Trace Analysis of Oxytetracycline and Tetracycline in Milk by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) method for the determination of oxytetracycline (OTC) and tetracycline (TC) residues in milk at levels as low as 10 ppb has been developed. Milk was acidified at pH 2.7 and extracted with acetonitrile. The extract was partly purified by treatment with ammonium sulfate solution and concentrated into a phosphate buffer, pH 8.2. Following addition of tetrabutylammonium reagent (TBA), tetracyclines were extracted as ion pairs with TBA into dichloromethane, reextracted into acid, and analyzed on a reversed-phase C_{18}, 5 μm, column. Overall recovery was found to be 72.7 ± 1.2% for OTC and 85.1 ± 1.3% for TC. The linearity was excellent for both compounds in the range examined (r = 0.9996, 23.7–190 ppb of OTC in milk; r = 0.09995, 26.5–212 ppb of TC in milk). Precision data based on within-day and among-days variation suggested an overall relative standard deviation of 5% for OTC and 4.2% for TC.

INTRODUCTION

Oxytetracycline (OTC) and tetracycline (TC) are widely used in the veterinary field for treatment of bovine mastitis. They are, also, frequently added at subtherapeutic concentrations to cattle feeds, primarily, for collective prophylaxis (*Feed Additive Compendium*, 1986). These uses have led to the need for methods to monitor residual levels of tetracyclines in milk, to ensure that it fulfils government requirements for tolerance limits. U.S. federal regulations have not set tolerance limits in milk (*Code of Federal Regulations*, 1986), but the World Health Organization has recommended a maximum allowable level of 100 ppb (Del Pozo, 1985).

TCs in milk have traditionally been regulated by bioassays (International Dairy Federation, 1987). These methods, which are based on testing for inhibition of microbial growth, are useful for initial screening of residues, but their sensitivity is poor, their precision appears to be variable, and their specificity is questionable. The occurrence in milk of natural inhibitory substances, such as lysozyme and lactoferrin, affects the accuracy of these assays (Carlsson and Bjorck, 1987). There has, therefore, been increasing interest in development of more reliable methods.

Several physicochemical methods including thin-layer chromatography (Ryan and Dupont, 1974), thin-layer chromatography and bioautography (Bossuyt et al., 1976; Hamann et al., 1979), electrophoresis and bioautography (Staudhouders et al., 1981; Lott et al., 1985; Pietrangeli et al., 1986), column chromatography and bioassay (Hamann, 1983), and gas–liquid chromatography (Hamann et al., 1979) have already been reported for the determination of TCs in milk. Most of them provide increased specificity and sensitivity and can be useful in classifying unknown residues in milk, but cannot be considered sufficiently sensitive or precise.

Considerable progress has been made recently on TC analysis by the use of high-performance liquid chromatography. Various HPLC methods allowing sensitive, accurate, and precise quantitation of TCs in tissues (Sharma and Bevill, 1978; Onji et al., 1984; Ashworth, 1985; Oka et al., 1985; Moats, 1986; Ikai et al., 1987; Kondo et al., 1988), eggs (Botsoglou et al., 1984), and honey (Sporns et al., 1986; Oka et al., 1987) were presented. However, such a method is not available for milk. A survey of the literature shows no definitive method on this subject.

This paper deals with the development of a sensitive, accurate, and precise method for the analysis of OTC and TC residues in milk by use of reversed-phase HPLC.

MATERIALS AND METHODS

Chemicals. Oxytetracycline and chlortetracycline (CTC), as their hydrochlorides, were purchased from Serva (Heidelberg, FRG), and tetracycline hydrochloride was supplied by Hoechst AG (Frankfurt, FRG). Tetrabutylammonium hydrogen sulfate (TBA-HSO_4) was obtained from Merck-Schuchardt (Munchen, FRG). All reagents used were of analytical grade.

Stock solutions of OTC and TC were prepared in methanol (ca. 1 mg/mL) and were stable for 1 month if stored at −25 °C. Dilute stock solutions (100 μg/mL) were also prepared in methanol immediately before use. Aliquots of the dilute stock solutions were further diluted with 0.1 M perchloric acid ($HClO_4$) to give working solutions containing each tetracycline in the range 0.040–1.6 μg/mL. Working solutions were protected from direct sun and artificial light throughout the analysis.

HPLC Apparatus. High-performance liquid chromatography was carried out on a Gilson system consisting of a Model 802 manometric module, a Model 302 piston pump, a Model HM/HPLC dual-beam variable-wavelength UV–vis spectrophotometer set at 355 nm, and a Model N1 variable-span recorder. A Hplc-technology Model TC 831 column oven, set at 35 °C, permitted temperature regulation. Injections were made on a Hichrom, 25 × 0.46 cm, column prepacked with Nucleosil 120, C_{18}, 5 μm, through a Rheodyne 7125 sample injector equipped with a 100-μL loop.

HPLC Procedure. The new Hichrom HPLC column was conditioned before use for 24 h by recycling through it, at a flow rate of 0.2 mL/min, a mixture of acetonitrile–water (50:50 v/v), containing 0.5 mg of CTC/mL. An overnight reconditioning of the column was made after each day's work.

The mobile phase used was a mixture of 240 mL of acetonitrile and 760 mL of 0.02 M phosphoric acid solution, pH 2.3. The mobile phase was degassed by using helium and delivered at a rate of 1.2 mL/min.

Milk Extraction. A 5-g sample was transferred to a 50-mL glass-stoppered centrifuge tube and acidified at pH 2.7 with 0.8 mL of 0.6 N sulfuric acid. A volume (10 mL) of acetonitrile was added, and the tube was vortexed for 15 s at high speed. After the precipitated proteins settled, the supernatant liquid was fil-

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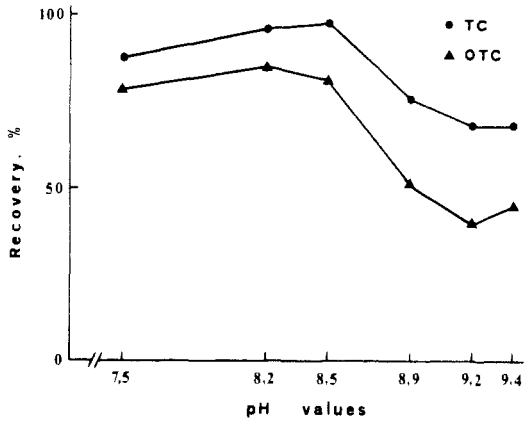

Figure 1. Effect of pH on extraction efficiency of OTC and TC as ion pairs with TBA reagent.

tered through a fritted glass disk funnel, and a 10-mL aliquot of the clear filtrate was pipetted to another tube.

Cleanup Procedure. A volume (3 mL) of saturated ammonium sulfate solution was added in the tube, and the suspension formed was vortexed for 30 s and centrifuged for 1 min at 1000*g*. The top layer separated was transferred to a 50-mL evaporating flask, and the remaining bottom layer was reextracted with 5 mL of acetonitrile, collecting the top acetonitrile layer into the same flask. A volume (2 mL) of 0.05 M phosphate buffer, pH 8.2, was added, and the resulting mixture was rotary-evaporated under reduced pressure at 35 °C to a volume of ca. 2 mL. Following the addition of 11 mL of dichloromethane and 2 mL of 0.4 M aqueous TBA-HSO_4 solution, adjusted at pH 8.2, the flask contents were shaken vigorously for 2 min, transferred to a 20-mL tube, and centrifuged for 1 min at 1000*g*. A 10-mL aliquot from the bottom organic layer separated was pipetted to a 15-mL tube and evaporated to a volume of 6 mL with nitrogen. A volume (0.5 mL) of 0.1 M perchloric acid was added in the remaining liquid, and the tube was vortexed for 1 min and centrifuged for 2 min at 2000*g*. A 100-μL volume from the top aqueous layer, equivalent to 0.575 g of milk, was used for high-performance liquid chromatography.

Calibration curves were constructed by plotting peak heights versus concentration from 100-μL injections of each of the prepared working solutions. The concentrations of OTC and TC in samples were calculated by reference to calibration curves and multiplication by an appropriate dilution factor.

RESULTS AND DISCUSSION

Treatment of milk samples with 2 volumes of acetonitrile was effective in precipitating proteins, since clear filtrates were consistently taken after a 3-min settling time. The extraction, however, of TCs was not quantitative, unless samples had been acidified at values lower than pH 3. This could be, possibly, due to incomplete dissociation of OTC and TC from milk proteins at higher pH values.

Some purification of the filtrates was effected by vortexing them with a saturated ammonium sulfate solution. By this treatment TCs were quantitatively recovered in the top acetonitrile layer, whereas a certain amount of interfering components was eliminated. Further purification was effected by mixing the acetonitrile layer with a phosphate buffer, evaporating acetonitrile, and extracting the aqueous buffer with dichloromethane.

As the partition coefficients of TCs prevent their extraction from aqueous into organic solvents (Ashworth, 1985), OTC and TC had to be converted, prior to dichloromethane extraction, to ion pairs with TBA cations that are readily extractable. This extraction was most efficient when the buffer solution had been adjusted at pH 8.2. Figure 1 indicates the influence of pH values on extraction efficiency of TC–TBA ion pairs. The dependence presented is not in agreement with that found by Eksborg et al. (1979), as it was reported that this extraction was most favorable at values higher than pH 9.

Since the presence in milk extracts of anionic components that could react with TBA reagent, decreasing its amount needed for ion pairing with the contained TCs, could not be excluded, an excess of this reagent was added in the buffer solution. Such an excess of TBA could result, however, in coextraction of unreacted reagent into the dichloromethane layer and, after the reextraction step, into the acidic layer, part of which was to be injected on to the column. This, in fact, was the case when 1 N phosphoric acid was used in the reextraction step; following injections, the retention times of TCs were gradually decreased, due apparently to the adsorption of the TBA cations on the chromatographic support. By use of 0.1 M perchloric instead of phosphoric acid in the reextraction step, such a problem was not noted, since TBA cations could be repartitioned into the dichloromethane layer as perchloric ion pairs. These findings confirm those previously reported by Eksborg et al. (1979).

The effectiveness of the cleanup procedure permitted chromatographic analysis of milk samples under isocratic conditions. When a Nucleosil C_{18} 5-μm stationary phase and aqueous acetonitrile containing dilute phosphoric acid as eluent were used, the chromatograms recorded were free of interfering extraneous peaks. However, baseline drift, poor resolution, and severe peak tailing were consistently noted. Since it has been assumed (Knox and Jurand, 1975; Ashworth, 1985) that these effects are due to the presence of residual free silanol groups on the support surface, and/or to metal ion contamination in the base silica of the bonded phases, attempts were made to develop another solvent system for TC elution that would be capable of blocking the reactive sites of the stationary phase, eliminating, thus, peak tailing and improving resolution.

Blocking agents added in mobile phase such as ethylenediaminetetraacetic acid (Mack and Ashworth, 1978; Ashworth 1985; Sporns et al., 1986; Chappell et al., 1986; Martinez and Shimoda, 1988) and/or alkyl-substituted ammonium compounds (Eksborg and Ekqvist, 1981; Moats, 1986; Khan et al., 1987) have been successfully used to reduce peak tailing. However, some authors have reported that such an addition was not needed (Eksborg et al., 1979; Hermansson, 1982; Onji et al., 1984; Botsoglou et al., 1984), whereas others have stated that new HPLC columns should be conditioned, before use, with chelating agents (Leenheer and Nelis, 1977; Sharma and Bevill, 1978). Since the above blocking agents could not completely eliminate peak tailing, use of another agent having similar chemical affinity to the TCs analyzed was investigated. It was found that, by overnight recycling through the HPLC column of an aqueous acetonitrile solution containing 0.5 mg of CTC/mL, peak responses were consistently high, no tailing was evident, and baseline separation was made possible.

Peak heights, although quite reproducible at a given mobile phase flow rate, varied greatly with it. With gradual reduction of the flow rate from 1.2 to 0.5 mL/min, peak heights could be decreased up to 25%, whereas increasing the flow rate from 1.2 to 2.0 mL/min had no effect on peak heights. These findings are not in agreement with the peak height dependence on flow rate reported by Kirkland (1974) for reversed-phase HPLC. Therefore, this inconsistency might be an indication that the predominant mechanism in TC chromatography is not the liquid–liquid partition.

Working solutions and milk extracts were monitored at 355 nm. At this wavelength OTC was eluted in 3.9 min

Table I. Raw Data and Regression Equation of Calibration Curve for OTC Determination by HPLC

concn of working solution, ng/100 μL[a]	peak height,[b] mm [mean ± SD (n = 6)]	% RSD
142.5	180.0 ± 2.4	1.3
95.0	116.2 ± 1.0	0.9
71.2	88.4 ± 0.5	1.1
35.3	41.9 ± 0.5	1.3
17.8	23.0 ± 0.7	3.1
8.9	11.2 ± 0.3	2.8
4.4	6.4 ± 0.4	6.6

[a] Volume injected. [b] Regression equation, $y = -0.438 + 1.252x$; correlation coefficient, $r = 0.9995$.

Table II. Raw Data and Regression Equation of Calibration Curve for TC Determination by HPLC

concn of working solution, ng/100 μL[a]	peak height,[b] mm [mean ± SD (n = 6)]	% RSD
159.0	143.3 ± 1.3	0.9
106.0	90.2 ± 2.2	2.5
79.5	67.8 ± 0.9	1.4
39.7	32.7 ± 1.0	3.0
19.9	17.1 ± 0.9	5.2
9.9	8.6 ± 0.6	7.4
5.0	5.8 ± 0.3	5.6

[a] Volume injected. [b] Regression equation, $y = -0.875 + 0.887x$; correlation coefficient, $r = 0.9988$.

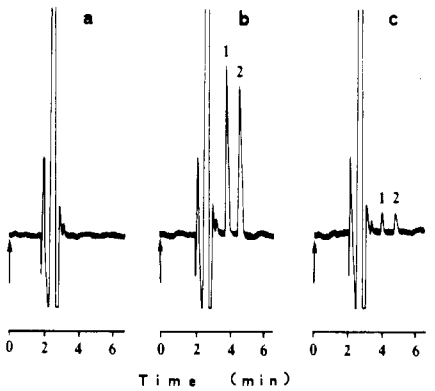

Figure 2. Typical chromatograms of a blank milk sample (a), a sample spiked with 93 ppb of OTC and 100 ppb of TC (b), and a sample spiked with 10 ppb of both compounds (c). Conditions: mobile phase, acetonitrile–0.02 M phosphoric acid solution, pH 2.3 (24:76 v/v); column, 25 × 0.46 cm, C_{18} (5 μm); temperature, 35 °C; flow rate, 1.2 mL/min; wavelength, 355 nm; recorder sensitivity, 0.020 AUFS; chart speed, 0.5 cm/min; injection volume, 100 μL.

and TC in 4.9 min. No changes in retention times were noted with continual column use. Regression analysis of the data obtained by running a series of working solutions of OTC (Table I) and TC (Table II) showed the response to be linear for both compounds in the range examined ($y = -0.438 + 1.252x$, $r = 0.9995$ for OTC; $y = -0.875 + 0.887x$, $r = 0.9988$ for TC, where y represents peak height (millimeters) and x the quantity (nanograms) of the compound injected).

Due to the absence of any interfering peaks in sample chromatograms (Figure 2), concentrations as low as 10 ppb of each compound could be readily determined (peak to noise ratio, 3). A survey of the literature shows that these limits are much lower than those reported in all previous bioassay and physicochemical procedures except that of Hamann (1983). This bioassay procedure is capable of quantitating levels of 10 ppb, but cannot distinguish between OTC and TC residues as the HPLC method does.

The accuracy of the method was studied by spiking milk samples at four fortification levels with standard OTC and TC and analyzing four replicates. Least-squares and regression analysis of the data presented in Tables III and IV showed that the relationship between "added" and "found" was adequately described, for both compounds, by a linear regression ($y = -0.13 + 0.727x$, $r = 0.9996$ for OTC; $y = 0.82 + 0.851x$, $r = 0.9995$ for TC). Therefore, the slopes (0.7271 ± 0.0122 for OTC; 0.8508 ± 0.0128 for TC) of these regression lines could be used as estimates of overall recovery for OTC (72.7 ± 1.2) and TC (85.1 ± 1.3) determination in milk.

Table III. Recovery Data for OTC Analysis in Milk

concn of OTC added, ppb	mean concn found,[a] ppb	mean rec, %
23.7	17.4 ± 0.6	73.2
47.5	33.8 ± 0.8	71.3
95.0	69.3 ± 1.4	72.9
190.0	137.9 ± 2.4	72.6

[a] Mean of four replicates ± SD.

Table IV. Recovery Data for TC Analysis in Milk

concn of TC added, ppb	mean concn found,[a] ppb	mean rec, %
26.5	23.5 ± 0.8	88.8
53.0	45.1 ± 1.6	85.1
106.0	91.9 ± 1.1	86.7
212.0	180.8 ± 3.9	85.3

[a] Mean of four replicates ± SD.

Table V. Precision Data for the Determination of OTC in Milk Samples Spiked with 23.7 ppb

day	concn of OTC found, ppb	mean value, ppb	SD	rel SD, %
1	17.8, 17.1, 17.4, 16.8, 16.5, 16.5	17.0	0.5	3.0
2	17.4, 17.4, 18.1, 16.7	17.4	0.6	3.3
3	16.5, 15.6, 15.8, 17.4, 15.7, 18.5	16.6	1.1	7.0

Variance Estimates	
source	rel SD, %
within day	4.9
among days	0.9
overall	5.0

Table VI. Precision Data for the Determination of TC in Milk Samples Spiked with 26.5 ppb

day	concn of OTC found, ppb	mean value, ppb	SD	rel SD, %
1	22.6, 22.0, 23.4, 23.0, 21.4, 22.6	22.5	0.7	3.2
2	24.0, 22.6, 24.4, 23.2	23.5	0.8	3.4
3	22.0, 22.2, 21.4, 23.8, 22.0, 21.8	22.2	0.8	3.7

Variance Estimates	
source	rel SD, %
within day	3.4
among days	2.5
overall	4.2

The precision of the proposed method was studied by assaying, on each of three different days, several milk samples spiked with OTC and TC at the 23.7 and 26.5 ppb levels, respectively. To estimate the components of variance, the concentrations found (Tables V and VI) were subjected to "analysis of variance and expected mean squares for the one way classification-unbalanced design" (Wernimont, 1987). The analysis of variance showed that the within-day and among-days precision, expressed as relative standard deviation (percent), were 4.9 and 0.9 for OTC and 3.4 and 2.5 for TC, respectively (Tables V and VI). It also suggested that the overall precision, which is in fact the overall uncertainty of a single determination, was 5.0% for OTC and 4.2% for TC.

Since other antibiotics or drugs might interfere with the analysis, an interference test was evaluated. Several

Table VII. Residues of OTC in Milk of a Dairy Cow Administered Intramuscularly an OTC Formulation

hours after injection[a]	concn of OTC[b] in milk, ppb	hours after injection[a]	concn of OTC[b] in milk, ppb
24	370.6	84	17.7
36	204.9	96	10.4
48	75.8	108	–[c]
60	38.4	120	–
72	20.1		

[a] Single injection of 5 mg of OTC/kg of body weight. [b] Values corrected for recovery. [c] Not detected.

compounds that are, frequently, added in feeds and/or used for treatment of mastitis, such as penicillin G, penicillin V, cloxacillin, furazolidone, nitrofurazone, oleandomycin, chloramphenicol, ampicillin, sulfathiazole, neomycin, erythromycin, streptomycin, chlortetracycline, and oxacillin, were dissolved in water at 1 ppm level and submitted to HPLC chromatography. It was found that none of the compounds tested interfered with the elution time of OTC and TC.

Characterization of the recorded peaks was based, solely, on the retention behavior of OTC and TC. Further characterization of the eluted peaks could be made possible by collecting column effluents (ca. 1.8 mL) corresponding to both peaks, adding 1.5 mL of saturated ammonium sulfate solution, and extracting the resulting mixture with 2 mL of acetonitrile. Following acetonitrile evaporation, TCs could be redissolved in ca. 30 μL of methanol and confirmed by using thin-layer chromatographic methods (Ryan and Dupont, 1974; Oka et al., 1984).

To validate the method with real samples, a trial was undertaken to quantitate residues in milk of a dairy cow administered intramuscularly a single dose of approximately 5 mg of OTC/kg of body weight. Drug formulation [10% OTC-HCl in poly(vinylpyrrolidone), Alfasan, Holland] was injected in the neck region at a point one hand's width before the shoulder and one hand's width below the ligamentum nuchae. The control milk sample, which was taken before the injection, and all other samples taken during the trial at 12-h intervals were stored at −25 °C until analyzed. The analysis data, presented in Table VII, showed that OTC persisted in milk for as long as 4 days after injection. These results cannot be considered inconsistent with those of a comparable experiment (Nouws et al., 1985) using bioassay, although the OTC concentrations that had been reported were relatively high and detectable residues could not be found in milk for more than 41 h. This inconsistency should be attributed to the decidedly better specificity and sensitivity of the proposed HPLC method. The results of the trial are, however, in direct contrast to those of another experiment using bioassay after a purification on amberlite column step (Hamann, 1983). The author reported that a single intramuscular injection of 1500 mg of OTC in dairy cows resulted in contamination of the produced milk by residues that persisted for more than 13 days after treatment. Since the sensitivity of this bioassay method is not better than that of the HPLC method, there is no obvious explanation for this inconsistency.

In conclusion, the results of the present study show that the proposed HPLC method is an efficient and reliable means of quantitating OTC and TC residues in milk. Although it involves a multistep extraction scheme, one analyst familiar with the method can easily process eight samples in 2 h. Therefore, the method should be useful for routine identification and confirmation of residues presumptively identified as TCs by other tests. The method should be, also, suitable to serve as a directly screening very sensitive test for OTC and TC contamination in milk, due to the increased sensitivity and specificity.

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