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Development of a Technique to Detect the Residue of Oxolinic Acid in the Serum and Muscle of Chinese Mitten Crab, *Eriocheir Sinensis*

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ABSTRACT

We analyzed the residual of oxolinic acid in the serum and muscle of the Chinese mitten crab by high-performance liquid chromatography (HPLC) coupled with a fluorescence detector. A standard solution of oxolinic acid was prepared by dilution with 0.05 M phosphate buffered saline (PBS) (pH 7.4). Serum and muscle samples were completely homogenized prior to addition of oxolinic acid. Samples were then transferred into an ultrasonic water bath and centrifuged. The supernatants of serum and water layer of hexane defatted muscle were passed through C-18 solid phase extraction columns. Finally, oxolinic acid in the column was eluted with methanol/NH_4OH (75:25 v/v), and then was evaporated to dry by nitrogen gas. Extracted oxolinic acid was determined by HPLC with a fluorescence detector (excitation/emission = 335 nm/378 nm) and a Cosmosil 5C18-MS reverse phase column (5 μm, 4.6 × 150 mm i.d.). A mixture of acetonitrile and 0.02 M PBS pH 3.0 (25:75) was used as the mobile phase at a flow rate of 1.0 mL/min. A peak with retention time around 8 min was observed. With the addition of 100 μg L^{-1} oxolinic acid treatments, the mean recoveries from serum and muscle were 92% and 82%, respectively. The detection limit was 1 ppb and standard curves spiked at different levels showed a good linear correlation coefficient (R^2 > 0.999 for oxolinic acid). Because residual oxolinic acid could not be detected in all tested crabs initially, five crabs were fed with oxolinic acid for 7 days by oral administration on purpose and the residual oxolinic acid in both sera and muscle were measured, respectively. The results showed that the residual oxoilinic acid in both sera and muscle could be quantified by this method. Therefore, our developed technique may be employed as a practical method for detection of residual oxolinic acid residue in Chinese mitten crabs or other aquaculture products.

Key words: oxolinic acid, HPLC, Chinese mitten crab, serum, muscle

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis*, has become a popular seafood for East-Asians and the market for this crab thus has grown enormously in Taiwan in recent years. Almost 99% of these crabs are imported from mainland China. Lakes in China like Yungzhanghu Lake and Taihu Lake[11], are the main culture farms of the Chinese mitten crab. The market for cultured Chinese mitten crab currently exceeds ten billion Taiwan dollars a year. High density culture is the most common culture type in China because it is more profitable. Given the size of the industry, there is obviously a potential for great economic loss in the event of infection by pathogens, such as bacteria[21], protozoa, and virus[25]. In order to reduce the loss due to pathogens usually, culture ponds were administrated with low doses of antimicrobial agents for long periods. An increasing number of reports have revealed that crabs are important bioindicators of aqua-environment pollutions[9,16]. Evidence of antibiotic residues from the culture environment has been found in many types of aquatic animals[10,14]. During the growth period, heavy metals, antibiotics and other chemical pollutants may accumulate in the crab tissues[2,17]. Excess usage of drugs poses a risk of toxicity in animals[10,23] which in turn may affect human health if the animals are consumed. The issue of residues of antibiotics like chloramphenical and oxytetracycline in Chinese mitten crabs for sale in Taiwan has frequently been addressed in the public news media. The problem of drug residue has had a heavy impact on the development of crab culture. There are currently few legal drugs for crustacean culture in Taiwan. One such drug is oxolinic acid, a powerful chemotherapeutic agent applied widely in many livestock, especially in aquatic animals[22]. It is one of the most commonly used quinolone antibiotics allowed by the government for use in treatment of diseased cultured shrimps. According to regulations guiding administration of aquatic animal pharmaceuticals[19], the administration period of oxolinic acid should not be more than 1 week and the period of withdrawal should be longer than 30 days.

Some studies have revealed that oxolinic acid used in fish farms had a potential to cause adverse effects in the aquatic environment. *Daphnia magna*, a freshwater

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crustacean presented chronic toxicity, and reproductive problems were also observed under oxolinic acid exposure[23]. Furthermore, overuse of oxolinic acid caused severe environmental pollution according to several studies. Oxolinic acid contamination was identified as a problem at a Mediterranean sparid fish farm[14]. The crab is known to be a biomarker of heavy metal pollution[9,16-17] and other pollutants[2] in water. Therefore, it is important to monitor water pollution which is a major public health issue. The culture period of the Chinese mitten crab is usually more than 2 years. After long term treatment with oxolinic acid, the amount of oxolinic acid in cultured crabs could still be at a high level, even the administration of the drug is discontinued prior to selling the crabs to markets. Harmful effects might be observed since the dose of oxolinic acid in the crabs is often above the acceptable range for human consumption.

Another serious problem caused by overuse of antibiotics is the development of drug-resistant microorganisms. *Vibrio* sp., *Pseudomonas* sp. and *Aeromonas* sp. in the water or sediments and shrimps of cultured ponds have been isolated and their drug resistance has been assessed[18]. The results indicated a high percentage of drug resistant strains to oxytetracycline and oxolinic acid. Therefore, other antimicrobial agents, should also be monitored in crab culture ponds. Reducing usage of chemical agents is the best way to avoid the problem of drug residue. However, preventative and therapeutic administration of antibiotics are still widely employed to minimize the economic loss due to disease outbreaks during the feeding period. To develop effective methods to detect the residual oxolinic acid in aquatic animals and crustacean has become an essential work today.

In order to analyze the residues of possible quinolones in food, a lot of extraction and clean-up procedures were developed[1,12-13]. According to sample characteristics, polar organic solvents or hydro-organic mixtures in acid or basic media were commonly used in the liquid-liquid[12] or solid-phase extraction procedures[1]. Various chromatographic methods for the analysis of extracted sample based on HPLC with UV or fluorescence detection[5,13] have been reported. Fluorescence detector may be the best choice for oxolinic acid detection[3-4,7]. HPLC is now being widely applied for the oxolinic acid determination in many animal tissues such as milk[7], chicken[24], fish[8,15] and shrimp[20]. HPLC based methods for analyzing the residue of oxolinic acid in different animals could support measurement of several drugs at the same time. However, lots of parameters such as pH, temperature, mobile phase, and wavelength could affect the sensitivity and recovery of multiple drugs determination. The optimal settings for synchronized detection technique may not be the best ones for single of them even using the same technique. Therefore, it should be noted that single drug detection usually has a better sensitivity than the multiple drug detection at the same time[4].

Although oxolinic acid is a commonly used chemotherapeutic agent for intestinal infections in fish[22], few studies have investigated the residue of oxolinic acid in crustacean's tissues. Since the Chinese mitten crab industry has rapidly grown recently, it is necessary to develop methods of detecting oxolinic acid and other legal antibiotics in cultured Chinese mitten crabs. Here, we developed a simple and rapid extraction procedure for oxolinic acid in crab tissues and constructed a sensitive HPLC separation and detection method combined with a fluorescence detector.

MATERIALS AND METHODS

I. *Reagents and Samples*

Standard solution of oxolinic acid (Sigma, St Louis, MO, USA) was prepared by dissolving 20 mg in 20 mL of methanol (Merck, Darmstadt, Germany) containing 0.1 N sodium hydroxide (Merck), and stored in the dark at 0-4°C. The working solutions were made by serial dilution of standard solution with 0.05 M potassium phosphate buffered saline (PBS) of pH 7.4. The concentrations of working solutions ranged from 0.001 to 1 mg/mL.

The Chinese mitten crabs, weighing from 100 g to 250 g, were bought from a local market from September to November, 2005. Usually, 6 to 10 mL of hemolymph were withdrawn from the joints and 2-5 g of muscle tissue were removed from each crab chelas and stored at -20°C for later use. After centrifugation (5 min, 4,000 ×g) of hemolymph in a 15 mL polypropylene tube, the serum was separated as the supernatant. The muscle tissue was homogenized in the laboratory blender, Model Stomach 80 (Seward, England, U.K.). Five mL of 0.05 M PBS (pH 7.4) were added to the homogenized muscle tissue samples (0.5 g) or serum samples (0.5 mL), and then they were put in an ultrasonic bath for 10 min. The samples were then centrifuged (10 min, 4,000 ×g) and the supernatants were transferred into clean tubes. The extraction procedure was performed twice for crab muscle samples and the two extracts were combined. Two mL of hexane was added to the muscle extract. After reverse shaking for 10 min, clean extracts from the muscle tissue in the water layer were collected and saved for later use.

Extract from serum or muscle tissue was applied to a C-18 solid phase extraction (SPE) cartridge (Varian, CA, USA) which was previously activated with 3 mL of methanol and 3 mL of nanopure water. After samples were loaded, the cartridge was washed with 3 mL of nanopure water and eluted with 5 mL of a mixture of methanol and 30% ammonium hydroxide (75:25 v/v). The elute was evaporated to dryness under streams of nitrogen gas at 40°C. 0.05 M PBS (pH 7.4) was added to the residue to reach 1 g for muscle sample or 1 mL for serum sample. The sample was filtered through a 0.45 μm, 13 mm PVDF Millex filter (Millipore, MA, USA). Finally, 20 μL of this solution was injected in the HPLC system for oxolinic acid analysis.

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

The intra-day accuracy and precision of the assay were assessed by spiking standards at three levels (10, 50 and 100 ppb) in triplicate. After repeating the above procedure on three consecutive days, the inter-day variability was determined. Our recovery test was preceded in triplicate by spiking standards at seven levels (1, 2, 5, 10, 20, 50 and 100 ppb) into individual homogenates of crab muscle and serum, respectively. The recovery was calculated by comparing the detected concentrations with spiked concentrations. The detection limit was calculated based on the ratio of the peak area of the oxolinic acid to that of the background noise. The ratio should be more than 3.

Crabs were bought from a market and prepared in our tanks. Five crabs were fed with the oxolinic acid in 0.05 M PBS (10 mg/ mL, pH 10.5) by a plastic I.V. catheter (1.7 × 45 mm i.d.; Becton Dickinson, Utah, USA). The administrated dose for each crab was around 50 mg/ kg-bw. After 7 days, samples were taken from the crabs and analyzed for oxolinic acid residue.

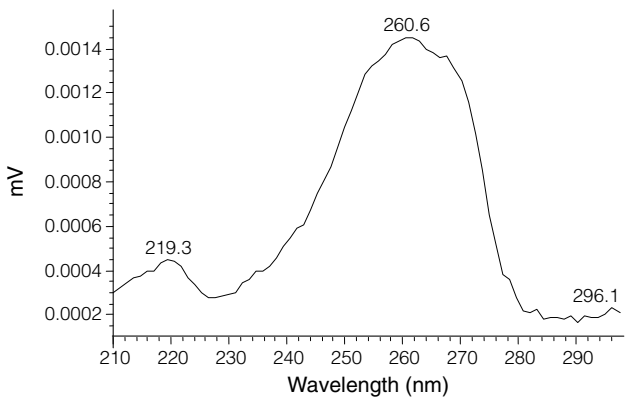

Figure 1. UV-absorption spectrum of 100 ppb oxolinic acid dissolved in methanol containing 0.1 N NaOH diluted with 0.05 M PBS (pH 7.4) using photodiode array detector.

II. *Apparatus*

Extracted oxolinic acid concentration was determined using Waters (Waters, MA, USA) instruments: a HPLC pump Model 600E, an automatic injector Model 717 and a multi λ fluorescence detector, Model 2475 or UV-Vis photodiode array detector Model 996 with Millennium software. A Cosmosil 5C18-MS reverse phase column (5 μm, 4.6 × 150 mm i.d.; Nacalai, Kyoto, Japan) was used for separation. The mobile phase was a mixture of acetonitrile and 0.02 M PBS, pH 3.0 (25:75 v/v). The flow rate was 1.0 mL/min. The excitation and emission wavelengths of fluorescence detector were 335 nm and 378 nm, respectively. The UV wavelength of photodiode array detector was set from 210 to 300 nm to scan the adsorption spectrum of oxolinic acid.

RESULTS AND DISCUSSION

In some Vietnamese shrimp ponds, concentrations of oxolinic acid in water and mud samples were as high as 2.50 ppm and 426.31 ppm, respectively[10]. Another study showed that bryophytes could also strongly accumulate oxolinic acid in its tissues in a freshwater environment[4]. The mean turnover time of this drug could be up to 59 days. Such high concentrations of antibiotic retention may have a damaging effect on ecosystems. Enhancement of the detection limit of drug analysis for livestock products may be of enormous benefit in preventing administration of illegal drugs and omission of drug withdrawal time. In order to achieve this, a detection technique with a high recovery extraction is needed. Although solid phase extraction costs more, it uses less solvent and has a higher recovery compared with other methods. Many methods are employed for analyzing drug residue in aquaculture

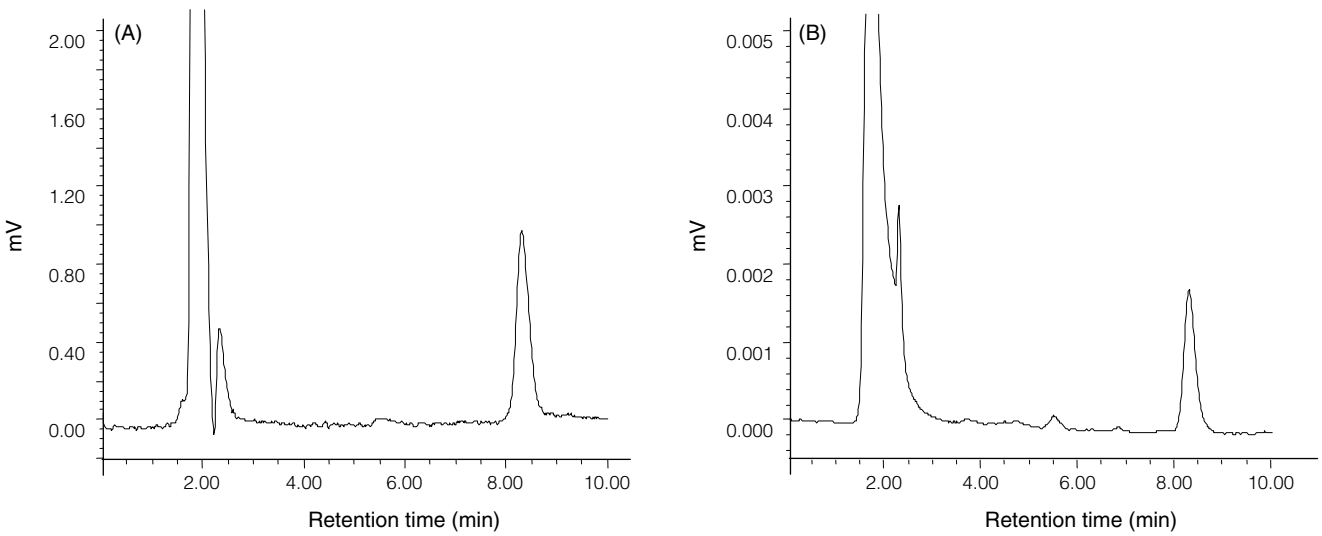

Figure 2. HPLC chromatographs for (A) 10 ppb oxolinic acid standard solution using fluorescence detector (excitation/emission = 335 nm/378 nm), and (B) 200 ppb oxolinic acid standard solution using UV detector (260 nm).

product by HPLC[13,20], HPLC mass-spectrometry (MS), and capillary electrophoresis (CE)[1,6]. HPLC is the most efficient among them due to the high cost of MS and false positive ratio of CE.

In order to confirm the absorbance peak of the oxolinic acid, the photodiode array detector was used to provide specific spectra identification for unknown peaks detected by the fluorescence detector. Oxolinic acid adsorption spectrum showed an absorbance peak appeared at 260 nm (Figure 1). Combined monitoring with fluorescence and photodiode array detectors showed sharp peaks with retention time around 8 min. The late retention time for oxolinic acid peak revealed less interference with tissue debris. Nevertheless, once it was necessary to move forward to the oxolinic acid peak, increase the ratio of acetonitrile will shorten the retention time. However, 25% of acetonitrile was the best ratio for oxolinic acid analysis since less solvent tended to give a wide and tailing peak (data not shown).

Compared with the oxolinic acid retention data obtained from cultured black tiger shrimps[20], the recovery from muscle tissue of Chinese mitten crabs was similar to both species with recoveries higher than 80%. This suggests that our technique was at least as effective as an established method. The detection mode used for oxolinic acid in shrimp muscle was the UV so a lower detection limit could be achieved in our study since fluorescence detector was employed. Cohen, *et al*. reported that the automated trace enrichment of dialysis-HPLC system with programmable fluorescence detection had a very low limit of detection, 0.2 ppb, for analyzing saraflooxacin, oxolinic acid, and flumequine in chicken liver tissue. Delépée and Pouliquen (2002) also established a HPLC method with fluorescence detector detection limit of 1 ppb for oxolinic acid in bryophyte. Both UV and fluorescence detectors were used to compare their limits of detection for oxolinic acid in crabs. Our results indicated that the fluorescence detector had much lower limitation than the UV detector. The peak area of 10 ppb drug under the excitation and emission wavelengths, 335 nm/ 378 nm (Figure 2B) was much higher than 200 ppb under the best UV absorbance wavelength, 260 nm (Figure 2A). At the same time, the excitation and emission wavelengths of fluorescence detector we used were better choice in terms of sensitivity than that used for detecting both oxolinic acid and flumequine[13], at 312 nm and 366 nm, respectively. As described above, the detected limit of oxolinic acid under these wavelengths was only around 5 ppb[13] which was higher than our limit of 1 ppb. The mean value of variation coefficient of intra- and inter- day precision of this method were 2.25% and 2.57%, respectively. The method was precise enough to detect oxolinic acid.

The standard curves spiked at different levels (1, 2, 5, 10, 20, 50 and 100 ppb) in triplicate showed a good linear correlation with the regression equation, Y = 1756.3X - 247.97 (R^2 = 0.9999) for oxolinic acid (Figure 3), where Y is the peak area and X is the concentration of oxolinic acid. Our data showed that the extraction of both crab serum (Figure 4A) and muscle (Figure 4C) were clean after SPE column treatment. The chromatograph of 2 ppb oxolinic acid in serum presented a clear and sharp peak (Figure 4B). However, the peak area of oxolinic acid from muscle extraction (Figure 4D) was higher than that from serum extraction (Figure 4B).

Although other materials like C-2 have been applied in separation of some quinolones[8], C-18 is a more suitable material for oxolinic acid binding and has been widely used in many reports[1,13,20]. It is a trend to use less organic solvent in HPLC analysis for economic and environmental considerations. Therefore, in order to keep the ratio of acetonitrile in the mobile phase as low as possible, the organic solvent used in studies these days is highly concentrated. For the same reason, the methanol and hexane used in extraction in our study were also controlled as necessary.

In this study, the recovery test was preceded in triplicate by spiking 0 (blank), 1, 2, 5, 10, 20, 50 and 100 ppb of oxolinic acid into crab serum and muscle samples. The data demonstrated that recoveries of oxolinic acid in crab serum and muscle were in the range of 71.9-134.8% and 75.0-126.8% with coefficients of variation between 0.25-3.97%, respectively (Table 1). However, in this study,

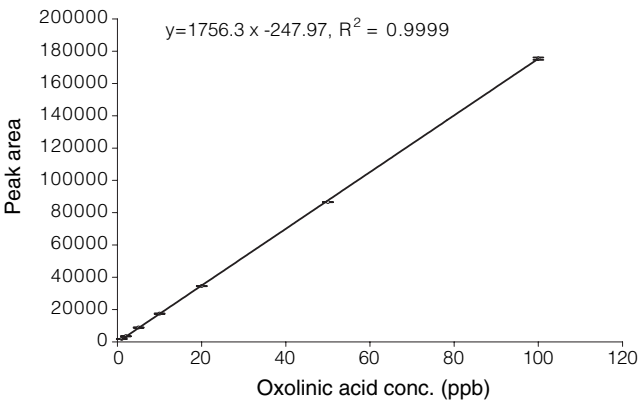

Figure 3. The standard curve of oxolinic acid (1, 2, 5, 10, 20, 50 and 100 ppb) by HPLC with fluorescence detector (mean ± S.D.).

Table 1. Recoveries of oxolinic acid spiked in crab serum and muscle, respectively

Spiked level	Crab serum (%)		Crab muscle (%)	
(ppb)	Recovery[a]	C.V	Recovery	C.V
1	125.98 ± 3.53	2.80	120.29 ± 3.73	3.10
2	134.80 ± 4.15	3.07	126.78 ± 2.85	2.25
5	108.55 ± 4.31	3.97	103.43 ± 1.84	1.78
10	78.88 ± 1.38	1.75	83.79 ± 1.09	1.30
20	71.90 ± 2.44	3.39	85.05 ± 0.21	0.25
50	93.38 ± 0.48	0.52	75.02 ± 1.30	1.74
100	92.23 ± 0.70	0.76	81.80 ± 0.58	0.70

[a]Data represent mean ± S.D. from triplicate analyses.

Journal of Food and Drug Analysis, Vol. 14, No. 4, 2006

lower level of oxolinic acid had a higher recovery, exceeding 100%. The results indicate that more effort is needed in the clean-up procedure of oxolinic acid in crab tissue. We used additional hexane in the muscle tissue samples to remove fat so the recovery of hexane-treated samples was significantly higher than that of the untreated ones (data not shown). In future studies, a higher volume of hexane might be used to improve the recovery rate in muscle samples.

We tested the suitability of our novel method to be used in field by screening Chinese mitten crabs bought from the market for residue of oxolinic acid. However, we did not detect any residue during the study period. Therefore, we fed oxolinic acid to five crabs by oral administration via plastic catheter for 7 days. The results suggested that the residues of oxolinic acid in both sera and muscles of the fed crabs could be detected by our technique (Figure 5).

CONCLUSIONS

In this study, a method for detecting oxolinic acid residue in Chinese mitten crabs tissues was developed. In the group treated with 100 ppb oxolinic acid, the mean recoveries from serum (92%) and muscle (82%) were both higher than 80%. The standard curves spiked at different levels (1-100 ppb), showing a good linear correlation with coefficient (R^2) higher than 0.999. Using HPLC with fluorescence detector to determine the residual oxolinic acid in the crab was efficient and highly sensitive, offering a detection limit as low as 1 ppb. This novel technique can provide a precise detection method of oxolinic acid residue in Chinese mitten crab tissues.

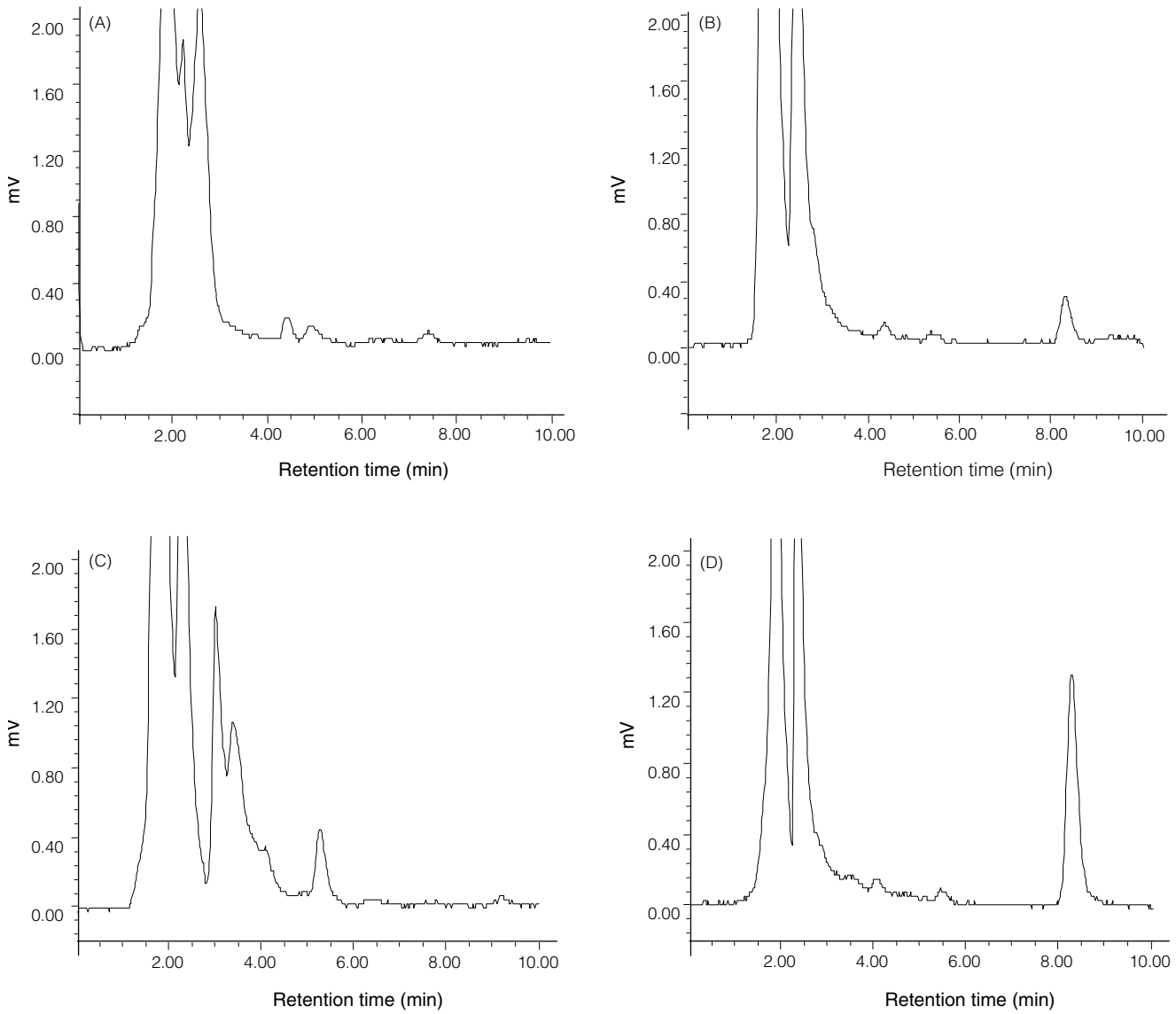

Figure 4. HPLC chromatographs for (A) crab serum, (B) crab serum spiked with 2 ppb oxolinic acid, (C) crab muscle, (D) crab muscle spiked with 2 ppb oxolinic acid.

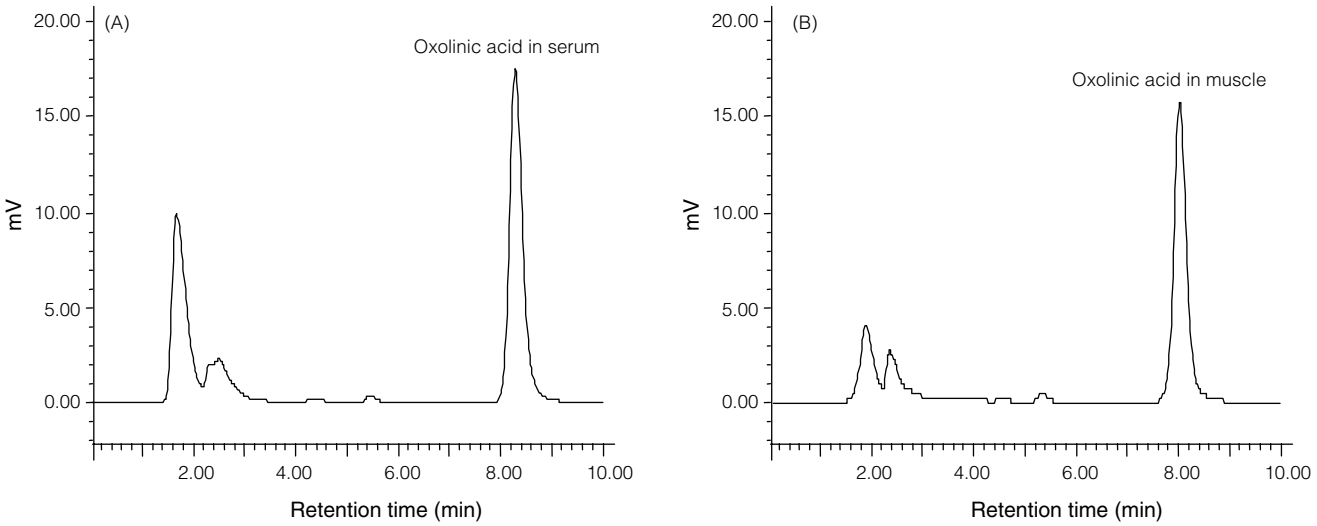

Figure 5. HPLC chromatographs for oxolinic acid residue in the serum (A) and muscle (B) of crab fed with oxolinic acid for 7 days. The concentrations of oxolinic acid in serum and muscle were 174.49 μg L^{-1} and 139.36 μg kg^{-1}, respectively.

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REFERENCES

1. Barrón, D., Jiménez-Lozano, E., Bailac, S. and Barbosa, S. J. 2003. Simultaneous determination of flumequine and oxolinic acid in chicken tissues by solid phase extraction and capillary electrophoresis. Analytica. Chimica. Acta. 477: 21-27.
2. Chen, M. H., Chen, C. Y., Chou, H. Y. and Wen, T. C. 2005. Gender and size effects of metal bioaccumulation on the rock crab, *Thalamita crenata*, in Dapeng Bay, southwestern Taiwan. Mar. Pollut. Bull. 50: 463-484.
3. Cohen, E., Maxwell, R. J. and Donoghue, D. J. 1999. Automated multi-residue isolation of fluoroquinolone antimicrobials from fortified and incurred chicken liver using on-line microdialysis and high-performance liquid chromatography with programmable fluorescence detection. J. Chromatogr. B 724: 137-145.
4. Delépée, R. and Pouliquen, H. 2002. Determination of oxolinic acid in the bryophyte *Fontinalis antipyretica* by liquid chromatography with fluorescence detection. J. Chromatogr. B 775: 89-95.
5. Garcia, M. A., Solans, C., Calvo, A., Hernandez, E., Rey, R., Bregante, M. A. and Puig, M. 2005. Determination of enrofloxacin and its primary metabolite, ciprofloxacin, in pig tissues. Application to residue studies. Biomed. Chromatogr. 19: 27-31.
6. Hernández, M., Borrull, F. and Calull, M. 2000. Determination of quinolones in plasma samples by capillary electrophoresis using solid-phase extraction. J. Chromatogr. B 742: 255-265.
7. Ho, C., Sin, D. W., Tang, H. P., Chung, L. P. and Siu, S. M. 2004. Determination and on-line clean-up of (fluoro) quinolones in bovine milk using column-switching liquid chromatography fluorescence detection. J. Chromatogr. A 1061: 123-131.
8. Ho, S. P., Cheng, C. F. and Wang, W. S. 1999. Pharmacokinetic and depletion studies of sarafloxacin after oral administration to eel (*Anguilla anguilla*). J. Vet. Med. Sci. 61: 459-463.
9. Hui, C. A., Rudnick, D. and Williams, E. 2005. Mercury burdens in Chinese mitten crabs (*Eriocheir sinensis*) in three tributaries of southern San Francisco Bay, California, USA. Envir. Pollut. 133: 481-487.
10. Le, T. X. and Munekage, Y. 2004. Residues of selected antibiotics in water and mud from shrimp ponds in mangrove areas in Viet Nam. Mar. Pollut. Bull. 49: 922-929.
11. Li, K. 1999. Management and restoration of fish communities in Lake Taihu, China. Fish. Manag. Ecol. 6: 71-81.
12. Pecorelli, I., Galarini, R., Bibi, R., Floridi, A., Casciarri, E. and Floridi, A. 2003. Simultaneous determination of 13 quinolones from feeds using accelerated solvent extraction and liquid chromatography. Anal. Chim. Acta. 483: 81-89.
13. Ramos, M., Aranda, A., Garcia, E., Reuvers, T. and Hooghuis, H. 2003. Simple and sensitive determination of five quinolones in food by liquid chromatography with fluorescence detection. J. Chromatogr. B 789: 373-381.
14. Rigos, G., Nengas, I., Alexis, M. and Troisi, G. M. 2004. Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms. Aquat. Toxicol. 69: 281-288.
15. Samuelsen, O. B., Ervik, A., Pursell, L. and Smith, P. 2000. Single-dose pharmacokinetic study of oxolinic

acid and vetoquinol, an oxolinic acid ester, in Atlantic salmon (*Salmo salar*) held in seawater and *in vitro* antibacterial activity against *Aeromonas salmonicida*. Aquaculture 187: 213-224.

16. Schuwerack, P. M. M., Lewis, J. W. and Jones, P. 2001. The potential use of the South Africa river crab, *Potamonautes warreni*, as a bioindicator species for heavy metal contamination. Ecotoxicology 10: 159-166.
17. Silvestre, F., Trausch, G., Péqueux, A. and Devos, P. 2004. Uptake of cadmium through isolated perfused gills of the Chinese mitten crab, *Eriocheir sinensis*. Comp. Biochem. & Physiol. A 137: 189-196.
18. Tendencia, E. A. and Peña, L. D. D. 2002. Level and percentage recovery of resistance to oxytetracycline and oxolinic acid of bacteria from shrimp ponds. Aquaculture 213: 1-13.
19. The Bureau of Animal and Plant Health Inspection and Quarantine. 2005. Oxolinic acid. In "Aquatic Animal Pharmaceuticals Administration Instructions". pp. 19-21. The Bureau of Animal and Plant Health Inspection and Quarantine Council of Agriculture, Executive Yuan, Taipei, Taiwan, R. O. C.
20. Ueno, R., Sangrungruang, K. and Miyakawa, M. 1999. A simplified method for the determination of several fish drugs in edible fish and shrimp by high-performance liquid chromatography. Food Res. Internat. 32: 629-633.
21. Vogan, C. L. and Rowley, A. F. 2002. Effects of shell disease syndrome on the haemocytes and humoral defences of the edible crab, *Cancer pagurus*, Aquaculture 205: 237-252.
22. Wang, W. S., Chen, M. H., Cheng C. F. and Liu, C. I. 1995. The antibacterial activities of fluoroquinolones against *Vibrio* spp. isolated from freshwater and saltwater fish *in vitro*. Taiwan J. Vet. Med. Anim. Husb. 65: 27-34.
23. Wollenberger, L., Halling-Sùrensen, B. and Kusk, K. O. 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia magna*. Chemosphere 40: 723-730.
24. Yorke, J. C. and Froc, P. 2000. Quantitation of nine quinolones in chicken tissues by high-performance liquid chromatography with fluorescence detection. J. Chromatogr. A 882: 63-77.
25. Zhang, S., Shi, Z., Zhang, J. and Bonami, J. R. 2004. Purification and characterization of a new reovirus from the Chinese mitten crab, *Eriocheir sinensis*. J. Fish Dis. 27: 687-692.