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Short communication

Determination of oxytetracycline levels in rainbow trout serum on a biphenyl column using high-performance liquid chromatography

Ron A. Miller*, Renate Reimschuessel, Mary C. Carson

US Food and Drug Administration, Center for Veterinary Medicine, Office of Research, 8401 Muirkirk Road, Laurel, MD 20708, USA

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Abstract

We developed a simple and sensitive high-performance liquid chromatography method on a biphenyl column to determine oxytetracycline (OTC) levels in rainbow trout serum. The assay used deproteination, filtration, and subsequent separation on a reverse-phase biphenyl column, with UV detection at 355 nm. OTC (7.8–7.9 min) was completely resolved from structurally similar riboflavin (10.4–10.5 min), a common feed supplement. Estimated limits of detection and quantitation of OTC were 0.01 and 0.04 μg/mL, respectively. The average recovery for OTC was 102% with a R.S.D. of 8.34%. Calibration standards were linear from 0.01 to 10 μg/mL.

Keywords: Oxytetracycline; HPLC; High-performance liquid chromatography; Riboflavin; Fish; Aquaculture; Rainbow trout

1. Introduction

Oxytetracycline (OTC) is one of only three antibiotics currently approved by the United States Food and Drug Administration and available for use in aquaculture. It is approved for use against selected indications in salmonids, catfish, and lobsters [1].

Tetracyclines are known to have low bioavailability and are poorly absorbed when administered with feed [2]. Elema et al. [3] showed feed pellets contained many metallic ions that formed complexes with OTC and reduced the amount of drug available for fish to absorb from the intestinal tract. To choose an appropriate therapeutic agent, it is essential to have information about the serum concentration, over time, at the site of infection. Additionally, the minimal inhibitory concentration (MIC) of the pathogen and clinical efficacy data are also important to predict therapeutic efficacy.

Using various methods, researchers have studied the pharmacokinetic profile of OTC in multiple species including shrimp [4], hens [5], sea turtles [6], fish [7], goats [8], sheep and calves [9], pigs [10], and humans [11]. Some of these methods evaluated drug activity- or microbiological-based methods to estimate OTC concentration in the blood, while others used analytical methods such as HPLC. Generally, HPLC is a more sensitive and precise method, and is especially useful when low concentrations of analyte are present. Very few, if any, studies have provided data resolving OTC from the structurally similar molecule riboflavin (Fig. 1) in serum or plasma. Riboflavin or vitamin B2, found in dairy products, eggs, vegetables, organ meats, whole grains, and wheat germ, is recommended as an aquaculture feed supplement (>20 mg/kg feed) to increase energy metabolism and promote physiological health [12]. Ichinose et al. [13] reported riboflavin concentrations ranging between 0.21 and 0.37 μg/mL in serum of black carp, gibel, and eel. Due to their similarities in structure and excitation range (riboflavin = 325–405 nm; OTC = 310–420 nm) [14], we compared the retention times of riboflavin and OTC on a polymer column. Riboflavin retention time was only 30 s longer than that of OTC (unpublished data). Low levels of riboflavin absorbed from intestinal contents may co-elute with OTC, increasing the area of the OTC peak. In pharmacokinetic studies, exclusively OTC must be quantified in serum or plasma to predict therapeutic efficacy and calculate the pharmacodynamic parameters: time above MIC, Cmax/MIC and AUC/MIC.

This is the first published method that uses a biphenyl column to quantify OTC in fish serum. This method resolves OTC from surrounding peaks, such as riboflavin.

* Corresponding author. Tel.: +1 301 210 4762; fax: +1 301 210 4685.
E-mail address: Ron.Miller@fda.hhs.gov (R.A. Miller).

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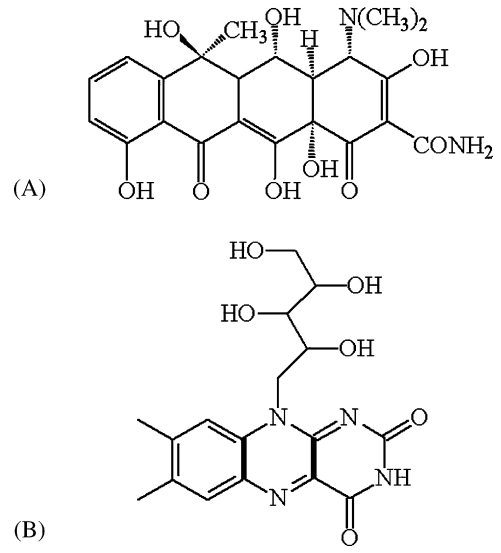

Fig. 1. Molecular structures of OTC (A) and riboflavin (B).

2. Experimental

2.1. Chemicals

All chemicals were reagent grade unless otherwise specified.

We obtained OTC from the United States Pharmacopeia (Rockville, MD). We prepared a stock OTC solution, 1.0 mg/mL, by solubilization of 100 mg in 100 mL methanol, and stored it up to 3 months at −20 °C.

We prepared McIlvaine buffer [15] by solubilization of 12.9 g anhydrous citric acid (Mallinckrodt Baker Inc., Phillipsburg, NJ) and 10.9 g dibasic sodium phosphate (Fisher Scientific, Fair Lawn, NJ) in 1 L water. We prepared McIlvaine–EDTA buffer by solubilization of 37.2 g EDTA disodium salt dihydrate (Sigma–Aldrich, St. Louis, MO) up to 1 L of McIlvaine buffer with gentle heating. Prior to use, we filtered McIlvaine–EDTA buffer through a 0.2 μm nylon filter and stored at 4 °C.

We used methanol (Mallinckrodt Baker Inc.) and acetonitrile (Honeywell Burdick and Jackson, Morristown, NJ), both HPLC grade, without further purification. We obtained oxalic acid dihydrate from Sigma–Aldrich. Water used throughout this study was purified by the Milli-Q plus Ultra-Pure Water System (Millipore Corporation, Bedford, MA).

2.2. Equipment

We used an Agilent Series 1100 (Agilent Technologies, Palo Alto, CA) Quaternary Pump, Vacuum Degasser, Autosampler, Thermostatted Column Compartment, and Variable Wavelength Detector for chromatographic separation. We completed data acquisition and analysis with a PE Nelson 900 Series Interface Controller and TotalChrom™ software (PerkinElmer Life and Analytical Sciences, Wellesley, MA).

2.3. Sample preparation

We collected control serum from 200 to 600 g rainbow trout obtained from a commercial source. Fish were held in tanks in our laboratory at 14–18 °C, and fed a standard diet which contained 35–45 mg riboflavin/kg feed according to the feed manufacturer (Rangen Inc., Buhl, ID). We euthanatized fish by immersion in a lethal dose of tricaine methanesulfonate (MS-222) (Crescent Research Chemicals, Phoenix, AZ). We stored serum at −80 °C until use. We used the stock 1.0 mg/mL OTC solution diluted in water to concentrations of 10 and 3.0 μg/mL, to fortify serum samples at concentrations of 3.0, 0.50, and 0.10 μg/mL.

We vortex mixed serum samples (600 μL) with an equal volume (600 μL) McIlvaine–EDTA buffer, incubated at 30 °C for 30 min, and centrifuged at 12,000 × *g* for 10 min to deproteinate. We removed supernates, and filtered through a Whatman (Florham Park, NJ) Puradisc™ 13 mm PVDF 0.2 μm syringe filter. We loaded the filtered samples into autosampler vials and injected a 100 μL aliquot onto the LC column.

We prepared chromatographic standards by serial dilution of the stock 1.0 mg/mL OTC solution in water to concentrations of 10, 3.0, 1.0, 0.30, 0.10, 0.03, and 0.01 μg/mL. We then diluted the standards 1:1 with McIlvaine–EDTA buffer. We analyzed chromatographic standards in a bracketed sequence (i.e. calibration standards + samples + calibration standards).

2.4. Column liquid chromatography

Separation was performed on an Allure™ biphenyl column 4.6 mm × 150 mm, 5 μm particle size, and 60 Å pore diameter (Restek Corporation, Bellefonte, PA). The column was maintained at 40 °C. We used mobile phases which were 0.2 μm nylon-filtered aqueous 0.01 M oxalic acid and acetonitrile. We employed gradient elution with initial conditions of 5% acetonitrile for 1 min, and linear changes to 30% acetonitrile at 10 min, 80% at 12 min, 80% at 13 min, 5% at 15 min, and 5% at 20 min. The flow rate was 1 mL/min.

2.5. Validation

We analyzed control and fortified serum samples to determine selectivity, accuracy, and precision. We extracted and analyzed

Table 1
Validation using control and OTC fortified rainbow trout serum

Sample	No.	Mean OTC concentration (μg/mL) ± S.D.	Mean recovery (%) ± % R.S.D.[a]
Control serum—0 μg/mL	8	–	–
Fortified serum—0.10 μg/mL OTC	8	0.11 ± 0.01	107 ± 12.3
Fortified serum—0.50 μg/mL OTC	8	0.51 ± 0.02	101 ± 3.06
Fortified serum—3.0 μg/mL OTC	8	2.9 ± 0.06	97.1 ± 2.13

[a] Mean recovery ± % R.S.D. in all samples = 102 ± 8.34.

four replicates of control serum and serum fortified at 3.0, 0.50, and 0.10 μg/mL on each of 2 different days, for a total of eight replicate analyses at each concentration.

We performed an exhaustive extraction procedure to assess accuracy with a real sample. In this experiment, we used incurred rainbow trout serum samples obtained from one fish fed an OTC-medicated aquaculture feed (Rangen Inc.). As described above, we extracted and filtered five replicates of the incurred serum sample (200 μL each) with an equal volume of McIlvaine–EDTA buffer (normal extract). We resuspended the remaining pellet after centrifugation in 400 μL of McIlvaine–EDTA buffer, vortex mixed, centrifuged, and filtered as described above (exhaustive extract). We loaded normal and exhaustive extracts into autosampler vials and injected a 100 μL aliquot onto the LC column.

3. Results and discussion

Table 1 summarizes the results of the validation study where the mean recovery of OTC was uniformly high (97.1–107%) at all concentrations tested. We did not find OTC in the unfortified rainbow trout serum control samples. Relative standard deviations were acceptably low at all three OTC concentrations tested, indicative of a precise method. Estimated lower limits of detection (LOD) and quantitation (LOQ) of OTC were 0.01 and 0.04 μg/mL, respectively. We calculated these values as the average background signal plus 3× the standard deviation (LOD) or plus 10× the standard deviation (LOQ) in analyses of rainbow trout control serum. Calculated LOD and LOQ values were lower than those reported in previous OTC pharmacokinetics studies where serum was analyzed on a C8 column (LOD = 0.05 μg/mL) [16] and plasma was analyzed on a C18 column (LOQ = 0.1 μg/mL) [17]. In addition, the mean percent recovery for all samples in this study (102 ± 8.34%) was greater than the same parameter measured previously by Doi et al. (76.16 ± 0.14%) [16] and Haug and Hals (83.0 ± 8.9%) [17].

We found excellent separation and recovery of OTC in fortified and incurred rainbow trout serum samples following simple deproteination and filtration (Fig. 2). From preliminary optimization trials, we found that riboflavin elutes at 10.4 min. Thus, in Fig. 2B–D presumed riboflavin was found to separate in rainbow trout serum samples at 10.4 min, and estimated concentrations were consistent with those reported previously by Ichinose et al [13]. These data suggest riboflavin can be present in fish serum at relatively high concentrations, and needs to be resolved from OTC when using HPLC methods.

We generated standard curves for OTC by linear (weighted $1/x^2$) regression of peak areas against their respective concentrations. We studied linearity by separate analysis in quadruplicate of the calibration curves, created using the standards (10–0.01 μg/mL). Regression analyses for each line resulted in R^2 values exceeding 0.9991.

Stable retention times were observed throughout the validation (Fig. 2). We were able to accurately determine OTC concentrations without interference from riboflavin (Fig. 2B–D) or other serum components.

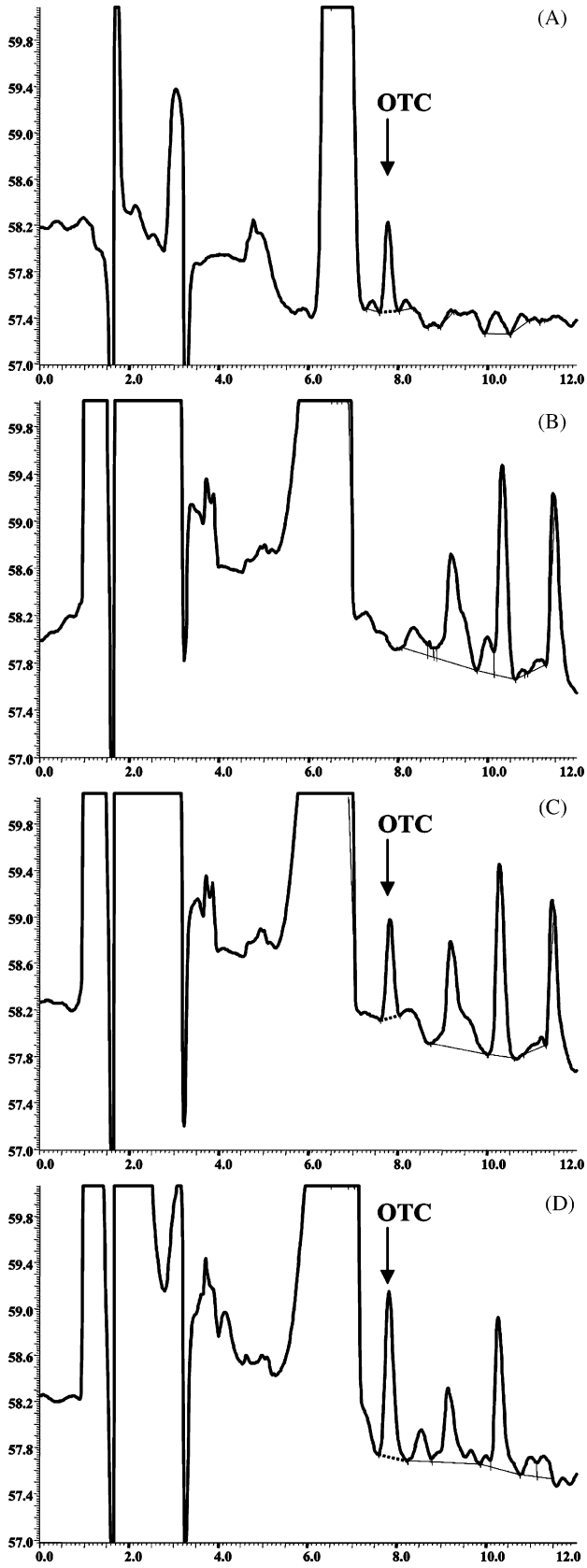

Fig. 2. Chromatograms of the (A) 0.10 μg/mL calibration standard, (B) rainbow trout control serum, (C) 0.10 μg/mL fortified rainbow trout serum, and (D) incurred rainbow trout serum (0.18 μg/mL).

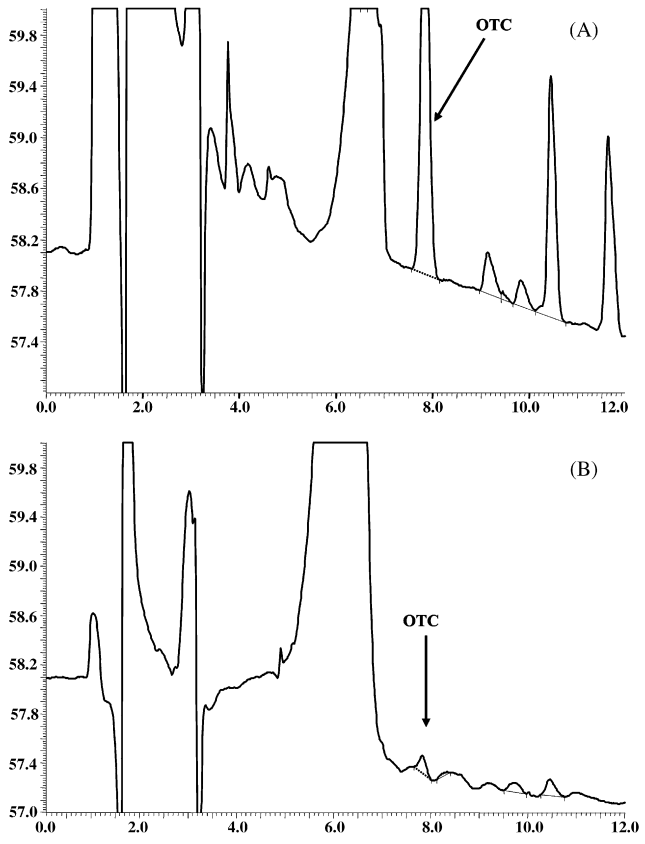

Fig. 3. Chromatograms of incurred rainbow trout serum (A) and an exhaustive extraction of the centrifuged serum components (B).

The mean normal extract concentration of five incurred serum replicates was 0.45 ± 0.01 μg/mL, while the mean exhaustive extract concentration was 0.02 ± 0.01 μg/mL, a value less than the LOQ of the method (Fig. 3). The normal extract of the incurred serum was 95.5% of the total extract (normal + exhaustive extracts), consistent with the near quantitative recoveries found in the fortified experiments.

4. Conclusion

The method we describe here uses simple extraction and chromatographic procedures that accurately quantify OTC concentrations in rainbow trout serum. We believe its selectivity for OTC makes it a highly valuable method for pharmacokinetics and pharmacodynamics studies which require accurate OTC concentrations.

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References

[1] Food and Drug Administration, Center for Veterinary Medicine. FDA-CVM Aquaculture website. <http://www.fda.gov/cvm/aqualibtoc.htm>, 2006 (accessed February 5, 2007).
[2] K.N. Agwuh, A. MacGowan, J. Antimicrob. Chemother. 58 (2006) 256.
[3] M.O. Elema, K.A. Hoff, H.G. Kristensen, Aquaculture 143 (1996) 7.
[4] S. Chiayvareesajja, A. Chandumpai, Y. Theapparat, D. Faroongsarng, J. Vet. Pharmacol. Ther. 29 (2006) 409.
[5] M. Singh, C. Varshneya, R.S. Telang, A.K. Srivastava, J. Vet. Sci. 6 (2005) 197.
[6] C.A. Harms, M.G. Papich, M.A. Stamper, P.M. Ross, M.X. Rodriguez, A.A. Hohn, J. Zoo. Wildl. Med. 35 (2004) 477.
[7] C.Y. Chen, R.G. Getchell, G.A. Wooster, A.L. Craigmill, P.R. Bowser, J. Aquat. Anim. Health 16 (2004) 208.
[8] M.A. Payne, J.G. Babish, M. Bulgin, M. Lane, S. Wetzlich, A.L. Craigmill, J. Vet. Pharmacol. Ther. 25 (2002) 25.
[9] A.L. Craigmill, R.E. Holland, D. Robinson, S. Wetzlich, T. Arndt, J. Vet. Pharmacol. Ther. 23 (2000) 345.
[10] P. Nielsen, N. Gyrd-Hansen, J. Vet. Pharmacol. Ther. 19 (1996) 305.
[11] J. Wojcicki, W. Kalinowski, B. Gawronska-Szklarz, Arzneimittelforschung 35 (1985) 991.
[12] C.B. Cowey, C. Young Cho, Proc. Nutr. Soc. 52 (1993) 417.
[13] N. Ichinose, K. Adachi, G. Schwedt, Analyst 110 (1985) 1505.
[14] I. Garcia, L.A. Sarabia, M.C. Ortiz, Anal. Chim. Acta. 501 (2004) 193.
[15] H. Oka, K. Uno, K. Harada, K. Yasaka, M. Suzuki, J. Chromatogr. 298 (1984) 435.
[16] A.M. Doi, M.K. Stoskopf, G.A. Lewbart, J. Vet. Pharmacol. Ther. 21 (1998) 364.
[17] T. Haug, P.A. Hals, Aquaculture 186 (2000) 175.