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RESEARCH ARTICLE

MICROBIOLOGICAL AND HPLC ASSAYS FOR DETECTION OF TETRACYCLINE RESIDUES IN CHICKEN MEAT

***[1]Ishraga G. Ibrahim, [2]Sabiel, Y. A., [1]Thoria, O. O., [3]Khalafalla, A. E., [1]Safa, O. S., [1]Khogali, M. E. Salwa, [1]Hala, A. Elrayah, [4]Manal H. Salih and [2]Muna, E. A.**

[1]Department of Biochemistry, Pharmacology and Toxicology, Veterinary Research Institute, Khartoum, Sudan
[2]Department of Bacteriology Veterinary Research Institute, Khartoum, Sudan
[3]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Khartoum, Khartoum, Sudan
[4]Department of pathology, Veterinary Research Institute, Khartoum, Sudan

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ABSTRACT

Antibiotics are used for control of infectious diseases, promote growth and increase feed efficiency in chickens. Incorrect use of these drugs are practiced deposits the presence of some residue in the products. Microbiological assay are generally used for residue screening as part of an integrated system with follow-up confirmatory analysis of suspicious samples. This research highlights the importance and existence of antibiotics residue in poultry meat. Eighty seven chicken meat samples were collected from different poultry slaughter houses and marketing centers in Khartoum State, Sudan. Samples were screened for the presence of residues of tetracycline by microbiological assay. Positive results were identified and confirmed by using HPLC. The results showed that more than 5% of the meat samples had noticeable antibiotic residues. Residues level quantified between 150 to 500 µg/kg of oxtetracycline drugs. Result are relevant as they indicated that antibiotics may be improperly used by producers, and that there is a strong need for heightened surveillance through regular mandatory testing of chickens sold for human consumption.

INTRODUCTION

Antibiotics are used extensively in poultry industry for treatment and prevention of several diseases and to promote growth and increase feed efficiency in less than ideal sanitary conditions (Mc Evoy 2002; Di Corcia and Nazari, 2002). Regulatory control of drugs used for therapeutic and prophylactic purpose in livestock has gained widespread attention in recent years. Because of their use in food producing animals, the risk of occurrence of unwanted residues in edible products exists in the exposure to low levels of antibacterial drugs over prolonged periods. These residues may pose a health threat to consumers in varying degrees depend on type of food and the amount of the residue resulting in microbial resistance to antibiotics and other health problems. This has stimulated monitoring and surveillance effort by government throughout the world (Mitchell *et al*., 1998, Mc Evoy 2002; Di Corcia and Nazari, 2002). Different techniques are available for detection of drugs residues in raw ex-farm products including microbiological, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and recently a multi-class method has been developed by using liquid chromatography for detection of antibiotics in chicken meat (Justavson *et al*., 2002; Kotretsu, 2004; Ramos *et al*., 2003, Bousova *et al*., 2012). Microbiological methods used for detection of antibiotics in animal issues relay on their ability to inhabit the growth of sensitive bacteria like *Bacillus subtilis*. Maximum residue level (MRL) has been established for all antibiotics used as veterinary drugs in food of animal origin has been proposed as a new evaluation procedure taking into account the presence of metabolites for the regulation of veterinary drug residues (Anadon and arrana,1999). These procedures can only be proven with fully validated chromatographic techniques such as HPLC or hyphenated techniques such as GC-MS. In this study, the presence of antibiotic residues in meat of chicken was detected with microbiological test. Positive samples are confirmed and identified by HPLC.

Corresponding author: Ishraga G. Ibrahim
Department of Biochemistry, Pharmacology and Toxicology, Veterinary Research Institute

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade from Merck (Darmstadt, Germany). Tetracycline standards (Tetracycline,

oxytetracycline, demeclocycline and, doxycycline) were obtained from Sigma (St. Louis, MO, USA).

Sampling

Chicken meat samples were collected from different poultry slaughter houses and markets in Khartoum State. Chicken meats free from antibiotics were used as control samples.

Microbiological assay

Screen tests for antibiotic residues in muscle of chicken were done by microbiological assay *Bacillus subtilis* ATCC6633 as the test organisms according to modification of the method described by Ellerborek (1991). In brief, AST medium No. 1 (Difco Laboratories), was prepared and sterilized by autoclaving. Agar was cooled to 46 °C, 100 µl of an overnight culture of *B. subtilis* was added to 100 ml of the agar medium to obtain approximately 10^3- 10^6 CFU/ml of the organism. Sterile Petri dishes were filled with 20 ml of the inoculated media and cooled. Apiece of muscle (0.5g) was placed on the agar surface of the medium. The plates were kept for 1 h at 20 °C and then incubated at 37 °C for 18-24 h. The width of the inhibition zone around muscle was measured in mm from the edge of the sample to the edge of the inhibition zone, including any partial inhibition. Each test sample was assayed in triplicate; the limit of quantization of the method was 0.2µg/kg.

HPLC analytical procedure

Preparation of tetracycline standards

Tetracycline drugs (oxytetracycline, tetracycline, demeclocycline and cholorotetracycline) Stock solutions (1 mg/ ml) were prepared in MeOH and stored at 20 °C. Working standard solutions were prepared in MeOH.

Samples preparation

A liquid-liquid extraction method similar in principle to method described by Schuster (1997) was adopted for the analysis of meat sample. Chicken meat samples stored at -20°C were thawed overnight in a refrigerator. Meat samples (50 g) were cut into small pieces and blended at 20000 rpm in a tissue blender (IKA, M 20, Germany) for 2 minutes. One grams of blended meat tissue was spiked with demeclocycline as internal standard at concentration of 100mg/kg for 10 minutes, then mixed with 100 mg citric acid and placed in 20 ml polypropylene centrifuge tube. 1 ml of nitric acid (30%) and 4 ml of methanol were added. The mixture was homogenized for 5 minutes using Ultra- Turrax T25 tissue homogenizer (IKA, Ultra-Turrax, T25 basic, Germany). Then the total volume was completed to 10 ml by distil water and centrifuged at 4100RCF for 15 minutes at 4 ºC. The aqueous layer was filtered through a disposable syringe filter (Mini Sart RC4, 0.45 µm Sartorius AG, Germany) and transferred to a HPLC**.** Since the MRL of tetracyclines in meat is 100 µg/ kg, each batch of samples was accompanied with a spike at the MRL level and a blank. The calibration line was based on fortified blank samples at five concentrations: 10, 50, 100, 150, 200 µg/ kg.

HPLC determination

Tetracyclines were determined according to the method described by Schuster (1997). For the UV detection reverse phase column type hypersil BDS ((100 Å, 8 mm, 250 X 4.6 mm id) was used. The pump and detector used for detection were from the same supplier [Cykam S1122 quaternary pump and Cykam S3210 UV/VIS detector). The mobile phase contained water, pH = 2.1 with sulfuric acid (A) and ACN (B). Isocratic solvent program was run [85%(A) : 15%(B), v/v The flow rate was set at 1 ml/ min and detector at 355nm.

RESULTS

As to results of microbiological assay, the presence of residues was manifested by the formation of a clear zone of inhibition at least 2 mm in size. Positive findings for presence of antibiotic were recorded in 3 samples. The results of chromatographic analysis (HPLC) as shown in Table 1 indicated that in all positive samples for microbiological assays the oxytetracycline was quantified between 150 to 500 µg/kg. It was detected in 355nm by an isocratic system in 6.5 minutes. The limit of detection is 50µg/kg and the overall recoveries range between 63-68% The results of this study showed that microbiological assays for detection of antibiotics residues in meat samples is a useful tool in preliminary characterization of antibiotic residues in animal tissues.

Table 1. The concentrations of oxytetracycline detected in the positive chicken samples

Sample No.	Mean Concentrations (µg/kg)
1	500
2	400
3	150
Mean	350

DISCUSSION

Antibiotics are used by the poultry industry to enhance the health and productivity of flocks, significant concentrations of the drug may be retained in edible tissues for various periods. This can result for many reasons, including poor records of treatment, prolonged drug clearance, products not used according to label directions, lack of advice on withdrawal period, and others (Jones and Seymour, 1988). The hazards of consuming meat, milk or eggs containing such residues include hypersensitivities to other drugs that may be needed therapeutically and the preferential selection of bacterial mutants that become resistant to drugs used in the treatment of human disease. Also teratogenic and mutagenic effects may ensue due of some drug residues (Kaya, 2004). In this study oxytetracycline residues level in chicken meat samples were found in 3 of the tested samples (3.4%) and ranged between 150 to 500µg/kg and average of 350µg/kg.

The result of the present study was higher than those recommended by MRL's standards. The acceptable Maximum Residue Limit (MRL) as recommended by the joint FAO/WHO Expert Committee on Food Additives (1999) is 0.2µg/kg for meat OTC residues exceeding the tolerance level result in human health problems including gastrointestinal problems, stain the teeth of young children, allergic reaction

and development of antibiotic resistance pathogens (Baker and Leyland, 1983; Schenk and Collery, 1998; Walton *et al*., 1994). The results were comparable with other studies where Shahid *et al*. (2013) detected OTC residues in 44.8% of the tested poultry meat samples, Okerman *et al*. (1998) reported 86% of tested poultry meat samples were positive for OTC residues. For escaping from these problem outcomes, basic and advanced education of poultry and livestock farm workers is necessary; attention must be paid to the control measures. This process requires increased responsibility in the evidence of animals treated within the period of breeding, as well as to comply with withdrawal periods set by the valid food legislation for each individual drug. More curations using analytical techniques are complement of reducing or eliminating drugs residue dangers (Pipová *et al.,* 1995). Methods developed for detecting of antibiotic residues include microbiological assays, immunoassays, and liquid chromatography (LC). Microbiological assay, which rely on the residue to inhibit growth of the test organism. These assays are generally used for residue screening as part of an integrated system with follow-up confirmatory analysis of suspicious samples by chemical methods. They are inexpensive and simple, and can reveal some information on the nature of the residue (Currie *et al.*, 1998); however, most lack specificity and few are quantitative. Assay procedures involving LC are the most expensive in terms of equipment and labor. Although, they are highly specific and detect residues in the part per billion (bbp) ranges, they are not practical for screening large numbers of samples. Their use is therefore restricted to confirmatory analysis (Currie *et al.*, 1998).

Conclusion

Results indicated that antibiotics may be improperly used by producers, and that there is a strong need for heightened surveillance through regular mandatory testing of chickens sold for human consumption.

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