An organic solvent-free and quick determining method for routine residue monitoring of tetracycline and 4-epi-tetracycline in cow's milk

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Abstract

Under organic solvent-free conditions, a quick, easy, small-scale sample preparation followed by an isocratic mobile phase HPLC for quantifying tetracycline (TC) and its 4-epimer, 4-epi-tetracycline (4eTC), in cow's milk was described in this paper. The sample preparation could be made only homogenization using a handheld ultrasonic-homogenizer with deproteining aqueous solution followed by filtration with disposable unit. For determination and identification of analyte, an Inertsil WP300 C4 and isocratic 100% aqueous mobile with photodiode array detector was used. The method validation data were well within the international analytical method acceptance criteria. The total time required for the analysis of one sample was less than 7 min. In all the process, no organic solvents were used. The present method may be proposed as an international harmonized analytical method for routine residue monitoring of TC and 4eTC in cow's milk.

Keywords: International Harmonized Analytical Method, Organic Solvent-Free, HPLC, Residue Monitoring, Tetracycline, 4-Epi-Tetracycline

Introduction

Tetracycline (TC) is a broad-spectrum antibiotic widely used in veterinary medicine for cost-effective prophylactic and therapeutic treatment in food-producing animals and also as growth-promoting substances in cattle. The possibility of the drug residues in foods derived from treated animals is a key issue for food safety which arouses great public concern. To prevent any health problem, the European Community (EC) set maximum residue limits (MRLs) in animal-derived foods for the sum of TC and its 4-epimer (4-epi-tetracycline, 4eTC (European Commission, 2009), which is micro biologically active, probably by re-conversion to the respective TC: residue analysis lacking consideration of the epimer fail to lack to measure the true TC concentration in the animal tissues (European Commission, 2000).

Cow's Milk is very important food because it is nutritious (well balanced), inexpensive, and readily available: it is the most familiar of animal products and is consumed at many family dining tables. Since consumers have no way of knowing what TC may be present in milk, they rely on the food inspection authorities to assure milk safety. The EU have established the MRL in milk of 0.1 µg/mL (European Commission, 2009). The strict residue monitoring of TC and 4eTC in cow's milk is therefore an important job to guarantee food safety, and a validated analytical method for the simultaneous determining TC and 4eTC is presently required.

In current international trading, as foods are produced and distributed throughout the world, food safety have become increasing concerns for consumers. To protect the health of consumers, there is a requirement for more diligent monitoring of foods for regulators, vendors and producers. Under these circumstances, the development of international harmonized methods to determine chemical residues in foods is essential to guarantee equitable international trade in these foods.

Whether in industrial nations or developing countries, an international harmonized analytical method (or world standard analytical method) for residue monitoring in foods is urgently–needed. The acceptable harmonized method must be easy and rapid, be economical in time and cost, cause negligible harm to the environment and analysts ("no uses organic solvents"), and be applicable to routine work at municipal health centers and health laboratories in major food trade countries.

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Several methods have been described in the literature for determining TC and 4eTC in in animal-derived foods, including milk (Cherlet et al., 2003; Gajda and Posyniak, 2015; Reddy et al., 2017; De Ruyck and De Ridder, 2007; Singh et al., 2015). However, these methods have following three crucial drawbacks:

1) Their sample preparation operations are complicated and labor intensive, which are time-and cost-consuming, do not permit the determination of large number of samples, and can give low reproducibility;

2) They consume large quantities of poisonous organic solvents, acetonitrile or methanol etc., as extraction solvents, purification eluents, and LC mobile phases. The solvents are toxic to humans and to the environment. Their disposal is costly and must be performed with ecological responsibility. Because discharging organic solvents is a serious problem, analytical methods for residue monitoring should avoid their use (Anastas and Warner, 1998; Iahibashi, 1996; Malish et al., 1992; Ogawa, 1996).

3) They are based on LC-MS or -MS/MS. LC-MS/MS systems are mainly available in a part of industrial nations because these are hugely expensive, and the methodologies use complex and specific. These systems are unavailable for routine work in a lot of laboratories, including small local analysis facilities, especially in developing countries. No optimal method that satisfies the aforementioned requirements has yet been identified.

As an optimal technique that can be recommended as an international harmonized analytical method for the routine residue monitoring of TC and 4eTC in cow's milk, this paper describes a quick, easy, small-scale sample preparation followed by an isocratic 100 % aqueous mobile phase HPLC system to determine the both compounds simultaneously.

Materials and Methods

Reagents and apparatuses

Tetracycline (TC) and 4-epi-tetracycline (4eTC) standards, distilled water (for HPLC), and trichloroacetic acid (TCA, 100%, w/v, for biochemistry) were purchased from FUJIFILM Wako Pure Chem. Corp. (Osaka, Japan). As an extraction/deproteinization solution, 10 % (v/v) TCA solution (diluted with distilled water) was used. 1-octanesulfonic acid sodium (OSA) and tetra-n-butylammonium phosphate (TBP) used as ion-pairing regents for HPLC mobile phase were from GL Science Inc. (Tokyo, Japan).

Stock standard solutions of TC and 4eTC were prepared by dissolving each compound in water followed by water to a concentration of 100 µg/mL. Each solution was stored at -20℃. Working mixed standard solutions of these two compounds were freshly prepared by suitably diluting the stock solutions with water on the day of the analysis.

The following apparatuses were used in the sample preparation: handheld ultrasonic-homogenizer (model HOM-100, 2 mm ID probe, Iwaki Glass Co., Ltd., Funabashi, Japan); a disposable syringe filter unit (DISMIC-25cs, 0.45 µm hydrophilic cellulose acetate membrane) (Advantec Toyo (Tokyo, Japan).

The HPLC system employed was: a model PU-980 pump equipped with a model DG-980-50-degasser (Jasco Corp., Tokyo, Japan), a model CO-810 column oven (Thosoh Corp., Tokyo, Japan) and a model MD-4017 photodiode-array detector (PAD) connected with a model LC-Net Ⅱ/AD interface box (Jasco). An Inertsil® WP300 C4 (5 µm, 4.6 × 150 mm) column (GL Sciences Inc., Tokyo, Japan) was used for the separation.

HPLC operating conditions

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The analytical column was an Inertsil WP300 C4 column using an isocratic 5 mM TBP mobile phase at a flow rate of 1.0 mL/min at 55 ℃. PAD was operated at 200 – 400 nm: the monitoring wavelengths were adjusted to 360 and 368 nm which represent maximums for TC and 4eTC, respectively. The injection volumes were 10 – 20 µL.

Preparation of Calibration Standards and Quality Control Samples

For method validation studies, calibration standards and quality control samples (QCs), terms defined in the FDA guideline (FDA/CDER, 1994), were prepared by spiking appropriate aliquots of the mixed standard solution in blank milk samples. Calibration standards were used to construct calibration curves from which the concentrations of analytes in unknown monitoring samples are determined practically. QCs used to evaluate the validation performances of the proposed method. In this study, the standards were prepared in the range of 0.05 – 1.0 µg/mL for both analytes. Three QC levels (For both analytes, QC1 = 0.1 µg/mL; QC2= 0.2 µg/mL; QC3 = 0.5 µg/mL) were prepared.

Sample preparation

An accurate 100 µL milk sample was taken into a 1.5 mL micro-centrifuge tube and homogenized with 500 µL of 10 % (v/v) TCA solution with a handheld ultrasonic-homogenizer for 30 s. After being homogenized, the mixture was filtered through a 0.45 disposal syringe filter unit. The filtrate was injected into the HPLC system.

Method validation

The performance of the developed method was validated in terms of many parameters from the Codex and FDA international guidelines for bio-analytical procedure (Codex alimentarius, 2001 and 2009; FDA/CDER, 1994).

Results and Discussion

Sample preparation and HPLC operating conditions

The procedure described in this paper is a very easy, quick, and small-scale technique that enabled effective extraction of TC and 4eTC from milk sample by the ultrasonic homogenization with 10 % TCA solution. In fact, the extraction/deproteinization provided fine recoveries of the target compounds and the prompt coagulation of protein in the milk.

To optimize the separation with an isocratic 100% aqueous mobile phase, i.e., a poisonous organic solvent-free mobile phase, and a more rapid separation, the author tested an Inertsil WP300 C4 column which has a low carbon content (3%) and weak retention capacity. This study used a 100% water and two ion-pair reagents, OSA for basic analytes and TBP for acidic analytes, because TC has three pK_a (3.3, 7.7 and 9.5) (Sigma-Aldrich, 2003) in the molecule, as the isocratic aqueous mobile phases and employed the operation conditions: mobile phases with a 100 % water, 1 – 20 mM OSA and 1 – 20 mM TBP, respectively; column temperatures ≥ 25 ℃; flow rate ≥ 0.5 mL/min; HPLC run times ≤ 10 min. As the HPLC separations were performed serially, the time/run was critical for routine residue monitoring. The short run time not only increased sample throughout for analysis but also affected the method-development time.

It was difficult to separate TC and 4eTC with an isocratic water and OSA mobile phases within the condition ranges examined in this test. An optimal chromatogram with the complete separation of TC and 4eTC, their sharp peaks, and their short retention times was obtained using the above Inertsil WP300 C4 (5 µm, 4.6 × 150 mm) column and a mobile phase of 5 mM TBP at column temperature of 55℃ and flow-rate of 1.0 mL/min.

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Fig. 1 illustrate that the resulting chromatograms were free of interfering compounds for the quantification and identification of TC and 4eTC by the HPLC with the PAD set at 364 nm, giving the maximum absorbance for TC and 4eTC. The present HPLC system accomplished good separation with the need for a gradient system to improve the separation and pre-column washing after analysis. This figure demonstrates that the present method can provide the quantification and identification of the analytes.

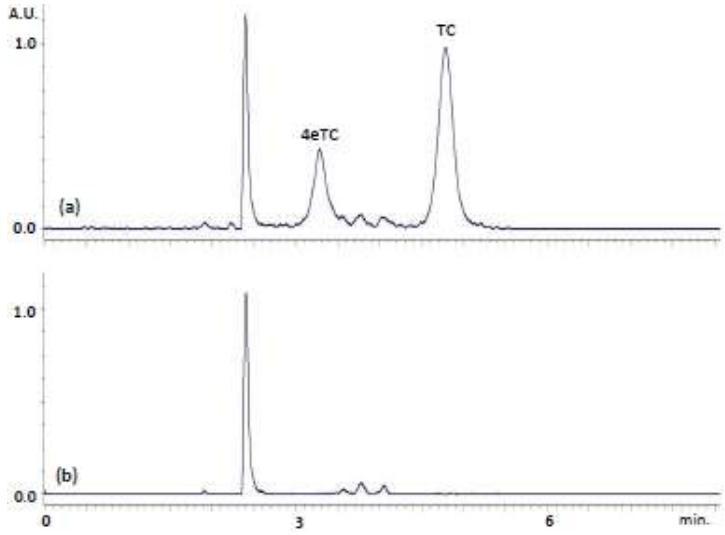

Figure 1. Chromatograms obtained from a spiked milk sample (0.5 µg/mL for TC and 4eTC, respectively) (a) and a blank milk sample (b). PAD set at 364 nm. Peak's retention times: 4eTC = 3.3 min; TC = 4.8 min.

Main Method Validation

Table 1 summarizes the main validation data. All the parameters were sufficiently satisfy the Codex's accepted and/or FDA's recommended criteria (Codex alimentarius, 2001 and 2009; FDA/CDER, 1994). The quantitative limits for TC and 4eTC were 0.033 and 0.054 µg/mL, respectively. The findings demonstrates high accuracy and reproducibility. The other validation findings are as follows:

Table 1: Method validation data

Parameter		4eTC	TC	Codex acceptance criterion[a]	FDA recommended value[b]
Linearity (r)[c]		0.9994	0.9992		≥ 0.999
Range (µg/mL)		0.05 - 1.0			
Accuracy[d] (%)		93.5	96.7	70 - 110	
Precision[e] (%)		3.3	2.1	≤ 20	
Sensitivity[f] (µg/mL)		0.054	0.033	0.1[g]	
System Suitability:					
a) Injection re-peatability[h](RSD, %)	retention time	0.13	0.25		≤ 1
	peak are	0.45	0.34		≤ 1
b) Resolution[i] (R_s)		3.01			> 2
c) Peak tailing factor (T)		1.2	1.3		≤ 2

[a] Codex's guidelines (Codex alimentarius, 2001).

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[b] Recommendations in FDA's guidelines (FDA/CDER, 1994)

[c] r is the correlation coefficient (P <0.01).

[d] Average recoveries from 18 replicates (= six replicates at three spiked levels: 0.1, 0.2, and 0.5 µg/mL for TC and 4eTC, respectively).

[e] Values are relative standard deviations (n= 18).

[f] Quantitative limit: the concentration of analyte giving a signal-to-noise ratio = 10.

[g] Maximum residue limit (µg/mL) for TC including 4eTC in milk (set by EU (European Commission, 2009)

[h] Data as the relative standard deviations calculated for 10 replicate HPLC injections of the prepared eluate for a spiked milk sample (0.5 µg/mL of 4eTC and TC, respectively).

[i] Rs is measure of how well two peaks are separated.

Selectivity and Specificity

The application of the proposed procedure to 10 blank milk samples demonstrated that no interference peak was presented around the retention times for TC and 4eTC in any of the sample examined. The present HPLC - PAD system easily confirmed the peak identity of target compound. The target analytes could be identified in a milk sample by their retention times and absorption spectra. As shown in Fig. 2, the 4eTC spectrum obtained from the spiked milk sample (Fig. 1-(a)) could be practically identified to that of the standard: similarity values (= correlation coefficients) obtained by the chromatogram data system for controlling the PAD were calculated as > 0.99 for TC and 4eTC. Because of the complete separations, PAD at trace levels is fully available. It is, therefore, instructive to demonstrate purification effectiveness of the sample preparation. The HPLC system did not require the use of MS or MS/MS, which is very expensive and is not widely available for routine analysis.

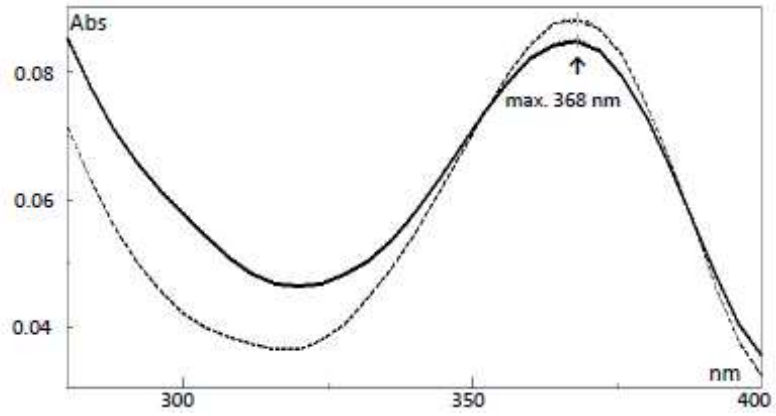

Figure 2. Absorption spectra for 4eTC peak in Figure 1-(a) (Solid line, max. 368 nm) and 4eTC standard (dashed line, max. 368 nm).

Cost and Time performances

The total time and budget required for the analysis of a single sample were less than 7 min and approximately 1.5 USD (= 1.3 EUR or 103 INR, as of December 13, 2019), respectively. The findings became a requirement required for the routine assay. The short time and low-cost quantitative method increased the sample throughput for actual routine monitoring work.

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Application to real milk samples

A total of 15 samples of commercial packed raw milk purchased from convenience stores in Osaka, Japan, were analyzed using the present method. No samples contained detectable concentrations of TC and 4eTC. All chromatograms were free from interferences.

Conclusions

The proposed HPLC-PAD procedure is useful for the routine residue monitoring of TC and 4eTC in cow's milk for the following reasons: 1) easy, direct HPLC analysis; 2) extremely short analysis time, total < 7 min/sample; 3) uses no organic solvents; 4) economical; 5) provides high reproducibility; 6) applicable to the local analysis facilities: in summary, the procedure may be recommended as an international harmonized analytical method for TC and 4eTC residue monitoring in real cow's milk.

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Conflicts of Interest

The author declares that he has no conflicts of interest.