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Note

Rapid high-performance liquid chromatographic method for the determination of oxolinic acid in fish serum employing solid-phase extraction

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There is a lack of information on the pharmacokinetics of antibacterial agents used in aquaculture, partly owing to a lack of analytical methods that measure precisely the drug concentration in fish serum or plasma Oxolinic acid (OA, 5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid) is an antibacterial agent that is frequently used in aquaculture Several methods for determining OA based on bioassay [1,2], fluorimetry [2] and high-performance liquid chromatography (HPLC) [3–9] have been described In contrast to the HPLC methods, the bioassay and fluorimetric methods are poor in sensitivity and non-specific towards active metabolites On the other hand, previous HPLC procedures involve systems that are less suitable for pharmacokinetic studies of OA in fish, owing to the presence of interfering endogenous substances in fish serum and low recoveries in small volumes of blood Hence the purpose of this work was to develop a simple HPLC assay for the determination of OA in fish serum

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EXPERIMENTAL

Chromatographic system and chemicals

A Shimadzu (Kyoto, Japan) SCL-6A modular HPLC system was used, consisting of an LC-6A solvent-delivery pump and a SIL-6A autoinjector, separation of injected compounds was achieved with a Nova-Pak C_{18} (4 μm) column (150 mm × 3.9 mm I.D.) (Millipore-Waters, Milford, MA, U.S.A.) fitted with a Supelcosil LC-18 (40 μm) guard column (2 cm) (Supelco, Gland, Switzerland) at room temperature. The column effluent was monitored at 258 nm with a SPD-6AV detector operating at 0.08 a.u.f.s.; peak areas and concentrations were calculated with an C-R3A on-line computer. Nalidixic acid (NA) (Sigma, St. Louis, MO, U.S.A.) was used as an internal standard. The mobile phase used for isocratic elution consisted of 40% (v/v) methanol (Fisons, Loughborough, U.K.) and 60% phosphate buffer containing 7.5 g/l $KH_2PO_4 \cdot 2H_2O$ and 2.5 g/l $Na_2HPO_4 \cdot H_2O$ (Merck, Darmstadt, F.R.G.). The phosphate buffer was adjusted to pH 8.2 with 1 *M* sodium hydroxide (Merck). Tetrabutyl ammonium phosphate (PIC A, 5 m*M*) (Millipore-Waters) was added to the mixture. The flow-rate was 0.6 ml min^{-1}. Solid-phase extraction was performed with Sep-Pak Accell cartridges and a Sep-Pak rack (Millipore-Waters). All reagents were of analytical-reagent grade.

Calibration graphs

Standards were prepared for each series of runs by adding 0.01, 0.1, 1.0, 5.0, 15.0 or 25.0 μg ml^{-1} OA (Sigma) to drug-free serum from rainbow trout (*Salmo gairdneri* Richardson) or Atlantic salmon (*Salmo salar* L.).

Extraction of OA from serum

Liquid-phase extraction was performed according to Cuisinaud et al. [4] with some modifications. To 100 μl of fish serum were added 100 μl of internal standard solution (5 μg ml^{-1}), 100 μl of 1 *M* hydrochloric acid (Merck) and 2 ml of chloroform–ethyl acetate (equal volumes) (Fisons). After mixing for 1.5 min and subsequent centrifugation (10 min at 1300 *g*), the organic phase was extracted with 2 ml of 0.2 *M* sodium hydroxide for 30 min. Following centrifugation (10 min at 1300 *g*), the aqueous phase was extracted with 1 ml of 1 *M* hydrochloric acid and 2 ml of chloroform–ethyl acetate as described above. The organic layer was removed and evaporated to dryness under nitrogen at 40°C. Residues were dissolved in 200 μl of mobile phase and 20 μl of this solution were injected into the HPLC system within 24 h.

Solid-phase extraction was performed by passing 100 μl of serum and 100 μl of internal standard through a Sep-Pak Accell cartridge that had been prewashed with 5 ml of 1 *M* sodium hydroxide. After subsequent washing with 4 ml of 1 *M* sodium hydroxide, 6 ml of 70% methanol in 0.03 *M* citrate buffer of pH 4.9 (Merck) were used to elute OA and the internal standard. The process

was performed applying a vacuum to a flow-through of 1–2 ml min^{-1} The effluent was evaporated to dryness, dissolved in the mobile phase and injected into the HPLC system as described above

RESULTS AND DISCUSSION

Chromatograms of blank serum and serum from fish after a single intravascular injection of OA (10 mg kg^{-1}) are shown in Fig. 1 The retention times were 4 5 and 8 6 min for OA and NA, respectively Strongly tailing peaks occurred with most of the chromatographic systems used; these systems were based on Radial-Pak CN, C_8 and C_{18} (Millipore-Waters) and Supelcosil LC-18-DB (Supelco) columns This is in accordance with previous reports on the determination of OA and NA in human serum and urine [4,10] The observed tailing was probably due to the interaction of OA and NA with residual silanol groups The end-capped C_{18} and a phenyl column (Brownlee Labs , Santa Clara, CA, U S A.) both gave narrow chromatographic peaks The paired-ion technique and the end-capped C_{18} column, however, gave a better separation; symmetrical chromatographic peaks were obtained when a high and constant con-

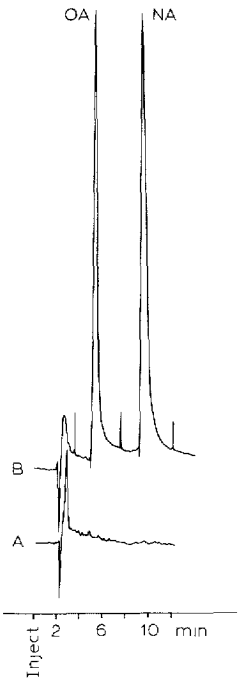

Fig 1 Chromatograms of (A) a blank fish serum extract and (B) fish serum extract from a sample taken 24 h after a single bolus intravascular injection of oxolinic acid (OA) The sample shown contained 6 μg ml^{-1} OA and 5 μg ml^{-1} nalidixic acid (NA) as internal standard

centration (>4 mM) of a counter ion was maintained in the mobile phase This was obtained by adding a cation, i.e , tetrabutyl ammonium phosphate, to the mobile phase, thus making a suitable pair of ions for adsorption on the stationary phase [11] The inter- and intra-day variations of OA determined from the same standard (5 0 μg ml^{-1}) with this method were 0 8 and 1 9%, respectively ($n=10$). There were no significant changes in the OA concentrations in dissolved residues stored at room temperature in the autoinjector for 24 h, in standards stored at 4°C for 21 days or in serum aliquots stored at -20°C for 3 months.

The calibration graph plotted as peak-area ratio of OA to the internal standard against the concentration of OA was found linear ($r>0.998$) over the OA concentration range studied (0 01–25 μg ml^{-1}) The detection limit (signal-to-noise ratio 3 1) was 1 ng ml^{-1}, which is lower [7] or equal to [4,8] those in previous studies.

Both extraction procedures gave extracts devoid of interfering endogenous substances. A less time-consuming liquid-phase extraction method which is suitable for chicken serum [7] was found to be suitable for human serum, but the extracts contained interfering substances when this method was applied to fish The mean recoveries and coefficients of variation (C V) of liquid-phase and solid-phase extraction procedures are listed in Table I Our liquid-phase extraction procedure gave slightly lower and the solid-phase extraction slightly higher recoveries than previously reported liquid-phase extraction procedures [4,7] The higher C.V in the solid-phase extraction method was probably due to practical problems in solvation of the solid phase between the washing and elution procedures, when using a single cartridge the C V was 4 5 and 2 3% ($n=10$) for the addition of 10 0 and 1 0 μg ml^{-1} OA, respectively Hence the Sep-Pak rack was not suitably constructed to ensure adequate solvation of all solid phases when eight cartridges at a time were used We recommend the solid-phase extraction procedure performed with single cartridges for further

TABLE I

OXOLINIC ACID ASSAY IN FISH SERUM

Extraction procedure	Concentration (μg ml^{-1})		Coefficient of variation (%)
	Added	Recovered (mean, $n=8$)	
Liquid-phase	1 0	0 89	2 9
	5 0	4 4	2 5
	15 0	13 9	3 2
Solid-phase	1 0	0 96	17 5
	5 0	4 9	11 6
	15 0	14 4	9 1

pharmacokinetic studies in fish owing to the high recovery and simplicity Provided that the capacity of the evaporation set-up is sufficiently high, the number of analyses per day may easily be more than doubled compared with a liquid-phase extraction procedure Further, the prepared samples are devoid of interfering substances, the chromatographic system is stable and the chromatographic peaks are narrow and symmetrical Finally, the method allows us the measurement of OA concentrations in small volumes of serum, which is required when sampling repeatedly from individual resting fish.

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