-times lower or higher than the medium dose according to the dose level spacing suggested by the Organization for Economic Cooperation and Development (OECD) Guideline 453 and the

Table 4
Mean plasma pharmacokinetic parameters of cyadox and its main metabolites in rat (n = 6) following p.o. administration at a dose of 100 mg/kg bw, 300 mg/kg bw and 500 mg/kg bw.

Compounds	Dose (mg/kg bw)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	$AUC_{(0-48h)}$ ((h·μg)/mL)	$AUC_{(0-\infty)}$ ((h·μg)/mL)	V_F (L/kg)	Cl_F (L/h·kg)	MRT (h)
Cy0	100	6.35 ± 1.56(a)a	2.00 ± 0.00(b)b	0.10 ± 0.01(b)b	0.51 ± 0.07(b)b	0.60 ± 0.09(b)b	903.3 ± 125.1(a)a	100.3 ± 12.1(ab)a	10.09 ± 2.18(a)b
	300	8.22 ± 3.33(a)a	3.33 ± 1.03(ab)b	0.16 ± 0.04(b)b	1.41 ± 0.32(b)b	1.47 ± 0.37(b)b	1675.9 ± 523.4(a)a	149.8 ± 35.6(a)a	12.7 ± 4.44(a)a
	500	12.44 ± 4.569(a)a	4.00 ± 0.00(a)c	0.37 ± 0.09(a)b	4.90 ± 1.17(a)b	5.18 ± 1.55(a)c	1555.4 ± 646.5(a)a	86.54 ± 22.10(b)a	17.94 ± 5.49(a)a
Cy1	100	–	–	–	–	–	–	–	–
	300	3.62 ± 1.05(a)a	7.33 ± 1.03(a)a	1.95 ± 0.66(a)a	15.07 ± 5.18(a)a	15.16 ± 4.35(a)a	98.6 ± 39.8(a)c	21.6 ± 7.71(a)c	9.59 ± 0.73(a)a
	500	7.61 ± 2.91(a)a	6.33 ± 0.82(a)b	2.71 ± 1.31(a)a	14.13 ± 5.46(a)ab	14.28 ± 4.37(a)ab	437.6 ± 267.4(a)b	38.52 ± 15.65(a)b	9.70 ± 1.95(a)a
Cy4	100	–	–	–	–	–	–	–	–
	300	4.82 ± 1.11(a)a	7.33 ± 1.03(a)a	1.34 ± 0.38(a)a	12.98 ± 3.58(a)a	13.06 ± 4.61(a)a	165.9 ± 60.3(a)bc	22.1 ± 6.31(a)c	12.03 ± 0.78(a)a
	500	6.85 ± 1.16(a)a	7.77 ± 0.82(a)ab	1.51 ± 0.30(a)ab	16.95 ± 4.52(a)a	17.25 ± 4.22(a)a	285.8 ± 75.6(a)b	29.11 ± 7.00(a)b	13.96 ± 1.48(a)a
Cy6	100	8.50 ± 2.32(a)a	8.00 ± 0.00(a)a	0.22 ± 0.04(b)a	2.28 ± 0.37(b)a	2.58 ± 0.42(b)a	389.5 ± 81.0(b)b	32.3 ± 3.6(b)b	17.08 ± 3.17(a)a
	300	8.43 ± 2.02(a)a	7.33 ± 1.03(a)a	0.29 ± 0.07(b)b	3.20 ± 0.60(b)b	3.24 ± 0.56(b)b	902.6 ± 226.6(a)b	74.4 ± 11.0(a)b	15.94 ± 2.15(a)a
	500	8.73 ± 1.38(a)a	8.00 ± 0.00(a)a	0.53 ± 0.13(a)b	7.16 ± 1.45(a)b	7.23 ± 1.33(a)bc	814.9 ± 138.5(a)ab	65.88 ± 14.43(a)ab	15.65 ± 1.51(a)a

(a), (b), (c): Pharmacokinetic parameters of the same metabolite in plasma of same animal at different doses are statistically significantly different ($P < 0.05$).
a, b, c, d: Pharmacokinetic parameters of the different metabolites in plasma of same animal at same dose are statistically significantly different ($P < 0.05$).

Table 5
Mean plasma pharmacokinetic parameters of cyadox and its main metabolites in pig (n = 8) following p.o. administration at a dose of 10 mg/kg bw, 40 mg/kg bw and 100 mg/kg bw.

Compounds	Dose (mg/kg bw)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	$AUC_{(0-48h)}$ ((h·μg)/mL)	$AUC_{(0-\infty)}$ ((h·μg)/mL)	V_F (L/kg)	Cl_F (L/h·kg)	MRT (h)
Cy0	10	–	–	–	–	–	–	–	–
	40	7.41 ± 4.78(a)ab	1.56 ± 0.62(a)c	0.04 ± 0.01(b)d	0.19 ± 0.04(b)b	0.20 ± 0.04(b)b	971.3 ± 316.9(a)a	105.46 ± 27.61(a)a	11.26 ± 6.70(a)ab
	100	6.89 ± 1.03(a)ab	1.75 ± 0.46(a)c	0.07 ± 0.01(a)c	0.47 ± 0.05(a)b	0.51 ± 0.06(a)b	1438.7 ± 210.6(a)a	145.03 ± 10.13(a)a	10.20 ± 1.19(a)bc
Cy1	10	2.99 ± 0.87(a)a	5.75 ± 0.46(b)b	0.07 ± 0.03(b)b	0.31 ± 0.11(b)b	0.33 ± 0.11(b)b	110.9 ± 36.7(a)a	25.56 ± 5.56(b)a	8.46 ± 0.93(a)b
	40	1.62 ± 0.47(a)b	7.38 ± 0.74(a)b	0.15 ± 0.05(b)bc	0.65 ± 0.24(b)b	0.69 ± 0.24(b)b	149.9 ± 87.3(a)bc	60.69 ± 20.02(a)ab	8.02 ± 0.53(a)b
	100	1.86 ± 0.55(a)c	5.75 ± 0.46(b)b	0.72 ± 0.19(a)ab	3.42 ± 1.10(a)ab	3.55 ± 1.12(a)b	81.6 ± 31.3(a)c	30.48 ± 8.23(ab)b	7.40 ± 0.72(a)c
Cy2	10	–	–	–	–	–	–	–	–
	40	4.51 ± 1.39(a)ab	8.38 ± 1.06(a)ab	0.06 ± 0.02(b)cd	0.35 ± 0.11(b)b	0.42 ± 0.14(b)b	540.8 ± 120.3(a)b	87.97 ± 27.64(a)a	12.85 ± 1.80(a)ab
	100	4.76 ± 1.99(a)abc	10.00 ± 0.00(a)a	0.29 ± 0.13(a)bc	2.90 ± 1.25(a)ab	3.15 ± 1.19(a)b	232.6 ± 109.6(b)bc	34.01 ± 13.44(b)b	14.23 ± 2.18(a)ab
Cy4	10	4.81 ± 0.96(a)a	7.00 ± 0.76(b)ab	0.15 ± 0.02(b)a	1.15 ± 0.23(b)a	1.31 ± 0.27(b)a	48.4 ± 8.4(a)b	7.19 ± 2.02(b)b	11.59 ± 0.92(a)a
	40	5.16 ± 1.82(a)ab	10.00 ± 1.07(a)a	0.25 ± 0.06(b)a	2.29 ± 0.58(b)a	2.56 ± 0.57(b)a	112.5 ± 35.5(a)c	15.52 ± 2.84(b)b	13.47 ± 2.02(a)ab
	100	4.23 ± 0.60(a)bc	10.00 ± 0.00(a)a	0.86 ± 0.38(a)a	6.65 ± 2.67(a)a	7.91 ± 3.06(a)a	78.2 ± 22.9(a)c	12.78 ± 3.73(ab)b	12.87 ± 0.88(a)ab
Cy6	10	3.64 ± 0.94(b)a	8.00 ± 0.93(ab)a	0.17 ± 0.04(a)a	1.26 ± 0.42(b)a	1.42 ± 0.46(b)a	38.3 ± 13.1(b)b	7.38 ± 2.40(b)b	11.35 ± 1.08(b)a
	40	9.04 ± 1.87(a)a	10.00 ± 0.00(a)a	0.17 ± 0.02(a)ab	2.02 ± 0.25(ab)a	2.35 ± 0.33(ab)a	189.8 ± 28.9(b)bc	14.97 ± 2.92(b)b	19.38 ± 2.60(a)a
	100	8.32 ± 2.42(a)a	7.25 ± 1.58(b)b	0.23 ± 0.04(a)bc	2.16 ± 0.32(a)b	2.46 ± 0.36(a)b	426.6 ± 106.8(a)b	36.78 ± 6.35(a)b	16.49 ± 3.38(ab)a

(a), (b), (c): Pharmacokinetic parameters of the same metabolite in plasma of same animal at different doses are statistically significantly different ($P < 0.05$).
a, b, c, d: Pharmacokinetic parameters of the different metabolites in plasma of same animal at same dose are statistically significantly different ($P < 0.05$).

Procedures for Toxicological Assessment of Food in China (OECD, 2018; GB15193.1, 2014). The results showed that the C_{max} of Cy0, Cy1, Cy2, Cy4, and Cy6 increased in plasma at different time points with increasing dosages in four animals (Fig. 4, Tables 4–7), which showed that the concentrations of CYA and its metabolites in plasma were dose-dependent. No such characteristics were observed in other pharmacokinetic parameters ($T_{1/2}$, T_{max}, $AUC_{(0-48h)}$, $AUC_{(0-\infty)}$, and MRT). Therefore, it could be concluded that although the concentration of CYA and its metabolites (Cy1, Cy2, Cy4, and Cy6) in plasma were apparently increasing, the elimination time of those compounds in plasma was not prolonged as CYA dosage increased. The results of this study revealed obvious species-specific differences in the kinetic

Table 6
Mean plasma pharmacokinetic parameters of cyadox and its main metabolites in chicken (n = 10) following p.o. administration at a dose of 50 mg/kg bw, 100 mg/kg bw and 200 mg/kg bw.

Compounds	Dose (mg/kg bw)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL	$AUC_{(0-48h)}$ ((h·μg)/mL)	$AUC_{(0-\infty)}$ ((h·μg)/mL)	V_F (L/kg)	Cl_F (L/h·kg)	MRT (h)
Cy0	50	–	–	–	–	–	–	–	–
	100	–	–	–	–	–	–	–	–
	200	2.91 ± 1.02a	0.78 ± 0.14a	0.034 ± 0.003a	0.07 ± 0.02a	0.08 ± 0.02a	5364.6 ± 606.7a	1410.1 ± 443.1a	4.46 ± 1.47a
Cy6	50	3.50 ± 1.18 (a)	1.25 ± 0.26 (a)	0.031 ± 0.002 (a)	0.06 ± 0.01(a)	0.07 ± 0.01(a)	1438.8 ± 175.6 (b)	320.5 ± 122.4 (b)	5.67 ± 1.71 (a)
	100	3.41 ± 0.93 (a)	1.30 ± 0.26 (a)	0.037 ± 0.004 (a)	0.09 ± 0.02(a)	0.10 ± 0.02(a)	2489.5 ± 305.6 (b)	527.9 ± 112.4 (b)	5.54 ± 1.27 (a)
	200	3.66 ± 0.82 (a)a	1.15 ± 0.24 (a)a	0.039 ± 0.005 (a)a	0.09 ± 0.02(a) a	0.10 ± 0.02(a) a	5138.1 ± 886.1 (a)a	994.3 ± 153.9 (a)a	5.84 ± 1.15 (a)a

(a), (b), (c): Pharmacokinetic parameters of the same metabolite in plasma of same animal at different doses are statistically significantly different ($P < 0.05$).
a, b, c, d: Pharmacokinetic parameters of the different metabolites in plasma of same animal at same dose are statistically significantly different ($P < 0.05$).

Table 7
Mean plasma pharmacokinetic parameters of cyadox and its main metabolites in carp (n = 5) following p.o. administration at a dose of 10 mg/kg bw, 20 mg/kg bw and 40 mg/kg bw.

Compounds	Dose (mg/kg bw)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (μg/mL)	$AUC_{(0-48h)}$ ((h·μg)/mL)	$AUC_{(0-\infty)}$ ((h·μg)/mL)	V_F (L/kg)	Cl_F (L/h·kg)	MRT (h)
Cy0	10	–	–	–	–	–	–	–	–
	20	–	–	–	–	–	–	–	–
	40	5.41 ± 1.15 b	3.20 ± 1.10a	0.063 ± 0.006a	0.49 ± 0.06a	0.51 ± 0.09 b	451.9 ± 52.0a	59.50 ± 11.49a	9.1 ± 1.6 b
Cy1	10	–	–	–	–	–	–	–	–
	20	5.09 ± 0.97 (a)b	5.20 ± 1.09 (a)a	0.051 ± 0.006 (b)a	0.39 ± 0.056(b) a	0.41 ± 0.089 (a)a	267.8 ± 47.7 (b)a	36.55 ± 3.28 (b)a	9.5 ± 1.1(a)b
	40	5.56 ± 0.94 (a)b	4.80 ± 1.10 (a)a	0.069 ± 0.004 (a)a	0.52 ± 0.04(a)a	0.53 ± 0.03(a) ab	419.8 ± 42.8 (a)a	53.04 ± 6.76 (a)a	5.56 ± 0.94 (b)c
Cy6	10	8.51 ± 1.63 (a)	5.60 ± 0.89 (a)	0.042 ± 0.002 (b)	0.35 ± 0.04(b)	0.39 ± 0.05(b)	173.1 ± 13.2 (c)	14.4 ± 2.0(c)	14.1 ± 1.3(a)
	20	8.33 ± 0.84 (a)a	4.80 ± 1.10 (a)a	0.050 ± 0.004 (b)a	0.43 ± 0.054(b) a	0.48 ± 0.072 (b)a	315.8 ± 27.7 (b)a	26.56 ± 4.15 (b)b	13.8 ± 1.5(a) a
	40	8.68 ± 1.09 (a)a	5.60 ± 0.89 (a)a	0.061 ± 0.007 (a)a	0.64 ± 0.07(a)a	0.67 ± 0.05(a)a	521.3 ± 67.3 (a)a	42.04 ± 6.75 (a)a	14.6 ± 1.5(a) a

(a), (b), (c): Pharmacokinetic parameters of the same metabolite in plasma of same animal at different doses are statistically significantly different ($P < 0.05$).
a, b, c, d: Pharmacokinetic parameters of the different metabolites in plasma of same animal at same dose are statistically significantly different ($P < 0.05$).

behavior of CYA and its metabolites, which may be related to clinical efficacy and toxicity, which needs further studies.

In general, pharmacokinetic parameters differ according to species, age, sex, body condition, drug formulation and administration route, all of which contribute to differences in drug efficacy (Canga et al., 2009). In this study, higher apparent volume of distribution (Vd) of Cy0 was observed at different dosages, indicating that Cy0 was the most widely distributed compound after p. o. administration in four species. The concentration vs. time curves of four animals obtained after p. o. administration revealed a rapid increase followed by a gradual decrease in the concentration of CYA and its metabolites, except in the case of not-detectable timepoint (Cy1 and Cy4 in rat plasma after p. o. administration of CYA at 100 mg/kg bw, Fig. 4). Furthermore, the maximum concentration (C_{max}) of Cy0 (CYA) in plasma was 0.034–0.37 μg mL^{-1} at 0.78–4.00 h following p. o. administration in four species, and compared with its metabolites, CYA showed lower concentrations and remained in plasma for relatively shorter time-periods in the same species (Tables 4–7), implying that CYA was quickly metabolized to its metabolites. The findings of this study were in accordance with those of previous studies conducted in pigs (Guo et al., 2011; Zhao et al., 2013) and beagle dogs (Sattar et al., 2016). Other members of this drug group, i.e., quinocetone and mequindox, exhibit the same type of response in pigs and chickens after oral administration (Zhong et al., 2011; Ding et al., 2012; Li et al., 2012). For the AUC and $T_{1/2}$, no significant difference observed for the main metabolites (Cy1, Cy2, Cy4, and Cy6) and those of Cy0 in the plasma of the four animals, implying that the CYA was transformed slowly into its metabolites. Similar results were observed during pharmacokinetic characterization of mequindox and quinocetone (Ding et al., 2012). Therefore, close attention should be paid to these metabolites when identifying the residue markers of CYA in animal tissues.

In summary, this study provides a complete insight in the pharmacokinetic properties of CYA and its five main metabolites in the plasma of rats, pigs, chickens, and carps following oral administration at three dosages. The results showed that species affected the formation, concentration, and elimination of CYA metabolites in plasma, while different dosage of the CYX and its metabolites had no significant effect on wash-out period. Cy0 was the most widely distributed compound in four species following oral administration through gavage. Cy0 and Cy6 were persisted for the longest time at lower concentration in the plasma of four species. Cy1 was not detected in chicken plasma after administration at three different dosages in this study. The present study described the pharmacokinetic and metabolism profiles of CYA and its main metabolites following oral administration at different dosages in different animals, which will not only improve our understanding of the pharmacology and toxicology of CYA but also ensure the safety and use of this compound in food-producing animals.

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CRediT authorship contribution statement

Sechenchogt Harnud: Conceived and designed the experiments, Writing – review & editing, Conceptualization, Methodology. **Shishan Fu:** Data curation, Writing – original draft. **Yulian Wang:** Visualization, Investigation. **Yuanhu Pan:** Software, Validation. **Aiqun Zhang:** Software, Validation. **Lingli Huang:** Supervision, Contribute reagents and materials, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Baggot, J.D., 1992. Clinical pharmacokinetics in veterinary medicine. Clin. Pharmacokinet. 22, 254–273. https://doi.org/10.2165/00003088-199222040-00002.

Canga, A.G., Prieto, A.M.S., Liebana, M.J.D., Martinez, N.F., Vega, M.S., Vieitez, J.J., 2009. The pharmacokinetics and metabolism of ivermectin in domestic animal species. Vet. J. 179, 25–37. https://doi.org/10.1016/j.tvjl.2007.07.011.

Causon, R., 1997. Validation of chromatographic methods in biomedical analysis. Viewpoint and discussion. J. Chromatogr. B 689, 175–180. https://doi.org/10.1016/s0378-4347(96)00297-6.

Cheng, G., Sa, W., Cao, C., Guo, L., Hao, H., Liu, Z., Wang, X., Yuan, Z., 2016. Quinoxaline 1, 4-di-N-oxides: biological activities and mechanisms of actions. Front. Pharmacol. 7, 64. https://doi.org/10.3389/fphar.2016.00064.

Cutler, D.J., 1978. Theory of the mean absorption time, an adjunct to conventional bioavailability studies. J. Pharm. Pharmacol. 30, 476–478. https://doi.org/10.1111/j.2042-7158.1978.tb13296.x.

Devane, C.L., 2010. Pharmacokinetics (2nd edn, revised and expanded), M. Gibaldi and D. Perrier (Vol. 15 of Drugs and the Pharmaceutical Sciences), Marcel Dekker, New York. Biopharm. Drug Dispos. vol. 4, 201-201. https://doi.org/10.1002/bdd.2510040213.

Ding, H., Liu, Y., Zeng, Z., Si, H., Liu, K., Liu, Y., Yang, F., Li, Y., Zeng, D., 2012. Pharmacokinetics of mequindox and one of its major metabolites in chickens after intravenous, intramuscular and oral administration. Res. Vet. Sci. 93, 374–377. https://doi.org/10.1016/j.rvsc.2011.07.007.

Ding, M., Wang, Y., Zhu, H., Yuan, Z., 2006a. Effects of cyadox and olaquindox on intestinal mucosal immunity and on fecal shedding of Escherichia coli in piglets. J. Anim. Sci. 84, 2367–2373. https://doi.org/10.2527/jas.2005-564.

Ding, M., Yuan, Z., Wang, Y., Zhu, H., Fan, S., 2006b. Olaquindox and cyadox stimulate growth and decrease mucosal immunity in piglets orally inoculated with Escherichia coli. J. Anim. Physiol. Anim. Nutr. 90, 238–243. https://doi.org/10.1111/j.1439-0396.2005.00593.x.

Fang, G., He, Q., Zhou, S., Wang, D., Zhang, Y., Yuan, Z., 2006. Subchronic oral toxicity study with cyadox in Wistar rats. Food Chem. Toxicol. 44, 36–41. https://doi.org/10.1016/j.fct.2005.06.003.

FDA, 2018. Guidance for industry: bioanalytical method validation, United States department of Health and human service. Available online at: https://www.fda.gov/media/70858/download.

GB15193.1 (2014), Procedures for Toxicological Assessment of Food in China.

Guo, J., Zhang, Z., Yuan, L., Yu, J., Hu, H., Deng, H., Liu, Y., 2011. Pharmacokinetics of cyadox and its mainly metabolites in swine. China anim. Husbandry vet. med. 38, 228–231. Available online at. http://en.cnki.com.cn/Article_en/CJFDTOTAL-GWXK201109061.htm.

Harnud, S., Zhang, A., Mao, Q., Yang, X., Yuan, Z., 2018. The elimination of cyadox and its two major metabolites in chickens. Chin. J. Vet. Drug. 1 (52), 35–45. https://doi.org/10.11751/ISSN.1002-1280.2018.01.06.

He, Q., Fang, G., Wang, Y., Wei, Z., Wang, D., Zhou, S., Fan, S., Yuan, Z., 2006. Experimental evaluation of cyadox phototoxicity to Balb/c mouse skin. Photodermatol. Photoimmunol. Photomed. 22, 100–104. https://doi.org/10.1111/j.1600-0781.2006.00199.x.

Huang, L., Yin, F., Pan, Y., Chen, D., Li, J., Wan, D., Liu, Z., Yuan, Z., 2015a. Metabolism, distribution, and elimination of mequindox in pigs, chickens, and rats. J. Agric. Food Chem. 63 (44), 9839–9849. https://doi.org/10.1021/acs.jafc.5b02780.

Huang, L., Xu, N., Harnud, S., Pan, Y., Chen, D., Tao, Y., Liu, Z., Yuan, Z., 2015b. Metabolic disposition and elimination of cyadox in pigs, chickens, carp, and rats. J. Agric. Food Chem. 63, 5557–5569. https://doi.org/10.1021/acs.jafc.5b01745.

Huang, L., Qiu, Y., Sun, L., Li, J., Pan, Y., Wang, Y., Yuan, Z., 2018. Dietary exposure assessment of cyadox based on tissue depletion of cyadox and its major metabolites in pigs, chickens, and carp. J. Vet. Pharmacol. Therapeut. 41, 125–136. https://doi.org/10.1111/jvp.12440.

Huang, L., Wang, Y., Tao, Y., Chen, D., Yuan, Z., 2008. Development of high performance liquid chromatographic methods for the determination of cyadox and its metabolites in plasma and tissues of chicken. J. Chromatogr. B 874, 7–14. https://doi.org/10.1016/j.jchromb.2008.08.013.

Ihsan, A., Wang, X., Huang, X.J., Liu, Y., Liu, Q., Zhou, W., Yuan, Z.H., 2010. Acute and subchronic toxicological evaluation of Mequindox in Wistar rats. Regul. Toxicol. Pharmacol. : RTP (Regul. Toxicol. Pharmacol.) 57 (2–3), 307–314. https://doi.org/10.1016/j.yrtph.2010.03.011.

Ihsan, A., Wang, X., Tu, H.G., Zhang, W., Dai, M.H., Peng, D.P., Wang, Y.L., Huang, L.L., Chen, D.M., Mannan, S., Tao, Y.F., Liu, Z.L., Yuan, Z.H., 2013. Genotoxicity evaluation of Mequindox in different short-term tests. Food Chem. Toxicol. : Int. J. pub. Br. Ind. Biol. Res. Assoc. 51, 330–336. https://doi.org/10.1016/j.fct.2012.10.003.

Li, G., Yang, F., He, L., Ding, H., Sun, H., Liu, Y., Shan, Q., Li, Y., Zeng, Z., 2012. Pharmacokinetics of mequindox and its metabolites in rats after intravenous and oral administration. Res. Vet. Sci. 93, 1380–1386. https://doi.org/10.1016/j.rvsc.2012.02.015.

Li, Y.F., Zhao, N., Zeng, Z.L., Gu, X.Y., Fang, B.H., Yang, F., Zhang, B.X., Ding, H.Z., 2013. Tissue deposition and residue depletion of cyadox and its three major metabolites in pigs after oral administration. J. Agric. Food Chem. 61, 9510–9515. https://doi.org/10.1021/jf4028602.

Lin, J.H., 1994. Dose-dependent pharmacokinetics: experimental observations and theoretical considerations. Biopharm Drug Dispos. 15, 1–31. https://doi.org/10.1002/bdd.2510150102.

Lin, J.H., Lu, A.Y., 1997. Role of pharmacokinetics and metabolism in drug discovery and development. Pharmacol. Rev. 49 (4), 403–449.

Liu, Q., Lei, Z., Cui, L., Zhang, J., Awais, I., Dai, M., Wang, X., Yuan, Z., 2017. A two-year dietary carcinogenicity study of cyadox in Sprague-Dawley rats. Regul. Toxicol. Pharmacol. : RTP (Regul. Toxicol. Pharmacol.) 87, 9–22. https://doi.org/10.1016/j.yrtph.2017.04.011.

Liu, Z., Huang, L., Dai, M., Chen, D., Tao, Y., Wang, Y., Yuan, Z., 2009. Metabolism of cyadox in rat, chickens and pig liver microsomes and identification of metabolites by accurate mass measurements using electrospray ionization hybrid ion trap/time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 23, 2026–2034. https://doi.org/10.1002/rcm.4106.

OECD, 2018. Test No. 453: Combined Chronic Toxicity/Carcinogenicity Studies, OECD Guidelines for the Testing of Chemicals, Section vol. 4, OECD Publishing, Paris. https://doi.org/10.1787/9789264071223-en.

Qiu Y.M., 2012. reportResidue Depletion of Cyadox in Pigs, Chickens and Carp. masters dissertation, Huazhong Agricultural University, Wuhan.

Sattar, A., Xie, S., Huang, L., Iqbal, Z., Qu, W., Shabbir, M.A., Pan Y, Hussain H.I., Chen, D., Tao, Y., Liu, Z., Iqbal, M., Yuan, Z., 2016. Pharmacokinetics and metabolism of cyadox and its main metabolites in beagle dogs following oral, intramuscular, and intravenous administration. Front. Pharmacol. 7, 236. https://doi.org/10.3389/fphar.2016.00236.

Singh, S., 2006. Preclinical pharmacokinetics: an approach towards safer and efficacious drugs. Curr. Drug Metabol. 7, 165–182. https://doi.org/10.2174/138920006775541552.

Theuretzbacher, U., 2012. Pharmacokinetic and pharmacodynamic issues for antimicrobial therapy in patients with cancer. Clin. Infect. Dis. 54, 1785–1792. https://doi.org/10.1093/cid/cis210.

Wang, X., Tan, Z., Cheng, G., Awais, I., Huang, L., Chen, D., Pan, Y., Liu, Z., Yuan, Z., 2015a. Two-generation reproduction and teratology studies of feeding aditoprim in Wistar rats. J. Appl. Toxicol. 35 (12), 1531–1538. https://doi.org/10.1002/jat.3121.

Wang, X., Zhou, W., Ihsan, A., Chen, D., Cheng, G., Hao, H., Liu, Z., Wang, Y., Yuan, Z., 2015b. Assessment of thirteen-week subchronic oral toxicity of cyadox in Beagle dogs. Regul. Toxicol. Pharmacol. 73, 652–659. https://doi.org/10.1016/j.yrtph.2015.09.023.

Wu, H., Li, L., Shen, J., Wang, Y., Liu, K., Zhang, S., 2012. In vitro metabolism of cyadox in rat, chicken and swine using ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. J. Pharmaceut. Biomed. Anal. 67–68, 175–185. https://doi.org/10.1016/j.jpba.2012.04.004.

Yamaoka, K., Nakagawa, T., Uno, T., 1978. Statistical moments in pharmacokinetics. J. Pharmacokinet. Biopharm. 6, 547–558. https://doi.org/10.1007/BF01062109.

Yang, X., Zhou, Y.F., Yu, Y., Zhao, D.H., Shi, W., Fang, B.H., Liu, Y.H., 2015. A physiologically based pharmacokinetic model for quinoxaline-2-carboxylic acid in rats, extrapolation to pigs. J. Vet. Pharmacol. Therapeut. 38, 55–64. https://doi.org/10.1111/jvp.12143.

Zhao, N., Wang, L., Lu, X., Jia, H., Fang, B., Zeng, Z., Ding, H.Z., 2013. Pharmacokinetics of cyadox and its major metabolites in swine after intravenous and oral

administration. J. Integr. Agr. 12, 495–501. https://doi.org/10.1016/S2095-3119(13)60250-6.

Zheng, M., Jiang, J., Wang, J., Tang, X., Ouyang, M., Deng, Y., 2011. The mechanism of enzymatic and non-enzymatic N-oxide reductive metabolism of cyadox in pig liver. Xenobiotica; the fate of foreign compounds in biological systems 41 (11), 964–971. https://doi.org/10.3109/00498254.2011.593207.

Zhong, J.L., Zhang, G.J., Shen, X.G., Wang, L., Fang, B.H., Ding, H.Z., 2011. Pharmacokinetics of quinocetone and its major metabolites in swine after intravenous and oral administration. Agric. Sci. China 10, 1292–1300. https://doi.org/10.1016/s1671-2927(11)60121-1.