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Note

High-performance liquid chromatographic determination of several quinolone antibacterials in medicated fish feed

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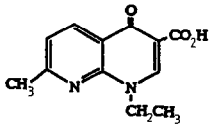
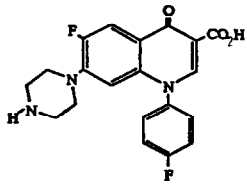
Aquaculture is growing as a commercial source of consumable fish products. During the growth stages of aquaculture, antibacterial agents are used to prevent the development of gram-negative bacterial infections in the fish.

Quinolone analogues of nalidixic acid[1] (I), 1-ethyl-1,4-dihydro-4-oxo-7-methyl-1,8-naphthyridine-3-carboxylic acid, are wide-spectrum antibacterial agents. Some of these are being developed for use in aquaculture. A validated analytical procedure was developed to measure various concentrations of the quinolones in feed. Feed–drug admixtures were studied to determine effectiveness and dosage levels for various fish species.



I



II

Although methodology for various quinolones have been reported in several matrices including fish tissue[2–6], none has been reported for feed. Animal feeds, in general, are an admixture of several grains and soy products containing numerous polar and non-polar compounds. This can often lead to the co-extraction of interfering components along with the drug of interest. In order to minimize this problem, it is preferable to extract the drug from the feed using a solvent of moderate polarity such as acetonitrile. Additionally the natural products present in the feed provide favorable binding sites for many drugs which often makes quantitative extraction difficult.

Sarafloxacin (II) is a quinolone which is a potent wide-spectrum antibacterial. Sarafloxacin is presently being used successfully in aquaculture trials.

Sarafloxacin is quite insoluble in water (0.3 mg/ml) and in most organic solvents (< 1 mg/ml) and therefore presents a formidable analytical problem when mixed with

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feed. Sarafloxacin will be commercially available as a pre-mix with soy flour which is then mixed with the appropriate fish feed (trout, salmon and catfish) before feeding. It was therefore necessary to develop analytical methodology for quantitating sarafloxacin in both the pre-mix and in the various fish feeds.

Due to the low solubility of sarafloxacin in organic solvents, it is impossible to use moderate-polarity solvents such as acetonitrile to extract the drug from the feed. As a result of an unusual synergistic effect, however, sarafloxacin is significantly more soluble in an equal mixture of acetonitrile and water (>4 mg/ml) than in either of the individual solvents. This equal mixture of acetonitrile and water was chosen as the extraction solvent, even though it resulted in the extraction of numerous other components from the feeds. The extraction was further complicated by the fact that sarafloxacin is amphoteric and has a tendency to bind to the feed.

This paper presents validated methods developed for both the soy flour pre-mix of sarafloxacin and the various fish feed admixtures.

MATERIALS AND METHODS

Instrumentation and reagents

A Polytron Model PT 35/4 homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) was used for sample extraction. The column liquid chromatography (LC) system used throughout this work consisted of a Model SIL-6A autosampler (Shimadzu, Kyoto, Japan) and a Spectroflow 783 variable-wavelength UV detector (Kratos Analytical Instruments). Chromatograms were processed using a Chromatopac Model C-R4A integrator (Shimadzu). A C_{18} chromatographic column (10 μm, 25 cm $\times$ 4.6 mm I.D.) was used (Alltech, Deerfield, IL, U.SA.). Chemicals and solvents were reagent grade and high-performance liquid chromatography (HPLC) grade, respectively. Bulk sarafloxacin and difloxacin were prepared at Abbott Laboratories, North Chicago, IL, U.S.A. Bulk ciprofloxacin and norfloxacin were purchased from Miles Pharmaceutical Division, New Haven, CT, U.S.A. and Merck, Sharpe and Dohme, West Point, PA, U.S.A., respectively. The catfish, salmon and trout feed mixtures were prepared at Clear Springs Trout Co., Buhl, ID, U.S.A. and Mississippi State University, Oxord, MS, U.S.A., respectively. *p*-Nitroacetophenone and *p*-bromoacetophenone used as internal standards were purchased from Aldrich, Milwaukee, WI, U.S.A.

Chromatographic conditions

Pre-mix analysis. The LC eluent was an aqueous buffer containing 0.02 *M* sodium citrate and 0.02 *M* citric acid (pH adjusted to 2.4 with perchloric acid) mixed with acetonitrile at a ratio 65:35. After filtering through a 0.45-μm nylon membrane (Cuno, Meriden, CT, U.S.A.) the eluent was pumped at 1.5 ml/min. The UV detector was set at a detection wavelength of 280 nm. The internal standard was *p*-nitroacetophenone.

Feed admixtures. The LC eluent was an aqueous buffer containing 0.1 *M* sodium dodecyl sulfate (pH adjusted to 4.0 with phosphoric acid) mixed with acetonitrile and tetrahydrofuran at a ratio 66.5:28.5:5.0. After filtering through a 0.45-μm nylon membrane, the eluent was pumped at 1.5 ml/min. The UV detector was set at a detection wavelength of 280 nm. The internal standard was *p*-bromoacetophenone.

Assay procedure

Pre-mix. An internal-standard solution was prepared by dissolving approximately 250 mg of *p*-nitroacetophenone accurately weighed in 1 l of acetonitrile–water (1:1). A standard was prepared by weighing accurately approximately 23 mg of sarafloxacin reference standard and dissolving in 100.0 ml of internal standard solution. This solution was then diluted 5:50 with eluent. Samples were analyzed by weighing accurately approximately 400 mg of pre-mix into a tall-form beaker and pipeting 100 ml of internal standard into the beaker. The sample was then blended at approximately 5500 rpm for 2 min. The mixture was allowed to settle for 10 min and a portion of the supernatant was centrifuged. A portion of the clear supernatant was then diluted 5:50 with eluent. Peak area ratios were determined and the sarafloxacin content was calculated *versus* the standard.

Feed admixtures. An internal standard solution was prepared by accurately weighing 18 mg of *p*-bromoacetophenone into 1.0 l of acetonitrile–water (1:1). A standard curve was prepared by weighing four individual 3-g samples of blank feed into separate tall-form beakers and spiking into the respective samples 0, 50, 100 and 150% of the anticipated amount of drug in the unknown feed samples. These samples were spiked by adding an appropriate volume of a methanol solution of sarafloxacin and evaporating to dryness. An appropriate volume of internal standard (50 ml for feeds containing < 500 ppm drug and 100 ml for feeds containing $\geqslant 500$ ppm drug) was pipeted into the sample. The mixture was blended in a high-speed homogenizer for 2 min at approximately 5500 rpm. The mixture was allowed to settle for 5–10 min, then a portion of the supernatant was centrifuged. Samples of 50 μl of each standard preparation were injected into the chromatograph and ratios of peak heights of the drug to internal standard were plotted *versus* the drug concentration (mg/g of feed) to produce a standard curve.

Samples of feed containing sarafloxacin were assayed by weighing 3 g into a tall-form beaker and extracting as described for the standard curve. Peak height ratios were determined for the samples and the sarafloxacin content was calculated from the standard curve.

RESULTS AND DISCUSSION

In this work, our purpose was to develop procedures for determining sarafloxacin in both soy flour pre-mix and in actual fish feed admixtures. Because of the large number of binding sites available in the natural products used and the amphoteric nature of sarafloxacin, neither prolonged, vigorous shaking nor sonification were sufficient to quantitatively extract the drug from either the soy flour or the feed. High-speed blending with a homogenizer at 5500 rpm was found to give recoveries ranging from 99 to 101% for spiked soy flour samples. In order to avoid errors due to solvent evaporation during the blending, an internal standard of *p*-nitroacetophenone was added in the extraction solvent.

Typical chromatograms for a sarafloxacin standard and pre-mix sample preparation are show in Fig. 1. The precision of the analysis was determined by performing the analysis several times on the same lot over a two day period. The measurements were made by two analysts and the results are shown in Table I. As shown, the relative standard deviation (R.S.D.) was $\pm$ 0.69%. The linearity of the

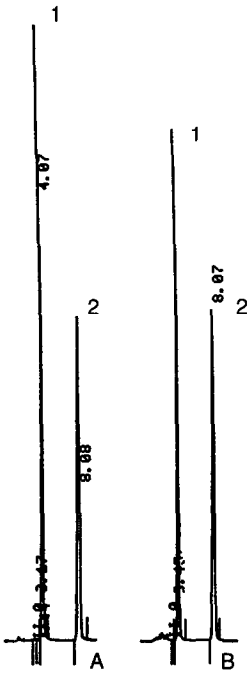


Fig. 1. Chromatograms of sarafloxacin. (A) Standard preparation; (B) pre-mix assay preparation. Peaks: 1 = *p*-nitroacetophenone; 2 = sarafloxacin. Retention times in min.

detector response was demonstrated by chromatographing solutions of sarafloxacin in internal standard solution containing concentrations from 2.4 to 24 μg/ml. A plot of peak area ratios *versus* concentration was linear. The regression line showed a *y*-intercept of 0.004 (not statistically different from the origin, $P \leqslant 0.05$) and a correlation coefficient of 0.9999.

A similar extraction was attempted on fish feed containing sarafloxacin but complete recovery was not obtained. When blank feed was added to a clear

TABLE I

PRECISION DATA FOR ANALYSIS OF SARAFLOXACIN IN PRE-MIX

	Analyst I (mg/g)	*Analyst II* (mg/g)
	45.0	45.8
	45.5	45.6
	45.3	45.8
	45.2	45.9
	45.4	45.9
Average	45.5	
S.D.	0.31	
R.S.D.	0.69%.	

TABLE II

PRECISION DATA FOR THE ANALYSIS OF SARAFLOXACIN IN FISH FEED

	Sample I (ca. 300 μg/g)		*Sample II (ca. 700 μg/g)*	
	Analyst I (μg/g)	*Analyst II (μg/g)*	*Analyst I (μg/g)*	*Analyst II (μg/g)*
	344.7	324.3	831.6	753.2
	338.7	327.6	836.5	812.7
	347.4	326.7	834.4	816.2
	338.7	335.4	837.9	831.6
	344.7	324.9	833.7	826.7
Average	335.3		821.5	
S.D.	8.87		25.4	
R.S.D.	2.6%		3.1%	

homogeneous solution of sarafloxacin, only 85% of the sarafloxacin remained in solution indicating that a partitioning of drug was occurring between the fish feed and the extraction solvent. Direct extraction of sarafloxacin from feed, using the HPLC eluent, which contained the surfactant sodium dodecyl sulfate, did not result in improved recoveries. Analysis of spiked blank feed with levels of sarafloxacin ranging from 100 to 765 μg/g were performed. Plotting the peak height ratios *versus* concentration of drug in feed gave linear relationships with correlation coefficients

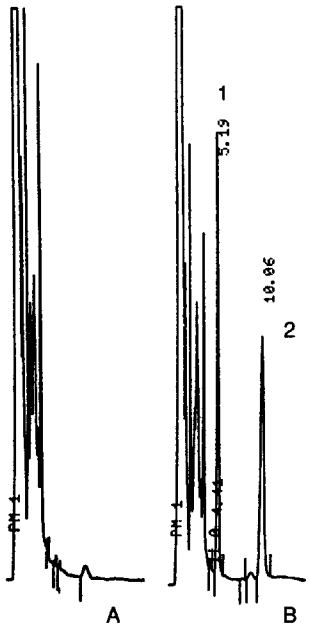


Fig. 2. (A) Chromatogram of blank fish feed. (B) Typical chromatogram of sarafloxacin assay preparation from fish feed. Peaks: 1 = *p*-bromoacetophenone; 2 = sarafloxacin.

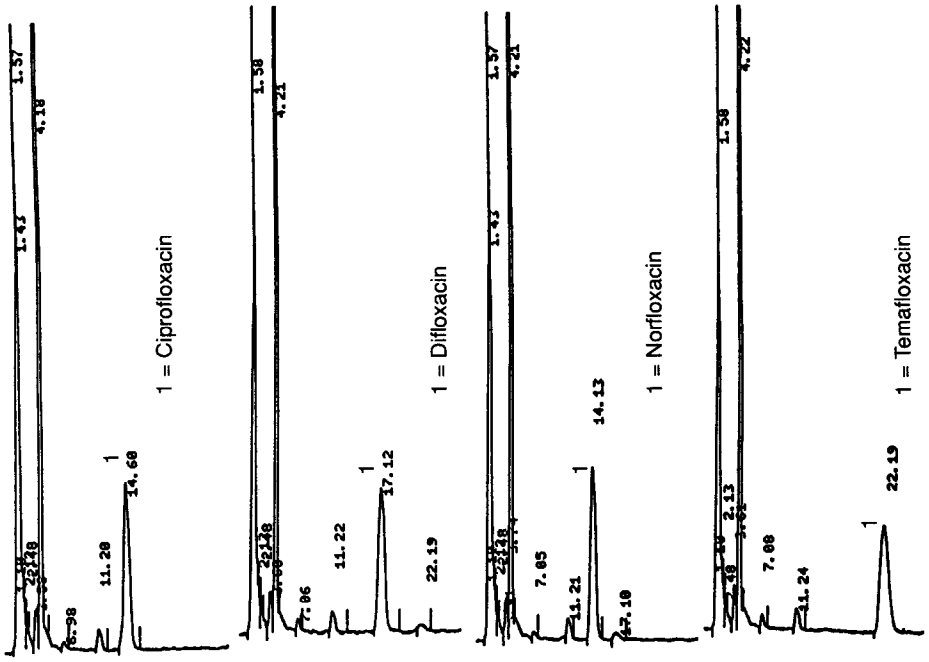
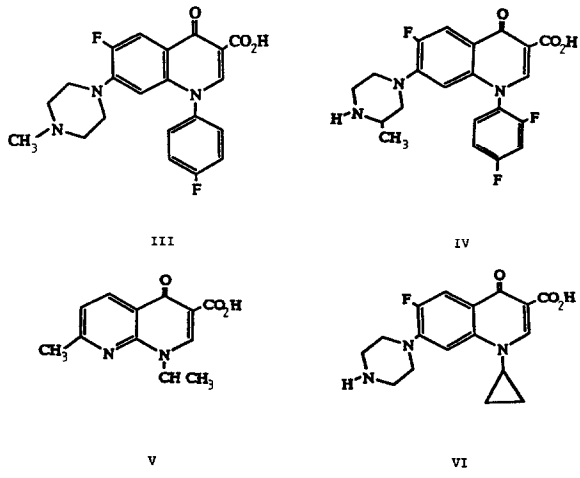


Fig. 3. Application of procedure to other quinolones in fish feed.

typically $\geqslant 0.999$. For this reason, a calibration curve was prepared as part of the analysis to determine the recovery and demonstrate its reproducibility. Samples were then analyzed *versus* the standard curve. Standard addition/recovery experiments using the procedure demonstrated good accuracy (100–105% of spike amount). Due to the co-extraction and co-elution of several other components from the feed, a change in eluent was made to increase the retention of sarafloxacin on the column and allow the other components to elute quickly.



III IV

V VI

The internal standard was changed to *p*-bromoacetophenone to avoid interference from the feed. The precision of the analysis was determined by performing the analysis several times on a single lot. Two analysts performed the analysis over a two-day period. The results are shown in Table II. Fig. 2 shows typical chromatograms for sarafloxacin in fish feed.

The present investigation focused on the determination of sarafloxacin in pre-mix and fish feed; however, the described technique is applicable to several other quinolones: difloxacin (III), temafloxacin (IV), norfloxacin (V) and ciprofloxacin (VI). Fig. 3 shows chromatograms for these compound. The correlation coefficients for standard curves of these quinolones in feed were all greater than 0.9999.

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