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Note

Determination of oxytetracycline in fish by high-performance liquid chromatography

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The use of antibiotics for therapy of bacterial fish diseases may lead to problems with residues in medicated fish and in the environment. Thus adequate methods for analysis of antibiotics are needed.

The aim of this study was to develop a simple high-performance liquid chromatographic (HPLC) method, which incorporates an internal standard, for determining oxytetracycline concentrations in serum and tissues of rainbow trout. The commonly used bacterial bioassay methods for determination of antibiotics are sensitive but unspecific, and they involve the risk of false positive results.

Several studies on HPLC separation of tetracyclines, their degradation products and impurities have been published [1–6]. Many methods have also been described for the HPLC determination of tetracyclines from urine, blood and tissues of humans and other mammals [7–15] and a few for tetracyclines in fish tissues [15,16]. None of these methods, however, involves the use of an internal standard to determine drug concentrations.

In the described method tetracycline is used as an internal standard.

EXPERIMENTAL

Chemicals and reagents

Methanol and acetonitrile (J.T. Baker, Deventer, The Netherlands) were of HPLC grade. Citric acid, disodium hydrogenphosphate, N,N-dimethylformamide, disodium ethylenediaminetetraacetate (Na_2EDTA) and oxalic acid (Merck, Darmstadt, F.R.G.) were analytical-grade reagents. The water used was distilled and purified with a Milli-Q reagent-grade water system (Millipore, Molsheim,

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France). Pure oxytetracycline, tetracycline, chlortetracycline and doxycycline, as their hydrochlorides, were purchased from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The HPLC system consisted of a Spectroflow 400 pump connected to a Spectroflow 757 variable-wavelength absorbance detector (Kratos, Ramsey, NJ, U.S.A.). The detector response was monitored with a Shimadzu C-R3A Chromatopac integrator (Kyoto, Japan). The samples were injected with a Rheodyne 7125 loop injector (Cotati, CA, U.S.A.). The analytical column (100 mm × 4.6 mm I.D.) and the guard column (30 mm × 4.6 mm I.D.) were packed with Spheri 5-μm reversed-phase octadecylsilica gel (Brownlee Labs., Santa Clara, CA, U.S.A.).

Standards

Methanolic stock solutions of the four tetracyclines were prepared at a concentration of 1 mg/ml and stored in the dark at $-20°C$. Working standards were prepared daily by dilution from the stock solutions.

Operating conditions

The mobile phase consisted of acetonitrile, N,N-dimethylformamide and 0.01 *M* aqueous oxalic acid (pH 2.1). The oxalic acid was passed through a 0.22-μm filter (Millipore). The mobile phase was degassed under reduced pressure and maintained at 25°C during the analysis. The flow-rate was 1 ml/min and the UV detector was set at 355 nm and 0.02 a.u.f.s. The sample volume injected on the column was 20 μl. The guard column was changed at intervals of 150–200 sample injections.

Conditioning of columns

When injected on new, unconditioned HPLC columns the tetracyclines were adsorbed to the column packing material and appeared as strongly tailing peaks. Therefore the new columns were conditioned by flushing with acetonitrile (2 h), an aqueous solution of 0.05 *M* Na_2EDTA adjusted to pH 7.0 with 1 *M* sodium hydroxide (10 h), acetonitrile–water (50:50, v/v) containing 0.1 mg/ml chlortetracycline (2 h), acetonitrile–water (50:50, v/v) (2 h) and the mobile phase (2 h).

Extraction and clean-up procedure

Serum samples (500 μl) from rainbow trout were spiked with the internal standard (0.5 μg tetracycline). A 10-ml volume of cold (+4°C) extraction buffer, containing 0.1 *M* citric acid and 0.2 *M* disodium hydrogenphosphate (62:38) (pH 4.0), was added. The samples were shaken and sonicated for 5 min and allowed to stand for 15 min at +4°C. The mixtures were transferred to Bond Elut C_{18} solid-phase extraction columns (Analytichem International, Harbor City, CA, U.S.A.), which had been prepared by flushing with 5 ml of methanol and 10 ml of 5% (w/v) aqueous Na_2EDTA. After the samples had been loaded, the columns were flushed with 30 ml of water, which was discarded. The samples were eluted

with 10 ml of 0.01 *M* methanolic oxalic acid and evaporated at 35°C under reduced pressure to 500 μl. After centrifugation for 2 min at 11 000 *g*, 20-μl aliquots were injected into the HPLC system.

Liver samples (2.0 g) from rainbow trout were spiked with the internal standard (4.0 μg of tetracycline) and homogenized on ice in 20 ml of cold extraction buffer using a high-speed blender. The samples were sonicated for 5 min and centrifuged for 15 min at 2500 *g*. The supernatants were passed through paper filters (MN 615, Macherey-Nagel, Düren, F.R.G.). The extraction was repeated twice and the filtrates were loaded on to Bond Elut C_{18} columns. The preparation and elution of the Bond Elut C_{18} columns and the subsequent treatment of the liver samples were done as previously described for serum samples.

Muscle samples (5.0 g) from rainbow trout were spiked with the internal standard, equivalent to 5 μg of tetracycline, and processed in the same way as the liver samples.

Calibration and recovery

Standard calibration curves for oxytetracycline in the range 0.2–20 μg/g were prepared in six replicates using drug-free serum, liver and muscle tissue from rainbow trout and tetracycline as the internal standard. Standard curves were drawn by plotting the known oxytetracycline concentrations against the ratio of oxytetracycline to internal standard peak height. Regression analysis was employed to determine the slope, intercept and correlation coefficient for oxytetracycline standard curves in serum, liver and muscle tissue. The oxytetracycline concentration in unknown samples was read from these standard curves.

The extraction recoveries of oxytetracycline, tetracycline, chlortetracycline and doxycycline were determined by comparing peak heights from the analysis of serum spiked at 1 μg/ml and liver and muscle tissue samples spiked at 1 μg/g with peak heights resulting from direct injection of methanolic standards.

RESULTS AND DISCUSSION

Chromatography

Oxytetracycline, tetracycline, chlortetracycline and doxycycline were separated by the described HPLC method using the mobile phase acetonitrile–N,N-dimethylformamide–0.01 *M* oxalic acid (22:6:72) pH 2.1 (Fig. 1A). For measuring oxytetracycline concentrations in fish tissues, with tetracycline as an internal standard, the mobile phase was acetonitrile–N,N-dimethylformamide–0.01 *M* oxalic acid (27:6:67) pH 2.1 (Fig. 1B). The two mobile phase compositions separated oxytetracycline and tetracycline equally well. The latter composition, however, gave sharper peaks with less tailing, and thus showed a better correlation between peak-height and drug concentration.

No endogenous material interfered with the drug peaks. The retention times for oxytetracycline varied from 3.1 to 3.4 min and that of the internal standard, tetracycline, from 3.8 to 4.1 min. Typical chromatograms of fish tissues are shown in Fig. 2.

The precision of the method was tested by analysing a sample eight times in

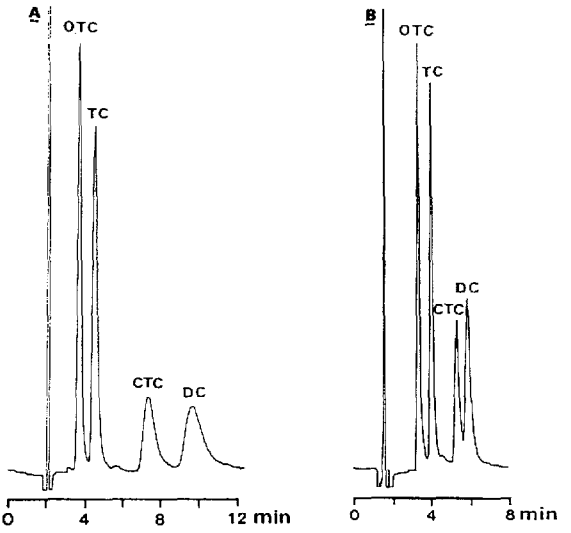

Fig. 1. HPLC separation of methanolic tetracycline standards, 0.1 μg of each. (A) Mobile phase, acetonitrile–N,N-dimethylformamide–0.01 *M* oxalic acid (22:6:72) pH 2.1; (B) mobile phase acetonitrile–N,N-dimethylformamide–0.01 *M* oxalic acid (27:6:67) pH 2.1. Peaks: OTC = oxytetracycline; TC = tetracycline; CTC = chlortetracycline; DC = doxycycline. For chromatographic conditions, see text.

the same run and over six days in separate runs. The intra-run relative coefficient of variation for the oxytetracycline peak height was 1.0% and for the tetracycline peak height 0.8%. The inter-run relative coefficient of variation was 4.7% for oxytetracycline and 4.1% for tetracycline.

Tetracycline proved to be an appropriate internal standard. Chromatograms of tissue samples spiked only with tetracycline gave no additional peaks, possibly resulting from impurities or epimerization products, that could interfere with the oxytetracycline peaks. Neither did any such peaks appear in chromatograms of tissues from fish treated with oxytetracycline at the tetracycline retention time. It is known that some tetracycline preparations can contain small amounts of other tetracycline compounds as impurities [3,11]. The veterinary preparations of oxytetracycline administered orally to cultured fish in Finland [Terramycin® (Pfizer, Karlsruhe, F.R.G.) and Orimycin® (Orion, Espoo, Finland] are, however, of the same purity used in preparations for humans, and do not contain related compounds in amounts of significant importance for the HPLC assay.

The detection limit of the described method was 0.05 μg/ml for rainbow trout serum, 0.05 μg/g for muscle tissue and 0.1 μg/g for liver.

It has been reported that Bond Elut C_{18} solid-phase extraction cartridges cannot be used successfully for the extraction of tetracyclines from fish tissues [15,17]. However, these cartridges were well suited to the analysis of tetracyclines in fish tissues under the conditions described. The recovery rates of the four tetracyclines from spiked rainbow trout serum and tissues are listed in Table I.

The standard calibration curves for oxytetracycline in rainbow trout serum and muscle were linear at least from 0.2 to 10 μg/g and in liver at least over the range

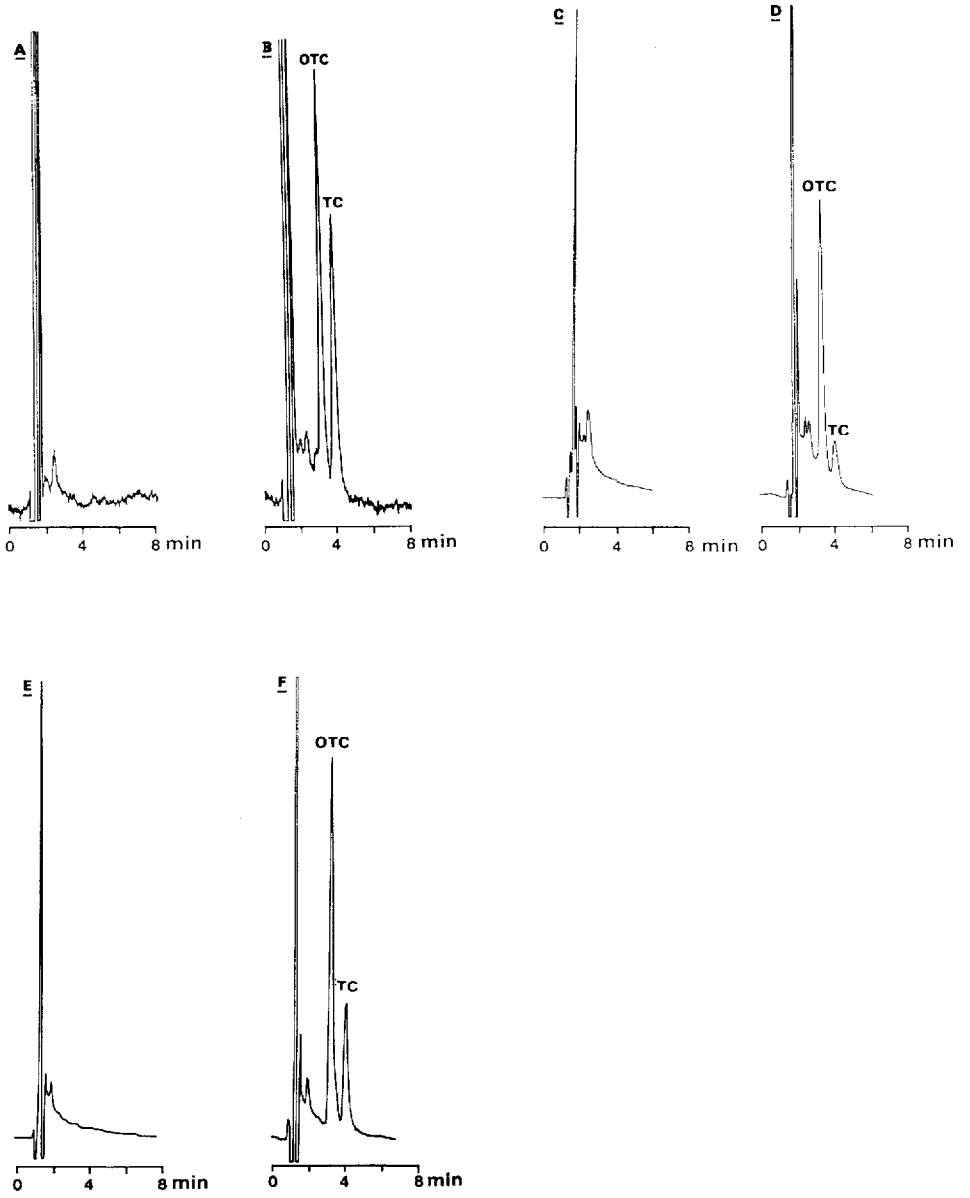

Fig. 2. Chromatograms of rainbow trout tissues. (A) Untreated serum sample; (B) serum sample (500 μl) from a rainbow trout sixteen days after a single oral dose of oxytetracycline (75 mg/kg body weight); the sample was spiked with 0.5 μg of internal standard, tetracycline; (C) untreated liver sample; (D) liver sample (2.0 g) from a rainbow trout four days after a single oral dose of oxytetracycline (75 mg/kg); the sample was spiked with 4.0 μg of internal standard; (E) untreated muscle sample; (F) muscle sample (5.0 g) from a rainbow trout sixteen days after a single oral dose of oxytetracycline (75 mg/kg); the sample was spiked with 5.0 μg of internal standard. Peaks: OTC = oxytetracycline; TC = tetracycline.

TABLE I

RECOVERY OF TETRACYCLINES FROM SPIKED RAINBOW TROUT TISSUES

Serum (500 μl) was spiked with tetracyclines at 1 μg/ml. Liver (2.0 g) and muscle (5.0 g) were spiked with tetracyclines at 1 μg/g. OTC = oxytetracycline; TC = tetracycline; CTC = chlortetracycline; DC = doxycycline. Values in parentheses are coefficients of variation (%).

Tissue	n	Recovery (%)			
		OTC	TC	CTC	DC
Serum	6	91.3 (4.1)	79.7 (4.1)	94.1 (3.3)	96.6 (2.5)
Liver	6	95.2 (2.3)	77.0 (4.3)	82.5 (3.4)	85.2 (2.7)
Muscle	8	87.6 (3.0)	78.1 (3.8)	78.3 (4.3)	87.1 (3.2)

0.2–20 μg/g. The equation of the regression line for the serum standard curve was $y = 1.376x - 0.029$, for the liver curve $y = 1.001x - 0.102$ and for the muscle curve $y = 1.610x - 0.054$. The correlation coefficient for the serum standard curve was 0.9997, for the liver curve 0.9991 and for the muscle curve 0.9999; thus the linearity is good.

The separation efficiency of the analytical column under the described conditions was still good after 1100 sample injections.

In conclusion, the HPLC method described is simple, rapid and sensitive, and thus suitable for pharmacokinetic and residue studies on oxytetracycline in fish.

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