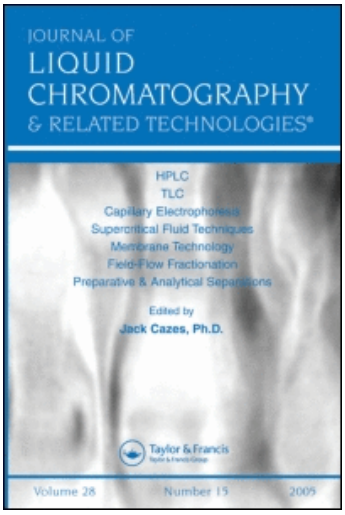

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Determination of Miloxacin and Its Metabolite in Fish by High Performance Liquid Chromatography with Fluorescence and UV Detection

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DETERMINATION OF MILOXACIN AND ITS METABOLITE IN FISH BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE AND UV DETECTION

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ABSTRACT

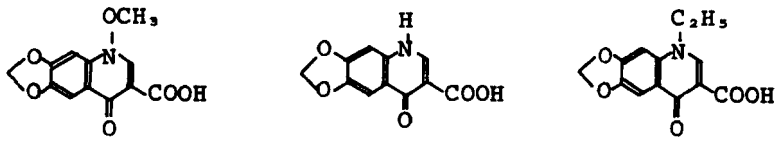
A simple and rapid method for the determination of miloxacin (MLX) and its principal metabolite, 5,8-dihydro-8-oxo-1,3-dioxolo [4,5-g]quinoline-7-carboxylic acid (M-1), in cultured fish by high performance liquid chromatography (HPLC) with fluorescence and UV detection was developed. The drugs were extracted from fish with 0.2% metaphosphoric acid-methanol (7:3), followed by the Bond Elut C_{18} clean-up procedure. The HPLC separation was carried out on a L-column ODS (15 cm x 4.6 mm i.d.) using 0.05M sodium dihydrogenphosphate (pH 4.5)-acetonitrile (65:35) as the mobile phase at a flow-rate of 0.5 ml/min. The calibration graphs were rectilinear from 1 to 50 ng for MLX and M-1. The recoveries of MLX and M-1 from various fishes fortified at 0.5 μ g/g were 84.5-87.9 and 74.8-77.6 %, respectively, with a coefficient of variation of 1.1-2.8 and 1.8-3.2%, respectively. The limits of detection were 0.01 μ g/g for both drugs.

INTRODUCTION

With the development of fish farming such as for eel and yellowtail, various antibiotics and synthetic antibacterials have been widely used for the prevention and treatment of various infectious diseases in fish. The use of antibacterial agents in food-producing animals makes it necessary to develop adequate methods for pharmacokinetic studies and analysis of drug residues in animal tissues. According to the Japanese Food Sanitation Law, no food should contain antibiotics and, in addition, meat, poultry, eggs, fish and shellfish should not contain any synthetic antibacterials. Therefore, a simple and reliable method is needed to monitor drug residues in edible tissues of fish.

Miloxacin (MLX; 5,8-dihydro-5-methoxy-8-oxo-1,3-dioxolo[4,5-g] quinoline-7-carboxylic acid, Fig. 1) is a quinolone derivative active against a broad spectrum of Gram-negative bacteria (1). The drug has found widespread use as a chemotherapeutic in fish farming in Japan. A microbiological method has been described for the analysis of MLX in biological fluids (2-3). However, the sensitivity and selectivity of the microbiological method is poor. Yoshitake *et al.*(4) reported a high performance liquid chromatographic (HPLC) method for the determination of MLX and a major metabolite, 5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid (M-1) (Fig. 1) in human serum and urine. This method is based on using an anion exchange column. Ueno *et al.*(5) reported an HPLC method for the determination of MLX and M-1 in yellowtail, but the method was either tedious or recoveries of M-1 were very low (11-22 %).

Because quinolone derivatives such as nalidixic acid, oxolinic acid and piromidic acid, appear as tailing peaks in reversed-phase



Miloxacin **M-1** **Oxolinic acid**

Fig. 1. Chemical structures of miloxacin, its metabolite M-1 and oxolinic acid.

chromatography, several techniques to avoid this effect have been attempted such as ion-pair chromatography (6-8), ion-exchange columns (9), wide-pore ODS columns (10), non-silica-based columns (11), derivatization of quinolone derivatives to methyl esters (12) and application of oxalic acid in the mobile phase (13-14).

This paper describes a simple, specific and rapid HPLC method for the determination of MLX and M-1 using a pure silica-based C_{18} column and Bond Elut C_{18} cartridges as a clean-up step.

MATERIALS AND METHODS

Caution : Sample and fortified extracts must be protected from light.

Materials and Reagents

The edible muscle tissues of eel, yellowtail, red sea bream and rainbow trout served as samples.

MLX was obtained from Sumitomo Pharmaceutical (Osaka, Japan). Bond Elut C_{18} (200 mg) cartridges were purchased from Varian (Harbor City, CA, U.S.A.). The cartridges were washed with 5 ml of methanol and then 10 ml of distilled water before use. Hyflo Super-Cel was purchased from Johns-Manville (Denver, CO, U.S.A.). Mueller-Hinton

agar was purchased from Nissui Pharmaceutical (Tokyo, Japan). Other Chemicals were of analytical-reagent or HPLC grade. Deionized or distilled water was used throughout all experiments.

Standard Solutions

A stock standard solution (100 μ g/ml) was prepared by dissolving 10.0 mg of MLX in 0.01M aqueous sodium hydroxide-methanol(2:8) in a 100-ml brown volumetric flask. Working standards solutions were prepared by diluting the stock solution with the HPLC mobile phase or 0.05 M phosphate buffer (pH 8.0). The standard solutions were stored in the dark at 5 ℃.

Preparation of M-1

M-1 was prepared from MLX by light decomposition. Since MLX in the stock solution (100 μ g/ml) was completely decomposed to M-1 by sunlight (*ca.* 20,000 lux, irradiation time; 60 min), the decomposed solution was used as the standard M-1 solution (89 μ g/ml). The degradation compounds were characterized by Thermospray liquid chromatographic-mass spectrometry (TSP/LC/MS).

Apparatus

The HPLC system consisted of a LC-6A solvent-delivery system, a SPD-6A UV detector operated at 260 nm, a RF-535 fluorescence detector operated at an excitation wavelength of 325 nm and an emission wavelength of 365 nm and a Chromatopac C-R3A data system, all from Shimadzu (Kyoto, Japan). Injection was carried out with a Model 7125 injection valve with 20 μ l loop from Rheodyne (Cotati, CA, U.S.A.). The separation was performed on a L-column ODS (150 x 4.6 mm I.D., Chemicals Inspection & Testing Institute, Tokyo, Japan)

with 0.05 M sodium dihydrogenphosphate-acetonitrile (65:35) as the mobile phase at a flow-rate of 0.5 ml/min. The phosphate solution was filtered through a Millipore GS 0.22 μ m filter (Milford, MA, U.S.A.). The chromatograph was operated at ambient temperature.

The mass spectrometer is a Shimadzu LCMS/QP 1000 quadruple mass spectrometer equipped with a Vestec thermospray interface (Houston, TX, U.S.A.). The filament off mode was used for ionization. The vaporizer temperature was 180 ℃, and the ion source block temperature was 270 ℃. The measurement mass range was m/z 150-400. The separation was performed on a L-column ODS with 0.1M ammonium acetate buffer (pH 4.5)-acetonitrile (7:3) as the mobile phase at a flow-rate of 0.8 ml/min at 35 ℃.

The other instruments used were a Model 650-40 spectrofluorometer (Hitachi, Tokyo, Japan) and a Model NS-50 Physcotron homogenizer (Niti-on, Chiba, Japan).

Sample Preparation

The sample preparation was done as follows based on a previous paper (10). A 5 g sample was homogenized with 100 ml of 0.2% metaphosphoric acid-methanol (7:3) used as a deproteinizing extractant at high speed for 2 min. The homogenate was filtered through *ca.* 2 mm Hyflo Super-Cel coated on a suction funnel. The filtrate was evaporated under reduced pressure at 50 ℃. Evaporation was interrupted when ca. 30 ml of solution remained in the flask. The flask contents were applied to a Bond Elut C_{18} cartridge. After washing with 10 ml of 5% methanol, the cartridge was eluted with 10 ml of methanol. The elute was evaporated to dryness under reduced pressure and the residue was dissolved in 1 ml of HPLC mobile phase. Ten μ l of the solution were then injected into the HPLC.

Light resistant brown bottle was used for handling MLX because of its instability toward of light.

Calibration Graphs

Standards at concentrations of 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μ g/ml of MLX and M-1 were prepared from stock standard solutions. A 10μ l volume of these solutions was injected into the column. Calibration graphs were obtained by measurement of peak heights.

Microbiological Assay

The antibacterial activities of MLX and M-1 were measured by a paper disk method with *Bacillus subtilis* ATCC 6633 and *Micrococcus luteus* ATCC 9341 as the test organisms. The assay procedure was carried out according to the Japanese Official Method (15).

RESULTS AND DISCUSSION

Stability of Miloxacin for Light

MLX is a quinolone derivative closely related structurally to oxolinic acid (Fig. 1). Oxolinic acid in aqueous solution is quite stable toward light, but the aqueous solution of MLX is reported to be unstable (16). Thus, in this study, the stability of 5 ppm MLX in aqueous solution toward light was examined. As shown in Fig. 2, MLX is extremely unstable in aqueous solution toward light, and it is rapidly converted to its demethoxy derivative, M-1. The decomposition rate of MLX was more rapid by sunlight than by fluorescent lamp at the same intensity of light illumination because of the stronger UV component of sunlight. MLX was also rapidly decomposed in methanol. Identification of M-1 was performed by liquid chromatographic-mass spectrometry.

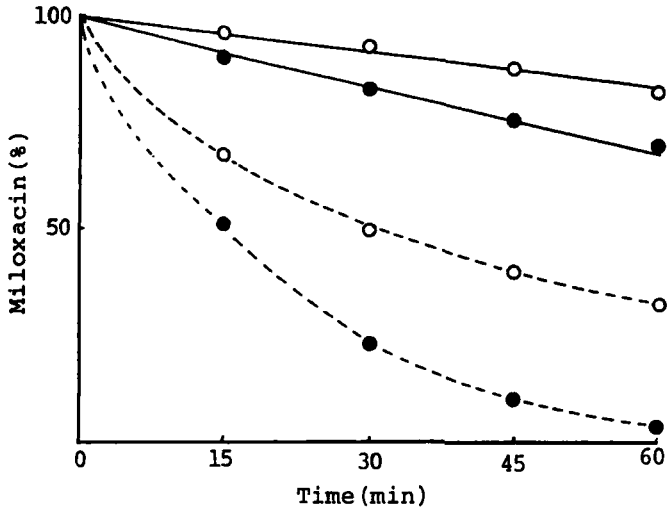


Fig. 2. Influence of light on stability of miloxacin in aqueous solution. ○——○: 500 lux fluorescent lamp; ●——●: 1000 lux fluorescent lamp; ○----○: 500 lux sunlight; ●----●: 1000 lux sunlight.

Antibacterial Activity of Miloxacin and M-1

Like other anti-bacterial agents, the potency of MLX was determined by microbiological assay. The antibacterial activity of MLX is considered to be comparable to its related compound, OXA. Therefore, the antibacterial activity of MLX and M-1 was examined using *Bacillus subtilis* ATCC 6633 and *Micrococcus luteus* ATCC 9341 as the test organisms, which are frequently used for the test of residual antibiotics in animal and meat. As shown in Table 1, the antibacterial activity of M-1 for both test organisms was extremely weak, and the sensitivity of MLX detection was not sufficient. In addition, the microbiological assays tended to lack specificity.

Quinolone such as MLX, oxolinic acid and nalidixic acid do not lend themselves to simple gas chromatographic assays because of their

TABLE 1

Antibacterial Activity of Miloxacin and Its Major Metabolite, M-1

Organism	MIC(μg/ml)[1]	
	Miloxacin	M-1
B. subtilis ATCC 6633	0.78	> 50
M. luteus ATCC 9341	> 50	> 50

1) MIC: minimum inhibitory concentration

low volatility without derivatization. For these reasons, HPLC is considered to be more useful for the assay of MLX for residual analysis and pharmacokinetic study. Also, the chemical method of analysis should be developed.

Chromatographic Conditions

It is generally known that quinolone derivatives such as oxolinic acid and nalidixic acid, which have similar chemical structures to MLX, give broad or tailing peaks in reversed-phase chromatography. Similarly, MLX and M-1 appeared as strong tailing peaks in reversed-phase chromatography using conventional C_{18} (ODS) columns. Residual siranol groups and metal impurities in column packing materials are known to be the cause of tailing in reversed-phase chromatography (13-14, 17-18). In a previous paper (14), we reported that the use of an oxalic acid in the mobile phase could be used for the purpose of masking metal impurities and inhibit tailing.

Highly purified silica gel containing low metallic impurities has been recently developed as the packing materials for HPLC columns, and it has been applied for the separation of coordination compounds (17-18). Thus, as shown in Table 2, conditions for separation of

TABLE 2

Physical Properties of ODS Columns Used in This Study

Column		Silica gel	Particle (μm)	Pore (Å)	Carbon (%)	End-capping
L-column ODS	(150 x 4.6mm)	pure	5	120	17	Yes
Wakosil-II 5C18HG	(150 x 4.6mm)	pure	5	120	15	Yes
Inertsil ODS-2	(150 x 4.6mm)	pure	5	150	18.5	Yes
Capcell Pak C18SG	(150 x 4.6mm)	pure	5	120	20	Yes
TSK-gel ODS-80T μ	(150 x 4.6mm)	pure	5	80	15	Yes
Inertsil ODS	(150 x 4.6mm)		5	100	14	Yes
Capcell Pak C18AG	(150 x 4.6mm)		5	120	20	Yes
Wakosil 5C18-200	(150 x 4.6mm)		5	200	12	Yes
RoSil C18-5	(150 x 4.6mm)		5	90	17	Yes
Nucleosil 5C18	(150 x 4.6mm)		5	100	14	Yes
LiChrosorb RP-18	(125 x 4.0mm)		5	100		No

Data from manufacture's specifications.

MLX and M-1 have been examined using pure silica-based ODS columns. MLX and M-1 were adsorbed on the column or showed broad tailing, as shown in Fig. 3, A and B, if residual siranol groups were not end-capped or metal impurities were present in the packing materials. However, when pure silica gel was used as the column packing material, broad tailing of the peak could be prevented. Peak shapes were the best with the L-column ODS, therefore, the L-column ODS was used exclusively in this study.

The water-acetonitrile solvent system, and phosphate buffer -acetonitrile system are frequently used in the reversed phase HPLC, and separation was examined using these solvent systems. As a result it was found that the phosphate buffer-acetonitrile system was better based on tailing.

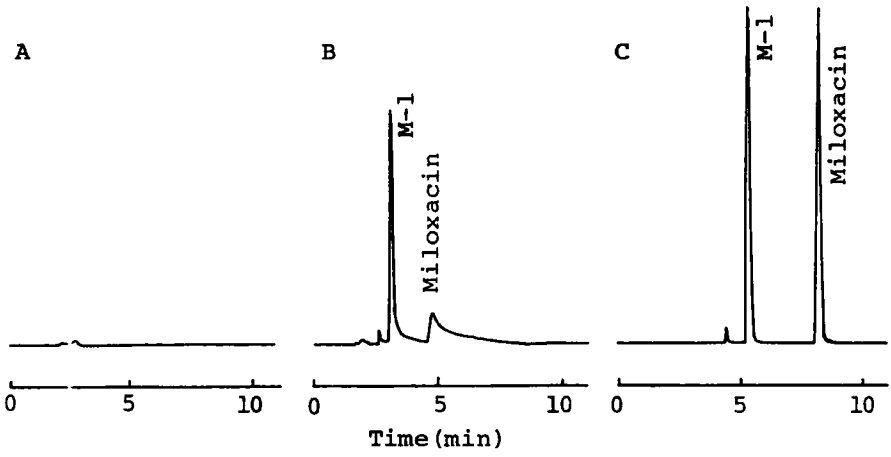


Fig. 3. Typical chromatograms of miloxacin and M-1.
LC conditions: column, (A) RoSil C18-5, (B) Wakosil 5C18-200, (C) L-column ODS; mobile phase, 0.05M sodium dihydrogen phosphate-acetonitrile(6:4); flow-rate, 0.5 ml/min; detection, UV(260 nm).

Based on a study with different mixing ratios of phosphate buffer and acetonitrile at various salt concentrations and pH, 0.05M sodium dihydrogenphosphate-acetonitrile (65:35) without pH conditioning was chosen as the mobile phase.

Strong UV absorption is caused by MLX and M-1, and these compounds are also strong fluorogenic ones. For better analytical results, both UV and fluorescence detectors were used in the HPLC. The best sensitivity for the fluorescence detector was achieved using an excitation wavelength of 325 nm and an emission wavelength of 365 nm. Fig. 4A shows the chromatogram of MLX and M-1 obtained under the established conditions.

Clean-up

Ueno *et al.* (5) reported residual analysis of MLX and M-1 using chloroform in liquid-liquid partition, and recovery of M-1 was rather

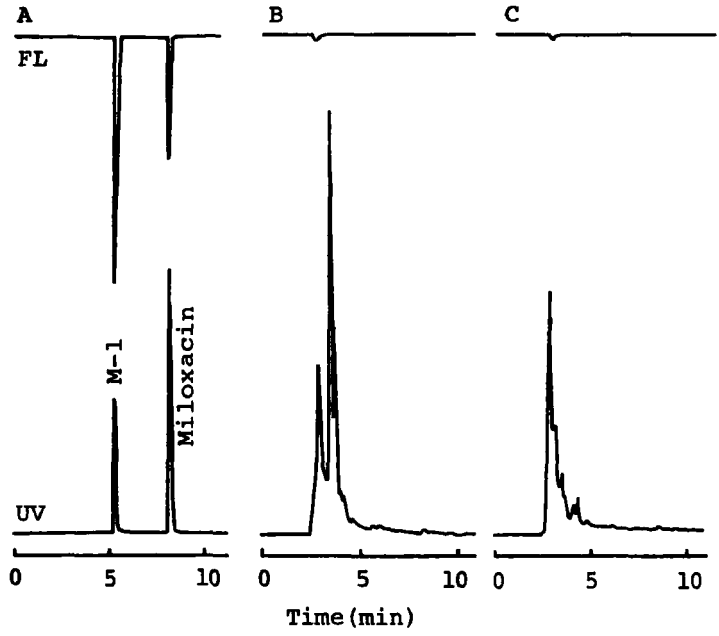


Fig. 4. Typical chromatograms of extracts from cultured fish. Top traces: fluorescence detection at the excitation wavelength of 325 nm and the emission wavelength of 365 nm. Bottom traces: ultraviolet absorbance (260 nm). (A) Standard mixture (M-1; 5 ng, Miloxacin; 10 ng). (B) Eel extract. (C) Red sea bream extract.

low (11.3-22.0 %). One of the causes of low recovery of these compounds might be that the polarity of M-1 is higher than that of the MLX precursor. A solid-phase extraction method have been extensively used for cleanup of biological material prior to quantitation analysis of trace amounts of contaminants in the matrices. Thus, the solid phase extraction method using an ODS cartridge was employed for better operation and reproducibility.

Retention profiles of MLX and M-1 on the ODS cartridge are shown in Table 3. MLX and M-1 are strongly adsorbed on the ODS cartridge,

TABLE 3

Comparison of Disposable Reversed-Phase C_{18} Cartridges

Cartridge	Recovery(%)[1] Miloxacin	M-1
Bond Elut C_{18} (200mg)	97.8	97.5
Baker C_{18} (200mg)	90.7	98.1
Toyo-Pak ODS (300mg)	83.6	92.8
Sep-Pak C_{18} (400mg)	76.9	78.9
Adsorbex RP-18(400mg)	71.5	37.3
Bond Elut C_{18} (500mg)	83.7	97.5
Baker C_{18} (500mg)	54.7	66.2
Easy Chromato C_{18}(500mg)	45.3	50.1

1) Recoveries of miloxacin and M-1 from 30 ml distilled water. Samples were added with 0.1 μ g/ml of both drugs. Results of three replicates.

and when the packing amount exceeded 500 mg, a part of the loaded compounds was not eluted. The retention profile was different for different manufacturers even with the same amount of compound packing on the column. Features of the ODS cartridge packing materials are the same as those of ODS for HPLC column, and retention might be increased by residual siranol and mixed metal impurities.

Bond Elut C_{18} (200 mg) was used for clean up. Impurity peaks were shown at the solvent front by UV monitoring, but they were not a problem for the present analytical purpose. More specific fluorescence detection showed no disturbing peak on the chromatogram, and more sensitive detection was accomplished.

Ozawa *et al.*(3) examined the stability of MLX under room light using microbiological assay, and reported that the stability was not the problem within the duration of the assay procedure. However, in this experiment, a brown container was not used, and the recovery

TABLE 4

Recoveries of Miloxacin and M-1 from Cultured Fish

Sample	Added (μg/g)	Recovery (%)[1]	
		Miloxacin	M-1
Eel	0.5	86.5 ± 1.8	74.8 ± 1.8
Yellowtail	0.5	84.5 ± 2.8	77.6 ± 3.2
Red sea bream	0.5	87.5 ± 1.1	76.3 ± 2.8
Rainbow trout	0.5	84.9 ± 1.6	75.7 ± 2.6

1) Values are mean ± S.D. (n=5).

test showed approximately 20% MLX decomposition to M-1 by room light. Therefore, a brown bottle must be used to prevent decomposition of MLX.

Recovery

Linear calibration graphs were obtained from 1 to 50 ng (equivalent to 0.02-1.0 μg/g) and the detection limits of MLX and M-1 were 0.5 ng (signal-to-noise ratio greater than 3). Table 4 summarizes the recoveries of the drugs from commercial samples of eel, yellowtail, red sea bream and rainbow trout fortified with 0.5μg/g of MLX and M-1. Although the recovery of M-1 was slightly low (74.8-77.6%), that of MLX was higher than 85%, with standard deviations within 5%. The detection limits of the method were 0.01μg/g for both drugs in fish and the time required for the analysis of one sample was less than one hour.

In conclusion, we have found the HPLC method suitable for the pharmacokinetic and residue studies of MLX in fish.

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