Simple and Rapid Method for Analysis of Oxytetracycline as a Residue in Milk Sample by HPLC.

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Received on: 14-07-2012; Revised on: 19-08-2012; Accepted on: 17-09-2012

ABSTRACT

A simple and rapid reversed phase high performance liquid chromatograph (HPLC) method for analysis of Oxytetracycline (OTC) was developed and applied in the determination of the antibiotic as a residue of antibiotic in milk samples fortified with known concentration of OTC. The estimation of OTC was done by Hitachi D-2000 Elite HPLC system manager using gradient pump system. From two pumps (L-2130) A containing mobile phase Methanol which was adjusted on 40% flow while the pump B containing Mobile phase buffer (Orthophosphoric acid + Triethylamine) on 60%. The flow rate was 1.0ml/min; the separation was done by using C-18 column and detected by UV-Visible Detector at 240 nm. The retention time was within 1.67-1.70 minutes. Standard curves were linear over the concentration range of 0.5 to 10µg/ml. The extraction recovery of OTC was within 94 and 99%. The proposed rate was 1.0ml/min; the separation was done by using C-18 column and detected by UV-Visible Detector at 240 nm. The retention time was within 1.67-1.70 minutes. Standard curves were linear over the concentration range of 0.5 to 10µg/ml. The extraction recovery of OTC was within 94 and 99%. The proposed method was found to be rapid accurate, repeatability and consistent. It was successfully applied for the analysis of the OTC. This was rapid from before reported method.

Keywords: Oxytetracycline, Residue, HPLC, Liquid Chromatography.

INTRODUCTION:

Tetracyclines (TCs), including mainly Oxytetracycline, tetracycline and chlorotetracycline, are broad-spectrum antibiotics widely used in treating infectious diseases in humans and animals, and used as growth promoters in modern animal husbandry. The usages of tetracycline applied in animals were 1,469,400 kg in the USA in 1999, 16,268 kg in the UK in 2000 and 3324 kg in Kenya in 1999. [1] These Antibiotics are widely used in food-producing animals for the treatment of disease and as dietary supplements. They may be administered orally as food additives or directly by injection. The use of antibiotics may result in drug residues in the meat, especially if they are not used according to label directions. The presence of antibiotic residues in meat, milk, etc. may cause allergic reactions in sensitive individuals [2].

Oxytetracycline (OTC), [4S-4a,4a,5a,5a,6b,12a]-4-(dimethylamino)-4,4a,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydoxy-6-methyl-1,11-dioxo-2-naphtacenecarboxamide (Figure:1), it was first prepared by the catalytic reduction of chlorotetracycline. TC has also been reported to be produced from the Strains of Streptomyces aureofaciens, Streptomyces avellanus, Streptomyces foefaciens, Streptomyces albolfavus and many others [3]. Among the TC group of antibiotics, OTC is the most widely used in therapeutics. It is commonly used for the prevention and treatment of diseases in livestock production. As a feed additive in sub therapeutic doses, it contributes to the maintenance of optimal health and thus promotes growth in food-producing animals. [4] Different methods is used for detection and quantification of antibiotic drugs and as a residue in milk and meat sample.

Liquid chromatography has become the most widely used separation technique for determining tetracycline antibiotics in edible animal products [5, 6]. Methods used to determine OTC residue levels include microbiological [7], mass spectrometric [8] and HPLC [9, 10]. Recently developments in the chromatographic techniques for determinations of antibiotic residues in milk have been reviewed [11], and [12]. In order to monitor compliance to these limits several analytical methods have been developed and validated [13, 14 and 15].

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samples on day-1 and day-2. The Accuracy was evaluated by the recovery determination.

PREPARATION OF SOLUTION:

Solvents Preparation:
(Orthophosphoric acid + Triethylamine). 100ml of 1M of Orthophosphoric acid was dissolved in 1000ml distilled water and the pH was adjust to 3.0±0.1 with Triethylamine. (Monopotassium Phosphate + citric acid ) 500ml of 0.075 M monopotassium Phosphate was well mixed with 500ml of 1.0 M citric acid. Methanol and Acetonitril used as directly. All the solvents were filtered through 0.45µm filter membrane and degass for 20 minutes by ultrasonic cleaner.

Standard Preparation.
A standard stock solution of OTC (100mg/100mL) was prepared by dissolving the drug in 40ml methanol and 60ml Buffer. The concentration of standard was 1mg/ml solution was further diluted with mobile phase in same ratio to 0.5, 1.0, 2.0, 3.0 and 5.0µg/ml. All the solutions were stored at 4°C and were brought to room temperature before use.

Sample preparation:
The sample solution was prepared by adding 200 µL of five standard solutions of OTC (1.0, 2.0, 3.0, 5.0 and 10.0 µg/mL) to separate 2.0 mL portions of the milk samples, followed by thorough mixing. The fortification was usually made by spiking. Fortification with a known amount of analyte which became 0.1, 0.2, 0.3, 0.5 and 1.0µg/mL respectively. These fortified samples were allowed to stand at 4 °C for 24 h after OTC addition. For extraction standard spiked milk were subjected to a deprotenizing chemical procedure using Trichlroaceticacid. A 2mL quotient of milk sample was placed into a 10mL test tube and shaken intensively with 3mL of 20% (v/v) TCA for 1 min. The mixture was centrifuged for 15 min at 5000rpm and supernatant was injected 10ul to HPLC system.

Optimization of Mobile Phase:
The mobile phase composition was optimized on the basis of CHP method. The aqueous phase were composed of Buffers (Orthophosphoric acid + Triethylamine) and (monopotassium Phosphate + citric acid) while the organic phase was selected from methanol, acetonitrile and their Mixture in different proportions. Many efforts were made on the adjustment of the ratios of the components of mobile phases. The best separation and recovery were made by using 40% methanol with 60% buffer. (Orthophosphoric acid + Triethylamine).

CHROMATOGRAPHY:
The optimized mobile phase was a methanol and Buffer. Pump A was adjusted at flow rate 40% for Methanol while pump B at 60% flow rate for Buffer. The flow rate was set at 1 ml/min. The injection volume was 10µL for samples and standards, which is injected to the column by autosampler. The separation was achieved using Column oven L-2300 at 40°C and column Intersil ODS-3 C18 (GL Sciences Inc. Tokyo Japan 5um, 250x4.6 mm) and the detection wavelength was set at 240 nm. The UV absorbance of the effluent was scanned by UV Spectrophotometer (optima S-3000 Kyoto, Japan) over the range of 200-400nm and was obtained by measuring the absorption of 0.1µg/ml solution, prepared from stock solution. This showed a maximum absorbance on 240nm.

RESULT AND DISCUSSION:

LOD and LOQ:
Limit of detection (LOD) and limit of quantification (LOQ) of the assay method were determined. Results showed that the detection limit of the tested drugs was 0.05 µg/mL. (figure:2) which was a good improvement of before reported methods. [15,16,17] For measurement, consideration was given only when the first condition was satisfied for ascertaining the presence of target compounds i.e. OTC with a signal/noise ratio of 3 (S/N = 3). The LOQ calculated was 0.1µg/ml. (figure:3) So, LOQ was started from this concentration. The LOQ was calculated on the basis of minimal accepted value of S/N =10.
Linearity:
Intra-day and inter-day precision was determined by injecting 10µl six standard solutions (n = 6). The mean of the recorded peak area of inter day and intra day is taken for calibration curve. (Table 1) The peak areas which were automatically measured by an integrator of HPLC instrument. The calibration curve obtained by plotting peak area against concentration of the standard in Graph 1 and of sample (Graph 2) which showed linearity in accordance to Beer's law over this range and the linearity equation was:

\[ y = 2121063x - 24934.9 \] for standard and 

\[ y = 195431x - 2134.7 \] for sample. The regression coefficient \( r^2 \) were in the range from 0.9993-0.9995 (n=6).

### Table 1: Peak Areas of OTC Standard

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration µg/ml</th>
<th>Peak Areas</th>
<th>Inter Day</th>
<th>Intra Day-2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>287352</td>
<td>287423</td>
<td>287387</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
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<td>557525</td>
<td>557469</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>1115671</td>
<td>1115814</td>
<td>1115743</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.0</td>
<td>1661640</td>
<td>1661710</td>
<td>1661675</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>2867838</td>
<td>2867899</td>
<td>2867869</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
<td>5678904</td>
<td>5678935</td>
<td>5678919</td>
<td></td>
</tr>
</tbody>
</table>

Each 10µl Injection of OTC.
Peak Area (automatically measured by an integrator of HPLC instrument)

### Table 2: Peak Areas of Milk sample contaminated with OTC.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration µg/ml</th>
<th>Peak Areas</th>
<th>Inter Day</th>
<th>Intra Day-2</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>2</td>
<td>0.2</td>
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<td>54712</td>
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<tr>
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<td>126325</td>
<td>126341</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>260444</td>
<td>261002</td>
<td>260723</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>557414</td>
<td>557243</td>
<td>557328</td>
<td></td>
</tr>
</tbody>
</table>

Each 10µl Injection of OTC.
Peak Area (automatically measured by an integrator of HPLC instrument)

Recovery and precision of Streptomycin.
The current method is valid and accurate. The Accuracy was evaluated by the recovery determination. Our results showed the amount obtained by this method were between 94% and 99 %. (Table 2) the absolute recoveries of streptomycin Sulphate were determined in triplicate by direct comparison of peak area form standard versus sample. (Figure 4-5). The data was analyzed statistically by calculating average mean RSD by using formula. [RSD = (S.D./mean of the recoveries) × 100%].

\[
\text{Conc. of Sample µg/ml = Peak area of Sample \times Conc. of Std \times Std potency / Peak area of Std Sample Potency}
\]
Table-3: Recovery and Precision of OTC for contaminated Milk sample.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Recovery Conc. (µg/ml)</th>
<th>Conc. Day 1 (µg/ml)</th>
<th>Conc. Day 1 (µg/ml)</th>
<th>Mean (µg/ml)</th>
<th>Recovery (%)</th>
<th>Precision RSD%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.0969</td>
<td>0.0919</td>
<td>0.0945</td>
<td>94.50</td>
<td>3.6023</td>
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<tr>
<td>2</td>
<td>0.2</td>
<td>0.1952</td>
<td>0.1936</td>
<td>0.1944</td>
<td>97.20</td>
<td>1.4684</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>0.2930</td>
<td>0.2950</td>
<td>0.2940</td>
<td>98.10</td>
<td>1.0521</td>
</tr>
<tr>
<td>4</td>
<td>0.5</td>
<td>0.4940</td>
<td>0.4958</td>
<td>0.4954</td>
<td>99.08</td>
<td>0.5207</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>0.9850</td>
<td>0.9810</td>
<td>0.9830</td>
<td>98.30</td>
<td>0.8767</td>
</tr>
</tbody>
</table>

Specificity:
The specificity of the method was ascertained by analyzing standard drug and sample. The retention time (RT) of streptomycin confirmed by comparing the RT with that of the standard, which was within 1.67-1.70 minutes. The presence of other substances in the formulation did not cause any interference with the OTC peak so specific for analysis of Oxytetracycline.

CONCLUSION:
The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection of OTC. Various methods are available for determination but having some disadvantages of being time consuming with poor recoveries and reproducibility. While the proposed method was found to be rapid, accurate, repeatable and consistent. It was successfully applied for the analysis of the drug in marketed formulation and could be effectively used for the detection and quantification of OTC residue in Milk, Meat and Honey. This was rapid from before reported method.

REFERENCES:

Source of support: Nil, Conflict of interest: None Declared