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Analysis of oxolinic acid in fish by high-performance liquid chromatography

HARRY V. BJÖRKLUND

Institute of Parasitology, Åbo Akademi, Porthansgatan 3, SF-20500 Åbo (Finland)

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ABSTRACT

A simple high-performance liquid chromatographic method for assaying oxolinic acid, a chemotherapeutic agent, in fish tissues has been developed. Nalidixic acid is used as an internal standard. The drugs are separated on an internal surface reversed-phase column. The sample clean-up is minimized. Serum samples are analysed by direct injection on the column; muscle and liver samples are analysed after solid-phase extraction. The recoveries of oxolinic acid from spiked rainbow trout serum, muscle tissue and liver are 99.7, 87.7 and 83.6%, respectively. The lowest measurable amount of the drug is 0.01 μg/g in all three tissues.

INTRODUCTION

Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid) is a chemotherapeutic agent used in serveral countries in the treatment of fish diseases caused by gram-negative bacteria. The use of chemotherapeutic agents in food-producing animals makes it necessary to develop adequate methods for pharmacokinetic studies and for analysis of drug residues in animal tissues.

Oxolinic acid has been determined by reversed-phase high-performance liquid chromatography (HPLC) using octyl or octadecyl silane columns [1–8]. Conventional reversed-phase columns are easily damaged by denaturating proteins and impurities in the injected samples. Therefore most of these methods involve tedious and time-consuming extraction procedures, such as liquid–liquid partition, and evaporation of large volumes of solvent prior to sample injection. Recently a method for analysing oxolinic acid in fish serum has been developed [9], based on direct plasma injection and on-line sample clean-up on a precolumn involving a column-switching technique. This advanced method, however, requires special instrumentation and large sample series in order to be effectively used.

The purpose of the present work was to develop a simple and rapid HPLC method for determination of oxolinic acid in fish serum and tissues. The internal surface reversed-phase (ISRP) analytical column used minimizes sample clean-up. Serum samples can be injected directly on the HPLC column without ex-

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traction or protein precipitation procedures [10–12]. The ISRP concept is based on size exclusion and internal surface partitioning. The packing material of the column consists of porous spherical silica particles. The stationary phase, glycine-phenylalanine-phenylalanine, is located in narrow pores on the inside of the particles. The outer surface of the particles, covered with glycine residues, is non-adsorptive. Thus macromolecules do not have access to the stationary phase but pass through the column unretained. Small molecules, such as drugs and their metabolites, penetrate the pores and are retained and separated by the stationary phase.

A time-saving solid-phase extraction method is described for analysing oxolinic acid in fish liver and muscle tissue.

EXPERIMENTAL

Chemicals

Acetonitrile, 2-propanol and methanol (Merck, Darmstadt, F.R.G.) were of HPLC grade. Potassium dihydrogenphosphate, sodium hydroxide, hydrochloric acid and orthophosphoric acid (Merck) were analytical-grade reagents. The water used in buffers and eluents was distilled and purified with a Milli-Q reagent-grade water system (Millipore, Molsheim, France). Oxolinic acid and nalidixic acid were purchased as pure standards from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The HPLC system consisted of a Spectroflow 400 pump, a Spectroflow 757 variable-wavelength absorbance detector (Kratos, Ramsey, NJ, U.S.A.) and 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, a Regis Pinkerton GFF 5 μm ISRP, 150 mm × 4.6 mm I.D. (Regis, Morton Grove, IL, U.S.A.), was equipped with a Regis ISPRP guard column and a Rheodyne column inlet filter, 0.5 μm × 3 mm.

Standards

Stock solutions of oxolinic acid and nalidixic acid were prepared at a concentration of 1 mg/ml in 0.03 *M* aqueous sodium hydroxide. Working standards were prepared by 1:10 dilution in acetonitrile or 1:100 dilution in water. The standards were stored in dark at +4°C.

Extraction and recovery

Unheparinized blood samples from rainbow trout (*Salmo gairdneri* R.) were allowed to clot for 30 min at room temperature and were then centrifuged for 2 min at 15 000 *g*. Serum samples (100 μl) were spiked with internal standard (0.1 μg nalidixic acid), shaken and centrifuged for 2 min at 20 000 *g*. Aliquots of 10 μl were injected into the HPLC column without extraction or deproteinization.

Muscle samples (5 g) from rainbow trout were spiked with internal standard, equivalent to 5 μg nalidixic acid. The samples were homogenized with a high-speed blender (Bamix, Switzerland) in 20 ml of 0.1 *M* aqueous potassium dihydrogenphosphate. Different pH values of the phosphate buffer, adjusted with 5 *M* sodium hydroxide, were tested in order to obtain the best recovery of the drugs. After sonication for 5 min in a bath sonicator (Telsonic, Switzerland) the samples were centrifuged for 15 min at 2500 *g*. The supernatants were passed through paper filters (MN 615, Macherey-Nagel, Düren, F.R.G.), and the muscle tissues were re-extracted twice with 20 ml of phosphate buffer. The combined and filtered supernatants where then purified and concentrated by passing through Bond Elut 6 ml solid-phase extraction cartridges (Analytichem International, Harbor City, CA, U.S.A.), attached to a Amprep vacuum Manifold-10 system (Amersham International, Amersham, U.K.). Two different types of extraction cartridge, C_2 and C_{18}, were tested. Before use they were flushed with 5 ml of methanol and 5 ml of phosphate buffer. After the samples had been passed, the extraction columns were flushed with 10 ml of water and the drugs were eluted with 5 ml of methanol–1 *M* aqueous orthophosphoric acid (90:10, v/v). The eluates were evaporated at 35°C under reduced pressure to 500 μl. After centrifugation for 2 min at 20 000 *g*, 10-μl aliquots were injected into the HPLC column.

Liver samples (2 g) from rainbow trout were spiked with 4 μg of internal standard, homogenized and extracted in the same way as samples from muscle tissue.

The recoveries of oxolinic acid and nalidixic acid from rainbow trout tissues were determined by comparing peak heights obtained by chromatography of spiked and extracted tissues with peak heights obtained by chromatography of pure drug standards.

Chromatographic conditions

The mobile phase consisted of 0.1 *M* aqueous potassium dihydrogenphosphate and acetonitrile. Different proportions of acetonitrile and different pH values of the mobile phase (adjusted with 1 *M* hydrochloric acid or 5 *M* sodium hydroxide) were tested in order to optimize the chromatographic conditions. The phosphate buffer was filtered through a Millipore GF 0.22-μm filter. The mobile phase flow-rate was 1 ml/min. The detector measured peak heights at 254 nm and 0.02 a.u.f.s. The chromatograph was operated at ambient temperature. Samples of 10 μl were injected into the column. The column inlet filter and the guard column were changed at intervals of 150–200 sample injections.

Standard graphs

Standard calibration graphs for oxolinic acid were obtained with six replicates of spiked drug-free serum, muscle tissue and liver from rainbow trout. Nalidixic acid was used as internal standard. Standard graphs were drawn by plotting known oxolinic acid concentrations against oxolinic acid/internal standard peak

heights. The slopes and correlation coefficients for the oxolinic acid standard graphs of serum, muscle tissue and liver were determined by linear regression analysis. The oxolinic acid concentration in analysed fish samples were read from these standard graphs.

RESULTS AND DISCUSSION

Extraction and recovery

The recoveries of oxolinic acid and nalidixic acid from spiked (1 μg/ml, $n=6$) rainbow trout serum after direct sample injection on the HPLC column were 99.7 and 89.3%, respectively. The respective coefficients of variation (C.V.) were 3.9 and 3.4%.

The recoveries of oxolinic acid and nalidixic acid from spiked rainbow trout muscle tissue, using two different solid-phase extraction cartridges and different pH values of the phosphate buffer used for extraction, are shown in Table I. The best recoveries of the drugs were obtained with a C_{18} cartridge in the pH region 7–8. At pH 8, however, the cartridges became clogged with particulate matter in the extracts. Use of phosphate buffer at pH 7 solved this problem. Thus phosphate buffer (pH 7) and C_{18} extraction cartridges were chosen for extraction of the drugs from muscle tissue.

Three extraction cycles, each with 20 ml of phosphate buffer, were required in order to obtain good recovery of the drugs. With one, two and three extraction cycles the recoveries were 50, 75 and 88%, respectively.

The recoveries of oxolinic acid and nalidixic acid from rainbow trout liver are shown in Table II. At pH 8 liver samples also caused clogging of the cartridges.

TABLE I

RECOVERY OF OXOLINIC ACID AND NALIDIXIC ACID FROM SPIKED RAINBOW TROUT MUSCLE TISSUE

Samples from muscle tissue were spiked with oxolinic acid (1.0 μg/g) and nalidixic acid (1.0 μg/g). OA = Oxolinic acid; NA = nalidixic acid. Values in parentheses are C.V. (%).

pH of 0.1 *M* potassium dihydrogen-phosphate (3 × 20 ml)	Recovery (%)			
	Bond Elut C_2		Bond Elut C_{18}	
	OA (n = 4)	NA (n = 4)	OA (n = 4)	NA (n = 4)
4.0	73.2 (1.6)	78.9 (1.6)	77.9 (2.4)	82.4 (3.2)
5.0	71.2 (2.5)	77.2 (2.5)	73.5 (3.6)	75.3 (4.2)
6.0	68.1 (1.8)	75.6 (2.2)	76.2 (2.8)	86.9 (4.6)
7.0	69.5 (1.4)	58.9 (3.9)	87.7 (2.6)	87.7 (3.0)
8.0	25.7 (18.5)	12.7 (12.2)	87.9 (3.4)	85.6 (4.6)

TABLE II

RECOVERY OF OXOLINIC ACID AND NALIDIXIC ACID FROM SPIKED RAINBOW TROUT LIVER

Liver samples (2.0 g) were spiked with oxolinic acid (1.0 μg/g) and nalidixic acid (1.0 μg/g). OA = Oxolinic acid; NA = nalidixic acid. Values in parentheses are C.V. (%).

pH of 0.1 *M* potassium dihydrogenphosphate (3 × 20 ml)	Recovery from Bond Elut C_{18} (%)	
	OA (n = 4)	NA (n = 4)
4.5	83.6 (4.6)	86.5 (6.5)
5.0	79.5 (1.3)	83.8 (0.5)
6.0	82.4 (1.7)	86.9 (1.0)
7.0	72.5 (6.1)	69.3 (4.8)
8.0	—[a]	—[a]

[a] The extracts caused frequent clogging of the solid-phase extraction cartridge.

Good recoveries of the drugs were, however, obtained in the pH region 4.5–6. Phosphate buffer at pH 4.5 was chosen for liver extractions because of good recovery and smoothest operation of the extraction cartridges.

Standard calibration graphs were linear and reproducible at least over the range 0.2–10 μg/ml in rainbow trout serum and over the range 0.2–20 μg/g in rainbow trout liver and muscle tissue. The correlation coefficients for serum, liver and muscle tissue standard graphs were 0.999, 0.997 and 0.994, respectively. The lowest measurable oxolinic acid concentration was 0.01 μg/ml in serum and 0.01 μg/g in liver and muscle tissue (signal-to-noise ratio greater than 5).

Chromatographic conditions

The effects of the percentage of organic modifier and the pH of the buffer in the mobile phase on the capacity factor are shown in Fig. 1. The best separation and peak shape were obtained with the mobile phase acetonitrile–0.1 *M* potassium dihydrogenphosphate buffer (10:90, v/v) at pH 2.0 (Fig. 2). Under these conditions the capacity factor for nalidixic acid was 4.2 and that for oxolinic acid was 5.4. The respective retention times for nalidixic acid and oxolinic acid were 8.9 min and 10.9 min. A lower percentage of acetonitrile and higher pH values of the mobile phase gave rise to severely tailing peaks and showed lower correlation between peak height and drug concentration (data not shown). Mobile phase compositions giving capacity factors of 3 or lower caused the drug peaks to coelute with endogenous compounds in fish tissues.

The main problem when analysing serum samples by direct injection is the accumulation of serum proteins in the HPLC column. In previous serum applications on Regis Pinkerton ISRP columns, the mobile phase has been adjusted to pH 6.8 to minimize the precipitation of proteins [10–12]. A neutral pH leaves

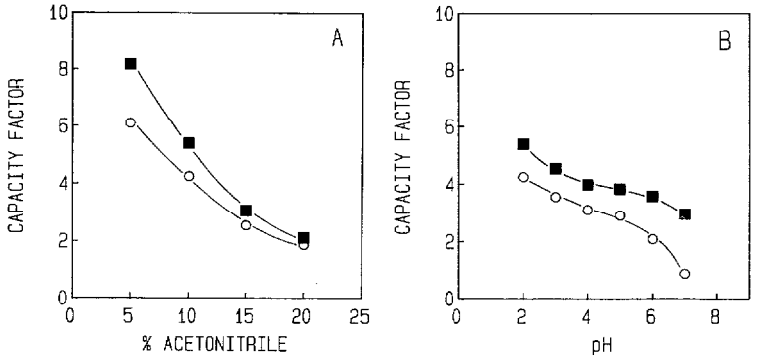

Fig. 1. Effect of (A) the percentage of acetonitrile and (B) the pH of the mobile phase on the capacity factor of (■) oxolinic acid and (○) nalidixic acid. For chromatographic conditions see text.

the serum proteins with a negative charge, which is also the case with the external glycine residues on the ISRP stationary phase. Consequently the proteins in the injected sample are repulsed by the residual glycines and are not retained by the stationary phase.

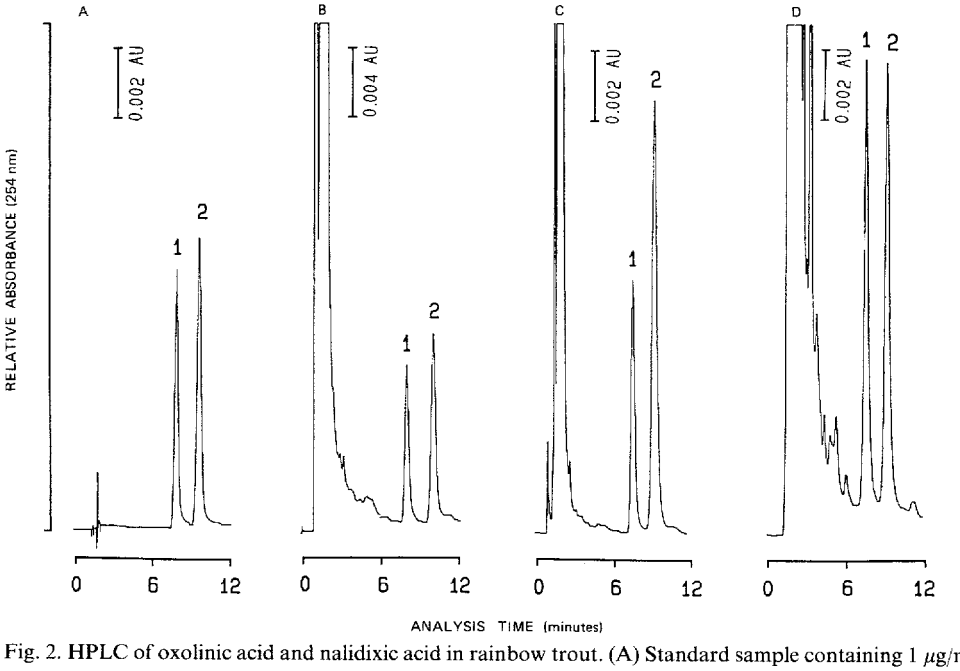

Fig. 2. HPLC of oxolinic acid and nalidixic acid in rainbow trout. (A) Standard sample containing 1 μg/ml each of oxolinic acid and nalidixic acid. (B) Serum sample containing 1 μg/ml oxolinic acid; the sample was spiked with 1 μg/ml internal standard (nalidixic acid). (C) Sample of muscle tissue containing 1.4 μg/g oxolinic acid; the sample was spiked with 1 μg/g internal standard. (D) Liver sample containing 1.9 μg/g oxolinic acid; the sample was spiked with 2 μg/g internal standard. The mobile phase was acetonitrile–0.1 *M* KH_2PO_4 (10:90, v/v) adjusted to pH 2.0. For chromatographic conditions see text. Peaks: 1 = internal standard (nalidixic acid); 2 = oxolinic acid.

In the present application, however, the optimal operating conditions were obtained at low pH only. Under these conditions precipitation of serum proteins can be expected. During analysis of a typical set of 30–50 serum samples the column back-pressure increased from the initial 80 bar to *ca.* 130 bar. This was caused by clogging of the stainless-steel frit in the column inlet filter, presumably by denatured proteins from the serum samples. This was solved easily by reversing the column system and flushing with 20 ml of mobile phase. This decreased the back-pressure to the original level and the analysis of serum samples could be continued.

When the column was to be taken out of service, it was first reversed and flushed with 100 ml of 0.1 *M* potassium dihydrogenphosphate buffer (pH 4.5) and then with 100 ml of the same buffer at pH 7. This was done in order to remove residues of serum proteins from the column. The column was then flushed with 100 ml of water and 100 ml of 2-propanol and stored containing 2-propanol. This procedure maintained the column in working order without an increase in back-pressure.

No increase in back-pressure was obtained when solid-phase extracts of muscle tissue and liver were analysed.

In the present study no irreversible binding of serum proteins to the ISRP stationary phase was found. This can probably be explained by the fact that at pH 2 the external glycine residues on the ISRP stationary phase are protonated, leaving them neutral. The pK_a of the bound glycine is presumed to lie between 2.3 and 3. The mobile phase at pH 2 leaves serum proteins with a net positive charge. Consequently no strong adsorption of the serum proteins to the ISRP stationary phase occurs. This possibility is discussed by the ISRP column developers [12] though it has not been tested.

After a total of 1400 injections of drug standards, serum samples and extracts of liver and muscle tissue the column performance is still good. The retention times of oxolinic acid and nalidixic acid are shortened by 0.5 min. The number of theoretical plates is 1200 (for nalidixic acid) compared with the initial value of 1300.

CONCLUSIONS

The described HPLC method for analysing oxolinic acid in fish serum and tissues is simple, rapid and requires no special instrumentation. The sample clean-up is minimized. The method is sensitive and reproducible and thus suitable for pharmacokinetic and drug residue studies. The low pH application in serum analyses by direct injection on ISRP columns is new. A proper column-cleaning procedure and regular replacement of column inlet filter and guard column prevent accumulation of residual proteins and maintains column performance.

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