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Validation/suitability of HPLC system with an isocratic 100% aqueous mobile phase for detecting tetracycline and its 4-epimer, 4-epi-tetracycline

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Abstract This paper introduces an organic solvent-free mobile phase HPLC system for simultaneous analysis of tetracycline (TC) and 4-epi-tetracycline (4eTC). The chromatographic separation was achieved an Inertsil® WP300 C4 column with an isocratic aqueous mobile phase and a photodiode array detector (PAD). The run-time was < 5 min/sample. The main validation performance parameters, linearity, range, and system suitability, were well within the international recommended criteria. The detection limits for TC and 4eTC were 0.005 and 0.014 μg/mL, respectively. The high repeatable, quick, and easy HPLC-PAD system may be further effective for the quantifying TC and 4eTC residues in animal-derived foods.

Keywords International harmonized analytical method; organic solvent-free; HPLC; tetracycline; 4-epi-tetracycline

1. Introduction

Tetracycline (TC) 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 TC and its 4-epimer (4-epi-tetracycline, 4eTC [1], 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 [2]. The determination of TC and 4eTC in the animal-derived foods 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 method for residue monitoring in foods is urgently–needed. The ideal harmonized method 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.

Several previously reported methods for detecting TC and 4eTC [3-8] have the following crucial drawbacks:

1) they consume large quantities of poisonous organic solvents, acetonitrile or methanol, in the mobile phases. 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. Eliminating the use of organic solvents is an important goal in terms of environmental conservation, human health and the economy [9];

2) 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 in a lot of laboratories for routine analysis, particularly in developing countries.

In order to establish an international harmonized method for the residue monitoring of TC and 4eTC, this paper describes an isocratic 100 % aqueous mobile phase HPLC conditions to detect the both compounds simultaneously.

2. Experimental

2.1. Reagents and equipment

Tetracycline (TC) and 4-epi-tetracycline (4eTC) standards and distilled water (HPLC grade) were purchased from FUJIFILM Wako Pure Chem. Corp. (Osaka, Japan). 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, Japa).

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).

The following twelve types of C1, TMS, or C4 non-polar sorbent (high-purity spherical silica-based) columns (5 μm, 4.6 × 150 mm) for HPLC separations were used:

Daisopak™ SP-200-5-C1-P (Osaka Soda Co., Ltd., Osaka, Japan); Developsil® TMS-UG-5 (Nomura Chemical Co., Ltd., Aichi, Japan); Hypersil™ SAS C1 (Thermo Fisher Scientific Inc., Waltham, MA, USA); Inertsil® TMS and Inertsil® WP300 C4 (GL Sciences Inc., Tokyo, Japan); Kaseisorb LC C1-300-5 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); Mightysil® RP-4GP (Kanto Chemical Co., Inc., Tokyo, Japan); SUPELCOSIL™ LC-1 (SUPELCO/ Sigma-Aldrich, Inc., Merck KGaA, Darmstadt, Germany); Spherisorb® C1 (Waters Corporation, MA, USA); Wakopak® Wakosil 5TMS and Wakopak® Wakosil 5C4-200 (FUJIFILM-Wako); ZORBAX TMS (Agilent Technologies Japan, Ltd., Tokyo, Japan). Table 1 lists the particle physical/chemical specifications.

Table 1: Physical/chemical specifications of the less-retentive reversed-phase columns used

Column[a]		Pore diameter (nm)	Surface area (m^2/g)	Carbon content (%)
Trade name	**bonded group**			
Daisopak™ SP-200-5-C1-P	methyl	20	200	3
Developsil® TMS-UG-5	methyl	14	300	4.5
Hypersil™ SAS C1	methyl	12	170	3
Inertsil®TMS	trimethylsilyl	10	450	3.5
Inertsil® WP300 C4	butyl	30	150	3
Kaseisorb LC-C1-300-5	trimethylsilyl	30	100	1
Mightysil® RP-4GP	butyl	12.5	350	4
SUPELCOSIL™ LC-1	methyl	12	170	2
Spherisob® C1	trimethylsilyl	8	220	2.2
Wakosil® 5TMS	trimethylsilyl	12	300	4
Wakosil® 5C4-200	butyl	20	200	5
ZORBAX TMS	trimethylsilyl	7	300	4

[a] All column sizes used are 5 μm, 4.6 × 150 mm.

2.2 Optimum operating HPLC conditions

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 (Fig. 1). The injection volumes were 10 – 20 µL.

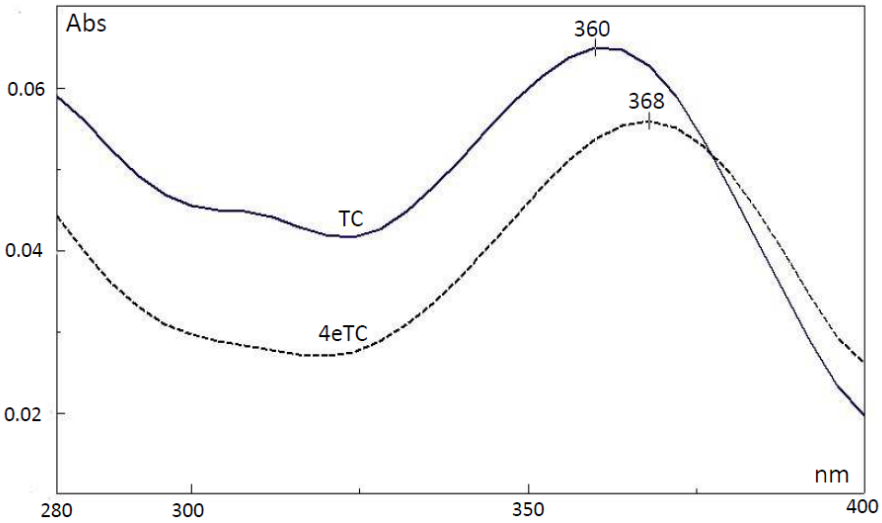

Figure 1: Typical absorption spectra of peaks for TC (solid line, max. 360 nm) and 4eTC (dashed line, max. 368 nm) standards in the HPLC chromatogram

2.3. Preparation of stock standards and working mixed solutions

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.

2.4. HPLC validation

Linearity: The calibration curve was generated by plotting peak areas ranging from 0.05 to 2.0 µg/mL versus their concentrations. The linearity was assessed from the linear regression with its correlation coefficient.

Detection limit: The detection limit should correspond to the concentration for which the signal-to-noise ratio. The value was defined as the lowest concentration level resulting in a peak area of three times the baseline noise.

System suitability test: The HPLC system suitability is an essential parameter of HPLC determination, and it ascertains the strictness of the system used. In the tests, the injection repeatability was evaluated as the relative standard deviations of peak areas and retention times calculated for 10 replicate injections of a mixed standard solution (0.5 µg/mL).

3. Results and Discussion

3.1. Optimum HPLC conditions

In order 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 twelve types of C1, TMS and C4 columns (Table 1). 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) [10] 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 ℃; the flow rates ≥ 0.5 mL/min; HPLC retention times ≤ 10 min (Table 2). 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.

The twelve columns were compared with regard to 1) elution from the column; 2) separation between TC and 4eTC; 3) sharpness of peaks obtained upon injection of equal amounts. The resulting chromatographic separation and peak form profiles within the conditions ranges examined are presented in Table 2.

Table 2: Resulting chromatographic 4eTC and TC separations obtained under the HPLC condition renges examined

Column (trade name)	100% water mobile phase			1-octanesulfonic acid sodium (> 1 mmol/L) mobile phase			tetra-n-butylammonium phospate (> 1 mmol/L) mobile phase		
	Eluted	**Separated**	**Peak form**	**Eluted**	**Separated**	**Peak form**	**Eluted**	**Separated**	**Peak form**
Daisopak SP-200-5-C1-P	×	—	—	×	×	—	◯	×	Tailing
Developsil TMS-UG-5	×	—	—	×	—	—	×	—	—
Hypersil SAS C1	×	—	—	×	—	—	×	—	—
Inertsil TMS	◯	×	Broadening	◯	×	Sharp	◯	×	Sharp
Inertsil WP300 C4	◯	×	Sharp	◯	×	Sharp	◯	◎	Symmetrical and sharp
Kaseisorb LC-C1-300-5	◯	×	Sharp	◯	×	Rounding	◯	×	Broadening
Mightysil RP-4GP	×	—	—	×	—	—	×	—	—
SUPELCOSIL LC-1	×	—	—	×	—	—	×	—	—
Spherisob C1	◯	×	Rounding	×	×	—	◯	×	Rounding
Wakosil 5TMS	×	—	—	×	—	—	×	—	—
Wakosil 5C4-200	×	—	—	×	—	—	×	—	—
ZORBAX TMS	×	—	—	×	—	—	×	—	—

It was extremely difficult to elute and separate TC and 4eTC with an isocratic 100% aqueous mobile phase, i.e., water, OSA or TBP alone. Only use of an "Inertsil WP300 C4" enabled valid separation of the two target compounds within the condition ranges examined in this study (Table 2). 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 a 5 mM TBP at a column temperature of 55℃ and a flow-rate of 1.0 mL/min.

Fig. 2 displays that the resulting chromatogram obtained from the HPLC system under the optimal conditions, with the PAD set at 364 nm (giving an average of maximum absorption spectra for TC and 4eTC). The two target peaks are clearly identified at 3.3 min for 4eTC and 4.8 min for TC, respectively, and enabled also the multiple sequential injections. Under the highly pH and lowly-concentrated ion-pair reagent of mobile phase and raised column temperature, large pore diameter of 30 nm and 3% carbon contents in the only applied column were necessary at least to obtain the findings.

The present HPLC-PAD method accomplished optimum separation in a short time (< 5 min/sample) without the need for a gradient mode to improve the separation and pre-column washing after an analysis. Furthermore, the PAD easily confirmed the peak identity of the target compound. The analyte can be easily identified by its retention time and absorption spectrum without using MS or MS/MS. The present HPLC-PAD system did not require the use of MS or MS/MS, which is very expensive, laborious to manage, and is unavailable in a number of laboratories for routine analysis.

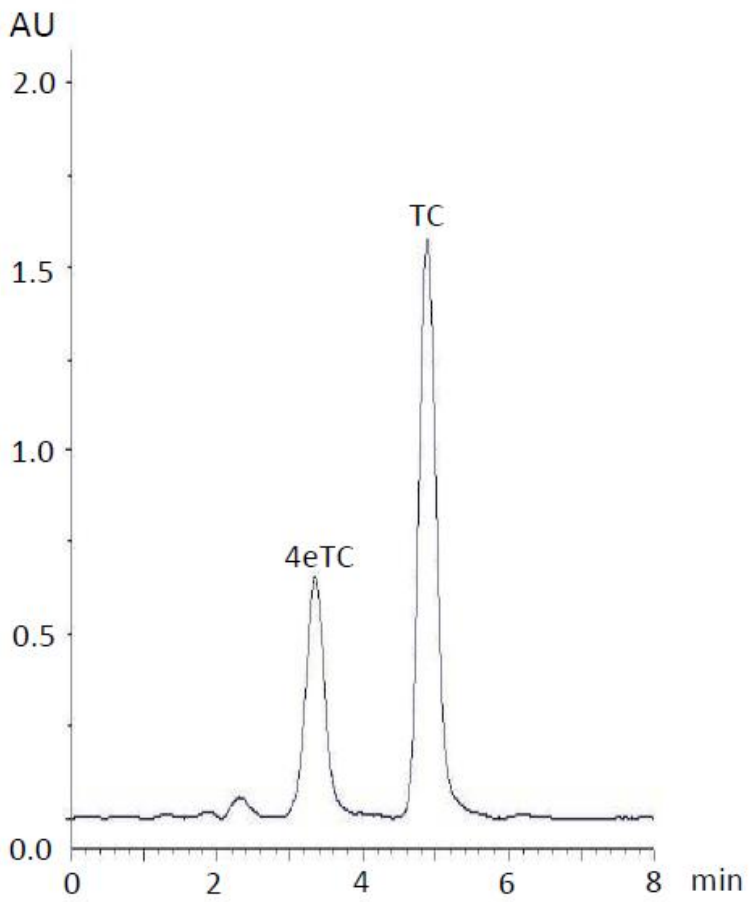

Figure 2: A typical chromatogram of a standard mixture (0.5 µg/mL) obtained from the HPLC system. Retention times, 4eTC = 3.3 min; TC = 4.8 min

3.2. HPLC validation

Table 3 summarizes the validation data for the present HPLC performances. The linearity and system suitability values were sufficiently satisfy the FDA's recommended criteria [11]. The detection limits for TC and 4eTC were 0.005 and 0.014 µg/mL, respectively. The findings demonstrates high accuracy and reproducibility.

Table 3: Chromatographic method validation data

	4eTC	**TC**	**Acceptance criterion[a]**
Linearity (*r*)[b]	0.9998	0.9996	≥ 0.999
Range (µg/mL)	0.05 - 2.0		
Detection limit[c] (µg/mL)	0.014	0.005	
System Suitability Specifications:			
Injection repeatability[d] (RSD, %)			
Retention time	0.14	0.28	≤ 1
Peak area	0.58	0.38	≤ 1
Resolution (Rs)	3.01		> 2
Peak tailing factor (T)	1.26	1.34	≤ 2

[a] Recommendations in the FDA guidelines [11].
[b] *r* is the correlation coefficient ($p < 0.01$) for calibration curve.
[c] Detection limit as the concentration of analyte giving a signal-to-noise ratio = 3.
[d] Data as the relative standard deviations calculated for 10 replicate injections of a mixed standard solution (0.5 µg/mL of 4eTC and TC, respectively).

4. Conclusion

A validated HPLC-PAD system for analysing TC and its metabolite, 4eTC, using an isocratic 100% aqueous mobile phase has been successfully established. This organic solvent-free system results in a low-cost and low-impact to the environment and to humans, particularly analysis employees, and has a short run-time and high system suitability. Such the short run-time and low-cost system increased the sample throughput for actual routine residue monitoring work. The present system may be proposed as the international harmonized method for simultaneous detection of TC and 4eTC.

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