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ORIGINAL ARTICLE

High performance liquid chromatographic apprasial of doxycycline residues in commercial broilers

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Abstract

Antibiotics are used for therapeutic purpose as well as animal feed additive in poultry feed. Doxycycline (DOX), a member of the tetracycline group of antibiotics has been extensively used as an antimicrobial growth promoter as well as in the treatment of diseases in poultry industry. This study was aimed to determine DOX residues in poultry meat and liver samples using high performance liquid chromatography. The maximum residual limit (MRL) of DOX in muscle and liver tissues of broiler was 200 and 600 µg/kg, respectively. The results showed that 89% of total samples (n=150) were DOX residue positive. Out of these 96% samples were below MRL while 4% samples were above MRL. From the meat samples (n=75), 91% samples were positive while only 3% samples had residual concentration above MRL. Similarly, from liver samples (n=75), 87% samples were positive for DOX residues where as 5% samples had residual concentration above MRL. The outcome of present study provides valuable baseline for health regulatory authorities to establish MRL for antibiotic growth promoters which are being used most extensively in commercial poultry farming.

Keywords

Antibiotics
Doxycycline
Maximum residual limit

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Introduction

The poultry business is one of the biggest and quickest developing agro-enterprises in the world though poultry meat is the second most broadly used meat. The significance of poultry industry is because of increase in demands of meat at low price. The worldwide production of poultry meat rising ceaselessly at the annual growth rate of 4% (Khalda et al., 2013). Production of poultry meat has been increased with the use of antibiotics as growth promoters. These antibiotics play an important role in increasing body weight of broilers by inducing the generation of vitamin B-complex in their digestive system (Wijayanti et al., 2011).

Tetracyclines including doxycycline (Dox), as antimicrobial growth promoters, are still used in USA for the treatment and prevention of various livestock diseases (Ramesh et al., 2010). It is given orally as feed additive in broilers it gets absorbed by the intestinal wall and then enters the systemic circulation and distributed across the whole body. The distribution of the drug can be measured by numerous pharmacokinetic parameters i.e. volume of distribution (V_d). The V_d can be calculated with the help of physicochemical properties of drug, tissue and plasma protein binding capacity of drug and physiological condition of the animal (Vandenberge et al., 2012). The unfriendly utilization of antibiotics raises the danger of multiple resistances to various groups of anti-microbials. Antimicrobial resistant genes in animal pathogens also transmitted to human pathogens (Dibner & Richards, 2005). Drug resistance is caused by residues of these drugs (Abedullah & Bukhsh, 2007).

Most countries in the world have laid down clear and detailed procedures for the establishment of maximum residue limits (MRL) of antibiotics in foodstuffs of animal origin. According to FAO/WHO

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standards, the maximal residual concentrations of tetracycline, oxytetracycline, chlortetracycline and DOX must not be greater than 200, 600, 1200 µg/kg in muscle, liver and kidney, respectively (Joint et al., 2004; Shalaby et al., 2011). At present, there are various methods used to detect antibiotic residues in animal-derived food, including microbiological, spectrophotometric, capillary electrophoresis, and high performance liquid chromatography (HPLC) methods as well as hyphenated techniques (De Wasch et al., 1998; Zhang et al., 2014). The detection of antibiotic residues in foodstuffs of animal origin also requires appropriate and robust sample preparation, which is a prerequisite for the accuracy and sensitivity of detection methods, prior to HPLC analysis.

In Pakistan, no strict rules are being followed regarding use of anti-microbials in food producing animals (Abedullah & Bukhsh, 2007). The aimless utilization of DOX without observing their withdrawal period may make risk for human wellbeing throughout the country. In the previous literature it has been shown that the residues of DOX might be accumulated in broiler meat and liver, so the present study was conducted in Faisalabad metropolis to scrutinize its residues in broiler meat for the safety of consumers.

Materials and Methods

Chemicals and reagents: Chemicals and reagents were deionized water, acetonitrile, trichloroactic acid, trifluoroacetic acid. Oxytetracycline internal standard (99% pure) was purchased from SHIFA Laboratories LTD, Lahore, Pakistan and DOX standards (99% pure) was purchased from NEUTRO Pharma LTD, Lahore.

Sample collection: Meat and liver samples of five birds were collected from various farms around Faisalabad, Pakistan and transferred to self sealing polythene bags and stored at -20°C till analysis.

Preparation of standard stock solution: A stock solution of 100 ppm was prepared in deionized water which was further used for making working dilutions from 1 to 0.001 ppm. Mobile phase was composed of acetonitrile and deionized water (50:50) while trifluoracetic acid was used to maintain pH 2.5. The wavelength 350 nm was used for detection (Castro et al., 2009).

Sample preparation: Muscle/liver sample (4 gram) was crushed using pestle and mortar for several minutes and transferred to 15 ml centrifugation tube (Elkholy et al., 2009). 1 ml of 10 µg/ml internal standard solution (oxytetracycline) was added in centrifuge tubes (Castro et al., 2009). Samples were mixed for one min with the vortex mixer and filtered using Buchner funnel. Then 1 ml of 10% trichloroacetic acid solution was added to crushed samples in centrifuge tube. This mixture was again vortexed for one minute. The mixture was then centrifuged for 10 minutes at 13000 rpm. After centrifugation the supernatant was collected and filtered (0.45 µm). The residues were ready for HPLC analysis (Elkholy et al., 2009). Representative chromatograms of DOX residues in broiler samples are shown in Figure 1a, and b. The residual concentrations of DOX were calculated in each sample using the formulae:

$$\text{Amount of in each sample}\left(\frac{\mu g}{kg}\right) = \frac{(y-b)\times V}{M\times W}$$

Where, y=peak area of extract sample; b=intercept of standard curve; V=volume of sample extract in ml; M=slope of standard curve; W=weight of sample in gram

Linearity and calibration curve: The linearity for DOX was obtained from the calibration graphs, which is composed of six points by plotting peak areas of DOX standards having concentrations from 0.001 to 1 ppm. The correlations of determination (R^2) for all DOX were more than 0.99 as presented in Figure 2. The calibration curve was developed by plotting peak area vs concentration with the help of Microsoft office 2013.

Precision: The repeatability of samples with proposed method was assessed by repeating the standard DOX solution (0.3 µg/g) at different time interval on same day and on three different days.

Recovery: In order to determine the recovery, 10 g of blended samples were spiked with 0.01 µg/g of standards DOX to the samples followed by homogenization for 1 min which range from 85.2 to 98.7 %. The % recovery was found to be satisfactory.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD was 0.009 µg/ml while LOQ was 0.028 µg/ml.

Results

The outcomes of this study showed that 133 (89%) samples were DOX residues positive while 17 (11%) samples were DOX residues negative. From the positive samples 5 (4%) sampels were above MRL whereas 128 (96%) samples were below MRL as presented in Table 1. The concentrations in meat and liver samples are presented in Figure 3. Concentration of DOX in muscle samples of 15 poultry farms was ranged from 7.15 to 204 µg/kg while in liver samples was ranged from 9.16 to 681.81 µg/kg. The main reason for the presence of DOX residues in positive samples is that DOX is used as antimicrobial growth promoter at sub therapeutic doses in broilers on daily basis. So irrespective of the time and day of sample collection, DOX residues would be present in maximum samples.

Table 1: Dox residual data in poultry bird samples.

Type of Tissue	Positive samples	Negative samples	Samples above MRL	Samples below MRL	MRL (µg/kg)	Con. range (µg/kg)
Meat	68/75 (91%)	7/75 (9%)	2 (3%)	66 (97%)	200	7.15-204
Liver	65/75 (87%)	10/75 (13%)	3 (5%)	62 (95%)	600	9.16-681.81
Total	133 (89%)	17 (11%)	5 (4%)	128 (96%)	-	-

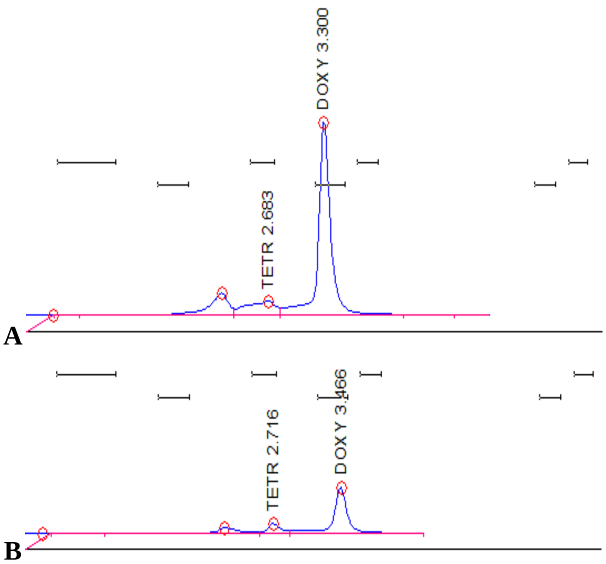

Figure 1: HPLC chromatograms of DOX residues (a) spiked liver sample (b) spiked meat sample from poultry birds.

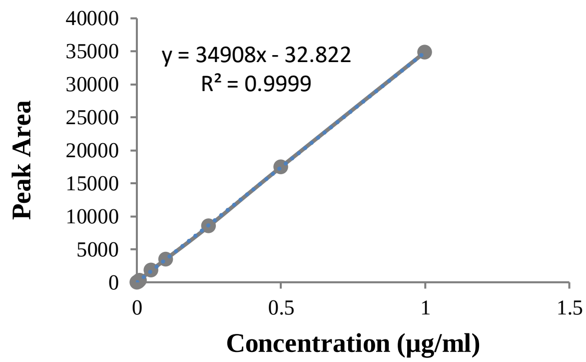

Figure 2: Graphical representation of standard curve of Dox.

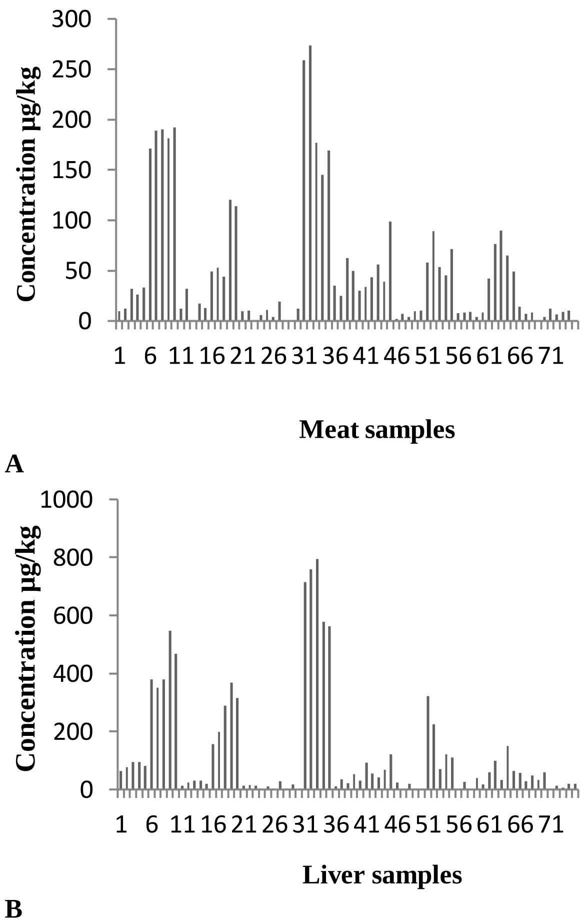

Figure 3: Dox residual concentrations in poultry (a) meat and (b) liver samples (n=75).

Discussion

Antibiotic consumption in food animals was first applied in the 1950s in order to fulfill the meat requirement (Allen et al., 2013). They are used therapeutically for the prevention of hazardous diseases as well as antimicrobial growth promoters in development and production of food animals (Taylor, 1999). Among various groups of antibiotics, tetra-cycline was the first class of broad spectrum antibiotics which was effective against both gram negative and gram positive bacteria. Tetracyclines have been sold as animal feed additives and for therapeutic purpose in food animals in USA for the years 2007 and 2009 (Abou-Raya et al., 2013). Recently there is an increasing awareness of the hazards of using food products with multiple drug residues. Many of these drugs are now categorized as toxic, carcinogenic and allergenic (Er et al., 2013). Consumption of such meat containing drug residues have serious health threats to human beings (Shareef et al., 2009). DOX, a memeber of tetracycline group, is a lipophilic drug and well absorbed from the gastro-intestinal tract (Yoshimura et al., 1991) from where it is widely distributed in the body tissues and found in higher concentration in the liver and kidney with high tissue binding (Santos et al., 1997).

The present study aimed to estimate the DOX residues quantitatively in raw chicken meat and liver using the HPLC-UV method. Our results showed that concentration of DOX residues in liver samples was greater than the concentration in muscle samples Figure 3a and b. DOX had higher residue concentrations in the liver compared to muscle which reflect feed/tissue transfer ratio (Atef et al., 2002; Ismail & El-Kattan, 2004). The results of the present study also showed that overall farm mean concentrations of DOX were 0.8-1.2 times greater than recommended MRL. The results of our study were similar to the other studies (Cetinkaya et al., 2012). As in our investigation overall 3% meat and 5% liver samples were above MRL however it is currently recognized that the incidence of residues violation should not be exceeded 1.0% of the samples and that levels above it indicate improper use of the antimicrobial agents, especially the failure to adhere the recommended withdrawal times (Tanner, 2000). Similar kind of studies also conducted to find the resiudes in the local chicken meat to find the antibiotic residual concentrations (Aslam et al., 2016; Nasim et al., 2016). The residual concentration of the DOX at the age of slaughtering indicating that this drug is continuously used throughout the production cycle.

These residual concentrations in the meat products may produce cross resistance in human pathogens. There are many reports showing that resistance to antibiotics may arise in animals which is being transferred to human pathogens due to irrational use of these antibiotics (Holmberg et al., 1984; Al-Ghamdi et al., 1999). In addition, this may also induce immunological response in susceptible individuals. So, overall the aim of the present study was to provide the base line for the residual concentrations for the awareness of the poultry producers and legislating authorities about the missuse of the anitbiotics and their side effects.

Conclusion: This study was focused on the importance of screening of DOX residues in food from animal origin for maintaining their safety and quality. The analytical method was rapid, reliable, sensitive, precise and accurate, which was applied for the screening of residues of DOX in animal tissues. Consequences which were obtained from broiler muscle and liver samples collected from vicinities around Faisalabad division showed that there is a risk of exposure of public to the residues of DOX.

Conflict of interest: All authors declare no conflict of interest.

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