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Short communication

One-step liquid chromatographic method for the determination of oxytetracycline in fish muscle

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Abstract

A one-step simple and rapid high performance liquid chromatography (HPLC) method was developed for the determination of oxytetracycline (OTC) in fish tissue. The method involves liquid extraction of muscle tissue, precipitation of proteins and reversed phase HPLC analysis with spectrophotometric detection. The limit of quantitation of OTC in spiked fish muscle was 0.04 μg/g and the method showed high linearity (r^2 = >0.999) in the working range of 0.04–2 μg/g. The precision (%R.S.D.) was between 1.9 and 7.5% for the concentration range 0.04–1.0 μg/g and there was no significant difference between the concentrations determined on three different test days for all four spiked concentrations. The percentage recovery over the spiked concentration range 0.04–1.0 μg/g was consistently within a narrow range of 33–35%. While the method had the advantage of high precision, sensitivity and linearity, the method's additional salient advantages included high sample through-put (60 individual preparations per day) and minimum amount of consumables, time and labour required to perform the analysis. The method was successfully applied to a pharmacokinetic study.
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1. Introduction

According to the FAO statistics on aquaculture species and the potential for growth [1], aquaculture represents one of the fastest growing food producing sectors. However, the intensive culture of aquatic organisms involves the risk of outbreaks of infectious diseases. Oxytetracycline (OTC) (Fig. 1) is an antibacterial agent used against disease outbreaks in aquaculture because of its broad antibacterial spectrum, economic advantages and legal availability. In general, research on OTC concentrations in fish using HPLC methodologies are applied to either pharmacokinetic studies, which require a precise and rapid method with large sample through-put capability, or residue depletion studies which have a requirement for method sensitivity. An HPLC method which can fulfil both pharmacokinetic and residue requirements is therefore ideal. However, the ability of a single method to accommodate both of these requirements is made difficult by high through-put needs conflicting with sophisticated methods required in order to achieve sensitivity. Thus, for the applied purpose of the method required, the most salient features of the HPLC method were considered to be precision, sensitivity, simplicity and large sample through-put capacity.

While microbiological assays are most commonly used for the measurement of OTC in foods, these methods are time consuming, have variable precision and may lack specificity [2–4]. At the other end of the specificity spectrum, LC–MS methodology is capable of detecting and identifying both chlortetracycline (CTC) and its epimer in muscle and kidney [5]. Chromatographic analysis of tetracycline antibiotics in foods was reviewed by Oka et al. [6] and this review included OTC analysis in fish tissue. Additional HPLC methodology not included in this review are Namdari et al. [7], Iwaki et al. [8] and Ueno and Aoki [9]. The majority of the described

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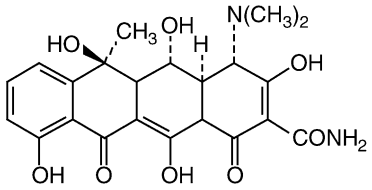

Fig. 1. Molecular structure of oxytetracycline.

methods have long, tedious and costly sample extraction and pre-treatment using liquid–liquid extraction [10,11], solid phase extraction (SPE) [12–16], matrix solid phase dispersion (MSPD) [17] or require additional expensive equipment such as on-line dialysis [18]. Normally these methods also include elution, evaporation and sample resuspension steps. Only one, one-step method has been published for OTC analysis in fish muscle [9]. In that study, the fish muscle was homogenised in 90% methanol containing EDTA followed by centrifugation and filtration of the sample. A recovery of 80% and a limit of quantitation (LOQ) of 0.1 μg/g were reported. However, in-house experience using this method was congealing of the muscle tissue around the Ultra-turrax blade resulting in a lengthy cleaning procedure between every sample homogenisation.

The difficulty associated with oxytetracycline isolation from biological matrices is the propensity of OTC to complex with inorganic ions and bind to protein. As a result, tissue extractants like the addition of EDTA to a mild acidic solvent or an acidic buffer has been formulated to overcome these problems and has been used by the majority of investigators [12,13,15,16,19,20]. In order to increase recovery, multiple extractions are commonly reported using either double or triple extraction. Solid phase extraction and MSPD matrix solid phase dispersion have been used as clean up procedure [12–17]. Solid phase extraction serves not only to remove interfering substances but also to concentrate samples and improve percentage recovery. However, SPE is known to give batch-to-batch variability, column clogging maybe experienced and is invariably time consuming to use, in addition to adding to the cost of sample preparation. While the current state of the art of HPLC analysis for OTC in fish muscle gives high percentage recovery, high precision and sensitivity, these methods have the disadvantage of being long, tedious and often imply costly sample pre-treatment procedures.

The objective of this study was to develop and validate a one-step, rapid, simple and precise method that is sensitive and has a large sample through-put capacity with good linearity.

2. Experimental

2.1. Reagents and chemicals

HPLC grade acetonitrile and dimethylformamide and PA grade disodium hydrogen phosphate monohydrate, potassium nitrate, citric acid monohydrate, trichloroacetic acid and disodium EDTA (Triplex III) were all obtained from E. Merck (Darmstadt, Germany) and OTC hydrochloride from Norsk Medisinaldepot (Bergen, Norway).

2.2. Chromatography

The HPLC system used consisted of a Spectra Physics SP 8800 ternary HPLC-pump (Spectra-Physics, San Jose, CA, USA) connected to a Gilson 234 Autoinjector (Gilson, Middleton, WI, USA) and a Spectra-Physics SP 8480 spectrophotometric detector operating at a wavelength of 353 nm. The integrator was the model SP-4270, from Spectra-Physics. The analytical column was a 150 mm × 4.6 mm Zorbax SB C_{18}, 5 μm (Agilent Technologies, Karlsruhe, Germany) connected to a C_{18} guard column (10 mm × 4.6 mm). A 100 μl sample injection volume was used. The column was operated at ambient room temperature. The mobile phase used was a modification of Knox and Jurand [21] mobile phase and consisted of 65% aqueous phase consisting of 0.001 M EDTA, 0.05 M citric acid, 0.013 M trisodium citrate and 0.1 M potassium nitrate with the addition of 25% dimethylformamide and 10% acetonitrile.

The aqueous and organic phases were mixed and degassing was achieved by sonication for 5 min, followed by a stream of He (1 l/min) for approximately 10 min. The mobile phase flow-rate was 1 ml/min, with a resultant operating pressure of approximately 13.8 MPa (1950 psi). Under these chromatographic operating conditions, the retention time of OTC was approximately 3.40 min.

2.3. Stock solution and standards

The stock 50 μg/ml OTC solution was prepared in millipore HPLC grade water. The working standards were prepared by diluting the stock solution volumetrically in millipore water. As OTC is photolabile, all solution containers were covered in aluminium foil.

Spiked standards were prepared by adding 20 or 40 μl of the OTC working standard to 1 g of Atlantic salmon (*Salmo salar*) muscle tissue and extracting with 980 or 960 μl, respectively, of 0.1 M EDTA MacIlvaine buffer pH 4 (0.1 M citric acid (61.45%) and 0.2 M disodium hydrogen phosphate (38.55%)).

2.4. Sample preparation

The sample used was the edible muscle of Atlantic salmon (*Salmo salar*). To 1 g of sample, 1 ml of 0.1 M EDTA in MacIlvaine buffer (pH 4.0) was added and homogenised using a high-speed blender (Ultra-Turrax, Bioblock, Illkirch, France) until completely homogeneous (approximately 30 s). The sample was centrifuged at 3000 × *g* (Kubota 8800, Kubota Corporation, Tokyo, Japan) for 10 min at 4 °C and the supernatant was removed. An aliquot of 300 μl was pipetted into an Eppendorf and 30 μl of trichloroacetic

acid (24%) was added to precipitate proteins. The sample was vortexed and left at the bench for 30 min in the dark, in order to obtain complete protein precipitation, followed by centrifugation at 16,000 × *g* in a Biofuge A centrifuge (Heraeus, Sepatech, Osterode am Harz, FRG) for 2 min and transfer of the supernatant to the HPLC injection vial.

2.5. Recovery and precision studies

The intra and inter-day precision of the method were examined by analysing five replicates of muscle samples spiked with 0.04, 0.1, 0.4 and1.0 μg/g OTC over three test days. Statistical analysis using the mean, standard deviation (S.D.), percentage relative standard deviation (%R.S.D.) and two-way ANOVA (SPSS.com) were used for data evaluation. The extraction recovery was determined by comparing the area from spiking muscle samples, 0.04, 0.1, 0.4 and 1.0 μg/g OTC with the area resulting from direct injection of standards.

2.6. Calibration and stability

A standard calibration curve for OTC in millipore water in the range 0.04–2.0 μg/ml and a spiked standard calibration curve using OTC-free muscle samples spiked with OTC in the range 0.04–2.0 μg/g were prepared in duplicate. The standard curves were constructed using a scatter plot with known OTC concentration of standard working solution or OTC spiked concentration in fish muscle versus integrated peak area and fitted with a linear regression line. Spiked OTC muscle standards were analysed for stability at the beginning and the end of an 8 h run at room temperature (20 °C).

3. Results and discussion

The chromatographic conditions resulted in a narrow, symmetrical OTC peak and no interfering peaks around the retention time of OTC. Fig. 2 shows typical chromatograms of (A) OTC free muscle tissue; (B) muscle tissue spiked with OTC (1.0 μg/g); and (C) incurred muscle tissue from a pharmacokinetic study in which OTC was orally administered to the fish.

While moderate recovery of 33% was obtained, this recovery was consistently within a narrow range of between 33 and 35% over the concentration range 0.04–1 μg/g (Table 1). This is low compared to previous investigators percentage recoveries of 60–100% [12,14,16–18]. It is suggested that this low percentage recovery was probably attributable to the small volume of buffer used for extraction. However, the precision of the method (Table 1) was found to be similar to that reported by Meinertz et al. [20] for OTC in Atlantic salmon muscle with R.S.D. values of 1.8 and 3.1% at 1.0 and 0.1 μg/g, respectively, by Bjørklund [13] in rainbow trout (*Oncorhynchus mykiss*) muscle with 3% R.S.D at the 1.0 μg/g level and by Moretti et al. [15] in channel catfish (*Ictalurus punctatis*) muscle with a R.S.D. of 6.7% at the 0.5 μg/g level.

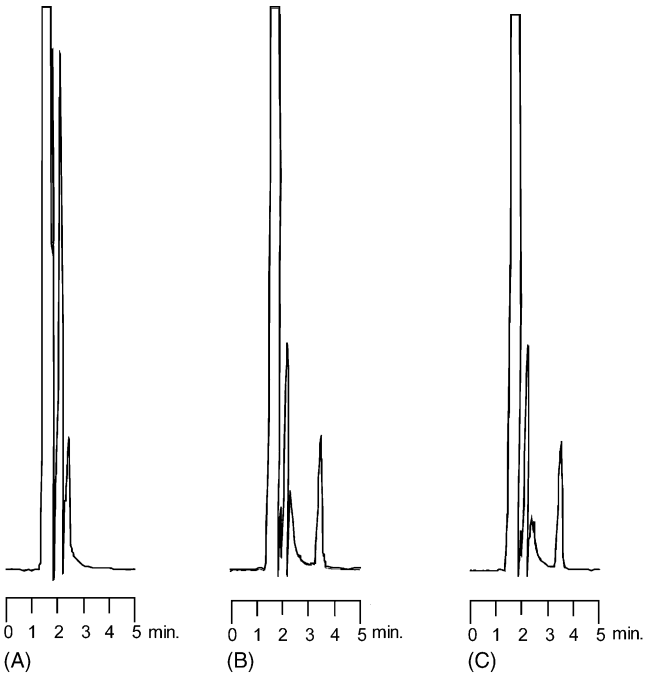

Fig. 2. Chromatograms of extracts from Atlantic salmon muscle: (A) drug free muscle; (B) muscle spiked with 1.0 μg/g of oxytetracycline; (C) muscle sample from fish orally medicated with a daily dose of 75 mg/kg oxytetracycline for 10 days. The sample was obtained 24 h following last medication. Integrator sensitivity: range = 0.002, attenuation = 16.

There was no significant difference (two-way ANOVA) between the OTC concentrations determined on days 1, 2 and 3 for the OTC spiked muscle at 0.04, 0.1, 0.4 and 1.0 μg/g (Table 1). The precision and reliability of the method maybe well be attributable to the small number of sample preparation

Table 1
Recovery and precision of the HPLC assay for OTC in fish muscle

	Day 1	Day 2	Day 3
OTC concentration in muscle (μg/g)	1.0 (n = 4)	1.0 (n = 6)	1.0 (n = 5)
Percent recovery	33	33	33
Mean and S.D.	0.99 ± 0.02	1.0 ± 0.05	1.0 ± 0.03
Precision (%R.S.D.)	1.9	5.4	3.2
OTC conc. in muscle (μg/g)	0.4 (n = 5)	0.4 (n = 5)	0.4 (n = 5)
Percent recovery	33	34	33
Mean and S.D.	0.41 ± 0.02	0.40 ± 0.02	0.41 ± 0.016
Precision (%R.S.D.)	4.8	5.6	3.9
OTC concentration in muscle (μg/g)	0.1 (n = 5)	0.1 (n = 5)	0.1 (n = 5)
Percent recovery	34	34	35
Mean and S.D.	0.12 ± 0.003	0.09 ± 0.007	0.12 ± 0.011
Precision (%R.S.D)	6.4	7.7	9.0
OTC concentration in muscle (μg/g)	0.04 (n = 5)	0.04 (n = 5)	0.04 (n = 5)
Percent recovery	34	33	35
Mean and S.D.	0.040 ± 0.003	0.039 ± 0.003	0.041 ± 0.005
Precision (%R.S.D.)	7.5	7.7	12.1

Percentage standard deviation (%R.S.D.) = standard deviation/mean × 100.

steps involved. Therefore, while a modest percent recovery was obtained in this work, the precision as demonstrated by using %R.S.D. value, was consistent and in agreement with the published literature. Furthermore, OTC was found to be stable in the sample matrix at room temperature (20 °C) as a spiked standard sample of 1.0 μg/g, injected at the start and at the end of a 8 h run gave a difference in integrated peak area of less than 1%.

The linearity of the method was established over the concentration range of 0.04–2.0 μg/g and was found to have a high correlation coefficient (r^2 = 0.999). The least square linear regression analysis of the data was used to calculate the equation of the line ($y = 1.327x + 0.00067$). The limit of quantitation of the method was 0.04 μg/g, which is within the range (0.005–0.1 μg/g) reported by other investigators for OTC in muscle tissue of fish [7–9,12–14,16,17]. The maximum residue limit (MRL) value is set by the EU to 0.1 μg/g totally for OTC and its 4-epimer [22]. However, Blanchflower et al. [5] reported that the epioxytetraycline and the parent compound were not separated using any of the described assays for their LC–MS system and there are currently no published methods specifically designed for the analysis of OTC and epioxytetracycline in fish tissue. Similarly in this work, the HPLC chromatograms gave only one peak with no other peaks visible around the OTC retention time, suggesting that the method presents the sum of the OTC and epioxytetracycline. Therefore, since the EU have recommended that mass related data (LC–MS), in order to increase specificity, is preferred for confirmatory analysis of OTC residues, it is suggested that the HPLC method described in this paper may have a value in screening laboratories. Furthermore, due to the large sample though-put capacity (approximately 60 individual preparation/day), simplicity, precision and linearity of this method, it is well suited for pharmacokinetic studies.

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