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Determination of oxolinic acid in feeds and cultured fish using capillary electrophoresis

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Abstract

A capillary electrophoretic method was developed for the determination of the antibiotic oxolinic acid. The electrolyte composed of a buffer solution (10 mM phosphate, pH 9.00) and methanol (9:1) was found to be the most suitable for this separation. The effect of type of buffer, its pH and concentration as well as injection times and applied voltage on the migration of oxolinic acid was also studied. Key analytical characteristics of the method are as follows: detection limit (signal-to-noise ratio 3), 0.08 μg ml^{-1}; linear range, 0.5–40 μg ml^{-1}; migration time, 5.3 min; relative standard deviation for within-day and day-to-day variation of 1.67 and 2.24%, respectively. The method, in conjunction with a solid phase extraction procedure, was successfully applied for the analysis of spiked oxolinic acid in fish feeds and fish muscles. The recoveries of oxolinic acid from spiked feeds and muscle tissues were 81.15 and 84.80%, respectively. © 2002 Published by Elsevier Science Ltd.

1. Introduction

Lately, aquaculture industry has undergone tremendous growth, and it is viewed as a key strategy to provide sufficiency in food supply to meet demands of the growing population by many countries of the world (Carignan, Larocque, & Svend, 1991; Takatsuki, 1992). The raising of large numbers of fish and prawns in confined space as in modern aquaculture practice necessitates the use of an extensive range of chemicals for the treatment and prevention of outbreak of diseases, and as well as to maintain the required water quality. Oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid (Fig. 1) is a common antibiotic that is used in aquaculture practices, and was reported to be effective against gram-negative bacteria (Bjorklund, 1990; Carignan et al., 1991; Rasmussen, Tonneson, Thanh, Rogstad, & Aanesrud, 1989; Samuelsen, 1990).

The drug can be administered orally, mixed in the feed at 12 mg/kg per day (Carignan et al., 1991). Alternatively, the drug may be administered as bath treatment for several days, at typical dosage of 200 mg/l (Samuelsen & Lunestad, 1996). The drug has also been marketed as a fungicide under the trade name Starner as it was also found to be effective in the control of agricultural disease cause by Pseudomonas and Erwina species in rice crops (Shiga & Matano, 1993). To date, existing data on drug residues in cultured fish is scarce when compared to domestic animals. Unlike livestocks, maximum residue limits for antibiotics in most seafood have not been established (Carignan et al., 1991). As an interim measure, many countries enforce a zero tolerance of antibiotics (Choo, 1998; Takatsuki, 1992). Zero tolerance actually reflects different residue levels, depending on the sensitivity of the analytical method.

Most of the analytical methods reported for the determination of oxolinic acid is reversed-phase high performance liquid chromatography, in conjunction with either ultraviolet (Bjorklund, 1990; Hustvedt, Salte, & Benjaminsen, 1989; Ueno & Aoki, 1996) or fluorescence (Carignan et al., 1991; Rasmussen et al., 1989; Samuelsen, 1990; Shiga & Matano, 1993; Thanh, Andresen, Agasorter, & Rasmussen, 1990) detector. Prior to the analytical separation, the samples were subjected to liquid–liquid extraction (Carignan et al., 1991; Samuelsen, 1990) or solid-phase extraction (Bjorklund, 1990; Rasmussen et al., 1989; Samuelsen &

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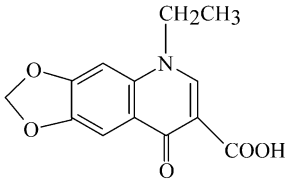

Fig. 1. Structure of oxolinic acid.

Lunestad, 1996). Oxolinic acid can also be reduced with sodium tetrahydroborate to yield a sufficiently volatile derivative. Takatsuki (1992) employed such strategies for the gas chromatographic/mass spectrometric determination of oxolinic acid residues in fish samples.

Capillary electrophoresis (CE) has emerged as a powerful analytical tool and has been applied to the analysis of food, forensic, pharmaceutical, environmental, clinical, molecular biological samples, etc (Barron, Jinenez-Lozanono, & Barbosa, 2000; Huang, Du, Marshall, & Wei, 1997). Inherent characteristics of the CE method such as speed of analysis, high efficiency, separation selectivity, small sample size capability and low reagent consumption has been well recognized within the scientific community. However, very limited work had been reported on the application of CE to the analysis of drug residues in foods (Huang et al., 1997; Oka, Ito, Yuko, & Matsumoto, 2000). The optimization of experimental parameters for the CE separation of a few quinolones was reported, but its application for real samples was not attempted (Barron et al., 2000). The suitability of the CE method for the determination of tetracycline antibiotic residues in catfish dosed at 37.5, 75.0 and 150 mg/kg for 10 days had also been demonstrated (Huang et al., 1997). In this work, we report the analytical method development for the separation and quantification of oxolinic acid using CE. The CE system was optimized with respect to important operating parameter such as the type of buffer, its pH and concentration. Finally, the developed method was applied towards the analysis of this drug in feeds and muscles of cultured fish. To the best of our knowledge, the determination of oxolinic acid using the CE approach had not been reported.

2. Experimental

2.1. Instrumentation

Analytical separation was carried out on a Waters capillary ion analyzer (Milford, MA, USA) which was interfaced to a Waters PC 800 workstation. The capillaries (75 μm internal diameter×60 cm) used were made of fused silica and were supplied by Waters. Direct UV detection was performed at 254 nm with a mercury lamp and a 254-nm optical filter. Samples were introduced into the capillary using 10-V electrokinetic injections. Oxolinic acid determinations were performed using a negative power supply with the applied voltage set, unless stated, at 10 kV and thermostated at 25 °C. Each day before starting with any analysis, the capillary was conditioned by purging with 100 mM potassium hydroxide solution and then followed by Milli-Q water for at least 5 min. Between each run, the capillary was rinsed with electrolyte for 2 min. At the end of the working day, the capillary was flushed and cleaned with Milli-Q water for 5 min. These steps are mandatory for the required reproducibility and effectiveness of the capillary electrophoretic separation.

2.2. Chemicals and reagents

Oxolinic acid, nalidixic acid and Tris buffer were purchased from Sigma (St. Louis, MO, USA), while methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). All solutions, including electrolytes, and standards were prepared using 18.2 M ohm-cm Milli-Q water generated by a Milli-Q Plus Water Purification System (Millipore, Bedford, MA, USA). Fresh working electrolytes and working standards were prepared daily, vacuum-filtered and degassed prior to use. Extract Clean C18 cartridges were purchased from Alltech, USA.

2.3. Test samples

A total of 11 fish feed samples, including six that were imported from USA, Japan, Taiwan and Thailand were used in the study. Ten cultured seabass specimens were purchased from wet markets from the northern states of Peninsular Malaysia, covering the states of Perlis, Kedah and Penang. The fish were immediately frozen upon reaching the laboratory. Before the analysis, the fish samples were thawed at room temperature for about 1 h.

2.4. Extraction and clean-up

The extraction and SPE procedure which was adapted from the work of Bjorklund (1990) was used in the present work. A known amount of nalidixic acid which was used as an internal standard was spiked to 5 g of sample. The sample was next homogenized with a blender using 30 ml of 10 mM phosphate buffer at pH 7.00. The slurry was sonicated for 5 min in a bath sonicator, centrifuged for 15 min at 2500 rpm. The supernatants were filtered and re-extracted twice with 30 ml of 10 mM phosphate buffer. The combined extracts were filtered and the supernatant was cleaned-up using SPE cartridges.

Prior to use, the SPE cartridge was conditioned by flushing with 5 ml methanol, followed by 5 ml 10 mM phosphate buffer (pH 3.0). A total of 5 ml of the extracts whose pH had been adjusted to 3.0 was then passed through the cartridge. It was next rinsed with 10

ml water and finally eluted with 5 ml 10 mM phosphate buffer (pH 9.0):methanol (9:1, v/v).

3. Results and discussion

The choice of buffers is a very important factor in electrophoretic separation as buffers by their very nature help to control the concentration of the ionic species when the pH of the buffer solution is close to the pK_a of the acid. The effect of a few common buffers and their pH on the migration time and peak area when injected with 10 μg ml^{-1} oxolinic acid is shown in Figs. 2 and 3, respectively. The use of phthalate buffer was found to be unsatisfactory due to its lower sensitivity (Fig. 3), eventhough its migration time is less than 5 min under the experimental conditions employed for the study. At a lower pH, the migration time is longer when phosphate or Tris buffer was used. The use of phosphate buffer was found to yield sharper peaks and reasonable migration time as compared to the use of Tris buffer. Thus the former buffer at pH 9.00 was used for further studies.

Buffers should normally be prepared at relatively low concentrations so that salts do not precipitate out of the mobile phase in the event that addition of organic solvent is required. Lower concentration of electrolyte is also desired to reduce heat build-up during the electrophoretic separation. The effect of concentration of the phosphate buffer (1–25 mM) on the migration time and peak area was studied at a fixed pH (9.00) and applied voltage (20 kV). Results for the injection of 10 ug ml^{-1} oxolinic acid indicated that there was neither much variation of peak area nor migration times over the concentration of the phosphate buffer studied. 10 mM phosphate buffer was adopted.

The effect of variation of applied voltage (5–25 kV) on the migration time of 10 μg ml^{-1} oxolinic acid was also studied. Operating at lower voltages resulted not only in longer migration times but also broader peaks. A voltage of 20 kV was chosen for further studies.

Hydrostatic sampling mode at a constant height of 10 cm and varying the injection times from 5 to 40 s was also investigated. Longer injection times was found to increase in peak area but changes in the migration time was minimum. An injection time of 30 s was used.

The addition of additives to the electrolyte was found to be beneficial as it offered improved peak shapes and sensitivity. In this work, we also evaluated the addition of methanol to the phosphate buffer. It was found that addition of 10% methanol improved the overall analytical characteristics, especially on the migration time. The use of methanol was also found to minimize clogging of the capillaries. The final CE operating parameters that were adopted for the application studies was a compromise between speed and sensitivity, and are summarized:

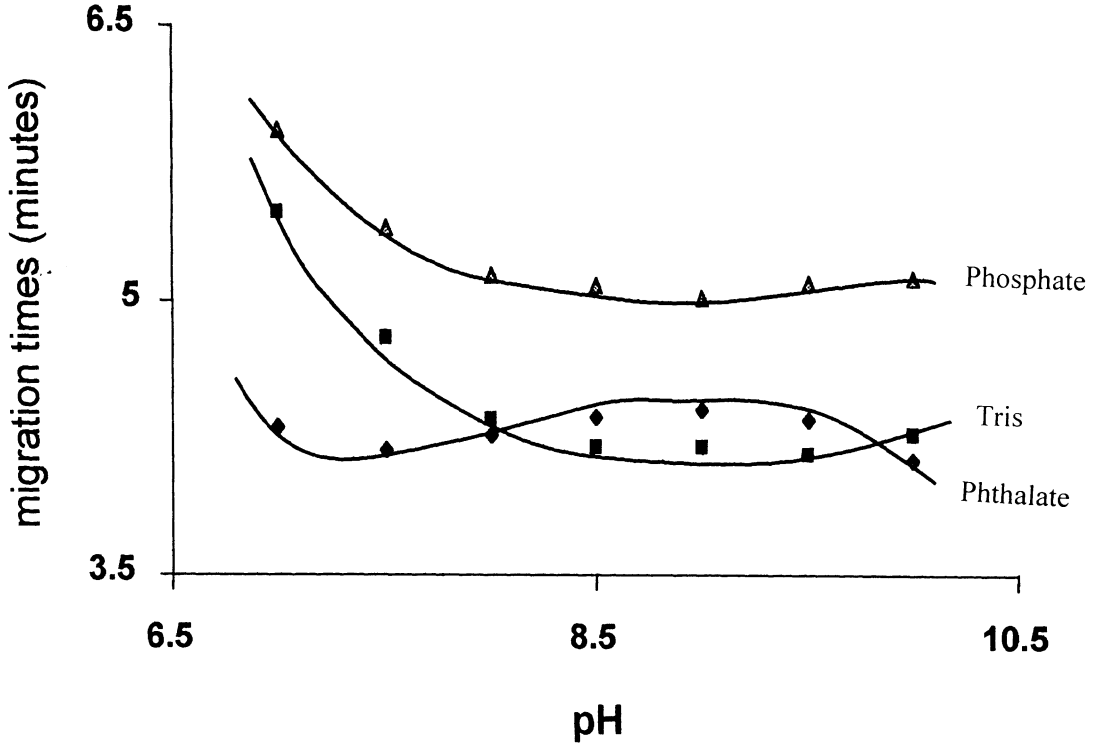

Fig. 2. The effect of type of buffer and its pH on the migration time of 10 μg ml^{-1} oxolinic acid. Conditions: buffer concentration, 5 mM; applied voltage, 20 kV; injection time, 30 s.

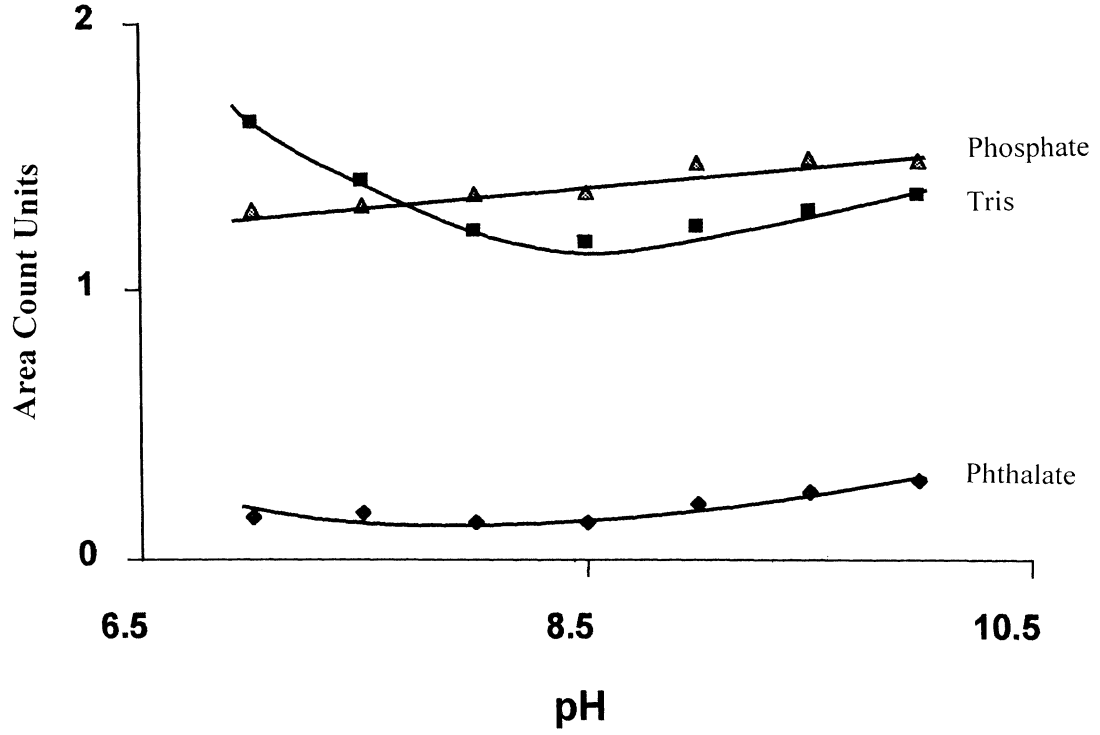

Fig. 3. The effect of type of buffer and its pH on the peak area of 10 μg ml^{-1} oxolinic acid. Conditions: buffer concentration, 5 mM; applied voltage, 20 kV; injection time, 30 s.

Electrolyte composition:	10 mM phosphate buffer, pH 9.00: methanol (9:1)
Applied voltage:	20 kV,
Injection time:	30 s

Calibration graphs plotted as peak area ratio of oxolinic acid to the internal standard was found to be linear ($r > 0.998$) over 0.5–40 μg ml^{-1} oxolinic acid. The detection limit was 0.08 μg ml^{-1} (signal-to-noise ratio 3) and the average migration time is 5.3 min. The relative standard deviation for the determination of 10 ug ml^{-1} oxolinic acid for within day and day-to-day over five consecutive days was 1.67 and 2.24%, respectively. The sensitivity of the method was slightly inferior when compared to some of the HPLC-spectrophotometric methods reported earlier (Bjorklund, 1990; Hustvedt et al., 1989; Ueno & Aoki, 1996; claimed detection limits of 0.01 μg ml^{-1}). This is mainly due to the capillary tubing's small diameter which yields smaller absorbance values as compared to those obtained in HPLC cells. However, the sensitivity problem can be further improved by using other capillary cell designs with longer pathlength (Harvey, 2000), or by using fluorescence detection. Additionally, the proposed method is more rapid (migration time of about 5.3 min) when compared to the earlier reports that were based on HPLC (Bjorklund, 1990; Samuelsen, 1990; Ueno & Aoki, 1996; retention time of about 7–13 min).

The method was applied for the analysis of oxolinic acid in fish feeds, with nalidixic acid being used as internal standard. Ten fish feed samples, including a medicated feed was treated as mentioned in the experimental section. Oxolinic acid was not detected in any of these samples using the proposed method. Here, it should be pointed out that it is a common practice among the Malaysian fish farmers to add oxolinic acid to non-medicated feeds just before feeding and this could explain why oxolinic acid was not detected in any of these samples.

The SPE method was also used for the clean-up of muscle samples. To test the viability of the adopted SPE and liquid–liquid extraction method, 5 μg ml^{-1} oxolinic acid was subjected to the entire procedure. An average recovery of 97.92% ($n = 3$) was obtained, indicating the suitability of the procedure. A total of ten fish samples

Table 1
Percentage recoveries and reproducibility of oxolinic acid that were spiked to feed and fish samples that were determined using the proposed method

Concentration of oxolinic acid spiked, μgml^{-1}	Type of sample	
	Fish feed	Fish muscle
1.00	87.85 (0.60)	94.01 (3.80)
5.00	77.42 (0.60)	80.72 (0.80)
8.00	78.19 (0.90)	79.66 (2.40)

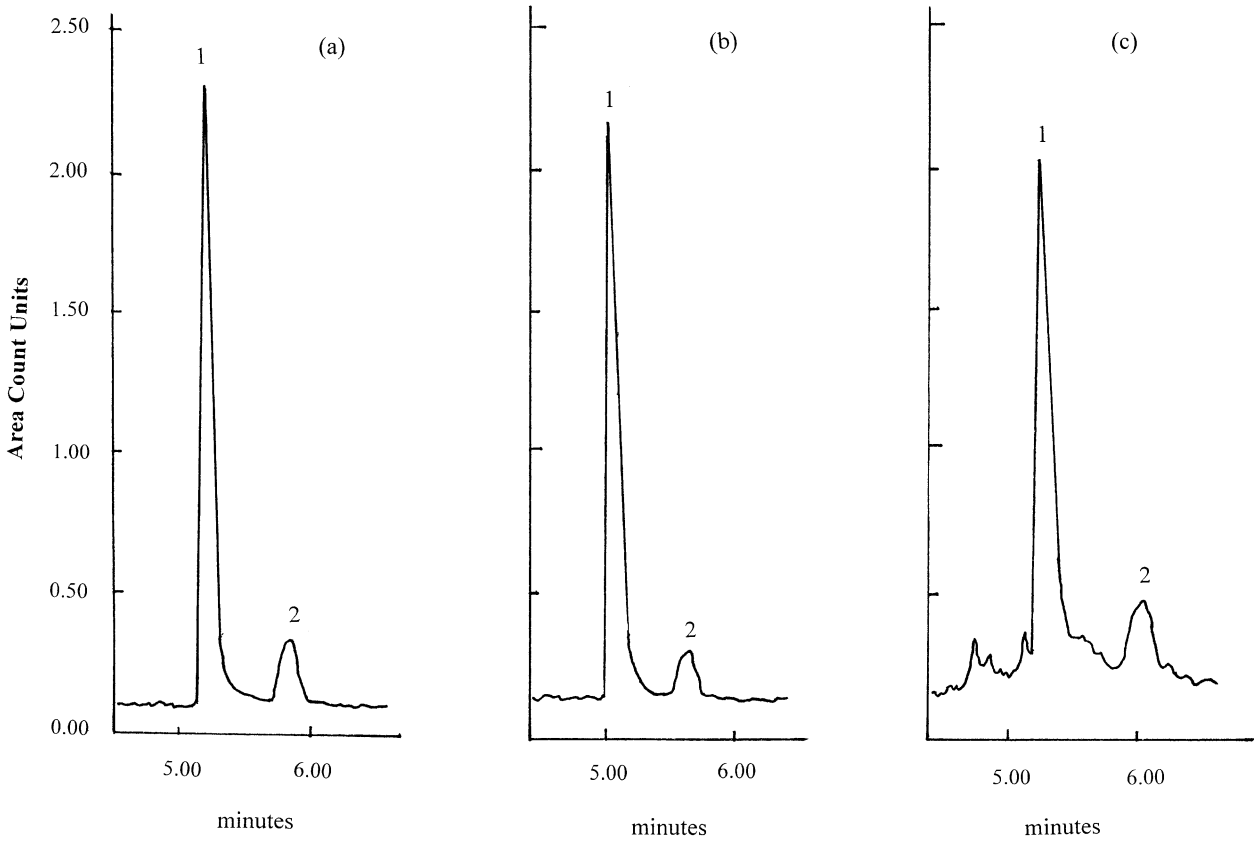

Fig. 4. Typical electropherograms of mixtures of 10 μg ml^{-1} naladixic acid and 1 ug ml^{-1} oxolinic acid, (a) standard mixtures, (b) spiked to fish muscle, and (c) spiked to fish feed sample. Peaks: 1, nalidixic acid (internal standard); 2, oxolinic acid.

were analysed and again no oxolinic acid was detected in any of these samples. Oxolinic acids at three different concentrations were then spiked both to a feed and fish muscle sample. Results for the determination are summarized in Table 1. The pretreatment procedures, as well as the CE method seemed suitable for the determination of oxalinic acid in fish and feed samples at such levels. A survey of the earlier chromatographic reports also revealed that most of the analysis were done on spiked samples at concentrations of oxolinic acid much higher than the sensitivity of the present CE work (Samuelsen, 1990; Thanh et al., 1992; Ueno & Aoki, 1996). Typical electropherograms obtained from the feed and muscle samples are shown in Fig. 4.

4. Conclusion

A CE method for the separation and quantitation of oxolinic acid was developed. The use of phosphate buffer (pH 9.00):methanol (9:1) was effective as the electrolyte. In conjunction with a SPE procedure, the CE method is suitable for the analysis of oxolinic acid in feeds and fish muscles. Even though the method is slightly inferior in terms of sensitivity but its excellent reproducibility, and superiority in terms of much less reagent consumption and small sample size required for the analysis negate the shortcoming. The proposed method is more rapid than other HPLC methods reported earlier.

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