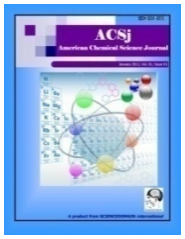

American Chemical Science Journal
3(4): 500-506, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org

A 100% Water Mobile Phase HPLC-PDA Analysis of Tetracycline Antibiotics

Naoto Furusawa[1*]

[1]*Graduate School of Human Life Science, Osaka City University, Osaka-558-8585, Japan.*

Author's contribution

This work was carried out in author alone. Author NF designed the study, performed the statistical analysis, wrote the protocol, and wrote the manuscript. 'The author managed the analyses and the literature searches of the study. The author read and approved the final manuscript.

Short Communication

Received 19th March 2013
Accepted 3rd July 2013
Published 27th August 2013

ABSTRACT

Aims: To develop a reserved-phase HPLC method for detecting oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) using a 100% water mobile phase.
Study Design: HPLC conditions.
Place and Duration of Study: Author's Lab., Osaka City University, Japan, between September and November 2012.
Methodology: Chromatographic separations were performed an Inertsil® WP300 C4 (100×4.6 mm, 5 µm) with a water mobile phase and a photodiode-array detector.
Results: The run time was < 5.5 min. The method shows high stability, significant linearity and satisfactory sensitivity. The detection limits were established in the range 0.01 – 0.059 µg/mL.
Conclusion: An organic solvent/reagent-free HPLC method for the simultaneous detection of OTC, TC, and CTC was developed and may be further applied to the quantification in foods.

Keywords: Oxytetracycline; tetracycline; chlortetracycline; high-performance liquid chromatography; 100% water mobile phase.

**Corresponding author: Email: furusawa@life.osaka-cu.ac.jp;*

1. INTRODUCTION

Antibiotic residues in foods pose a serious threat to public health. Tetracycline antibiotics are called "broad-spectrum" antibiotics, because they can be used to treat a wide variety of infections. These antibiotics are widely used in animal-derived food production site, however over use can lead to antibiotic resistance and food residues.

To assure the safety of food to consumers, U.S. Food and Drug Administration (FDA) has established tolerances for the sum of three tetracyclines (chlortetracycline, CTC; oxytetracycline, OTC; tetracycline, TC) residues for animal-derived foods as listed under Code of Federal Regulations (CFR) Title 21 [1]. The validated monitoring of OTC, TC, and CTC residue levels in food and animal products is, therefore, essential to guarantee the safety of the food supply and manage global health risks.

In response to the recent expansion in the internal food trade, the development of international harmonized methods to determine chemical residues in foods is essential to guarantee equitable international trade in these foods and ensure food safety for consumers. Whether in industrial nations or developing countries, an internal harmonized method for residue monitoring in foods is urgently-needed. The optimal harmonized method must be easy-to-use, economical in time and cost, and must cause no harm to the environment and analyst.

Several high-performance liquid chromatographic (HPLC) methods, previously summarized in a review paper [2], have been developed for the monitoring TCs with different detection modes such as UV-spectrophotometry [3,4], fluorescence [5,6], photo-diode array (PDA) [7,8], and mass spectrometry (MS) [9-12] . The AOAC International issued an official HPLC method for determination of in edible animal tissues, based on HPLC-UV detection [13]. However, these methods have crucial drawbacks:

1) All of the methods consume large quantities of organic solvents 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. Additionally, incineration for disposal of waste organic solvents has steadily increasing over the past ten-odd years and has spent huge amounts of money. Eliminating the use of organic solvents is an important goal in terms of environmental conservation, human health and the economy [14,15].
2) Most of the recent methods are based on LC-MS/MS. The facilities that LC-MS/MS system is available are limited to part of industrial nations because these are hugely expensive, and the methodologies use complex and specific. These are unavailable in a lot of laboratories for routine analysis, particularly in developing countries.

As the first examination problem in the establishment of an international harmonized method for the residue monitoring of OTC, TC, and CTC, this paper describes a 100% water mobile phase HPLC conditions to detect the three compounds without organic solvent /analytical reagent consumption.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Standards of oxytetracycline (OTC, $C_{22}H_{24}N_2O_9$; 460.434 g/mol), tetracycline (TC, $C_{22}H_{24}N_2O_8$; 444.435 g/mol), and chlortetracycline (CTC, $C_{22}H_{23}ClN_2O_8$; 478.88 g/mol) and distilled water (HPLC grade) were purchased from Wako Pure Chem. Ltd. (Osaka, Japan). These standards were greater than 99% purity.

2.2 Equipments and Operating Conditions

The HPLC system, used for method development, included a model PU-980 pump and DG-980-50-degasser (Jasco Corp., Tokyo, Japan) equipped with a model CO-810 column oven (Thosoh Corp., Tokyo, Japan), as well as a model SPD-M10A $_{VP}$ photodiode-array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan). The following six types of non-polar sorbent columns were used in this study, and Table 1 lists the particle physical specifications.

Table 1. Physical/chemical specifications of the reversed-phase silica columns[a] used and chromatographic OTC, TC, and CTC separations obtained under the HPLC conditions examined[b]

Column		d_p (µm)	Pore diameter (nm)	Pore volume (mL/g)	Surface area (m^2/g)	Carbon load (%)	HPLC separations
Type	Trade name						
(A) C1	Daisopak®SP-200-3-C1-P	3	20	1.1	200	3	NS[c]
(B) C1	Develosil®TMS-UG-3	3	14	1.05	300	4.5	NS
(C) C1	Kaseisorb®LC C1-300-5	5	30	0.95	100	1	NS
(D) C1	Wakosil®5TMS	5	12	1.0	300	4	NS
(E) C4	Inertsil®WP300 C4	5	30	1.05	150	3	Separated
(F) C4	Mightysil®RP-4 GP	5	12.5	1.05	350	4	NS

[a]4.6×100 mm. Column (A): Daiso Co., Ltd., Osaka, Japan; (B): Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; (C): Nomura Chemical Co., Ltd., Aichi, Japan; (D): Wako; E: GL Sciences; (F): Kanto Chemical Co., Inc., Tokyo, Japan.

[b]Mobile phase of water, column temperatures ≥ 20 ℃ flow-rates ≥ 0.5 mL min-1, and HPLC retention times ≤ 15 min.

[c]Not separated (between OTC and TC standards).

The analytical column was an Inertsil® WP300 C4 column (GL Sciences, Tokyo, Japan) using a water mobile phase at a flow rate of 1.0 mL/min at 25℃. PDA detector was operated at 190 – 600 nm: the monitoring wavelength was adjusted to 282 nm which represents an average maximum for all the analytes.

2.4 Preparation of Stock Standards and Working Mixed Solutions

Stock standard solutions of OTC, TC, and CTC were prepared by dissolving each compound in water to a concentration of 10 µg/mL. Working mixed standard solutions of these three

compounds were prepared by suitably diluting the stock solutions with water. These solutions were kept in a refrigerator (5ºC).

2.5 HPLC Validation

2.5.1 Linearity

The calibration curve was generated by plotting peak areas ranging from 50 to 5,000 ng/mL versus their concentrations. The linearity was assessed from the linear regression with its correlation coefficient.

2.5.2 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.

2.5.3 Standard solution Stability

The stability of stock solutions of the target compounds were evaluated at room temperature, 25ºC, for 24 h and 5ºC for 10 days, respectively. After completion of the storage time, the stability was tested by comparing the HPLC response with that of freshly prepared solutions. In the same manner, a working mixed standard solution (1 µg/mL of each compound) was tested.

2.5.4 Robustness

Changes of ±5% units of the flow rate (1.0 mL/min) and the column temperature (25℃) were determined. The effect on the peak areas and the validations in the retention times were evaluated.

2.5.5 System suitability test

The HPLC system suitability 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 areas and retention times calculated for 20 replicate injections of a mixed standard solution (0.1 µg/mL of each compound).

3. RESULTS AND DISCUSSION

3.1 Optimum HPLC Conditions

In order to achieve the separation with a 100% water mobile phase, this study tested six types of non-polar sorbent columns. The physical and chemical specifications are listed in Table 1. This study examined column temperatures (≥ 20℃) and flow rates (≥ 0.5 mL/min). The six columns were compared with regard to the separation among the four target compounds and the sharpness of the peaks peak obtained upon injection of equal amounts. The chromatographic separations within the conditions ranges examined are also presented in Table 1.

The complete separation of OTC, TC, and CTC and their symmetrical peaks were obtained by a Column E and a 100% water mobile phase with a column temperature of 25°C and a flow rate of 1.0 mL/min. Fig. 1 displays that the resulting chromatogram obtained from the HPLC. The three target peaks are clearly distinguished within 5.16 min (Fig. 1). The present HPLC analysis accomplished optimum separation in a short time without the need for a gradient system to improve the separation and pre-column washing after an analysis. From the data shown in Table 1, it is difficult to prove the criterial parameter in the column with regard to the retentions of the target compounds and their peak forms.

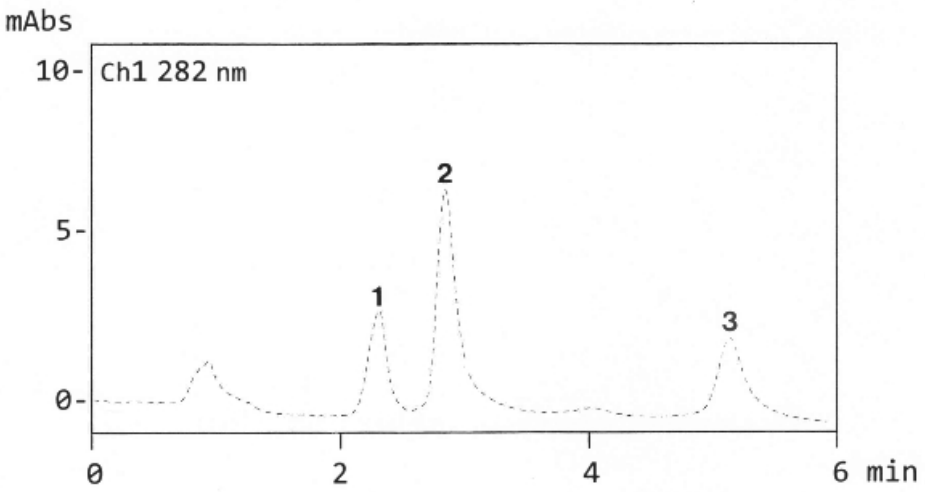

Fig. 1. Typical chromatogram of a standard mixture (OTC 0.5 µg/mL, TC or CTC 1.0 µg/mL) obtained from the HPLC system. Peaks, 1= OTC (retention time, R_t= 2.32 min); 2= TC (R_t= 2.86 min); 3= CTC (R_t= 5.16 min)

3.2 HPLC Validation

3.2.1 Main validation data

Table 2 summarizes the validation data for the main performance parameters (linearity, detection limit, standard solution stability, and system suitability). The present standard stabilities and system suitability were well within the international acceptance criteria [16-18].

3.2.2 Robustness

Changes of ±5% of the flow rate and the column temperature had no effect on the peak areas, whereas the variations in the retention times were obtained with the flow rate and the column temperature. Normal retention times for OTC, TC, CTC were 2.32, 2.86, and 5.16 min, respectively. At +5% the flow rate, the three retention times were decreased, ranging between 1.5 and 4.9% and at -5%, the times were increased ranging between 5.0 and 8.1%. By changing the column temperature by +5%, decreasing retention times obtained were 1.7 – 7.7%, however, no significant variations were observed with -5%. During these studies, all the target compounds were separated.

Table 2. HPLC Validation Data

Parameter	*OTC*	*TC*	*CTC*
Linearity (r)[a]	0.9995	0.9990	0.9961
Range (µg/mL)		0.05–5	
Detection limit[b](µg/mL)	0.010	0.028	0.059
Standard stability[c]:			
a) At 25ºC for 24 h			
Stock		99.2–100.7 (0.4–1.1)	
Working		99.6–101.0 (0.7–1.2)	
b) At 5 ºC for 14 d			
Stock		99.0–100.9 (0.7–1.0)	
Working		99.5–102.1 (0.8–1.2)	
System suitability[d]:			
Retention time	0.11	0.14	0.20
Peak area	0.68	0.75	0.93

[a] *r is the correlation coefficient (p<0.01) for calibration curve.*
[b] *Detection limit as the concentration of analyte giving a signal-to-noise ratio = 3.*
[c] *The chromatographic peak area (%) after completion of the store time: data are expressed as average area ranges (n=5, for each compound); relative standard deviations (RSDs) in parentheses.*
[d] *Data as RSDs calculated for 10 replicate injections*

4. CONCLUSION

A breakthrough HPLC-PDA method for detecting OTC, TC, and CTC using a 100% water mobile phase has been successfully established. A water mobile phase method, which has never happened before, is harmlessness to the environment and to humans and has a short run time and high sensitivity. For the quantification in various foods, the proposed HPLC method will be applicable enough by performing a suitable sample preparation technique.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. FDA Code of Federal Regulations, Title 21, Volume 6, Revised as of April 1, 2012, 21CFR556.720.
2. Oka H, Ito H, Matsumi H. Review, Chromatographic analysis of tetracycline antibiotics in foods. Journal of Chromatography A. 2000;882:109–133.
3. Meinertz JR, Stehly GR, Gengerich WH. Liquid chromatographic determination of oxytetracycline in edible fish fillets from six species of fish. Journal of AOAC International. 1998;81:702–708.
4. Wen Y, Wang Y, Feng YQ. Simultaneous residue monitoring of four tetracycline antibiotics in fish muscle by intube solid-phase microextraction coupled with high-performance liquid chromatography. Talanta. 2006;70;153–159.
5. Peres GT, Rath S, Reyes FGR. A HPLC with fluorescence detection method for the determination of tetracyclines residues and evaluation of their stability in honey. Food Control. 2010;21(5):620-625

6. Agasoster T, Rasmussen KE. Online dialysis liquid-chromatography and postcolumn reaction detection of oxytetracycline in salmon muscle extracts. Journal of Pharmaceutical and Biomedical Analysis. 1992;10(5):349–354.
7. Cristofani E, Antonini C, Tovo G, Fioroni L, Piersanti A, Galarini R. A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection. Analytical Chimica Acta. 2009;637(1-2):40-46.
8. Furusawa N. Isolation of tetracyclines in milk using a solid-phase extracting column and water eluent. Talanta. 2003;59(1):155-159.
9. Cetinkaya F, Yibar A, Soyutemiz GE, Okutan B, Ozcan A, Karaca MY. Determination of tetracycline residues in chicken meat by liquid chromatography-tandem mass spectrometry. Food Additives & Contaminants B. 2012;5(1):45-49.
10. Dasenaki ME, Thomaidis NS. Multi-residue determination of seventeen sulfonamides and five tetracyclines in fish tissue using a multi-stage LC-ESI-MS/MS approach based on advanced mass spectrometric techniques. Analytical Ch Chimica Acta. 2010;672:93–102.
11. De Alwis H, Heller DN. Multiclass, multiresidue method for the detection of antibiotic residues in distillers grains by liquid chromatography and ion trap tandem mass spectrometry. Journal of Chromatography A. 2010;1217(18):3076-3084.
12. Guo L, Chen YQ, Zhang LY, Yang WJ, He PL. Development and Validation of a Liquid Chromatographic/Tandem Mass Spectrometric Method for Determination of Chlortetracycline, Oxytetracycline, Tetracycline, and Doxycycline in Animal Feeds. Journal of AOAC International. 2012;95(4):1010-1015.
13. AOAC International. AOAC Official method 995.09. 2000. Accessed 12 September. Available: http://img.21food.cn/img/biaozhun/20100108/177/11285241.pdf.
14. Anastas PT, Warner JC. Green Chemistry; Theory and Practice. Oxford University Press, Oxford, United Kingdom; 2003.
15. Yoshimura T, Nishinomiya T, Homda Y, Murabayashi M. Green Chemistry – Aim for the Zero Emission-Chemicals. Sankyo Publishing Co. Ltd. Press, Tokyo, Japan; 2001.
16. Du L, Musson DG, Wang AQ. Stability studies of vorinostat and Its two metabolites in human plasma, serum and urine. Journal of Pharmaceutical and Biomedical Analysis. 2006;42(5):556-564.
17. FDA Reviewer Guidance, Validation of Chromatographic Methods. Center for Drug Evalution and Research (CFDER); 1994.
18. FDA Code of Federal Regulations, Title 21, Volume 6, Revised as of April 1, 2012;21CFR556.720.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=242&id=16&aid=1939