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Simultaneous Determination of Tetracyclines Residues in Bovine Milk Samples by Solid Phase Extraction and HPLC-FL Method

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ABSTRACT

Introduction: Tetracyclines (TCs) are widely used in animal husbandry and their residues in milk may resultin harmful effects on human. The aim of this study was to investigate the presence of TCs residues in various bovine milk samples from local markets of Ardabil, Iran. ***Methods***: One hundred and fourteen pasteurized, sterilized and raw milk samples were collected from markets of Ardabil. Tetracycline, Oxytetracycline and Chlortetracycline (TCs) residues extraction carried out by Solid Phase Extraction method. Determination of TCs residues were performed by high performance liquid chromatography (HPLC) method using Fluorescence detector. ***Results***: The mean of total TCs residues in all samples (114 samples) was 97.6 ±16.9ng/g and that of pasteurized, sterilized and raw milk samples were 87.1 ± 17.7, 112.0 ± 57.3 and 154.0 ± 66.3ng/g respectively. Twenty five point four percent of the all samples, and 24.4%, 30% and 28.6% of the pasteurized, sterilized and raw milk samples, respectively had higher TCs residues than the recommended maximum levels (100ng/g). ***Conclusion***: This study indicates the presence of tetracycline residues more than allowed amount. Regulatory authorities should ensure proper withdrawal period before milking the animals and definite supervisions are necessary on application of these drugs.

Introduction

In modern farming practice, drugs are using in a large scale and are applied in animal husbandry for different reasons. They are used to prevent disease, cure animals, or as feed additive to promote growth. All drugs administered to milk-producing animals may lead to residues in the milk. In addition to the drug dosage, the levels of those residues depend on the period between administration and collection of the animal products, which is called withdrawal period. [1]

Antibiotic residues like other drugs, remain in animals body even after slaughtering, if there has been no enough time to their repel. [2] Residues of antibiotics in foodstuffs from animal origin could represent a hazard for the consumer of these products. Allergic reactions maybe produced in susceptible or sensitized individuals. [2-7] The other hazardous effect is development of resistant strains of bacteria following the prescription of subtherapeutic doses of antimicrobials. It may include transferring of resistance gene of plasmid from nonpathogenic microorganisms to pathogenic microorganisms, which then will not respond to normal drug treatment. [2,4,8]

Tetracyclines (TCs) including Tetracycline (TC), Oxytetracycline (OTC) and Chlortetracycline (CTC) are broad-spectrum antibiotics widely used in animal husbandry for both prevention and treatment of diseases and feed additives to promote growth. In food-producing

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animals, tetracyclines may be administrated orally through feed or drinking water, parenterally, or by intra-mammary infusion. Due to entero-hepatic circulation, a small amount of administrated dosage may persist in the body for a long time after administration. [1] The rate of metabolism of TCs in dairy cows has been estimated 25-75% and a significant percentage of the administrated TCs are excreted in bovine milk. If these antibiotics administrate improperly or if the withdrawal time for the treated cows has not been passed, TCs and their degraded products may be present in milk and may cause harmful effects on consumers. [9]

The antibiotic residues in food could influence the bacterial composition of the intestinal microflora, their metabolic activity and the metabolism of endogenous compounds.[10] Tetracyclines in milk potentially may stain teeth of young children.[9,10] The world health organization (WHO) and the food and agriculture organization (FAO) have set standards for acceptable daily intake (ADI) and maximum residue levels (MRLs) in foods, in order to protect humans from harmful effects of drug residues in milk. WHO, European Union (EU) and Chinese ministry of agriculture have established a MRL of 100ng/g for Tetracycline (TC), Oxytetracycline (OTC) and Chlortetracycline (CTC). [9, 10, 11,12] The U.S. food and drug administration (FDA) has set the MRL of 300ng/g for total residues of tetracyclines (TC, OTC and CTC).The acceptable MRL for tetracyclines (singly or in combination) as recommended by the Joint FAO/WHO Expert Committee on Food Additives is 100ng/g for bovine milk. [11]

Studies conducted in Kenya showed samples of milk to contain TCs at levels exceeding the established MRL. [12] Studies in Kuwait showed that 18% of milk samples had tetracyclines residues above the permitted limit.[13] In Czech Republic, studied showed presence of tetracycline residues in all and oxytetracycline residues in 50.6% of analyzed raw cow milk samples. [10]

Some different methods, such as microbiological and chromatographic methods have been described for monitoring tetracyclines 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.[9] 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 tetracyclines in milk and animal tissues. [9]

The aim of this study was to investigate the presence of TCs residues in various bovine milk samples from internal markets of Ardabil city of Iran. Ardabil is the capital of Ardabil province in North West of Iran.

Material and Methods

Chemicals and reagents

Chemicals, analytical standards of TCs and Oasis HLB cartridge (WAT094226) purchased from Merck (Germany), Sigma and Waters (USA) respectively. Methanol and acetonitrile were HPLC grade from Caledon, Canada. The double distilled and de-ionized water was prepared through a Millipore water purification unit.

Sampling procedure

Sampling performed by stratified random sampling method. In this method, 114, either processed (pasteurized or sterilized) or raw, milk samples were purchased from supermarkets in Ardabil city. There are five suppliers for pasteurized milk and only one sterilized milk manufacturer in Ardabil city and the people consume pasteurized milk much more than sterilized and raw milk.

Therefore, 90 pasteurized, 10 sterilized and 14 raw milk samples were collected for this study. The samples were at least 50 ml in sterile polypropylenebags and were kept in-70˚C deep freezer (Snijders Scientific, Holland) until analysis.

Preparation of standard curves

Calibration standard solutions were prepared freshly by dissolving tetracyclines in a mixture of methanol, acetonitrile and 50 mM oxalic acid (10: 20: 70%). Serial dilutions were prepared for TCs (TC, OTC and CTC entirely) to give the following concentrations: 50000, 10000, 5000, 1000, 500, 100, 50 and 10ng/g. Then mixed standard solutions were prepared for simultaneously calibrating and calculating of TCs residues. Figure 1 shows the chromatogram of mixed standard solution of TCs (3333ng/g).

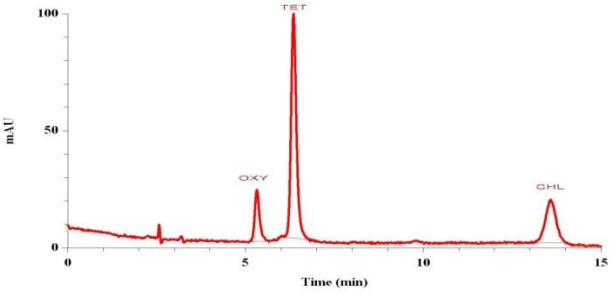

Figure 1. The chromatogram of mixed standard solution of TCs (3333 ng/g).

Sample preparation

A15 ml of milk sample was homogenized and mixed with 25 ml Mcllvaine Buffer (mixed citrate/phosphate, pH 4.1 with EDTA) in a 50 ml plastic centrifuge bottle. The solution was agitated for 1 minute using a vortex and followed by centrifugation at 10000g for 12 minutes at 4˚C. Any floating lipid layer and the precipitate were disposed and the remaining supernatant processed using solid-phase extraction (SPE) cartridges.

The SPE cartridge (Oasis HLB- WAT094226) conditioned by 3ml of methanol at a flow rate of 3 ml/min, and then rinsed by 2 ml of de-ionized water. [16] The prepared mixture (centrifuged sample solution) was loaded in SPE cartridge at a flow rate of 5 ml/min. The cartridge treated (washed) with 1.5 ml of 5% methanol in de-ionized water. Elution performed with 2 ml of HPLC grade methanol at a rate of 4 ml/min. Samples dried by lyophilizing, followed by reconstituting with 1 ml mobile phase. [8, 14-17]

HPLC analysis

The HPLC system was a KNAUER (Berlin, Germany) system plus RF-551 fluorescence detector and a Spark Triathlon Autosampler. The mobile phase was a mixture of methanol, acetonitrile and 50mM oxalic acid (10: 20: 70% V/V). The mobile phase was mixed and filtered through a 0.45 µ filter (Nalgene, USA), and was degassed by sonication for 5 minutes. Theex citation and emission wavelengths were 255 and 365 nm, respectively. HPLC column was Phenomenex Luna 5µ C18 (250 ×4.6 mm) and the flow rate was 1 ml/min, with an injection volume of 20 µl.[17]

Quantitation was made based on the linear (R^2>0.999) calibration curves between the concentrations and peak area of standard solutions. HPLC chromatograms of two milk samples containing tetracycline and chlortetracycline residues are shown in Figure 2.

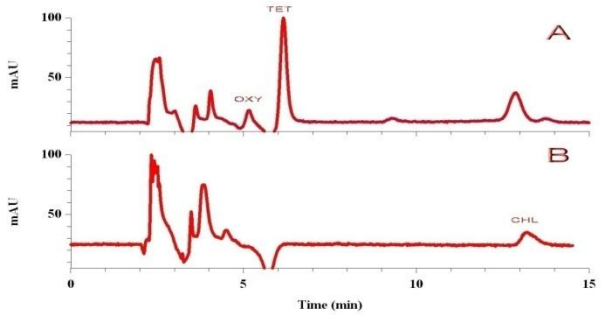

Figure. 2. The HPLC chromatograms of a milk sample containing tetracycline and oxytetracycline residues(A), and a milk sample containing chlortetracycline residues (B).

The limit of detection (LOD) and the limit of quantification (LOQ) for TCs were obtained 2.2, and 6.6ng/g, respectively. Results for recovery, inter-assay and intra-assay variations of the method were calculated and shown in Table1.

Table 1. Recovery, inter-assay and intra-assay variations of the method used for detection of TCs.

	Oxytetracycline	Tetracycline	Chlortetracycline	Total mean
Mean recovery (%)	82.5 n=9	75.3 n=9	78.4 n=9	78.7 n=27
Intra-assay variation mean C.V.%	2.9 n=9	3.4 n=9	4.0 n=9	3.4 n=27
Inter-assay variation mean C.V.%	4.1 n=9	4.5 n=9	5.0 n=9	4.53 n=27

n = sample number, C.V. = Coefficient of Variation

Results

The results show that in analyzed samples, the mean amount of oxytetracycline was 32.9 ng/g and 71.1% of samples lacked measurable amount of oxytetracycline residues. The mean oxytetracycline residues in pasteurized and raw milk samples were 24.1 and 40.0ng/g, respectively. Fifty seven point one percent of raw milk samples had detectable oxytetracycline residues.

The mean amount of tetracycline residues in all the milk samples was 17.8ng/g and 93.9% of samples lacked measurable amount of tetracycline residues. The mean tetracycline residues in the pasteurized and raw milk samples were 18.3 and 22.3ng/g, respectively. 5.6% of pasteurized and 7.1% of raw milk samples had detectable tetracycline residues.

The mean amount of chlortetracycline residues in all the milk samples was 329.1 ng/g, and 0.9% of samples lacked measurable amount of chlortetracycline residues. The mean chlortetracycline residues in the pasteurized, sterilized and raw milk samples were 330.9, 287.1 and 340.8 ng/g, respectively. All of (100%) of the

pasteurized and raw samples and 90% of sterilized milk samples had detectable chlortetracycline residues.

The mean amount of TCs in all milk samples was 97.6 ± 16.9ng/g and 14.9% of samples lacked measurable amount of TCs. The mean TCs residues in the pasteurized, sterilized and raw milk samples were 87.1 ± 17.7, 112.0 ± 57.3 and 154.7 ± 66.3, respectively. TCs residues were detectable in 90, 50 and 90% of pasteurized, raw and sterilized samples, respectively.

The results for Mean of tetracyclines residues and their proportion in different samples are shown in Table 2 and Table 3, respectively.

Table 2. Mean TCs residues in pasteurized, sterilized and raw milk samples

	Oxytetracycline(ng/g)	Tetracycline (ng/g)	Chlortetracycline (ng/g)	Mean of total TCs residues (ng/g)
Pasteurized milk	24.1 ± 6.16	18.3 ± 9.9	44.7 ± 14.3	87.1 ± 17.7
Sterilized milk	-	-	3.4 ± 2.4	112.0 ± 57.3
Raw milk	40.0 ± 20.9	22.3 ± 12.3	92.4 ± 61.7	154.7 ± 66.3
Total mean	32.9 ± 7.6	17.8 ± 8.3	46.9 ± 13.6	97.6 ± 16.9

Table 3. Percent of milk samples with TCs residues above WHO standard MRL (100 ng/g).

	Oxytetracycline	Tetracycline	Chlortetracycline	Total TCs
		n(percent of samples>100ng/g)		
Pasteurized milk	8 (8.9%)[a]	5 (5.6%)	10 (11.1%)	22 (24.4%)
Sterilized milk	3 (30%)	0 (0%)	0 (0%)	3 (30%)
Raw milk	2 (14.3%)	1 (7.1%)	2 (14.2%)	4 (28.6%)
All samples	12 (10.5%)	6 (5.2%)	12 (10.5%)	29 (25.4%)

n= sample number, a = percent of samples >100ng/g

Discussion

The overall average recovery of the analytical procedures for TCs was 75-82% that is less than reported recovery, 88.1-93.5%, by Navratilova et al [10], 80.3-93.3% by Cinquina et al[18] and 88-100% by Zhao.[19] The average detection limit of TCs were obtained 2.2 ng/g, whereas detection limits of oxytetracycline, tetracycline and chlortetracycline were reported 5, 5 and 20ng/g respectively by Navratilova et al [10] and 2ng/g by Fritz et al. [9]

Ten point five percent of total samples and 8.9% of pasteurized, 30% of sterilized and 14.3% of raw milk samples had oxytetracycline residue levels above the WHO standard (100ng/g). Tetracycline residues more than 100 ng/g were detected in 5.2, 5.6 and 7.1% of total samples, pasteurized and raw milk samples, respectively. About 10.5% of the all milk samples and 11.1% of the pasteurized and 14.2% raw ones had chlortetracycline residue levels, above the WHO standard. Altogether 25.4% of total milk samples and 24.4% of the pasteurized, 30% of the sterilized and 28.6% of the raw milk samples had TCs (sum of OTC, TC and CTC) residue levels more than the WHO standard level (100 ng/g).

Mean of total TCs residues in raw milk samples was more than sterilized ones, and that of sterilized samples was more than pasteurized milk samples. It may be due to processing effect on TCs residues. Further processing of milk can bring on the lowering on the concentrations of tetracycline antibiotics. For instance, the temperature of 62ºC over a period of 30 min caused a reduction in CTC content by 16%, in that of OTC by 23% while at the temperature of 72ºC the reduction was by 27% and 35%, respectively. [10]

The number of samples with TCs residues more than WHO MRLs was more than most of similar studies in other countries.[10, 13] It may be due to use of tetracyclines as feed additives widely to prevent or treat mastitis and metritis in cows.[19] Regulatory authorities should constantly conduct surveillance on withdrawal period before milking.

The results indicate the presence of TCs residues in various bovine milk samples from local markets of Ardabil city. Most samples contained more intentioned

drug residues than MRLs. These antibiotic residues may result drug resistance, digestive and allergic effects in consumers.[6] It would also changes organoleptic specifications in some milk samples and affects some dairy products processing. There are not enough studies in Iran about drug residues in milk.

Conclusion

The present study indicates the presence of TCs residues in various bovine milk samples from internal markets of Ardabil city. Therefore, the bovine milk samples did not have desired conditions because of presence of tetracyclines residues more than Maximum Residue Limits (MRLs). Other studies are necessary to evaluate other drug residues in milk samples and to evaluate the hazards of these residues in relation with daily intakes and other related factors.

Ethical issues

None to be declared.

Conflict of interests

The authors declare no conflict of interests.

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