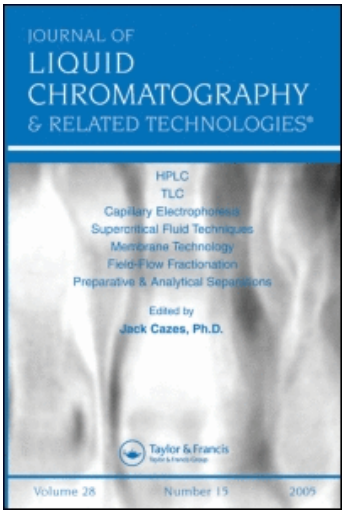

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DETERMINATION OF OXOLINIC ACID IN MARINE SEDIMENT BY HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

A high performance liquid chromatographic (HPLC) method based on fluorescence detection was developed for determination of oxolinic acid in marine sediment. Sediment was extracted with a mixture of sodium hydroxide and dimethyl sulphoxide and cleaned up by solid phase extraction (SPE). The limit of detection was 1.6 μg/kg. The recoveries were 85 ± 6% (mean ± SD) at a level of 10 μg/kg and 82 ± 4% (mean ± SD) when samples containing < 1.6-190 μg/kg oxolinic acid were spiked with 50 μg/kg oxolinic acid. The relative repeatability standard deviation was 5.5% at levels of 2-10 μg/kg and 2.5% at a level of 25 μg/kg.

INTRODUCTION

Oxolinic acid is a quinolone in common use for the treatment of infectious diseases in fish farming. The drug is administered as an ingredient in feed pellets, which may result in a significant loss to the environment. Generally, contam-

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ination of the environment with drugs is of increasing concern because the drugs may be transferred to the food chain and expose humans to undesirable effects and promote the development of resistant organisms. Sensitive methods are, therefore, necessary in checking the environment for drug residues.

Although several methods have been reported for determination of oxolinic acid in fish tissue,(1-11) only a few high performance liquid chromatographic (HPLC) methods have been reported for determination of oxolinic acid in marine sediment. Methods based on extraction with sodium hydroxide and direct injection into an HPLC system,(12) or injection after additional cleaning up by liquid-liquid extraction with chloroform and ethyl acetate,(13) have been published. In both cases ultraviolet (UV) detection was applied.

This paper describes a simple and reliable HPLC method for determination of oxolinic acid in marine sediment, based on solid phase extraction (SPE) and fluorescence detection. The method has improved recovery and sensitivity characteristics in comparison to previous reported methods and does not apply ozone-depleting agents.

EXPERIMENTAL

Chemicals and Reagents

Oxolinic acid was purchased from Sigma (St. Louis, MO., USA). Acetonitrile of chromatography grade, dimethyl sulphoxide (DMSO), phosphoric acid, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Water was purified through a Millipore Milli-Q Plus system (Bedford, MA, USA).

The mobile phase system for HPLC was prepared by diluting 120 mL acetonitrile to 1000 mL with 0.02 M phosphate buffer pH 3.0. The phosphate buffer was prepared from phosphoric acid and sodium hydroxide.

Standard Solutions

Stock solution of oxolinic acid was prepared at a concentration of 1000 μg/mL by dissolving the pure substances in a 0.03 M NaOH solution. This was stable for at least one month when stored at 5±2°C. A standard solution containing 1000 ng/mL of oxolinic acid was prepared by dilution of a stock solution with 0.03 M NaOH solution. Calibration standards containing 5, 50, 100, 200, and 300 ng/mL were prepared by diluting aliquots of the 1000 ng/mL solution with mobile phase. The calibration standards were stable for at least two weeks when stored at 5 ± 2°C.

Materials

Solid phase extraction cartridges, Oasis HLB (divinylbenzene-co-N-vinylpyrrolidone polymeric sorbent) 60 mg, were obtained from Waters (Milford, MA, USA). Acrodisc 13 PVDF, 13 mm × 0.45 μm disposable filter units were obtained from Gelman Sciences (Ann Arbor, MI, USA). Centrifuge tubes of 15 mL and 50 mL capacity and HPLC vials were made of polypropylene. Marine sediments covering a range from sandy to muddy texture were used for method validation.

Instrumentation and Chromatographic Conditions

The instruments used were a Sigma centrifuge model 2-15 (Osterode, Germany), a VF2 test tube shaker (IKA, Staufen, Germany), a HS500 horizontal shaker (IKA), a vacuum manifold for SPE cartridges (Waters), a pH meter PHM 93 (Radiometer, Copenhagen, Denmark), and a temperature-controlled heating block with a manifold for nitrogen flow (Mikrolab Aarhus, Aarhus, Denmark).

The liquid chromatography system consisted of a Waters pump gradient system 600, a Waters 470 fluorescence detector, and a Waters 717 autosampler. Reverse-phase liquid chromatography was carried out on a XTerra RP_{18} column (3.5 μm, 150 mm × 4.6 mm I.D.) (Waters). The operation of the chromatographic system and acquisition of data were controlled by Waters Millennium 32 software.

The injection volume was 100 μL and the mobile phase flow rate was set at 1.0 mL/min. The column temperature was kept at 25 ± 1°C. The excitation and emission wavelengths were 325 nm and 360 nm respectively.

Sample Preparation

A mass of 5.0 g sample was weighed into a 50 mL centrifuge tube. Volumes of 16.0 mL NaOH solution (0.2 M) and 4.0 mL DMSO were added and the tube was shaken horizontally (250 strokes/min) for 30 min. The mixture was centrifuged at 3000 *g* for 5 min and the supernatant was collected. The extraction was repeated twice. The supernatants were combined and diluted to 100 mL with water. A volume of 20 mL extract was adjusted to pH 3.0 with phosphoric acid. An SPE cartridge was washed with 2 mL methanol followed by 5 mL water. The pH-adjusted extract was pulled through the cartridge at a flow rate of 1-2 mL/min. The column was washed with 5 mL water and dried by suction for 1 min. The oxolinic acid was eluted with 3.0 mL acetonitrile. The eluate was collected in a 15 mL polypropylene tube and evaporated to bare dryness at 45-50°C

under a stream of nitrogen. The residue was redissolved in 100 µL 0.03 M NaOH solution. A volume of 900 µL mobile phase was added and the solution was filtered through a 0.45 µm filter.

Ruggedness

The influence of sample pH on recovery of oxolinic acid from SPE was investigated in the pH range 3.0-8.0 using contaminated sediments.

The capacity of the SPE cartridge was checked by connecting two cartridges in series and applying 25% surplus volume of extract from contaminated sediment containing oxolinic acid at a level of 190 µg/kg. The amounts of oxolinic acid adsorbed to each cartridge were determined according to the procedure.

The elution profile was determined on contaminated sediments containing oxolinic acid at levels of 180-250 µg/kg. Aliquots of 0.5 mL acetonitrile was used for elution.

The stability at 5-7°C of final sample extracts of sediments containing oxolinic acid at levels of 2.3, 10, and 25 µg/kg and standard solutions containing 5 and 300 ng/mL, was tested over a period of two weeks.

Limits of Detection

The limits of detection (LODs) were determined on 20 different blank control sediments. To obtain realistic LODs, the samples were spiked prior to extraction with oxolinic acid to a peak height on chromatograms corresponding to ca. three times the short term baseline variation. The samples were, thus, spiked with oxolinic acid to a level of 1.0 µg/kg. The spiked samples were mixed and stored 16-20 h at 5-7°C before extraction. The detection limits were determined as the mean results plus three times the standard deviation (SD) of the 20 measurements.

Precision and Recovery

The repeatability standard deviation (i.e. variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation (i.e. variability of independent analytical results obtained on the same test sample, in the same laboratory by different operators under different experimental conditions) were determined on

contaminated sediments containing oxolinic acid at levels of 2.3, 10, and 25 μg/kg. The samples were analysed in duplicate on each of 8 days. Calculation of repeatability was done in accordance with ISO standard 5725-2, 1994.(14) The intra-laboratory reproducibility was calculated by the same principle used for determination of reproducibility.(14) The recovery was determined on 15 different blank control sediments spiked with oxolinic acid to a level of 10 μg/kg, and 31 different sediments spiked with 50 μg/kg oxolinic acid. The spiked samples were mixed and stored 16-20 h at 5-7°C before extraction.

Determination of Total Solids and Organic Matter

A mass of 5 g sample was transferred to a porcelain crucible and dried at 105 ± 3°C for 20 h to determine the content of total solids (TS). The residue was then ignited at 550 ± 25°C for 2 h to give the content of fixed residue. The difference was defined as the content of organic matter in total solids (OMTS).

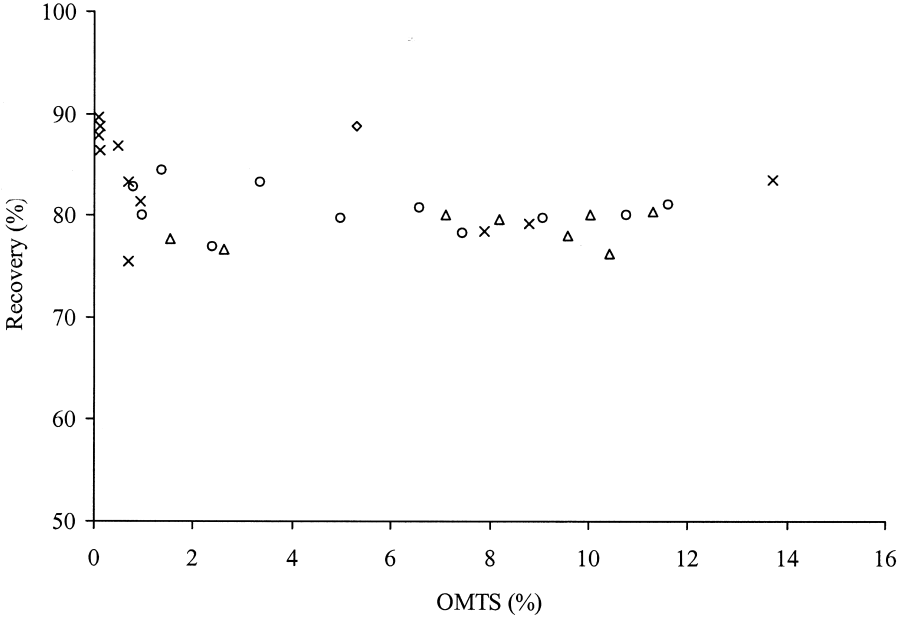

Figure 1. Correlation between OMTS and recovery from sediment spiked with 50 μg/kg oxolinic acid. The original content of oxolinic acid was less than 1.6 μg/kg (×), 2-10 μg/kg (o), 10-40 μg/kg (Δ), and 190 μg/kg (◊).

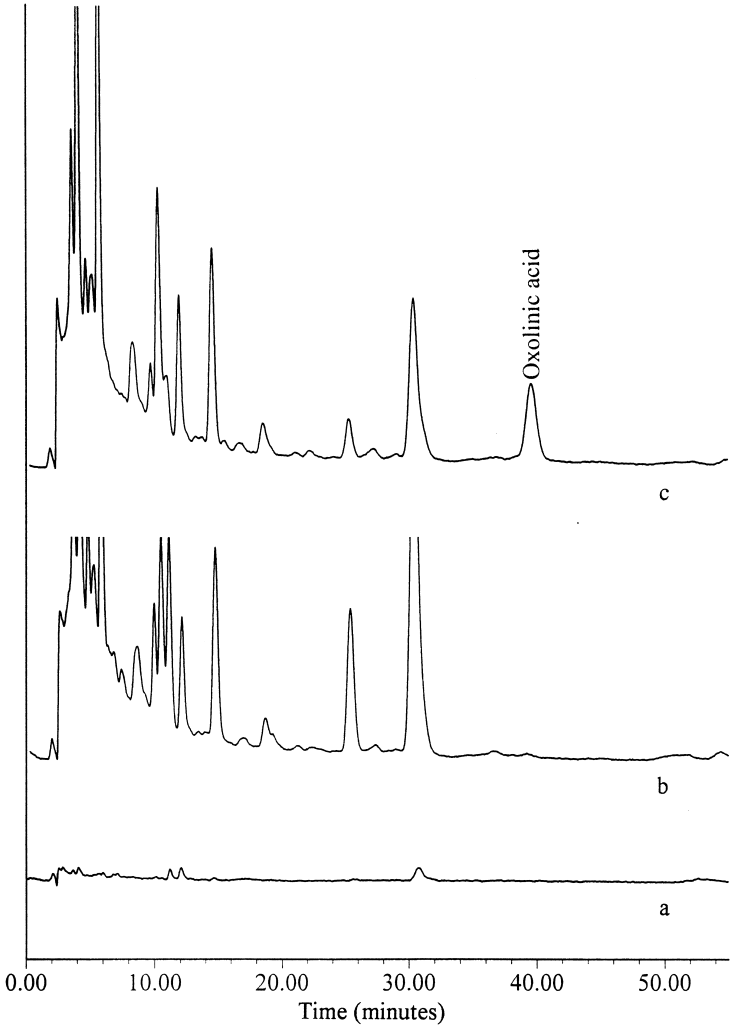

Figure 2. Chromatograms of blank control sandy sediment containing 0.1% OMTS: (a), blank control muddy sediment containing 9.5% OMTS (b) and contaminated sediment containing 7.1% OMTS and 10 µg/kg oxolinic acid (c).

RESULTS AND DISCUSSION

Extraction of oxolinic acid from sediment with 0.1-0.2 M NaOH solution has previously been reported.(12,13) The extraction solution was modified in the present study to comprise of 80% 0.2 M NaOH and 20% DMSO. This modifica-

tion increased the extraction yield from contaminated samples by ca 50%. The recovery of oxolinic acid from sediments spiked with 50 μg/kg was 82 ± 4% (mean ± SD) (n=31). The recovery did not depend on the content of OMTS in the range 0.5-14% (Figure 1). The recovery of oxolinic acid from blank control sediments (OMTS range 0.1-14%) spiked to a level of 10 μg/kg, was 85 ± 6% (mean ± SD) (n=15).

Typical chromatograms of blank control sediments and sediment containing 10 μg/kg oxolinic acid are shown in Figure 2. The XTerra RP_{18} analytical column was selected because this column resulted in fewer interfering peaks in comparison with traditional silica-based C_{18} packings.

The result obtained on 20 blank control sediments spiked with oxolinic acid to a level of 1.0 μg/kg was 1.09 ± 0.16 μg/kg (mean ± SD). From this the LOD was calculated as 1.6 μg/kg. The TS and OMTS range were 21-83% and 0.1-14%, respectively. The results obtained in the precision study are summarised in Table 1. The relative repeatability standard deviation (RSD_r) was 5.5% in the concentration range 2-10 μg/kg and 2.5% at a level of 25 μg/kg (Table 1).

The calibration curves forced through the origin were linear in the tested range up to 300 ng/mL. The slope converting concentration (ng/mL) to peak area (μV s) was $7.19 \times 10^4 \pm 0.16 \times 10^4$ (mean ± SD) in the precision study. The mean standard error of slope estimates was 7.21×10^2. The coefficients of determination (R^2) were 0.9991-0.9999.

The capacity of the SPE cartridge was checked by applying 25% surplus volume of extract from sediment containing 190 μg/kg oxolinic acid and 8% OMTS to two cartridges connected in series. Less than 1.0% of oxolinic acid content was detected in the eluate from the second cartridge (n = 4). The recovery from SPE was dependent on the pH of the sample extract, with highest yield obtained at pH 3.0 (n=4).

A volume of 3.0 mL acetonitrile was sufficient for complete elution of oxolinic acid from SPE cartridges (n=4). Less than 0.5% of the oxolinic acid was found in the 2.0-3.0 mL fraction.

Table 1. The Relative Repeatability Standard Deviation (RSD_r) and Intra-laboratory Reproducibility Standard Deviation ($RSD_{R,intra}$) Determined on Contaminated Samples

Conc. Level (μg/kg)	TS (%)	OMTS (%)	RSD_r[a] (μg/kg)	$RSD_{R,intra}$[a] (μg/kg)
2.3	66	1.6	5.0	9.2
10	45	2.6	5.9	7.8
25	30	5.3	2.5	3.9

[a]One duplicate analysis at each level was conducted on each of 8 days.

The stability at 5-7°C of final extracts of sediments containing oxolinic acid at levels of 2.3, 10, and 25 μg/kg, and standard solutions containing 5 and 300 ng/mL were investigated over a period of 14 days. No significant changes in response were observed.

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