

DETERMINATION OF MINOCYCLINE RESIDUES IN CHICKENS USING HPLC

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Abstract: Residues of veterinary drugs in poultry meat have serious health effects on humans (e.g., increase antimicrobial resistance, carcinogenicity, mutagenicity and hypersensitivity) which make the control of veterinary drug residues an important parameter in ensuring consumer protection. This work was performed to quantitatively determine minocycline residues in different tissues of chickens (kidneys, liver, muscles and fat) and sera following multiple oral doses of the drug using High Performance Liquid Chromatography (HPLC). Moreover, the study aimed to estimate the withdrawal time of this drug in chicken tissues. Twenty five healthy chicks (Cobb 500) were used. Twenty one chickens were given minocycline directly into the stomach at a dosage of 7 mg/kg body weight once daily for five successive days. Samples were analyzed at 1st, 3rd, 7th, 14th, 21st and 28th day after last oral dose. The results indicated a widespread distribution of minocycline in the tissue samples, which remained within the detectable limit till the 3rd day (49.20-135.20 µg/kg) in all tested tissues. While in kidneys, minocycline remained till the 7th day (11.80 µg/kg) following the last oral administration of the drug. Therefore, it is recommended to pay attention to the proper withdrawal periods before marketing to ensure the hygienic suitability of broilers edibles for safe human consumption.

Key words: minocycline; residues; chickens; HPLC

Introduction

In Egypt, broad spectrum tetracyclines are the most common used antibiotics in poultry husbandry. They are used for therapeutic and prophylactic purposes against both aerobic and anaerobic bacteria and some protozoa due to their relatively low cost, easily accessible and availability. They can be administered either in feed, in drinking water or by injection. Tetracyclines include a wide number of various types of compounds as oxytetracycline,

chlortetracycline, doxycycline and minocycline (1). About 60% of an ingested dose of oxytetracycline is absorbed from the gastrointestinal tract (GIT) and widely distributed in the body, particularly to liver, kidney, muscles, bones and teeth (2).

Minocycline is an effective semi-synthetic, second-generation tetracycline that was used for treatment of certain sexually transmitted diseases (3), comparable with oxytetracycline, it is more active against sensitive micro-organisms especially *Staphylococci*, *Mycopla-*

sma pneumonia and Chlamydia (4). This may be due to its highly infiltration into the microbial cells (5) or its higher lipid solubility which leads to improved antimicrobial activity and antibiotic absorption and enhanced its distribution in the body (6).

The potential harmful human health effects presented by the presence of antimicrobial residues or their metabolites in poultry meat may be due to intensive uncontrolled use of antimicrobials in the poultry industry especially if the withdrawal times are neglected (7). To ensure human safety, World Health Organization/Food Agriculture Organization (WHO/FAO) (8) set a tolerance or Maximum Residue Limits (MRLs) for tetracycline and their metabolites at a level of 200µg/kg in muscle, 600µg/kg in liver and 1200µg/kg in kidney. Hazard of some tetracyclines` residues is decreased by heat treatment but complete removal of these antibiotic residues in broiler meat could not be achieved (9).

The use of High Performance Liquid Chromatography (HPLC) is an effective, accurate, rapid and sensitive separation technique for the analysis of minocycline residues (identification, confirmation and quantification) in the serum and tissue (10), varying its efficiency according to chromatographic condition and type of detector used (11). Different detectors have been described for analysis of minocycline residues in chicken tissues as ultraviolet (UV), diode- array detection (DAD), fluorescence (FLD) and mass spectrometry (12). Therefore, this study was planned in Egypt to determine minocycline residues and their withdrawal times from different chicken tissue parts (liver, kidneys, muscles and fat) and sera after administration of last oral drug dose (7mg/ kg BW) using HPLC technique.

Material and methods

Drug

Minocycline (Minocine) was supplied by Lederle Company for Medical Products, USA. It was prepared by dissolving the powder, bright yellow-orange amorphous solid, in normal saline.

Experimental design

The study was approved by the Committee of Animal Welfare and Research Ethics Faculty of Veterinary Medicine, Zagazig University, Egypt. A total of 25 apparently healthy live unsexed one-day old broiler chicks (Cobb 500) were acquired from Mansoura Poultry farm at Dakahlia Governorate. They were reared until 21 days old before the start of the experiment. They were fed on balanced commercial ration free from any medication, and the water was provided *ad libitum*. They were kept under clean condition during the investigation period. The chicks were randomly allocated into two unequal groups; group 1 (negative control, n=4) was administered sterile normal saline and group 2 (n=21) was orally administered 7mg/kg BW minocycline directly into the mouth through a stomach tube once daily for 5 consecutive days. The dose was calculated according to Paget and Barnes (13). Five samples (liver, kidney, breast muscle, fat and serum) were collected from each slaughtered bird (n=3) at six withdrawal periods (1st, 3rd, 7th, 14th, 21st and 28th day) following the last oral medication dose.

Chemicals and reagents and apparatus used for HPLC analysis (14)

Chemical and reagents used throughout the study as Methanol HPLC, Acetonitrile HPLC trifluoroacetic acid and potassium hydroxide (Fisher Scientific Co, Fairlawn, NJ, UK); Triethylamine and phosphoric acid (Sigma-Aldrich Co, USA); Sodium hydroxide (Honeywell Co, Germany); Granular potassium dihydrogen phosphate monobasic (KH₂PO₄) (Sham Lab, Syria); Ammonium acetate (Fisher Scientific Co, Fairlawn, NJ, UK); Dithioerythritol, iodoacetamide, potassium chloride (Sigma Aldrich Co, UK); Calcium chloride (Merk Co, Darmstadt, Germany); Sodium borate (Lobachemie PVT,Ltd., India); Deionized water (Milford, MA, USA) and McIlvaine buffer.

Agilent Series 1200 quaternary pump, Series 1200 auto sampler, Series 1200 UV Vis detector, Column (Agilent ZORBAX SB-C8 250mm × 4.6mm, 5µm), SPE vacuum manifold and solid-phase extraction (SPE) columns C18

(500 mg, 3 or 6 ml; Varian, Les Ulis, France) and nitrogen evaporator were used.

HPLC analysis

Preparation of the samples

Blood and tissue samples were collected from slaughter birds after 24 h from the last dose. Blood were gathered in sterile glass tubes and placed in a slant position for 20 min at 25°C to be coagulated then centrifuged at 3000 rpm for 10 minutes to obtain the serum which stored with chicken tissues (liver, kidney, breast muscle and fat) at -20 °C until used for HPLC investigation (14).

Extraction and determination of drug residues was performed as described previously (14). Briefly, 5 g of homogenize sample were extracted with 20 ml of 0.1 mol/l Na₂EDTA-McIlvaine buffer solution (60.5g Na₂EDTA. 2H₂O (J.T. Baker, England) was added to 1625 mL McIlvaine buffer). The later was prepared by addition of 1000 ml of 0.1M citric acid (J.T. Baker, England) to 625 ml of 0.2 M disodium hydrogen phosphate (J.T. Baker, England), the reagents were mixed and PH adjusted to 4.0±0.05 with NaOH or HCl as needed. The extract was vortexed for 1 min followed by 10 min ultrasonic extraction in an ice bath, centrifugation was done at 3,000 rpm for 5 min in a cooling centrifuge (below 15 °C). The supernatant was filtered and the extract was saved in a clean tube. The extraction was repeated twice with 20 ml and 10 ml 0.1 mol/l Na₂EDTA-McIlvaine buffer solution, successively. The supernatant (50 ml in 0.1 mol/l Na₂EDTA-McIlvaine buffer solution) was collected, centrifuged at 4,000 rpm for 10 min (below 15 °C) and filtered with fast filter paper.

Solid phase extraction

Ten mL extract (equivalent to a 1g sample) was passed through the SampliQ OPT cartridge (Varian, Les Ulis, France) at a speed of 1 ml/min which was previously conditioned with 5ml of methanol HPLC (Fisher Scientific Co, Fairlawn, NJ, UK) then 5ml of a 10mmol/l trifluoroacetic acid (TFA) solution. (Fisher Scientific Co, Fairlawn, NJ, UK). After the sample effusion, the cartridge was washed with 3 ml of water (pH adjusted to 4.5 with TFA), the entire effluent was discarded and the cartridge was dried under negative pressure below 2.0 kPa for 3 minutes. Finally, minocycline was eluted with 10ml of 10mmol/l oxalic acid in methanol, collected and dried under nitrogen below 40°C. The resulting residue was dissolved and made to a constant volume of 0.5ml using the methanol/10 mmol/l TFA solution (1/19) then filtered through a 0.45-µm filter membrane (p/n 5185-5836) and analyzed (14).

Liquid chromatography operating conditions

Flow rate, 1.5 ml/min; column (Agilent ZORBAX SB-C8 250mm × 4.6mm, 5µm) temperature, 30°C; injection volume, 100 µL and detector (Series 1200 UV Vis detector) wavelength, 350nm were adjusted for HPLC analysis. Moreover, the mobile phase; Methanol-acetonitrile-10 mmol/l TFA solution, gradient elution and its gradient is shown in Table (1).

Quantification of residues

Quantification of the antibiotic residues in the samples was obtained and calculated from the area under curves extrapolated automatically by the HPLC 2D Chemstation Software (Hewlett-Packard, Les Ulis, France).

Table 1: Composition of gradient mobile phase

Time (minutes)	Methanol %	Acetonitrile %	10 mmol TFA %
0	1	4	95
7.5	6	24	70
13.5	7	28	65
15	1	4	95

TFA: trifluoroacetic acid

Standard curve

A reference standard, minocycline powder, was obtained from Sigma-Aldrich Co, USA. The stock solution (1mg/ml) was prepared in methanol, kept in the freezer at -20°C for 6 months in the dark. Intermediate standard solution (100 $\mu\text{g/ml}$) was prepared from stock solution in methanol. Working solutions at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 $\mu\text{g/g}$ were prepared using the stock solution diluted with a mixture of methanol/10 mmol/l trifluoroacetic acid solution (1/19). The working solutions were prepared daily.

Spiked samples of minocycline

Minocycline standard prepared at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 $\mu\text{g/gm}$ and homogenized with 5 g of control chicks muscles (blank samples) then treated according to the abovementioned extraction procedure. The calibration curve was calculated by linear regression equation method as $y = 0.10391546 x + 3.4880808$ where y symbol indicated area under peak and x symbol indicated concentrations of minocycline.

Statistical analysis

Data was analyzed by using computerized SPSS (Statistical Package for Social Sciences) program version 25 (IBM Corp., Armonk, NY). Statistical evaluations of the results were done by using methods as mixed model analysis of variance (ANOVA) followed by Tukey test. Significant difference (Tukey's HSD) test as post hoc test was used. The level of significance was taken as $P < 0.01$.

Results

High performane liquid chromattography analysis recorded that the corresponding peak

responses (area under peak) of minocycline standard concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 $\mu\text{g/gm}$ as illustrated in Table (2) and Figure (1). Linearity existed witin range of 0.025 and 5 $\mu\text{g/gm}$ with a correlation coefficient ($r^2=0.99982$). The percentage recovery of spiked samples ranged from 95-98%. The limit of detection for minocycline was 0.0025 $\mu\text{g/ml}$, while, LOQ (limit of quantification) was 0.01 $\mu\text{g/ml}$.

Minocycline distribution in chicken tissues (liver, kidney, breast muscles and fat) and serum was represented in Table (3). The typical chromatogram of minocycline is shown in Figure (2). There was a widespread distribution of the minocycline in the tested tissues and serum. The mean concentration of minocycline residues were 139.6 ± 1.4 , 157.4 ± 2.5 , 89.2 ± 2.2 , $100.6 \pm 2.6 \mu\text{g/kg}$ and $294.6 \pm 3.7 \mu\text{g/kg}$ at the 1st day after the last dosage in liver, kidney, breast muscles, fat and serum, respectively. In the 3rd day of slaughter, the residues minimized in all organs to be 73.4 ± 1.3 , 135.2 ± 1.8 , 63 ± 1.4 , 49.2 ± 1.7 and $68.4 \pm 1.2 \mu\text{g/kg}$ for liver, kidney, breast muscles, fat and serum respectively. Moreover, in the 7th day post treatment the residues disappear in all organs except kidney ($11.80 \pm 0.97 \mu\text{g/kg}$), while minocycline residues were not detected in all organs after the 7th day post treatment.

From the obtained results it was found that the highest minocycline residual level was detected in kidney followed by liver while the lowest level was recorded in muscle and fat samples. There was a highly significant difference ($P < 0.01$) in detection levels of minocycline residues in serum and tissues (kidney and that of liver and muscle) and along the investigation periods. Our detectable levels of minocycline residues in kidney, liver and muscle samples were lower than the established MRL of WHO/FAO.

Table 2: The concentrations of minocycline spiked tissues ($\mu\text{g/gm}$) and their corresponding peak response automatically using HPLC

RT	Level	Amount ($\mu\text{g/gm}$)	Area
7.331	1	0.025	7.640
	2	0.050	9.386
	3	0.100	14.850
	4	0.250	32.359
	5	0.500	55.571
	6	1.000	101.140
	7	2.500	268.160
	8	5.000	521.680

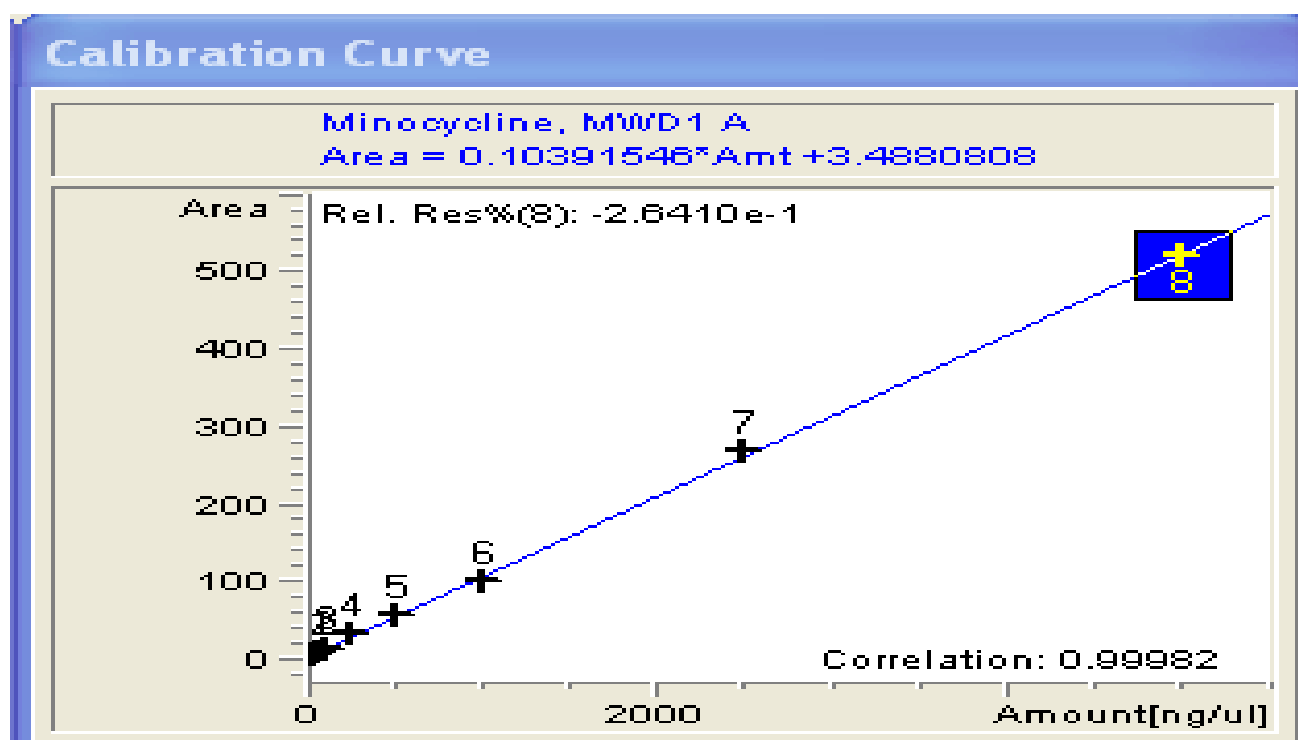
RT: Retention Time.

Table 3: The concentrations of minocycline in tissues of slaughtered chickens at various intervals following treatment with 7 mg/kg BW once daily for 5 consecutive days ($n=3$) automatically using HPLC

Tissue	concentration ($\mu\text{g/kg}$) after minocycline administration					
	Mean $\pm$ SE					
	1 st day	3 rd day	7 th day	14 th day	21 st day	28 th day
Liver	139.60 $\pm$ 1.40 ^c	73.40 $\pm$ 1.30 ^f	ND	ND	ND	ND
Kidney	157.40 $\pm$ 2.50 ^b	135.20 $\pm$ 1.80 ^c	11.80 $\pm$ 0.97 ⁱ	ND	ND	ND
Breast muscle	89.20 $\pm$ 2.20 ^e	63.00 $\pm$ 1.40 ^g	ND	ND	ND	ND
Fat	100.60 $\pm$ 2.60 ^d	49.20 $\pm$ 1.70 ^h	ND	ND	ND	ND
Serum	294.60 $\pm$ 3.70 ^a	68.40 $\pm$ 1.20 ^{fg}	ND	ND	ND	ND

Means with different superscripts were statistically significant according to Tukey test.

ND: Not Detected

**Figure 1:** HPLC chromatograms standard curve of minocycline

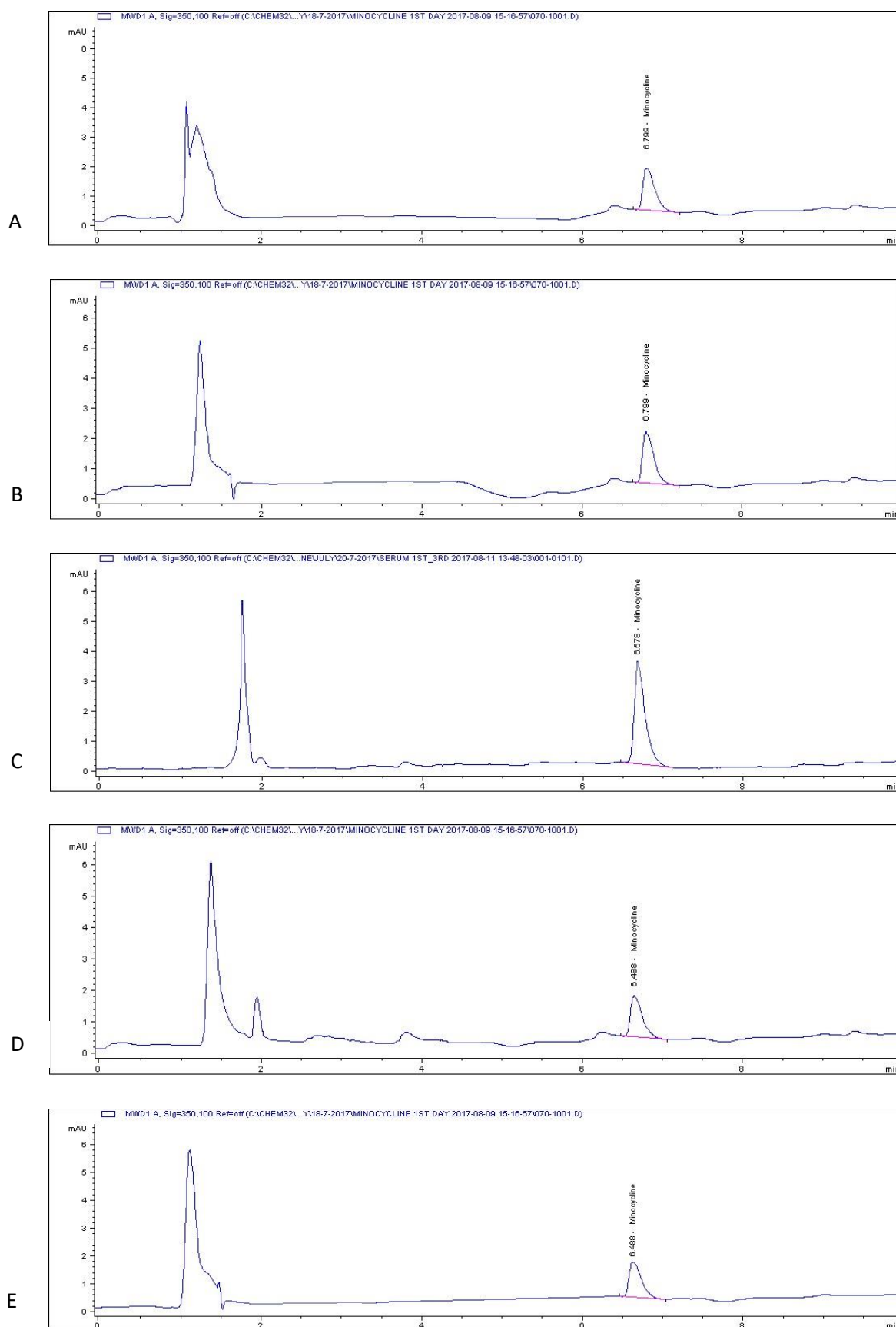


Figure 2: HPLC chromatograms of minocycline concentration in chicken samples (at 1st day following last oral dose (7 mg/kg BW). A: Liver; B: Kidney; C: Breast muscle; D: Fat and E: Serum

Discussion

Minocycline is a semi-synthetic, second generation tetracycline closely related to doxycycline, which could be used as an antimicrobial and growth-promoter in feed for livestock and poultry. This may be due to the positive control effect on intestinal microbial population, which is potentially fatal (15); its low poisonous quality or impact ahead against Gram-positive bacteria, Gram-negative bacteria and Mycoplasma (16). Minocycline is similar to doxycycline; rapidly and highly absorbed from GIT; has longer half-life (15-22 h); widely distributed through the body with the highest level in the kidney and liver; relatively high liposolubility (5 to 10 fold increase if compared to older tetracycline) which make it an effective antibacterial agent so its price in the Egyptian market is high compared with the tetracycline group (17).

It was clear from this study that the highest minocycline residual level was detected in kidney followed by liver while the lowest level was recorded in fat and muscle samples. Similar findings of tetracycline residues were reported in previous studies (18-20) in which higher tissue levels of doxycycline and tetracycline were reported in liver and kidney than muscle samples. The higher antibiotic residues in liver and kidney could be attributed to the role of liver and kidney in drug metabolism (detoxification, filtration and blood purification); unpaid attention to the withdrawal period because long-term administration of these antibiotic in feed, while the lower antibiotic residues in muscle due to most of the antibiotic are eliminated from the body via the kidney and bile (17).

In our study, we found that liver, kidney and muscle samples were lower than the MRL of WHO/FAO for tetracycline group. A several studies have been reported that levels of the tetracycline residues in muscle, liver and kidney (19-20) were exceeded above the recommended MRL of WHO/FAO in Egypt, also it was reported in many countries such as Mexico (21), Belgium (22), Saudi Arabia(23), Iran(18) and Pakistan(24).

From the obtained results it was found that minocycline remained in all tissue and serum till 3rd day post treatment but not detected in all organs or serum after this time except in kidney as the drug residues were detected at 7th day post drug treatment. The effective withdrawal period for doxycycline is 7 days to ensure that no harmful residues remain in food products after slaughter. The antibiotic concentrations in the edible tissues were detected to be below the MRLs (25) and did not enter the human food chain as reported previously (26). The possible human hazards related to antibiotic residues have been reported including; allergic/toxic reactions, chronic toxic effects (microbiological, carcinogenicity, reproductive and teratogenic effects) which may occur with prolonged exposure to low levels of antibiotic residues and development of antibiotic-resistant bacteria in treated animal (27). So, the microbiological ADI of 3µg/kg of minocycline/kg BW×60kg (standard body weight of human) =180 µg/person was established as the overall ADI for minocycline, which can be ingested by human over a life time without appreciable risk (28).

Conclusion

The results confirmed the presence of minocycline residues in chicken samples. Higher level of minocycline residues usually observed in kidney and liver rather than in muscle due to their role in metabolism and excretion of antibiotics. This may pose potential hazard to public health. Thus, it is recommended that rules should be taken to ensure observing proper withdrawal periods before marketing and drug control in veterinary use. In addition, a monitoring policy should be implemented to ensure the conformity of poultry meat sold in Egypt with international standards.

Conflict of interest

None of the authors have any conflict of interest to declare.

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