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SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FLUMEQUINE AND OXOLINIC ACID IN SALMON PLASMA

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SUMMARY

Two methods for determination of oxolinic acid and flumequine in salmon plasma are described The first method applies sample pretreatment on C_2 disposable solid-phase extraction columns The second method is based on direct plasma injection and on-line sample clean-up on a polystyrene–divinylbenzene precolumn After column-switching, the analytes are separated on a polystyrene–divinylbenzene analytical column and detected with a fluorescence detector Validation of the methods showed good sensitivity, precision and reproducibility Both methods are well suited for determination of plasma levels of the drugs in pharmacokinetic studies in Atlantic salmon

INTRODUCTION

Flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1*H*,5*H*-benzo[*i*,*j*]quinolizine-2-carboxylic acid) and oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid) are quinolone derivatives active against a broad spectrum of Gram-negative bacteria Both compounds have found widespread use as chemotherapeutics in fish farming of Atlantic salmon (*Salmo salar* L.). In order to carry out pharmacokinetic and toxicological studies of these compounds in fish, a rapid and selective method is needed for the analysis of large series of plasma samples.

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Microbiological, fluorimetric and high-performance liquid chromatographic (HPLC) methods with UV detection have been described for analysis of flumequine and oxolinic acid in plasma from humans and animals [1–5] However, the sensitivity and selectivity of the microbiological and fluorimetric methods are poor. Common to the HPLC procedures is the need for sample pretreatment Although liquid–liquid extraction may be sufficient for some applications, these plasma clean-up methods are too lengthy for routine analysis of large series of plasma samples.

The use of solid-phase extraction in the sample pretreatment is efficient and time-saving. Both an off-line procedure based on sample pretreatment on disposable solid-phase extraction columns and a totally automated on-line procedure based on column switching have been developed. The off-line procedure is based on Bond Elut^{TM} C_2 solid-phase extraction columns, and the column-switching procedure involves a polystyrene–divinylbenzene column for sample concentration and clean-up. The HPLC analysis is carried out with a polystyrene column, which eliminates the problems with peak-tailing frequently observed on silica-based columns. The methods have been used successfully for the analysis of more than 1000 plasma samples from Atlantic salmon.

EXPERIMENTAL

Chemicals

Oxolinic acid and nalidixic acid were obtained from Sigma (St Louis, MO, U S.A.) and flumequine was purchased from Solchem Italiana (Mulazzano, Italy) HPLC-grade acetonitrile, methanol and tetrahydrofuran were from Rathburn (Walkerburn, U.K.). Orthophosphoric acid, sodium dihydrogenphosphate and sodium hydroxide were supplied by E. Merck (Darmstadt, F.R.G). HPLC-grade water was obtained by purifying distilled water in a Milli-Q (Millipore, Bedford, MD, U.S.A.) filtration system

Preparation of standards

Standard solutions of flumequine (0 50 mg/ml) and oxolinic acid (0.25 mg/ml) were prepared in 0.01 *M* sodium hydroxide. Working standards (50 and 25 μg/ml) were prepared by dilution with water and were used for preparation of development standards in water, mobile phase or acetonitrile (0.01–10 μg/ml). Spiked salmon plasma standards ranging from 0.01 to 10 μg/ml of the drugs were prepared from the working standards. Drug-free plasma and plasma standards were kept at $-20\,^{\circ}C$ until analysis.

Solid-phase extraction with Bond Elut columns

Apparatus and columns A Vac Elut^{TM} vacuum manifold and Bond Elut columns from Analytichem International (Harbor City, CA, U.S.A.) were used to clean-up plasma samples prior to the HPLC analysis. The extraction col-

umns investigated were packed with 100 mg of C_2, C_8, C_{18}, CH and PH bonded phases.

High-performance liquid chromatography The liquid chromatograph was an LC 6A (Shimadzu, Kyoto, Japan) equipped with a Model RF 530 (Shimadzu) fluorescence detector with a 12-μl flow-cell operated at an excitation wavelength of 262 nm and an emission wavelength of 380 nm. Peak heights were recorded with a Chromatopac C-R3A (Shimadzu) Samples were injected with a Model 7125 injector (Rheodyne, Berkley, CA, U.S A) equipped with a 20-μl loop The column (150 mm$\times$4 6 mm I.D.) was packed with 5 μm particle diameter polystyrene–divinylbenzene PLRP-S (Polymer Labs., Churchtretton, U K.). The mobile phase was acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (20 15 65, v/v), and the flow-rate was 0 7 ml/min. The solvent mixture was filtered under vacuum (0 45-μm filter) prior to use

Solid-phase extraction The C_2 extraction column was used as follows It was conditioned with two column volumes of methanol followed by one column volume of 1.0 *M* orthophosphoric acid Then 250–500 μl of plasma and 250 μl of aqueous internal standard solution (2 0 μg/ml in water) were added to each column. Flumequine was used as internal standard for the determination of oxolinic acid and oxolinic acid as internal standard for the determination of flumequine. The mixture was drawn through the column using the vacuum manifold. The column was washed with 500 μl of water and then with 250 μl of 1.0 *M* orthophosphoric acid The compounds were eluted with 250 μl of acetonitrile–methanol–1.0 *M* orthophosphoric acid (80 10 10, v/v) and collected in 1.5-ml polypropylene sampling tubes. Each collection tube was thoroughly mixed before 20 μl of the eluate were injected into the liquid chromatograph

Evaluation of extraction columns Extraction columns packed with C_2, C_8, CH and PH were evaluated. After conditioning with methanol and orthophosphoric acid, an aqueous standard containing oxolonic acid and flumequine (1 μg/ml) was added. The analytes were eluted with 250 μl of mobile phase, and nalidixic acid (2.5 μg/ml) as internal standard was then added to the eluates. After mixing, 20 μl were injected and the recovery of flumequine and oxolinic acid was determined. The same columns were again eluted with two 250-μl volumes of the mobile phase, and the recovery of each fraction was determined

Validation of the Bond Elut procedure The precision, recovery and linearity of the method were determined by analysis of spiked plasma in the concentration range 0 5–5 μg/ml for flumequine and 0.25–5 μg/ml for oxolinic acid

On-line solid-phase extraction

HPLC system The HPLC system consisted of a Waters Assoc. (Milford, MA, U S A) Model 590 programmable solvent-delivery system and a Model 712 WISP sample processor for automated injection A Waters Model 6000 A was used as auxiliary pump. The on-line extraction and clean-up were carried out using a Waters WAVS automated valve station, which accomplished sol-

vent-switching between the precolumn and the analytical column. A Shimadzu Model RF 530 fluorescence detector, equipped with a 12-μl flow-cell, was operated at an excitation wavelength of 262 nm and an emission wavelength of 380 nm. The peak heights were calculated on an SP 4270 integrator (Spectra Physics, San Jose, CA, U.S A.).

The preconcentration column was a Chrompack (Middelburg, The Netherlands) cartridge with a cartridge precolumn (10 mm$\times$2 mm I D.) packed with 36 μm particle size polystyrene–divinylbenzene particles (Dynospheres, Dyno Particles, Lillestrøm, Norway). Precolumns packed with 40 μm particle size Sepralyte^{TM} C_{18}, C_8, C_2, CH and CN (Analytichem International) were also evaluated Sample concentration and clean-up was achieved with 0.001*M* orthophosphoric acid as mobile phase at a flow-rate of 0 8 ml/min The samples were back-flushed on to the PLRP-S analytical column with a mobile phase of acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (225 175 600, v/v) for determination of flumequine. For determination of oxolinic acid the mobile phase was acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (200 150 650, v/v). The flow-rate of mobile phase was 1 0 ml/min.

Column switching with WAVS Aliquots of 20 μl of plasma were injected into the precolumn Flumequine and oxolinic acid were retained whereas unwanted plasma components passed through to waste. Using WAVS, the solvent was switched to the mobile phase of the analytical column after 4 min and directed to back-flush The compound of interest was eluted from the precolumn onto the analytical column. After 7 min the solvent was switched back to the aqueous mobile phase to prepare the precolumn for the next analytical run. A new injection was performed after 15 min. The precolumn was repacked after 150–200 injections of 20 μl of plasma, making a total of 3–4 ml plasma injected

Validation of the on-line clean-up procedure The precision and the recovery of the method were determined by spiking drug-free plasma. The linearity of the calibration curve was tested in the range 0 1–5 00 μg/ml for flumequine and 0 05–10 μg/ml for oxolinic acid. The stability of spiked plasma samples was tested by storage at 20 °C in the autoinjector for 24 h

Comparison between the Bond Elut procedure and the on-line procedure

Both spiked plasma and plasma samples from pharmacokinetic studies containing flumequine or oxolinic acid were separately analysed with the Bond Elut procedure and the on-line column-switching procedure The correlation between the two methods was determined.

RESULTS AND DISCUSSION

Chromatographic conditions

Both oxolinic acid and flumequine show strong fluorescence in acidic aqueous solutions To make use of the increased specificity and sensitivity offered by

the fluorescence detector, orthophosphoric acid was used as a mobile phase additive. Mobile phases for cross-linked polystyrene–divinylbenzene often contain an organic modifier, such as acetonitrile mixed with tetrahydrofuran [6,7]. The presence of tetrahydrofuran has been shown to reduce peak tailing and to increase the number of theoretical plates. The mobile phase was therefore optimized with respect to acetonitrile, tetrahydrofuran and orthophosphoric acid. Acceptable retention of both oxolinic acid and flumequine was obtained using 35–40% (v/v) organic modifier. With acetonitrile–0.02 *M* orthophosphoric acid (40 60, v/v) as mobile phase the number of theoretical plates of oxolinic acid and flumequine was 800 and 1850, respectively. With acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (20 15 65, v/v) as mobile phase the number of theoretical plates for oxolinic acid and flumequine increased to 2970 and 2600, respectively. Variation of the concentration of orthophosphoric acid in the range 0.001–0 05 *M* had no effect on the retention. The maximum number of theoretical plates was obtained with 0 02 *M* orthophosphoric acid.

Bond Elut extraction

Prior to method development with Bond Elut extraction columns, an attempt was made to precipitate plasma proteins with acetonitrile followed by direct injection of the supernatant after centrifugation. Acceptable recovery (90%) and precision (2.0%) were obtained for flumequine but the chromatogram showed many impurities, which accumulated on the analytical column and caused increasing back-pressure. These problems were avoided with solid-phase extraction. Fig. 1 shows the recovery of flumequine and oxolinic acid after three subsequent elutions with mobile phase from the extraction columns investigated

Acceptable recovery from the first elution from C_2, C_8 and CH extraction columns was obtained for oxolinic acid, whereas only C_2 gave an acceptable recovery for flumequine. Sample pretreatment for both flumequine and oxolinic acid was therefore optimized with C_2 solid-phase material. Optimization of the washing and elution steps was carried out with spiked plasma. Washing with water and orthophosphoric acid gave 'clean' extracts. Mixtures of acetonitrile, methanol, tetrahydrofuran and orthophosphoric acid were systematically tested as elution solvents. Complete recovery of both flumequine and oxolinic acid was obtained using a single 250-μl elution with acetonitrile–methanol–*1 M* orthophosphoric acid (80 10 10, v/v). Chromatograms of a drug-free plasma and a plasma standard containing flumequine and oxolinic acid are shown in Fig. 2. The recovery and reproducibility of the procedure are shown in Table I. The standard curves based on peak-height measurements were linear in the concentration range 0.25–5.0 μg/ml of plasma for oxolinic acid with flumequine as internal standard ($r=0.9999$) and in the concentration range 0 5–5 μg/ml for flumequine with oxolinic acid as internal standard ($r=0$ 9999).

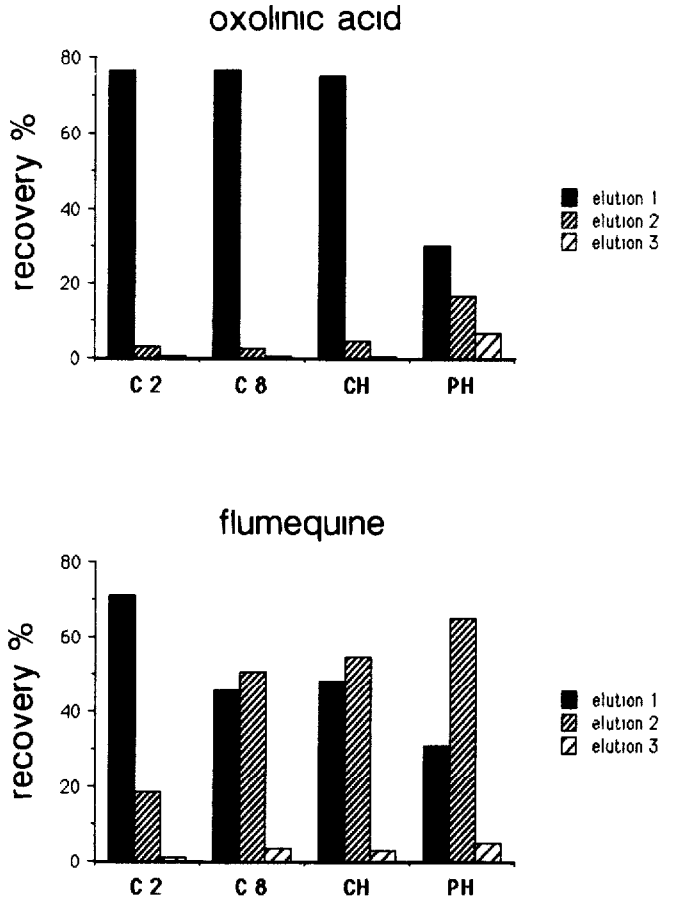

Fig 1 Recovery of oxolinic acid and flumequine from C_2, C_8, CH and PH solid-phase extraction columns after three consecutive elutions with 250 μl of acetonitrile–tetrahydrofuran–0 02 *M* orthophosphoric acid (20 15 65, v/v)

Column-switching procedure

Silica phases modified with C_{18}, C_8, C_2 and CN were initially tried in the concentration column Each column was conditioned with methanol before introduction into the precolumn cartridge. Peak tailing was observed for both oxolinic acid and flumequine when C_{18} and C_8 were used, whereas C_2 and CN gave symmetrical peaks. The recovery was low, however· less than 70% for C_2 and less than 50% for CN. Furthermore, polystyrene–divinylbenzene was tried out as packing material in the precolumn and gave complete recovery of both flumequine and oxolinic acid. No interfering peaks were detected by fluorescence detection when drug-free plasma was injected Wash times were varied from 1 to 10 min and flow-rates between 0.5 and 1.0 ml/min. Variation of these

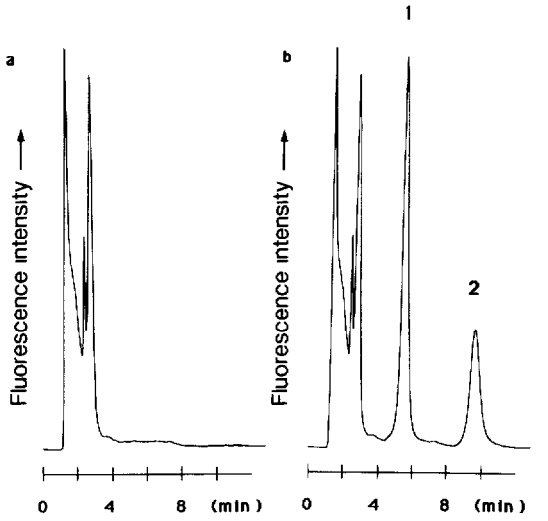

Fig 2 Chromatograms of (a) a drug-free plasma and (b) a plasma sample with 0 4 μg/ml oxolinic acid and 1 0 μg/ml flumequine as internal standard after sample pretreatment on a C_2 extraction column Analytical column, PLRP-S, mobile phase, acetonitrile–tetrahydrofuran–0 02 *M* orthophosphoric acid (20 15 65, v/v), detector, fluorescence, attenuation, 32 Peaks 1 = oxolinic acid, 2 = flumequine

TABLE I

RECOVERY AND REPRODUCIBILITY OF OXOLINIC ACID AND FLUMEQUINE IN PLASMA AFTER SAMPLE PRETREATMENT ON C_2 SOLID-PHASE EXTRACTION COLUMNS

Compound	Concentration added (μg/ml)	Mean recovery (n=6) (%)	Coefficient of variation (%)
Oxolinic acid	0 5	103 6	4 2
	1 0	100 1	2 5
	2 5	100 4	2 8
	5 0	98 7	1 2
Flumequine	0 5	103 2	2 8
	1 0	97 8	2 1
	2 5	103 2	2 5
	5 0	99 2	1 6

parameters did not affect the chromatograms to a large extent. However, in continuous overnight analysis with a flow-rate of 0.5 ml/min through the pre-column, wash times less than 4 min gave an increase in back-pressure on the analytical column. A flow-rate of 0.8 ml/min and wash times of 3 min were therefore used routinely. By optimizing the mobile phase for each analyte sep-

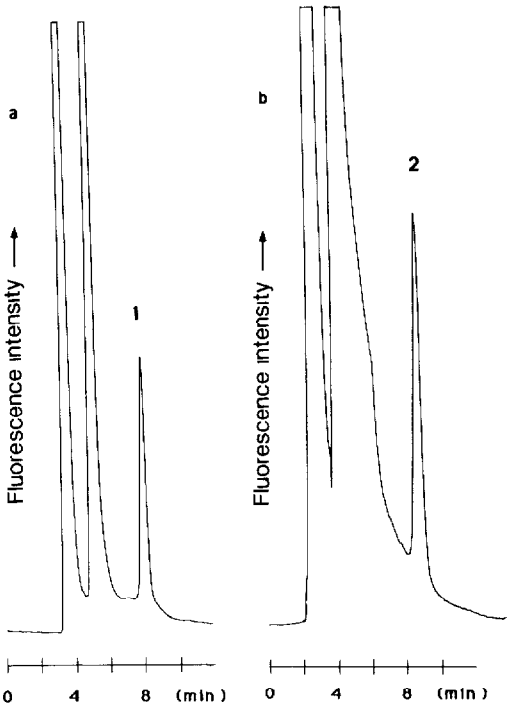

Fig 3 Chromatograms of a plasma sample with (a) 0 5 μg/ml oxolinic acid and (b) 1 0 μg/ml flumequine after injection of 20 μl of plasma and column switching Precolumn, polystyrene-divinylbenzene, mobile phase, 0 001 *M* orthophosphoric acid, analytical column, PLRP-S, mobile phase for oxolinic acid, acetonitrile–tetrahydrofuran–0 02 *M* orthophosphoric acid (200 150 650, v/v), mobile phase for flumequine, acetonitrile–tetrahydrofuran–0 02 *M* orthophosphoric acid (225 175 600, v/v), detector, fluorescence, attenuation, (a) 64, (b) 16 Peaks 1 = oxolinic acid, 2 = flumequine

arately, good chromatograms were obtained as shown in Fig 3 No degradation was observed after storage of plasma samples for 24 h in the autosampler.

Results from validation of the assay are shown in Table II. The calibration graphs obtained by plotting peak height against concentration were linear for flumequine over the concentration range 0 1–5.0 μg/ml with $r = 0.9998$ and for oxolinic acid in the concentration range 0 05–10.0 μg/ml with $r = 0.9999$. The limits of detection in plasma, with an injection volume of 50 μl and fluorescence detection, was 10 ng/ml for flumequine and 5 ng/ml for oxolinic acid (signal-to-noise ratio 2)

Comparison of the Bond Elut procedure and the column-switching procedure

Ten plasma samples containing flumequine (0.5–10 μg/ml) and ten plasma samples containing oxolinic acid (0.1–5 μg/ml), were analysed with the Bond Elut procedure and the column-switching procedure. The results were com-

TABLE II

RECOVERY AND REPRODUCIBILITY OF OXOLINIC ACID AND FLUMEQUINE IN PLASMA AFTER COLUMN SWITCHING

Compound	Concentration added (μg/ml)	Mean recovery ($n=6$) (%)	Coefficient of variation (%)
Oxolinic acid	0 05	97 5	2 7
	0 5	97 2	1 0
	1 0	98 1	1 1
	2 5	98 1	1 9
	5 0	95 7	1 0
Flumequine	0 1	95 3	5 3
	0 5	93 0	1 8
	1 0	97 4	1 2
	2 5	99 7	1 1
	5 0	103 7	0 8

pared by using linear regression, with y = concentration obtained with the Bond Elut procedure and x = concentration obtained with the column-switching procedure The regression lines, $y=1.064x+0.070$ with $r=0.998$ for flumequine and $y=0.972x+0.004$ with $r=0.998$ for oxolinic acid, show a good correlation between the two methods.

CONCLUSION

The Bond Elut procedure and the automatic column-switching procedure have been used successfully for the analysis of flumequine and oxolinic acid in more than 1000 plasma samples Both methods are rapid and easy to carry out and have the necessary sensitivity and reproducibility for pharmacokinetic studies. Results from pharmacokinetic studies of flumequine and oxolinic acid in Atlantic salmon will be published [8] The Bond Elut procedure is mostly used for random analysis of a small number of samples, whereas the column-switching procedure is used for analysis of large plasma series Only 20–50 μl plasma are injected in the column-switching procedure. This is a great advantage because plasma volumes available from fish are often limited.

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