The Effect of Heating on Multiple Residues of Tetracyclines in Milk

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Abstract

The change by heating on residues of oxytetracycline (OTC), tetracycline (TC), and chlotetracycline (CTC) in milk is evaluated. The residues are measured using high performance liquid chromatography (HPLC) with a UV detector. Milk spiked with OTC, TC, and CTC at 200, 200, and 400 ppb, respectively, are heated to 63°C for 30 min. OTC residues were significantly ($p \leq 0.05$) reduced 79.36-86.77%. TC residues were significantly ($p \leq 0.05$) reduced 22.97-54.75%. No significant ($p>0.5$) reduction of CTC was found. Results showed that normal pasteurization procedure (63°C for 30 min) causes a reduction in OTC, TC and CTC residues in milk, but it does not completely eliminate all the residues from milk.

Key Words : oxytetracycline, tetracycline, chlotetracycline, milk, heat treatment, antibiotic residues, HPLC.

Introduction

The tetracyclines (TCs) are a group of antibiotics commonly used as veterinary medicines and as a growth promoter in food-producing animals. In bovine, oxytetracycline (OTC), tetracycline (TC), and chlotetracycline (CTC) are the major drugs of choice in this group of antibiotics for prevention and treatment of bacterial infection and feed additive. Because of their wide application, there is a concern about the presence of residues in milk which may result from any of the following: excessive use, improper use, or a shortened withdrawal time. Despite their low toxicity, residues of TCs in milk should be avoided because they may pose potential health threats to consumers which could be extremely serious. One of the most concerning problems associated with TCs residues is idiosyncratic reactions especially in hypersensitive consumers [1, 2]. Like other antibiotics, TCs may be agents for the selection of antibiotics-resistant organism [3]. In addition some residues can interfere with the starter cultures used in processed milk products [4].

To protect the consumer, many countries have set up maximum residue limits (MRLs) of some TCs in milk. In Thailand the MRLs is 100 μg/l for OTC [5].

Since milk is always heated before consumption, a few reports have been published about the effect of heating on the stability of TCs residues in milk [6, 7, 8]. However, most earlier studies with drug residues in heated milk were analyzed using microbiological assays. Also, little or no information is available concerning the stability of multiple antibiotic residues in milk as affected by heat treatments. Microbiological assays are usually based on growth inhibition of a sensitive organism. These assays detect a broad spectrum of antibiotics but can not either distinguish antibiotics from one another or detect multiple antibiotics simultaneously. For identification and quantitation of suspected residues, more specific methods such as HPLC are needed. Therefore, the objective of this study is to examine the effect of heat treatment on multiple TCs residues presence in milk by HPLC. The selected heating condition (30 min at 63°C) is one of the normal pasteurization procedures used for milk production.

Materials and Methods

Reagents

(a) Chemicals.-All chemicals used were analytical grade, unless otherwise specified. Oxytetracycline (OTC), tetracycline (TC), and chlotetracycline (CTC) were purchased from Sigma (USA). Disodium ethylenediaminetetraacetate dihydrate was purchased from Merk (Germany). Citric acid monohydrate, sodium chloride, and disodium hydrogen phosphatedihydrate were purchased from Carlo Erba (Italy).

(b) Sovents.-Acetonitrile and methanol were HPLC grade (Lab-Scan,Thailand).

(c) McIlvaine-EDTA-NaCl buffer. Prepared according to the method of AOAC [9].

Apparatus

(a) Refrigerated centrifuges (Universal 16R, Hettich, Germany)

(b) Vortex mixer (model G-560E, Genie Scientific, USA)

(c) Water bath (model FED 115, WTB Binder, Germany).

(d) Thermocouple

(e)High performance liquid chromatograph (Thermo Separation Products, USA) consists of a Spectral System Binary Pump model P2000, a Vacuum membrane Degasser, a Spectral System Detector model UV 2000 set at 350 nm, a Rheodyne 7125 sampling valve with a 100-μL loop, and a Computor for control and data handling. A Spherisorb column, ODS2, 250 x 4.6 nm id. 5 μm particle size (Phenomenex) equipped with a guard column OSD2, 10 μm particle size, 10 x 4.6 mm. The integration was carried out using the software program PC1000

Preparation of standard solutions

(a) Stock standard solutions were prepared by dissolving 25 mg of each standard in a separate 25 mL volumetric flask with methanol and mixed thoroughly. These stock solutions were stored in refrigerator.

(b) Mix standard solutions were prepared by measuring desired amounts of standard from each stock solution into the same volumetric flask and diluted to volume with distilled water. These solutions were stored in refrigerator.

(c) Working solutions of standard were prepared on the day of its analysis by proper dilution of the mix standard solution with Mc II Vaine-EDTA-NaCl buffer to obtain concentrations of 20-200 ppb for OTC and TC, and 200-3000 ppb for CTC. From these solutions, 100 μL aliquots were used for the construction of the calibration curve.

Milk Samples

Antibiotics-free milk was prepared by reconstituting antibiotics-free whole milk powder (Dumex, NewZealand) with distilled water. This milk was used as blank milk.

Eighty raw milk samples were obtained from the Dairy Farming Promotion Organization of Thailand, Saraburi.

Effect of heating on OTC, TC, and CTC residues in milk

A 2.0 ml of blank milk was spiked with mixed standard at 100-300 ppb for OTC and TC and 750-1500 ppb for CTC. The spiked milk samples were then heated to 65°C for 30 min in water bath, removed, and cooled rapidly to room temperature prior to the sample extraction procedure.

Sample Extraction

The procedure by Thomas [10] was used for analysis of OTC, TC, and CTC , with some modifications. A 2.0 mL of Mc II Vaine-EDTA-NaCl buffer was added to 2.0 mL of test milk sample. The mixture was mixed on a vortex-mix for 10 min, centrifuged at 5000 rpm for 30 min at 10°C. The supernatant was collected and filtered through a 0.45 μm nylon membrane into vials for HPLC analysis.

HPLC Analysis

Injected 100 μL sample into HPLC system. Separation was achieved by gradient elution using mobile phase; (A) acetonitrile and (B) methanol : acetronitril : 0.01M Oxalic acid (1:1.5:4.5) , and a flow-rate of 1 mL/min. A gradient program was from 0A-100B to 35A-65B in 11 min, maintained this composition for 2 min followed by return to initial condition in 2 min and maintained at initial condition for 5 min. Quantitation was based on peak area. All separations were done at room temperature (25°C).

Recovery of added OTC, TC, and CTC

A 2.0 mL of blank milk was spiked with mixed standards at 100-300 ppb for OTC and TC and 750-1500 ppb for CTC, mixed

thoroughly, and carried through the sample extraction procedure.

Statistic analysis

Analysis of the data was carried out using ANOVA (SPSS program) to assess the effect of heating (non-heated milk and heated milk). Differences between means were tested using the Duncan's multiple range test.

Results and Discussion

Typical chromatograms of extracts of blank milk and blank milk spiked with OTC at 200 ppb, TC at 200 ppb, and CTC at 400 ppb are shown in Figure 1. The chromatographic system chosen was satisfactory, with minimized background and optimized resolution for all three drugs in milk within a reasonable time. The retention times were 6.14, 7.16, and 10.47 min for OTC, TC, and CTC, respectively. However, care must be taken during running the LC system. Because this LC system used gradient elution to separate all three drugs, problems from shift in retention times could arise if regeneration of the column to the initial solvent between runs was not achieved. In addition, sometimes there were two small endogenous peaks appeared between OTC and TC, which could interfere with the chromatograms, therefore appropriate baselines determined should be checked. The concentration of CTC detected in this study was set at higher level than OTC and TC due to a longer time required to elute CTC from the column causing its peak to be so broad that at very low concentration it was indistinguisable from the base line. The recovery data for spiked milk are shown in Table 1. Average recoveries were 83.16% for OTC and 65.51% for TC over the concentrations of 100-300 ppb, and 83.49% for CTC over the concentrations of 750-1500 ppb. The extraction procedure used was satisfactory, with sacrifice to minimize the time of analysis and no hazardous waste being generated. Approximately 80 milk samples were analyzed by this method. No samples showed any detectable amounts of TCs.

The effect of heating on OTC, TC, and CTC residues in milk is shown in Table 2. There were significant differences ($p \leq 0.05$) in OTC and TC concentration between non-heated and heated milk, while no significant differences ($p > 0.05$) in CTC concentrations between non-heated and heated milk was obtained. The reductions of OTC spiked in milk at 200 and 300 ppb were 86.77 and 79.36%, respectively (Table 2). The reduction of OTC spiked in milk at 100 ppb could not be estimated because peak from heated milk became indistinguisable from the base line, which made it difficult to obtain an accurate result. The reductions of TC spiked in milk at 100, 200 , and 300 ppb were 54.75, 22.97, and 37.45% respectively. For CTC, although no significant differences ($p > 0.05$) was obtained, it was found that heated milk had a lower concentration of CTC residues than non-heated milk, suggesting that the reductions of CTC also occurred but with a slow rate. The reductions of CTC were 3.71-9.75% over the concentrations of 750-1500 ppb. These findings indicated that OTC was degraded more rapidly than TC. CTC was the most stable of this study. These results also suggested that heating under normal pasteurization condition could cause a reduction of drug residues, but it did not completely eliminate OTC, TC, and CTC residues from heated milk. Shahani [6, 7] studied heat stability of drug residues in milk, and also reported that OTC was less heat stable than CTC and both were not completely reduced by 30 min at 62°C and 71°C, being only 23.6-35.6% reduction for OCT and 16.6-27.6% reduction for CTC. The author found that OTC was completely reduced by 190 min at 71°C and CTC was completely reduced by 15 min at 121°C, indicating that longer heating or more severe heating was required to completely reduce antibiotic residues in milk. Khopaibool [8] investigated the effect of heating on inhibition zone of TC added in milk using microbiological disk assay and also found that TC residues in milk was not inactivated by 17.2 sec at 74-100°C, but totally inactivated by 7.9 sec at 155°C.

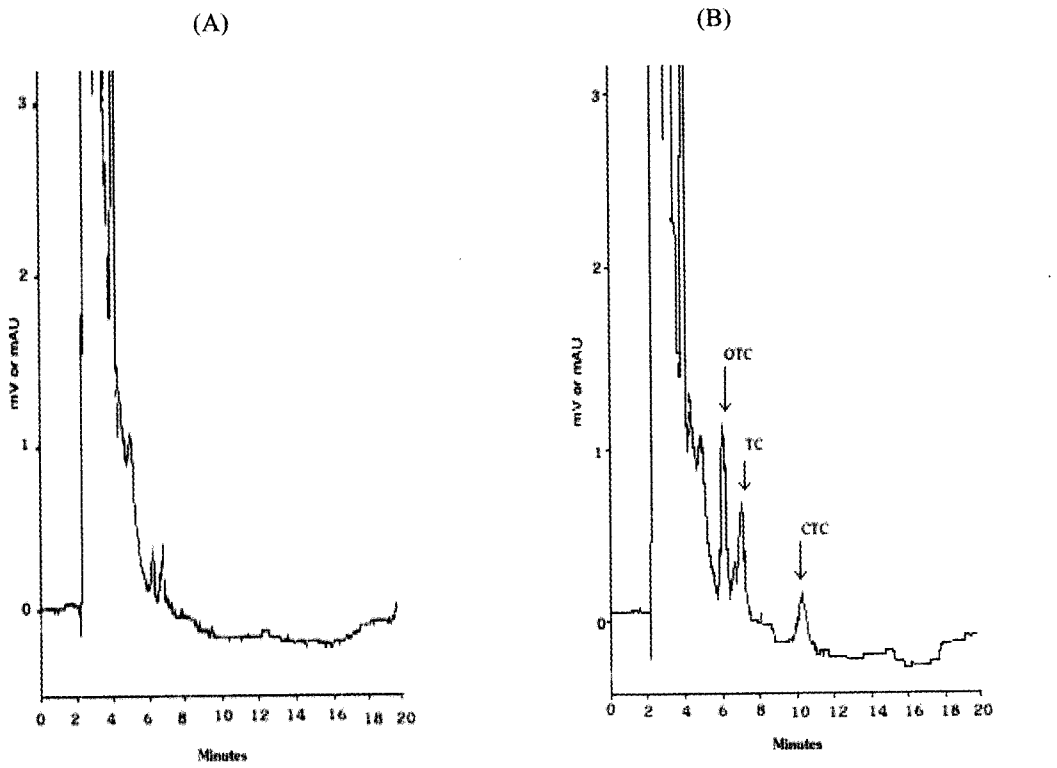

Figure 1. Typical chromatograms of milk extracts: (A) blank milk, (B) blank milk spiked with OTC, TC, and CTC at 200, 200, and 400 ppb, respectively.

Table 1 Recovery of oxytetracycline (OTC), tetracycline (TC), and chlotetracycline (CTC) from milk spiked at various concentrations.

Antibiotic	Amount added (ppb)	Recovery (%) (Mean ± SD)*
OTC		
	100	91.02 ± 7.69
	200	78.57 ± 11.16
	300	79.89 ± 1.65
TC		
	100	51.54 ± 9.94
	200	67.62 ± 4.10
	300	77.37 ± 19.15
CTC		
	750	87.27 ± 2.25
	1000	87.96 ± 11.55
	1500	75.24 ± 6.58

*Mean of three replicates.

Table 2 Effect of heat treatment at 63°C for 30 min on stability of tetracyclines residues in milk.

Antibiotic	Added (ppb)	Found (ppb)*		Rate of degradation (%)
		Non-heated	Heated	
OTC				
	100	77.79 ± 6.92	ND	ND
	200	145.18[a]± 22.57	19.21[b]± 10.51	86.77
	300	209.04[a]± 4.11	43.14[b]± 1.82	79.36
TC				
	100	44.64[a]± 9.32	20.20[b]± 4.81	54.75
	200	118.11[a]± 7.17	90.98[b]± 5.60	22.97
	300	176.49[a]± 21.37	110.40[b]± 12.41	37.45
CTC				
	750	745.11[a]± 6.69	673.79[a]± 54.18	9.57
	1000	576.11[a]± 75.65	547.97[a]± 74.84	4.88
	1500	985.53[a]± 86.22	948.96[a]± 75.89	3.71

*Mean ± SD of three replicates, no corrections for recovery loss were made.
[a-b]Means in the same row, followed by different superscripts, are significantly different at $p \leq 0.05$ by Duncan's multiple range test.
[ND]Not detected.

Conclusion

Normal pasteurization procedure by 30 min at 63°C caused a reduction in OTC, TC and TCT residues in milk. However, it could not completely eliminate all the residues from the milk. The heat stability of residues depend on type of drugs. OTC was the most easily degraded by heating. OTC was relatively heat stable.

Acknowledgements

Financial support was provided by the research budget of Thammasat University. Special thanks to the Dairy Farming Promotion Organization of Thailand, Saraburi for giving some raw milk. Also thank you to Tiparut Tomongkol for technical assistance.

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