Occurrence of levofloxacin, clarithromycin and azithromycin in wastewater treatment plant in Japan

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Abstract Residual pharmaceutical products in sewage and other water environments have recently become a serious social problem in advanced countries. Among these pharmaceutical products, antibiotics have attracted special attention due to their serious impact on the ecosystem and connections to the emergence of drug-resistant bacteria. Our research intended to develop a new method to analyse the three antibiotics estimated to be released out of the body in large amounts in Japan; levofloxacin (LVFX), clarithromycin (CAM) and azithromycin (AZM), and survey the state of pollution in the sewerage. The concentrations of the water-phase antibiotics LVFX, CAM and AZM were measured in each process of activated sludge process in six wastewater treatment plants. Liquid chromatography tandem mass spectrometry (LC/MS/MS) was used to analyse solutions of the antibiotics after pretreatment with a solid phase extraction. The limits of quantification and the average recoveries for these antibiotics in the influent were 1.2 to 29 ng/L and 46 to 93%, respectively. In the influent, LVFX, CAM and AZM were detected at concentrations of 552, 647 and 260 ng/L, respectively, while their removal efficiencies were 42, 43 and 49%, respectively. Although the CAM and AZM concentrations decreased as the treatment progressed, it was shown that the LVFX concentration increased in activated sludge reactors in some cases. Despite differences in octanol-water partition coefficients among LVFX, CAM and AZM, their removal efficiency showed no major difference. This indicates that this removal phenomenon cannot be explained by simple adsorption by the activated sludge.

Keywords Analytical method; azithromycin; clarithromycin; levofloxacin; wastewater treatment plants

Introduction

Antibiotic substances in sewage and in other water environments have become a serious social problem in many advanced countries. It has been reported that antipyretics, painkillers, and other antibiotic substances for human and veterinary use are detected in the discharge from sewers (Hirsch *et al.*, 1999; Golet *et al.*, 2001; Kolpin *et al.*, 2002; Mcardell *et al.*, 2003) and in river water (Hirsch *et al.*, 1999; Kolpin *et al.*, 2002; Calamari *et al.*, 2003) on the order of ng/l to μg/l. If these substances are insufficiently removed in the sewerage and discharged into water environments, they can impact the aquatic life living in these environments in a variety of ways (Yasojima *et al.*, 2004). If residual pharmaceuticals eventually circulate into drinking water, humans will be exposed to these drugs at very low concentrations. Antibiotic substances, among these pharmaceutical products, particularly need attention as, in addition to the problem described above, they are suspected to be linked to the emergence of drug-resistant bacteria (Seino *et al.*, 2004). Japan is known to use a very large amount of antibiotics compared with other countries in the world. Although investigations and research on antibiotics in water environments and sewers are actively conducted in some European countries and in the United States, almost no information on the status of antibiotics

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in Japanese water environments or sewer systems is available. It is important to grasp the status of antibiotics in such environments in Japan considering the large consumption of antibiotics and the different uses than in other countries. Considering these points, the authors chose three antibiotics estimated by our calculations to be extensively released from humans in Japan, namely levofloxacin (LVFX, 14–16t/y), clarithromycin (CAM, 5.4–11 t/y) and azithromycin (AZM, 0.3–0.9 t/y), and aimed at developing a method to analyze these drugs in terms of their presence in wastewater in sewage. In addition, the presence of these antibiotics was observed in each process involved in one of the typical treatment methods in wastewater treatment plants in Japan, the conventional activated sludge process.

Materials and methods

Analytical methods for levofloxacin, clarithromycin and azithromycin

EDTA was added to sample water to a volumetric ratio of 1 g per litre. 100 mL of the sample water was filtered using a glass fibre filter (Whatman GF/B, 1 μm pore size), and then analysed. The subject material was maintained by flowing the sample water at a flow rate of 10 mL per minute to Oasis HLB (Waters, filling capacity of 200 mg) preconditioned with 3 mL of methanol and 3 mL of purified water. The Oasis HLB was then aspirated and dehydrated, and the subject material was eluted with 6 mL of methanol. The eluate was concentrated and dry-solidified using a gentle nitrogen stream, and the solid was then redissolved into a 1 mL solution made up of a 0.5% formic acid solution and acetonitrile mixed at a ratio of 3 to 7 to prepare samples for measurement. For LVFX, the sample for measurement was prepared by adding 180 μL of a 1% formic acid solution to 20 μL of the redissolved eluate.

Qualification and quantification of these three materials were performed using an LC/MS/MS method. Agilent 1100 (Agilent) and AP14000 (Applied Biosystems) instruments were used for the HPLC and MS/MS, respectively. The columns used for analysis were XDB-C18 (Agilent, 2.1 × 150 mm) for LVFX and LUNA C8 (Phenomenex, 2.0 × 150 mm) for CAM and AZM. The column temperature was 40 °C. The injection volume was 5 μL, and the ESI-positive mode was set for ionisation. Measurement conditions are shown in Table 1 for LVFX and in Table 2 for CAM and AZM.

Description of wastewater treatment plants

The wastewater sampled in our research was from six wastewater treatment plants in Japan where a typical treatment system, the conventional activated sludge process, is used. Table 3 outlines these plants. At each of these plants, water was sampled from each process every 2 h at the sampling points shown in Figure 1, except that no water was sampled from the reactors in plants C, D, E and F. These 24-h flow-proportionate composite samples prepared from water samples taken every 2 h were used for the measurements. The water-sampling period was from July to October 2004.

Table 1 HPLC solvent gradient for the measurement of LVFX

Time (min)	Solvent A (%)	Solvent B (%)
0	2	98
15	70	30
15.1	100	0
18	100	0
18.1	2	98
28	2	98

Solvent A: acetonitrile with 0.02 M formic acid. Solvent B: Milli-Q water with 0.02 M formic acid

Table 2 HPLC solvent gradient for the separation of CAM and AZM

Time (min)	Solvent A (%)	Solvent B (%)
0	74	26
8	0	100
13	0	100
17	74	26
22	74	26

Solvent A: Acetic acid was added to 900 mL water containing 3.6 mmol/L NH_3 until a pH 5.7 was obtained. 100 mL of acetonitrile was added to this. Solvent B: 200 mL solvent A + 800 mL acetonitrile

Results and discussion

Optimization of the LC/MS/MS condition

The ions to be measured in the LC/MS/MS experiments were determined by observing a standard solution of LVFX, CAM and AZM. From this observation, the monitor ions were selected for LVFX, CAM and AZM with the $[M + H]^+$ parent ions m/z = 362.1, 748.5, and 749.6, respectively, and product ions m/z = 318.2 ($[M + H{-}CO_2]^+$), 158.2 ($[\text{desosamine} + H]^+$), and 591.5 ($[M + H{-}\text{desosamine}]^+$), respectively. The dynamic range for these antibiotics was 0.5 to 50 μg/L for LVFX, and 1 to 100 μg/L for CAM and AZM ($R^2 > 0.998$).

Recoveries and precision

The limits of detection (LOD) and the limits of quantification (LOQ) for LVFX, CAM and AZM in the influent and secondary effluent were calculated to be three and 10 times the signal-to-noise (S/N) ratio of the chromatograms of the said influent and effluent. The LOD in the influent was between 0.37 and 8.8 ng/L, while the LOQ was between 1.2 and 29 ng/L (Table 4). The LOD in the secondary effluent was between 0.24 and 4.9 ng/L, while the LOQ was between 0.79 and 16 ng/L (Table 4). The decrease in the antibiotics present in these solutions due to pretreatment was evaluated by a recovery test ($n = 5$) on the influent and secondary effluent. The average recovery for the influent and the secondary effluent was between 46 and 93% and 93 and 108%, respectively (Table 5). The fact that the recovery for CAM was lower in the influent is probably the result of some ionisation suppression due to the matrices contained in the sewer sample. The coefficient of variation (CV) of CAM in the recovery was a sufficiently low 9.3%, and the subject material appeared as an apparent single peak when the influent was measured. All of these results indicate that our developed analytical method is applicable to the analysis of sewer samples.

Occurrence of levofloxacin, clarithromycin and azithromycin in wastewater treatment plants

The levels of LVFX, CAM and AZM measured in the influent at the wastewater treatment plants investigated were in the range of 307 to 981, 492 to 883 and 199 to

Table 3 Outlines of six wastewater treatment plants (WWTPs)

WWTP	Service area (ha)	Population served	Capacity (m^3/day)	HRT (h)	SRT (day)
A	1,200	75,000	42,000	12	9
B	460	30,000	24,000	10	7
C	5,700	957,000	680,000	7	7
D	3,900	811,000	700,000	7	7
E	4,400	743,000	225,000	6	8
F	3,500	678,000	450,000	4	5

From Statistics of Sewerage in Japan, Public Administration Version, 2003

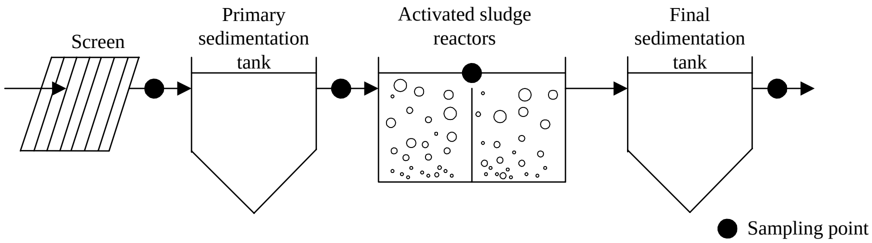


Figure 1 Sampling points in wastewater treatment plants

371 ng/L, respectively. The averages of the above ranges were 552, 647, and 260 ng/L, respectively. In the secondary effluent, the measurement ranges for LVFX, CAM and AZM were 189 to 400, 266 to 444, and 88 to 219 ng/L, respectively, with averages of 301, 359, and 138 ng/L, respectively (Figure 2). The removal efficiencies for these compounds (average $\pm\sigma$) were 42 ± 13, 43 ± 14 and 49 ± 7%, respectively. To the best of our knowledge, no previous study has measured AZM in the influent and secondary effluent. For LVFX and CAM, only a few studies have reported concentrations in the secondary effluent (Hirsch *et al.*, 1999; Giger *et al.*, 2003; Mcardell *et al.*, 2003; Yasojima *et al.*, 2004). Our results were compared with the past reported measurements of LVFX and two other quinolonic antibiotics (LVFX is a quinolonic antibiotic), ciprofloxacin (CPFX) (Golet *et al.*, 2001, 2003; Reverté *et al.*, 2003) and norfloxacin (NFLX), CAM, AZM (Yasojima *et al.*, 2004), erythromycin-H_2O (EM), and roxithromycin (RXM) (Golet *et al.*, 2001; Giger *et al.*, 2003; Mcardell *et al.*, 2003), which are macrolidic antibiotics, as shown in Table 6.

The LVFX concentration in the influent and primary effluent measured in our research was slightly higher than that in the other report with CPFX and NFLX in the influent. The LVFX concentration in the secondary effluent measured in our research was 3 to 4 times higher than the previously reported concentrations of CPFX and NFLX in secondary effluent. The CAM concentration in the secondary effluent in our research was higher than the measured CAM values reported outside of Japan and, with one exception, was also higher than those of EM and RXM. Our measurement of the AZM concentration was within the range of concentrations reported in the past. The removal efficiency for both CPFX and NFLX up to the secondary effluent was approximately 80%, whereas that for LVFX was 42%. These values, both ours and the reported ones, came from plants operating activated sludge process. Differences in the removal efficiency of LVFX and CPFX are present before and after reactors at these plants, presumably because of the differences in behaviour resulting from the physical properties of the antibiotics in the reactors. A positive correlation in the removal efficiency was observed between LVFX and AZM and SRT, as shown in Figure 3. This indicates that differences in the operating conditions regarding the activated sludge, as represented by SRT, may create these differences.

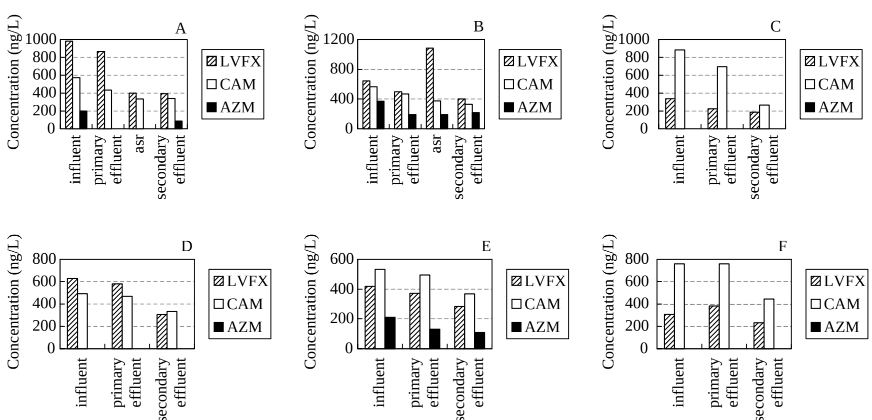
Table 4 LOD and LOQ for the determination of LVFX, CAM and AZM in influent and secondary effluent (ng/L)

	Influent		Effluent	
	LOD	**LOQ**	**LOD**	**LOQ**
LVFX	8.8	29	4.9	16
CAM	1.6	5.2	1.4	4.6
AZM	0.37	1.2	0.24	0.79

Table 5 Average recoveries and standard deviations (S) of LVFX, CAM and AZM in influent and secondary effluent ($n = 5$) (%)

	Influent		Effluent	
	Recovery	**S**	**Recovery**	**S**
LVFX	93	3.4	105	2.8
CAM	46	4.3	108	7.7
AZM	80	2.6	93	5.4

In our research, the concentrations of CAM and AZM decreased at every plant investigated with the progress of treatment. The concentration of LVFX also decreased at these plants with treatment as in the case of CAM and AZM, although LVFX concentrations almost doubled in the reactors at one plant. Although the cause of this rise is unknown, probable causes include influences of returned sludge, deconjugation of conjugates due to aeration, or desorption from sludge. Eighty-five percent of LVFX taken by a person is excreted out of the body as an unaltered substance in the urine (Pharmaceuticals and Medical Device Agency). Likewise, 30–50% of CAM and 9% of AZM leave the body as unaltered substances in the urine (P. M. D. Agency). Based on this information, since the excretion of LVFX conjugates is considerably smaller than the quantity of LVFX as an unaltered substance, deconjugation is believed to be less contributable for the concentration increase. It is necessary to further study the behaviour of antibiotics as well as the sludge used in these plants in order to clarify sorption and desorption between the water phase and the sludge. Considering the octanol-water partition coefficients, which are important factors in sorption to activated sludge, being 0.553, 7.18, and 4.02 for LVFX, CAM and AZM, respectively, the removal efficiency by sorption to activated sludge is predicted to be, in descending order, CAM, AZM and LVFX. Our research, however, indicated that the removal efficiencies for these substances were almost the same. Therefore, the removal of these antibiotics from the water phase was assumed to be dependent on other factors such as biodegradation. Although some studies have reported that biodegradation of CPFX at reactors was minor (Ahmad *et al.*, 1999; Golet *et al.*, 2003), no information on LVFX, CAM or AZM exists. No knowledge on the dependence of the octanol-water partition coefficient on pH is available either. It is thus necessary to acquire additional knowledge regarding these points in the future.



*asr; activated sludge reactors

Figure 2 Concentrations of LVFX, CAM and AZM in six WWTPs

Table 6 Comparison of this research and previous reports (ng/L)

Points	Quinolone			Macrolide			
	LVFX	CPFX	NFLX	CAM	AZM	EM	RXM
Influent	552*	427 ± 69[a] 580[b]	431 ± 45[a]	647*	260*	–	–
Primary Effluent	487*	331 ± 53[a] 249–405[c]	383 ± 61[a] 270–367[c]	559*	163*	–	–
Asr**	742*	–	–	709*	192*	–	–
Secondary Effluent	301* 152–323[g]	95 ± 15[a]	69 ± 15[a]	359* 240[d] 57–328[e] 57–328[f] 303–567[g]	138*	2,500[d] ND-199[e] ND-287[f]	680[d] ND-31[e] ND-72[f]
Tertiary Effluent	–	71 ± 11[a] 45–108[c]	51 ± 7[a] 48–120[c]	–	–	–	–

*This study (average)
**Activated sludge reactors
[a]Golet *et al.*, 2003; [b]Reverté *et al.*, 2003; [c]Golet *et al.*, 2001; [d]Hirsch *et al.*, 1999; [e]Mcardell *et al.*, 2003; [f]Giger *et al.*, 2003; [g]Yasojima *et al.*, 2004

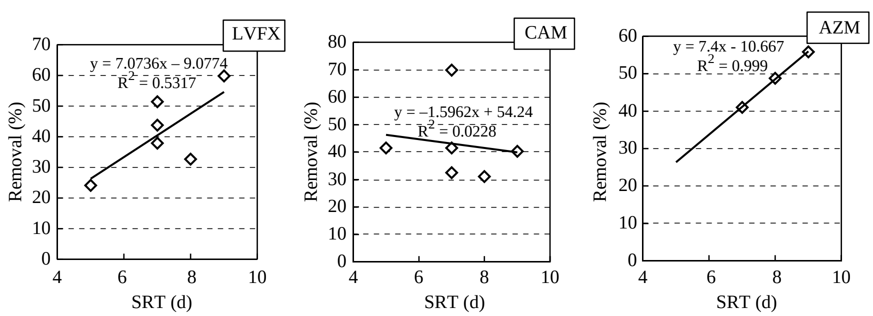


Figure 3 Correlation of SRT and removal efficiencies in six WWTPs

Conclusions

A method to analyse three antibiotics, LVFX, CAM and AZM, in wastewater treatment plants was developed, and the following results obtained:

(1) Analysis of selected antibiotics was performed by using LC/MS/MS. LOD values in the influent for LVFX, CAM and AZM were 38, 55 and 57 ng/L, respectively, with average recoveries of 93, 46 and 80%, respectively.

(2) The concentrations of LVFX, CAM and AZM in the influent and secondary effluent of wastewater treatment plants were 552, 647 and 260 ng/L, respectively, and 301, 359 and 138 ng/L, respectively. Their removal efficiencies were 42 ± 13, 43 ± 14 and 49 ± 7%, respectively.

(3) The LVFX and AZM concentrations in the secondary effluent were in the range of those previously reported. The CAM concentration in the secondary effluent was higher than those reported in other countries.

(4) Although the CAM and AZM concentrations decreased with treatment, that of LVFX increased in the reactors in some cases.

(5) The removal efficiencies of the three antibiotics from the water phase due to sorption as predicted from the octanol-water partition coefficients was in the descending order of CAM, AZM and LVFX. However, the experimental results indicated that the three antibiotics had almost the same removal efficiency. This indicated that other factors were involved in the removal of these substances.

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