Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.052

RESEARCH ARTICLE

Estimation of Florfenicol Residues in Layer Meat and Egg Samples using High Performance Liquid Chromatography

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ARTICLE HISTORY (17-234)

Received: July 06, 2017
Revised: April 01, 2018
Accepted: April 20, 2018
Published online: June 26, 2018

Key words:
Eggs
Florfenicol
Layer
Meat
Residues

A B S T R A C T

Antibiotics are widely used in the poultry industry to enhance the health and productivity of flocks which may have adverse effects on consumer's health. It is necessary to screen food products from animal origin for antimicrobial residues to safeguard the consumer's health. The present study was aimed to detect florfenicol (FF) residues in meat and egg samples of layer birds. For this purpose 150 meat and eggs samples were collected in equal ratio. High performance liquid chromatography (HPLC) was used to determine residual concentration of FF in meat and egg samples at wavelength of 223 nm. Ethyl acetate and phosphate buffer saline solution were used for extraction of FF from the samples. The mobile phase contained acetonitrile and water (27:73 v/v). Mean residual concentrations of FF as 61.56±13.19 and 281.08±57.46 µg/kg in meat and egg samples was detected. This study also showed that 80% (60) meat and 72% (54) egg samples were FF residue positive, out of these 86.7% (52) meat and 55.6% (30) egg samples were found to have residual concentrations above maximum residual limits. This contaminated meat may cause public health issues. There is a need to develop legislation about residual concentration of drugs in animal food products in Pakistan as well as to inform formers about the detrimental effects of drug residues on human health.

To Cite This Article: Fahim A, Aslam B, Mohsin M, Raza A, Faisal MN and Hussain A, 2018. Estimation of florfenicol residues in layer meat and egg samples using high performance liquid chromatography. Pak Vet J, 38(3): 329-332. http://dx.doi.org/10.29261/pakvetj/2018.052

INTRODUCTION

The poultry business is one of the main ventures of Pakistan. Form last few years, poultry meat production has increased at a rate of 20-25% per annum. In Pakistan, poultry industry is providing meat which contributes 19% of the total meat production. Lack of a disease control program is one of the desolate problems that is being faced by this industry (Shah and Korejo, 2012). The food produced by animals carries antibiotic residues. Parent therapeutic compound, metabolites and conjugates can accumulate in tissues of animals known as residues (Adewuyi *et al.,* 2011; Sajid *et al.,* 2016). The continuous usage of antibiotics in poultry farms causes major health problems in consumers. Allergies, resistance of microbes to drugs, carcinogenic effect and the potential adverse effect on human intestinal microflora can occur by consuming the low doses of antibiotics for long periods (Nasri *et al*., 2012; Boamah *et al.,* 2016).

Chloramphenicol (CAP), florfenicol (FF) and thiamphenicol (TAP) are broad-spectrum antibiotics from class Amphenicol (AP). APs are extensively used in veterinary practices for the cure of bacterial infections. FF is safer as compared to CAP. Now-a-days, food producing animals are treated with FF for improving the antibacterial activity, which is an alternative to CAP (Tao *et al.,* 2013). In the poultry industry, FF is preferred over some antibiotics because of its good pharmacological and pharmacokinetics characteristics (Shaheen and El-Far, 2013). It shows its activity at smaller concentrations as compared to CAP and TAP. The occurrence of FF residues in tissues may increase the resistance to bacteria. It can produce deleterious effects on human health (Wu *et al.,* 2008). The administration of this drug should not be allowed in eggs producing animals (Fodey *et al.,* 2013).

Due to the continuous administration of AP to animals available for human consumption, residues of AP in edible tissues can produce harmful effects on human health (Evaggelopoulou and Samanidou, 2013). It is needed to protect the health of consumer against possible deleterious effects of residues of veterinary medicines (Mamani *et al.,* 2009). A maximum residue limit (MRL)

has been set by the EU as the tolerance level of the compound to maintain the safety for food production. FF has a MRL of 100 µg/kg for chicken muscle and 2500 µg/kg for chicken liver. However, the EU has not provided the MRL value for FF in eggs (Xie *et al.,* 2012).

As food matrices are complex, there is need to develop highly sensitive and selective detection method. In food matrices, high performance liquid chromatography (HPLC) is used for the determination of antibiotic residues (Sadeghi and Jahani, 2013; Aslam *et al.,* 2016). In Pakistan, availability of excessive amount of antibiotic residues in meat is due to lack of enforcement of regulation by government (Mumtaz *et al.,* 2000). There is no proper detection system, which can determine the level of antibiotics in animals and public in a short period. Therefore, this study aims to determine FF residues in poultry products by using the simple and efficient detection method.

MATERIALS AND METHODS

Area for sampling: Faisalabad is a big district of Pakistan and has a large number of poultry farms therefore Faisalabad was chosen for sampling with the aims to determine the presence of FF residues in layer's meat and egg samples.

Collection and storage of samples: Random sampling method was used to collect the samples. Fifteen layer farms were selected randomly throughout the Faisalabad, then five birds (for meat samples) and five egg samples were randomly collected from each farm. After aseptic collection of samples these were transferred to self-sealing polythene bags and were labeled properly. These samples were then moved to pharmacology laboratory, Department of physiology and pharmacology, University of Agriculture Faisalabad under chilled conditions and stored at -20ºC and 4ºC for meat and eggs respectively till analysis.

Equipment and Instrumentation: The chromatographic system consisted of BDS Hypersil C_{18} column (250 mm, 4.6 µm, 5 µm), UV-visible detector, Solvent delivery system, sonicator, Homogenizer, Centrifuge machine and Syringe filters.

Preparation of standard stock solution and working dilution: FF stock of 1 mg/ml was prepared in acetonitrile. Then serial dilutions were prepared from this stock solution to get concentrations between 10-100 µg/ml in acetonitrile. Each dilution (20 µl) was injected in the HPLC system for analysis. The best fit of line was calculated by equation of line. Linearity was evaluated through correlation coefficient. Standard calibration curve of FF is shown in Fig. 1.

Sample Preparation: The analytical procedure of Wang *et al*. (2009) was carried out on both meat and eggs samples.

Thawing: Firstly, the meat samples were thawed properly. Then the samples were crushed for several minutes with the help of pestle and mortar. Egg samples were homogenized with the help of Homogenizer.

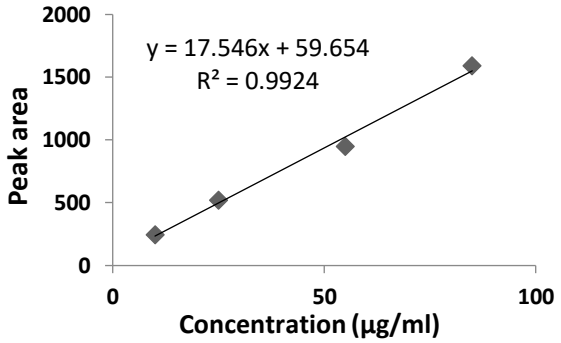

Fig. 1: Standard curve of florfenicol (10-100 µg/ml) using HPLC (n=5).

Pretreatment of sample: 5 g of meat and egg samples were weighed separately and placed into 40ml centrifugation tube. Then, 5 ml of Phosphate Buffer Saline (PBS) solution and 20 ml of ethyl acetate (EA) were added into that centrifugation tube. The mixture was homogenized for 3 minutes for mixing all contents thoroughly. Homogenization of sample was performed at temperature below 4ºC.

Extraction of residues: The mixture was then centrifuged at 1500 g for 20 minutes. The supernatant was taken in a clean test tube. The extraction step was repeated twice to take an adequate volume of extraction. Then, evaporation of extracts was carried at 60ºC by using steam of nitrogen and dried contents were left in centrifugation tube.

Cleanup process: The dried contents were reconstituted with 3 ml of mobile phase and 2 ml of n-hexane and solution was homogenized to mix all the contents. The defating of extracting solution was done with n-hexane. The solution was centrifuged at 4000 g for 10 minutes for separating the organic layer from aqueous layer. The resulting aqueous solution was filtered through a cellulose nitrate membrane syringe filter having a diameter of 13mm with pore size of 0.45 µm placed in filtration assembly.

Analysis: HPLC with UV-visible detector was used for analysis of eluted sample of FF. 20µl of the solution was taken and injected into the HPLC system. C18 column was used for separating the analyses. The flow rate was kept at 1.0 ml/min. The UV detector was adjusted at wavelength of 223nm (Wang *et al.,* 2009). The representative chromatograms of standard, meat and egg are shown in Fig. 2. The mobile phase was having acetonitrile and water (27:73 v/v).

Formula for calculation of concentration: The concentration of FF was determined by using the formula:

$$FF(\mu g/kg)=\frac{(y-b) \times V}{m \times W}$$

Where, y = Peak area of extracted sample, b = Intercept of standard curve, V = Volume of sample extract in ml, m = Slope of standard curve, W = Weight of sample in grams.

RESULTS

In present study, random sampling of layer's meat and eggs was performed from the different poultry farms

in vicinities of the Faisalabad district. Meat and egg samples contaminated with residues of FF were taken as positive samples. Whereas, those samples in which FF residues were not detected, were taken as negative samples. The results demonstrated that 114 (76%) out of 150 samples were having FF residues. Out of these 60 (80%) positive meat samples, 52 (86.7%) samples were having residual concentration above the MRL as shown in Table 1. There has not been established MRL for FF in poultry egg. But, based on the MRL residual concentration of FF in meat, the obtained results showed that egg samples have high concentrations of FF which may produce potential risk for a consumer's health. 54 (72%) egg samples positive for FF residues and out of these 30 (55.6%) egg samples had residual concentration above the MRL, and 24 (44.4%) egg samples were having residual concentration below the MRL. The result obtained from the present study indicated that high concentration of FF residues prevails in layer's meat and eggs. The meat and egg samples showed an average mean concentration of FF 281.08±57.46 and 61.56±13.19 µg/kg respectively shown in Table 1. The concentration of FF analyzed in all 15 commercial poultry farms have been shown in Table 2. The result described that the average mean concentration of FF in meat samples are much higher than average mean concentration of FF in egg samples.

DISCUSSION

Layers are important source of protein in the form of eggs and food for the consumers. FF is veterinary drug which is widely administered to chickens for therapeutic purpose (Xiao *et al*., 2015). Although it is safer drug as compared to other members of AP, but may lead to accumulation of drugs in animal tissues. The residues of FF and its metabolites can deposit in the eggs laid by hens after continuous administration of the drug (Sattar *et al*., 2014; Barreto *et al*., 2016). The safety of the consumer's health is maintained by adjusting the elimination period of FF. Only those eggs are safe to consume for humans, which are laid after a prescribed elimination period of antibiotic.

All of egg samples have a concentration of FF which ranges between 51.45-201.83 µg/kg. Two poultry farms have shown unusual high concentrations which indicate that these two poultry farms might be administrated FF irrationally. According to the literature, residues of FF were detected in egg samples where the mean concentration of drugs was much higher than the MRL (Xie *et al*., 2011). But according to this study, the maximum 176 mean concentration of FF is lower as compared to previous study, but it is also much higher which make it unfit for human consumption. In this study, 72% of egg samples have shown positive results, whereas only 28% of samples presented negative results. Another study showed the presence of FF in eggs as 57% of the drug was eliminated from egg yolk (Filazi *et al.*, 2014).

All of meat samples have concentration of FF ranged between 106.1-707.28 µg/kg. The results of the present study can be confirmed by comparing with data from previous study (Nasim *et al.*, 2016). According to current study, the residual concentration of FF is 164 much higher as compared to the previous study conducted by Zhang *et al*. (2008). The current study shows that 80% of meat

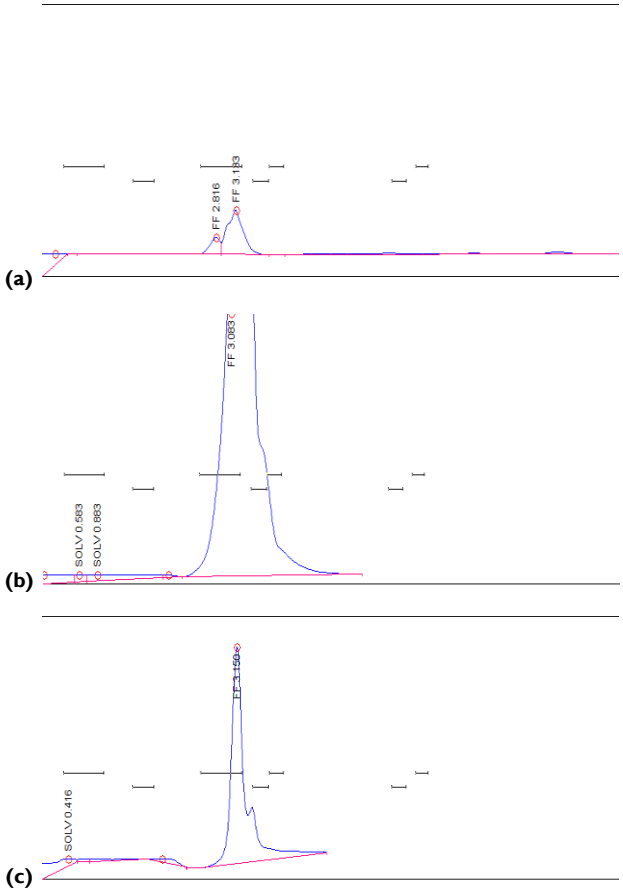

Fig. 2: HPLC chromatograms (UV detector at 223 nm) for (a) florfenicol standard (100 µg/kg) (b) spiked meat sample (c) spiked egg sample from layer chicken analyzed by HPLC.

samples are positive, this percentage is much higher than result obtained from study conducted by Tao *et al.*, (2013). The high mean concentration of drug is shown by the present study which is supported by previous literature of Shen *et al.*, (2009). The residual concentration obtained from the present study is much higher as compared to a previous study which was conducted by Kowalski *et al.*, (2011) on the samples of chickens collected from the local market of Poland.

The percentage of positive samples of egg is slightly low as compared to positive meat samples. This shows that the tendency of accumulation of drug in eggs is same as in other tissues. The albumin and yolk of egg have accumulation of FF which depends upon the duration of administration and its nature. Due to lipophilic nature, FF can pass easily through egg albumin and yolk. According to one of preliminary study, the concentration of the drug increased slowly in the highest value in yolk after single administration of the drug orally (Giorgi *et al.*, 2000). In a current study, a whole egg (yolk + albumin) was taken in analyzing the residues of FF where the residues of drug may be equally distributed throughout the albumen and the yolk.

The non-observance of elimination period of drug, usage of overdose of antibiotics, lack of implementation of legislative regulations, usage of antibiotics without following label, easy accessibility of antimicrobials to lay man and the lack of cognizance of consumer related to human health which is affected by usage of animal producing food containing residues of drugs may become the cause of the prevalence of residual concentration of drug which is higher than the MRL. This study shows that

Table 1: Florfenicol residual data in layer chicken samples analyzed by HPLC (n=150)

Type of Tissue	Positive samples	Negative samples	Samples above MRL	Samples below MRL	Concentration of FF	
					Mean	SD
Meat	60/75 (80%)	15/75 (20%)	52 (86.7%)	8 (13.3%)	281.08	57.46
Egg	54/75 (72%)	21/75 (28%)	30 (55.6%)	24 (44.4%)	61.56	13.19
Total	114 (76%)	36 (24%)	82 (72%)	32 (18%)	-	-

Table 2: Concentrations (mean ± SD) of florfenicol in meat and egg samples obtained from 15 poultry farms in district Faisalabad

Farms	Meat sample (µg/kg)		Egg sample (µg/kg)	
	Mean	SD*	Mean	SD*
1	106.10	06.93	51.45	08.56
2	258.90	26.17	177.78	43.23
3	707.28	347.19	11.28	01.72
4	126.65	25.66	57.45	06.23
5	168.30	30.42	32.78	06.54
6	193.56	39.26	43.19	07.82
7	265.67	34.70	94.60	05.94
8	340.44	77.04	54.83	21.15
9	221.00	22.78	52.47	05.70
10	153.37	51.85	52.68	10.56
11	438.44	60.88	46.27	19.55
12	451.36	54.48	23.22	09.51
14	363.23	54.66	201.83	40.06
15	421.97	29.99	23.49	11.26

*SD: Standard deviation (n=5).

28% of meat samples have residual concentration lower than the prescribed maximum residue limit while 72% of samples present concentration of FF higher than the MRL. The estimated values of concentration of FF indicate high percentage of deviation from the prescribed MRLs. Therefore, there is need to prolong the elimination period and adjust the dosage of FF for laying chickens.

As FF is highly lipophilic therefore, the chance of the prevalence of residues of antibiotic in tissues of layer's meat and egg is increased. The chances of presence of residues of FF in the liver are more as compared to those in muscles because a large amount of FF undergoes metabolism through cytochrome P450 and its major metabolite, FF amine is formed. FF can persist in bile and require long periods to remove from the bile with feces. The factors which affect the residual concentration of drug in tissues of poultry are a pH of the body, temperature of body, quantity of dose of the drug and nature of diet and water intake. The irrational usage of FF in poultry farms of layers is indicated by high prevalence of residues of antibiotic in muscles and egg tissues. FF residues are bounded with tissues in the form of FF amine. The FF amine is required to eliminate from muscles, liver and kidney. This can be done with increasing the withdrawal period of drug by taking the vitamin C in feed that will enhance the performance of the kidney.

Conclusions: In conclusion, the present study indicated that FF is present above the MRL obtained from most of commercial poultry farms that may cause public health threat. So, there is a need to develop legislation about residual concentrations of drugs in animal food products in Pakistan as well as to run awareness campaigns among farmers, veterinarians and end users. There is also a need of continuous monitoring of other antimicrobial residues in poultry tissues in cooked and raw form.

Authors contribution: AF and AR performed the whole experiment. BA, MNF and MMG conceived and designed the experiment. AR analyzed the data and drafted the manuscript. All the authors read and approved the final version of the manuscript.

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