Journal of Chromatography, 530 (1990) 452–457
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5371

Note

Simple and rapid method for the determination of flumequine and oxolinic acid in salmon (*Salmo salar*) plasma by high-performance liquid chromatography and fluorescence detection

OLE BENT SAMUELSEN

Department of Pharmacology and Toxicology, School of Medicine, University of Bergen, Amauer Hansens hus, 5021 Bergen (Norway)

(First received February 27th, 1990; revised manuscript received April 28th, 1990)

With the development of commercial aquaculture several antibiotics and chemotherapeutic agents have been used for the prevention and treatment of various infectious diseases in fish. Both flumequine {fluoro-6,7-dihydro-5-methyl-1-oxo-1*H*,5*H*-benzo[*ij*]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, which have found widespread use in farming of Atlantic salmon (*Salmo salar*).

In order to carry out metabolic and pharmacokinetic studies of these drugs, a rapid, simple and selective extraction and analytical method is crucial for the analysis of large series of plasma samples. Several methods to determine flumequine and oxolinic acid in plasma from humans and animals have been described. Bioassay [1,2] and fluorometry [3,4] methods are poor in selectivity and sensitivity and earlier high-performance liquid chromatographic (HPLC) methods required time-consuming and complex sample pretreatment [3,5–7]. Recently, several time-saving solid-phase extractions, including both off-line procedures based on sample pretreatment on disposable solid-phase extraction columns [8,9] and a totally automated on-line procedure based on column switching [9], have been described. However, the off-line procedures still involve several steps and the on-line procedure requires expensive additional equipment for the HPLC system. Because flumequine and oxolinic acid appear as tailing peaks in reversed-phase HPLC, several techniques to avoid this effect have been employed, the most successful being the use of non-silica-based columns [9], a combination of end-capped C_{18} columns and a paired-ion technique [8], ion-exchange columns [10,11], wide-pore ODS columns [12] and C_{18} columns using citric acid buffer and tetrahydrofuran in the mobile phase [13].

This paper describes a method with a silica-based C_8 column, which minimizes

0378-4347/90/$03.50

the tailing by the use of a gradient and oxalic acid and tetrahydrofuran in the mobile phase. The pretreatment of the samples is limited to protein precipitation with acetonitrile–zinc sulphate followed by centrifugation.

EXPERIMENTAL

Chemicals and reagents

Flumequine and oxolinic acid were kindly supplied by the late Prof. E. Egidius (Institute of Marine Research, Bergen, Norway). Methanol, acetronitrile, tetrahydrofuran (HPLC grade) and oxalic acid dihydrate and zinc sulphate (p.a. grade) were all from Merck (Darmstadt, F.R.G.).

Chromatography

The HPLC system consisted of a Spectra-Physics SP 8800 ternary HPLC pump (Spectra-Physics, San Jose, CA, U.S.A.) connected to a Spectra-Physics SP 8780 X R autosampler and a Shimadzu RF-535 fluorescence detector (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 325 nm and an emission wavelength of 360 nm. The integrator was the Model SP-4270 from Spectra-Physics.

The HPLC system was programmed to inject 20-μl samples into the 150 × 4.6 mm I.D. analytical column, packed with 3-μm particles of MOS-Hypersil (C_8) (Shandon Southern Products, Cheshire, U.K.) in our laboratory using a Shandon column-packing machine. The column was operated at room temperature. The mobile phase contained (A) 0.025 *M* oxalic acid (pH 3.2)–acetonitrile–methanol–tetrahydrofuran (80:2.5:15:2.5, v/v) and (B) oxalic acid (pH 3.2)–acetonitrile–methanol–tetrahydrofuran (50:20:25:5, v/v). The solutions were filtered through a 0.2-μm Millipore filter. The elution profile was a follows: 0–5 min, 0–100% B linear gradient; 5–10 min isocratic 100% B; 10.1–15 min isocratic 100% A. The flow-rate was 1 ml/min and the pressure *ca.* 16.1 MPa.

Standards

Stock solutions of flumequine and oxolinic acid were prepared at a concentration of 0.1 mg/ml in 0.1 *M* NaOH and stored in the dark at −20°C. Working standards were prepared by dilution from the stock solutions with 0.1 *M* NaOH.

Sample preparation

To 100 μl of fish plasma, 10 μl of a 10% w/v solution of zinc sulphate and 10 μl of a 0.1 *M* NaOH solution containing oxolinic acid or flumequine (0.1 μg) as internal standard were added. Upon mixing, the plasma turned milky, but the protein precipitation was not complete. Then 80 μl of acetonitrile were added, followed by vigorous shaking for 1 min. After centrifugation at 14 930 *g* in a Biofuge A table-centrifuge (Heraeus Sepatech, Osterode am Harz, F.R.G.) for 2 min a clear supernatant was obtained, ready for analysis. Flumequine was used as

internal standard for the determination of oxolinic acid, and oxolinic acid was used as internal standard for the determination of flumequine.

Calibration

Standard calibration curves for flumequine and oxolinic acid in the range 0.05–1.0 μg/ml were prepared in four replicates using drug-free plasma and oxolinic acid or flumequine as internal standard. Standard curves were drawn by plotting the known drug concentration against the ratio of drug to internal standard peak height. These curves were also used to calculate the detection limits.

Recovery and precision studies

The extraction recoveries of flumequine and oxolinic acid were determined by comparing peak heights from the analysis of plasma samples spiked with 0.05 μg/ml and 1.0 μg/ml flumequine or oxolinic acid with peak heights resulting from direct injection of the standards.

To determine the within-run precision, five replicates of a plasma sample spiked with 1.0 μg/ml drug and 1.0 μg/ml internal standard were analysed.

RESULTS AND DISCUSSION

When the previously described isocratic HPLC system [13] was used to determine flumequine and oxolinic acid in fish plasma, no baseline separation was obtained of the flumequine peak and a peak occurring in front of it. Some adjustment of the method [13] was therefore deemed necessary.

Both flumequine and oxolinic acid show strong fluorescence in acidic aqueous solutions. To make use of the increased specificity and sensitivity offered by the fluorescence detector, and at the same time improve the peak shape by effectively masking the residual silanol groups in the stationary phase, oxalic acid was used as a mobile phase additive [14]. The presence of tetrahydrofuran in the mobile phase has also been shown to reduce peak tailing of the different quinolone derivatives and to increase the number of theoretical plates of the column [13,15]. An organic modifier such as methanol and/or acetonitrile is commonly used in reversed-phase HPLC. Hence, the mobile phase was optimized with respect to acetonitrile, tetrahydrofuran, methanol and oxalic acid. Variations in the oxalic acid concentration (0.01–0.05 *M*) had only a minor effect on the peak shape and retention times of the peaks. Changing the pH from 2.2 [14] to 3.2 and using a C_8 analytical column instead of a C_{18} column [13] narrowed the large peak between the oxolinic acid and the flumequine peaks and improved the separation. Gradient elution improved both the separation and the peak shape.

Chromatograms of blank plasma and plasma from fish treated with flumequine (20 mg/kg fish per day for 4 days) are shown in Fig. 1. The retention times were 6.6 and 8.9 min for oxolinic acid and flumequine, respectively. No significant changes were found in the oxolinic acid or flumequine concentrations in

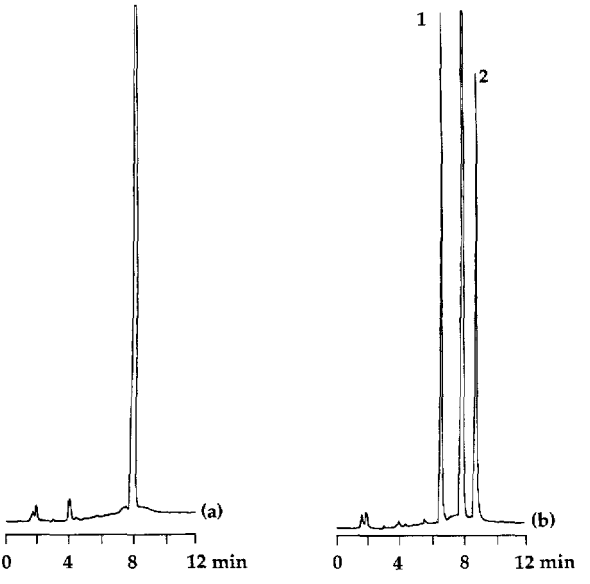

Fig. 1. Chromatograms of (a) a drug-free plasma sample, (b) a plasma sample from a fish treated with flumequine. Oxolinic acid (1 μg/ml) was used as internal standard. Fluorescence detection attenuation, 32. Peaks: 1 = oxolinic acid, 2 = flumequine.

dissolved residues stored in the dark at 4°C in a refrigerator or at room temperature in the autoinjector for 24 h. The extraction procedure gave extracts devoid of interfering endogenous substances.

The calibration graphs plotted as peak-area ratio of oxolinic acid or flumequine to the internal standard against concentration of oxolinic acid of flumequine were found to be linear over the range studied (0.05–1.0 μg/ml): $y=0.56x+0.21$, $r=0.999$ for oxolinic acid; and $y=0.65x+0.16$, $r=0.996$ for flumequine. The within-run coefficient of variation of the flumequine peak was 2.1% and that of oxolinic acid 1.0%. The limits of detection at a signal-to-noise ratio of 3 were 0.003 and 0.005 μg/ml for oxolinic acid and flumequine, respectively, with an injection volume of 50 μl. These values are lower [9] or slightly higher [8,16] than those in previous studies. The mean recoveries and reproducibilities of the procedure for both flumequine and oxolinic acid are listed in Table I.

A one-step procedure for the determination of oxolinic acid in chicken plasma using acetonitrile as precipitating agent followed by centrifugation and HPLC [17] was reported to produce interfering substances and impurities that accumulated on the analytical column causing increasing back-pressure when applied to fish plasma [8,9]. However, when the supernatant was left overnight (4°C in the dark) an additional precipitation of proteins occurred and when this was removed by centrifugation a clear supernatant that remained clear for at least 24 h

TABLE I

RECOVERY AND REPRODUCIBILITY OF FLUMEQUINE AND OXOLINIC ACID IN FISH PLASMA

Drug	Concentration (μg/ml)		Coefficient of variation (%)
	Added	Recovered ($n=6$)	
Flumequine	0.050	0.045	3.5
	1.00	0.89	1.3
Oxolinic acid	0.050	0.047	5.6
	1.00	0.92	0.6

at room temperature was the result. This procedure, adding 90 μl of acetonitrile and 10 μl of internal standard solution (0.1 *M* NaOH) to 100 μl of plasma was found to be linear over the range studied (0.05–1 μg/ml), and gave slightly higher recoveries but a poorer overall precision and was more time-consuming than the procedure using zinc sulphate already described. When zinc sulphate was used [18] in addition to acetonitrile and internal standard solution, supernatants free of additional precipitation for at least 24 h at room temperature were produced immediately.

Interfering substances did occur in the area of the chromatograms in which oxolinic acid and flumequine appeared when a UV detector at 280 nm [8] or the fluorescence detector at an excitation wavelength of 260 nm and an emission wavelength of 380 nm [9] was used. Changing the wavelengths to 325 nm (excitation) and 360 nm (emission) and use of a gradient caused the interfering peaks to disappear, or else changed the retention times sufficiently to separate them from the peaks of interest. Changing the wavelengths reduced the sensitivity to oxolinic acid by a factor of *ca.* 5, but that of flumequine was only slightly affected ($<5\%$ decrease in peak height).

The procedure also worked well with 50 μl of plasma and an equivalent reduction of the amount of acetonitrile, zinc sulphate and internal standard solution. The analytical column showed minor signs of increasing back-pressure and peak-broadening after *ca.* 100 injections. A routine repacking of the top 3–4 mm of the column after 100–120 injections, following the method described by Ueland and Solheim [19], prevented both increasing back-pressure and peak-broadening.

In conclusion, the extraction and HPLC method described is simple and sensitive and very suitable for pharmacokinetic studies of oxolinic acid and flumequine in fish plasma, where analysis of large series of plasma samples is necessary.

ACKNOWLEDGEMENTS

The author is grateful to The Norwegian Research Council for Fisheries

Science and the Norwegian Enviromental Protection Agency (Statens Forurensingstilsyn) for financial support. The excellent technical assistance of Mr. Audun Høylandsskjær is highly appreciated.

REFERENCES

1 G. Ziv, *Am. J. Vet. Res.*, 37 (1976) 513.
2 S. M. Ringel, F. J. Turner, S. Roemer, J. M. Daly, R. Slatanoff and B. S. Swarts, *Antimicrob. Agents Chemother.*, 7 (1967) 486.
3 L. I. Harrison, D. Schuppan, S. R. Rohlfing, A. R. Hansen, C. S. Hansen, M. L. Funk, S. H. Collins and R. E. Ober, *Antimicrob. Agents Chemother.*, 25 (1984) 301.
4 P. T. Mannisto, *Clin. Pharmacol. Ther.*, 19 (1976) 37.
5 Y. Kasuga, A. Sugitani and F. Yamada, *J. Food Hyg. Soc. Jpn.*, 23 (1982) 344.
6 D. Decolin, A. Nicolas and G. Siest, *J. Chromatogr.*, 414 (1987) 499.
7 L. Ellerbroek, *J. Chromatogr.*, 495 (1989) 314.
8 S. O. Hustvedt, R. Salte and T. Benjaminsen, *J. Chromatogr.*, 494 (1989) 335.
9 K. E. Rasmussen, F. Tønnesen, H. H. Thanh, A. Rogstad and A. Aanesrud, *J. Chromatogr.*, 496 (1989) 355.
10 L. Shargel, R. F. Koss, A. V. R. Chain and V. J. Boyle, *J. Pharm. Sci.*, 62 (1973) 1452.
11 D. L. Sondack and W. L. Koch, *J. Chromatogr.*, 132 (1977) 352.
12 M. Horie, K. Saito, Y. Hoshino and N. Nose, *J. Chromatogr.*, 402 (1987) 301.
13 O. B. Samuelsen, *J. Chromatogr.*, 497 (1989) 355.
14 Y. Ikai, H. Oka, N. Kawamura, M. Yamada, K.-I. Herada, M. Suzuki and H. Nakazawa, *J. Chromatogr.*, 477 (1989) 397.
15 R. M. Smith and D. R. Garside, *J. Chromatogr.*, 407 (1987) 19.
16 G. Guisinade, N. Ferry, M. Seccia, N Bernard and J. Sassard, *J. Chromatogr.*, 181 (1980) 399.
17 K. Hamamoto, *J. Chromatogr.*, 381 (1986) 453.
18 S. Lam and G. Malikin, *J. Liq.Chromatogr.*, 12 (1989) 1851.
19 P. M. Ueland and E. Solheim, *J. Chromatogr.*, 276 (1983) 157.