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Screening of oxytetracycline residue in cattle milk collected from Jaipur, Rajasthan

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Abstract
In India, antibiotics are being used in large quantities for decades, until the existence of these drugs in the milk has received a little concern. The prolonged or inappropriate usages of antibiotics are leading to residues, which pose the risk of human health hazards and are also interfering with the processing of the milk and milk products. The separation of Oxytetracycline antibiotic was achieved on an Acclaim C18 analytical column (150 mm x 4.6 mm x 5 µm). The mobile phase was a mixture of.01M oxalic acid, acetonitrile and methanol (70:20:10 v/v) at a flow rate of 1ml/min at 30^0C temperature. Milk samples were collected from different places of Jaipur. Antibiotic residues were detected in most of the samples. It was found that there was an increase in the residual level of milk up to three days of treatment after that the concentration of the antibiotic residue starts declining.

Keywords: milk, oxytetracycline, HPLC, residue, health

1. Introduction
The presence of antimicrobial substances in raw milk could have serious toxicological and technical consequences [1]. The indiscriminate use of antibiotics for treatment practices is the main cause for the occurrence of antibiotic residues in food product of animal origin including milk, egg and meat. The presence of drugs or antibiotic residues in various food products above the maximum acceptable level has been recognized worldwide by various public authorities [2]. The reason for antibiotic contamination of milk was reported to be due to intramammary infusions of antibiotics for treating mastitis (92%), injections (6%), and other causes 2% [3].
The widespread usage has created potential residual problems in the milk and milk products. There is too much concern about antibiotic residues due to their adverse effects on people allergic to antibiotics, potential build up of antibiotic-resistant organism in humans and inhibition of starter cultures used to produce cultured milk products such as yogurt and cheeses[4]. Antibiotic residues may occure due to ignorance of mandatory with drawl period of antibiotics or due to faulty dosage levels. The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have set standards for acceptable dose of drugs and maximum residue limit (MRLs) of each antibiotics in milk.
Tetracycline is a wide spectrum of antimicrobial agent, apart from gram-positive bacteria and gram-negative bacterias, they are also effective against mycoplasmas, chlamydia, Rickettsias, spirochetes, actinomycetes, and some protozoa[5]. The sum action of tetracycline is bacteriostatic. The antibacterial action of tetracycline is through protein synthesis inhibition. They bind to the bacterial 30S ribosomal subunit and present attachment of aminoacylt RNA to the ribosomal receptor site [6]. In cattle, tetracycline is used when treating general, respiratory, urinary, and local infections. A specific indication for administering tetracycline in cattle is infectious mastitis. To prevent any harmful health effects on consumers, Food and Agricultural Organization, World Health Organization and European Union (EU) have established the maximum residue limits (MRL) for veterinary drugs (Council Regulation 2377/90/EEC). The maximum residual limit set by the EU legislation for oxytetracycline (OTC) in raw cow milk is set to 100ppb (0.1 mg/kg).
Various different methods, such as microbiological and chromatographic methods are being used for monitoring tetracycline in milk and other food samples. The microbiological assay techniques are relatively fast and simple, but in the same time, they provide only semi-quantitative measurements of residues detected. Bioassay techniques are less specific and

sometimes, they produce false positives results. Chromatographic techniques, such as thin layer chromatography (TLC), capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), have been developed for the quantitative, accurate and reliable measurements of tetracycline in milk and animal tissues [7].

Keeping in the view of the public health importance of antibiotic residue present study was done to investigate the presence antibiotic residue, level of oxytetracycline residue and their withdrawal time in cattle milk samples collected from Jaipur city of Rajasthan state.

2. Materials and Methods

2.1 Chemicals and reagents

The solvent used for the experiment were methanol and acetonitrile with gradient grade HPLC by SRL chemical. The reagent used was oxalic acid of analytical grade. The double distilled and de-ionized water was prepared through a Millipore water purification unit installed in the lab. The vitamin standard used was OTCs was purchased from Hi Media. A Thermo-Scientific UHPLC system equipped with UltiMate™ HPG-3400SD standard binary pump, UltiMate™ ACC-3000 autosampler (1 to 2000µL), UltiMate™ UV 3000 detector was used. Chromatographic separation was done on a Thermo Scientific™ Acclaim™ 120 C18 column (150mmx4.6mmx5µm). Data were integrated using Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System (CDS) software. The mobile phase was a mixture of .01M oxalic acid, acetonitrile and methanol (70:20:10 v/v). An ultrasonic cleaner (for degassing of mobile phase, labconco), solid phase extraction (Supelco), centrifuge (thermo scientific), concentrator, cold trap (Labconco) and vortex mixture was used for sample preparation.

2.2 Sampling procedure

Milk samples were collected from cattle's of different dairies and veterinary hospitals of Jaipur district, which were treated with oxytetracyline antibiotic for different clinical symptoms. Samples were collected from the next day of injection until that day when the antibiotic residue were absent in milk. The antibiotic treatment was given upto 3 days for all the cattles. At least 50 ml of milk samples were collected from the each teat in sterile polypropylene bottles and were kept in -70 ˚C deep freezer until analysis.

2.3 Preparation of standard calibration curves

Chromatographic peaks were obtained for samples and identified by comparing with those of standards. The working standard solution (c = 1 mg/ml) was prepared by dissolving accurately weighed 10.0 mg of OTC into 10.0 ml methanol (HPLC grade) in a volumetric flask on each day of the validation. Serial dilutions were prepared for oxytetracyline to give the following concentrations: 1.00, 0.50 and 0.25 mg/ml in separate amber vials. The calibration curve was constructed by plotting the peak area of the standard against its analyte.

2.4 HPLC condition

The chromatographic separation was performed on a reversed-phase Thermo Scientific™ Acclaim™ 120 C18 column (150mmx4.6mmx5µm) at 30°C using .01M oxalic acid, acetonitrile and methanol (70:20:10 v/v) as a mobile phase at a flow rate of 1 ml/min. The injection volume was set at 10 µL, depending on the type of the preparation. The detection of the OTC was carried out at 360 nm.

2.5 Sample preparation

In a 50 ml plastic centrifuge tube 15 ml of homogenized milk sample and 25 ml Mcllvaine Buffer (pH 4.1) was taken and agitated for 1 minute using a vortex. This was followed by centrifugation at 10000rpm for 12 minutes at 4˚C. Supernatant lipid layer was disposed and the remaining was processed using solid-phase extraction (SPE) cartridges. Before putting the centrifuged sample, the SPE cartridge was conditioned by 3ml of methanol at a flow rate of 3 ml/min and after then rinsed by 2 ml of de-ionized water. After that prepared mixture (centrifuged sample solution) was loaded in SPE cartridge at a flow rate of 5 ml/min. The cartridge was then treated (washed) with 1.5 ml of 5% methanol in de-ionized water. Elution was performed by 2 ml of HPLC grade methanol at a flow rate of 4 ml/min. Samples were dried by lyophilizing, followed by reconstituting with 1 ml mobile phase i.e. 0.01M oxalic acid, acetonitrile and methanol (70:20:10 v/v).

3. Results

The result of this study was interpreted based on the result of the standard calibration curve and the standard chromatogram represented in fig 1 & 2. The peak of the standard was found at RT 2.9 and the coefficient of correlation of the curve was (r^2) 0.999. Concentration of unknown sample was determined based on the peak area and concentration data.

Total 90 milk samples were collected from 18 cows (five samples from each cow) from dairies and veterinary hospitals of Jaipur for the analysis of oxytetracycline residue. The samples were taken in 50 ml sterile polypropylene bags and were kept in freezer until analysis. Most of the samples had shown positive results for antibiotic residue but it was negative for few of the samples. The samples which showed positive result for oxytetracycline residue, the concentration antibiotic residue was not more than the MRL value of oxytetracycline i.e. 100 ppb. Antibiotic residues were not detected in milk samples of three (16.67%) cattle's and were detected in rest fifteen (83.33%) cattles. In the positive samples there was an increase in the residual level of oxytetracyclin antibiotic in milk upto third day of treatment after that the concentration of the antibiotic residue started declining. Table no. 01 is showing the results of this study in which the concentration of antibitics is increasing from 1st day to the 3rd day and from the 4th day antibiotic concentration starts declining in the milk samples and on the 7th day the antibiotic residues were not detected in the milk samples.

4. Discussion

Milk and milk products which are contaminated with antibiotics beyond a given levels, are considered as unfit for consumption human beings[8]. It is reported that at least seventy five of veterinary drugs are being used more or less extensively in animals for therapeutic and prophylactic purposes [9]. More than 80 veterinary drugs are likely to produce residues in animal-derived food as identified by USA Food and Drug Administration (FDA) [10], and excessive or improper use of these drugs is of great concern for the presence of residues in food of animal origin. Countries where rules on the use of veterinary drugs are not strictly followed, the residues in milk may cause adverse effects on consumers' health.

The antibiotics were given to the animal for three days, and the samples were collected from the next day of injection till the antibiotic residues were not detected in the milk, that is

why the study was conducted for 7 days only. There is lot of variation in the concentration of the antibiotic residue in milk, because of the different amount of antibiotics given to the animal as in this study the dose was not standardized. The result shows the indiscriminate use of antibiotics by the practitioners without proper dose calculation.

In few cattles (16.67%) the antibiotic residue were not detected the reason behind this may be due to the low dose or appropriate dose of antibiotics given to the cattle. This also shows that the practitioners are not calculating the antibiotic dose or the practitioner are not been properly trained for the calculation of drug dosing. The antibiotic residue was detected in 15 cattles (88.33%) and the concentration of the residue was increasing upto three days and after that the concentration starts declining and on the 7th day the antibiotic residue was not detected in most of the samples. Increase in the concentration antibiotic residue was due to the increase in the concentration of drug in the circulatory system of the cattle. When the antibiotic treatment was stopped the concentration of antibiotic decreased in the circulation and theirfore its concentration started declining in the milk. The same result was also observed by Jevinova *et. al*., (2003) [11], they had given intramammary OTC to cattles for 5 days and found maximum residual concentration in milk on 5th day and after that the concentration started declining and ended up in 3 days. Rathe and Shaha, (2017) [12] had administered antibiotic for four days and found no antibiotic residue in milk after 3rd day of stopping the treatment in the cattle milk. The findings were similar to Sudershan and bhat, (1995) [13] who also found antibiotic residues in milk samples in India. Oxytetracycline residues found in milk samples collected ranged from .0975 to 79.75ppb. This finding was lower as compared to Owens *et al.*, (1991) [14] who found oxytetracycline concentration within range of 13-106ppb in USA. Mehran *et al.*, (2011) [15] reported high amount of oxyteteracyclin residue in commercial milk product.

Drug residue especially in milk is a major problem in most countries, many countries of the world are regulating use of drugs in food producing animals and setting up limits. Presence of antibiotic residues above tolerance level induced allergic reaction which is a potential risk for public health safety. Antibiotic residue poses a significant threat to public health when present in sufficiently high concentrations in food (McEwen and McNab, 1997) [16]. Antibiotic residues in milk are of great public health concern since milk is being widely consumed by infants, youngster and adults throughout the globe (Khaniki, 2007) [17]. Considering the issue of public health hazards, milk and milk products contaminated with antibiotics and other chemical contaminants beyond a given residue levels, are considered unfit for human consumption (Goffova *et al*., 2012) [18]. Occurrences of veterinary drug residues pose the broad range of health consequences in the consumers. The residues of antibacterials may present pharmacological, toxicological, microbiological and immune pathological health risks for humans (Drackova *et al*., 2009) [19]. It can also changes organoleptic specifications in some milk samples and affects some dairy products processing. Indiscriminate and irrational use of antibiotics in livestock without following withdrawal period may result in unexpected residues in food supplies and could cause serious health hazards to consumers.

5. Conclusion

This study showed higher prevalence of oxytetracycline residues in milk of Jaipur city. It suggest that the present status of oxytetracycline contamination is within the specified limit but needs to be continuous monitoring to take timely remedial action to prevent its detrimental effects on public health. Indiscriminate use of oxytetracycline antibiotic for treatment of diseases in dairy farms, treatment of diseased cows by owners without professional advice, poor knowledge about drug dosing can be the major contributing factors. This study suggests that the regulatory authorities should ensure proper training of drug dosing, avoid indiscriminate usage of antibiotics and following of the withdrawal period of antibiotics before milking the animals and definite supervisions are necessary on application of oxytetracycline antibiotics.

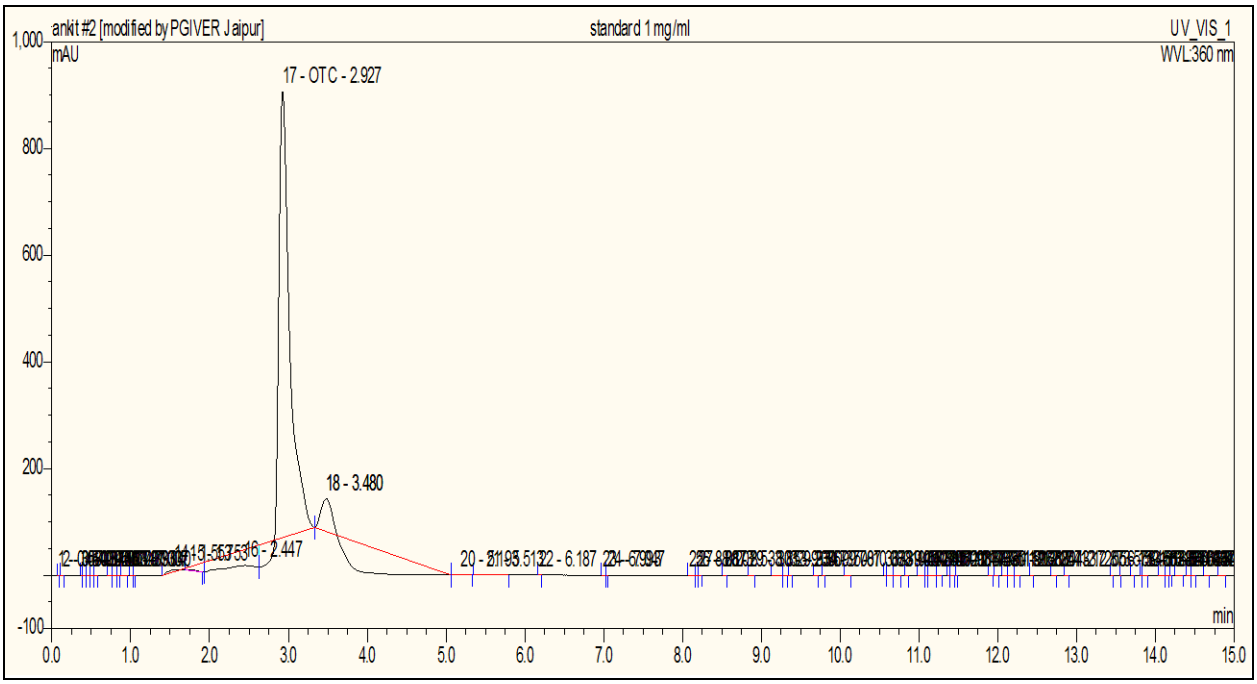

Fig 1: Chromatogram of the standard

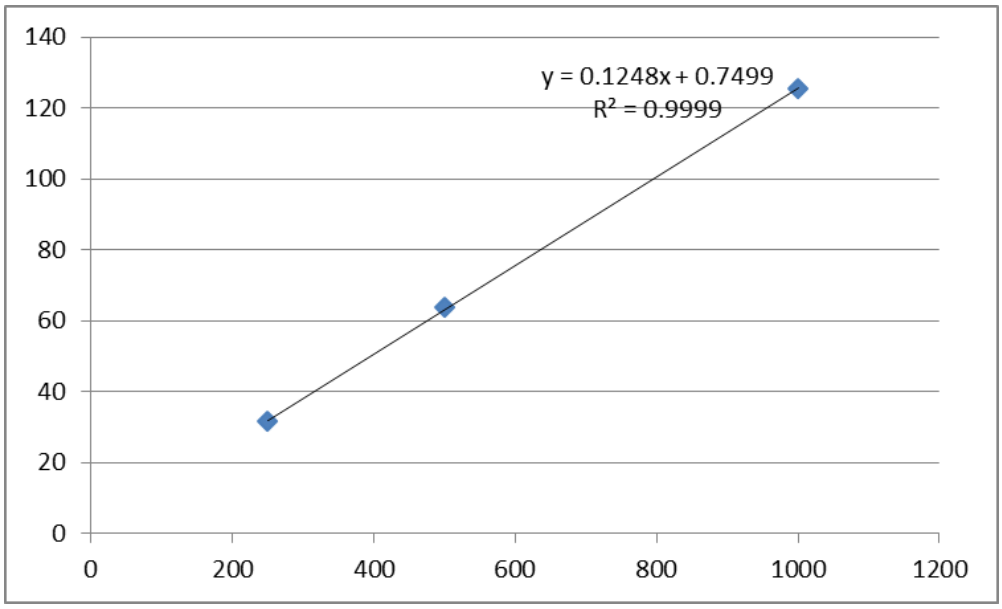

Fig 2: Calibration curve for oxytetracycline standard

Table 1: Concentration (ppb) of residue in different samples

Days	Cattle 1	Cattle 2	Cattle 3	Cattle 4	Cattle 5	Cattle 6	Cattle 7	Cattle 8	Cattle 9	Cattle 10	Cattle 11	Cattle 12	Cattle 13	Cattle 14	Cattle 15	Cattle 16	Cattle 17	Cattle 18
1st	ND	ND	8.31	1.59	ND	6.38	ND	26.38	3.19	ND	4.87	ND	2.39	5.58	1.59	7.17	6.38	7.24
2nd	ND	ND	18.34	2.59	ND	13.55	6.38	41.78	4.62	5.73	7.97	6.79	8.77	6.38	10.36	16.74	7.97	10.25
3rd	ND	ND	79.75	4.27	ND	14.56	7.88	61.82	8.77	8.77	31.10	12.76	20.73	18.34	8.23	15.24	8.25	26.31
4th	ND	ND	26.83	3.92	ND	3.52	2.49	59.81	7.17	7.23	10.36	ND	8.77	15.15	ND	8.29	2.21	19.58
5th	ND	ND	7.79	2.39	ND	ND	1.97	9.57	5.23	3.41	8.77	ND	7.97	23.22	ND	ND	1.79	10.55
6th	ND	ND	3.57	ND	ND	ND	ND	4.32	ND	ND	2.59	ND	ND	10.77	ND	ND	ND	6.34
7th	ND	ND	ND	ND	ND	ND	ND	1.57	ND	ND	ND	ND	ND	5.34	ND	ND	ND	ND

ND: Not detected

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