

VALIDATION OF A SIMPLE ISOCRATIC HPLC ASSAY FOR THE DETECTION OF OXYTETRACYCLINE RESIDUES IN CHICKEN MEAT

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ABSTRACT

One of the major concerns in poultry rearing has been the use of antibacterials for the control of infections. The antibacterial drugs often find their way into the edible part of chicken, thus making it an issue of human food safety. Screening of chicken meat for antibacterials is thus an important part of ensuring food safety. Simple sensitive methods which could be adopted easily at laboratories of research institutes and testing centres are a need of the hour. In this study, one such assay method for the assay of oxytetracycline, a popularly used antibiotic in poultry farms, in broiler chicken meat standardized using High-performance liquid chromatography (HPLC) is described. The HPLC assay method consisted of a C_{18} column as the stationary phase and a mobile phase consisting of 0.2M oxalic acid: Acetonitrile: Methanol (3.5:1:1, v/v) pumped in with the flow rate of 1.5 ml/min and assayed at 360nm for detection of OTC. The Limit of Detection and Limit of Quantification was 0.141 $\mu\text{g/ml}$ and 0.465 $\mu\text{g/ml}$ respectively. The total elution time was less than 4 minutes with a recovery rate of 105%. The results suggest that this method, with its high detection throughput and remarkable sensitivity and specificity, can be applied successfully for the field screening of oxytetracycline residue in chicken meat samples.

Keywords: Broiler chicken meat, HPLC LOD and LOQ, Oxytetracycline Residue, Recovery, Repeatability

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INTRODUCTION

India has become one of the rapidly growing poultry producers globally, during the last decade. Chicken meat is highly consumed in India because of its widespread acceptance and relatively low cost compared to other meats. The increased demand in production can be met through the intensive system of

rearing. The stress due to high animal density in the intensive farming system makes the chicken much more vulnerable to diseases. To combat the diseases, antimicrobials are commonly used to control infections caused by bacteria and also improve feed efficiency, productivity, and growth.

Worldwide, India has been one of the top consumers of animal antibiotics that accounts for 3% of global consumption in 2010 and this use is projected to increase by about two-thirds in 2030 (Meena and Kumar, 2019). The use of antibiotics in chickens is estimated to triple in India by 2030 (Sivaraman, 2018). Tetracyclines are the most widely used antibiotics in veterinary medicine in India due to their broad spectrum of antimicrobial activity, availability, and low cost (Biswas *et al.*, 2007). This group of antibiotics has been reported to affect the growth of bones and teeth in children. The presence of oxytetracycline is also associated with development of resistance in the bacteria. Globally, there has been a surge in awareness among the public about the quality of food. It thus becomes necessary to develop a simple validated assay method to screen the meat for antibiotic residue.

Hence in this study, it was proposed to develop and validate a simple isocratic method for the determination of oxytetracycline residues in chicken meat which is sensitive and easily applicable in the field.

MATERIALS AND METHODS

Instrumentation

The chromatographic system used, Waters® (USA) consists of an isocratic pump,

sample injector, column, thermostat, and UV-VIS detector. The above chromatographic system is controlled using Pentium 1 with Empower Software.

Chemicals and reagents

All the chemicals used were of analytical grade and a few of them were of HPLC grade. Acetonitrile and Methanol of HPLC grade were purchased from Merck. Highly purified Milli-Q water from the Millipore system was used in all procedures. The standard Oxytetracycline hydrochloride was purchased from Sigma Aldrich (Germany).

Chromatographic conditions

The chromatographic separation was carried out on a Thermo Fisher Scientific RP-C₁₈ column with particle size 5 µ, 250 mm x 4.3 mm. The mobile phase contained 0.2M oxalic acid (pH 2.5): Methanol: Acetonitrile (3.5:1:1, v/v) at a flow rate of 1.5 ml/min (Ueno *et al.*, 1989). The injection volume was 10 µl. The experiment was performed at ambient temperature. The absorbance measurements were held at 360 nm and the elution time was less than 4 minutes.

Preparation of solutions

The stock solution was prepared in 10mg/10ml concentration by dissolving 10mg of OTC standard in 100 µl of 0.01M HCl (Tauber *et al.*, 2015) and 9.9 ml of Milli-Q water was added. The solution was stored in dark at 4°C until use. The standard calibration solutions with concentrations 0.5, 1.0, 2.0, 5.0, 10, 20, 50, and 100 µg/ml were made using Milli-Q water.

Samples and sample preparation

For chicken meat matrix, broiler chicken meat samples were obtained from local retail meat shops and stored at -20°C until use. One gram of meat sample was weighed in a polypropylene tube and finely chopped and then homogenized with 1ml of McIlvaine- EDTA buffer(buffer composition: 12g citric acid monohydrate, 10.9g disodium hydrogen phosphate, and 37.2g EDTA in 1L water and adjusted to pH 3.8 using HCl) using a tissue homogenizer (Al-Bahry *et al.*, 2013; Pokrant *et al.*, 2018). It was then followed by centrifugation at 12,000 x g (9500 rpm) for 15 minutes at 4°C and the supernatant was collected.

The supernatant was spiked with OTC standards in 1:9 ratio to get matrix-based standards in the concentrations of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 µg/ml. To the various standards of OTC, acetonitrile was added to it in the ratio of 1.5:1, vortexed, and centrifuged at 6300 x g for 5 minutes. The supernatant was mixed with water in 1:1, filtered, and injected into HPLC.

Validation of method

The HPLC method for the determination of Oxytetracycline in chicken meat was validated according to ICH guidelines (ICH, 2005). The assessed parameters were specificity, linearity, the limit of detection, the limit of quantification, precision, and recovery.

Specificity

The specificity of Oxytetracycline was checked by calculating the retention time and area of the peak.

Linearity

For linearity assessment, 8 varying concentrations of matrix standards 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 µg/ml were injected and the respective peak areas were noted. The calibration curve was obtained by graphical representation of Oxytetracycline concentrations versus area of peak.

Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of drug that can be reliably detected with a given analytical method. LOD is calculated using calibration curve by the following formula:

$$\text{LOD} = 3.3 \times \text{Standard Error} / \text{Slope of the calibration curve}$$

Limit of quantification

LOQ is defined as the lowest concentration of an analyte that can be quantitatively detected with acceptable accuracy and precision. The LOQ can be approximated by multiplying the LOD by 3.3 (Lister, 2007).

Precision

The precision of the analytical method was determined by evaluating repeatability (intra-day) and intermediate (inter-day) precision. Intra-day variation was determined by assaying three replicates of four concentrations (0.05, 0.1, 0.5, and 2.0 µg/ml). Inter-day variation was determined by assaying seven concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 µg/ml) on three different occasions at least 24 hours apart between each assay. The precision of the proposed method

was expressed as the percent of co-efficient of variation (CoV).

Analytical recovery

Analytical recovery refers to the capability of the method to detect the compound without any loss that could happen due to the matrix effect or the methodology used. . It is expressed as a ratio or per cent. Analytical recovery was calculated as a ratio of the peak areas obtained for meat-based standards and those obtained for water-based standards. Percent recovery was calculated according to the regression formula. Four different concentrations of OTC (One lower concentration, two intermediate concentrations and one higher concentration) were prepared in the matrix. The samples were processed and the final concentration was detected according to the regression formula. The recovery was calculated as below.

where,

X = concentration of drug spiked

Y = concentration found by assay method (recovery x concentration spiked)

n = number of observations

RESULTS

The HPLC method suggested had a retention time of 3.9 minutes, which is very convenient for large scale screening (Fig. 1). The overlay report of Oxytetracycline at varying concentrations is shown in Fig. 2. The calibration curve obtained was very reliable with a R^2 value of 0.997.

The calculated limit of detection and limit of quantification for Oxytetracycline in meat samples was 0.141 $\mu\text{g/ml}$ and 0.465 $\mu\text{g/ml}$ respectively. The calibration curve for linearity is shown in Fig. 3 and the equation of the line was as follows,

$$Y = 4.86e+003 X + 4.33e+002; R^2 \text{ value: } 0.998.$$

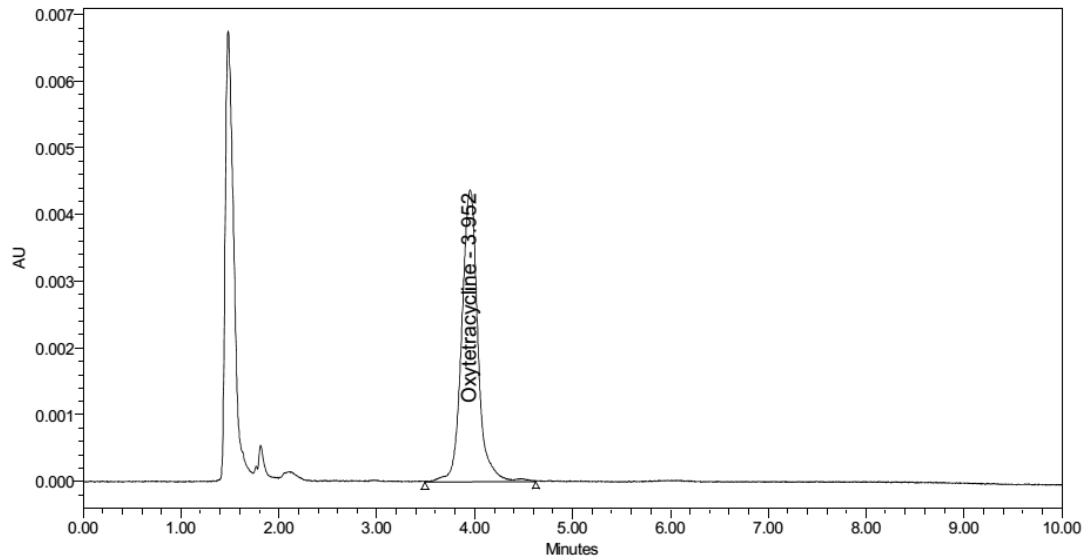
Intra-day and inter-day precisions were shown at different concentrations (Tables 1 and 2). The results of recovery calculations suggest that losses due to the process were minimal and the method enjoyed complete recovery (Table 3).

Table 1. Results for repeatability

Concentration in chicken meat ($\mu\text{g/ml}$)	Intra-day precision (CV %)
0.05	9.08
0.1	10.84
0.5	1.42
2.0	0.98

Table 2. Results for intermediate precision

Concentration in chicken meat ($\mu\text{g/ml}$)	Inter-day precision (CV %)
0.1	12.36
0.2	8.22
0.5	10.82
1.0	5.76
2.0	1.23
5.0	2.78
10.0	2.27



	Peak Name	RT	Area	% Area	Height	Amount	Units
1	Oxytetracycline	3.952	49948	100.00	4379	10.000	µg/ml

Fig. 1. Chromatogram of OTC Standard 10 µg/ml

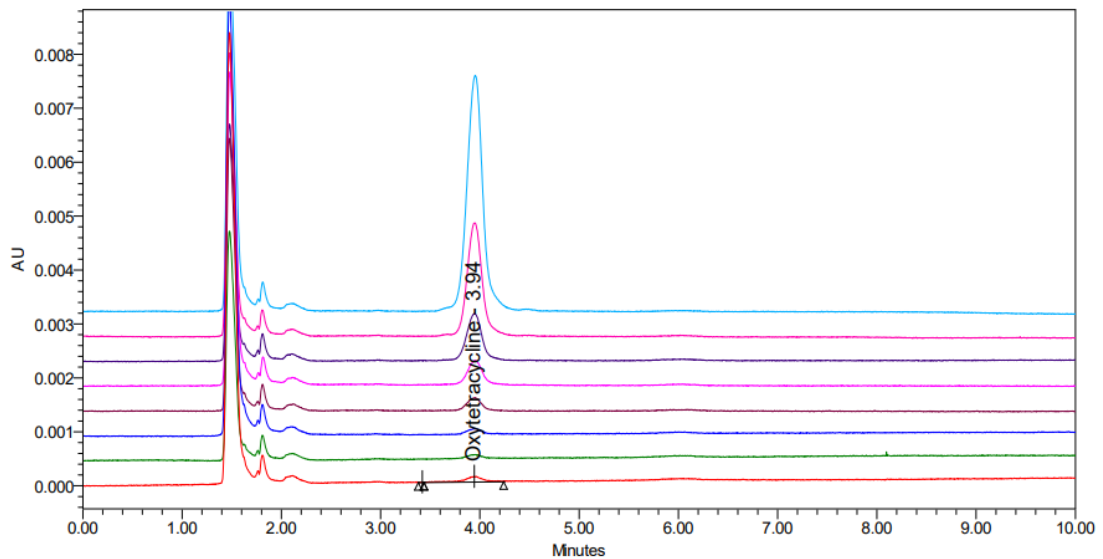


Fig. 2. Overlay chromatogram of oxytetracycline

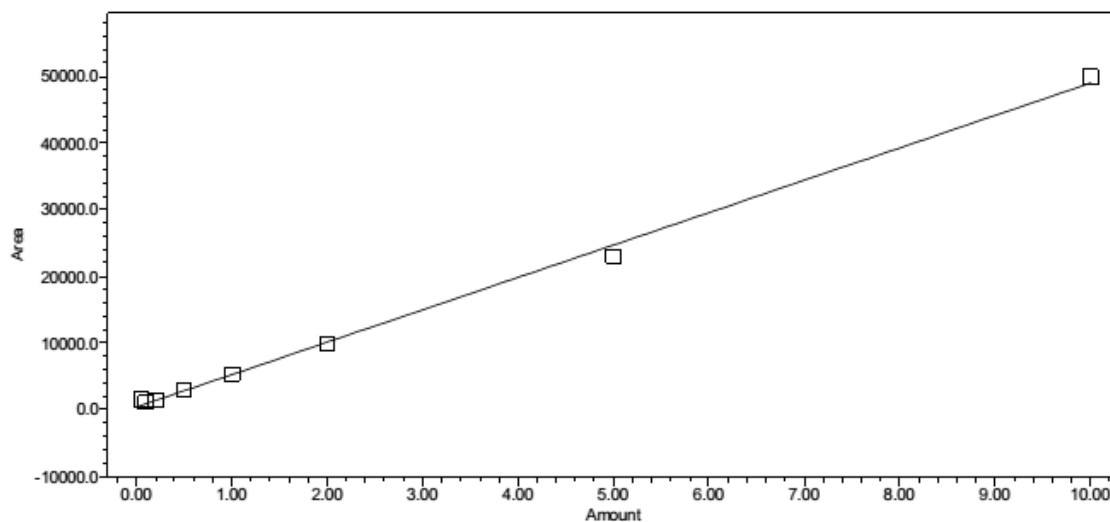


Fig. 3. Calibration curve of OTC

Table 3. Results for recovery

Concentration spiked ($\mu\text{g/ml}$)	Oxytetracycline in meat			
	0.05	0.1	0.5	2.0
Recovery (range)	0.4427 - 1.2671	0.7269 - 0.8507	1.272 - 1.320	1.012 - 1.136
Concentration found (range, $\mu\text{g/ml}$)	0.022 - 0.063	0.0727 - 0.0851	0.636 - 0.660	2.024 - 2.273
Mean $\pm$ SE	0.0476 $\pm$ 0.0128	0.0807 $\pm$ 0.004	0.6485 $\pm$ 0.0069	2.1173 $\pm$ 0.0783
Percent recovery	105.78 %			

DISCUSSION

Oxytetracycline is one of the commonly used drugs in poultry. As there is increasing awareness about the drug residues among the consuming public, laboratories in universities and institutes will be expected to standardize a methodology for the assessment of OTC residue in chicken meat.

Here we report a simple isocratic method for the determination of oxytetracycline in chicken meat samples. The constant retention time for OTC elution during the entire standardization process ensures the specificity of analyte. The calibration curve R^2 value of 0.997 indicates the linearity. The biggest advantage of this method is the very short retention time of about four minutes. This indicates that the method will be suitable for running a large number of samples per day. This factor will be very useful for screening laboratories. Since, the limit of detection is 0.141 $\mu\text{g/ml}$, this method can also detect at low concentrations below the MRL of the OTC (200 ppb) for chicken meat.

Both intra-day and inter-day variations showed a very high precision with a $\text{CoV} < 15\%$ at different concentrations expresses the reliable repeatability of this method.

The recovery calculation in this method indicates complete recovery. Normally methods with simpler extraction processes suffer from poor recovery. Any method that gives $> 80\%$ recovery from a tissue is appreciable. Here we have about 100% which is complete recovery and this means there is no loss of drug owing to tissue effect or the method effect. A longer complicated extraction

increases input costs and also increases sample process time. In this method, the complete recovery with one-step extraction is a big advantage.

Though several methodologies like HPTLC, ELISA, and LC-MS/MS (Huong-Anh *et al.*, 2020; Kumar *et al.*, 2020), are available for estimation of OTC residue, however HPLC method is the widely accepted for the determination of residue due to its high specificity, precision, and reliability (Yuan *et al.*, 2001). Mass spectrophotometry is out of reach of most laboratories because it is less suited for routine laboratory work and difficult to perform quantitative determinations and also difficult to maintain (Pitt, 2009), whereas HPLC is more easily available. ELISA, on the other hand, suffers from the issues of false-positive results (Fischer and Michael, 1997) and also commercial kits of ELISA, are often prohibitively costly. HPTLC has an open system which requires stringent condition like dust free environment for operation (Bairy, 2015), whereas HPLC is a closed system. HPTLC is often non-specific and less sensitive compared to HPLC.

In this method, an isocratic elution is used which is better than gradient elution. Isocratic elution is cost-effective, simple for the separation of analytes, and there is no need to re-equilibrate with the initial mobile phase between consecutive sample injections (Schellinger and Carr, 2006).

The method can also be improved to suit the simultaneous detection of other tetracyclines. The method can also be adopted for other matrices such as egg, milk etc.

CONCLUSION

The proposed method fulfills the need for a simple sensitive assay method for the detection of oxytetracycline in chicken meat. Oxytetracycline assay in chicken meat samples was done using RP- C₁₈ column in HPLC with UV-Vis detection. The method was highly selective, specific, and accurate with a recovery rate of 105 %. This method is rapid because it involves a short elution time of 3.9 minutes. This method will be handy for the detection of OTC residues in chicken meat samples by research and testing laboratories.

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