



## SHORT COMMUNICATION

### Reduced Resident Time and Tissue Residues of Synergistic Florfenicol-Thiamphenicol Combination in Leghorn Chickens

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#### ABSTRACT

*In vivo* synergistic antimicrobial effects against pathogens in chickens have been reported for florfenicol (FF) and thiamphenicol (TAP) at a ratio of 1:2. The present study evaluates the pharmacokinetics and tissue residues of a single combined intramuscular treatment of FF+TAP at 1/6 and 1/3 of their respective recommended doses in 5-week old broiler chickens. The drug concentrations were determined by LC/MS/MS. Significant reductions were observed in the  $C_{max}$  (0.045 and 2.64  $\mu\text{g/mL}$  for FF and TAP, respectively) and AUC (0.13 and 7.19  $\text{h}\cdot\mu\text{g/mL}$  for FF and TAP, respectively) compared to corresponding values reported for the recommended dose (up to two orders of magnitude lower). Tissue FF (10.6-88.4  $\mu\text{g/kg}$ ) and TAP concentrations (6.5-147.0  $\mu\text{g/kg}$ ) declined below the maximum residue limit within only one day except for TAP residue in skin/fat. This experiment provided supporting evidence for a complete study that synergistic FF-TAP has potential benefits over monotherapy since drug residues could be greatly reduced; much shorter withdrawal period was strongly indicated.

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#### INTRODUCTION

While new drug development is time and cost consuming, one promising way to reduce the amount of drug use and to slow down the development of resistance without compromising the efficacy is to use synergistic drug combination. Our previous publications have demonstrated that combination of FF and TAP produces synergistic actions *in vitro* against isolates of *Staphylococcus aureus* (Wei *et al.*, 2016a) and *Pasteurella multocida* (Wei *et al.*, 2016b) from chickens, cattle and pigs. FF-TAP synergism has also been demonstrated *in vivo* through bacterial challenge experiments in mice, wherein treatment with FF-TAP (10+10 mg/kg) afforded complete protection against experimental *S. aureus* infection compared to lower survival rates with higher doses of FF or TAP administered alone (Wei *et al.*, 2016a). Similarly, treatment with FF-TAP (5+10 mg/kg) provided complete protection against *P. multocida* infection in chickens (Wei *et al.*, 2016b). In view of the potential to maintain or

improve antimicrobial efficacy with lower doses of FF and TAP compared to monotherapy with either agent, it is reasonable to assume that combination therapy may reduce net drug exposure with concomitant reductions in tissue residues and withdrawal times as well as reduced risks of toxicity or human food safety concerns. In order to address these possible advantages, the preliminary study was carried out in chickens to evaluate the pharmacokinetic characteristics and drug residues in kidney, liver, skin with fat and breast muscle 24 h following combined treatment with FF and TAP. The results of this study provide critical information on plasma pharmacokinetics and tissue residues, which should prove useful for developing dosing regimen and withdrawal time for combination FF-TAP therapies.

#### MATERIALS AND METHODS

Five-week old Leghorn chickens (2 kg), which were fed an antibacterial-free diet with free access to water, were used for this study. The animal study protocol was

approved by the Institutional Animal Care and Use Committee at National Chung Hsing University (IACUC approval No: 101-104<sup>R2</sup>). Three chickens were given a single intramuscular injection of FF-TAP (5+10 mg/kg body weight, 1/6 and 1/3 of the recommended doses of FF and TAP, respectively) and blood samples (1.5 mL) were collected at 5, 10, 15 min, 1, 2, 4 and 6 h following drug administration. Plasma was separated by centrifugation at 1610 x g for 15 min, removed and stored at -20°C until analysis of FF and TAP by validated LC/MS/MS method at the Advance Instrument Center, National Chung Hsing University, Taiwan. Briefly, plasma was extracted by ethyl acetate in 1N NaOH, evaporated to dryness and reconstituted in mobile phase for analytical injection. Liquid chromatographic separations were carried out in a Thermo Accure™ C18 column (100 x 2.1 mm, 2.6 mm) using a model 1100 LC system (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of 0.1% acetic acid and acetonitrile in a gradient elution. The mass spectra were obtained by a Thermo TSQ Quantum Ultra EMR (Thermo-Scientific, San Jose, CA, USA) operated using electrospray ionization (ESI) sources in positive mode. Pharmacokinetic parameters were analyzed by non-compartment analysis using PKSolver 2.0.

## RESULTS AND DISCUSSION

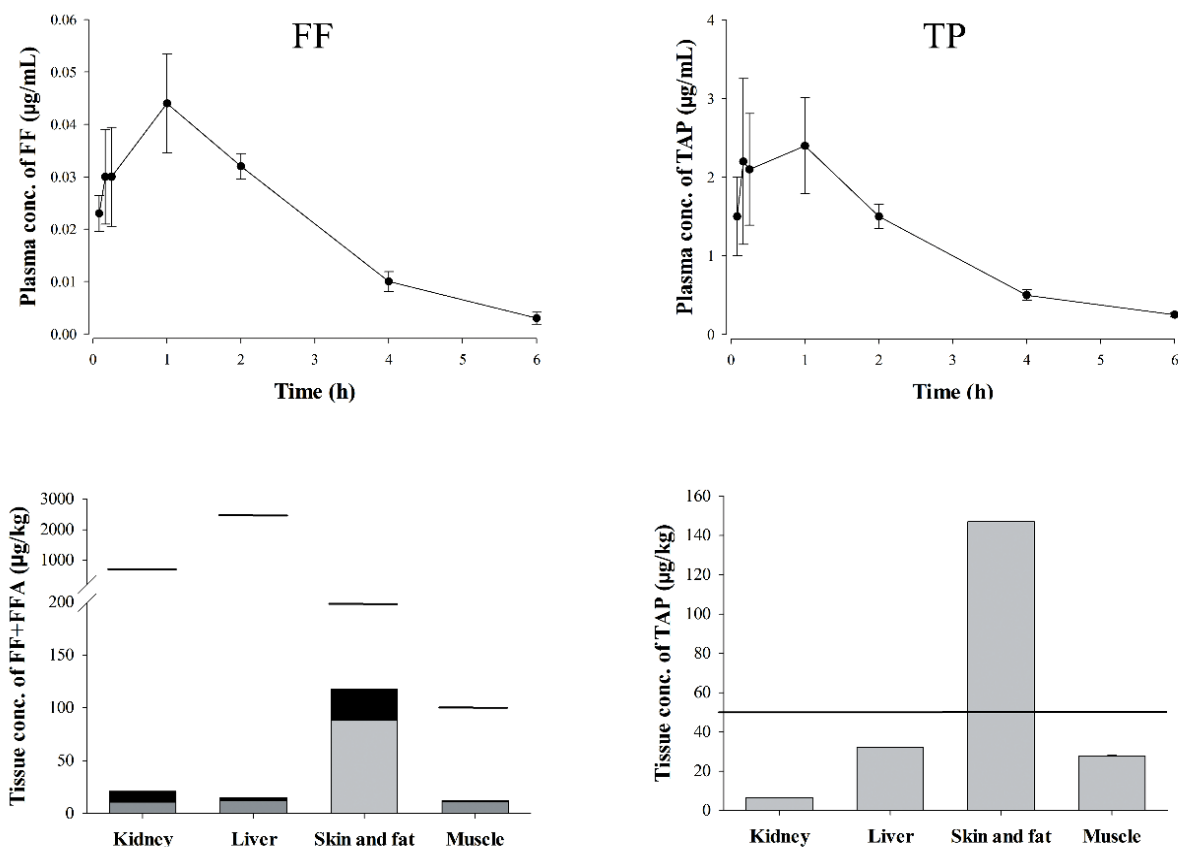
The pharmacokinetic study (Fig. 1, upper panel) revealed that combined intramuscular injection of FF-TAP (5+10 mg/kg) produced a  $C_{max}$  of 0.045 µg/mL and AUC of 0.13 h·µg/mL for FF (Table 1). By comparison, at the recommended dose for FF alone (30 mg/kg), the reported values for  $C_{max}$  and AUC are approximately two orders of magnitude higher with ranges of 3.82-8.85 µg/mL and 17.84-35.56 h·µg/mL, respectively (Afifi and Abo el-Sooud, 1997; Shen *et al.*, 2002; Ismail and El-Kattan, 2009). Likewise, the mean residence time (MRT) and the terminal phase half-life ( $t_{1/2\lambda z}$ ) of FF in this study (2.19 h and 1.15 h, respectively) were also shorter than those of the recommended dose (5.21 h and 2.15-3.40 h, respectively). Similar albeit smaller differences were detected for TAP when administered in combination with FF. In the current study,  $C_{max}$  and AUC for TAP were estimated as 2.64 µg/mL and 7.19 h·µg/mL (Table 1). By comparison, Guo *et al.* (2010) reported a  $C_{max}$  of 6.43 µg/mL and AUC of 19.02 h·µg/mL following oral administration of TAP at recommended dose of 30 mg/kg. Although the reductions in  $C_{max}$  and AUC are much greater for FF versus TAP, the differences are somewhat expected since the dose rate difference for combination versus single drug treatments was two-fold greater for FF (5 vs 30 mg/kg) than TAP (10 vs 30 mg/kg) and their concentration-time profiles were quite parallel, with the  $T_{max}$  around 1 h and the slope of the terminal phase about 0.5 h<sup>-1</sup>. This would be desirable for synergistic drug combination as their ratio for effectiveness could be maintained with minimum deviation.

In order to determine whether the reductions in  $C_{max}$  and AUC were associated with lower drug residues in tissues, a trial was conducted to assess drug concentrations in different tissues at 24 h following intramuscular administration of FF-TAP (5+10 mg/kg) combination. FF residue in the liver, kidney, breast

muscle, and skin with fat at 24 h post treatment were 12.0, 10.6, 10.8 and 88.4 µg/kg, respectively. Since the marker residue of FF in the tissues is FF parent compound plus its metabolite florfenicol amine (FFA) based on European Medicines Agency, we estimated the amount of FFA by using FF to FFA ratio at one day determined from a similar study (Anadón *et al.*, 2008) and the summations of FF plus estimated FFA were shown in Fig. 1 (lower panel). Concentrations of FF+FFA in liver (15.1 µg/kg), kidney (20.9 µg/kg), skin with fat (117.7 µg/kg), and breast muscle (12.2 µg/kg) were all substantially below the maximum residue limits (MRL) of 2500, 750, 200, and 100 µg/kg for each tissue, respectively. By comparison, tissue residues of FF+FFA following administration of the recommended dose have been reported to be approximately one to two order of magnitude higher; for example, the average 24 h FF+FFA level in kidney, liver, skin with fat, and muscle of chickens following 40 mg/kg FF administered orally for three days were 1250, 3006, 1040, and 569 µg/kg, respectively (Anadón *et al.*, 2008). Similar results were obtained with TAP, wherein the average tissue concentrations 24 h post drug treatment in kidney (6.5 µg/kg), liver (32.2 µg/kg) and breast muscle (27.9 µg/kg) were all below the accepted tissue MRL of 50 µg/kg established by European Medicines Agency. In contrast, TAP residue level in skin with fat (147.0 µg/kg) remained above the MRL (50 µg/kg) 24 h following drug treatment (Fig. 1, lower panel). Given that the FF residues were too low, the withdrawal time of the combination would be mostly dependent on TAP level. As the  $C_{max}$  and AUC of TAP in this study were approximately 2-3 times lower than those of the recommended dose (Guo *et al.*, 2010) while the elimination half-life was about the same, it was most likely that the withdrawal time of this combination might be shortened by at least 1 week (original withdrawal time 21 days according to Council of Agriculture (2014)). In fact, the one-day tissue residue data for both drugs indicated that most tissue levels were already below MRL after 1 day.

It should be emphasized that when used in combination, the doses of both FF and TAP could be decreased without compromising the antimicrobial efficacy as demonstrated by our previous studies (Wei *et al.*, 2016a, 2016b). Interestingly, further reductions in the doses of FF-TAP (either 2+4 mg/kg q 24 h or 5+10 mg/kg q 48 h) were found to be equi-effective to the recommended dose regimens of FF (either 5 mg/kg q 24 h or 15 mg/kg q 48 h) in preventing *P. multocida* infection in pigs (data not shown). In view of the synergistic actions of FF-TAP combination and the ability to use lower doses, the resultant reductions in plasma and tissue drug concentrations as well as its MRT and  $t_{1/2\lambda z}$  provide a clear advantage with respect to improved food safety owing to reductions in drug residues in edible tissues.

Although the withdrawal time of the combination was not determined in this preliminary study, the present study did indicate a few possible advantages of using synergistic FF-TAP combination in chickens as a means to achieve more rapid reductions in tissue drug residues. Consequently, further study with oral administration and design to estimate withdrawal time of the combination is justified.



**Fig. 1:** Plasma concentration-time profiles of FF and TAP (upper panel) when used in combination (5+10 mg/kg) and tissue residues of FF (grey) plus the estimated FFA (black) and TAP at 1-day post administration in different organs (lower panel). The horizontal lines indicate the MRL of the respective organs.

**Table 1:** Plasma pharmacokinetic parameters of FF and TAP in chickens after single IM administration of FF-TAP combination (the results were expressed as means±SD)

PK parameters <sup>1</sup>	FF-TAP (5+10 mg/kg)	
	FF	TAP
$\lambda_z$ (h <sup>-1</sup> )	0.61±0.11	0.45±0.09
$t_{1/2\lambda_z}$ (h)	1.15±0.20	1.61±1.37
$T_{max}$ (h)	1.0±0.0	0.47±0.46
$C_{max}$ (µg/mL)	0.045±0.010	2.64±0.75
$AUC_{0-\infty}$ (h·µg/mL)	0.13±0.02	7.19±0.96
$V_z/F$ (L/kg)	65.42±1.26	3.29±1.03
$CL/F$ (L/kg/h)	39.98±7.71	1.41±0.18
$MRT$ (h)	2.19±0.09	2.52±0.42

<sup>1</sup> $\lambda_z$ , slope of the terminal phase;  $t_{1/2\lambda_z}$ , half-life of terminal phase;  $T_{max}$ , time to maximum concentration;  $C_{max}$ , maximum concentration;  $AUC_{0-\infty}$  (the "∞" should be in the same line as "AUC<sub>0-</sub>"), area under the concentration-time curve extrapolated to infinity;  $V_z/F$ , volume of distribution relative to bioavailability;  $CL/F$ , clearance relative to bioavailability;  $MRT$ , mean residence time.

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**Authors contribution:** CCC conceived and designed the study and revised the manuscript. TR and MKH executed the experiment and analyzed the data and drafted the

manuscript. TWV revised the manuscript. All authors interpreted the data, critically revised the manuscript and approved the final version.

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