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Journal of Chromatography B, 663 (1995) 167–171

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Determination of oxytetracycline in the live fish feed *Artemia* using high-performance liquid chromatography with ultraviolet detection

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First received 31 May 1994; revised manuscript received 19 September 1994

Abstract

A high-performance liquid chromatographic analytical method was developed for the determination of oxytetracycline in *Artemia* nauplii. A solid-phase extraction protocol was used to recover oxytetracycline and the internal standard tetracycline, from the *Artemia* samples. Oxytetracycline was analyzed using a 150 × 4.6 mm I.D. Hypersil-ODS column, a mobile phase of acetonitrile–tetrahydrofuran–0.01 *M* oxalic acid buffer (pH 3.0) (15:3:82, v/v), and an ultraviolet detection wavelength of 365 nm. The calibration curve of oxytetracycline in *Artemia* was linear ($r^2 = 0.9998$) from 0.1 to 6.4 μg/g of tissue. Using a signal-to-noise ratio of 4:1 the oxytetracycline detection limit was 10 ng/g of tissue. Mean recovery of oxytetracycline amounted to 97%, while intra-assay variability was 1.5%. Quantitative data from an in-vivo feeding study indicated an excellent uptake of oxytetracycline by *Artemia*, as its levels reached 25.6 μg per g of nauplii.

1. Introduction

The importance of aquaculture in the provision of fish and other aquatic organisms is continuously increasing [1]. However, intensive culture of fish larvae implicates the problem of infectious diseases [2,3]. Among the bacterial pathogens producing disease in marine fish larvae, the most important are the gram-negative bacteria [1].

A new and efficient method for the prevention of infectious diseases in fish consists of using *Artemia* nauplii, which are used as live feed, supplemented with therapeutic agents by the technique of bioencapsulation [4]. This method has been employed successfully for the encapsulation of sulphonamides in *Artemia* [4,5] and the subsequent delivery of medicated *Artemia* to fish larvae [6,7].

No attempt has been made, however, for the administration of water-soluble antibiotics to *Artemia* nauplii, probably due to their toxicity at the therapeutic doses frequently recommended [3] and their possible leaching into the environment. A simple and effective way of protecting *Artemia* and fish larvae from such toxic effects, as well as minimizing the risk for environmental pollution, could be the use of antibiotic-loaded

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0378-4347/95/$09.50
SSDI 0378-4347(94)00434-X

liposomes [9]. Among the water soluble antibiotics currently used for the treatment of fish diseases, oxytetracycline has been proven to be the drug of choice, as it is highly effective [2].

Since the method of bioencapsulation became feasible only recently [10,11], no analytical methods are available for the determination of oxytetracycline in *Artemia* nauplii and the monitoring of its incorporation efficiency. The method described in this paper uses a solid-phase extraction with reversed-phase liquid chromatography and UV detection at 365 nm.

2. Experimental

2.1. Materials

Oxytetracycline (OTC), tetracycline (TC), ethylenediaminetetraacetic acid (EDTA) and lipase (EC 3.1.4.3.) were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Farmitalia (Carlo Erba, Milano Italy). Ethyl acetate, hexane, tetrahydrofuran (THF) (E. Merck, Darmstadt, Germany) and oxalic acid (Fluka, Buchs, Switzerland) were analytical grade reagents.

2.2. Apparatus

The HPLC system consisted of a Gilson M305 isocratic pump, a Rheodyne 7125 valve injector with a 100-μl loop, an SSI Model 500 UV-Vis variable wavelength detector and a Varian 4290 integrator. The column was a 150 × 4.6 mm I.D. Hypersil octadecylsilane (ODS) with a particle size of 5 μm (Alltech, Deerfield, IL, USA). The mobile phase was acetonitrile–tetrahydrofuran–0.01 *M* oxalic acid buffer pH 3.0 (15:3:82, v/v) and was delivered isocratically at a flow-rate of 1.0 ml/min. The mobile phase was filtered prior to use with an Alltech HPLC solvent filtration system and Alltech 47-mm and 0.45-μm membrane filters. The solid-phase extraction cartridges were C_{18} Extract-Clean from Alltech.

2.3. Preparation of standard solutions and reagents

Stock oxytetracycline and tetracycline solutions of 1 mg/ml were prepared daily by dissolving standard compounds with HPLC grade methanol and diluting to the desired μg/ml levels with methanol. The standard solutions used for the standard curves were freshly prepared by adding 10 μl of 10, 20, 40, 80, 160, 320, 640 μg/ml of oxytetracycline, 10 μl of a 200 μg/ml tetracycline solution in individual vials and diluting them with mobile phase to give a final volume of 1 ml. An aliquot (50–100 μl) from each vial was injected onto the HPLC apparatus and analyzed. To each tissue sample, 100 μl of the internal standard working solution were added, resulting in a concentration of 20 μg per g of tissue.

2.4. Extraction procedure

Samples of *Artemia* nauplii (1 g) were homogenized in 3 ml of ethyl acetate and spiked with the internal standard (20 μg tetracycline). Then, 2 ml of 0.01 *M* EDTA (pH 3.0) were added and the homogenate was transferred to a rotary evaporator flask. After evaporation of the ethyl acetate the aqueous phase was transferred to a vial and was combined with 1 ml of 0.01 *M* EDTA used to wash the rotary evaporator flask. Lipase (10 IU) was added to the samples and they were left overnight at room temperature in the dark, for digestion of the lipids. After centrifuging the samples (5 min at 5000 *g*), the supernatant was applied onto a C_{18} Alltech Extract-Clean column, which was preconditioned with methanol (5 ml), water (5 ml) and 0.01 *M* oxalic acid buffer (pH 3.0) (5 ml). The column was washed twice with 5 ml of *n*-hexane for the removal of lipids and the drugs were eluted with 8 ml of ethyl acetate–acetonitrile (6:2, v/v). The resulting eluent was evaporated to dryness under vacuum (40°C). The residue was reconstituted in 2 ml of mobile phase and a portion (50–100 μl) was analyzed by HPLC.

2.5. Calibration curve, assay precision and recovery

A calibration curve was prepared from *Artemia* nauplii samples (1 g) to which 100 μl of the internal standard solution (200 μg/ml) and 10 μl of the appropriate OTC standard solutions were added to give a final concentration of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μg per g of tissue. The calibration curve was constructed by plotting the ratios of the peak areas of OTC to the peak areas of the internal standard TC, against the known concentrations of OTC. Recovery (%) of oxytetracycline was calculated as (found concentration/added concentration) · 100. Column efficiency (N) and band asymmetry (As) were calculated in the presence and absence of tetrahydrofuran in the mobile phase.

2.6. Bioencapsulation study

Instar I *Artemia* nauplii were kept at a density of 100 individuals/ml in artificial seawater (salinity 35 g/l, pH 8.75) under continuous aeration. Two rations of OTC-loaded liposomes were administered to the nauplii at t_0 and $t_0 + 6$ h, with t_0 being the onset of incubation. A time period of 24 h was allowed for the uptake of the liposomes by the nauplii. A blank was prepared by administrating liposomes containing no OTC to the nauplii under the above described conditions. Then the nauplii were collected, washed thoroughly and stored at −40°C until used for further analysis.

3. Results and discussion

Representative chromatograms of a blank *Artemia* extract and an *Artemia* extract containing oxytetracycline and the internal standard tetracycline are shown in Fig. 1. No endogenous material interfered with either the drug or the internal standard peaks. Oxalic acid has been employed frequently in mobile phases used for the analysis of tetracyclines [12] bearing optimum results. The use of organic modifiers, such as tetrahydrofuran, in the mobile phase has also been reported [13,14]. We found that the use of tetrahydrofuran in the mobile phase improved peak shape of oxytetracycline and tetracycline (Fig. 1). Moreover, the addition of tetrahydrofuran in the mobile phase greatly improved the column efficiency from 2668 plates/m to 11 018 plates/m for oxytetracycline, and had a profound impact on the asymmetry factor of the two peaks, which in the absence of tetrahydrofuran was 2.6 and 2.0 for OTC and TC respectively, while it became 1.5 for both peaks in the presence of tetrahydrofuran.

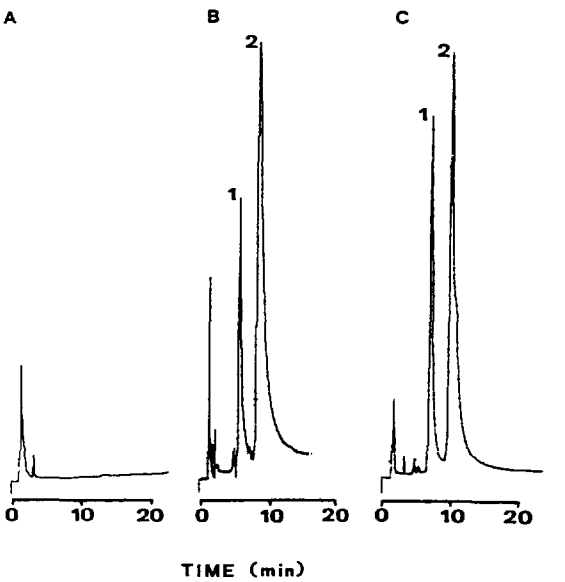

Fig. 1. Representative chromatograms from (A) a blank *Artemia* nauplii extract, (B) and (C) *Artemia* nauplii extract to which OTC has been added (3.0 μg/g). Chromatographic conditions: (A,C): Hypersil-ODS 5 μm (150 × 4.6 mm I.D.) column; mobile phase acetonitrile–THF–0.01 *M* oxalic acid buffer pH 3.0 (15:3:82, v/v); flow-rate 1.0 ml/min; ultraviolet detection wavelength 365 nm. (B): mobile phase acetonitrile–0.01 *M* oxalic acid buffer pH 3.0 (15:85, v/v). All other conditions as above. Peaks: 1 = tetracycline, 2 = oxytetracycline.

The equation of the standard curve obtained by analyzing spiked samples was $y = 0.017 + 0.047x$ ($r^2 = 0.9998$). The corresponding equation for the standard curves obtained by direct injection of the analyte plus the internal standard (in mobile phase) was $y = 0.016 + 0.046x$ ($r^2 =$

0.9992). The fact that the slopes of the two sets of standard curves are in good agreement suggests a similar behaviour of the drug and its internal standard in the course of sample pretreatment. Moreover, tetracycline has previously been reported to be an appropriate internal standard for the analytical determination of oxytetracycline in fish [15]. It has been reported that the C_{18} solid-phase extraction cartridges are suitable for the extraction of tetracyclines from fish tissues [14,15] and from milk using matrix solid-phase dispersion (MSPD) [16]. These cartridges were found to be well suited to the analysis of OTC in *Artemia* nauplii under the conditions described, as they provided excellent recovery data. The results for the standard addition/recovery of oxytetracycline from spiked samples are listed in Table 1. The mean recovery of OTC was found to be 97% over the concentration range 0.1–6.4 μg per g of tissue. It has been reported [17] that the use of ethyl acetate in combination with phenylbutazone greatly improves the extraction of tetracyclines. However, when phenylbutazone was added during the extraction procedure of *Artemia*, it resulted in the crystallization of the samples upon storage at 4°C. Thus the phenylbutazone treatment was not found to be suitable for the extraction of OTC from *Artemia* samples. The recovery of OTC compares favourably with those previously reported for fish muscle [18,19]. The intra-assay coefficient of variation was found to be 1.5%. A 0.8 μg per g of tissue *Artemia* extract was injected six times. The average calculated concentration was 0.81 μg per g of tissue. Using a signal-to-noise ratio of 4:1, the detection limit was 10 ng per g of tissue.

The bioencapsulation study was used in conjunction with the HPLC assay method to determine the efficacy of liposome-loaded *Artemia* nauplii to serve as carriers of water soluble antibiotics to fish larvae, minimizing the risk of leaching or decomposition of the drugs in the environment. The nauplii appeared to assimilate the liposomes present in their incubation medium and when observed under a microscope, their gut was loaded with liposomal vesicles. The use of different concentrations of liposomal OTC in the enriching medium resulted in an increase of OTC levels in the animals (Fig. 2). It has been reported that 70 to 80% of the orally administered OTC remains in the environment and there is a high persistence of antibiotics in sediments from fish farms [13]. Thus, a method for the safe delivery of water soluble drugs, such as tetracyclines, to fish larvae appears to be of essential importance.

The fact that the OTC levels observed in

Table 1
Standard addition/recovery of OTC in *Artemia* nauplii

Concentration (μg/g tissue)		Recovery (mean ± S.D.) (%)	C.V. (%)
Added	Found (mean ± S.D.)		
0.10	0.09 ± 0.01	98.2 ± 18.3	8.35
0.20	0.19 ± 0.01	95.8 ± 8.9	9.29
0.40	0.39 ± 0.04	98.1 ± 10.7	10.9
0.80	0.79 ± 0.05	99.1 ± 6.3	6.35
1.60	1.49 ± 0.06	93.6 ± 4.6	4.91
3.20	3.09 ± 0.18	96.8 ± 5.8	5.99
6.40	6.14 ± 0.23	96.0 ± 3.7	3.85

Correlation coefficient of standard curve = 0.9998
Inter-assay variability (%) = 7.1 ± 2.5
Intra-assay variability (%) = 1.5

Recovery (%) was taken as (found concentration/added concentration) · 100. Each value is the mean of five determinations at each sample concentration. The values in parentheses are the coefficients of variation (%).

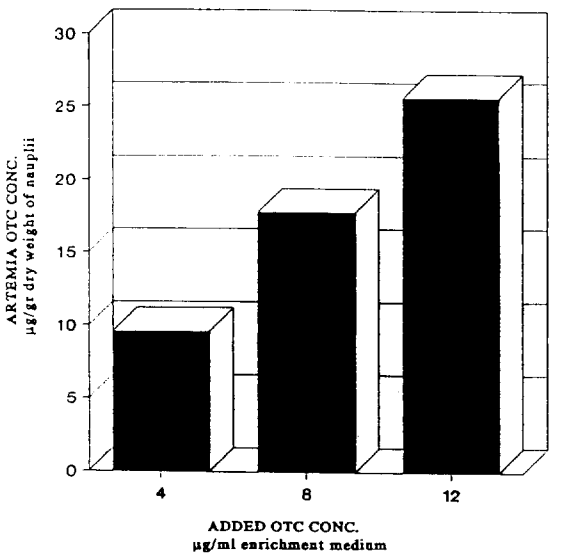

Fig. 2. Relationship between levels of OTC in *Artemia* nauplii and concentration of OTC in the enrichment medium. Each value is the mean of three determinations.

Artemia nauplii were quite satisfactory, favours an optimistic prediction regarding the efficacy of the bioencapsulation method using liposomes as a tool for prophylactic or therapeutic chemotherapy in larviculture. Work is in progress to extend the present analytical method to the analysis of OTC in fish larvae fed either directly or indirectly through *Artemia*, on OTC-loaded liposomes.

Acknowledgement

This study was supported by European Community AIR2 Research grant No. CT 93-1600.

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