Rapid HPLC Determination of Tetracycline Antibiotics in Milk

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A previously described method using on-line concentration and gradient elution was modified for more rapid isocratic analysis. Milk (5 mL) was extracted/deproteinized with 1 mL of 1 N HCl and 15 mL of acetonitrile. The resulting filtrate (12 mL) was evaporated directly, or the water layer resulting from addition of hexane and methylene chloride was evaporated. The extract could be concentrated to about 1 mL without significant degradation of tetracyclines. The concentrates were filtered. For analysis, a Polymer Laboratories PLRP-S column was used with a mobile phase of 0.02 M H_3PO_4 and 0.01 M sodium decanesulfonate–acetonitrile, 72 + 28 for oxytetracycline and tetracycline and 68 + 32 for chlortetracycline. The injection volume was 200 μL with UV detection at 380 nm. Average recoveries were greater than 80% with detection limits of 2–4 ppb.

Keywords: *Oxytetracycline; tetracycline; chlortetracycline; milk; HPLC determination*

INTRODUCTION

Some past surveys of the commercial milk supply in the United States indicated widespread contamination with low levels of the tetracycline group of antibiotics (Brady and Katz, 1988; Collins-Thompson et al., 1988). These results were based on a then newly introduced screening test, the Charm II receptor assay, which was substantially more sensitive than tests used previously for the tetracycline group of antibiotics. At the time, no HPLC confirmatory tests of comparable sensitivity were available, so it was not possible to confirm whether or not the suspected residues were real or an artifact of the testing procedure. Since that time, several HPLC methods capable of detecting residues at levels of ≤10 ppb have been reported (Carson, 1993; Fletouris et al., 1990; Farrington et al., 1991; White et al., 1993a). Procedures using cleanup methods gave markedly reduced recoveries of tetracyclines (Carson, 1993; Fletouris et al., 1990; Farrington et al., 1991). White et al. (1993a) found that tetracyclines could be effectively separated from interferences in milk extracts by adding an alkyl sulfonate (sodium decanesulfonate) as an ion pair. This eliminated the need for cleanup. White et al. (1993a) used direct on-line concentration of the water layer formed by adding organic solvents to the acetonitrile filtrates, thus eliminating the need for evaporative concentration. The tetracyclines were then eluted with an acetonitrile gradient for analysis. Gradient elution is, however, a lengthy procedure which has both advantages and disadvantages as compared with isocratic analysis.

The present study was undertaken to determine if the milk extracts prepared according to the method of White et al. (1993a) could be concentrated by evaporation and analyzed by a simpler and faster isocratic procedure.

MATERIALS AND METHODS

Chemicals. Acetonitrile, hexane, and methylene chloride were of HPLC grade (EM Omnisolv or equivalent, EM Industries, Gibbstown, NJ). Other chemicals were of reagent grade. Sodium decanesulfonate (99%) was purchased from Aldrich Chemical Co., Milwaukee, WI. Oxytetracycline (OTC), tetracycline (TET), and chlortetracycline (CTC) were purchased from Sigma Chemical Co., St. Louis, MO. Tertiary butyl alcohol (Mallinckrodt, AR; St. Louis, MO) was diluted with $^1/_{10}$ volume of acetonitrile to prevent crystallization. The tetracycline standards were prepared with corrections for purity by dissolving 1 mg/mL in 0.01 N HCl. Dilutions of 100, 10, and 1 μg/mL were prepared in 0.01 N HCl. The standards were stable for at least a month when refrigerated.

Equipment. A Buchler (Ft. Lee, NJ) vortex evaporator was used to concentrate samples under reduced pressure in conical centrifuge tubes. For evaporation in side-arm flasks, a shallow tray (5 × 25 × 50 cm) was placed on a thermostated hot plate. Glassware required included 50 mL graduated cylinders, 250 mL glass-stoppered side-arm flasks, plastic-coated lead rings (to weight flasks), 15 mL conical graduated centrifuge tubes (calibrated to 1 mL), 250 mL separatory funnels with Teflon stopcocks, 125 mL conical flasks, and 50 mm narrow short-stem funnels. All glassware was cleaned in special detergent (Micro, International Products, Trenton, NJ) and rinsed in ca. 0.01 M HCl or H_2SO_4 and deionized water.

HPLC Apparatus. The equipment used consisted of a Varian (Sugarland, TX) 9010 pump, a Varian 9090 autosampler, a Waters (Milford, MA) 481 UV–visible detector, and a Varian Model 654 data system. A Polymer Laboratories PLRP-S column, 4.6 × 150 mm, 100 Å pore size, 5 μm particle size, with matching guard cartridge was used. After use, the column was flushed with water and 60:40 acetonitrile–water for storage.

Sample Preparation. Five milliliters of milk was measured into a 125 mL conical flask, and 1 mL of 1 N HCl and 15 mL of acetonitrile were added; the flask was swirled after each addition to mix the contents. The final volume was 20 instead of 21 mL, because of decreased volume when water and acetonitrile are mixed . After 5 min, the supernatant liquid was decanted through a plug of glass wool in the stem of a funnel, and 12 mL of filtrate was collected and concentrated by one of the following procedures.

Direct Evaporation. The filtrate was transferred to a 250 mL glass-stoppered side-arm flask. The flask was weighted with a plastic-coated lead ring and placed in a shallow (2 cm) water bath heated to 40–50 °C on a hot plate. This was attached to a water aspirator and evaporated under reduced pressure to 2–4 mL. The liquid in the flask boiled briefly and then evaporated quietly. The contents of the flask were transferred to a graduated centrifuge tube calibrated to 1 mL with several small water rinses (0.5 mL). The contents were evaporated to 1 mL or slightly less (but not to dryness) on the vortex evaporator under reduced (water pump) pressure. The

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volume was adjusted to 1 mL with water, and the concentrate was filtered through a 13 or 25 mm, 0.45 μm, PVDF filter cartridge into autosampler vials.

Separatory Funnel–Evaporation. The filtrate (12 mL) was transferred to a separatory funnel, and 10 mL of hexane and 10 mL of methylene chloride were added. The water layer that formed was collected in a graduated conical tube calibrated to 1 mL. The organic layer in the separatory funnel was washed with 0.5 mL of water, which was combined with the first water fraction. About 0.5 mL of *tert*-butyl alcohol was added to prevent foaming or bumping, and the mixture was evaporated to 1 mL or slightly less in the vortex evaporator. The volume was adjusted to 1 mL and filtered through a disposable filter cartridge as in the direct evaporation procedure.

Analysis. The mobile phase was 0.02 M H_3PO_4 and 0.01 M sodium decanesulfonate–acetonitrile (70 + 30). For specific compounds, a ratio of 72 + 28 was used for oxytetracycline and tetracycline and 68 + 32 for chlortetracycline. The flow rate was 1 mL/min with detection by UV at 380 nm. The injection volume was 200 μL, which is equivalent to 0.6 mL of original milk. Recoveries were based on peak areas, which were more consistent than peak heights.

Recovery Experiments. Five milliliters of milk was spiked with 50 μL of the 100, 10, and 1 μg/mL dilutions to give 1, 0.1, and 0.01 ppm. Recoveries were determined by comparison with 5 mL portions of 0.01 N HCl spiked in the same manner and injected directly for HPLC analysis.

RESULTS AND DISCUSSION

With on-line concentration as described by White et al. (1993a), any volume of sample extract can be loaded on the column. The analytes can then be eluted with a solvent gradient for analysis. Gradient elution has the advantage that both strongly and weakly retained compounds can be eluted in the same procedure. It also has the advantage that peak shapes of late eluting compounds remain as sharp as those of compounds eluting earlier. However, the time required (about 50 min) was a disadvantage, particularly for analysis of multiple samples. The gradient tended to generate system peaks, giving a somewhat uneven base line.

For isocratic analysis, the maximum volume that could be loaded without excessive spreading of peaks was about 200 μL. It was therefore necessary to concentrate the sample extract to get the same amount of analyte on the column. Preliminary results showed that the tetracyclines were stable in milk extracts during evaporative concentration unless the extracts were taken to dryness. Two methods of evaporative concentration were used. In the first method, the acetonitrile filtrates were evaporated directly. The initial volume (12 mL) was too large to evaporate directly in conical tubes. Evaporation in side-arm flasks under reduced pressure was found to work as well as procedures requiring more expensive equipment. It required little bench space. The evaporation was completed in conical calibrated centrifuge tubes in the vortex evaporator. Some lipid material that coextracted precipitated when the acetonitrile was removed. This was readily removed by filtration through disposable filter cartridges. This procedure had the advantage that the total organic solvent required was only 15 mL.

Evaporation under a stream of air or nitrogen took much longer and required more heating of the sample. This procedure is not recommended.

In the second procedure, hexane and methylene chloride were added to the acetonitrile filtrate as described by White et al. (1993a). The water layer was then evaporated in a tube to 1 mL using the vortex evaporator. This was somewhat faster but required more organic solvent.

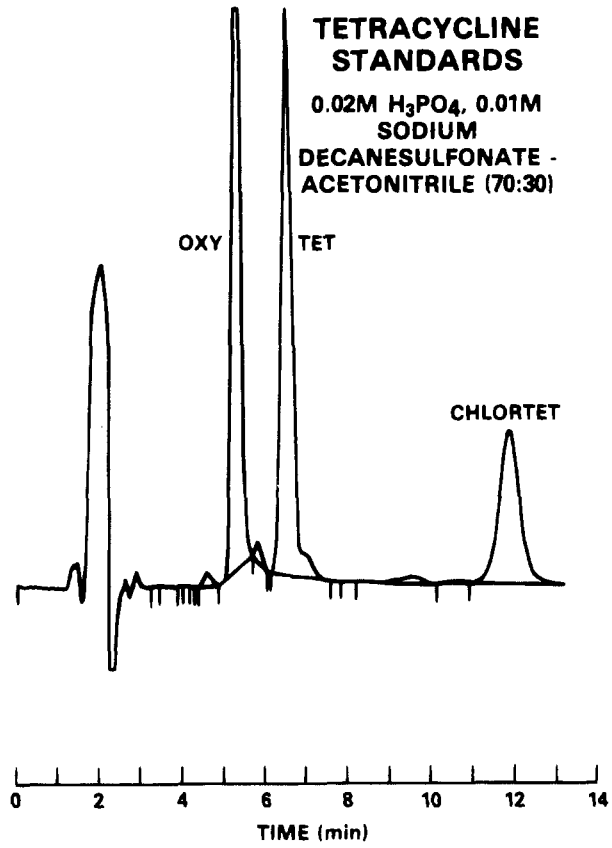

Figure 1. Isocratic analysis of tetracycline standards (1 μg/mL): Polymer Laboratories PLRP-S column; flow, 1 mL/min; detection, UV 380 nm; 200 μL injected.

For analysis, a Polymer Laboratories PLRP-S column was used. The advantages of this type of polymeric packing for chromatography of tetracyclines as compared with silica-based columns have been discussed by White et al. (1993b). However, nominally similar polymeric columns from other manufacturers were markedly less satisfactory. The mobile phase used was 0.02 M H_3PO_4 and 0.01 M sodium decanesulfonate–acetonitrile.

Separation of tetracycline standards using a ratio of 70 + 30 buffer–acetonitrile is shown in Figure 1. Under these conditions, oxytetracycline eluted rather close to interferences in sample extracts (Figure 2). This may interfere with accurate quantitation. When the acetonitrile concentration was slightly reduced to 28%, separation of OTC and TET from interferences and each other was improved (Figure 3). However, retention of CTC was correspondingly increased (not shown). For determination of CTC only, a buffer–acetonitrile ratio of 68 + 32 was satisfactory. Other tetracyclines could probably be determined according to this procedure. Minocycline eluted between TET and CTC. Doxycycline eluted after CTC.

The use of oxalic acid in place of phosphoric acid was also investigated. However, this mobile phase did not separate oxytetracycline from interferences.

The recoveries by both evaporation procedures were comparable and were generally satisfactory (Tables 1 and 2). Recoveries were generally greater than 90%, except for the lower level spikings with tetracycline when the separatory funnel procedure was used. The recoveries are markedly better than reported by other investigators using more elaborate cleanup procedures.

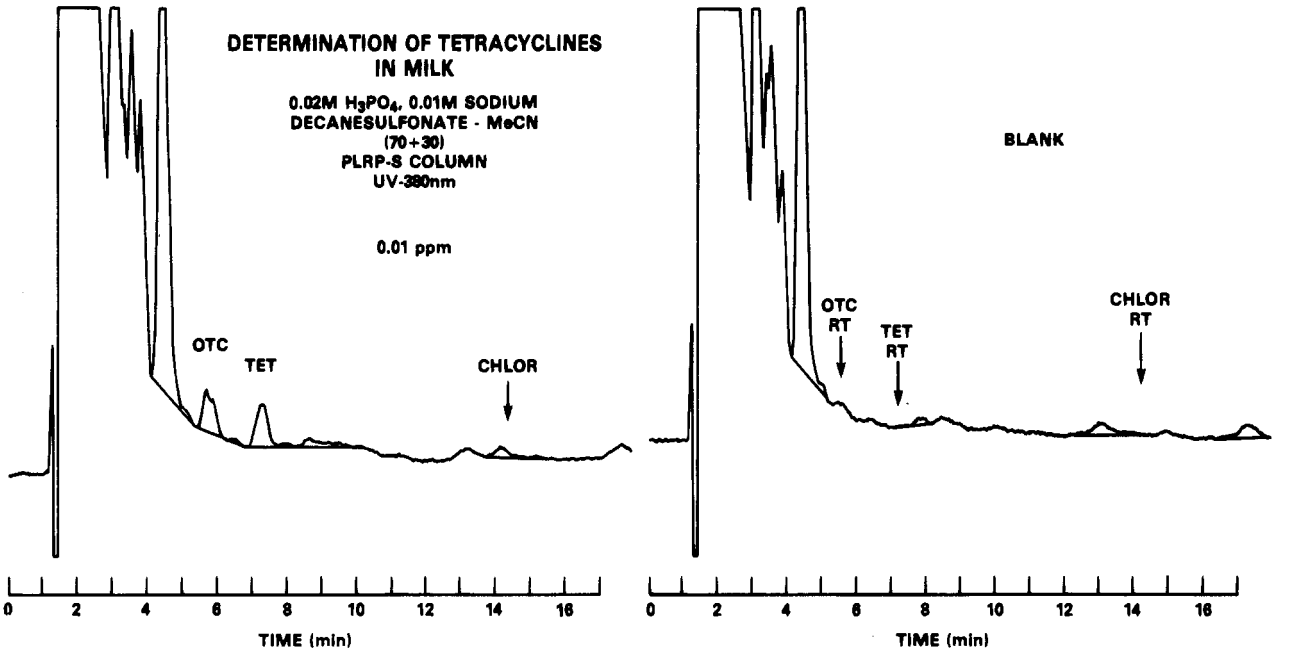

Figure 2. Isocratic analysis of milk for tetracyclines. Conditions were as in Figure 1.

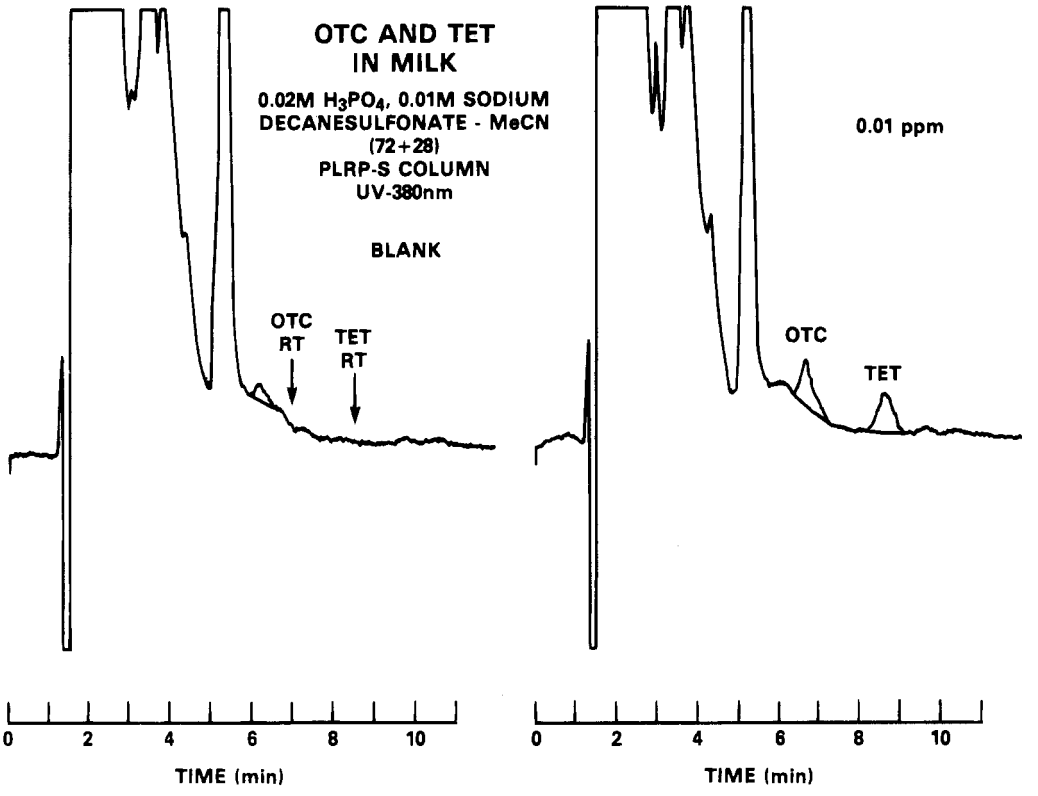

Figure 3. Isocratic analysis of milk for oxytetracycline and tetracycline using a decreased acetonitrile concentration (72 + 28).

The sensitivity of the method depended on the detector used. The best UV–visible detectors were more sensitive than the best diode array detectors. With the system used, 1 ng of tetracycline and oxytetracycline and 2 ng of chlortetracycline on the column gave a measurable peak well above background. Since the equivalent of 0.6 mL of milk was injected, this corresponds to about 2 and 4 ppb, respectively, in milk. It was possible to further increase sensitivity by doubling the sample size used in the direct evaporation procedure. With premixed mobile phase, detector noise was slight. Separation from interferences in milk extracts

Table 1. Recovery of Tetracyclines Added to Milk by Separatory Funnel–Evaporation Procedure

antibiotic	amt added (ppm)	% recovery determination 1	2	3	4	mean ± SD
oxytetracycline	1	100	101	102		101 ± 1
	0.1	90	89	105	105	97 ± 8
	0.01	95	93	101	105	99 ± 5
tetracycline	1	88	96	95		93 ± 3
	0.05	78	83	81		81 ± 2
	0.01	77	87	86		83 ± 4
chlortetracycline	1	97	84	97		93 ± 6
	0.1	102	105	101		103 ± 2
	0.01	126	91	83		100 ± 19

Table 2. Recovery of Tetracyclines Added to Milk by Direct Evaporation of Filtrate

antibiotic	amt added (ppm)	% recovery determination 1	2	3	4	mean ± SD
oxytetracycline	1	102	114	106	95	104 ± 7
	0.1	100	95	127a	113	103 ± 8
	0.01	116	94	110	105	106 ± 8
tetracycline	1	108	89	105		101 ± 8
	0.1	88	83	94	103	92 ± 7
	0.01	87	57a		87	
chlortetracycline	1	82	89	85		85 ± 3
	0.1	102	88	92		94 ± 6
	0.01	55a	107	112		110 ± 3

[a] Not included in data analysis.

was excellent, as shown in Figures 2 and 3. The base line was completely flat in the area where tetracyclines eluted.

There are some published reports in which luminescent (Duggan, 1991) and/or fluorescent detection (Haagsma and Scherpinesse, 1993) was used. These alternative methods may be useful in enhancing sensitivity and specificity and confirming the identity of suspect peaks.

The absence of a chromatographic peak of the proper retention time provides unequivocal evidence that a compound is absent at or above the detection limits of the method. However, suspect peaks may require further confirmation. It is worth noting that, of a number of cartons of milk purchased at a local convenience store for use as controls, none contained detectable tetracyclines. Carson (1993) made a similar observation.

In summary, tetracyclines can be extracted from milk with a rapid acid–acetonitrile procedure. By using ion pair liquid chromatography, residues can be effectively separated from interferences without further cleanup. Three alternative approaches to sample preparation for analysis are described: on-line concentration as described by White et al. (1993a) and two evaporative concentration methods. All give excellent recoveries and sensitivities for three tetracyclines. The methods are sufficiently rapid to permit processing of a substantial number of samples in a day.

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