The roles of cytochrome P450 and P-glycoprotein in the pharmacokinetics of florfenicol in chickens

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Summary

The effects of three selective oral inhibitors, fluvoxamine (FLU), ketoconazole (KET), and verapamil (VER), on the pharmacokinetics (PK) of florfenicol (FFC) were investigated in chickens. The chickens were administered orally with saline solution (SAL), FLU (60 mg/kg), KET (25 mg/kg), or VER (9 mg/kg) for 7 consecutive days. Florfenicol was given to the chickens at a single dose of 30 mg/kg orally. Blood samples were collected from each chicken at 0 to 12 h post-administration of FFC. The plasma concentration of FFC was analyzed by high-performance liquid chromatography (HPLC). The AUC of FFC increased and the CL_s of FFC decreased with oral co-administration of KET in chickens, and the C_{max} of FFC increased with VER. While the AUC, the CL_s and the C_{max} of FFC were all invariable with FLU. These data suggested that CYP 3A played a key role in the PK of FFC in chickens, however, P-glycoprotein (P-gp) and CYP 1A did not. The results imply that the adverse drug-drug interaction may occur in the use of FFC if the co-administrated drugs are the substrates, inducers or inhibitors of CYP 3A or/and P-gp.

Key words: CYP 1A, CYP 3A, Florfenicol, P-glycoprotein, pharmacokinetics

Introduction

Florfenicol (FFC) (2,2-dichloro-N-[(1R,2S)-3-fluoro-1-hydroxy-1-(4-methylsulfonylphenyl) propan-2-yl] acetamide), a synthetic broad-spectrum antibiotic, offers the advantages of low toxicity and a broad antimicrobial spectrum (Shin *et al*., 2005; Wei *et al*., 2016). Therefore, FFC has been widely used in husbandry industry to cure and prevent infections for sensitive bacteria (Soback *et al*., 1995; Atef *et al*., 2001; Verner-Jeffreys *et al*., 2017). In poultry farms, FFC is used for the treatment of gastrointestinal and respiratory tract infections (Shen *et al*., 2003). Although the pharmacokinetics (PK) of FFC has been extensively studied in chickens (Afifi and AboEl-Sooud, 1997; Anadón *et al*., 2008; Poźniak *et al*., 2017), the primary enzymes and transporters that are involved in the PK of FFC remain unclear.

Pharmacokinetics is a branch of pharmacology dedicated to analyzing the fate of substances administered to living organisms. It describes how the body affects a specific xenobiotic/chemical after exposure through the mechanisms of absorption and distribution, as well as the metabolic changes of the substance in the body (e.g. by metabolic enzymes such as cytochrome P450 (CYP 450) or glucuronosyl-transferase enzymes), and the routes of metabolites excretion of the drug (Azizi *et al*., 2013). Cytochrome P450 is a super-family of enzymes and mainly responsible for catalyzing the metabolism of many drugs. The CYP 1A subfamily mainly participates in the metabolism of aromatic compounds, while the CYP 3A subfamily metabolizes most of the therapeutic drugs (Tsuji *et al*., 2007; Zhou, 2008; He and Feng, 2015). P-glycoprotein (P-gp) is a class of important transporters which are expressed in the intestine along with CYP 3A and forms a barrier of the transmembrane transportation of drugs (Pal and Mitra, 2006; Lee *et al*., 2017). P-glycoprotein and⁄or CYP 3A play an important role in the disposition of FFC in rabbits (Liu *et al*., 2011). Florfenicol could be useful in controlling common bacterial infections in different avian species, but no data are available for the primary enzymes that are involved in the FFC metabolism in chickens. It has been documented that the disposition kinetics of FFC represent obvious differences between different species and should not be extrapolated for rabbits to chickens without pharmacokinetic data (Ismail and El-Kattan, 2009). Chicken accounts for a large share of human food consumption and FFC is extensively metabolized in chickens, and its main metabolite, florfenicol amine (FFA) may be a risk to human health (Filazi *et al*., 2014). Therefore, understanding the roles of CYP 1A, CYP 3A and P-gp in the PK of FFC is important to explore the PK profile, avoid the possible adverse drug-drug interactions and prevent the harm

caused by FFA residues in the edible tissues (Wang *et al*., 2013).

Fluvoxamine (FLU), a selective serotonin reuptake inhibitor, is regarded as a potent CYP1A2 inhibitor (Yasui-Furukori *et al*., 2004). Ketoconazole (KET), an imidazole anti-fungal drug, is an inhibitor of CYP 3A. Ketoconazole has been shown to inhibit testosterone hydroxylation in kidney in bobwhite quail (Cortright *et al*., 2006; Davidson Peiris and Wusirika, 2017). Verapamil (VER) is a calcium channel blocker and is commonly used to control hypertension, chest pain and arrhythmia (Ledwitch *et al*., 2016). Verapamil is also a potent and selective inhibitor of P-gp inhibitor which can increase the concentrations and the AUC in serum of many drugs (Zhang *et al*., 2017). To aid in determining the role of CYP 3A, CYP1A2 and P-gp in FFC metabolism in chickens, inhibition studies were conducted using KET, FLU and VER on the PK of FFC to investigate the roles of CYP 450 and P-gp in this paper.

Materials and Methods

Chemicals

Florfenicol (purity 99.6%) was provided by Biok Biology Co., Ltd. (Hangzhou, Zhejiang, China), and was dissolved in polyethylene glycol-300 (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) to a concentration of 50 g/L for the animal experiments immediately prepared before oral administration. Chloramphenicol analytical standards (purity 99%) were purchased from Schering-Plough Corporation (Shanghai, China) and Merck Corporation (Darmstadt, Germany). Maleic acid FLU tablets were purchased from Netherland Solvay Pharmaceuticals, Inc. (Chendu China). Ketoconazole tablets and VER hydrochloride tablets were purchased from Xi'an-Janssen Pharmaceutical Co., Ltd. (Xi'an, China). Acetonitrile was purchased from Merck Corporation (Darmstadt, Germany) and was of high-performance liquid chromatography (HPLC) grade. All other reagents that were used for extraction and analysis were analytical reagent grade or better, and were commercially available.

Chickens

One-day-old male AA broiler chickens provided by Hewei Agricultural Development Share Co., Ltd. (Xuancheng, Anhui, China) were used in this study. The animals were fed with a balanced ration of feed (antibacterial-free) and water *ad libitum* before starting the experiments. This investigation (animal study protocol No. 201207003) was approved by the Institutional Animal Care and Use Committee of College of Animal Science and Technology, Henan University of Science and Technology. According to the recommendations of the NRC (1994), the temperature, humidity, and light were controlled under suitable conditions.

Experimental design

Twenty-eight day old chickens (0.96 ± 0.24 kg, male) were divided into four groups (6 chickens per group). The chickens in the first group were given the volume of saline solution (SAL) as the other groups via oral administration once a day during the study. Those in the second, third, and fourth groups were administered FLU (60 mg/kg), KET (25 mg/kg), and VER (9 mg/kg) by oral gavage, respectively, for 7 consecutive days. At the end of day 7 of administrating, all chickens were given a single dose of 30 mg/kg of FFC by oral gavage administration at 30 min post final administration.

Firstly, the brachial wing vein of chickens was obtuse separated. Just before and at 0.08, 0.17, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12 h post-FFC administration, 1 ml blood samples were taken from the left brachial wing vein of each chicken and were collected in heparinized tube. Plasma was separated from sample after centrifugation (3500 rpm for 10 min) at room temperature and stored at -20°C until analysis.

Analytical method

Apparatus and chromatographic conditions

The quantitative analysis of FFC was performed with a HPLC (Agilent 1200 series; Agilent Technologies Co., Ltd., Palo Alto, Santa Clara, CA, USA). The HPLC system was equipped with a G1311A quaternary pump, a G1328B MAN injector, a G1314B ultraviolet wavelength detector set at 224 nm, a G1316A column temperature box set at 40°C, and an instrument 1 online/offline analysis software. HPLC analysis was carried out on a 5-μm Eclipse XDB-C18 column (250 × 4.6 mm). The mobile phase solution consisted of 50 mmol/L aqueous KH_2PO_4–acetonitrile (77:23 [vol/vol]). The elution flow rate was 0.9 ml/min.

Sample preparation

The frozen plasma samples were defrosted at room temperature and centrifuged for 5 min at 3500 rpm before analysis. A 200 μL supernatant of plasma was accurately collected and transferred to a new polyethylene centrifuge tube. Immediately, 5 μL of the internal standard solution (1 mg/ml chloramphenicol) and 600 μL ethyl acetate were added to the tube successively. After vortexing for 5 min, the tube was centrifuged at 12,000 rpm for 10 min. The upper layer was transferred to another centrifuge tube, and the underlying layer was re-extracted once with 600 μL ethyl acetate. The two extracts were pooled together and dried under a stream of nitrogen at 45°C, then dissolved in 200 μL of the mobile phase and centrifuged for 10 min at 12,000 rpm. Finally, 15 μL of the supernatant was injected into the HPLC system for analysis.

Quantification

Stock standard solutions (4000 μg/ml) of FFC standards were prepared by accurately dissolving 40 mg of the compounds into 10 ml of acetonitrile. The working standard solutions were prepared through serial dilution

of the stock solutions with mobile phase solution to 400, 200, 80, 40, 20, 8, 4, 2, 1, 0.4, and 0.2 µg/ml. 1 mg/ml chloramphenicol solution was used as an internal standard. Both the stock and working standard solutions were stable for 1 month at -20°C in the dark.

180 µL blank plasma samples were supplemented with 20 µL working standard solutions of FFC and 5 µL internal standard to prepare plasma standard samples (containing FFC 0.02-40 µg/ml correspondingly). The plasma standard samples were analyzed according to the sample preparation items as mentioned above. Calibration curve was constructed by plotting peak area ratios (FFC to chloramphenicol) versus the corresponding FFC concentrations. The FFC concentrations of the unknown samples were calculated from the calibration curve.

Method validation

Specificity

The specificity was evaluated by comparing blank individual plasma samples from chickens, blank plasma samples spiked with the FFC and internal standard, and plasma samples after oral administration of FFC.

Linearity

Calibration curves were prepared and assayed in duplicate in three consecutive days to assess linearity. Linearity was evaluated by calculating the FFC/internal standard peak area ratio versus FFC concentration using a weighted least-squares linear regression (weighting factor, $1/x^2$). The lowest to the highest concentration of FFC was the linearity range of the calibration curve.

Limits of detection and limits of quantification

The limits of detection (LODs) and limits of quantification (LOQs) were estimated from the signal of the FFC peak in spiked samples. They were defined as the concentrations that resulted in a detectable peak area 3 and 6 times the noise level, respectively.

Precision and accuracy

The intra-day accuracy and precision were determined by analyzing five replicates of the three level samples-spiked with high, middle, and low concentrations of FFC (the final concentrations were 8, 1, and 0.125 µg/ml, respectively) on the same day. The inter-day accuracy and precision were measured by analyzing the respective spiked samples on five consecutive days. The intra- and inter-day precisions were defined by the relative standard deviation (RSD), respectively. The accuracy expressed as the approximate extent of the detected FFC concentrations to the true concentrations, which was calculated by comparing the detected concentration of drug in spiked plasma samples with the true concentrations

Extraction recovery

The extraction recovery of FFC from plasma were determined by comparison of the peak areas of the analytes and internal standard in plasma experienced the complete preparation procedure with those spiked into the prepared blank plasma.

Pharmacokinetic data analysis

Pharmacokinetic analysis of plasma concentration-time data of FFC was carried out using a practical pharmacokinetic 3p87 program (Wang *et al*., 2013). A pharmacokinetic compartment model was selected by application of Akaike's information criterion (AIC method) and weighting factor (Yamaoka *et al*., 1978; Suo *et al*., 2007; Wang *et al*., 2013).

Lag time corresponds to the finite time taken for FFC to appear in systemic circulation following oral administration:

V/F: The apparent distribution volume

AUC: The area under the plasma concentration-time curves calculated by means of the trapezoidal rule

C_{max} and T_{max}: The peak plasma drug concentration and the time required to attain peak concentration, respectively

The apparent clearance (CL/F) was determined as:

$$CL/F = Dose/AUC$$

Following oral administration, the plasma concentration-time data of FFC for each broiler was fitted in one-compartment open models according to the following equations:

$$Ct = Error! \times (e^{-Ke \cdot t} - e^{-Ka \cdot t})$$

In the above formula,

Ct: The plasma drug concentration at time

t: Ka means the absorption rate constants

e: The base of natural logarithm

K_e: Elimination rate constants

F: The absolute percentage bioavailability

X_0 and V: The dose of administration and the volume of distribution

The absorption half-life (t1/2Kα) is given by the expression:

$$t1/2K\alpha = Error!$$

The elimination half-life (t1/2β) is given by the expression:

$$t1/2\beta = Error!$$

Statistical analysis

The results were presented as mean ± standard error (mean±SE). The data were statistically analyzed using the one-way ANOVA with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). P-values of <0.05 were considered to be statistically significant.

Results

A specific and sensitive HPLC method was developed for quantitation of FFC in chicken plasma. Using this analytical method to analyze blank samples, spiked samples, and samples from broilers administered FFC, the retention time of FFC and the internal standard was free of interference. The LOD and LOQ of FFC was 0.02 and 0.04 µg/ml in HPLC method, respectively. The calibration curve prepared from the chicken plasma

Table 1: Accuracy, precision, and extraction recovery data of the method

Spiked concentration (µg/ml)	Detected concentration (µg/ml)	Accuracy (%)	Intra-day	Inter-day	Extraction recovery (%)
			RSD (%)	RSD (%)	
0.125	0.129 ± 0.011	103.2 ± 8.8	4.9	3.5	90.8 ± 7.7
1	1.054 ± 0.023	105.4 ± 2.3	4.1	4.6	103.5 ± 2.3
8	8.389 ± 0.595	104.9 ± 7.4	3.2	2.4	105.2 ± 7.5

Values are presented as mean±SE (n=5)

Table 2: Pharmacokinetic parameters of FFC following-oral administration in broiler chickens [a]

Parameter	Unit	FFC + SAL	FFC + FLU	FFC + KET	FFC + VER
A	µg/ml	15.17 ± 0.70	16.21 ± 1.84	25.73 ± 2.23**	16.52 ± 0.31
t1/2ka	h	0.51 ± 0.03	0.66 ± 0.04*	0.59 ± 0.02	0.42 ± 0.003
t1/2ke	h	1.14 ± 0.14	1.41 ± 0.02	1.55 ± 0.12*	1.09 ± 0.02
T_{max}	h	1.07 ± 0.09	1.35 ± 0.05**	1.32 ± 0.03*	0.94 ± 0.001
C_{max}	µg/ml	4.33 ± 0.33	4.44 ± 0.26	8.66 ± 0.18**	5.55 ± 0.22**
AUC	mg·h/L	13.8 ± 2.18	17.62 ± 1.51	35.04 ± 2.11**	15.96 ± 0.80
CL/Fs	L/kg·h	2.28 ± 0.34	1.73 ± 0.14	0.86 ± 0.06**	1.89 ± 0.10
V/F	L/kg	3.63 ± 0.19	3.51 ± 0.25	1.91 ± 0.03**	2.97 ± 0.10*

[a] Note: FFC (30 mg/kg b.wt.) was co-administered orally with SAL, FLU (60 mg/kg), KET (25 mg/kg) or VER (9 mg/kg) (mean±SE, n=6) (mean±SE, n=6). * P<0.05 and ** P<0.01 compared with the FFC + SAL group

spiked with known amounts of FFC was linear between 0.02 and 40 µg/ml. The correlation coefficient (r^2) of the calibration curve was >0.9999. As shown in Table 1, analytical accuracy, intra-day precision, inter-day precision, and extraction recovery data of FFC (n=5) all met the requirements at three levels (0.125, 1, and 8 µg/ml) in chicken plasma. The assay was suitable to study the pharmacokinetic characteristics following oral dose of FFC extract in chickens.

No significant adverse effects were observed in broilers when FFC was co-administered orally with SAL, FLU, KET or VER. The pharmacokinetic profile of FFC is illustrated in Fig. 1. The concentration of FFC is best described by a one-compartment open model. As shown in Table 2, the AUC and CL_s of FFC in FFC + SAL group were 13.8 ± 2.18 µg/ml·h and 2.28 ± 0.34 L/kg/h, respectively. No significant difference of the AUC and CL_s of FFC was found when CYP 1A was inhibited by FLU (P>0.05). However, compared with the FFC + SAL group, the inhibition of CYP 3A by KET significantly increased the AUC to about 3 times, and decreases the CL_s of FFC (P<0.01, AUC = 35.04 ± 2.11 µg/ml·h, P<0.01, CL_s = 0.86 ± 0.06 L/kg/h). In addition, the C_{max} of FFC in the FFC + VER group was significantly increased, but the AUC was not changed relative to a control group.

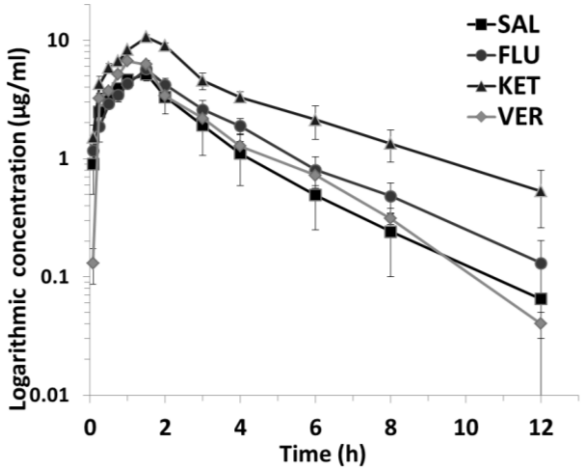

Fig. 1: Mean plasma concentration of FFC (µg/ml) following oral administration in broilers. FFC (30 mg/kg b.wt.) was co-administered orally with SAL, FLU (60 mg/kg), KET (25 mg/kg) or VER (9 mg/kg) (mean±SE, n=6)

Discussion

Florfenicol is used alone or with other drugs to cure and prevent bacteria infections in veterinary clinical practice (Ghoddusi *et al*., 2015; Razmyar and Zamani, 2016). When the drug combination is used, the PK drug-drug interactions may occur. In addition to chemical factors, physiological and biochemical factors such as metabolism enzymes, transporting proteins also may result in these interactions. Liu *et al*. (2011) reported that an obvious increase of T1⁄2Ke was found in the FFC + VER group, but not in the FFC + FLU group or FFC + KET group. The CL/F in the FFC + VER group or the FFC + KET group, but not in the FFC + FLU group, was remarkably slower than that of FFC alone group in rabbits. P-glycoprotein and/or CYP 3A have been found to affect the disposition of FFC in rabbits (Liu *et al*., 2011). It is a little different in rabbits metabolism, the present study showed that when CYP 3A was inhibited by KET, the AUC of FFC significantly increased and the CL_s of FFC significantly decreased. However, when CYP 1A was inhibited by FLU, neither the AUC nor the CL_s of FFC was changed. In general, VER should increase the C_{max} in serum and the AUC of many drugs. Our results showed the C_{max} of FFC was increased, but the AUC was not changed when P-gp was inhibited by VER. It suggested that CYP 3A play a key role in the PK of FFC in broiler chickens, the P-gp should be involved in the process and CYP 1A may not be important. The result implied that the adverse drug-drug interaction may occur in the use of FFC if the co-administered drugs are

the substrates, inducers or inhibitors of CYP 3A or/and P-gp.

In human and animal, CYP 3A is very abundant in the liver and the intestine, and metabolizes severaldrugs including some synthetic steroids and most macrolide antibiotics (Lee *et al*., 2017). In addition, P-gp is expressed in the intestine along with CYP 3A and form a transport barrier to drug absorption (Pal and Mitra, 2006). If the drug was a substrate of P-gp or P-gp was inhibited, the absorption of the drug would be increased and results in an increase of the drug concentration in the blood. However, the activity of CYP 3A was not decreased and a greater amount of drug was eliminated from the body which may not result in the AUC of the drug increasing. This may explain why the C_{max} of FFC was increased while the AUC of FFC was not changed when co-administered with VER. On the other hand, when CYP 3A was inhibited by some co-administered drugs, the metabolism of FFC was decreased, and the drug was accumulated in the animal body which resulted in augmentations of the C_{max} and the AUC of FFC. These may further cause a risk of toxicity to animals that are exposed to FFC and the prolonged withdrawal time of the edible tissues. Conversely, if CYP 3A was induced, the C_{max} and the AUC of FFC decreased which may lead to a failure of therapy.

Now, our study showed that FLU, KET and VER are not the specific inhibitors in the pharmacokinetics of FFC in chickens. They are the potent and selective inhibitors of CYP enzymes or/and P-gp (Yasui-Furokori *et al*., 2004; Zhou, 2008; Athukuri ans Neerati, 2017). Therefore, some other factors in the PK of FFC in chicken cannot be ruled out, and more and further study should be performed.

In conclusion, CYP 3A may play a key role in the metabolism of FFC and P-gp is likely involved in the absorption of FFC in chickens. To avoid the adverse drug-drug interactions, more attention should be paid when the substrates, inducers or inhibitors of CYP 3A and/or P-gp are co-administrated with FFC in poultry farms.

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Conflict of interest

The authors declare that they have no competing interests.

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