Research Note: Study on the residue depletion of febrifugine and isofebrifugine in broiler chicken

Ping Ping Li, Lei Zhang, and Jian Ping Wang[1]

College of Veterinary Medicine, Hebei Agricultural University, Baoding, Hebei 071000, China

ABSTRACT In this study, 105 broiler chickens were fed with dietary feeds containing different contents of *Dichroae Radix* extract for 10 consecutive days. Then the residue depletions of its main alkaloids (febrifugine and isofebrifugine) in muscle, kidney and liver samples at different withdrawal times were determined by an ultra-performance liquid chromatography method. Results showed that the 2 alkaloids were mainly at tissue-bound formation. At withdrawal period of 0 d, their concentrations in all samples were high but decreased rapidly after 1 day of cessation (35−91%). After 5 to 7 days of cessation, their residues in muscle and kidney were not detectable, and after at least 10 days of cessation they were not detectable in liver. These results indicated that an appropriate withdrawal time for *Dichroae Radix* preparation was required if it is licensed as a new drug, and the best target tissue for monitoring its residue was liver.

Key words: *Dichroae Radix*, febrifugine, isofebrifugine, residue depletion, chicken

2021 Poultry Science 100:101390
https://doi.org/10.1016/j.psj.2021.101390

INTRODUCTION

At present, the extensive use of chemical drugs to prevent and treat the diseases in food producing animals inevitably leads to their residues in foods of animal origin. Therefore, the finding appropriate alternative to replace the commonly used chemical veterinary drugs is very important. During the past decades, many traditional Chinese herbs have been proved to promote chicken growth and egg production, enhance the function of immune system, and improve antioxidant function (Li et al., 2017; Fu et al., 2018; Yang et al., 2019).

Dichroae Radix (Changshan) is a traditional Chinese herb that has been used to treat the fevers caused by malaria parasites in human being for several centuries, and its main medical ingredients are febrifugine and isofebrifugine. Recently, the extract and powder of *Dichroae Radix (Changshan)* have been proved to show high anticoccidial activity (Zhang et al., 2012; Guo et al., 2013) and be safe for chicken (Guo et al., 2020), so *Dichroae Radix (Changshan)* and its main ingredients are the promising coccidiostats. However, febrifugine and isofebrifugine have been proved to show some side effects (nausea, vomiting, and liver toxicity) (Jiang et al., 2005), so they have not been licensed as drugs so far. Still, the centuries of using this herb encourages many pharmacologists to develop potential new drugs with febrifugine as the lead compound (Sen and Chatterjee, 2013; Mai et al., 2014; McLaughlin et al., 2014).

However, if a natural herb and its ingredients are absolutely safe for food producing animals is unknown, and if they will cause their residues in foods of animal origin is also unknown. To the best knowledge of the authors, there has been no article reporting the residue depletion of herbal ingredient in food producing animal so far. In the present study, *Dichroae Radix (Changshan)* extract was used to feed broiler chickens at dietary feeds, and the residues of its main alkaloids febrifugine and isofebrifugine in 3 types of tissue samples were determined by ultra-performance liquid chromatography method (**UPLC**).

MATERIALS AND METHODS

Reagents and Chemicals

The standards of febrifugine and isofebrifugine were purchased from Huzhou Zhanshu Biological Technology Company (Zhejiang, China). *Dichroae Radix* extract was purchased from Zhengzhou Yinghe Biology Company (Henan, China), and the content of *Dichroa alkali* (febrifugine + isofebrifugine) was 4.0%. The standard stock solutions of febrifugine and isofebrifugine (100 μg/mL) and their working solutions (1.0−1,000 ng/mL) were prepared with mobile phase and stored at 4°C.

Received March 18, 2021.
Accepted July 20, 2021.

[1]Corresponding author: chinawangjp@hotmail.com

Animal Experiments

All the animal experiments were carried out at Animal Experiment Center of College of Veterinary Medicine, Hebei Agricultural University, and approved by the Ethics Committee for Laboratory Animal of Hebei Agricultural University. All the experiments were carried out by the professional operators according to the Regulation Guideline for Experimental Animals, Ministry of Science and Technology of China. One hundred and twenty 3-d Jinfeng broiler chickens were purchased from Dawu Poultry Industry Co., Ltd (Baoding, Hebei China), and they were fed with the homemade standard complete formula feeds for 10 days for acclimation. The animal room was equipped with continuous ventilation and heating system, and the temperature was adjusted according to their age. Their general health conditions were monitored by a veterinarian.

After acclimation, these chickens were divided into 4 groups randomly: group 1 (15 birds) as control animals, group 2 (35 birds) as low dosage animals, group 3 (35 birds) as medium dosage animals and group 4 (35 birds) as high dosage animals. The birds of group 1 were fed with no-medicated feeds. The birds of group 2, 3, 4 were fed with the medicated feeds containing 50, 100, and 200 mg of *Dichroae Radix* extract per kilogram of feed for 10 consecutive days respectively. During the medication period, all the chickens had free access to feed and water. During the experiment, the feed consumptions of group 2, 3, 4 were 7.99, 8.93, 8.29 kg, respectively, so the average dosages of *Dichroae Radix* extract for each bird in group 2, 3, and 4 were 11.4, 25.5, and 47.4 mg, respectively, and the *Dichroa alkali* (febrifugine + isofebrifugine) intakes were 0.456, 1.02, and 1.896 mg, respectively.

After the medication period, all birds were feed with nonmedicated feeds. At days 0, 1, 3, 5, 7, 10, and 14 after withdrawal of the extract, 5 birds from each medication group were selected randomly and euthanized by CO_2 asphyxiation. Immediately after slaughtering, the muscle, kidney, and liver samples were collected separately and stored at −20°C. These samples from the birds of group 1 were also collected as blank samples.

Sample Preparation

Before extraction, all the tissue samples were homogenized. For extraction, 2.0 g tissue homogenate, 2 mL water, and 2 mL trypsin (25 mg/mL) were added into a centrifuge tube, and the pH was adjusted to 7.6 with 10% sodium carbonate solution. The tube was put into a 40°C water bath to be stayed overnight. After that, 10 mL ethyl acetate was added to be stirred for 5 min and centrifuged at 8,000 rpm for 5 min. The ethyl acetate phase was transferred into a clean tube and 5 mL ammonium acetate solution (0.125 mol/L) was added to be stirred for 5 min. After centrifugation at 8,000 rpm for 5 min, the lower liquid phase was transferred into a clean tube and 10 mL *n*-hexane was added to be stirred for 1 min. After centrifugation at 5,000 rpm for 3 min, the lower liquid phase was used for the subsequent solid phase extraction.

The HLB cartridge was preconditioned by washing with 3 mL methanol, 3 mL water, and 3 mL ammonium acetate solution (0.125 mol/L). Then the sample extract was loaded onto the cartridge and passed through the cartridge at a flow rate of 1.0 mL/min. The cartridge was washed with 3 mL water and eluted with 8 mL methanol. The eluate was evaporated to dryness with cold nitrogen gas. The dry residue was dissolved in 0.4 mL methanol and filtered with a 0.22-μm membrane for UPLC analysis.

UPLC Conditions

The UPLC system contained a ACQUITY H-CLASS liquid chromatography, a fluorescence detector (**FLD**) and a BEH C_{18} column (2.1 × 50 mm, 1.7 mml; Waters, Milford, MA). The mobile phase consisted of acetonitrile, water, acetic acid, and triethylamine (5: 94: 0.35: 0.75, v/v). The operation conditions were as follows: injection volume, 10 μL; flow rate, 0.3 mL/min; excitation wavelength 270 nm and emission wavelength 335 nm. The qualitative analyses were according to the retention times of the standards of febrifugine and isofebrifugine respectively, and the quantifications were according to the chromatogram peak areas of the 2 standards.

The extracts from the blank samples were used to evaluate the UPLC method. The limits of detection (**LOD**) and the limits of quantification (**LOQ**) for the 2 analytes were defined as the concentrations equivalent to signal/noise of 3 and 10, respectively. The 2 analytes were fortified into the 3 types of blank samples to evaluate the intraday recoveries (mean result of 6 repetitive injections in a single day) and the interday recoveries (mean result of duplicate analysis on 6 successive days) respectively.

RESULTS AND DISCUSSIONS

Sample Extraction Method

There has been no article reporting the detection of febrifugine and isofebrifugine in animal tissues so far. Therefore, the extraction method for the 2 analytes was optimized firstly. During the experiments, the muscle samples from the high medication group at withdrawal time of 0 d were used as representative samples to optimize the sample extraction method. Results showed that the residue levels of febrifugine and isofebrifugine when using ethyl acetate were higher than that when using methanol, acetone and acetonitrile, so ethyl acetate was selected as the optimal extraction solvent.

For verification if there were tissue-bound febrifugine and isofebrifugine, their residues in the representative muscle samples when using and without using trypsin were compared. Results showed that the concentrations of febrifugine (540 ng/g) and isofebrifugine (409 ng/g) after enzymolysis were much higher than that without using enzymolysis (febrifugine 43 ng/g, isofebrifugine

Table 1. Residual concentrations of febrifugine and isofebrifugine in chicken samples at different withdrawal times (ng/g).

Group	Analyte	Sample	0 d	1 d	3 d	5 d	7 d	10 d	14 d
High medication group	Febrifugine	muscle	543	67	56	34	ND	ND	ND
		kidney	1109	97	54	39	ND	ND	ND
		liver	230	73	68	64	47	27	ND
	Isofebrifugine	muscle	406	72	45	23	ND	ND	ND
		kidney	1046	83	46	21	ND	ND	ND
		liver	282	80	67	62	49	37	ND
Medium medication group	Febrifugine	muscle	369	65	48	26	ND	ND	ND
		kidney	724	91	46	21	ND	ND	ND
		liver	176	67	63	54	35	21	ND
	Isofebrifugine	muscle	114	57	38	ND	ND	ND	ND
		kidney	634	71	36	ND	ND	ND	ND
		liver	132	75	63	51	33	20	ND
Low medication group	Febrifugine	muscle	181	62	40	20	ND	ND	ND
		kidney	452	71	31	22	ND	ND	ND
		liver	107	56	50	40	33	22	ND
	Isofebrifugine	muscle	69	40	36	ND	ND	ND	ND
		kidney	167	39	24	ND	ND	ND	ND
		liver	92	60	50	42	23	ND	ND

Abbreviation: ND, not detectable by this method.

46 ng/g), indicating about 90% of the 2 alkaloids were at tissue-bound formation. For verification of if the 2 alkaloids were metabolized in vivo, the ^{14}C-labeled febrifugine and isofebrifugine should be used to perform the special residue depletion experiments, which remained to be studied.

Method Performances

During the experiments, the matrix matched febrifugine and isofebrifugine solutions at series of concentrations were used to develop the calibration curves. Results showed that a good linearity in the range of 20 to 1,000 ng/mL for the 2 analytes was obtained. The LOD for febrifugine in muscle, kidney, and liver were 6, 7, and 6 ng/g, respectively, and the LOQ was 20 ng/g. The LOD for isofebrifugine in muscle, kidney, and liver were 7, 7, and 5 ng/g, respectively and the LOQ was 20 ng/g. For evaluating the accuracy and precision, febrifugine and isofebrifugine were fortified into the 3 types of blank samples for analysis respectively (10, 100, and 1,000 ng/g). The fortification levels were defined according to the maximum residue limits of halofuginone (a derivative of febrifugine that has been licensed as a coccidiostat): chicken muscle 100 μg/kg, liver 130 μg/kg, and kidney 130 μg/kg. Results showed that the recoveries of the 2 analytes from the standard fortified blank samples were in the range of 62.6 to 84.2%, and the coefficients of variation were in the range of 7.2 to 16.1%.

Residue Depletions

There has been no article reporting the residue depletions of febrifugine and isofebrifugine in food producing animals so far. Therefore, *Dichroae Radix* extract was used to feed broiler chickens at three medication levels to study the residue depletions of febrifugine and isofebrifugine in broiler chicken in the present study. The residual concentrations in muscle, liver, and kidney samples at different withdrawal times are shown in Table 1, and their depletion curves in these samples are shown in Figure 1.

For high medication group, the residual concentrations of febrifugine and isofebrifugine in these samples were high at withdrawal time of 0 d, and the highest concentrations were in kidney samples (Table 1). At withdrawal time of 7 d, the 2 analytes in muscle and kidney samples were not detectable, and after a 14-d withdrawal time the 2 analytes were not detectable in liver.

For medium medication group, their residues in these samples were also high at withdrawal time of 0 d, and the highest concentrations were also in kidney samples (Table 1). Similar to the results of high medication group, the 2 analytes were detectable in liver after 10 days of cessation, and were not detectable after a 14-d withdrawal time. Different from the results of high medication group, isofebrifugine was not detectable in muscle and kidney samples at withdrawal time of 5 d.

For low medication group, their residues in the 3 types of samples were also high at withdrawal time of 0 d, and the highest concentrations were also in kidney (Table 1). After 7 days of cessation, febrifugine was not detectable in muscle and kidney samples, and after 14 days of cessation it was not detectable in liver, which was similar to the results of high and medium medication group. However, isofebrifugine was not detectable in muscle and kidney samples at withdrawal time of 5 d, and was not detectable in liver at withdrawal time of 10 d.

As shown in Figure 1A, the depletion curves of febrifugine in the three types of samples at the 3 medication groups were very similar. Its residue levels decreased rapidly after 1 day of cessation, and the concentrations decreased about 66 to 88% in muscle, 85 to 91% in kidney and 48 to 69% in liver. After then, its residues decreased slowly. As shown in Figure 1B, the depletion curves of isofebrifugine in the 3 types of samples at the 3 medication groups were similar to that of febrifugine. At withdrawal time of 1 d, its residues decreased rapidly

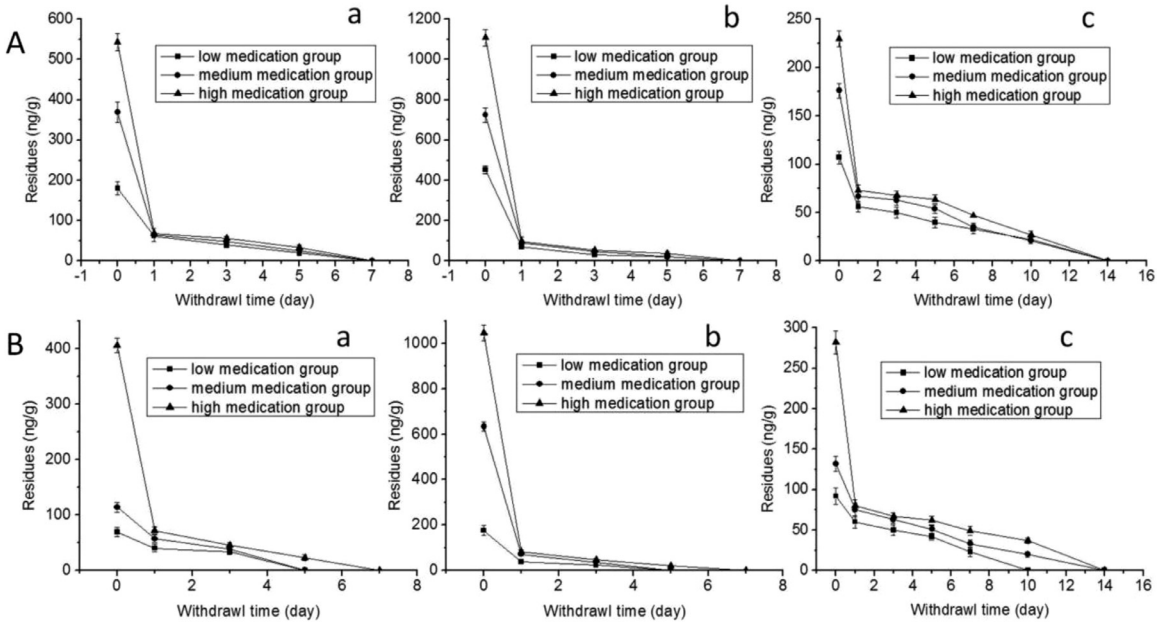

Figure 1. Depletion curves of (A) febrifugine in (a) muscle, (b) kidney and (c) liver, and that of (B) isofebrifugine in (a) muscle, (b) kidney, and (c) liver.

(42−82% in muscle, 78−92% in kidney, and 35−72% in liver), and after then its residues decreased slowly.

Based on the results of Table 1 and Figure 1, the following 5 points were concluded. First, the residue depletions of febrifugine and isofebrifugine in broiler chicken were comparable. Second, the 2 alkaloids were depleted rapidly within the 24 h after withdrawal of medication. Third, the 2 alkaloids were depleted rapidly in muscle and kidney but slowly in liver. Fourthly, liver sample was the best target tissue for inspection of their residues. Fifth, an appropriate withdrawal time for the 2 alkaloids should be set if they are licensed as new drugs in the future, which remained to be studied.

Dichroae Radix (Changshan) has been proved to show high anticoccidial activity. As its main ingredients, febrifugine and isofebrifugine have been extensively studied with the aim of developing a new drug. This paper for the first time studied the residue depletions of febrifugine and isofebrifugine in broiler chicken, and the obtained results provided some bases to evaluate their safeties for foods of animal origin and their possibilities being licensed as new drugs for food producing animals.

ACKNOWLEDGMENTS

The authors are grateful for the financial support of Hebei Layer/Broiler Industry Technology System (HBCT2018150210)

DISCLOSURES

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

REFERENCES

Fu, C., Y. Zhang, and Q. Jing. 2018. Effect of Chinese herbal medicine on growth performance, immune organ index and antioxidant functions in broiler chickens. Int. J. Agric. Biol. 20:1677–1681.

Guo, Z., J. Liang, X. Wei, R. Shang, W. Guo, X. Wang, and B. Hao. 2013. Curative effect of Radix dichroa extractive on coccidiosis in chicken artificially infected by Eimeria tenella. Chin. J. Vet. Sci. 33:1083–2085 (in Chinese).

Guo, Z., L. Wang, K. Zhang, K. Zhang, H. Liu, H. Xu, J. Li, and J. Liang. 2020. Target animal safety test of Changshan powder in chickens. Chin. Vet. Sci. 50:1176–1182 (in Chinese).

Jiang, S., Q. Zeng, M. Gettayacamin, A. Tungtaeng, S. Wannaying, A. Lim, P. Hansukjariya, C. O. Okunji, S. Zhu, and D. Fang. 2005. Antimalarial activities and therapeutic properties of febrifugine analogs. Antimicrob. Agents Ch. 49:1169–1176.

Li, C., Y. Dong, Z. H. Li, R. Zhang, R. Qin, W. Li, and W. Shi. 2017. Effects of traditional Chinese herbal medicines on egg production, egg quality and antioxidant enzyme activities in aged laying hens. Indian J. Anim. Res. 51:327–331.

Mai, H. D. T., G. V. Thanh, V. H. Tran, V. N. Vu, V. L. Vu, C. V. Le, T. L. Nguyen, T. D. Phi, B. N. Truong, V. M. Chau, and V. C. Pham. 2014. Synthesis and biological evaluation of febrifugine analogues. Nat. Prod. Commn. 9:1717–1720.

McLaughlin, N. P., P. Evans, and M. Pines. 2014. The chemistry and biology of febrifugine and halofuginone. Bioorga. Med. Chem. 22:1993–2004.

Sen, D., and T. K. Chatterjee. 2013. Pharmacophore modeling and 3D quantitative structure-activity relationship analysis of febrifugine analogues as potent antimalarial agent. J. Adv. Pharm. Technol. Res. 4:50–60.

Yang, C., Y. Liao, F. Wang, J. Yang, and Z. Wu. 2019. A Review of Chinese herb medicine additives on chicken growth performance. Agr. Biotechnol. 8:48–51.

Zhang, D. F., B. B. Sun, Y. Y. Yue, Q. J. Zhou, and A. F. Du. 2012. Anticoccidial activity of traditional Chinese hebal Dichroa febrifuga Lour extract against Eimeria tenella infection in chickens. Parasitol. Res. 111:2229–2233.