Detection of Antiviral Drugs Oseltamivir Phosphate and Oseltamivir Carboxylate in Neya River, Osaka, Japan

Ryohei TAKANAMI*, Hiroaki OZAKI**, Rabindra Raj GIRI*, Shogo TANIGUCHI*, Shintaro HAYASHI**

*New Industrial R & D Center, Osaka Sangyo University, 3-1-1 Nakagaito, Daito City, 574-8530 Osaka, Japan

**Department of Civil Engineering, Osaka Sangyo University, 3-1-1 Nakagaito, Daito City, 574-8630 Osaka, Japan

ABSTRACT

Presence of the antiviral drug oseltamivir in considerable concentrations in surface waters especially in seasonal and pandemic influenza cases has raised concerns on its possible consequences in the environment and human health. This investigation aimed to elucidate concentration levels of the drug in Neya River in Osaka during 2009/2010 seasonal influenza. Oseltamivir phosphate was detected for the first time in Neya River suggesting the presence of the drug in phosphate form in surface waters is significant only in influenza pandemic cases. Oseltamivir carboxylate concentrations in Neya River were as high as 15-fold the concentrations in Yodo River in 2007/2008 and 3-fold the concentrations in a sewage treatment plant effluent in Kyoto in 2008/2009. The highest oseltamivir carboxylate concentration in Neya River was detected at ST-2 (864.8 ng/L) followed by ST-3. This was possibly due to the inefficiency of the treatment plant upstream and low river water flowrate. Based on the limited information available on the possible environmental risks of the drug in surface waters, the detected concentrations in Neya River may not be an immediate threat to the environment. However, detailed risk assessment studies are essential to clarify the potential environmental risk issue.

Keywords: environmental risk, influenza index, oseltamivir, sewage treatment plants, surface water

INTRODUCTION

Oseltamivir phosphate (OP), also known as “Tamiflu”, is the most commonly used antiviral drug for treatment and prevention of influenza “A” and “B”, but Zanamivir (also known as “Relenza”) is more commonly prescribed against seasonal influenza viruses in Japan. As thousands of H1N1 influenza cases were reported in the major Japanese cities in the middle of 2009 and World Health Organization (WHO) declared H1N1 influenza pandemic worldwide later in the year, the Japanese government stockpiled OP and Zanamivir for about 12 and 12.7 million people, respectively, which were respectively 2.8 and 6.7-folds larger than those of the previous year, to be used in the forthcoming winter season (Ministry of Health, Labor and Welfare, Japan, 2010).

Oseltamivir phosphate itself is not effective against influenza viruses. It is hepatically hydrolyzed to oseltamivir carboxylate (OC) in the human body after ingestion. Therefore, OC rather than OP is really responsible for the prevention and treatment of influenza. About 80% of OP is converted to OC due to hepatic metabolism (Taylor *et al.*, 2008; Genentech USA Inc, 2009; Ghosh *et al.*, 2010 cited Sweetman, 2007), while no further change in OC occurs in the human body (Genentech USA Inc, 2009; Straub, 2009). Moreover, OP and OC are excreted mainly through the renal pathway in a ratio of approximately 1:4 (Straub, 2009). It is therefore apparent that the excreted drugs (i.e.,

Address correspondence to Ryohei Takanami, New Industrial R & D Center, Osaka Sangyo University,
E-mail: r-nami@cnt.osaka-sandai.ac.jp

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OP and OC) are discharged through mainly domestic sewage.

Oseltamivir phosphate is believed to be converted to OC in the water environment by some natural chemical processes. However, OC is not degraded or removed in conventional sewage treatment plants (Fick *et al.*, 2007), and it ultimately reaches to various surface water bodies. Furthermore, OC in water is not substantially degraded by direct photolysis using solar radiation (Accinelli *et al.*, 2007; Fick *et al.*, 2007; Bratels and Wolf, 2008). It is thought that indirect photolysis and microbial metabolism may be responsible for the degradation of OC in the environment (Accinelli *et al.*, 2007; Bartels and Wolf, 2008). Some recently published articles highlighted the detection of large OC concentrations in natural water bodies, especially rivers receiving effluents from sewage treatment plants (Soderstrom *et al.*, 2009; Ghosh *et al.*, 2010; Prasse *et al.*, 2010). The predicted environmental concentrations of OC in surface waters for seasonal and pandemic influenza scenarios are as high as 98.1 µg/L, and the values for sewage treatment plants are as high as 348.0 µg/L (Straub, 2009). However, to date, there is no information on the fate of OP in sewage treatment plants and natural water bodies.

An environmental risk assessment for River Lee catchment in the UK and lower Colorado basin in the USA (Straub, 2009) concluded that oseltamivir does not pose a significant risk to surface waters or sewage works during both regular seasonal use and high pandemic use of the drug (≤ 98.1 µg/L). One concern raised today is the environmental risk of the drug in water environment. It is suspected that the presence of the drug in considerable concentrations in water bodies for considerable periods may result to the development of oseltamivir-resistant influenza viruses. Furthermore, Japan is at the top of the list of countries for per capita consumption of Oseltamivir (Soderstrom *et al.*, 2009), and it has also the highest rate of emerging resistance of influenza virus to this drug (Fick *et al.*, 2007).

Oseltamivir carboxylate concentrations as high as 293.3 ng/L were detected in the effluents from sewage treatment plants in Kyoto, Japan, from November 2008 to February 2009 (Ghosh *et al.*, 2010). Very high concentrations of antiviral drugs including oseltamivir can be expected in surface water bodies, especially those receiving effluents from sewage treatment plants, during the winter season of 2009/2010 in Japan owing to H1N1 influenza pandemic and drastic increase in antiviral drugs consumption. It is therefore worthy to monitor the drugs in surface waters in the winter season to get more insight of whether the drugs really exist in alarming concentrations. This research aimed to elucidate on OP and OC concentration levels in Neya River, which receives effluents from several sewage treatment plants in Osaka, and the significance of these drugs during the period of seasonal influenza and H1N1 influenza pandemic (September 2009 to February 2010).

MATERIALS AND METHODS

Chemicals and Reagents

Oseltamivir phosphate (CAS-RN: 204255-11-8) was obtained from F. Hoffmann-La Ltd., Switzerland. Oseltamivir carboxylate (CAS-RN: 187227-45-8) was obtained from Toronto Research Chemicals Inc., Canada. Oseltamivir carboxylate labeled with deuterium (OC-D₃) as internal standard of OC was also obtained from Toronto Research

Chemicals Inc., Canada. LC/MS grade acetonitrile and methanol were purchased from Wako Pure Chemical Industries Ltd., Japan. All other chemicals including formic acid and ammonia solution were also obtained from Wako Pure Chemical Industries Ltd., Japan. Stock solutions of OP and OC (1.0 mg/L each) were prepared in methanol and stored in refrigerator (4°C) prior to usage.

Description of Sampling Sites

Fig. 1 shows the location of the sampling sites with the map of Japan inset. The Neya River is located in Osaka Prefecture of Japan as shown in the figure. Details of the sampling stations and dates are illustrated in Table 1. The stations along the river extend between 34° 40' 45.75"N and 34° 46' 13.96"N latitudes, and 135° 28' 3.42"E and 135° 36' 40.44"E longitudes. No sewage treatment plants are located in the upstream vicinity of ST-1, ST-3, ST-5 and ST-6. Only one ordinary wastewater treatment plant exists between ST-1 and ST-2. However, five treatment plants are located between ST-3 and ST-4. Out of the five plants, three plants used advanced treatment techniques while the other two used simple techniques.

Sample Collection and Pretreatment

Grab samples were taken from the six stations on six dates as shown in Table 1. The samples were collected in clean plastic containers and immediately stored in a refrigerator at 4°C. The stored samples were used within a few days.

One liter of water sample was filtered through glass microfiber filter (Whatman, GF/F, pore size: 0.7 µm). The filter paper containing the residue was placed on a cleaned watch glass, methanol was poured slowly over it and was sonicated for 5 min to dissolve any undissolved portion of the target compounds. The methanol after sonication was slowly poured into the filtrate without disturbing the residue on the filter

Fig. 1 - Location of study area and sampling stations

Table 1 - Sampling stations, points and dates

Station	Sampling point (City)	Date (Week of the year)
ST-1	Gokuraku Bridge (Neyagawa)	Sept. 01, 2009 (36); Sept. 29, 2009 (40); Nov. 05, 2009 (45); Dec. 01, 2009 (49); Jan. 04, 2010 (1) and Feb. 05, 2010 (5)
ST-2	Kamikayashima Bridge (Neyagawa)	
ST-3	Suminodouohashi Bridge (Daito)	
ST-4	Kyoubashi Bridge (Osaka)	
ST-5	Tenzinbashi Bridge (Osaka)	
ST-6	Ajigawa Tunnel (Osaka)	

paper. The process of dissolving the target components in the filtered residue was repeated three times to ensure their complete dissolution. Then, the pH of the sample was adjusted to 2.8 using formic acid, and an internal standard OC-D₃ (50 ng) was added to the sample for calculating the recovery rate. Solid phase extraction (SPE) was performed using Oasis MCX cartridge (6 cc, 150 mg), Waters Co., USA. The cartridge was preconditioned by passing 10 mL methanol followed by 10 mL distilled-deionized water (ddw) containing 0.1% formic acid. The sample was passed through the cartridge at 5 mL/min flow rate. The cartridge was then rinsed with 10 mL ddw containing 2% formic acid (v/v) followed by another rinsing with 10 mL methanol. The analytes in the cartridge were then eluted using 6 mL 5% ammonia solution in methanol. The sample was then dried to 0.5 mL in a gentle flow of nitrogen gas at 40°C. Finally, the sample volume was adjusted to 1.0 mL using acetonitrile for analysis.

Sample Analyses

The pretreated samples were analyzed for OP, OC and OC-D₃ using Waters ACQUITY ultra performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS). The UPLC system was equipped with a binary pump and ACQUITY UPLC BEH HILIC column (100 mm × 2.1 mm, 1.7 µm). Eluent “A” consisted of 10 mM ammonium acetate in ddw containing 5% acetonitrile (v/v) adjusted to pH 5 using acetic acid. Eluent “B” was the same as eluent “A” except for the acetonitrile content, which was 95% (v/v). The eluent flow rate, sample injection volume and column temperature were 0.7 mL/min, 1.0 µL and 30°C, respectively. The UPLC analysis started with 100% eluent “B”, which continued until 1.0 min then it linearly decreased to 80% until 3.5 min. It linearly decreased again to 50% until 4.0 min, and then the analysis ended.

Electro spray ionization (ESI) in positive mode was the ion source, and mass detection was carried out in multiple reactions monitoring (MRM) mode. The monitored mass number (*m/z*) values for parent ions of OP, OC and OC-D₃ were 313.14, 285.20 and 288.10, respectively. Similarly, mass numbers for product ions in the same order were 225.04, 197.00 and 200.06, respectively. Quantifications of OP, OC and OC-D₃ were based on calibration curves from 0 to 1000 ng/L. The limit of detection (LOD) and limit of quantification (LOQ) values for OP, OC and OC-D₃ in this investigation were 0.75, 7.5, 7.5 and 2.5, 25.0, 25.0 µg/L respectively.

Influenza Cases

Influenza index values for ST-2 from the 34th week of 2009 to the 7th week of 2010 period were obtained from Osaka Prefectural Institute of Public Health (2010). An index value gives the total number of influenza cases (average) in a hospital in a week. The

numbers of influenza cases for ST-2 in this investigation were calculated by multiplying the index values and total number of major hospitals in the area (which was 23).

RESULTS AND DISCUSSION

Oseltamivir Phosphate in River Water

No earlier publications in our knowledge have reported detection of oseltamivir phosphate in sewage treatment plants and surface waters, but the drug (i.e. OP) was detected in Neya River at significantly large concentrations in this investigation. Oseltamivir phosphate concentrations at the six sampling stations and dates (Table 1) are illustrated in Fig. 2. The OP concentrations in river water gradually increased with the approaching influenza season in 2009, the peak values of OP concentrations were observed in December 01, 2009 samples, and then the values gradually decreased at later dates. Station-2 exhibited the highest OP concentrations followed by ST-3. The peak of OP concentrations at ST-2 (154.2 ng/L) and ST-3 (132.4 ng/L) were respectively about 5 and 4-folds larger than those at the other stations.

The OP concentrations at the six sampling stations can possibly be related to sewage treatment plants located in the upstream vicinity as similar earlier studies mentioned a link between treatment plants and OC concentrations in receiving waters (Fick *et al.*, 2007; Ghosh *et al.*, 2010; Prasse *et al.*, 2010). The sources of OP at ST-1 are unknown as no treatment plants exist in the upstream side. However, direct discharge of domestic sewage and /or hospital wastes combined with very low river water flow rate (daily average = 4,104 m³/day, Feb. 17, 2010) could be possible reasons for the considerable OP concentrations at ST-1. The largest OP concentrations at ST-2 could possibly be attributed to the plant upstream as the effluent discharge from the plant (yearly average = 142,500 m³/day for 2009) appeared to be more than 30-folds larger than the river flow at ST-1. Despite the absence of treatment plants between ST-2 and ST-3, OP concentrations at the latter point were the second highest among the six stations, which can be attributed to the very high OP concentrations upstream (i.e. ST-2) and possibly a little dilution between ST-2 and ST-3 (average daily river water flow rate at ST-2 on Feb. 17, 2010 = 129,600 m³/day). The section between ST-3 and ST-4 is characterized by the highest number of treatment plants (i.e. five) and the largest distance. However, OP

Fig. 2 - Oseltamivir phosphate concentrations at the sampling stations

concentrations at ST-4 were almost four-folds smaller than those in ST-3, and this may be attributed to higher OP removal efficiency of the plants combined with large increase in river water flow rate resulting to OP dilution. Similar OP concentrations at ST-4 and ST-5 may be due to the absence of treatment plant between them and possibly no significant OP dilution in the section. It is to be noted here that no river discharge data downstream are available, and the river downstream flows in reverse direction during high tide.

Oseltamivir Carboxylate Concentration Levels

Oseltamivir carboxylate (i.e. OC) concentrations at the six stations and dates are shown in Fig. 3. Though OC concentrations were larger than the corresponding OP concentrations, concentration distribution patterns for both compounds were almost the same. Similar to OP, ST-2 exhibited the largest OC concentrations followed by ST-3. Moreover, OC concentration drastically increased at ST-2 and ST-3 in November 05, 2009, peak values (864.8 and 629.1 ng/L respectively) were observed in December 01, 2009, and then the values decreased drastically at later dates. The highest OC concentrations at ST-2 and ST-3 were respectively about 5.5 and 4.1-folds larger than those in other stations.

The distribution of OC at the six sampling stations and dates (Fig. 3) may be correlated with sewage treatment plants discharge into the river upstream (Fick *et al.*, 2007; Soderstrom *et al.*, 2009; Ghosh *et al.*, 2010; Prasse *et al.*, 2010). As discussed in the preceding section, the highest OC concentrations at ST-2 may be attributed to the inefficiency of the treatment plant located upstream in removing the compound. The second highest OC concentrations at ST-3 could mainly be attributed to the short distance between ST-2 and ST-3 combined with very low flowrate of water into the river resulting in no significant dilution of the drug in the section. The drastic decrease in OC concentration at ST-4 and its downstream stations may indicate higher efficiencies of the plants located between ST-3 and ST-4. Furthermore, as river water flow greatly increased on the upstream of ST-4, dilution factor also might have played a role for those low OC concentrations.

The highest OC concentration detected in Neya River during 2009/2010 influenza

Fig. 3 - Oseltamivir carboxylate concentrations at the sampling stations

season was about 15-fold larger than the value for Yodo River (Kyoto and Osaka) during 2007/2008 influenza season (Soderstrom *et al.*, 2009). The highest concentration in Neya River was about 3-fold larger than the highest OC concentration detected in the effluent from a sewage treatment plant in Kyoto during 2008/2009 influenza season (Ghosh *et al.*, 2010). However, the predicted environmental concentrations of oseltamivir in surface water (highest values) for Western Europe (5.9 µg/L) and River Lee catchment (98.1 µg/L) for pandemic influenza (Straub, 2009) were respectively about 6.8 and 113.4-fold larger than the largest OC concentration detected in Neya River. It may be apparent from this discussion that the large OC concentrations detected at ST-2 and ST-3 in Neya River compared to the values (58.0 ng/L and 19.0 ng/L respectively) reported for Yodo River and Katsura River in Osaka and Kyoto area, respectively (Soderstrom *et al.*, 2009), during seasonal influenza in previous years can be attributed to drastic increase in oseltamivir consumption during 2009/2010 influenza season. Moreover, the concentrations at ST-2 and ST-3 were quite below the predicted highest oseltamivir concentrations for Western Europe and River Lee catchment for influenza pandemic scenario.

Oseltamivir (i.e. Tamiflu) is rapidly absorbed in the gastrointestinal tract and converted to OC via hepatic and/or intestinal esterases (Genentech USA Inc, 2009). About 60% to 80% of an oral dose of oseltamivir is excreted in urine as OC while less than 5% is recovered unchanged (i.e. as OP) in the urine (Bartels and Tumpling, 2008). The orally administrated OP is first hydrolyzed to oseltamivir ethylester in the digestive tract before its absorption, and the hydrolyzed OP and its active metabolite OC are excreted mainly by the renal pathway in a ratio of about 1:4 (Straub, 2009). However, there are no reported studies on OP in sewage and surface waters until now. Oseltamivir phosphate and OC concentrations and their ratios at the six stations in the samples of December 01, 2009 from Neya River are shown in Fig. 4. The OC:OP ratio from ST-1 through ST-5 varied between 1:4.5 and 1:5.6, which were closer to the ratio (1:4) mentioned in Straub (2009). As no OP (Fig. 2) and OC (Fig. 3) were detected at ST-6 on the date, a zero value is assigned in this case. It is apparent from the results presented here and those (Soderstrom *et al.*, 2009; Ghosh *et al.*, 2010) published earlier that OP may be detected in receiving waters only when the drug is heavily used (i.e. influenza pandemic cases).

Influenza Cases and Oseltamivir in River Water

Fig. 5 illustrates the number of influenza cases together with OP and OC concentrations at ST-2, which exhibited the highest drug concentrations among the six sampling stations. The only sewage treatment plant located in Hirakata City serves the whole Hirakata City and Katano City. The treated sewage is discharged at a point upstream of ST-2. The 23 major hospitals taken into account for the reported influenza cases shown in Fig. 5 are located within these two cities. The trends in oseltamivir concentrations and the number of influenza cases at the selected dates (Fig. 5) were closely related. Furthermore, the peak OC concentration values on the 40th, 45th, 49th week of 2009 and 1st week of 2010 were slightly lagging behind the corresponding peak for influenza cases if closely observed. This phenomenon possibly indicated the time lag between the drug administration and its appearance in the river water. Nevertheless, the figure clearly indicated good correlation between OC/OP concentrations at ST-2 and reported influenza cases within the coverage of sewage collection and treatment.

Fig. 4 - Oseltamivir concentrations in Dec. 01, 2009 (i.e. 49th week) at the stations

Fig. 5 - Oseltamivir concentrations at ST-2 and reported influenza cases within the coverage of sewage collection and treatment

Significance of OP and OC in Neya River

Many researchers are concerned about the possible consequences of oseltamivir in surface waters while only very few published information on the issue are available to date. The predicted environmental concentrations of oseltamivir in surface water and sewage works for River Lee catchment area and Western Europe were $\leq 98.1 \mu\text{g/L}$ and $\leq 348.0 \mu\text{g/L}$, respectively (Straub, 2009). The highest oseltamivir concentrations detected in Neya River water were well below $98.1 \mu\text{g/L}$. Moreover, the predicted ineffective concentration for algae, daphnia and fish was $\geq 1.0 \text{ mg/L}$ and the concentrations in Neya River were about 1000-fold smaller than this value. On the basis of this information, it may be safely mentioned that the detected OP and OC concentrations in Neya River during the pandemic influenza of 2009/2010 do not pose serious threats to the water environment. However, very little is known about the consequences to date, and hence, further investigation is essential to clarify the matter.

CONCLUSIONS

Oseltamivir in phosphate form was detected for the first time in river water during

seasonal influenza of 2009/2010. It appeared that the presence of oseltamivir phosphate in surface waters is significant only in seasonal influenza pandemic cases. The highest oseltamivir carboxylate concentration in Neya River during the 2009/2010 influenza season (864.8 ng/L) was about 15-fold larger than the highest concentration detected in Yodo River during 2007/2008 influenza season, and 3-fold larger than the highest concentration detected in a sewage treatment plant effluent in Kyoto during 2008/2009 influenza season. The largest oseltamivir concentrations at ST-2 were possibly the results of treatment plant inefficiency upstream and very small river water flow between ST-1 and ST-2. Though concerns have been raised on the presence of oseltamivir in significant concentrations in surface water bodies, the concentrations detected in Neya River may not be a threat to the environment based on the very limited information available to date on the environmental risk of the drug. However, more risk assessment studies are necessary to clarify the potential environmental risk of the drug in water.

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