

A trace analysis of oxytetracycline and tetracycline residues in pasteurized milk supplied in Tehran: a one-year study (April 2011-March 2012)

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Abstract:

BACKGROUND: Tetracyclines (TCs) are broad-spectrum antibiotics that are widely used in veterinary medicine. The presence of TCs residues in milk is a public health concern all over the world. **OBJECTIVES:** This study aimed to determine TCs residuals in pasteurized milk marketed by some dairy companies in Tehran from April 2011 to March 2012. **METHODS:** 432 pasteurized milk samples were purchased from supermarkets supplying the milk products of 12 major dairy companies in Tehran (3 samples from each company every month), and they were stored at -20 °C until analysis. Oxytetracycline (OTC) and Tetracycline (TC) residues in each sample were extracted by a liquid - liquid phase procedure and quantitated using a high performance liquid chromatographic (HPLC) method. Chromatographic conditions included a mobile phase as oxalic acid buffer- acetonitril (80: 20) with a flow rate of 1 mL/min and UV-detection at 355 nm. **RESULTS:** TCs residuals in most milk samples were lower than 100 ppb, maximum residue level (MRL); however, in seven samples (1.62%) the total residues of OTC and TC were more than MRL. In the latter milk samples, the median total TCs residue was 625 ppb, ranging between 274 and 1270 ppb. **CONCLUSIONS:** Because of the presence of TC residues above the MRL level in a limited number of milk samples, it is concluded that more studies and supervision of health authorities are needed in this field.

Introduction

Tetracyclines are broad-spectrum antibiotics with a bacteriostatic effect on a wide range of gram-negative and gram-positive bacteria and are widely used in veterinary medicine and in humans. They are used for prevention or treatment of a great number of diseases and for growth promotion in animal husbandry. These antibiotics are produced by *Streptomyces* spp., and the mode of action is exerted by binding to 30S ribosomal subunits of susceptible bacteria, which in turn inhibits their protein synthesis (Chopra, 1981).

The use of TCs in dairy farms may have serious adverse effects due to the potential presence of antibiotic residues in milk. Antibiotic residues are small amounts of drugs or their active metabolites that remain in milk or meat after treating the cows. Problems associated with antibiotic residues in milk include the risk of allergic reactions, increased resistance of pathogens against antibiotics, and inhibition of bacterial starter cultures used in dairy production. Low levels of these residues in milk when consumed over a period of time can lead to the development of drug-resistant microorganisms. The occurrence of drug residues mainly results from

failure to implement the mandatory withdrawal periods; illegal or extra-label use of drugs and incorrect dosage levels are hazardous (Ivona et al., 2002).

In order to reduce the risks of TC residues in milk on public health, international organizations such as World Health Organization (WHO), US Food and Drug Administration (FDA), and the European Union (EU), Codex Alimentarius Commission (CAC) proposed 100ng/mL of parent drugs, separately or in combination, as MRL (Kaplan et al., 1962; WHO, 1990; Commission of EC, 1991; Rassouli et al., 2010).

The MRL level for each compound in any specified foodstuff is not a fixed value throughout the world, and it may vary in different countries according to their dietary habits as well as the policies and authorities of their public health organizations. Although the primary purpose of establishing MRLs is to ensure the food safety and to protect the health of the consumer against possible harmful effects resulting from exposure to residues, it should be uniformly established and harmonized to facilitate the marketing and free trade of foodstuffs of animal origin in national and international levels.

A number of methods including microbiological and physicochemical ones are used to determine TCs residues in biological matrices (Iwaki et al., 1992; Jacques et al., 1998; Furusawa, 1999; Ding et al., 2000; Fritz and Zuo, 2007). However, HPLC methods are the most sensitive and specific techniques in this regard. Therefore, in the present study, a rapid and easy HPLC procedure was adopted and modified for determination of TCs residue levels in milk.

This project was carried out due to the widespread use of TCs in lactating cows in Iran and occasional debates between public health and veterinary officials regarding the presence and the levels of antibiotic residues in foods of animal origin as well as the lack of long-term studies (covering an entire year) in milk industry.

Materials and Methods

Milk sample collection and preparation: Four hundred and thirty two pasteurized milk samples were purchased from Tehran supermarkets supplying the products of 12 major dairy companies from April

2011 to March 2012 (three milk samples from each company every month and in total 36 samples in each month). The milk samples were collected once in the middle of each month, and then they were transferred to the laboratory of the Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran, and were stored at -20 °C until analysis.

OTC and TC residues in milk were extracted by a liquid-liquid phase procedure (described briefly below) and quantified by an HPLC method. This technique was used just after validation of the HPLC method for analysis of both antibiotics including limits of detection (LOD), limits of quantification (LOQ), recovery rates, and linearity of calibration curves.

100 µL of NaOH (0.1 g in 100 mL H₂O) was added to 1.0 mL of milk and was mixed using a vortex mixer. 1.5 mL of acetonitril (HPLC grade, Merck, Germany) was added and mixed again. The whole solution was centrifuged at 3000 rpm for 5 min, and then 1.0 mL of saturated Na₂SO₄ solution was added. 300 µL of supernatant was transferred into another tube and 600 µL of 0.01 M oxalic acid buffer (pH, 7.0) was added and mixed. Finally, it was filtered into an autoinjector vial using 0.45 µm membrane filter (Millipore, USA) to make the final solution for HPLC analysis.

Determination of TCs levels: All samples were analyzed by HPLC system (Knauer, Germany) after their preparations, using the HPLC method of Fritz and Zuo (2007) with some modifications. The chromatographic conditions included a mobile phase as 0.01 M oxalic acid buffer - acetonitril (80: 20) running through a C18 column (Eurospher 100; 5 µm, 4.0*300 mm) isocratically with a flow rate of 1.0 mL/min and UV- detection at 355 nm. Chromatographic data including peak areas were recorded and analyzed by Chromgate software (Knauer, Germany).

OTC and TC residue levels in milk samples were calculated using their corresponding peak areas and calibration curve formula of each antibiotic by Microsoft Office Excel 2007.

Data analysis: Descriptive statistics were used for TCs residue data analysis. Median and the range of total TCs residues in milk samples containing residues above MRL were presented. The MRL value in this study was adopted according to the MRLs established by Codex Alimentarius Commission, i.e.,

100ng/mL of total TCs in milk or 100ppb.

Results

The HPLC method validation data: The retention times for OTC and TC were 2.1 and 2.5 min, respectively, without any interference in the retention times (Figure 1). The recovery rates for the OTC and TC residues in milk were 85.5 +/- 3.4% and 77.9 +/- 5.2 %, respectively. The linearity of calibration curve and curve formula for OTC levels at 0.05 - 10 µg/mL were $R^2=0.999$ and $Y= 613327 X+ 46850$, respectively. The linearity and calibration curve formula for TC levels between 0.05 and 10 µg/mL were $R^2=0.999$ and $Y = 775277 X + 47631$, respectively. The limit of detection (LOD) and limit of quantification (LOQ) for OTC analysis were 5 and 16 ng/mL, respectively, and for TC analysis they were 4 and 13 ng/mL, respectively.

TCs levels in milk: In 418 milk samples out of 432, the total residue levels of OTC and TC were lower than LOQ. In seven milk samples, although the TCs levels were quantifiable, total TCs residue contents were lower than 100 ng/mL (MRL). However, in seven milk samples (1.62 %), the total TCs residue levels were more than MRL. The specifications of the milk samples in which the amounts of total TCs residues were more than MRL are shown in Table 1.

TCs residues had not been detected in the samples collected in spring and summer seasons at all and all seven samples with TC residue levels above MRL were collected in autumn and winter seasons, 2 samples in October and 5 samples in February. The other seven milk samples with quantifiable TCs levels, but lower than MRL, had been collected in November (4 samples), January (1 sample), and February (2 samples).

Discussion

Public health concerns in connection with drug residues in milk are growing all over the world including Iran, in which occasional debates occur between public health sectors and veterinary officials. Recent cross-sectional studies regarding the TCs residue in milk in different parts of Iran indicate the presence of these antibiotics in marketed milk

Table 1. The specifications and amounts of oxytetracycline and tetracycline residues in seven pasteurized milk samples in which the total TCs residues were above MRL(100 ng/mL) in a one-year study (April 2011-March 2012). OTC: Oxytetracycline; TC: Tetracycline.

Sample No.	Month	OTC level (ng/mL)	TC level (ng/mL)	Total TCs Level (ng/mL)
7-5-3	Oct	483	-	483
7-12-2	Oct	526	706	1232
11-1-2	Feb	123	151	274
11-1-3	Feb	474	151	625
11-2-1	Feb	071	290	361
11-2-2	Feb	566	593	1159
11-8-1	Feb	-	1270	1270

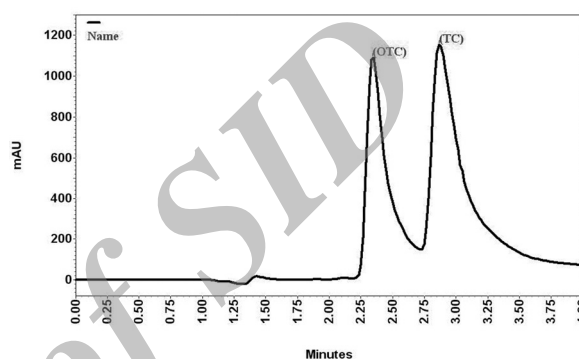


Figure 1. The chromatogram of mixed standard solution of oxytetracycline and tetracycline (1µg/mL of each).

samples (Rassouli et al, 2010; Mesgari Abbasi et al., 2011). In a similar fashion, the present one-year study suggests that a limited number of milk samples (1.62%) have TCs residues above MRL. Regarding the relationship between seasonal changes and occurrence of residues in milk samples, it was notable that all milk samples with detectable TCs residues had been collected in cold seasons, autumn and winter.

Initial residue studies in milk were done in the 1950s by US-FDA. More studies on the crude and pasteurized milk supply in USA, Canada, UK, and South Africa between 1955 and 1959 revealed that approximately 3 to 5% of tested samples contained drug residues. Therefore, the US-FDA proposed plans in 1960 to prevent drug residues in milk, which included the establishment of withdrawal (withholding) times for a number of drugs used in food-producing animals and established maximum residues limits (MRLs) in food stuffs of animal origin including MRL for OTC and TC in milk (0.1µg/mL).

It has been reported that in 92% of cases, the cause

for the incidence of the antibiotic residues in milk was due to their administration in mastitis therapy (Schmidt et al., 2003). The occurrence of antibiotic residues in milk is strongly associated with certain variables such as milk production rate at the time of treatment, the type and amount of antibiotic used, the type of vehicle used in antibiotic formulations, and the disease state of the animal (Mercer et al., 1970).

Antimicrobials, anti-inflammatory, and hormones are the pharmacologically active substances most used for these purposes; however, an illegal or unsuitable use increases the risk of introducing harmful residues into human food chain. Adverse effects in consumers are connected with the intrinsic toxicity of a drug and/or its metabolites. Hence, the use of antimicrobial agents in food animals has made a lot of concerns regarding their impacts on human health.

The main applications of tetracycline in animal husbandry are for prophylaxis of bacterial infections and increasing the growth rates. Although the public health risks are difficult to define, it is accepted that antimicrobial drug residues may induce allergic reactions in sensitized individuals and may have negative effects on the composition of the human intestinal flora. In general, the excessive use of antimicrobials has led to the development and prevalence of multi-drug resistance in animal and human pathogens (Sarmah et al., 2006). Furthermore, milk contaminated with even low concentrations of antimicrobial residues may also create problems in the production process of fermented milk by-products, because such compounds may inhibit the growth of the starter cultures.

The first study on antibiotic residues in milk in Iran was carried out by Khavari (1961) in which 20 dairy cattle with clinical mastitis received an intramammary product containing antibiotics. Using microbiological tests, Khavari showed that 13 out of the 20 milk samples had antibiotic residues. However, in another study, using microbiological tests, Abedi et al. (1984) did not find antibiotic residues in 325 milk samples collected from pasteurized dairy companies in Fars province. Another study assessed 200 raw milk samples in Shiraz (Fars province) for antibiotic contamination using the microbiological four-plate test (Liaghat et al., 1998). They reported that 10% of the raw milk samples from dairy companies and 13%

of those from local markets were positive for antibiotic residues. However, they found no antibiotic residues in 100 pasteurized milk samples.

Desalegne (2008) collected 400 bulk milk samples randomly from dairy farms in Ethiopia. All samples were qualitatively screened for antibiotic residues by Delvotest SP assay. Out of 400 samples analyzed for antibiotic residue, 34 (8.5%) milk samples were positive for antibiotic residues. Then residue levels of the common antibiotics in positive samples were determined by HPLC. The antibiotic-residue positive samples which showed OTC residues higher than 100 ng/mL were 24 out of 34 (70.58%). Regarding penicillin G residual above MRL of 4 ng/mL, they were 7 (20.58%).

Rassouli et al. (2010), in a cross-sectional study, collected ninety milk samples during five sequential days from the products of six major dairy companies in Tehran in 2007. OTC and TC residues were extracted and quantified by an HPLC method with an ultraviolet detector. TCs residual were detected in seven (7.8%) milk samples. The OTC and TC in almost all samples were lower than 100 ng/mL. However, just in one milk sample out of 90 milk samples tested, the total residue levels of OTC and TC was more than MRL, 138.8 ng/mL.

Khosro khavar et al. (2011) studied OTC residues in infant formula in Tehran. They reported that the samples had no residues of OTC in infant formula from different companies.

Mesgari Abbasi collected 114 pasteurized, sterilized, and raw milk samples from markets of Ardabil (Mesgari Abbasi et al., 2011). Tetracycline, oxytetracycline, and chlortetracycline (TCs) residues extraction was carried out using solid phase extraction (SPE) method. The mean of total TCs residues in all samples (114 samples) was 97.6 ± 16.9 ng/mL and the means of pasteurized, sterilized, and raw milk samples were 87.1 ± 17.7 , 112.0 ± 57.3 and 154.0 ± 66.3 ng/mL, respectively. 24.4%, 30%, and 28.6% of the pasteurized, sterilized, and raw milk samples, respectively, had higher TCs residues than the recommended MRL (100 ng/mL).

The method that was used in the present study for determination of TCs residues in milk was easy to validate, simple to perform, and more economical compared to the methods that used SPE cartridges. According to the results of this study, there were

detectable TCs residues in 14 out of 432 (3.2%) of pasteurized milk samples; however, in seven cases (1.62%), the TCs levels were greater than the MRL. It was also noted that all milk samples containing detectable TCs residuals were collected in fall and winter in which it seems mastitis occur more frequently due to climatic changes, and as a result antibiotic therapy is carried out more often. It should be reminded that the milk samples analyzed in the present study represent the milk of a large number of dairy cows that were mixed together in milk tanks in dairy farms and further in dairy companies, and these processes greatly dilute the initial drug residues in milk.

In summary, with regard to the presence of TC residues above MRL in a limited number of milk samples, it is concluded that more studies on drug residues in food animal and the establishment of suitable regulations and inspection systems are needed to reduce the risks of antibiotic and other drug residues for public health.

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References

- Chopra, I., Howe, T.G.B., Linton, A.H., Linton, K.B., Richmond, M.H., Speller, D.C.E. (1981) The tetracyclines: Prospect at the beginning of the 1980s. *J Anti Chem.* 8: 5-21.
- Ding, X., Mou, S. (2000) Ion chromatographic analysis of tetracyclines using polymeric column and acidic eluent. *J Chromatogr A.* 897: 205-214.
- Fritz, J.W., Zuo, Y.G. (2007) Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. *Food Chemistry.* 105: 1297-1301.
- Furusawa, N. (1999) Rapid and simple determination of oxytetracycline in milk products. *J AOAC Int.* 82: 770-772.
- Ivona, K., Mate, D. (2002) Evaluation of the sensitivity of individual test organisms to residual concentrations of selected types of drugs. *Slov Vet Res.* 9: 78-82.
- Iwaki, K., Okumura, N., Yamazaki, M. (1992) Determination of tetracycline antibiotics by reversed-phase high performance liquid chromatography with fluorescence detection. *J Chromatogr.* 623: 153-158.
- Jacques, F.M.N., Loeffen, G., Schouten, J., Egmond, H.V., Keukens, H., Stegeman, H. (1998) Testing of raw milk for tetracycline residues. *J Dairy Sci.* 81: 2341-2345.
- Kaplan, M.M., Abdussalam, M., Bijlenga, U. (1992) Specific and non-specific sensitizing agents FAO/WHO milk hygiene. WHO monograph series. 48: 56-58.
- Khosrokhavar, R., Hosseini, M., Shoeibi, S., Jannat, B. (2011) Detection of oxytetracycline residue in infant formula by high performance liquid chromatography (HPLC). *Iran J Pharm Res.* 10: 221-224.
- Mercer, H.D., Geleta, J.N., Wright, W.W. (1970) Milk out rates for antibiotics in intramammary infusion products used in the treatment of bovine mastitis: Relationship of somatic cell counts of milk production levels and drug vehicle. *Am J Vet Res.* 31: 1549-1560.
- Mesgari Abbasi, M., Babaei, H., Ansarin, M., Nourdadgar, A., Nemati, M. (2011) Simultaneous determination of tetracyclines residues in bovine milk samples by solid phase extraction and HPLC-FL method. *Adv Pharm Bull.* 1: 34-39.
- Rassouli, A., Abdolmaleki, Z., Bokaei, S., Kamkar, A., Shams, G.R. (2010) A cross-sectional study on oxytetracycline and tetracycline residues in pasteurized milk supplied in Tehran by an HPLC method. *Int J Vet Res.* 4: 1-3.
- Sarmah, A.K., Meyer, M.T., Boxall, A.B. (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics in the environment. *Chemosphere.* 65: 725-759.
- Schmidt, R.H., Rodrick, G.E. (2003) *Food Safety Handbook.* John Wiley & Sons, New Jersey, USA.
- WHO technical report series No. 799. Report of the joint FAO/WHO expert committee on food Additives, WHO, Switzerland 1990.

ردیابی کمی بقایای اکسی تتراسایکلین و تتراسایکلین در شیرهای پاستوریزه مصرفی شهر تهران در طول سال ۱۳۹۰

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چکیده

زمینه مطالعه: تتراسایکلین‌ها آنتی بیوتیک‌های وسیع الطیفی هستند و به طور گسترده در دامپزشکی مصرف می‌شوند. حضور بقایای تتراسایکلین‌ها در شیریک نگرانی بهداشت عمومی در سراسر جهان به شمار می‌رود. **هدف:** بررسی میزان بقایای تتراسایکلین‌ها در شیرهای پاستوریزه مصرفی شهر تهران در ماه‌های مختلف سال ۱۳۹۰. **روش کار:** ۴۳۲ نمونه شیر از محصولات ۱۲ شرکت عمده تولید کننده شیر پاستوریزه شهر تهران (۳ نمونه شیر از هر شرکت و در مجموع هر ماه ۳۶ نمونه) از فروشگاه‌های محلی خریداری و تا زمان آنالیز در فریزر 20°C نگهداری شد. بقایای اکسی تتراسایکلین و تتراسایکلین از طریق یک روش استخراج مایع - مایع از نمونه‌های شیر جدا شد و با استفاده از سیستم کروماتوگرافی مایع با کارکرد عالی تعیین مقدار گردید. شرایط کروماتوگرافی شامل فاز متحرک، بافر اسید اگزالیک - استونیتریل (۲۰:۸۰)، میزان جریان ۱ mL/min و با دتکتور UV در طول موج ۳۵۵ nm ردیابی گردید. **نتایج:** میزان تتراسایکلین‌ها در اکثر نمونه‌ها کمتر از میزان حد مجاز (۱۰۰ ppb) بود و تنها در ۷ نمونه شیر (۱/۶۲٪) مجموع بقایای تتراسایکلین و اکسی تتراسایکلین بالاتر از حد مجاز به دست آمد. در نمونه‌های اخیر، میانگین بقایای تتراسایکلین‌ها برابر با ۶۲۵ ppb و با دامنه ۲۷۴ تا ۱۲۷۰ ppb بود. **نتیجه‌گیری نهایی:** با توجه به حضور بقایای تتراسایکلین در تعدادی از نمونه‌های شیر مصرفی در مقادیر بالاتر از حد مجاز نیاز به مطالعه گسترده‌تر و نظارت بیشتر مسئولین بهداشتی در این زمینه احساس می‌شود.

واژه‌های کلیدی: کروماتوگرافی مایع با کارکرد عالی، شیر، بقایای دارویی، تتراسایکلین‌ها

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