DETECTING ANTIBIOTIC RESIDUES BY HPLC METHOD IN CHICKEN AND CALVES MEAT IN DIET OF A MILITARY CENTER IN TEHRAN

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ABSTRACT

Nowadays different kinds of antibiotics, due to their availability and low cost are using as therapeutic, prophylactic and nutritive purposes in livestock and poultry industries. Incorrect use of this drugs leads to deposit some residues in animal products, that is potentially hazardous for human health as it may cause allergic reactions in human and antibiotic resistance in pathogenic microorganisms. This study designed to investigate the occurrence of 4 common antibiotic residues in broiler chicken tissues, calves muscles of a military center staffs diet. 100 samples from chickens and calves were taken from slaughterhouses of Tehran which supply personnel’s food of Tehran military center in 2012. Oxytetracycline, chloramphenicol, enrofloxacin and penicillin residues measured by high performance liquid chromatography (HPLC) method and data is analysed by statistical softwares. Results show that the percentage of contamination and the mean level of enrofloxacin in poultry samples were 88% (0.73 μg/kg) and 100% (2.57 μg/kg) in summer and winter (MRL=75 μg/kg) respectively. This rates were 100% for chloramphenicol in both seasons with means of 1.34 μg/kg and 13.9 μg/kg in summer and winter (MRL=0) respectively. In beefs, positive sample percent and mean level of penicillin in summer and winter were 76% (0.87 μg/kg) and 92% (1.3 μg/kg) respectively (MRL=4 μg/kg). Oxytetracyclin were detected in 100% and 72% of samples, which their mean value was 3.5 μg/kg and 4.61 μg/kg in summer and winter respectively (MRL=100 μg/kg). This study indicates the presence of different levels of antibiotic residues in 99% of samples. But non of samples were above MRLs except chloramphenicol that’s prohibited in poultry industry and cause serious hazards for militaries. Moreover, gathering few amounts of different antibiotic seems to be a health threat for military force staffs.

Key words: Antibiotic Residues, Chicken, calves meat, military center, Tehran.

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Introduction

The term of antibiotic refers to a wide range of chemical substances derives from a natural, semisynthetic or synthetic way that affect antibacterial activity, by killing or inhibiting the growth of bacterial pathogens(1). Nowadays Antibiotics are widely prescribed for both therapeutic and prophylactic aims against microbial infections and as a growth promoting substances in animal and poultry farms.

The incorrect use of these drugs in veterinary field can lead to the existence of residues in animal derived foods that affect human health(2,3). It may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or may leads to bacterial resistance to different kinds of antibiotics(4).

In this sense, the control of antibiotic residues in edible animal tissues is mandatory. Therefore, simple and reliable analytical methods like HPLC is required to monitor antibiotic drug residues in human foods especially in people masses who Physical strength plays an important role in their Working Life.

In 2010 the EU established safe maximum residue limits (MRLs) for residues of veterinary drugs in foodstuffs with animal origin [e.g., animal
tissues (muscle, liver, kidney, fat), milk or eggs which enters into the human food chain[1].

Among common antibiotics, some of them, such as the followings, are more frequently prescribed by veterinary clinicians in many countries because of their efficacies. Oxytetracycline is a natural tetracycline compound that is derived from the fungus Streptomyces rimosus. It is a wide-spectrum antibiotic with bacteriostatic activity against both gram-positive and gram-negative bacteria, such as the species of Spirochete, Actinomyces, Rickettsia and Mycoplasma(4, 5). But it’s poorly metabolized in target animals and excreted in its parent form, due to its high water solubility(6). The residues of this antibiotic can cause allergic reactions in some hypersensitive individuals if consumed for a long time(7). A maximum residue limit (MRL)/tolerance of 100 μg kg-1 in muscle of all food-producing species has been officially established by the EU(8).

Penicillin, a non-toxic class of antibiotics which are commonly used in food-producing animals, may give rise to residues in meat products that might be harmful for the consumers (allergic reactions). To ensure human food safety, maximum residue limits (MRLs) have been fixed to 50 mg kg-1(9).

Enrofloxacin is the second generation of fluoroquinolone with bactericidal activity. After application, it is well absorbed and distributed in tissues and metabolized in the liver. Generating it’s major active metabolite may lead to the emergence of drug-resistant bacteria. The maximum residue limits (MRLs) established by the European Union is 100 μg kg-1 in meat(10).

Chloramphenicol is widely used in poultry industries, due to its broad spectrum antimicrobial activity as well as its remarkable penetration into the tissues. Although it is known to exert several side effects in humans such as bone marrow depression and grey syndrome in newborns(10).

The main objective of this study is to monitor residues of four prevalent antibiotics (Oxytetracycline, Enrofloxacin, penicillin and chloramphenicol) in central part of Iran in winter and summer by HPLC method. It is worth to mention that this is the first report from chicken and beef meat which has been used in diet by the military center in Tehran.

Materials and methods

In this cross-sectional study sampling were done by a systematic random sampling method, 100 of the samples (50 chiken and 50 beef) were taken from slaughterhouses of Tehran which supply military centers food in summer and winter of 2012.

Sample preparation

100gr of each samples aseptically collected in separate sterile sample containers and transported in ice box packed with ice to the baqiyatallah university of medical sciences laboratory and all of them kept in 20 Degrees centigrade before analysis has been started. So after defrosting in 25 degrees centigrade, 50gr of each sample were homogenized by grinder. Then 10gr of each sample were added to 10ml of chloridrich acid 0.15 molar and centrifuged at 4400 rpm for 20 min at 4°C. The extraction step was repeated twice and supernatants were pooled and finally filtered by 0.2µm syringe filter(14, 15).

Both detection and analysis of antibiotic residues were done by using High Performance Liquid chromatography (HPLC).

Sample cleanup by solid-phase extraction

An SPE cartridge (SPE-pack vac 1cc (100 mg) was conditioned with 2.5 ml of methanol and 2.5 ml of HPLC grade water. The final extract (14 ml) was applied into the cartridge. When the extraction was completed, the cartridge was washed consecutively with 3 ml of HPLC grade water, 3 ml of 0.2M Na2HPO4 (pH=9) and 5 ml of HPLC grade water. The cartridge was subsequently dried by air aspiration.

Each antibiotic was eluted with 3.5 ml of MeOH. The eluted antibiotic was evaporated to dry under nitrogen stream, The dry residue was redissolved in 200 ml of 0.2 ml Na2HPO4 (pH=9) and 5 ml of HPLC grade water. The test tube was vortex- mixed for 30s and then centrifuged at 4400 g for 5 min at 4°C. The supernatant was transferred to an injection vial and 30ml was injected into the HPLC system.

Antibiotic determination was performed by means of an HPLC system consisting of a waters prep LC 4000 (USA) and a Spectroflow 783 UV-Vis detector (WATERS tm 486, tunable
absorbance, USA) a 125 mm×4mm i.d. LiChrospher 100°C 18 HPLC column (5 m) from waters was used. All the data analysed by personal computer software (millennium v 12.15)\(^{(15,16,17)}\).

**HPLC method**

The mobile phase used, was water- HPLC grade acetonitrile (CAN) -triethylamine (TEA) (83:14:0.45 v/v), pH was adjusted to 2.3 with 85% H3PO4 before adding CAN. The flow-rate was 1 ml/min. HPLC analysis of the samples was performed in 15 minutes for each one. For antibiotic level detecting on the base of ppb and evaluated HPLC standards.

**Data analysis**

All data analysed by SPSS14 and Paired Sample T-test statistical method were used. The P<0.05 were considered statistically significant and MRL level was obtained from FDA and WHO standards. It considered for penicillin, oxytetracyclin, Chloramphenicol and enrofloxacin, 4,100,0,75 μg kg-1 respectively.

**Results and discussion**

Analysis of chicken and beef with HPLC showed that the majority of the samples had variable amounts of enrofloxacin, chloramphenicol, oxytetracyclin and penicillin residues.

**Chicken samples**

In the case of Enrofloxacin the rate and range of pollution in summer and winter were 88% (22/25), (0-1.8 μg/kg) and 100% (25/25), (1.2-4.8 μg/kg) respectively, but no sample was detected exceeding the maximum Residue Limit (MRL=100 μg/kg) (table 1).

The difference between residue levels, mean and percentage of positive samples related to the enrofloxacin is significantly higher in winter than summer (p<0.05) but the percentage of samples, which value of their residue exceed MRL, were not significantly different (p>0.05) (Fig. 1, 2).

**Table 1**: The mean, range and numbers of positive samples for Chloramphenicol and Enrofloxacin residues.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>Positive 25/25(100%)</td>
<td>25/25(100%)</td>
</tr>
<tr>
<td></td>
<td>Mean (μg/kg)</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>Range(μg/kg)</td>
<td>0.48-2.9</td>
</tr>
<tr>
<td></td>
<td>Std.deviation</td>
<td>0.65 ±</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>Positive 22/25(88%)</td>
<td>25/25(100%)</td>
</tr>
<tr>
<td></td>
<td>Mean(μg/kg)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>Range(μg/kg)</td>
<td>0-1.8</td>
</tr>
<tr>
<td></td>
<td>Std.deviation</td>
<td>±0.45</td>
</tr>
</tbody>
</table>

**Figure 1**: Enrofloxacin concentrations in summer chicken samples (μg/kg) MRL=75.

**Figure 2**: Enrofloxacin concentrations in winter chicken samples (μg/kg) MRL=75.

**Figure 3**: Chloramphenicol concentrations in summer chicken samples (μg/kg) MRL=0.

In the case of Chloramphenicol, the rate and range of pollution in summer and winter were 25/25(100%),(0.48-2.9 μg/kg) and 25/25 (100%), (0.87-42.32 μg/kg) respectively. All samples were detected higher than Maximum Residue Limit (MRL=0 μg/kg) (Fig. 3, 4). The mean±SD and range of pollution in about this antibiotic were sig-
significantly higher in winter than summer (p<0.05) but no statistical differences were observed in positive samples which showed residues of Chloramphenicol above MRL between seasons (p>0.05) (table 1).

In addition, HPLC analysis results show that in summer, 88% (22/25) of samples contain both of Chloramphenicol and Enrofloxacin residues but in winter all samples were contaminated with both antibiotics.

### Beef samples

Out of totally 50 samples analysed in this study, 19/25 (76%) and 23/25 (92%) that collected in summer and winter respectively, had detectable levels of penicillin but no one were above MRL=4 μg/kg.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Positive</th>
<th>Summer</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>19/25 (76%)</td>
<td>23/25 (92%)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.87</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-2</td>
<td>0-3.15</td>
<td></td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>±0.55</td>
<td>±0.83</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxytetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Mean (µg/kg)</td>
</tr>
<tr>
<td>Range (µg/kg)</td>
</tr>
<tr>
<td>Std. Deviation</td>
</tr>
</tbody>
</table>

Table 2: The mean, range and numbers of positive samples for Penicillin and Oxytetracycline residues.

The mean ± SD of penicillin residues were about (0.87±0.55 µg/kg) and (1.30±0.82 µg/kg) with the range of (0-2 µg/kg) and (0-3.15 µg/kg) in summer and winter respectively (table 2). Data analysis shows significant differences between mean, range and number of positive samples between seasons (P<0.05), but no statistical differences were observed in a number of high risk samples that contain high levels of antibiotic residues (P>0.05) (Fig. 5, 6).

The number of oxytetracyclin positive samples in winter and summer were 25/25 (100%) and 18/25 (72%) but no one determined over MRL standards=100 µg/kg (Fig. 7, 8). The mean ± SD of oxytetracyclin residues was reported about (3.05±0.86 µg/kg) and (4.61±4.35 µg/kg) µg/kg with the range of (1.1-4.8 µg/kg) and (0-14.2 µg/kg) in winter and summer respectively and there was no significant difference between any parameters in the seasons (table 2).

Results show that 19/25 (76%) of summer collected samples and 17/25 (68%) of winter collected samples were positive in the existence of both oxytetracyclin and penicillin residues, but only 1 (4%) sample were reported as non-polluted in the case of any antibiotics.
The determination of antibiotic residues in food-producing animals has received enormous worldwide attention by international regulatory and public health agencies today.

It states the importance of the issue and its great impact on public health specially in military forces that play an important role in establishing security and order in each society and as a defining force for foreign threats. So delineating a national MRL is a necessary scale for food industries in each country to prevent irregular antibiotic usage and faults in animal farms. Unfortunately there is no national MRL in Iran as a source of judgment on the base of local farm properties, kinds of common antibiotics and measuring devices for antibiotic residues so other standards like European community scales and WHO are used as an indicator now.

About chicken samples, data analysis shows 50/50 (100%) of chicken samples are contaminated with chloramphenicol that is prohibited in chicken farms by Iranian veterinary consul and other international health organizations (MRL=0). Because of serious side effects like aplastic anemia, grey baby syndrome, digestive disorders and bone marrow problems. There are a few studies on chloramphenicol residues in chicken meats in Iran. Tajik et al in 2010 compared FPT, Elsia and HPLC method for detecting Chloramphenicol residues in chicken meats in Iran. The determination of antibiotic residues in food-producing animals has received enormous worldwide attention by international regulatory and public health agencies today.

Against our results about Enrofloxacin residues that shows a range of (0-4.8 μg/kg) with a mean of 2.57±1.07 μg/kg and 0.73±0.45 μg/kg in winter and summer, with no sample higher rate than standard levels (MRL=75 μg/kg). There are many studies about Enrofloxacin specially in chicken with different results. For example Salehzade et al in a study in 2006 in Tehran, Iran expressed that all 270 samples got from kidney, liver and muscles of chickens contained some antibiotic levels but 8.8% of them were above MRL standards with Mean of 18.32±32.29 (ng/g). This problem is not only dedicated to Iran. Some other studies in middle east countries like Saudi Arabia by AL-Mustafa showed that Norfloxacin rate, at 35.0% of positive muscle samples was about 2.7-34.3-fold higher than this country MRL. (30ng/g) This reference reveals widespread misuse of Norfloxacin in poultry farms of Persian gulf border countries, which may pose a major risk to public health including possible stimulation of bacterial resistance, disorders of intestinal flora and hypersensitivity reactions to fluoroquinolones. But according to the analyzed data that non of samples contaminated higher than MRL levels in this study. It seems that there is no risk to the army men about Enrofloxacin.

In Food industry Penicillin is a nontoxic antibiotic that is common in veterinarian prescibiptions but inmethodical usage leads to remain high residue levels in animal tissue that may cause allergic reactions. In our study, all samples were in the range of 0-3.15 μg/kg so there was not any residues above the MRL (4μg/kg). It can be resulted by implementation of recommended withdrawal times by farmers, if not even freezing to -75 degrees centigrade for 3 month by consumers cannot significantly reduce amounts of penicillin in calves meat.

We reported all the summer taken beef samples and 78% of winter samples, positive about Oxytetracycline but against other researches like Hunde Bedada in 2012 in Ethiopia that were declared 71.3% of calves meats were contained some amounts of oxytetracyclind and 48% that reported above MRL standards. (100 μg/kg) We have not reported any sample higher than MRL but there was some similarity in positive contaminated prevalence.

Moreover, according to studies that Mesgari Abbasi et al in 2012 operated in Ardebil, Iran on 110 samples of slaughtered calves, about 25.8% of muscles were contaminated with (176.3±46.8) and (405.3±219.6) μg/kg in triceps and gluteal muscles.
that is more than MRL rates\(^{(15)}\). He also declared that in 2009 among 380 samples of muscle, liver and kidney tissues of calves that analysed by HPLC, 21.7% of them were above MRL of WHO with a mean of 163.1, 63.4, 166.7μg/kg in triceps, gluteal and diaphragmatic muscles. It shows evidence of failure to comply withdrawal time for meats\(^{(16)}\). The comparison of mentioned results, in the case of oxytetracyclin, shows that all the Consumable meats in the military centre are insured proper withdrawal period before slaughtering of the animals (28 days for Oxytetracycline)\(^{(18)}\) and prepared suitable quality for providing health indexes.

Despite of reporting all antibiotic residues under MRL in this survey, multiple residues of different antibiotics, except chloramphenicol, can be as an important alert for military personnel and soldiers health. This is because of the fact that, gathering some antibiotic remains in animal tissues can lead to threat human health by such issues like: antibiotic resistant bacteria, Immunopathological effects and Allergy (penicillin), Autoimmunity, Carcinogenicity (Oxytetracycline), Mutagenicity, Hepatotoxicity (Enrofloxacin), Bone marrow toxicity (Chloramphenicol)\(^{(26)}\).

Other aims of this study was comparing antibiotic rates in summer and winter, that statistical analysis shows significant increase in winter samples, that may relate to outbreak some infectious diseases and compression of flocks and irregular utilization of antibiotics in winters in order to prevent diseases and improving growth\(^{(25)}\). These results confirmed by vahedi et al investigation in 2008 in Mazandaran province, Iran that was operated on chicken carcasses by F.P.T method cleared that there is a significant increase in antibiotic residue amount in winter slaughtered chickens\(^{(26)}\).

**Conclusions**

Taking the achieved results into consideration, (99/100) 99% of calves meat and chicken samples were containing at least one kind of examined antibiotics. so it confirmed widespread use of antibiotic in livestock and chicken farms especially in Iran. The research is also showed the importance of implementation of recommended withdrawal times to control its residues in animal derived foods. Although chloramphenicol residues cannot overlook in society foods specially in military personnel that must be considered.

**References**


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