

Original Research Article

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Determination of Tetracycline Residues in Milk by High Performance Liquid Chromatography

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ABSTRACT

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A Reverse phase high-performance liquid chromatographic method (RP-HPLC) for the simultaneous determination of tetracycline, Oxytetracycline and chlortetracycline residues in milk has been developed. The determination of these antimicrobials was carried out using HPLC UV-VIS with a C₈ hybrid column elution with a mobile phase composed of solvent A (water: formic acid as 1000:1 v/v) and solvent B (water: acetonitrile: formic acid as 100:900:1). Sample preparation involved protein precipitation followed by solid-phase extraction using a C₁₈ cartridge. The method was validated and applied for the analysis of different type of milk samples commercialized in Hisar, Haryana. The limits of quantitation for all antimicrobials were below the maximum residue limit, which indicates that the method is appropriate for the determination of these antimicrobials in milk.

Introduction

Milk is an important constituent of human diet. It is consumed by all age groups particularly children and elderly people. India is the first ranker in milk production in the world with a total annual production of 146.3 million tonnes (18.5%) and per capita availability 337 g/day (NDDDB, 2016). The per capita availability of milk in Haryana is 835 g/day (DAHD, 2016). It is therefore important to provide due attention to the quality of milk produced and distributed to the consumers.

Antimicrobial agents mainly tetracycline's are widely used in food of animal origin for

therapeutic purpose to treat diseases and control and used as a additive for the growth promotion and productive efficiency of the animal. The antimicrobial residues present in the food of animal origin above the established maximum residue limits (MRLs) indicates that good veterinary practices were not established and may lead to resistance to bacteria in humans. In milk, however, their presence may cause allergic reactions i.e. photosensitivity and risk of teratogenicity when administered during the first trimester of pregnancy in sensitive individuals and may interfere with starter cultures for cheese and other dairy products (Schenk and Callery,

1998). Moreover, primary and permanent teeth discoloration often occurs when milk is consumed by infants. Tetracyclines, in particular chlortetracycline, have been routinely employed to prevent and treat mastitis in lactating dairy cows (JECFA, 2002). Chlortetracycline and Oxytetracycline are licensed as growth promoters for livestock in the United States (Meyer *et al.*, 2000).

Throughout the world antimicrobial drug residues are known to be present in food but the milk is of greater concern to the human beings specially children that may lead to the resistance of microorganisms in gutflora (Ekuttan *et al.*, 2007). Apart from the direct toxic effects on the consumers, antimicrobial residues cause propagation of antimicrobial drug resistant bacteria in the food chain, environment and the body system of humans as well as animals.

The rampant and indiscriminate use of antibiotics among the small-scale livestock keepers increases the possibility of transfer of antibiotic resistant bacteria from animals to humans and that may lead to various chronic diseases among the users of milk and milk products. Antimicrobial residues result in development of drug resistance in the gut flora in human beings.

Avoidance of antimicrobial residues in milk needs an important focus on the dairy industry. Lack of awareness of withdrawal times or increased use may lead to elevated levels of drug residues in the milk. The most common causes of occurrence of drug residues in milk are insufficient identification of treated cows, lack of knowledge about withdrawal periods and the failures of the hired staff.

The increasing use of antibiotic consumption in India is reflected by the emerging drug resistance problem while the regulations

concerning the use of antibiotics in human and animals are still very poor. In addition to the health issue, the presence of antibiotic residues in milk may interfere with starter culture in the production of cheese and other fermented dairy products resulting in significant economic losses to the producers of milk and milk products (Katla *et al.*, 2001).

The committee also recommended MRLs in milk of 100µg/l. The information on the occurrence of antibiotic drug residues in India is available only in the form of very limited academic research papers. Few such studies have demonstrated the presence of tetracycline residues in milk (Das *et al.*, 2014; Gaurav *et al.*, 2014; Kalla *et al.*, 2015).

Different kinds of analytical methods are in practice to identify and quantify antibiotic residues in milk. While rapid screening methods (immunological or microbial inhibition assays) are commonly used to detect the presence of antimicrobials in food, more accurate chromatographic methods are required by the governmental regulatory agencies to identify and confirm the presence of these compounds. Such methods are always aimed at detection of individual analyte at a concentration lower than the specified MRL.

Conventional sample treatment protocols involve protein precipitation, centrifugation and analyte extraction, followed by clean-up of the extract over solid-phase cartridges. Many liquid chromatographic methods have been published for the determination of tetracyclines (Oka *et al.*, 2000; Andersen *et al.*, 2005) but the work done in India is very limited. This paper focuses on the development and validation of a simple HPLC-UV method for the simultaneous determination of TC, OTC and CTC in milk which could be applied to quality control in the routine analysis.

Materials and Methods

Collection of samples

A Total 100 milk samples were collected from Hisar and nearby areas. Among these, 40 raw milk samples were collected from local vendors and another 40 raw milk samples were collected from mini dairies (private milk collection centers). Twenty pasteurized milk samples of different brands were also collected from various retail shops. For each collected raw milk sample a quantity of about 100mL was collected in a labeled sterilized bottle and stored at -20°C till analysis.

HPLC instrumentation and condition

A Shimadzu prominence UFLC system equipped with DGU-20A5R degasser, SIL-20A HT autosampler and LC-20AD pump connected to C_8 column (Enable 4.6 mm x 250 mm porosity 5 μm) housed in CTO-10AS column oven with SPD-20A UV-VIS detector was used throughout the experiment. The system was controlled by Lab Solution Software.

Chemicals and reagents

The analytical standards of antimicrobials viz. tetracycline, Oxytetracycline and chlortetracycline, all having purity more than 98%, were procured from Sigma-Aldrich. SupelcleanTM LC-18 SPE Tube having bed wt. 500 mg and volume 3 mL were also procured from Sigma-Aldrich. All HPLC grade solvents namely methanol, acetonitrile and iso-propyl alcohol (IPA) were procured from Fisher Scientific whereas anhydrous sodium sulphate was procured from Qualigens. HPLC grade water was prepared in the laboratory using Millipore (Bedford, MA, USA) Milli-Q system to give a resistivity of at least 18.2 $\text{M}\Omega\text{ cm}$.

Preparation of reagents

Mobile phase

Mobile phase used for the instrumental analysis of tetracycline was composed of solvent A (water : formic acid as 1000:1 v/v) and solvent B (water : acetonitrile : formic acid as 100:900:1 v/v/v).

Preparation of standards reagent solutions

The primary standard solution of each antimicrobial was prepared by dissolving neat standards of TETs in methanol by using class A glassware (Final volume 25 ml) so that effective concentration remained more than 100 $\mu\text{g}/\text{mL}$. Standard solutions of TETs were stored at -18°C . For preparation of individual secondary standard solutions, the maximum residue limits (MRLs) prescribed by European Union Commission (EU, 2010) and Codex Alimentarius Commission of WHO (Codex, 2015) for all antibiotics were considered. Based on these MRL values, a linearity range (50, 100, 150, 200, 250 $\mu\text{g}/\text{kg}$) was selected to cover the lowest MRLs for all the analyte molecules. Then, appropriate quantity of primary standard solution(s) was diluted to the required volume with same solvent to prepare individual secondary standard solution as well as standard mix.

HPLC analysis

HPLC-UV technique was standardized for detection of TETs viz. tetracycline, oxytetracycline and chlortetracycline from milk as per method described by Stolker *et al.*, (2008) with slight modification. Mobile phase used for the instrumental analysis of tetracycline was composed of solvent A (water : formic acid as 1000:1 v/v) and solvent B (water : acetonitrile : formic acid as 100:900:1 v/v/v). The flow rate was 1ml/min. Detection of Tetracyclines was performed at UV detector at 280nm wavelength.

Sample preparation

The spiked milk samples for linearity as well as recovery studies were prepared by fortification of proportional quantities of ground blank milk samples were with standard mix at various concentrations viz. 50, 100, 150, 200 and 250 µg/kg and then subjected to extraction and cleanup procedure. Milk samples collected from market were processed as such.

Results and Discussion

In the present study, an analytical technique using high pressure liquid chromatography with UV (HPLC-UV) detector was standardized as per the sample processing method proposed by Stolker *et al.*, (2008) with slight modifications. Standardized Tetracyclines compared with blank milk samples showed in chromatogram in Figure 1. Tetracyclines (TETs) such as tetracycline, oxytetracycline, chlortetracycline were detected by HPLC-UV technique. The standardized method was validated as per the ICH Harmonized Tripartite Guidelines (ICH, 1998). This validated method was used for analysis of 100 randomly collected milk samples for the detection and quantitation of TETs.

Standardization and validation studies

i) System Precision: The system precision was evaluated by studying the reproducibility of the instrumental response with respect to retention time and area of an analyte. The percent relative standard deviation (RSD) for analyte was found to be in range of 0.64-1.12% for retention time of TETs. The percent relative standard deviation (RSD) for analyte was found to be in range of 3.01-12.68 for area of TETs. The percent Relative Standard Deviation (%RSD) for all analyte was found to be less than 0.07 percent for area and 0.02 percent for retention time.

ii) Specificity: It was evaluated by visual observation of chromatograms of blank sample matrix and sample matrix spiked with standard mixture. For milk, chromatographic signals at the retention times of TETs were absent in blank sample matrix.

iii) Linearity: The standard calibration curves of the analyzed TETs standards presented a good regression line ($r^2 > 0.99$) in the range of explored concentrations i.e. from 50 to 250 µg/kg. The graphs showing calibration curve of these standards, revealed that all concentrations of the TETs under study were collinear and thus calibration curves were further employed for the detection of analytes under study.

iv) Limit of detection (LOD) and limit of quantitation (LOQ): LOD and LOQ were determined on the basis of standard deviation of the blank. Measurement of the magnitude of the analytical background response was performed by the analysis of 10 blank samples and calculating the standard deviations of these responses. Table 1 summaries the LOD and LOQ obtained for each analytes of TETs group. Perusal of tables clearly indicates that the LOD and LOQ for individual analytes were well below their respective MRLs indicating that the method was able to detect the given antibiotics at sufficiently low level.

v) Accuracy: The accuracy in terms of percent recovery of each analytes of TETs group at five different fortification levels (50, 100, 150, 200 and 250 µg/kg) was evaluated for milk and the results are presented in Table 2. Satisfactory results were found in almost all instances. Recoveries for all analyte-matrix combinations ranged between 71- 110% in milk. However, in general, the antibiotics gave acceptable recoveries within the mentioned validation interval as per legislation (EU, 2002) between 70 and 110 percent.

vi) Precision: The precision was assessed, at five concentration levels (50, 100, 150, 200 and 250 µg/kg) by the recovery studies. Repeatability and intermediate precision values, expressed as relative standard deviation (CV percent) were found less than 10.51 for all analytes of TETs (Table 3).

Overall the multiresidue method followed for multiresidue detection and quantification of TETs antibiotic residues in milk was subjected to rigorous validation parameters. The system precision values indicated a good consistency in response by the HPLC instrument used during present study. A good linearity was noted for standards and spiked milk samples. Absence of interfering peaks in

blank samples indicates good specificity of extraction and clean up method. Accuracy and precision of the method were in accepted range in comparison with international guidelines. These results of validation studies clearly demonstrated that the present method is suited for routine analysis of TETs in milk.

Determination of residues of TETs in milk

After successful standardization and validation of techniques for detection of TETs residues the extraction, detection and quantification were carried out on 100 samples of milk collected from Hisar city. The overall occurrence of TETs residues is presented in Table 4.

Table.1 Limit of detection (LOD) and limit of quantification (LOQ) for TETs

Group of antimicrobials	Analytes	LOD (µg/kg)	LOQ (µg/kg)
Tetracyclines	Oxytetracycline	48	98
	Tetracycline	44	94
	Chlortetracycline	8	23

Table.2 Accuracy of method for detection of TETs residues in milk

Analytes	Accuracy (% Average Recovery ± SD) at various spiked Concentrations(µg/kg)				
	50	100	150	200	250
Oxytetracycline	109.6±4.6	110.8±8.48	107±11.79	105 ± 3.84	109.6 ± 9.67
Tetracycline	108± 3.68	101±7.02	98± 10.36	101 ± 5.52	87.1 ± 7.41
Chlortetracycline	65.5±5.40	73.5 ±6.18	71.18±7.27	61 ± 1.81	64.6 ± 6.14

Table.3 Precision of method for detection of TETs residues in milk

Analytes	Precision (% Relative Standard Deviation) at various spiked Concentrations(µg/kg)				
	50	100	150	200	250
Oxytetracycline	4.26	7.65	9.7	3.66	8.81
Tetracycline	3.41	6.93	9.49	5.51	8.48
Chlortetracycline	8.24	8.41	9.8	2.99	9.46

Table.4 Mean concentration of TETs residues in milk

Group of antimicrobials	Analytes	Mean concentration (µg/Kg)			
		Raw milk-Vendor (n=40)	Raw milk-Dairy (n=40)	Pasteurized milk (n=20)	Total (n=100)
Tetracyclines	Oxytetracycline	25.49	-	11.26	12.45
	Tetracycline	2.78	-	-	1.11
	Chlortetracycline	16.68	2.55	-	7.69

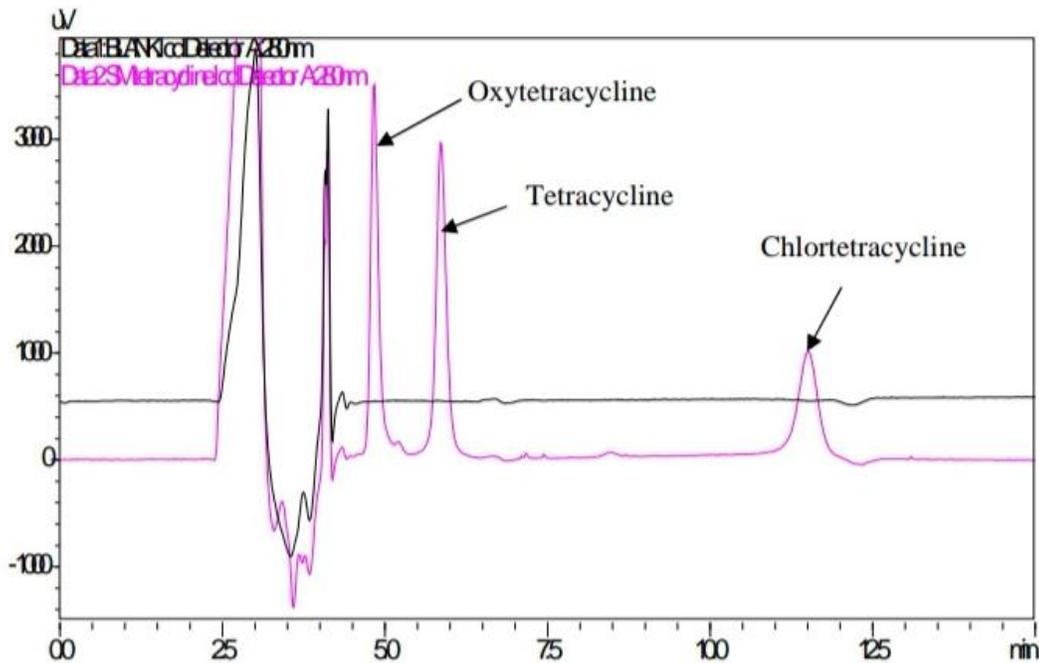
Table.5 Comparison of TETs residue levels in milk samples with the national and International MRLs

Analyte	International MRLs		Number of samples violating MRLs	
	EU (2010) (µg/kg)	Codex (2015)(µg/l)	EU	Codex
Oxytetracycline	100	100*	5	5
Tetracycline	100		0	0
Chlortetracycline	100		0	0

NE = Not established

*This tolerance includes both the sum and the individual residues of chlortetracycline, oxytetracycline and tetracycline. The sum of the tetracyclines present should not exceed 100 µg/l (Codex, 2015)

Fig.1 Chromatogram of solvent blank and standard mix of tetracyclines



In the present study, maximum numbers of milk samples were found to be contaminated with CTC (9%) residues followed by oxytetracycline (6%) and tetracycline (3%). Raw milk samples from vendors showed presence of all three TCs while the milk samples from mini dairies showed presence of all except oxytetracycline residues. Pasteurized milk samples showed presence of only oxytetracycline. This trend shows the effect of mixing and dilution of antimicrobial residue contaminated milk with uncontaminated milk.

Mean concentration for each analyte is provided in Table 4 in milk samples. The results revealed that absolute mean concentration of tetracyclines was found to be 21.25 µg/kg which was mostly contributed by oxytetracycline (12.45 µg/kg) followed by chlortetracycline (7.69 µg/kg) and tetracycline (1.11µg/kg). Alomirah *et al.*, (2007) reported that 29.1% of the analyzed fresh milk samples were above the MRL for tested residues, with tetracycline as the dominant residue. Similarly, in the study conducted by Bilandzic *et al.*, (2011), the highest tetracycline level detected was 49.5 µg/kg. However, the mean tetracycline concentration (2.83 µg/kg) was more than 35 times lower than the MRL level. Whereas, in the present study, the mean concentration of tetracycline residues was found to be less than the values obtained in the above study.

On the contrary to the findings of present study, TCs were detected with much higher frequency in other states of India. In general, out of all samples, 5% samples were found to be exceeding the MRL with respect to tetracyclines (Table 5). Sudershan and bhat (1995) found OTC residues in 9% of market milk samples and 73% in individual animal samples in Hyderabad. Gaurav *et al.*, (2014) studied the occurrence of tetracycline residues in milk samples collected from various part of Punjab by ELISA method and found the

residues in 13% samples. Kalla and coworkers (2015) in Andhra Pradesh studied the prevalence of antibiotic residues in raw milk and found 51% milk samples positive for TETs. However, Nirala *et al.*, (2017) found tetracycline residues in only 3.3% milk samples from various districts of Bihar which is comparatively in lesser proportion than the present study.

Occurrence of tetracycline residues has also been reported from various parts of the world. In Brazil, Bando *et al.*, (2009) and Zanella *et al.*, (2010) reported significantly high presence of TC antibiotic with 41 (27.2%) and 48 samples (18.5%) contaminated with TC residues. Ahmed *et al.*, (2015) found tetracycline residues in Egypt in 30% milk sample with the mean concentration of 23.62 ± 7.01 µg/L. Syit (2011) reported OTC residues in Ethiopia in 70.58% milk samples above MRL and the mean residue level of OTC was 142.00µg/L. TC residues were detected in the milk from various parts of the world with much higher frequency than the present study (Navratilova *et al.*, 2009; Abbasi *et al.*, 2011 and Elizabeta *et al.*, 2011).

Rasooli *et al.*, (2014) examined the presence of tetracycline residues in 432 pasteurized milk samples in Iran and found 7 samples above the MRL. All the samples above tolerance limits were recorded in association with vendor milk and some of the dairy or pasteurized milk sample was found to have antimicrobial concentration above MRLs. This might be due to the dilution effect of bulking on residue concentration in dairy and pasteurized milk samples i.e. mixing of positive milk with negative milk at a larger level in dairy plants.

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