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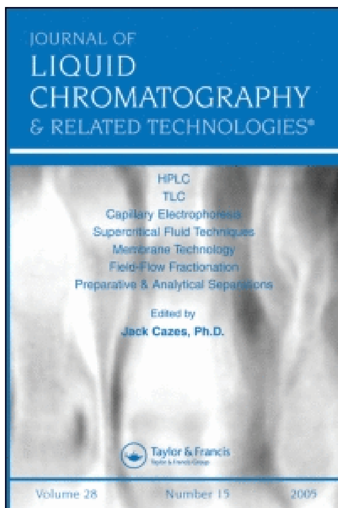

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Simple and Rapid Method for the Determination of Oxolinic Acid in Turbot (*Scophthalmus maximus*) Serum by High Performance Liquid Chromatography with Fluorescence Detection

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SIMPLE AND RAPID METHOD FOR THE DETERMINATION OF OXOLINIC ACID IN TURBOT *(SCOPHTHALMUS MAXIMUS)* SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A simple high performance liquid chromatographic method for the determination of oxolinic acid, a chemotherapeutic agent, in turbot serum has been developed. Nalidixic acid was used as an internal standard. The drugs were separated by using a 5 μm Supelcosil ABZ+Plus® reversed phase cartridge, a mobile phase of acetonitrile, tetrahydrofuran and 0.001 M orthophosphoric acid solution (22:10:68 v/v), pH 3.4 with a fluorescence detection. The sample clean-up was reduced to the use of precipitating agents and centrifugations because the solid-phase extraction procedure was not suited to the analysis of oxolinic acid in turbot serum.

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Linearity and precision were checked over the concentration range 0.020-2.500 µg/mL. Limits of detection and determination were respectively 0.005 and 0.015 µg/mL. Recoveries of oxolinic acid from turbot serum were between 103.89 and 110.71 %.

INTRODUCTION

The quinolone 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 several countries in the treatment and prevention of infectious fish diseases. Pharmacokinetic and residues studies should be developed in fish species to which oxolinic acid is administered. Some studies are available about the pharmacokinetics of oxolinic acid, mostly in salmonid fish.[1-12] No report has been previously published about pharmacokinetic studies in turbot (*Scophthalmus maximus*).

Analytical methods for pharmacokinetic studies should be specific, rapid, and simple because blood samples may contain metabolites of the drug and the number of samples is large. The microbiological techniques for determining oxolinic acid in fish blood are less sensitive and less specific than the high performance liquid chromatographic (HPLC) methods.[13]

Several HPLC methods for analysing oxolinic acid in fish blood have been described.[8,11,14-21] Common to these methods mostly is the need for sample preparation by liquid-phase[11,14,15,19] or solid-phase extractions.[8,16,17,20]

The purpose of the present work was to develop a simple, rapid, and accurate HPLC method for analysing oxolinic acid in turbot serum. The need for this method was the ability to study pharmacokinetics of oxolinic acid in turbot.

MATERIALS AND METHODS

Chemicals

Acetonitrile (Merck, Darmstadt, Germany) and tetrahydrofuran (Fluka, Buchs, Switzerland) were of HPLC-grade. Methanol, orthophosphoric acid 85% and 0.1 M sodium hydroxide solution (Merck) were analytical-grade reagents. Zinc sulphate heptahydrate (Panreac, Barcelona, Spain) was of analytical-grade. The water used in buffers and eluents was desionised with a

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Nanopure apparatus (Barnstead, Dubuque, IO, USA). Oxolinic acid and nalidixic acid were purchased as pure standards from Sigma (St. Louis, MO, USA).

Apparatus

A Merck HPLC system was used consisting of a L-6 200 A solvent-delivery pump, a AS 2 000 autosampler, a F 1 000 fluorescence detector and a Deskpro 386S/20 computer (Compaq, Houston, TX, USA). Peak heights and concentrations were calculated with the D-6 000 HPLC Manager Software System (Merck).

The analytical cartridge, a 5 µm Supelcosil ABZ+Plus®, 150 X 4.6 mm I.D. (Supelco, Bellefonte, PA, USA), was equipped with a 5 µm Supelcosil ABZ+Plus® guard cartridge, 20 X 4.6 mm I.D.

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Chromatographic Conditions

The mobile phase consisted of acetonitrile, tetrahydrofuran and 0.001 M orthophosphoric acid solution (22:10:68 v/v), pH 3.4. The orthophosphoric acid solution was filtered with a Millipore HPLC solvent filtration system (Millipore, Bedford, MA, USA) and Nalgene 47 mm, 0.20 µm nylon filters (Nalge Company, Rochester, NW, USA).

The chromatographic experiments were performed at a temperature of 24°C. The operating flow rate was 0.8 mL/min. The fluorescence detector was set at an excitation wavelength of 327 nm and an emission wavelength of 360 nm.

The sample volume injected onto the cartridge was 10 µL. The guard cartridge was removed at intervals of 500 sample injections.

The cartridge was conditioned prior to use by flushing with: (1) a mixture of 50 % of acetonitrile:water (50:50 v/v) and 50 % of mobile phase (30 min) at a flow rate of 0.4 mL/min; (2) mobile phase (30 min) at a flow rate of 0.4 mL/min; (3) mobile phase (30 min) at a flow rate of 0.8 mL/min. Moreover, the cartridge was flushed for 2 h after each day of operation with acetonitrile and water (50:50 v/v) at a flow rate of 0.2 mL/min.

Standard Solutions

Stock solutions of oxolinic acid and nalidixic acid were prepared in a 0.03M sodium hydroxide solution at a concentration of 1 mg/mL and were stable for one month when stored at + 4°C. Working standard solutions were prepared by dilution in water immediately before use.

Extraction Procedure

Serum samples (10, 20, 50, or 200 µL) from turbot were spiked with nalidixic acid as internal standard (0.200 µg/mL). A 20 µL-volume of a 10 % w/v aqueous solution of zinc sulphate, a 20 µL-volume of a 0.1 M aqueous sodium hydroxyde, and a 400 µL-volume of acetonitrile were added. After homogenization for 1 h (Rotator Drive, Heidolph, Keilheim, Germany) and centrifugation at 18,000 g for 15 min at + 4°C (Biofuge 15 R, Heraeus, Hanau, Germany), the supernatant was transferred in a 12 mL glass tube and evaporated to dryness under nitrogen stream at + 40 °C.

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The extract was dissolved in 200 µL of mobile phase, homogenized for 30s (Top-Mix 94323, Heidolph), sonicated for 3 min (Transonic 570, Elma, Munich, Germany), and centrifugated at 4,000 g for 10 min at + 4°C (Biofuge 15 R). The supernatant was transferred into a 0.2 mL vial before injection onto the cartridge.

Validation Assay

Standard calibration curves for oxolinic acid (range: 0, 0.020, 0.100, 0.500, and 2.500 µg/mL) were obtained by analysing three replicates of each spiked serum for five days. They were drawn by plotting the known oxolinic acid concentrations against the ratio of oxolinic acid to internal standard peak heights. These curves were used to study linearity, regression, and precision and to calculate the limits of detection and determination. The precision of the limit of determination was studied by spiking serum samples at this limit and analysing five replicates for two days.

The extraction recoveries of oxolinic acid were determined by comparing peak heights obtained by chromatographing spiked and extracted serums with peak heights obtained by chromatographing the working standard solutions. A 280 days-study of stability was performed by analysing samples spiked at a concentration of 0.100 µg/mL with oxolinic acid and stored at -20°C.

RESULTS AND DISCUSSION

Chromatographic Conditions

Reversed phase HPLC has often been used for determining quinolones antibiotics in fish plasma and serum and gave good performance in terms of plate numbers.[11,16,19-20] In our experiment, using a Supelcosil ABZ+Plus® prepacked cartridge instead of a LiChroSpher 100-RP 18E® (Merck) prepacked cartridge narrowed the oxolinic and nalidixic peaks and improved both the resolution and the peaks symmetry. This is in accordance with previous reports about the importance of using endcapped materials in order to reduce the interactions of oxolinic and nalidixic acids with residual silanol groups of the stationary phase.[11,16,19-20]

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All the previous workers using reversed phase systems added an organic modifier to the predominantly aqueous eluent.[8,11,14-21] Comparisons of the three organic modifiers - methanol, tetrahydrofuran, and acetonitrile - indicated that peaks were more symmetrical by using a mixture of acetonitrile and tetrahydrofuran (data not shown). The presence of tetrahydrofuran in the mobile phase also reduced peak tailing and increased the number of theoretical plates of the cartridge.

Both oxolinic and nalidixic acids show strong fluorescence in acid aqueous solutions.[17,19] Orthophosphoric acid was used as aqueous eluent in order to make use of the increase specificity and sensitivity given by the fluorescence detection and to improve the peak shape by masking the residual silanol groups of the stationary phase.[8,17,20] Variations in the orthophosphoric acid concentration (0.001 - 0.020 M) had only a minor effect on the peak shape and retention times of oxolinic and nalidixic acids.

Nevertheless, the stationary phase Supelcosil ABZ+Plus® was stable when the pH of the mobile phase was between 3.0 and 7.5. Therefore, a 0.001 M orthophosphoric acid solution was choosen in order that the pH of the mobile phase was above 3.0.

The highest efficiency was obtained with acetonitrile, tetrahydrofuran and 0.001 M orthophosphoric acid solution (22:10:68 v/v), pH 3.4. Lower proportions of acetonitrile and tetrahydrofuran and higher pH values of the mobile phase gave higher retention times and lower peak heights (data not shown).

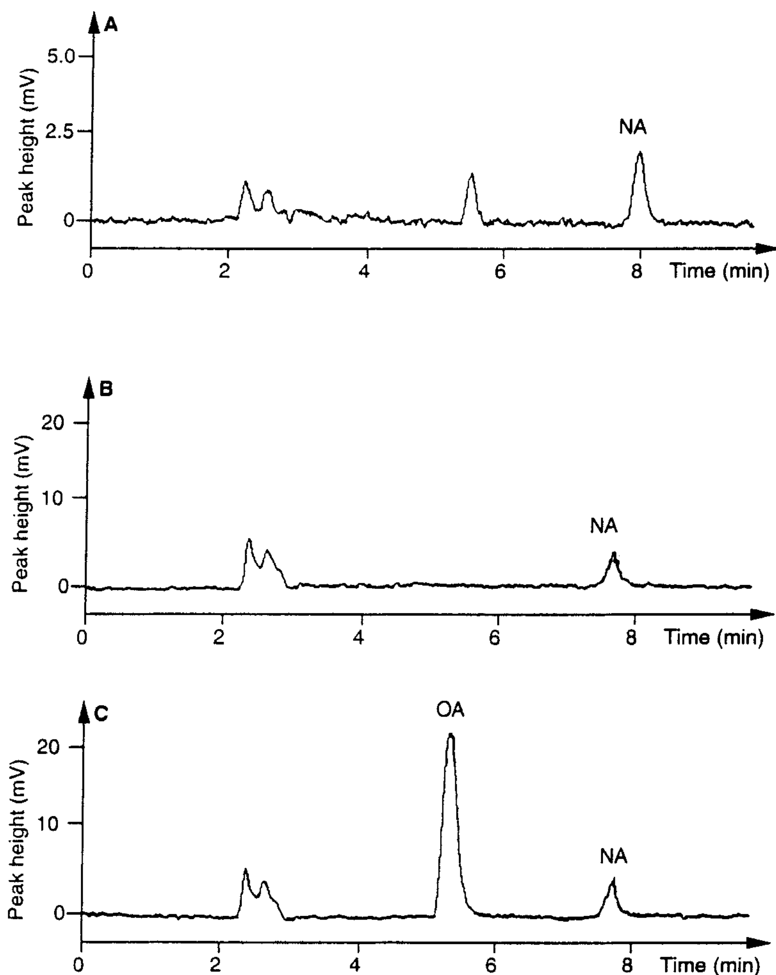

Figure 1. HPLC chromatograms of Turbot Serum A, containing Nalidixic Acid (NA) at a concentration of 0.20µg/mL after solid phase extraction on a C_{18} cartridge; B, containing NA at a concentration of 0.20 µg/mL after liquid phase extraction; C, containing Oxolinic Acid (OA) at a concentration of 0.50 µg/mL and NA at a concentration of 0.20 µg/mL after liquid phase extraction. Conditions: analytical cartridge, Supelcosil ABZ + Plus 5µm; mobile phase, Acetonitrile,Tetrahydrofuran and 0.001 M Orthophosphoric Acid solution (22:10:68 v/v), pH 3.4; Flow rate, 0.8 mL/min; Detection, fluorescence; Injection volume, 10 µL.

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Under the operating conditions, oxolinic and nalidixic acids were eluted in 5.0 and 7.2 min respectively (Figure 1). No changes in retention times were noted with continual cartridge use. No additionnal peaks, possibly resulting from impurities or degradation products, that could interfere with the oxolinic or nalidixic acids peaks, were noted on the chromatograms.

Ending each day of operation, recycling with acetonitrile and water (50:50 v/v) through the HPLC cartridge for 2 h allowed the cartridge life to increase, because reversed phases are unstable at low pH values. Under these conditions, there was no loss of bonded layer and cartridge could be used for about 1,500 injections of samples without any significative change in its performances. After 500 injections, the guard cartridge was saturated with turbot serum components and gave a drifting baseline.

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Extractions and Recoveries

When oxolinic acid was extracted from rainbow trout (*Oncorhynchus mykiss*) or salmon (*Salmo salar*) plasma and serum, better recoveries were obtained by using a solid-phase extraction procedure rather than a liquid-phase extraction procedure.[8,16,17,20] In a preliminary study, a solid-phase extraction procedure was optimized in order to adapt it to turbot serum.

Many solid-phase extraction cartridges were used: Bond Elut® Octyl, Octadecyl and SAX Quaternary Amine (Analytichem International, Harbor City, CA, USA) and Chromabond® Octyl, Octadecyl, and Octadecyl End-Capped (Macherey-Nalgel, Düren, Germany). Different solvents or mixtures of solvents were tested for conditioning (methanol, acetonitrile, oxalic acid solution, orthophosphoric acid solution, phosphate buffers), washing (water, oxalic acid solution, orthophosphoric acid solution), and eluting (acetonitrile, methanol) the cartridges. The best recoveries (90.0 %) were obtained with octadecyl solid-phase extraction cartridges, methanol, and 0.008 M orthophosphoric acid solution as conditioning solvents, water and 0.008 M orthophosphoric acid solution as washing solvent and acetonitrile as elution solvent (data not shown). Whatever the cartridge and the solvents may be, the chromatograms of a blank turbot serum extract always showed a peak at the retention time of oxolinic acid (Figure 1 A). This result indicated that the solid-phase extraction procedure was not well suited to the analysis of oxolinic acid in turbot serum, probably because of an interfering substance resulting from interactions between a turbot serum component and the stationary phase of the solid-phase extraction cartridges.

Table 1

Recoveries of Oxolinic Acid From Turbot Serum Spiked With Oxolinic Acid From 0.02 to 2.50 μg/mL.

	0.02μg/mL (n=14)	0.10 μg/mL (n=15)	0.05 μg/mL (n=13)	2.50 μg/mL (n=14)
Mean (%)	110.71	105.80	104.35	103.89
Standard Deviation (%)	8.52	8.76	6.41	5.06

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Table 2

Linearity and Regression Data for the Calibration Graphs Obtained From Turbot Serum Spiked With Oxolinic Acid From 0.02 to 2.50 μg/mL[a]

	Day 1 (n=13)	Day 2 (n=15)	Day 3 (n=15)	Day 4 (n=14)	Day 5 (n=14)	Mean (n=71)
Slope	16.107	17.632	19.276	18.610	17.799	17.822
Intercept	0.153	0.080	-0.115	0.341	-0.047	0.104
Correlation Coefficient	0.999	0.995	0.999	0.997	0.9923	0.994
Comparison Intercept/0 (t-Test)	0.754*	0.143*	0.543*	0.890*	0.065*	0.398*
Slope Existence (F-Test)	9303**	1285**	10774*	2243**	828**	5930**
Calibration Validity (F-Test)	0.117*	0.005*	0.785*	0.380*	0.054*	0.551*

[a] y = ax + b; y = Ratio of oxolinic acid to nalidixic acid peak height; X = oxolinic acid concentration (μG/Ml); A = Slope; b = Intercept.

* Non signicative value at the 0.05 level.

** Significative value at the 0.05 level.

A liquid-phase extraction procedure was optimized in order to extract oxolinic acid from turbot serum. The highest recoveries were achieved using zinc sulphate, sodium hydroxide, and acetonitrile as precipitating agents followed by a high-speed centrifugation. Under these conditions, interfering substances did not occur in the area of the chromatograms in which oxolinic and nalidixic acids appeared (Figure 1 B et C). The mean recoveries of oxolinic acid from turbot serum were between 103.89 % and 110.71 %, over the concentration range 0.020-2.500 µg/mL (Table 1). These recoveries were above those obtained in fish serum and plasma (92.00 - 99.70 %) by other authors.[8,11,14-21]

Linearity, Regression, Precision, and Limits of Detection and Determination

A linearity and regression study was performed for each calibration curve separately (Table 2). The high values of the correlation coefficients (0.993 to 0.999) indicated good correlations between oxolinic acid concentration and ratio of oxolinic acid to internal standard peak height. The intercepts of the five calibration curves were not significantly different from 0 at the 0.05 level. Moreover, for each calibration curve, the statistical data showed the existence of a slope and of a valid calibration at the 0.05 level. Slopes and intercepts of the calibration curves were not significantly different between the five days at the 0.05 level (data not shown); therefore, a mean calibration curve was determined (Table 2).

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The relative standard deviations of repeatability for spiked serum were between 2.02 and 4.17 % (Table 3). The relative standard deviations of reproducibility were between 4.17 and 8.77 % (Table 3). The detection and determination limits of oxolinic acid in turbot serum were respectively 0.005 and 0.015 µg/mL. This determination limit was accepted (relative standard deviation = 22.8 % with $n = 10$) because the mean value of the ratio of oxolinic acid to internal standard peak height was significantly different from 0 at the 0.05 level and greater than three standard deviations.[22,23] The detection and determination limits were lower than those reported in previous studies with an injection volume of 10 µL.[16-21]

Stability Study

A study of the stability of oxolinic acid in turbot serum spiked at a concentration of 0.100 µg/mL and stored at - 20°C was performed. All the

Table 3

Precision Data Obtained From Turbot Serum Spiked With Oxolinic Acid From 0.02 to 2.50 μg/mL

	0.02 μg/mL (n-14)	**0.10 μg/mL (n=15)**	**0.50 μg/mL (n=13)**	**2.50 μg/mL (n=14)**
RSD* of Repeatability (%)	3.85	4.12	2.02	4.17
RSD* of Reproducibility (%)	8.15	8.77	6.15	4.17

* Relative Standard Deviation.

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recoveries of oxolinic acid from the samples stored at -20°C felt within the average limits calculated using the recoveries results from the validation.[22,23] Moreover, there was no significant decrease of the oxolinic acid recoveries from samples maintained at -20°C during 280 days.

CONCLUSION

The described method provides a selective, reliable, and precise mean for the rapid determination of oxolinic acid in turbot serum. It does not require time-consuming, complex extraction or derivatization techniques. An analyst familiar with the method could easily process thirty samples a day.

This method is, therefore, suitable for pharmacokinetics studies on oxolinic acid in turbot.

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REFERENCES

1. J. P. Cravedi, G. Choubert, G. Delous, Aquaculture, **60**, 133-141 (1987).

2. T. Endo, M. Onozawa, Nippon Suisan Gakkaishi, **53**, 551-555 (1987).

3. T. Endo, M. Onozawa, Nippon Suisan Gakkaishi, **53**, 557-562 (1987).

4. T. Endo, M. Onozawa, M. Hamaguchi, R. Kusuda, Nippon Suisan Gakkaishi, **53**, 1493 (1987).

5. H. V. Björklund, G. Bylund, Xenobiotica, **21**, 1511-1520 (1991).

6. S. O. Hustvedt, R. Salte, Aquaculture, **92**, 297-303 (1991).

7. S. O. Hustvedt, R. Salte, V. Vassvik, Aquaculture, **95**, 193-199 (1991).

8. S. O. Hustvedt, R. Salte, O. Kvendset, V. Vassvik, Aquaculture, **97**, 305-310 (1991).

9. S. O. Hustvedt, T. Storebakken, R. Salte, Aquaculture, **92**, 109-113 (1991).

10. H. V. Björklund, A. Eriksson, G. Bylund, Aquaculture, **102**, 17-27 (1992).

11. K. M. Kleinow, H. H. Jarboe, K. E. Shoemaker, K. J. Greenless, Can. J. Fish Aquat. Sci., **51**, 1205-1211 (1994).

12. B. Martinsen, T. E. Horsberg, Antimicrob. Agents Chemother., **39**, 1059-1064 (1995).

13. J. S. Giles, H. Hariharan, S. B. Heaney, Microbiologica, **17**, 155-158 (1994).

14. R. Ueno, Y. Horiguchi, S.S. Kubota, Nippon Suisan Gakkaishi, **54**, 479-484 (1988).

15. R. Ueno, M. Okumura, Y. Horiguchi, S.S. Kubota, Nippon Suisan Gakkaishi, **54**, 485-489 (1988).

16. S. O. Hustvedt, R. Salte, T. Benjaminsen, J. Chromatography, **494**, 335-339 (1989).

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17. K. E. Rasmussen, F. Tønnesen, H. H. Thanh, A. Rogstad, A. Aanesrud, J. Chromatography, **496**, 355-364 (1989).

18. H. V. Björklund, J. Chromatography, **530**, 75-82 (1990).

19. O. B. Samuelsen, J. Chromatography, **530**, 452-457 (1990).

20. T. Agasøster, K. E. Rasmussen, J. Chromatography, **564**, 171-179 (1991).

21. R. Ueno, T. Aoki, J. Chromatography B, **682**, 179-181 (1996).

22. J. R. Lang, S. Bolton, J. Pharm. Biomed. Anal., **9**, 357-361 (1991).

23. J. R. Lang, S. Bolton, J. Pharm. Biomed. Anal., **9**, 435-442 (1991).

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