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Validated quantitative analysis method for routine residue monitoring of oxytetracycline and its 4-epimer in milk: Pipetting sample preparation and isocratic mobile phase HPLC-diode array under organic solvent-free conditions

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Abstract
The author introduces an organic solvent-free, small-scale, time shortening, economical procedure for quantitation of oxytetracycline (OTC) and its 4-epimer, 4-epi-oxytetracycline (4eOTC), in cow's milk. Sample preparation was performed by homogenization using a handheld ultrasonic-homogenizer with water followed by Mono Tip® C18 pipette tip contains silica monolith bonded with octadecyl group with water eluent. The HPLC separation was achieved a C4 column with an isocratic aqueous mobile phase and a photodiode array detector (PDAD). The total time required for the analysis of one sample was less than 6 min. The method validation data were well within the international analytical method acceptance criteria. The limits of quantitation for OTC and 4eOTC were 0.035 and 0.048 µg/mL, respectively. The present technique may be proposed as an international standardized analytical method for routine residue monitoring for OTC and 4eOTC in milk.

Keywords: International standardized analytical method, pipette tip organic solvent-free, oxytetracycline, 4-epi-oxytetracycline, residue monitoring

Introduction
Oxytetracycline (OTC) is a broad-spectrum antibiotic widely used in veterinary medicine for cost-effective prophylactic and therapeutic treatment and also as growth-promoting substances in food-producing animals. 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 OTC and its 4-epimer (4-epi-oxytetracycline, 4eOTC) [1] (Fig.1), which is micro biologically active, probably by re-conversion to the respective OTC: residue analysis lacking consideration of the epimer fail to lack to measure the true OTC concentration in the animal tissues [2]. The determination of OTC and 4eOTC in the animal derived foods is therefore an important job to guarantee food safety, and a validated analytical method for the simultaneous determining OTC and 4eOTC is presently required.
In response to the recent expansion and multilateral food trade, the development of "international standardized analysis methods" to monitor chemical residues in foods that can be applied in all countries, without developed or developing, is essential to guarantee equitable international trade in these foods and ensure food safety for consumers. The recommended standardized analytical method for the routine monitoring of chemicals such as OTC in foods must be easy-to-use, economical in time and cost, no harm to the environment, and applicable to routine work at municipal health centers and health laboratories in major food trade countries.

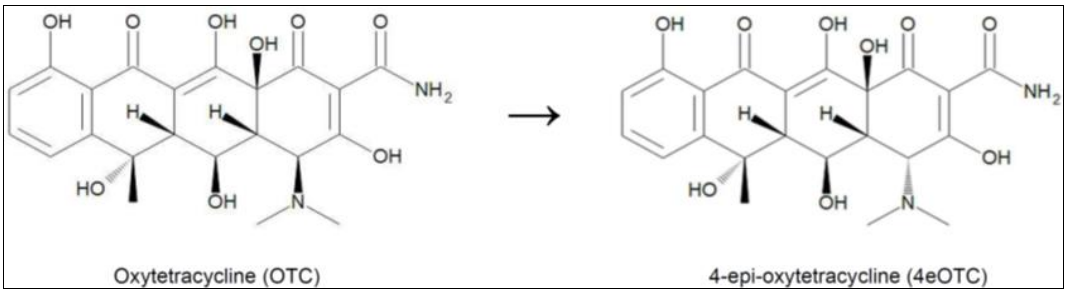

Fig 1: Chemical structures of OTC and its epimer, 4eOTC (both M.W. = 460.43 g/mol)

Although there have been significant developments in techniques based on HPLC for determination/identification of OTC and 4eOTC in foods of animal origin in recent years [3-6], these techniques have the following crucial drawbacks as the international standardized analytical method:

(i) They consume organic solvents in the HPLC and LC-MS/MS mobile phases as well as for extraction and deproteinization in sample preparation. The risk associated with these solvents extend beyond direct implications for the health of humans and wildlife to affect our environment and the ecosystem in which we all reside. Additionally, incineration for disposal of waste organic solvents has steadily increased, and huge amounts of cost have been spent. Eliminating the use of organic solvents is an important goal in terms of environmental conservation, human health and the economy [7];

(ii) They involve labor intensive pretreatment operations, resulting in time- and cost-consuming that do not permit the determination of large number of samples;

(iii) They are based on LC-MS/MS. LC-MS/MS systems [3-6] 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 in a lot of laboratories for routine analysis, particularly in developing countries. No adequate method that satisfies the aforementioned requirements has yet been identified.

In order to establish an international standardized analytical method for the residue monitoring of OTC and 4eOTC in milk, this paper describes a simple, time shortening and economical, organic solvent-free procedure to determine them simultaneously. Milk, which is the most familiar in animal-derived foods, is a very important and basic food because it is highly nutritious (well balanced), inexpensive, and readily available.

Materials and Methods

Reagents and apparatus

Oxytetracycline (OTC) and 4-epi-oxytetracycline (4eOTC) standards, and distilled water (for HPLC) were purchased from FUJIFILM Wako Pure Chem. Corp. (Osaka, Japan). Tetra-n-butylammonium phosphate (TBP) used as an ion-pairing regent for HPLC mobile phase were from GL Science Inc. (Tokyo, Japan). Stock standard solutions of OTC and 4eOTC were prepared by dissolving each compound in water followed by water to a concentration of 50 µg/mL. This solution was stored at -20℃. Working standard solutions of this compound 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); micro-centrifuge (Bio fuge® fresco, Kendo Lab. Products, Hanau, Germany); Mono Tip® C18 pipette tip (packed with silica monolith that consists of continuous through-pores and octadecyl bonding); sample throughput volume ≤ 200 µL; through-pore diameter of 10 - 20 µm; meso-pores of 20 nm; surface area of 200 m^2/g (GL Sciences, Inc., Tokyo, Japan). The HPLC system, controlled with *ChromNAV*® chromatography data system, included a model PU-4180 pump and DG-4580 degasser (Jasco Corp., Tokyo, Japan) equipped with a model CTO-10AS $_{VP}$ column oven (Shimadzu Scientific Instruments, Kyoto, Japan), as well as a model MD-4017 photodiode-array detector (PDAD) connected with a model LC-Net II/AD interface box (Jasco).

HPLC operating conditions

The analytical column was an Inertsil® WP300 C4 (5 µm, 4.6 × 150 mm) column (GL Sciences Inc., Tokyo, Japan) using an isocratic 7.5 mM TBP mobile phase at a flow rate of 1.0 mL/min at 50 ℃. PDAD was operated at 200 – 400 nm: the monitoring wavelengths were adjusted to 362 nm which represents an average maximum absorption spectrum for OTC and 4eOTC. The injection volumes were 10 – 20 µL.

Pipette tip operating procedure

After attaching a Mono Tip C18 pipette tip to a micro pipette, preconditioning of the tip was carried out by drawing and ejecting (to waste) 50 µL of distilled water to reduce background noise. A 50 µL aliquot of the sample was drew into the conditioned Mono Tip C18 tip, and ejected back into another sample tube. This series of IN and OUT operations was defined as one pipetting operation in this study.

Sample preparation

An accurate 50 µL milk sample was taken into a 1.5 mL micro-centrifuge tube and homogenized with 750 µL of water with a handheld ultrasonic-homogenizer for 20 s. The mixture was filtered through a 0.2-µm disposable syringe filter unit. A 50 µL aliquot of the filtrate was aspirated into the conditioned Mono Tip C18 pipette tip and dispensed back into the sample tube. The eluate was injected into the HPLC-PDAD system.

Method validation

The performance of the developed method was validated in terms of many parameters from the Codex and FDA international guidelines for the residue analytical techniques [8, 9].

Results and Discussion

Sample preparation

The advantage of the present procedure is that OTC and 4eOTC in milk are pretreated compactly, quickly and economically, without using organic solvents. The ultrasonic-homogenization enabled the satisfactory extraction of melamine from a milk sample of 0.05 mL with water of 0.75 mL without bumping. The extract from milk did not form an

emulsion that would hinder melamine recoveries. After being homogenized, the extract obtained was filtered through a disposable filter unit and purified by the monolithic pipetting tip, Mono Tip C18. The Mono Tip C18 treatment is not only used for deproteinization but can also be used for defatting. As a preliminary study, a 50 µL of a mixed standard solution (each compound 5 µ/mL in water) was applied to the Mono Tip C18 and the recoveries of OTC and 4eOTC from the pipette tip was examined. The Mono Tip C18 gave satisfactory recoveries (average > 97.3%) and repeat abilities (RSD < 2.3%) for OTC and 4eOTC. The extract obtained above was easily purified by the pipette tip, which was performed by one pipetting operation. The quick and easy procedure resulted in high recovery and reproducibility with great saving time and cost. The resulting extract was free from interference, as can be seen in HPLC traces of blank (Fig.2a) and spiked milk sample (Fig.2b). These findings demonstrate that the extraction and purification worked well.

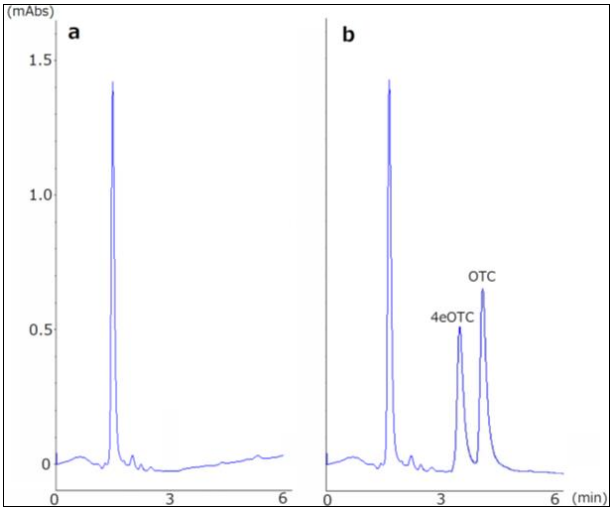

Fig. 2: Chromatograms obtained from a blank milk sample (a) and a spiked milk sample (each target 0.5 µg/mL). PDAD set at 362 nm. Peak's retention times: 4eOTC = 3.6 min; OTC = 4.2 min

HPLC conditions

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 an ion-pair reagent, TBP for acidic analytes, because OTC has three pKa (3.57, 7.49 and 9.44) [10] in the molecule, as the isocratic aqueous mobile phases and employed the operation conditions: mobile phases with 1 – 20 mM TBP; 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.

An optimal chromatogram with the complete separation of OTC and 4eOTC, their sharp peaks, and their short retention times was obtained using the above C4 column and a mobile phase of 7.5 mM TBP at column temperature of 50 ℃ and flow-rate of 1.0 mL/min.

Fig.2 illustrate that the resulting chromatograms were free of interfering compounds for the quantification and identification of 4eOTC and OTC by the HPLC with the PAD set at 362 nm, giving an average maximum absorption spectrum for 4eOTC and OTC. The present HPLC system achieved reproducible separation in < 4.5 min without the need for a gradient system with poor reproducibility and requiring time for conditioning during continuous analysis. This figure demonstrates that the present method can provide the quantification and identification of the analytes.

Table 1: Method validation data

Parameter		4eOTC	OTC	Criteria	
				Codex [a]	FDA [b]
Linearity (r)[c]		0.9992	0.9996		≥ 0.999
Range (µg/mL)		0.05 - 1.0	0.05 - 1.0		
Accuracy [d] (%)		92.9	94.1	70 - 110	
Precision [e] (%)		3.1	2.2	≤ 20	
Sensitivity [f] (µg/mL)		0.048	0.035	0.1[g]	
Injection repeatability [h] (%)	retention time	0.19	0.24		≤ 1
	peak are	0.42	0.33		≤ 1

[a] Codex's acceptable criteria [8].

[b] Recommendations in FDA's guidelines [9].

[c] r is the correlation coefficient (p<0.01) for the calibration curve of analyte in spiked milk sample.

[d] Average recoveries from 18 replicates (= six replicates at three spiked levels: 0.1, 0.2, and 0.5 µg/mL for OTC and 4eOTC, 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 OTC including 4eOTC in milk (set by EU [1]).

[h] = System suitability: data as the relative standard deviations (RSDs) calculated for 10 replicate HPLC injections of the prepared eluate for a spiked milk sample (0.5 µg/mL of OTC and 4eOTC, respectively).

Method validation

Table 1 summarizes the main method validation parameters. The system-suitability evaluation is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. The suitability was evaluated as the relative standard deviations of peak area and retention time calculated for 10 replicate HPLC injections of the prepared eluate for a spiked milk sample (0.5 µg/mL of OTC and 4eOTC, respectively). The values were estimated to be ≤ 0.24% for retention times and ≤ 0.42% for peak area, respectively. Including this system-suitability, the linearity, accuracy, and precision are within the international acceptance criteria (Table 1) [8, 9]. The limits of quantitation in milk samples were 0.035 µg/mL for OTC and 0.048 µg/mL for 4eOTC, respectively. The values are less than the EC's MRL (0.1 µg/mL for milk) [1]. In terms of selectivity, the present HPLC-PDAD system easily confirmed the peak identity of target compound. The analyte was identified in a milk sample by its retention time (Rt) and absorption spectrum. As can be seen from Fig.3, the OTC spectra obtained from the milk sample was practically identical to that of the standard: since the correlation coefficient (= 0.9944, calculated by the chromatography data system for controlling the PDAD) indicating the degree of similarity between the two absorption spectra was extremely high and the Rts are also the same (= 4.2 min), the OTC peak in Fig.2b could be identified as OTC. Similar results were obtained for 4eOTC. Because of the interference-free complete separations, PDAD at trace levels was fully available. The system did not require the use of MS/MS, which is very expensive and is not widely available for routine work.

Cost and time performances

The total time and budget required for the analysis of a single

sample were less than 6 min and approximately 6.4 USD (as of 26 November 2022), respectively. These findings became term required for the routine assay. The short time and low-cost quantitative method increased the sample throughput for actual routine monitoring work.

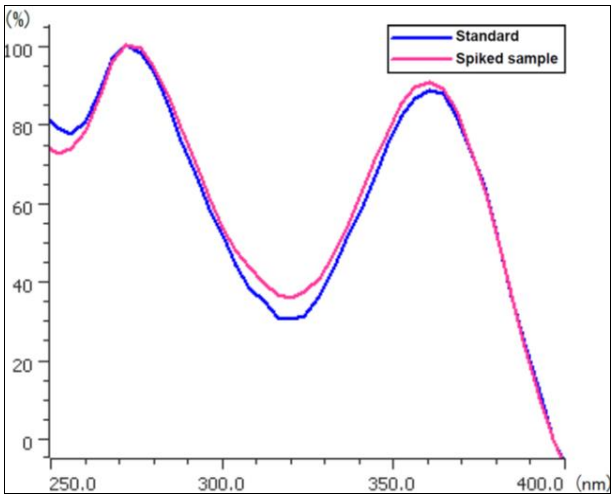

Fig. 3: Absorption spectra of peaks at 4.2 min for OTC in a chromatogram (Fig.2b).

Red line = standard; Blue line = spiked milk sample.
The correlation coefficient (= 0.9944) indicating the degree of similarity between the two absorption spectra

Conclusion

A pipetting sample preparation with water eluent followed by an isocratic aqueous mobile phase HPLC-PDAD system for quantitation of residual OTC and 4eOTC in milk without using organic solvent has been successfully established. The method validation data were well within the international method acceptance criteria. This procedure is organic solvent-free and easy operation only, resulted in high recovery and repeatability with considerable saving of analysis time/cost. In particular, the present technique may be proposed as an international standardized analytical method for routine residue monitoring of OTC and 4eOTC in milk.

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Author's Contribution

Not available

Conflict of Interest

Not available

Financial Support

Not available

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