

Investigation of some veterinary drug residues in sea water, sediment, and wild fishes captured around fish farms in the Aegean Sea: Oxytetracycline, ivermectin and emamectin

Emine BAYDAN¹, Sezai KAYA¹, Haşmet ÇAĞIRGAN², Ebru YILDIRIM³, Levent ALTINTAŞ¹, Begüm YURDAKÖK¹, Hüsamettin EKİCİ³, Farah Gönül AYDIN¹, Aslı Gül KÜÇÜKOSMANOĞLU¹

¹Ankara University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ankara; ²Ege University, Faculty of Fisheries, Department of Aquaculture, Izmir; ³Kırıkkale University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Kırıkkale, Turkey.

Summary: Veterinary drug residues and their metabolites in food are regularly investigated by local authorities; however their fate in the environment is still unknown. Despite the importance of the aquaculture industry and the widespread use of antibiotics; limited scientific information regarding their residue in natural fish, sediment and sea water are available in TURKEY. The current study; which is the first study in this area, was undertaken to determine the oxytetracycline (OTC), ivermectin (IVM) and emamectin benzoate (EMA) residues from samples of wild fishes (*Oblada melanura*, *Mullus barbatus*), sea water and sediment collected in four different months, caught around the fish cages near Salihli Island in Bodrum, Aegean Sea. Samples were analyzed by High Pressure Liquid Chromatography (HPLC) followed by the validation for each matrix. No residues were found to be above the Limit of detection (LOD) levels of the validated methods in the screened samples. In order to understand the possible risk of veterinary antibiotics, especially for low dose accumulation, to the ecosystem for sustainable aquaculture, conduction of more screening analysis with expanded possible matrices would be beneficial.

Keywords: Aegean Sea, emamectin, HPLC, ivermectin, oxytetracycline, residue.

Ege Denizinde balık çiftlikleri etrafında avlanan doğal ortam balıkları ile çevredeki su ve sedimentin bazı veteriner ilaç kalıntıları yönünden incelenmesi: Oksitetrasiklin, ivermektin ve emamektin

Özet: Besinlerdeki ilaç ve metabolitlerinin kalıntıları ilgili yerel makamlar tarafından düzenli olarak araştırılmaktadır; ancak bu kalıntıların çevredeki akıbeti bilinmemektedir. Akvakültür endüstrisinin giderek artan önemi ve antibiyotiklerin bu endüstri ile birlikte kullanımının giderek yaygınlaşmasına rağmen doğal balık, sediment ve deniz suyu kalıntıları ile ilgili araştırmalar Türkiye’de sınırlıdır. Bu araştırmada oksitetrasiklin, ivermektin ve emamektin benzoat kalıntıları; ilaç kullanımının daha fazla olduğu dört ay içerisinde, Ege Denizi, Bodrum, Salihli Adası çevresindeki balık çiftlikleri etrafından avlanan doğal ortam balıkları (*Oblada melanura*, *Mullus barbatus*), deniz suyu ve sediment örneklerinde Yüksek Basıncılı Sıvı Kromatografisi (YBSK) yöntemi kullanılarak ve her bir matriksin validasyonunu takiben araştırıldı. Numunelerde, valide edilmiş metodlardaki tespit limiti üzerinde herhangi bir kalıntıya rastlanmadı. Akvakültürde kullanılan veteriner antibiyotiklerinin ekosistem üzerinde özellikle düşük dozlarda akümülyasyona bağlı oluşabilecek potansiyel risklerinin değerlendirilebilmesi için, farklı analitlerde tarama testlerinin yaygınlaştırılması ve artırılması yararlı olacaktır.

Anahtar sözcükler: Ege Denizi, emamektin, ivermektin, kalıntı, oksitetrasiklin, YBSK.

Introduction

Antimicrobial agents have been extensively applied in aquaculture to prevent and control disease. Although they can be metabolized after administration; up to 80% of antibiotics administered, could be excreted through urine or feces without complete decomposition in fish. Depending the chemical composition, these antibiotics could be present in the sediments especially under fish farms which could be then transferred by underwater currents for human consumption through various routes (16,19).

Oxytetracycline (OTC), a broad spectrum antibacterial agent is used against disease outbreaks in aquaculture because of its economic advantages and legal availability commonly. In most cases in fish farming, OTC is given to the fish through medicated feeds. Uneaten pellets normally fall through fish cages and settle on bottom of sediments (13). They form chelate with divalent and trivalent cations (i.e. magnesium, calcium and iron) readily, resulting a decrease in their absorption and efficacy; which increase the stability in sediment (21). It is suggested that leakage to the sediments, may be the

main factor for the traces of OTC residues in sediments and OTC resistant bacteria strains isolated from wild fishes. The half-life of OTC in fish farm sediments varied between 9-419 days; where under stagnant anoxic conditions, it could be very persistent in sediments (11). Ivermectin (IVM) and emamectin benzoate (EMA), the main compounds of avermectins, are veterinary antihelminthic drugs belonging to the family of macrocyclic lactones that are highly effective against a number of arthropod and nematode infestations mainly for the control of sea lice in farmed salmon and trout (23). Ivermectin is rapidly photolysed, but when buried in sediments and incorporated into particles, it breaks down slowly in the dark and persist for weeks or months in the sediments, further increasing their environmental risks (17).

Despite the importance of the aquaculture industry, to our knowledge, there are no studies regarding the OTC, IVM and EMA residues in seawater, sediment and wild fishes (striped bream, red mullet) caught around the fish cages in Turkey. This study was aimed to determine the OTC, IVM and EMA residues from the samples of wild fishes (striped bream, red mullet), sea water and sediment collected in four different months (March, April, September, October) selected according to the drug application period, around the fish cages near Salihli Island in Bodrum, Aegean Sea, Turkey followed by the validation of each matrix.

Materials and Methods

Reagents and chemicals: HPLC grade solvents and analytical chemicals were obtained from Merck (Darmstadt, Germany) and OTC hydrochloride, IVM and EMA standards were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Ultra-pure water was obtained from a Millipore distillation system (Millipore, Molfheim, France). Disposable syringe filters were purchased from Cronus (PVDF, 13 mm, 0.2 µm).

Chromatographic conditions: Chromatography was performed on a Shimadzu LC-20AT prominence system (Shimadzu, Tokyo, Japan) and a SPD-M20A photodiode array UV-VIS detector (Shimadzu Company, Japan) for OTC analysis and RF-20A fluorescence dedector for EMA and IVM analysis. Separation was achieved on Luna C₁₈ column (150 mm X 4.6 mm i.d., 5 µm) for OTC, Hyperclone ODS column C18 (250mm X 4.6 mm i.d., 5 µm) for EMA and IVM analysis, both purchased from Phenomenex (CA, USA). Column temperature was maintained at 25 °C and the detector of HPLC was set at 360 nm for wavelength for OTC analysis (3,14). For EMA and IVM analysis; column temperature was set at 35 °C and 30 °C with fluorescence dedector wavelength settings at 365-470 nm and 365-465 nm for exitation-emission, respectively (18, 20, 22, 24, 26, 27).

Standard stock and working solutions: The stock 50 µg/mL OTC solution was prepared in methanol and working standards were prepared by diluting the stock solution volumetrically in methanol (eight dose between 3.125-400 ppb). Spiked samples were prepared six (Table 1) doses. Since OTC is photolabile, extra care has been taken and all solution containers were protected from direct sun and artificial light throughout the analysis. Since the recovery values from results of our preliminary experiments did not vary between different fish species, experiments were conducted from the selected fish; red mullet. Standard stock solutions for EMA and IVM analysis were prepared in acetonitrile and doses for the spiked samples ranged between 4.17-300 ppb (EMA-fish:50-300 ppb, EMA-sediment: 20-100 ppb, EMA-sea water: 4.17-133.33 ppb; IVM-fish: 10-160 ppb, IVM-sediment: 8.33-133.33 ppb, sea water: 4.17-66.67 ppb).

Sample collection: Striped bream, red mullet, sediment and sea water samples were collected from four different regions (GPS - Global Positioning System coordinates recorded) in Bodrum, Salih Island region on September-October 2011 and March-April 2012, around 20-30 m close to the fish farms, with the permission from the Republic of Turkey Ministry of Food, Agriculture and Livestock and Coast Guard. The coordinates were as follows: Region A-North: 37/07/297, East: 27/28/029; Region B-North: 37/08/336, East: 27/28/479; Region C-North: 37/09/330, East: 27/28/975; Region D-North: 37/09/870, East: 027/29/437. Sediment samples were collected from 50 m depth, over 0-20 cm of ground surface and sampling of water was conducted one meter above the place where the sediment samples were taken with the help of professional divers. Fish samples were collected from local fishermen on boats around the region. The dissolved oxygen and temperature were measured with oxygenometer (WTW-530), and the pH values were determined by pH indicator sticks (IsoLab, Wertheim, Germany) which were then confirmed by a pH meter at the laboratory. Sediment and water samples were collected randomly and stored in pre-cleaned light preserved bottles and were directly transported to the laboratory at cold chain and stored at -20 °C until analysis. For fish samples, the skin and muscles were dissected and placed in separate containers which were then stored in the freezer until the time of analysis. A total of 32 *Oblada melanura* and 32 *Mullus barbatus* and 16 sea water and sediment samples (4 different regions, 4 different periods) were analyzed.

Sample preparation: Fish and sea water extraction for OTC analysis were followed by the method described by Coyne et al. (14); whereas for the sediment Agilent Technologies Method (3) was followed with slight modifications. For EMA detection in seawater, analysis were performed according to the method described by Hicks et al. (18); for sediment by Thomas (26) and for

respectively; for EMA, LOD and LOQ values for sea water, sediment and red mullet were found as 6.62-20.05, 11.13-33.73, 15.94-48.32 ppb; and for IVM, these values for the mentioned matrices were found as 3.51-10.65, 7.07-21.43 and 2.63-7.96 ppb, respectively. Regression equations revealed good correlation coefficients (0.9925-0.9996) over the examined range for all drugs and analytes.

Recovery and precision studies: Accuracy and the precision of the method were determined by the recovery values for the spiked samples at various concentrations (Table 1). Repeatability and reproducibility (inter-day/intra-day) results for OTC, EMA and IVM are shown in Table 2. According to these results, the recovery values (%) of the methods for sea water, sediments, red mullet and striped bream were found as 85.30-91.77, 78.19-91.08, 83.88-89.15, 86.89-92.38 for OTC; 83.98-95.79, 70.77-96.32, 85.94-94.56 for EMA and 72.33-98.90, 65.54-85.14, 85.28-97.23 for IVM, respectively.

Sample analysis: No residual contamination were observed over the LOD values for OTC, EMA and IVM. One OTC suspected sediment sample (October, A

Region) were re-analyzed in Izmir, Bornova Veterinary Control Institute for confirmation by LC-MS MS analysis; where no contamination was detected.

Discussion and Conclusion

Aquaculture is a major contributor of antibiotic residues to the environment. Each year, millions of tons are released globally to the aquatic environment (8,15).

OTC is poorly absorbed through the intestinal tract of fish which cause consequently a slow excretion of large amounts (21). Also, uneaten medicated feed around the cultured fish production areas, may possibly be consumed by nontarget organisms; where OTC has been found in varying amounts for up to 2 weeks after treatment in invertebrates near net pens (oysters, crabs) at trace levels. It is possible that such organisms eventually reintroduce biologically available OTC to the environment through their excrement (12).

Currently 45 antimicrobial preparations are licenced for aquaculture in Turkey (7). Since the method is validated in our laboratory for sediment, striped bream

Table 2. Repeatability and reproducibility (inter-day/intra-day) results for oxytetracycline (OTC), Emamectine (EMA) and ivermectine (IVM).

Tablo 2. Oksitetrasiklin (OTC), emamektin (EMA) ve ivermektin (IVM) için sonuçların (günler arası/gün içi) tekrarlanabilirlik ve uyarlılık özelliği.

	Spiked concentration (ppb)			Intra-day % Relative Standard Deviation			Inter-day % Relative Standard Deviation		
	OTC	EMA	IVM	OTC	EMA	IVM	OTC	EMA	IVM
Sea water	12.5	4.17	4.17	2.21	4.72	2.78	1.55	10.34	9.82
	25	8.33	8.33	2.38	1.82	5.33	3.01	9.69	4.22
	50	16.67	16.67	3.90	0.01	6.30	2.65	4.17	8.78
	100	33.33	33.33	3.76	0.05	7.97	5.21	0.09	3.33
	200	66.67	66.67	1.36	1.81	3.78	1.99	3.95	5.86
	400	133.33	-	1.73	1.42	-	0.15	4.26	-
Sediment	12.5	20	8.33	5.78	6.25	4.10	0.97	6.56	3.58
	25	40	16.67	0.93	2.75	1.35	6.75	1.47	1.05
	50	60	33.33	5.21	6.93	8.86	0.31	1.53	7.53
	100	80	66.67	0.62	7.73	9.48	2.78	4.03	8.97
	200	100	133.33	1.35	1.58	9.96	0.26	7.28	7.41
	400	-	-	0.84	-	-	1.04	-	-
Red mullet	12.5	50	10.00	1.24	7.05	4.89	1.12	3.65	4.89
	25	100	20.00	0.89	1.24	7.60	0.99	5.28	3.68
	50	150	40.00	2.05	4.05	2.98	1.06	4.37	0.71
	100	200	80.00	0.33	7.58	8.48	0.75	8.76	3.46
	200	250	160.00	1.04	2.41	4.48	0.32	8.10	5.49
	400	300	-	0.49	2.67	4.89	1.06	6.71	4.89
Striped bream	12.5	-	-	1.27	-	-	6.15	-	-
	25	-	-	1.27	-	-	1.48	-	-
	50	-	-	0.73	-	-	1.52	-	-
	100	-	-	6.51	-	-	0.78	-	-
	200	-	-	1.27	-	-	4.44	-	-
	400	-	-	0.74	-	-	1.36	-	-

and sea water and even high recovery values were obtained with other fishes such as striped mullet, more data is expected to be provided in future for ecomonitoring studies. Scottish Environment Protection Agency (SEPA) (5) reported the occurrence of IVM and EMA residues in marine fish farm sediments collected from different fish farms in 2010, which were found in between 0.26-1.08 µg/kg dry weight, 0.09-2.69 µg/kg wet weight, respectively. In another report by SEPA (6), sediment samples taken from areas in three sea lochs (Loch Linnhe, Loch Ewe and Loch Nevis) during 2009 were screened, where no IVM residue was detected whereas EMA was found to be the most frequently detected of the substances tested, with the highest concentration in one sample as 44 µg/kg wet weight. In the current study, no residual contamination were observed over the LOD values for OTC, EMA and IVM in sampling field.

A major indicator of water quality where the aquatic life is dependant, is the dissolved oxygen. Lower amounts could be due to a pollution related to the excessive algae growth caused by phosphorus and is a major stress for fish (4). Dissolved oxygen threshold for healthy farms have a value over 4.0 mL/L; where as levels below 2.5 mL/L are considered to be critical (1,25). Water temperature is important because it not only establishes the maximum oxygen-holding capacity of water, but also has direct influence on rates of biochemical reactions and transformation processes occurring within the water column and in the sediment bed (4). The results of the current study; where the lowest temperature and dissolved oxygen values were recorded in March (14 °C, 6.05-6.44 mg/L, respectively) and the highest in October 2011 (28.8 °C, 10.78-11.55 mg/L, respectively) are found to be in accordance with the control and clean region stations of earlier studies in the Aegean Sea (2,9,10).

In conclusion, the current study was undertaken to determine the OTC residues from samples of wild fishes, sea water and sediment collected around the fish cages in Bodrum, Aegean Sea. Physicochemical properties of the sea water and the absence of residues indicate the investigated area, defined by authorities as offshore, is not yet contaminated to pose a risk. Although OTC, IVM and EMA residues were not detected (levels below LOD and thus below the maximum residue limits) in this study, the risk for low dose accumulation should be taken under consideration by using more screening analysis especially for the sediments since OTC is stable in order to understand the contamination risks for the ecosystem.

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Address for correspondence:

Prof.Dr. Emine Baydan
Ankara University, Faculty of Veterinary Medicine,
Department of Pharmacology and Toxicology,
06110 Dışkapı, Ankara, Turkey.
e-mail: baydan@veterinary.ankara.edu.tr