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Note

Determination of oxytetracycline in plasma from rainbow trout using high-performance liquid chromatography with ultraviolet detection

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Oxytetracycline is one of the most common drugs administered against bacterial infections in fish farming. In order to find optimum conditions for treatment with oxytetracycline, pharmacokinetic studies in fish have to be performed. This requires a selective and rapid method for the analysis of large series of plasma samples. Several methods have been described for the determination of tetracyclines in blood, serum and plasma of humans and animals using high-performance liquid chromatography (HPLC) with C_{18}, C_8 and phenyl columns [1–3]. These methods, however, were not found to be suitable for the determination of oxytetracycline in plasma samples from rainbow trout, owing to endogenous compounds in fish plasma that coeluted with oxytetracycline.

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Sample work-up procedures described for the determination of oxytetracycline in fish and animal tissue [4–6] were too complicated and time-consuming for the analysis of oxytetracycline in plasma.

This paper describes a rapid and selective method for the determination of oxytetracycline in plasma from rainbow trout, based on protein precipitation with trifluoracetic acid (TFA) and HPLC analysis on a cyano column. Up to 80–90 samples can be analysed per day and the method is well suited for pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Oxytetracycline hydrochloride was supplied by the Norwegian Medicinal Depot (Oslo, Norway). Trifluoroacetic acid was of Uvasol grade from E. Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade from E. Merck.

Chromatographic conditions and apparatus

The analyses were performed on a Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.), consisting of a Model 590 pump and a Model Wisp-712 auto-injector with a cooling module. The injector was operated at 15°C. The detector was a Model Lambda-max 481 equipped with a 14-μl flow-cell and was operated at 350 nm. Peak heights were calculated on an SP 4270 integrator (Spectra Physics, San Jose, CA, U.S.A.). The attenuation was 8 mV.

The column was a 5 μm Cyano Spheri-5 cartridge MPLC™ (100 mm × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.) equipped with a 5 μm Cyano Spheri-5 MPLC precolumn (30 mm × 4.6 mm I.D.).

The mobile phase was 0.02 *M* oxalic acid (pH 2)–methanol–N,N-dimethylformamide (DMF) (950:50:50, v/v). The pH of the oxalic acid solution was adjusted with a 25% (w/v) ammonia solution. The mobile phase was filtered and degassed prior to use.

The chromatographic experiments were performed at room temperature (22–23°C) and at a flow-rate of 1.0 ml/min.

Plasma samples

Blood samples were collected from the caudal vein, using Venoject blood sampling equipment (40 × 0.9 mm needles, 10-ml tubes) with heparin. Plasma was isolated by centrifugation of the blood at 13 840 *g* for 10 min and stored at −20°C.

Sample pretreatment

TFA (1.5 parts) was added to the plasma (10 parts). The samples were kept in a water-bath at 37°C for 10 min to obtain complete precipitation. The tubes

were agitated in a Whirlmixer for 10 s and centrifuged for 10 min at 13 840 *g*, and 20–100 μl of the supernatant were injected onto the column.

Standard solutions

Standard solutions in plasma (0.2–3.0 μg/ml) were prepared by spiking drug-free plasma with a stock solution of oxytetracycline (1 mg/ml) in distilled water. The calibration line was based on peak-height measurements. The linearity was checked in the range 0.2–3.0 μg/ml. The correlation coefficient was 0.9992. A typical equation of the calibration line was $y = 2.34x + 0.013$. Owing to the good and reproducible recoveries, an internal standard was not incorporated. The retention time of oxytetracycline was 4.3 min.

RESULTS AND DISCUSSION

The selectivity for oxytetracycline on a C_8 or C_{18} column was not satisfactory owing to coeluting endogenous compounds. These compounds were not retained on a cyano column (Fig. 1) and good selectivity was obtained. Oxytetracycline was eluted as an asymmetrical peak with a mobile phase of 0.02

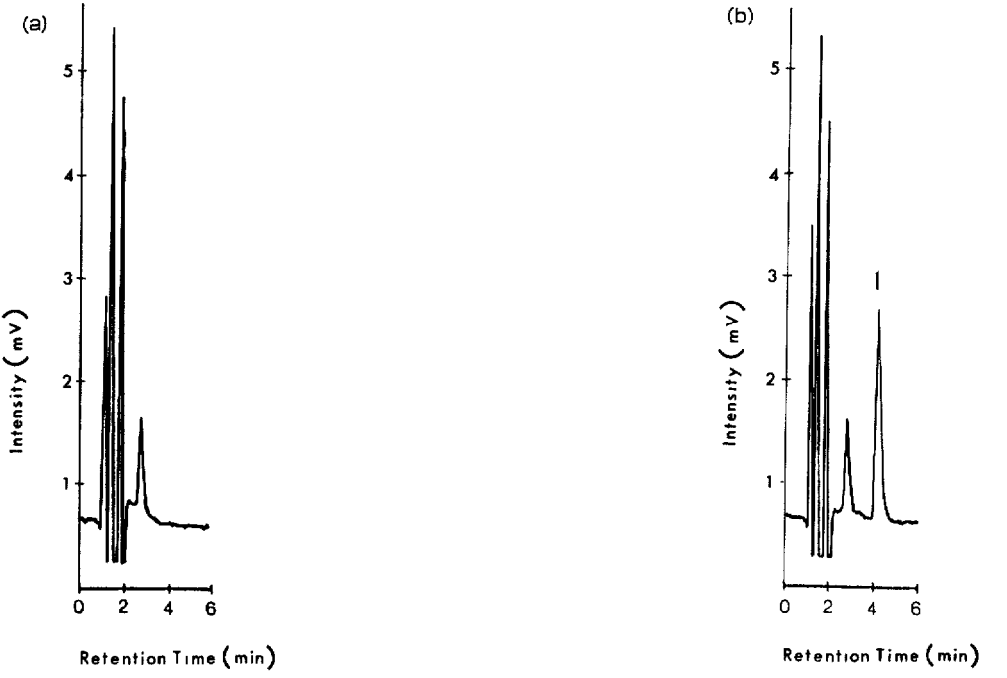

Fig. 1. Chromatograms obtained using a cyano column for the analysis of (a) drug-free plasma from rainbow trout and (b) plasma from rainbow trout spiked with 1.0 μg/ml oxytetracycline. Peak 1 = oxytetracycline (retention time = 4.3 min). For chromatographic conditions see text.

M oxalic acid–methanol (950:50, v/v). Oxalic acid has been reported to increase the peak symmetry of tetracyclines on C_{18} and C_8 columns [7], and DMF has been reported to be a more effective organic modifier than acetonitrile or methanol [8,9]. The asymmetry factor (A_s) decreased from 3.0 to 1.3 when 2.5–5.0% (v/v) of DMF was added to the mobile phase. Variation of the oxalic acid concentration between 0.01 and 0.05 *M* and the pH between 1.0 and 6.0 showed no effect on the peak shape. The capacity ratio (k'), however, showed an optimum at pH 2.0 and decreased slightly with increasing concentration of oxalic acid. The optimum conditions with respect to k' and A_s were obtained with a mobile phase of 0.02 *M* oxalic acid–methanol–DMF (950:50:50, v/v), and this mobile phase was used for further work.

TFA was chosen as the deproteinizing agent because tetracyclines are more stable in TFA solution than in perchloric acid solution [2]. The recovery of oxytetracycline from rainbow trout plasma was evaluated by analysing plasma spiked with oxytetracycline at three different concentrations (0.4, 0.7 and 1.2 μg/ml) ($n=6$). The recovery varied between 97.9 and 98.8%, with coefficients of variation (C.V.) between 4.2 and 5.2%. Plasma samples stored at −20°C showed no degradation after ten weeks.

The temperature in the autosampler operated without a cooling module was 30–35°C. The stability of oxytetracycline plasma samples (0.4, 0.7 and 1.2 μg/ml) stored in the autosampler at 35 and 15°C was studied by repeated injections over 10 h. Approximately 14% degradation was observed for all samples stored at 35°C for 10 h. No degradation was observed when the samples were stored at 15°C. An operating temperature of 15°C in the autosampler is therefore recommended.

The precision of the method was evaluated by determining the intra-day variations by analysing plasma spiked with oxytetracycline (0.4, 1.2 and 3.0 μg/ml) and by analysing real samples (0.3 and 0.8 μg/ml). It was also investigated for different plasma volumes (100, 250, 500 and 1000 μl). The intra-assay C.V. varied between 1.5 and 4.5% ($n=6$). The detection limit at a signal-to-noise ratio of 3.0 was 4 ng of oxytetracycline.

After ca. 400 injections of plasma samples the pre-column was saturated with plasma components and gave a drifting baseline. The analytical column was used for more than 3000 injections of plasma samples with no change in column performance. Variation in performance of different cyano columns delivered from the same manufacturer has been observed. The optimum conditions were, however, easily obtained by changing the concentration of DMF, the amount of methanol or the amount of oxalic acid solution in the mobile phase.

Fig. 2 shows an example of an absorption curve obtained after an oral dose of 150 mg/kg. Oxytetracycline was given to rainbow trout in a gelatine capsule, which was implanted into the stomach. The absorption study was performed in fresh water at 8°C. Blood samples were taken from five fish per day and the results are given as the mean value.

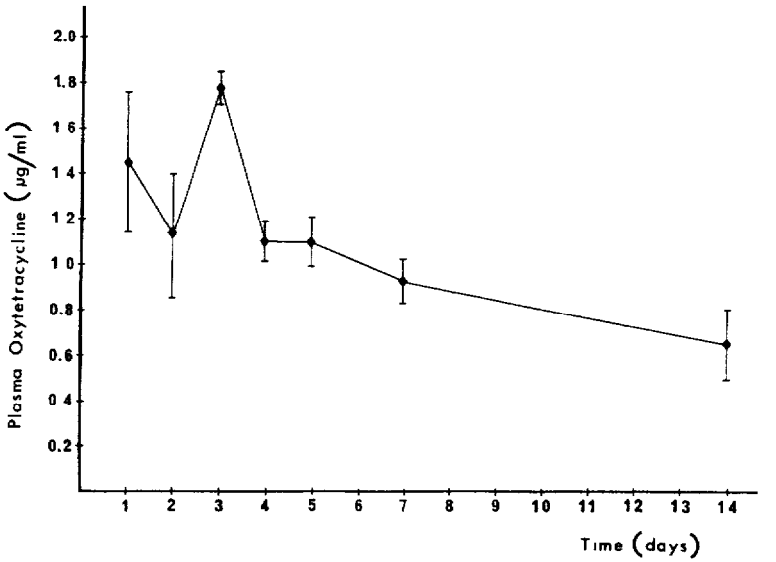

Fig. 2. Plasma levels of oxytetracycline in rainbow trout after an oral dose of 150 mg/kg oxytetracycline. The results are given as the mean value of five fish. The error bars are included and are calculated as the standard error of the mean.

The method has been used routinely in our laboratory for more than a year in a study of the pharmacokinetics of oxytetracycline in rainbow trout. Up to 80–90 samples have been analysed per day. The method is selective, reliable, reproducible and rapid. Plasma samples down to 100 µl may be analysed, which makes the method suitable for pharmacokinetic studies in fish of nearly every size.

Drug-free plasma from salmon has also been analysed according to the method described. The chromatograms showed no endogenous compounds that coeluted with oxytetracycline.

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