

LC-MS/MS Analysis of Anti-Infectives In Raw and Treated Sewage

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Introduction

“Anti-infectives” is a general term that refers to several classes of biologically active compounds used to treat or prevent infections. Therapeutic agents such as antimicrobials (synthetic) and antibiotics (natural or semi-natural) are examples of anti-infectives.

The widespread utilization of anti-infectives in urban centers as well as their resistance to biodegradation or elimination in wastewater treatment plants (WWTPs) has led to their appearance in effluents and surface waters^[1-3]. In the last few years there has been a growing concern about the environmental fate and the possible effects of these agents on the aquatic environment^[4,5].

The first report on the occurrence of anti-infective traces in the aquatic environment was published as early as 1983^[6]. A later study^[7] acknowledged that pharmaceuticals would enter the water cycle mainly via a “domestic route” (*i.e.* by the excreta of individuals taking medication at homes, hospitals or clinics). It is therefore important to know the amounts of these substances released in the aquatic environment to be able to evaluate potential effects.

A sensitive and robust method was developed for the determination of some of the most prescribed anti-infectives in trace amounts (lower nanogram-per-liter range) in raw and treated wastewaters.

Goals

- Quantify several anti-infectives at the lower nanogram-per-liter level in raw and treated wastewaters.
- Apply two specific single reaction monitoring mode (SRM) transitions and their peak ratio to avoid the presence of false positives.

Method

Raw sewage (north and south influent) was collected and treated (effluent) 24-h composite samples at the municipal wastewater treatment plant of the City of Montréal (Québec, Canada). This plant has physico-chemical treatments only and its effluent is one of the largest in North America. We analyzed six of the most prescribed compounds (sulfamethoxazole, trimethoprim, ciprofloxacin, levofloxacin, clarithromycin and azithromycin) (Figure 1), by using solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The compounds were selected based on drugstore sales.

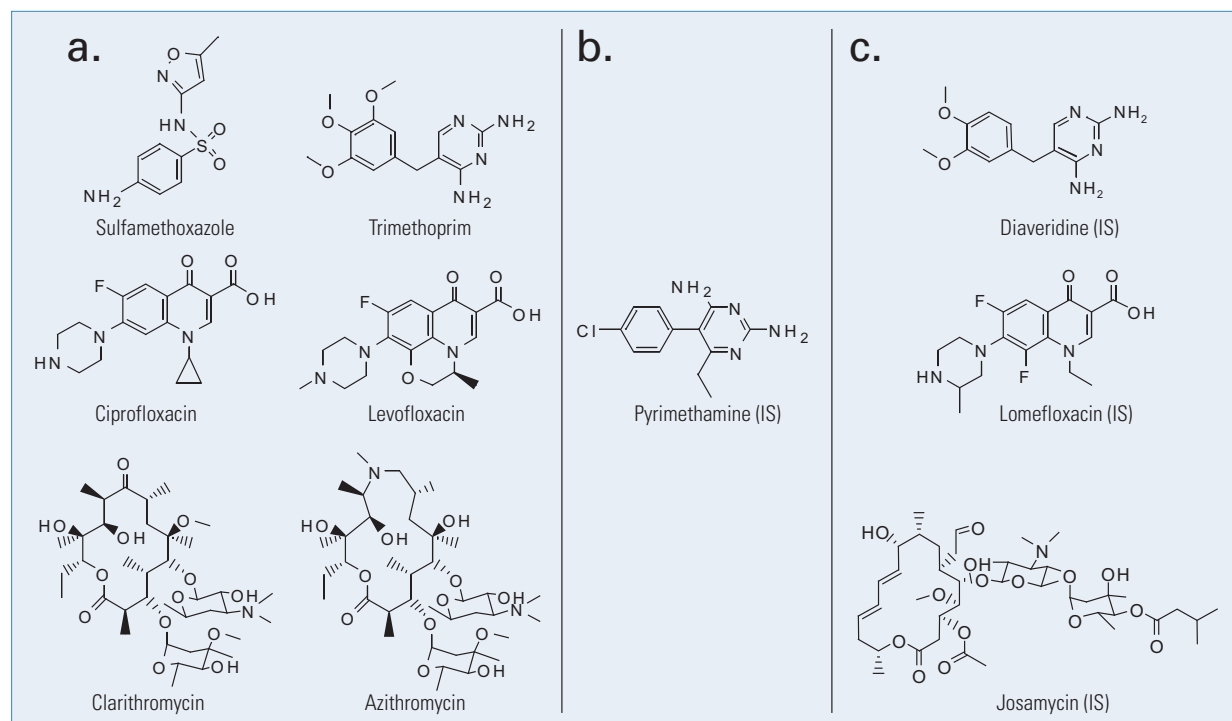


Figure 1: Molecular structures of the anti-infectives studied (a), the surrogate standard (b), and the internal standards (c).

Key Words

- TSQ Quantum Ultra™
- Surveyor HPLC™
- Antibiotics
- SPE

Sample Preparation

Wastewater samples were filtered using 1.2 µm pore-size fiber glass filters and then 0.45 µm pore-size mixed cellulose membranes. 50 mM of formic acid and 1 mL of a 5% Na₂EDTA (w/v) solution were added to 250 mL of wastewater and the pH adjusted to 3 with NaOH 1.0 M. Pyrimethamine was used as a surrogate standard and spiked at a concentration of 500 ng L⁻¹.

Analytes were pre-concentrated and extracted using a 200 mg reversed phase polymeric SPE cartridge on top of a 200 mg mixed mode polymeric SPE cartridge. Retained analytes were eluted from the cartridges using 2 × 2.5 mL ACN: MeOH 1:1 (reversed phase) and 2 × 2.5 mL 5% NH₃ in ACN: MeOH 1:1 (mixed mode). The eluates were recovered from both cartridges and were collected on the same conical-bottom centrifuge tube and then evaporated to dryness with N₂(g). Extracted analytes were reconstituted to 250 µL with 0.1% formic acid in 90% H₂O/5% MeOH/5% ACN solution containing the internal standards (diaveridine, lomefloxacin and josamycin).

LC-MS/MS Conditions

HPLC separation was done with a Thermo Scientific Surveyor HPLC system. Detection and quantification of the analytes was performed with a Thermo Scientific TSQ Quantum Ultra using the single reaction monitoring mode (SRM) (Table 1). Two specific single reaction monitoring (SRM) transitions were used for each compound as well as their peak area ratios to reliably confirm the presence of the targeted anti-infectives. This reduced the possibility of false positives given that some interfering matrix components are co-extracted with the analytes and could have the same SRM transition.^[8]

Results and Discussion

MS/MS in the SRM mode proved to be highly selective. Instrument response was linear ($r^2 \geq 0.99$) in the dynamic range (25–1000 ng L⁻¹) in spite of the presence of high concentrations of organic as well as inorganic interferences in the matrix. Limits of detection ranged from

Table 1: Instrument Parameters

| HPLC | | MS | |
|--------------------|--|------------------------------------|-----------|
| Column | Thermo Scientific BetaBasic™ C18 (50 × 2.1 mm, 3 µm) | Ionization mode | ESI+ |
| Column temperature | 30°C | Spray voltage | 3500 V |
| Mobile phase A | 0.1 % formic acid/H ₂ O | Ion transfer capillary temperature | 350 °C |
| Mobile phase B | 0.1% formic acid/MeOH:ACN 1:1 | Sheath gas pressure | 21 mTorr |
| Injection volume | 20 µL | Auxiliary gas pressure | 4 mTorr |
| Flow rate | 200 µLmin ⁻¹ | Collision gas pressure | 1.5 mTorr |
| Gradient | t=0 min, A=90%, B=10% t=2 min, A=80%, B=20% t=15 min, A=75%, B=25% t=17 min, A=50%, B=50% t=20 min, A=5%, B=95% t=25 min, A=5%, B=95% t=30 min, A=90%, B=10% | Source CID | -12 V |

Table 2: SRM transitions used for detection and quantification (SRM #1) and confirmation (SRM #2)

| Compound | SRM #1 | | CE (V) | SRM #2 | | CE (V) | Tube Lens |
|-------------------------------|--------|--------|--------|--------|--------|--------|-----------|
| Pyrimethamine | 249.10 | 177.07 | 40 | | | | |
| Sulfamethoxazole [†] | 254.08 | 92.11 | 36 | 254.08 | 108.10 | 37 | 70 |
| Diaveridine | 261.15 | 123.11 | 34 | | | | |
| Trimethoprim [†] | 291.16 | 123.10 | 33 | 291.16 | 230.17 | 34 | 91 |
| Ciprofloxacin [†] | 332.16 | 231.07 | 49 | 332.16 | 288.15 | 27 | 82 |
| Lomefloxacin | 352.17 | 265.13 | 34 | | | | |
| Levofloxacin [†] | 362.17 | 261.12 | 35 | 362.17 | 221.05 | 43 | 92 |
| Clarithromycin [*] | 748.55 | 590.36 | 19 | 748.55 | 115.99 | 35 | 96 |
| Azithromycin [*] | 375.33 | 82.96 | 25 | 749.54 | 158.04 | 38 | 74/112 |
| Josamycin | 828.53 | 108.87 | 46 | 828.53 | 173.96 | 47 | 126 |

[†]Quantified using diaveridine as the internal standard, [‡]Quantified using lomefloxacin as the internal standard, ^{*}Quantified using josamycin as the internal standard

Table 3: Analytical method parameters

| Compound | r ² matrix [*] | Limit of Detection (ngL ⁻¹) | Standard SRM ratio±SD [†] | Sample SRM ratio±SD [‡] | SRM ratio difference [^] |
|------------------|------------------------------------|---|------------------------------------|----------------------------------|-----------------------------------|
| Sulfamethoxazole | 0.9995 | 22 | 1.53 ± 0.03 | 1.6 ± 0.2 | -2.6 |
| Trimethoprim | 0.9998 | 7 | 4.2 ± 0.1 | 4.39 ± 0.07 | -3.3 |
| Ciprofloxacin | 0.9996 | 21 | 5.5 ± 0.8 | 6.59 ± 0.05 | -18.9 |
| Levofloxacin | 0.9996 | 4 | 3.65 ± 0.07 | 3.83 ± 0.06 | -5.0 |
| Clarithromycin | 0.9997 | 0.3 | 1.67 ± 0.04 | 1.59 ± 0.09 | 4.3 |
| Azithromycin | 0.9900 | 12 | 1.2 ± 0.1 | 0.44 ± 0.1 | 6.4 |

^{*}Determination coefficient of the calibration curve made using the WWTP effluent diluted by a factor of 10; ^{**}Calculated from the effluent data based on a S/N=3; [†]Standards spiked WWTP effluent diluted by a factor of 10, n=4; [‡]WWTP effluent, n=3; [^]Percentage difference between the standard and sample SRM ratio.

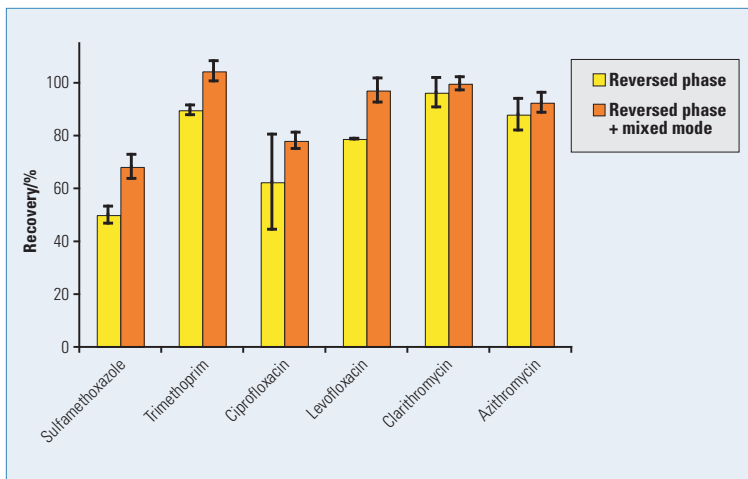
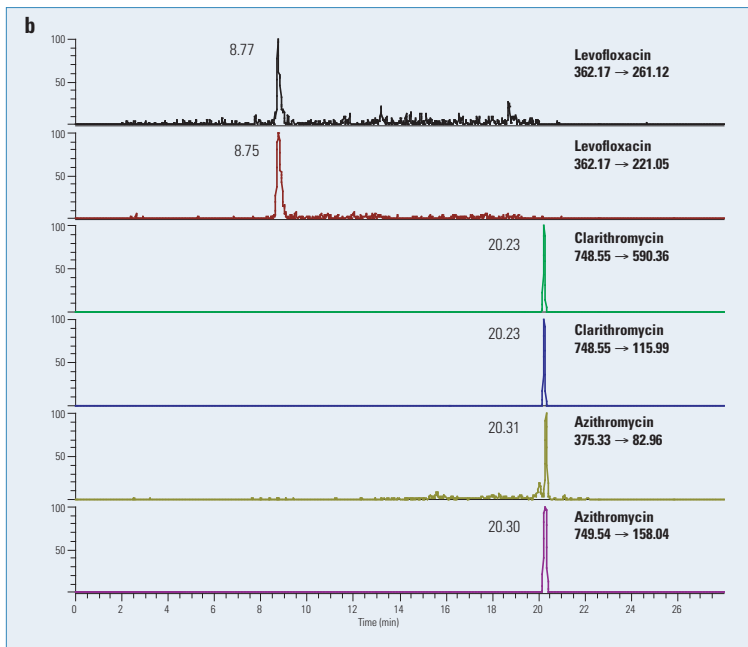
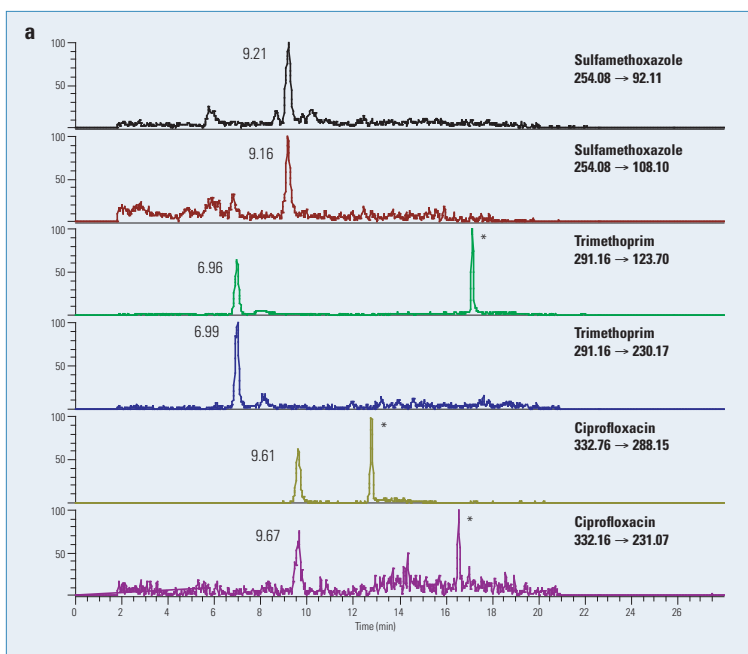


Figure 2: Analytes mean percentage recovery (spiked in the effluent at 500 ng L⁻¹, n=2)



0.3 to 22 ng L⁻¹ (Table 3). As suggested by Hernandez^[8], the use of two SRM transitions in the analytical method (Figure 3) as well as their peak ratios effectively and unambiguously confirmed the presence of the studied anti-infectives in all the samples. SRM peak ratios were reproducible (RSD <10%) and differences with SRM peak ratios of spiked standards were not higher than 20% except for AZI (64%).

The tandem-SPE approach utilized to pre-concentrate and extract the analytes from untreated and treated sewage improved the recovery on all six analytes (Figure 2).

The combination of reversed-phase and ion-exchange surface chemistry proved to be a suitable way to extract compounds having different chemical properties such as pK_a and pK_{ow}.

All targeted anti-infectives were found in the wastewater samples in concentrations ranging from 39 ± 1 to 276 ± 7 ng L⁻¹ (Figure 4).

Anti-infective daily mass flows in the St. Lawrence River were estimated using the flow of the sampling day (35 m³ s⁻¹) (Table 4). These results show that while anti-infective concentration in urban wastewaters are typically in the low nanogram-per-liter range, their daily discharged inputs in surface waters can be substantial.

Figures 3a-b: Chromatograms showing two SRM transitions of the studied compounds in treated wastewater. Peaks due to interferences are marked by asterisks(*).

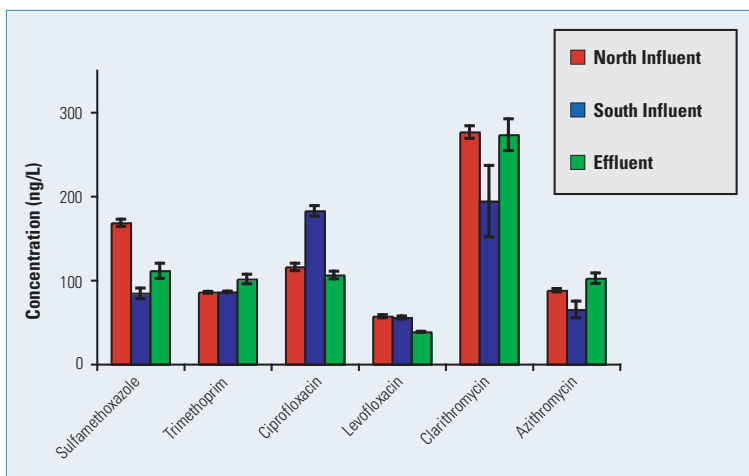


Figure 4: Occurrence of the studied anti-infectives in the dissolved phase of raw and treated sewage of the City of Montréal (n=3)

Table 4: Removal efficiency of the Montréal wastewater treatment plant and average mass flow of the studied anti-infectives.

| Compound | Mean mass flow in the St. Lawrence River (g day ⁻¹) |
|------------------|---|
| Sulfamethoxazole | 340 ± 30 |
| Trimethoprim | 310 ± 20 |
| Ciprofloxacin | 320 ± 10 |
| Levofloxacin | 118 ± 2 |
| Clarithromycin | 830 ± 60 |
| Azithromycin | 310 ± 20 |

Conclusions

The developed analytical method allowed the extraction, detection and quantification of six of the most used anti-infectives in untreated and treated sewage. Detection limits ranged from 0.3 to 22 ng L⁻¹ and instrument response was linear ($r^2 \geq 0.99$) in the dynamic range (25–1000 ng L⁻¹). The use of two specific SRM transitions and their peak area ratios proved to be a reliable and effective way to reduce false positives and confirm the presence of targeted substances. All the studied anti-infectives were found in the wastewater samples in concentrations ranging from 39 to 276 ng L⁻¹. More studies are necessary to elucidate the fate of these anti-infectives after they are discharged into the St. Lawrence River as well as their effects on aquatic biota and the environment.

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