Optimisation of the analysis of anti-influenza drugs in wastewater and surface water

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We optimised the simultaneous analysis of four analytes, namely three anti-influenza drugs (oseltamivir (OS), zanamivir (ZAN) and amantadine (AMN)) and one metabolite of OS (oseltamivir carboxylate (OC)), in sewage treatment plant (STP) influent and effluent by verifying the types and conditions of solid phase extraction (SPE) appropriate for LC-MS/MS analysis. In summary, these target analytes were extracted from aqueous samples (30–50 mL) by using strong cation-exchange SPE cartridges (500 mg adsorbent) under acidic conditions (pH 3–4). After washing of the cartridges with acidified water (pH 3.0, 3 mL) and methanol (3 mL), the analytes were eluted with a mixed solvent (2 mL) of 10% (v/v) triethylamine in a 1:1 (v/v) mixture of acetone and water. Application of this technique to the target compounds should yield a comprehensive understanding of the occurrence and fate of anti-influenza drugs in the water environment.

Keywords: LC-MS/MS; oseltamivir; pharmaceuticals; water environment; zanamivir

1. Introduction

Pharmaceutical and personal care products (PPCPs) have the potential to enter the water environment [1,2], and much attention is now being given to the risk assessment of their bioactive properties [3,4]. Among the environmental issues associated with this wide range of PPCPs, environmental problems evoked by the excretion of anti-influenza drugs – especially Tamiflu (oseltamivir, OS) and its active metabolite (oseltamivir carboxylate [OC], which is formed by the action of an esterase in the liver [5]) – during potential influenza pandemics are of public and scientific concern [6,7]. Therefore, much work has been focused on the processing effectiveness of sewage treatment plants (STPs) [8,9], the fate of the drugs in the water environment [10–13], and the potential hazards of transmission of new types of influenza virus from the water environment via aquatic birds [6,9,14,15].

In Japan, in addition to Tamiflu, Relenza (zanamivir, ZAN) and Amantadine (AMN) are used as anti-influenza drugs. Measurement of ZAN in the aquatic environment simultaneously with OS and OC is, however, difficult because of its highly polar structure [16], and only a few trials measuring AMN have been performed [8,12]. To analyse seven antiviral drug components present in poultry muscle, including the four described above, Berendsen et al. developed a column-switch liquid chromatography – mass spectrometry method by combining two different types of tandemly arranged solid-phase extraction (SPE) cartridges and separation using two
different types of analytical columns before introducing the sample into a mass spectrometer [17]. Separately, in the course of monitoring the time-dependent dynamics of influenza drugs in the water environment, we succeeded in detecting the clear convex dynamic profiles of OS, OC and ZAN, which were well synchronised with the numbers of influenza patients treated with the drugs in a seasonal influenza outbreak in 2011. We used a combination of single SPE cartridge pretreatment and liquid chromatography with tandem mass spectrometry (LC-MS/MS) [18]. A fixed level of AMN was, in contrast, detected year-round [18]. At that time we used a strong-cation SPE cartridge for pretreatment, without any steps for its selection. To make the simultaneous estimation method more reliable, further verification of the simultaneous measurement system seems necessary.

The objective of this study was therefore to establish a sensitive and robust method for quantifying the four analytes, namely the three anti-influenza drugs (OS, ZAN and AMN) and one metabolite of OS (OC), by thoroughly optimising the properties of the pretreatment cartridges and treatment conditions used in a combination of SPE and LC-MS/MS. We used the method to analyse a comprehensive range of environmental samples (e.g. river water and STP influent and effluent) for the target compounds.

2. Experimental

2.1 Chemicals and reagents

Oseltamivir (purity 98%) (OS; C_{16}H_{28}N_{2}O_{4}, [M + H]^+ 312.40) and oseltamivir carboxylate (purity 99%) (OC; C_{14}H_{24}N_{2}O_{4}, [M + H]^+ 284.35) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Zanamivir (purity 98%) (ZAN; C_{12}H_{20}N_{4}O_{7}, [M + H]^+ 332.31) was provided by GlaxoSmithKline (Stevenage, UK) and Santa Cruz Biotechnology, Inc. (California, USA). Amantadine (purity 99%) (AMN; C_{10}H_{17}N, [M + H]^+ 151.24) was purchased from LKT Laboratories (St. Paul, MN, USA). The chemical structures and physico-chemical properties of the anti-influenza drugs examined in this study are shown in Table S1.

LC-MS-grade solvents (methanol, acetone and acetonitrile), formic acid, hydrochloric acid, ascorbic acid, ammonia solution (25%) and triethylamine were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Individual standard stock solutions of OS, OC, ZAN and AMN at 1 mg 100 mL^{-1} were prepared in methanol and stored at −30°C.

2.2 Sampling

A total of 8 river water samples from 8 sites and 10 sewage samples (5 influent and 5 effluent) from 5 STPs were collected from the Yodo River basin in the Kansai area of Japan during the influenza season (January to February) in 2011. Conventional activated sludge processes and chlorination disinfection were used in all STPs. One-litre samples were collected in 1-L glass bottles containing ascorbic acid (1 g) as a preservative and kept in a refrigerator in darkness.

2.3 Evaluation of SPE cartridges

First, on the basis of the recovery rates obtained by using the standard stock solution, we optimised the combination of SPE cartridge and elution solvent for simultaneous analysis of the analytes. We evaluated 15 SPE cartridges. In the cartridges we tested two hydrophilic-lipophilic-balanced sorbents, Oasis HLB (200 mg; carrier: divinylbenzene-N-vinylpyrrolidone copolymer, Waters, Milford, MA, USA) and ISOLUTE ENV+ (500 mg; carrier: hydroxylated polystyrene-divinylbenzene copolymer, Biotage, Uppsala, Sweden); one anion exchange
adsorbent, Bond Elut SAX (200 mg, carrier: silica-based trimethylaminopropyl, Agilent Technologies); and 12 cation exchange adsorbents, Oasis MCX (150 mg; carrier: the same as that of the HLB but with added benzenesulfonic acid, Waters), Bond Elute SCX (100 mg, 500 mg and 1000 mg; carrier: silica-based benzenesulfonic acid, Agilent Technologies, Santa Clara, CA, USA), Bond Elute PRS (200 mg; carrier: silica-based propylsulfonic acid, Agilent Technologies), and Bond Elute Plexa SCX (200 mg; carrier: styrene-divinylbenzene copolymer-based benzenesulfonic acid, Agilent Technologies); Discovery DSC SCX and Supelclean LC SCX (both in 500 mg; carrier: silica-based benzenesulfonic acid, SUPELCO, St. Louis, MO, USA), Strata SCX (500 mg; carrier: silica-based benzenesulfonic acid, Phenomenex, Madrid Avenue Torrance, CA, USA), Strata X-C (500 mg; carrier: styrene-divinylbenzene copolymer-based benzenesulfonic acid, Phenomenex, Madrid Avenue Torrance, CA, USA), ISOLUTE SCX (500 mg; carrier: silica-based benzenesulfonic acid, Biotage, Uppsala, Sweden), InertSep LSC SCX2 (500 mg; carrier: silica-based propylbenzenesulfonic acid, GL SCIENCE, Tokyo, Japan), HyperSep SCX (500 mg; carrier: silica-based benzenesulfonic acid, Thermo Fisher Scientific, 81 Wyman Street Waltham, MA, USA), and HyperSep Retain CX (500 mg; carrier: styrene-divinylbenzene copolymer-based benzenesulfonic acid, Thermo Fisher Scientific, 81 Wyman Street Waltham, MA, USA). To condition the SPE cartridges containing ≤200 mg adsorbent, the cartridges were washed with 2 mL each of methanol and acidified water (MilliQ water pre-adjusted to pH 3 with 1 N HCl), in that order. Twice the volume was used to wash the cartridges containing 500 mg adsorbent before use. Enrichment of analytes onto the SPE cartridges was performed with a concentrator (Sep-Pak Plus, Waters).

From the standard stock solution, we made up a 2-mL solution containing 20 ng each of OS, OC, ZAN and AMN in 1 g L\(^{-1}\) ascorbic acid solution. The solution was applied to each conditioned cartridge and the materials adsorbed onto the cartridges were eluted with six kinds of solvent: (A) methanol containing 2% ammonia; (B) 10% triethylamine in a 1:1 (v/v) mixture of acetone and water; (C) 10% triethylamine in a 1:1 (v/v) mixture of methanol and water; (D) 10% ammonia in a 1:1 (v/v) mixture of acetone and water; (E) 10% ammonia in a 1:1 (v/v) mixture of methanol and water; and (F) methanol containing 2% acetic acid.

Each eluted solution was mildly concentrated under a gentle stream of nitrogen gas at 37–40°C. The residue was re-dissolved in 200 μL of a 4:1 (v/v) mixture of aqueous 0.1% formic acid solution and methanol, and 10 μL of this solution was subjected to LC-MS/MS analysis.

We then examined the effects of the following factors on the recovery rates of OS, OC, ZAN and AMN: pH of the elution solvent (pH 1–4); application flow rate of samples (5–20 mL min\(^{-1}\)); amount of adsorbent in the SPE cartridge (100–1000 mg); sample volume applied to the SPE cartridge (30–400 mL); volume of the elution solvent (2–8 mL); and procedure used to condition the SPE.

### 2.4 Analysis of anti-influenza drugs in river water and in STP influent and effluent

Water samples were analysed by using a combination of strong-cation solid-phase extraction cartridge (Bond Elut SCX, 500 mg; Agilent Technologies, Santa Clara, CA) and liquid chromatography – tandem mass spectrometry (LC-MS/MS) after the filtration of 50-mL portions through a glass fibre filter (GF/B, 1-μm pore size, Whatman, Maidstone, UK). Only filtered water samples were used for the analysis, because the adsorption of these drugs on particulate matter in sewage and river water is negligibly small [9,18,19]. The concentrations of OS, OC, ZAN and AMN were determined by subtracting the blank data from the data given by the addition of each spiked compound to account for matrix effects [1,20,21] and loss during sample extraction [1,21]. Previously 50 ngL\(^{-1}\) was used as each spiked sample [1,20,21], but 20 ngL\(^{-1}\) was used throughout this study to fit for the present samples.
2.5 **LC-MS/MS conditions**

A binary pump system (Waters Acquity Ultra Performance LC, UPLC) coupled to a Quattro Micro API MS (Waters Corp., Milford, MA, USA) equipped with an electrospray ionisation source and interface was used for estimation of OS, OC, ZAN and AMN. Instrument control, data acquisition and quantification were performed with Mass Lynx 4.1 software (Waters). A column of UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm, Waters) was used at 60 °C under a gradient elution program with a mixed solvent system of 0.1% formic acid (v/v) in water (A) and acetonitrile (B) at a flow rate of 0.35 μL/min under a program of 0.00–4.00 min (5% B), 4.00–4.30 min (25% B), 4.30–5.80 min (80% B), 5.80–6.00 min (80% B) and 6.00–8.00 min (5% B) to condition the column. The MS system was operated in positive ion mode, and product ions were generated with collision energies of 10, 10, 10 and 14 eV for OS, OC, ZAN and AMN, respectively. Under these conditions the elution times of OS, OC, ZAN and AMN were 4.92, 3.30, 0.67 and 3.00 min, respectively. Although unspecified matrix effects from compounds that show little retention on chromatographic columns remained and the retention time of ZAN was close to the void volume of the column, no clearly detectable peak appeared at this elution time without external addition of ZAN, and this peak assignable to ZAN by the MS/MS analysis was exclusively detected in the sampled environmental waters. This is the basis of our use of a single SPE/LC-MS/MS system to evaluate ZAN simultaneously with OS, OC and AMN.

2.6 **Method validation**

To quantify OS, OC, ZAN and AMN, seven-point calibration was applied in a concentration range from 0.5 to 500 ng mL⁻¹ in a 4:1 (v/v) mixture of 0.1% formic acid solution : methanol. Linear calibration curves given for these anti-influenza drugs all had \( r^2 > 0.999 \). Quantification was then performed by using each regression curve. The standard deviation (σ) of the lowest concentration was calculated from five independent standard curves (n = 5; coefficient of variation was below 5%) and used to calculate the limit of detection (LOD, 3 σ) and the limit of quantification (LOQ, 10 σ) [6,21] at signal to noise (S/N) ratios of 3 and 10, respectively, according to the methods used for PPCPs [1] and anti-influenza drugs [6]. The data at S/N < 3 and 3 ≤ S/N < 10 were expressed as not detected (N.D.) and not quantified (N.Q.), respectively. The LOD and LOQ values of OS, OC, ZAN and AMN were 0.1, 0.2, 0.4 and 0.1 ng L⁻¹, and 0.2, 0.7, 1.3 and 0.2 ng L⁻¹, respectively. The relative standard deviations (reproducibility values) for OS, OC, ZAN and AMN, assessed by injecting MilliQ water spiked at 20 ng/L (n = 3), were 2.3%, 4.0%, 1.8% and 0.8%, respectively.

Reproducibility was also assessed in the ranges of 1.4–8.0% (OS), 1.4–2.0% (OC), 5.4–8.2% (ZAN) and 1.0–1.3% (AMN), respectively by analysis of river water or STP influent or effluent spiked at 20 ng L⁻¹ (n = 3). The main method characteristics are summarised in Table 1.

Table 1. Validation of the method characteristics for analysis of anti-influenza drugs.

|               | Calibration range (ng L⁻¹) | Correlation coefficient (\( r^2 \)) | LOD (ng L⁻¹) | LOQ (ng L⁻¹) | Reproducibility* (%) (n = 3) |
|---------------|----------------------------|-----------------------------------|--------------|--------------|-----------------------------|
| OS            | 0.5–500                    | 0.999                             | 0.1          | 0.2          | MilliQ water: 2.3, River water: 4.0, STP effluent: 8.0, STP influent: 4.9 |
| OC            | 0.5–500                    | 0.999                             | 0.2          | 0.7          | MilliQ water: 4.0, River water: 1.4, STP effluent: 1.9, STP influent: 2.0 |
| ZAN           | 0.5–500                    | 0.999                             | 0.4          | 1.3          | MilliQ water: 1.8, River water: 8.1, STP effluent: 5.4, STP influent: 8.2 |
| AMN           | 0.5–500                    | 0.999                             | 0.1          | 0.2          | MilliQ water: 0.8, River water: 1.0, STP effluent: 1.3, STP influent: 1.0 |

* Spiked level 20 ng L⁻¹
3. Results and discussion

3.1 Optimisation of solid-phase extraction cartridge

To develop a suitable and practical solution for recovering OS, ZAN and AMN, we first performed preliminary experiments using a hydrophilic-lipophilic-balanced cartridge previously employed for adsorption of OC in aquatic environments at pH 3.2–3.4 \cite{6}. However, there was irreversible adsorption of ZAN onto the cartridge and the compound could not be recovered by using several types or combinations of solvents (Table S2). This may have been attributable to the highly polar nature of ZAN, as indicated by Lindegardh et al. \cite{16}.

We then attempted to select an SPE cartridge to develop a new method for simultaneous quantification of the four target analytes (OS, OC, ZAN and AMN). Fifteen SPE cartridges including two hydrophilic–lipophilic-balanced sorbents, one anion exchange adsorbent, and 12 cation exchange adsorbents were evaluated in terms of recovery of analytes. A strong acid-type cartridge, Bond Elut SCX, previously applied without specifications \cite{18}, was included as one of the choices. The data listed in Table S2 show the recovery rates of all drugs; we used these data to select a combination of Bond Elut SCX with elution solvent B (10% triethylamine in a 1:1 (v/v) mixture of acetone : water) as the best choice.

Comparison of the results with the values shown in Figure 1 revealed differences among OS, OC and AMN of 1–2%; these were reasonably within the relative standard deviations (reproducibility values) for OS (4.0%), OC (2.3%), ZAN (1.8%) and AMN (0.8%), as assessed by injecting MilliQ water spiked at 20 ng L$^{-1}$ ($n = 3$) (see 2.6 Method validation). In the case of ZAN the recovery rate shown in Table S2 appeared somewhat higher. Although the exact reason is not known, this difference may have been derived from a difference in the pH adjustment procedures: ascorbic acid was used to obtain the data in Table S2, whereas HCl was used for artificial pH adjustment for the data in Figure 1. Additional elution with different solvents such as methanol or methanol containing 2% ammonia did not improve recovery rates (data not shown). AMN was recovered at high rates (67–68%) by using the combinations of Bond Elut SCX with elution solvent B and Bond Elut PRS with elution solvent C (10% triethylamine in a 1:1 (v/v) mixture of methanol and water), the carrier in both SPEs being silica-based sulfonic acid (see Section 2.3). AMN also showed a high affinity for hydrophilic-lipophilic-balanced SPE cartridges such as Oasis HLB. The recovery rate of ZAN, however, was higher than 50% only when a combination of Bond Elut SCX and elution solvent B was used. Although ZAN is

![Figure 1](image-url). Effects of pH of sample water (50 mL of MilliQ-based water) on recovery rates of target compounds.
highly hydrophilic and is zwitterionic because of its guanidyl and carboxyl residues, ionic interactions owing to the presence of the guanidyl group seem to be important in increasing the recovery rate. Elution solvent B has been used to recover ZAN from human plasma [22]. Like ZAN, OC is zwitterionic, but it was hydrophobic enough to interact with the hydrophobic carriers in the many cartridges we tested (Table S2). In the case of OS, both ionic and hydrophobic interactions occurred with the SPE cartridges, giving a wide spectrum of applicability to the cartridges. These results indicated that a combination of the strongly acidic Bond Elut SCX and elution solvent B was the best for simultaneous analysis of the four target analytes.

Previously, a combination of Strata X-C SPE cartridge (100 mg, solid phase) and 0.75% ammonia in methanol (pH 12), as an eluent was used for a similar purpose by Berendsen et al. [17]. Although the exact recovery rates were not shown in their report, the values obtained with our present combination using Bond Elut SCX (500 mg, solid phase) and 10% triethylamine in a 1:1 (v/v) mixture of acetone : water as an eluent seemed substantially superior to their combination. The recovery data given by using a similar SPE cartridge, Strata X-C (500 mg, solid phase), are not worthy of special mention. These discrepancies may be attributable to differences in the pretreatment conditions and in the analytical method used.

3.2 Optimisation of speed of sample loading onto the solid-phase extraction cartridge, sample preparation, and elution from the cartridge

3.2.1 Effect of pH of the applied solution on recovery rate

Because of the strong cation exchange nature of Bond Elut SCX, for trapping purposes the pH of the MilliQ-based water had to be lower than the $pK_a$ (3.8, see 2.1) of all analytes. The effect of pH variation (1–4) of the MilliQ-based water (50 mL) on the adsorption and recovery of OS, OC, ZAN and AMN on and from the cartridge was evaluated. There was instability of ZAN recovery (~5%) at low pH (pH 1–2), but relatively high rates of recovery of all analytes at pH 3–4 (Figure 1).

3.2.2 Effect of speed of loading of sample onto cartridge on recovery

Optimisation of the speed at which the samples were loaded onto the Bond Elut SCX cartridge was then performed by using a concentrator. The recovery rates of ZAN and AMN showed a slight tendency to decrease with increasing rate of flow (5–20 mL min$^{-1}$) of the 50 mL of MilliQ sample water containing 1 g L$^{-1}$ of ascorbic acid and with the pH adjusted to 3 with 1 N HCl through the SPE cartridge (Figure S1). From the results, we chose a flow rate of 5–10 mL min$^{-1}$ for practical analysis.

3.2.3 Effect of adsorbent volume in the cartridge and elution solvent volume on recovery rate

To apply the new method to environmental samples, we needed to optimise two other factors, i.e. the capacity of the adsorbent in the SPE cartridge and the application volume. We first examined the capacity of the adsorbent (added in the currently available amounts of 100 mg, 500 mg, or 1000 mg) in the Bond Elut SCX cartridge by applying secondary effluent (50 mL) from an STP. The recovery rate of ZAN was improved efficiently from 0.2% with 100 mg of adsorbent to 19% and 24% with 500 mg and 1000 mg of adsorbent, respectively (Figure 2). In contrast, the recovery rates of OS, OC and AMN tended to decrease with increasing adsorbent volume in the cartridge. Because the exact reason for the reproducible decrease in recovery with
increasing amounts is not known, to limit solvent consumption and the time needed for evaporation we selected cartridges containing 500 mg of adsorbent for practical analysis of the water samples. We then analysed the effects of the volume of eluting solvent (2–8 mL) on the recovery rates from Bond Elut SCX cartridges containing 500 or 1000 mg of adsorbent. There were no significant differences in recovery rates with the use of different volumes. Furthermore, we checked for breakthrough of analytes from the cartridge by connecting a new 500-mg cartridge in a tandem way. The results clearly showed that breakthrough was negligible (data not shown).

3.2.4 Effect of sample volume on recovery
We examined the effects of the volume of influent water applied (10–50 mL) on the recovery rate from the Bond Elut SCX cartridge with 500 mg of adsorbent. Only the recovery rates of OS and ZAN decreased with increasing sample water volume (Figure S2). The recovery rate was not improved by dilution of the sample water several fold with MilliQ-based water (data not shown). To accurately analyse the drug contents of the environmental water samples by LC-MS/MS, we chose the combination of a 30-mL sample volume and 500 mg of adsorbent. When a treated water sample (50–400 mL) collected at an STP was applied, a similar decrease in recovery rate was observed with increasing application volume of the samples. In light of these results, we set the volume of effluent or river water applied to the 500-mg cartridge at 50 mL.

3.2.5 Effects of clean-up treatment on recovery
To improve the recovery rates, we tried cleaning up the cartridge after the application of water samples and before elution of the analytes from the cartridge. Three millilitres of the acidified MilliQ water (pH 3.0) and methanol were used in this order as clean-up solvents. This clean-up treatment eliminated further the matrix effects, resulting in improvement of the recovery rates of OS, OC, ZAN and AMN. Basically, the recovery rates of OS, OC, ZAN and AMN obtained without the clean-up treatment were 38%, 55% 18% and 38% for river water, 35%, 56%, 21% and 35% for STP effluent, and 14%, 46%, 6% and 7% for influent, respectively. On the basis of these values, the percentages increments after clean-up treatment were estimated as 24%, 11%,

![Figure 2. Effects of amount of adsorbent added to the Bond Elut SCX cartridge on the recovery rates of target compounds in 50 mL of secondary effluent.](image)
21% and 38% (river water), 29%, 11%, 16% and 35% (STP effluent), and 22%, 17%, 17% and 36% (STP influent), respectively.

3.2.6 Determination of recovery rates from environmental samples

The absolute recovery rates of the four target analytes from STP influent and effluent and from river water were examined. The results indicated that the method could be used for simultaneous analysis of all analytes in these waters (Figure 3), although the recovery rates varied among the drugs. For OS and ZAN we observed a significant difference at $P < 0.01$ between influent and effluent waters in addition to river waters. In addition, a significant difference at $P < 0.05$ was detected in the cases of OS and ZAN between the same sets of waters. The decreases in recovery rates of OS, OC, ZAN and AMN in the influent samples may be ascribed to the presence of contaminating matrix materials such as organic matter, as previously reported for anti-influenza drugs [20], PPCPs [1] and endogenous oestrogen [21].

In a previous simultaneous multiple antiviral-drug analysis, the respective recovery rates of OS and OC were reported to be about 70% and 40% for river water, 40% and 30% for STP effluent, and 20% and 10% for influent [20]. The corresponding values in our study were superior to the reported values for OS and OC by 24% and 37%, respectively, for STP effluent and 16% and 53% for influent; for OC they were superior by 26% for river water. They were inferior only in the case of OS (~8% for river water).

The rates of recovery of ZAN from influent, effluent and river waters ranged from 24–39%. This range is higher than that from human serum (25–30%) [22]. The recovery rates of OS, OC, ZAN and AMN (with coefficients of variation of repeatability in parentheses) were 61.6% (1.4), 66.2% (1.4), 39.2% (8.1) and 75.4% (1.0) in river water, 64.4% (8.0), 66.5% (1.9), 36.2% (5.4) and 69.9% (1.3) in STP effluent, and 36.4% (4.9), 62.7% (2.0), 23.3% (8.2) and 62.9% (1.0) in

![Figure 3. Absolute recovery rates of the four anti-influenza drugs from environmental waters (means ± SD, n = 8 (river water), n = 5 (STP effluent and influent)). Significant differences (P < 0.05) among each set of the environmental waters for each anti-influenza drug were indicated by different superscript letters. Asterisk shows significant difference (P < 0.01) between influent and effluent waters in addition to river waters.](image-url)
influent, respectively. Further matrix effects for OS, OC, ZAN and AMN were estimated as 7.1%, 10.8%, 12.6% and 0% in river water, 4.3%, 10.5%, 15.6% and 3.7% in STP effluent, and 32.3%, 14.3%, 28.5% and 10.7% in influent, respectively. These results indicate that our method is sufficiently accurate for the simultaneous analysis of OS, OC, ZAN and AMN present in the water environment.

3.3 Simultaneous analysis of the four analytes in sewage and river waters in the influenza season

We applied the new method for simultaneous analysis of the four target analytes in eight river water samples, five STP influent samples, and five effluent samples, all from the Yodo River basin. We optimised the LC-MS/MS analysis by using a 500-mg Bond Elut SCX cartridge; a 30–50-mL sample volume adjusted to pH 3–4; 3 mL each of acidified MilliQ water (pH 3) and methanol as clean-up solvents; and 2 mL of 10% triethylamine in a 1:1 (v/v) mixture of acetone : water as an elution solvent.

Water samples were collected in the influenza season (on 19 January 2011). OS, OC, ZAN and AMN were detected at concentrations up to 704.3 ng L\(^{-1}\) (Table 2), after correction of the original data as described in Section 2.4. The exact values of OS, OC, ZAN and AMN varied in the order of 1.7–80.7 ng L\(^{-1}\), 8.4–704.3 ng L\(^{-1}\), N.D.–23.1 ng L\(^{-1}\) and 7.8–258.4 ng L\(^{-1}\), respectively. The results supported the previous finding that OC could not be removed efficiently at STPs by using the conventional activated sludge process followed by the chlorination disinfection process. In addition, our results strongly indicated that OS and ZAN were not removed at conventional STPs and were being discharged into the river system. In future, this simultaneous analysis method may be useful as a basis for estimating the environmental effects of discharged anti-influenza drugs.

| Water sample | STP influent (n = 5, ng L\(^{-1}\)) | STP effluent (n = 5, ng L\(^{-1}\)) | River water (n = 8, ng L\(^{-1}\)) |
|--------------|----------------------------------|----------------------------------|-----------------------------------|
|              | Average | Min | Max | Average | Min | Max | Average | Min | Max |
| OS           | 51.5    | 35.2| 72.4| 59.9    | 36.7| 80.7| 7.3      | 1.7  | 26.2 |
| OC           | 336.8   | 186.7| 704.3|374.1    | 208.0| 568.8|35.5      | 8.4  | 128.6|
| ZAN          | 11.9    | N.D. | 23.1| 2.5     | N.D.| 12.7| 1.5      | N.D. | 6.3  |
| AMN          | 131.4   | 105.1| 160.9|152.5    | 89.1| 258.4|28.2      | 7.8  | 97.6 |

4. Conclusions

We developed a new method for simultaneous analysis of four analytes (three anti-influenza drugs (OS, ZAN and AMN) and one metabolite of OS (OC)) in river water and in STP influent and effluent by optimising the types and conditions of SPE for LC-MS/MS analysis.

Key factors in the analysis were the type and volume of SPE adsorbent, sample pH, volume of sample applied to the SPE cartridge (including flow rate), and elution and clean-up solvents used with the SPE. This method could offer new opportunities for scientists to gain a comprehensive understanding of the occurrence and fate of anti-influenza drugs in the water environment.
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Supplemental material
Supplemental material relating to this article is available online.

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