A cross-sectional study on Oxytetracycline and Tetracycline residues in pasteurized milk supplied in Tehran by an HPLC method

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Abstract

Tetracyclines (TCs) are broad-spectrum antibiotics that are used widely in veterinary medicine. The present study was carried out to trace the residues of oxytetracycline (OTC) and tetracycline (TC) in pasteurized milk that is marketed in Tehran with the use of a High Performance Liquid Chromatography (HPLC). Ninety milk samples were collected during five sequential days from the products of six major dairy companies. OTC and TC residues were extracted and quantified by an HPLC method with an ultraviolet radiation detector. TC residues were found in seven (7.8%) milk samples. The OTC and TC in almost all samples were lower than 100 ppb (parts per billion), which is the maximum residue level advised. However, the total residue of OTC and TC in one milk sample was 138.8 ppb. In conclusion, due to the presence of a significant amount of TC residues in a number of milk samples, more studies and further supervision of the quality of milk products are required.

Introduction

Tetracyclines (TCs) are broad-spectrum antibiotics that are used widely in human and veterinary medicine. They are used for the prevention and treatment of a great number of diseases, as well as growth promoters in animal husbandry (Ameri, 1992; Abdolmaleki, 2008). The use of TCs in dairy farms may have serious adverse effects due to the potential presence of antibiotic residues in milk. These may cause toxic effects and/or allergic reactions in some individuals. Low levels of these residues in milk that is consumed over a period of time can lead to the development of drug-resistant microorganisms. 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), proposed that 100 ppb (parts per billion) should be the maximum residue limit (MRL) (Kaplan *et al*., 1962; WHO, 1990; Commission of EC, 1991; Abdolmaleki, 2008).

There are a number of methods for the measurement of TC residues in biological matrices, such as microbiological and physicochemical methods (Abedi Shirazi, 1984; Iwaki *et al*., 1992; Jacques *et al.*, 1998; Furusawa, 1999; Ding and Mou, 2000; Furusawa, 2003). However, high performance liquid chromatography (HPLC) methods are the most sensitive and specific techniques. In the present study, a rapid and easy HPLC procedure was adopted for the determination of the level of TC residues in milk with certain modifications to the method described by Batto *et al.* (Batto *et al.,* 1999). Therefore, the HPLC method was set up and validated first and then the amount of TC residues in milk samples were determined.

Materials and Methods

Chemicals and instruments

Tetracycline and oxytetracycline (OTC) standards, HPLC grade methanol and acetonitril, hydrochloric acid, Na_2-EDTA, phosphoric acid (85%, w/v), KOH, hexane and dichloromethane were all from Merck, Germany. The HPLC system, which included the pump (model K1001), online degas, ultraviolet (UV) detector (model 2600), and the data-collection and system control software (Chromgate software) were all purchased from Knauer, Germany. The C_{18} column (Eurosphere 100, 4×300 mm, 5 μm), ultrasonic bath (Powersonic 505, S. Korea), and membrane filter (0.45 μm; Millipore, USA) were all acquired separately.

Milk sample collection

Ninety milk samples were collected from six major dairy companies that provide pasteurized milk to Tehran supermarkets. Samples were collected over five sequential days in October 2007. They were transferred to the laboratory of the Department of Pharmacology, Faculty of Veterinary Medicine, University of Tehran,

and were stored at -20ºC until the analysis.

Residue analysis

OTC and TC residues in milk were extracted by a liquid-liquid phase procedure and quantified by an HPLC method, as described previously by Batto *et al.,* (1999) with some modifications. This technique was only used after validation experiments were performed on the method. In brief, 5 ml milk, 1 ml HCl (1.0 M) and 24 ml acetonitril were mixed and shaken manually in a decanter for 5 min. The supernatant was then filtered and added to another decanter, where it was mixed with 30 ml hexane and 15 ml dichloromethane and fully shaken for a further 5 min. After the decanter was allowed to stand for 5 min, the aqueous phase (the lower part) was removed and poured into a graded glass tube. Its final volume was made up to 4 ml by adding distilled water. The extract was then filtered through a membrane filter (0.45 µm) and stored at -20ºC before it was analyzed by HPLC.

The chromatography conditions included phosphoric acid solution (pH: 2.3), acetonitril (24-76%) as the mobile phase, with an injection volume of 1000 µl, a C_{18} column with a flow rate of 1 ml/min, and UV detection set at 355 nm. Data from the chromatograms were collected and analyzed by Chromgate software, Knauer.

Results

Results of the HPLC set up and validation

The retention times for OTC and TC were 4.6 and 5.1 min, respectively. The recovery rates for the OTC and TC residues in milk were 88.2 +/- 5.4 and 70.0 +/- 8.0 %, respectively. The linearity and curve equations of the OTC calibration curve between 50 and 200 ng/ml were Y=10.6, X + 78.4, and r^2= 0.996. For TC at levels between 50 and 500 ng/ml, they were Y=0.98, X + 4.6, and r^2= 0.988. The limit of detection (LOD) and limit of quantification (LOQ) for OTC analysis were 3 and 10 ng/ml, respectively, and they were 9 and 30 ng/ml, respectively, for TC analysis. The precision of this method for the OTC analysis as intra- and inter-day CV% was 2-6% and 3-7%, respectively, and they were 2- 8% and 10-14%, respectively, for the TC analysis.

Amounts of TCs residues in milk

Of the 90 milk samples that were examined, the total residues of OTC and TC in six milk samples (6.7%) were at levels that were lower than 100 ppb (MRL); they were more than MRL (138.8 ppb) in one sample (1.1%), and the amount of residues were lower than the LOQ of the method in 83 samples (92.2%; 10 and 30 ng/ml for OTC and TC, respectively). The amount of OTC and/or TC residues in the milk samples are shown in Table 1.

Table 1: The amount of TC residues in pasteurized milk out of 90 milk samples supplied by six major dairy companies in Tehran, Iran.

Sample No.	OTC concentration (ppb)	TC concentration (ppb)	Total TCs concentration (ppb)
1-11	LLQ	LLQ	LLQ
12	44.3	94.5	138.8
13-40	LLQ	LLQ	LLQ
41	LLQ	19.6	19.6
42-51	LLQ	LLQ	LLQ
52	9.1	37.8	46.9
53	LLQ	64.2	64.2
54	12.9	73.3	86.2
55-61	LLQ	LLQ	LLQ
62	20.4	LLQ	20.4
63-73	LLQ	LLQ	LLQ
74	54.0	LLQ	54.0
75-90	LLQ	LLQ	LLQ

LLQ: lower than limit of quantification of the analytical method; OTC: oxytetracycline; TC: tetracycline; ppb: parts per billion.

Discussion

Public health concerns about the existence of drug residues in milk are not new issues, and studies in this regard were initiated in the 1950s by FDA (Abdolmaleki, 2008). In preliminary studies in the USA, it was shown that drug residues were present in a high percentage of the milk sold in that country. More studies on the crude and pasteurized milk supply in the USA, Canada, the United Kingdom, and South Africa between 1955 and 1959 revealed that approximately 3-5% of tested samples contained drug residues. Therefore, the USFDA 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.

The first study on antibiotic residues in milk in Iran was carried out by Khavari (Khavari, 1961) in which 20 dairy cattle with clinical mastitis that had received intramammary ointment containing antibiotics were studied. He showed that 13 out of the 20 milk samples had antibiotic residues with the use of microbiological tests. In another study in Iran, Abedi (Abedi *et al.,* 1984) did not find antibiotic residues in 325 milk samples that were collected from pasteurized dairy companies in the province of Fars, also with the use of microbiological tests.

Another study assessed 200 raw milk samples from cows in Shiraz (Fars province) for antibiotic contamination using the microbiological four plate test (Liaghat and Khan Nazer, 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 milk samples that had been pasteurized. Furusawa (Furusawa, 2004) studied OTC residues in pasteurized milk samples in Osaka, Japan. He reported that the amount of antibiotic residues were lower than

the MRL of 100 ppb.

The method that was used for the determination of TCs residues in milk in the present study was easy to validate and simple to perform. The treatment of milk samples with HCl and liquid-liquid extraction with hexane and dichloromethane produced comparable results when compared to the methods that used solid phase extraction cartridges. However, the methods used in our study were more economical (Furusawa, 2003).

According to the results of this study, there were detectable TC residues in 7.8% of pasteurized milk samples supplied for sale in Tehran and, in one case, this level was greater than the MRL, despite the fact that milk from a large number of dairy cows are mixed together by dairy companies, and this process should greatly dilute the initial drug residues in milk.

Conclusions

In conclusion, with regards to the increasing use of antimicrobial agents in dairy farms, more studies on drug residues in food animals, as well as the establishment of suitable regulations and inspection systems, are needed to reduce the risks of antibiotic and other drug residues for public health issues.

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