Status of Oxytetracycline Residues in Chicken Meat in Rawalpindi/Islamabad Area of Pakistan

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Abstract: In present study, status of Oxytetracycline (OTC) residues in chicken meat was determined in Rawalpindi/Islamabad area of Pakistan. Preliminary screening of samples for the presence of antibiotic residues was performed by a microbiological assay using Bacillus subtilis as test organism. OTC in positive samples was detected and quantified using High Performance Liquid Chromatography (HPLC) following extraction of the analyte with McIlvaine buffer and Solid Phase Extraction (SPE) system. A linear calibration curve was obtained with correlation coefficient of 0.9981 while average recoveries were greater than 91% with RSD values between 1.64 to 2.07%, while the Limit of Detection (LOD) was 0.01 µg mL⁻¹. Out of 29 meat samples that were analyzed for OTC residues, 13 (44.8%) had detectable residue levels for OTC and 6 (20.7%) had higher residue levels than the recommended maximum residue level (0.2, 0.6 and 1.2 µg g⁻¹) for muscles, liver and kidney, respectively. The method described in this study would be useful for routine monitoring of OTC residues in chicken meat.

Keywords: Oxytetracycline, residues, Bacillus subtilis, solid phase extraction, HPLC

INTRODUCTION

Tetracyclines are broad-spectrum antibiotics because they are active against gram positive and gram negative bacteria. They also act against some pathogenic agents unaffected by other antibiotics e.g., rickettsiae, certain large viruses belonging to psittacosis group in animals and lymphogranuloma venereum group in humans. Tetracyclines have activity against mycoplasmas, spirochetes and actinomyces at high doses some antiprotozoal activity has also been observed. Tetracyclines undergo metabolism to various degrees. The most frequently identified substance in urine, feces and tissue is the parent tetracycline. As much as 30% is excreted unchanged in feces. Tetracyclines are reversibly bound to plasma proteins and are widely distributed. Tetracyclines diffuse throughout the body and are found in highest concentrations in kidney, liver, spleen and lungs. They are also deposited at active sites of ossification (Riviere and Spoo, 2001).

Oxytetracycline (OTC) is commonly used in livestock and poultry for prevention and treatment of various diseases. However, the use of this compound may result in residues in animal derived food products, especially if withdrawal times are not observed. These residues may pose a health threat to consumers, depending on the type of food and the amount of residue present. The acceptable Maximum Residue Limit (MRL) for OTC as recommended by the joint FAO/WHO Expert Committee.

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on Food Additives (1999) is 0.2, 0.6 and 1.2 μg g⁻¹ for meat, liver and kidney, respectively. OTC residues that exceed the tolerance level may be of toxicological concern. Human health problems resulting from intake of sub-chronic exposure levels of oxytetracycline include gastrointestinal disturbances (Baker and Leyland, 1983), teratogenic risk to the fetus, allergic reactions (Schenk and Collery, 1998) and development of resistant pathogens for human and animals (Boggard and Stobberingh, 2000). Tetracyclines in meat may potentially stain the teeth of young children (Walton et al., 1994).

Microbial resistance to antibiotics is a worldwide problem in human and veterinary medicine. It is generally accepted that the main risk factor for the increase in antibiotic resistance is an extensive use of antibiotics. This has led to the emergence and dissemination of resistant bacteria and resistance genes in animals and humans. In both populations antibiotics are used for therapy and prophylaxis of infectious diseases. The main sector of resistance-increasing medicine usage, in regard to human health, lies within the health care sector. The antimicrobial agents used in animal care are also significant, not only in increasing the resistance in animal pathogen, but also in bacteria transmitted from animals to humans (Boggard and Stobberingh, 2000).

Resistance to members of tetracycline group has been reported for some strains of following bacteria: *Escherichia*, *Aerobacter*, *Salmonella cholerasuis*, *Salmonella typhimurium*, *Salmonella dublin*, *Salmonella pullorum*, *Salmonella gallinarum*, *Chlamydia psittaci*, *Proteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Pasturella multocida*, *Klebsiella pneumoniae* and *Beta-haemolytic Streptococci* (Kunin, 1993).

To present knowledge in Pakistan, so far no research has been reported on OTC residues in marketed meat. So, present study was planned in which Swab Test on Animal Food (STAF) was coupled with High-performance Liquid Chromatography (HPLC) for identification and quantification of oxytetracycline residues from chicken meat samples.

MATERIALS AND METHODS

Screening of Chicken Meat Samples by Microbiological Assay

A total of 100 chicken meat samples (33 liver, 33 kidney and 34 muscles) were collected from local market of Rawalpindi and Islamabad, Pakistan, during 2006. All the samples were transported to National Veterinary Laboratories, Islamabad, Pakistan under cold conditions. Preliminary screening of meat samples for the presence of antibiotic residues was performed using a microbiological assay called Swab Test on Animal Food (STAF) as described by Shahtid, 2006. Briefly, a locally isolated *Bacillus subtilis* was used as test organism on nutrient agar with 0.4% dextrose at pH 7. One milliliter spore suspension of *Bacillus subtilis* having 2×10⁷ spores ml⁻¹ was used in 100 ml of nutrient agar to make STAF plates. Sterile cotton swabs immersed in tissue samples were placed aseptically over STAF plates having Neomycin 5 μg antibiotic filter paper disc as a positive control and incubated at 37°C for 18 h. An inhibition zone greater than 2 mm around swab samples was considered as positive; while that less than 2 mm was taken as negative for antibiotic residues.

HPLC Method

The chicken meat samples determined as positive by STAF test were further analyzed by HPLC for Oxytetracycline identification and quantification. All the experiments were performed during 2006 in National Veterinary Laboratories, Islamabad, Pakistan.

Preparation of Standard Curve

Peak areas were recorded for OTC chromatographic standard solutions. Standard curve was prepared using peak areas vs. concentration of standard solutions (0.015, 0.062, 0.125, 0.250, 0.375, 0.50, 0.750 and 1 μg ml⁻¹). The best fit of data was determined using linear regression from Microsoft Excel that uses the following equation:
Y = mx + b

Where,
Y = Peak area
x = OTC concentration (μg kg−1)
m = Slope of curve
b = Intercept of y

From measured peak heights of analyte, OTC concentrations were calculated using regression slope and intercept values.

Extraction and Cleanup Procedure

Meat sample (5 g) mixed with 20 mL McIlvaine buffer-EDTA solution in a 50 mL polypropylene centrifuge tube was blended for 30 sec with homogenizer (IKA-Werke Ultra-Turrax T25 basic, Italy) at 1100 rpm and centrifuged (Sigma 3K30, Rotor # 1250H, Germany) for 10 min at 2500 x g. Supernatant was poured into second 50 mL centrifuge tube. No tissue was transferred. To the first tube 20 mL McIlvaine buffer-EDTA was added and the tissue plug was resuspended using Vortex mixer (IKA yellow line TSS2, USA). It was again centrifuged for 10 min at 2500 x g and supernatant was added to the supernatant in second tube. Finally 10 mL McIlvaine buffer-EDTA solution was used and all the steps were repeated until the supernatants from all 3 extractions were collected. The combined supernatant fluid was applied to the Solid Phase Extraction (SPE) apparatus (Lichrolut) using C18 SPE cartridge (6 mL, 500 mg, B and I). The cartridge was conditioned with 10 mL methanol followed 10 mL deionized water at a flow rate of 1 mL min−1. The combined supernatant fluid was passed through the column and eluted at the same flow rate. The analyte was extracted from the column by pouring 10 mL Methanolic oxalic acid. The solvent was evaporated using rotary evaporator (BUCHI rotavapor, R-200, Germany) at 45°C. The sample was dried completely using Nitrogen flow. One millilitre mobile phase was used to reconstitute the dried sample and this solution was later analyzed by HPLC system (Hitachi D-7000 series) for determination of OTC residues.

Chromatographic Conditions

HPLC analyses were carried out by using HPLC system (Hitachi D-7000 series, Japan) with Column Oven (L-7300), UV Detector (L-7400), Autosampler (L-7200), Pump (L-7100), Degasser (L-7610) and Interface Module (D-7000); data acquisition was controlled by Hitachi D-7000 Chromatography Data Station Software (HPLC System Manager) Version 4.0. Injections were automatically made on a Reversed phase Teknokroma Kromasil 100, C8 column (250×4.6 mm, 5 μm). The mobile phase was citric acid (25 mM): Acetonitrile (8:2 v/v). Injection volume was 10 μL of all standards and sample extracts. The analyses were carried out on column temperature 35°C under isocratic conditions and a flow rate of 1 mL min−1. Eluted peaks were detected at 270 nm.

Recovery Test

The meat sample, which was negative for Oxytetracycline, was selected for preparation of spiked tissue samples by confirming on HPLC to be free from Oxytetracycline. Oxytetracycline spiked tissue samples were prepared, considering the MRL of 0.2, 0.6 and 1.2 μg g−1 for meat, liver and kidney, respectively (recommended by EU, FAO/WHO, FDA of USA) ranging in concentration from 0.2 to 0.05 μg g−1 (0.2, 0.15, 0.1 and 0.05 μg g−1) by adding 1000 μL of 1.2, 0.9, 0.6, 0.3 μg mL−1 standard solutions of Oxytetracycline. Fortified samples were allowed to stand at 4°C for 12 h after standards addition and then mixed prior to work up. The percent recovery for each spiked sample was measured using the following relation:

\[
\text{Percent recovery of OTC} = \frac{\text{OTC concentration recovered}}{\text{OTC concentration in sample}} \times 100
\]
Repeatability Test

The OTC residues were detected in meat samples (blank) spiked with OTC standard at the rate of 0.1 µg g⁻¹ of the sample. The procedure was repeated three times in three different days to determine the precision and validity of the sample preparation and HPLC analyses.

RESULTS AND DISCUSSION

Out of 100 chicken meat samples examined by STAF test, 13 (39.4%) liver, 9 (27.3%) kidney and 7 (20.6%) muscle samples were detected positive for antibiotic residues. Twenty nine STAF positive chicken meat samples that were analyzed by HPLC for OTC residues, 13 (44.8%) had detectable levels for OTC. Similar results were observed by Ökemarm et al. (1998). They reported that the majority of positive results on plates seeded with B. subtilis, 77 of 89 (86%) samples tested, contained tetracycline antibiotics. In present study, the highest concentration was 3.880 µg g⁻¹ in kidney sample and lowest was 0.030 µg g⁻¹ in a muscle sample (Table 1). These findings are in line with Murray et al. (1998).

In case of liver samples, 3 out of 13 (23.1%) had detectable level of OTC residues, 6 out of 9 (66.7%) kidney samples had OTC residues while 4 out of 7 (57.1%) muscle samples had detectable levels of OTC. The mean residues levels in liver, kidney and muscle samples were 1.274, 2.119 and 0.051 µg g⁻¹, respectively. The results obtained are similar to the findings of Muriuki et al. (2001) who studied the presence of tetracycline residues in beef samples in Nairobi. Out of the 250 samples that were analysed for tetracycline residues 114 (45.6%) had detectable tetracycline residues. Of the 114 samples with detectable tetracycline residues, 60 (24%) were liver samples, 35 (14%), were kidney samples and 19 (7.6%) were muscle samples. Iqbal (2000) conducted a study in Faisalabad to check the presence of tetracycline residues in poultry meat and eggs by HPLC. The highest concentration of tetracycline was 81.3 µg g⁻¹ and lowest was 21.32 µg g⁻¹. It showed that incidence was 100%. Similarly all egg samples were also positive showing a range from 74.41 to 167.13 µg mL⁻¹.

The calibration graph was obtained by plotting peak area against concentration of OTC in standard solutions. The correlation coefficient was 0.9981. This is similar to the finding of Pena et al. (1999). The limit of detection of OTC was 0.01 µg mL⁻¹ (signal to noise ratio >5) which was well below the MRL (0.2, 0.6 and 1.2 µg g⁻¹ for muscle, liver and kidney, respectively).

Extraction of OTC residues from meat samples was performed using Mellaune buffer-EDTA solution. Clean up was done by using Solid Phase Extraction system (SPE) C18 cartridges. The average recoveries >91% with Relative Standard Deviations (RSD) between 1.64 to 2.07% indicated the efficiency of extraction and clean up procedure. Similar results were obtained by Sokol et al. (1994), Chet et al. (2003), Pena et al. (2003) and Cinquina et al. (2003).

The mean recoveries of OTC from meat samples at four different spiking levels (0.05, 0.1, 0.15 and 0.2 µg g⁻¹) are summarized in Table 2. Results obtained showed the average recoveries >91% with Relative Standard Deviations (RSD) between 1.64 to 2.07%. Recoveries obtained are similar to those determined by Mulders et al. (1989) for tetracyclines in animal tissues. It indicates high efficiency of the extraction cleanup procedure used. These results are also in line with the findings of Semyuya et al. (2000).

Table 1: Tetracycline concentrations in chicken meat samples determined by HPLC method

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Positive samples/ total No.</th>
<th>Percentage of OTC positive samples (µg g⁻¹)</th>
<th>Concentration of OTC (µg g⁻¹)</th>
<th>MRL (µg g⁻¹)</th>
<th>No. of samples above *MRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>4/7</td>
<td>57.1</td>
<td>0.030-0.085</td>
<td>0.051</td>
<td>0.026</td>
</tr>
<tr>
<td>Liver</td>
<td>3/13</td>
<td>23.1</td>
<td>0.567-1.940</td>
<td>1.274</td>
<td>0.087</td>
</tr>
<tr>
<td>Kidney</td>
<td>6/9</td>
<td>66.7</td>
<td>0.638-3.880</td>
<td>2.119</td>
<td>1.209</td>
</tr>
</tbody>
</table>

*Maximum Residue Limit
Table 2: Recoveries for oxytetracycline fortified meat samples

<table>
<thead>
<tr>
<th>Spiked (µg g⁻¹)</th>
<th>No. of replicates</th>
<th>Mean conc. Recovered (µg g⁻¹) ±SD</th>
<th>Mean recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>3</td>
<td>0.0459±0.0009</td>
<td>91.7±1.9</td>
<td>2.07</td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
<td>0.0913±0.0015</td>
<td>91.3±1.5</td>
<td>1.64</td>
</tr>
<tr>
<td>0.15</td>
<td>3</td>
<td>0.1414±0.0027</td>
<td>94.2±1.8</td>
<td>1.91</td>
</tr>
<tr>
<td>0.2</td>
<td>3</td>
<td>0.1840±0.0038</td>
<td>92.5±1.92</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Table 3: Precision data for meat samples spiked to 0.1 µg g⁻¹

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>0.0935</td>
<td>0.0895</td>
<td>0.0904</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>0.0912</td>
<td>0.0916</td>
<td>0.0882</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>0.0885</td>
<td>0.0879</td>
<td>0.0923</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0911</td>
<td>0.0897</td>
<td>0.0930</td>
</tr>
<tr>
<td>SD</td>
<td>0.0025</td>
<td>0.0018</td>
<td>0.0020</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>2.74</td>
<td>2.00</td>
<td>2.22</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>91.1</td>
<td>89.7</td>
<td>90.3</td>
</tr>
</tbody>
</table>

Fig. 1: HPLC chromatograms (UV detector set at 270 nm). (A) Standard (0.1 µg mL⁻¹); (B) Blank tissue sample; (C) Spiked meat sample (0.05 µg g⁻¹). Peak 1 = Oxytetracycline (retention time = 5.3 min)

In the present method, the HPLC UV detector was used. OTC was identified in the sample by retention time (Fig. 1). The maximum absorption wavelength of OTC standard solution occurred at both 270 and 352 nm using an UV-Vis spectrophotometer; however, the wavelength adjusted to 270 nm was used for detection of OTC in HPLC (Fig. 2-4). These results are in agreement with those reported by Sokol et al. (1994), Moretti et al. (1994) and Seryuyu et al. (2000).

The precision of the method was also studied by assaying on each of the 3 different days. Several meat samples were spiked with OTC (0.1 µg g⁻¹). To improve the precision, the analyses were carried
Fig. 2: UV scan of mobile phase (200-1100 nm)

Fig. 3: UV scans of Oxytetracycline standard (200-1100 nm); peak absorbance can be seen at 270 and 352 nm.

out by the same operator using the same material and performing three trials each day during 3 days. Results are shown in Table 3.

Reverse phase column was used in present study. The suitability of the column was evaluated by calculating the retention time of a standard solution of OTC (0.1 μg mL⁻¹) after every 40 sample injections. The mean of the observed retention time was 5.3±0.2 min (Fig. 1). Similar results were obtained by Poulignier et al. (1997), Furusawa (2002) and Cherlet et al. (2003) using reverse phase column.

The HPLC method used in this study was found to be sensitive, precise, specific and convenient analytical method for the detection and quantification of oxytetracycline residues in chicken meat samples. One of the major advantages over microbiological method is that the lower detection limit of 0.01 μg mL⁻¹ makes it a highly precise instrument.
Fig. 4: UV scan of Oxytetracycline standard dissolved in mobile phase (200-1100 nm)

CONCLUSIONS

Results of this study indicated that 44.8% of the total number of samples analyzed by HPLC for OTC residues had detectable residue levels where 20% had higher residue level than MRL. Therefore it is the need of time that regulatory authorities should constantly conduct surveillance to monitor the antibiotic residue levels in order to safeguard human health.

REFERENCES


