Preliminary investigation on the occurrence of several sulfonamide antibiotics in the Haihe River Basin of China

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Abstract. Several samples collected from lakes, rivers and reservoirs in Haihe river basin of China were analyzed for 8 sulfonamide antibiotics by using solid-phase extraction and liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). All water samples were enriched with HLB extraction cartridges. The antibiotics were separated by gradient elution with methanol as the mobile phase adding 0.1% formic acid. The eluate was then analyzed by the mode of multiple reaction monitoring (MRM). The limits of detection (LOD) and quantification (LOQ) were 0.4-1.0 ng/L and 1.0-3.0 ng/L respectively. The method was used for the analysis of 13 samples from Haihe river basin in China. The results showed that sulfamethoxazole was present in all water samples with maximum concentration of 107.59 ng/L. Sulfadiazine was also frequently detected, concentrations ranging from 2.81 ng/L to 85.35 ng/L. Other sulfonamide antibiotics were not detected in most water samples, especially for those samples from drinking water resources.

1. Introduction
Water pollution is one of the most serious environmental problems in northern China. In this area, several industrial pollutants have been monitored for many years. However, some new types of pollutants are still unknown in the surface waters of northern China. Antibiotics are one of the most popular pharmaceuticals. The occurrence of antibiotics in aquatic environments has led to an increasing concern about the environmental risks associated with these compounds [1-3]. Sulfonamide antibiotics (SAs) are widely used antibiotics in modern human and veterinary medical practices. The concentrations of sulfonamide antibiotics measured in surface water samples typically are in the tens of nanograms per liter [4, 5]. Recent studies showed the presence of residual antibiotics in surface waters [6-10]. Although the concentrations are still lower than typical maximum concentrations (in the tens of micrograms per liter) reported before, the effect of continuous exposure to antibiotics on aquatic biota is unknown [11]. In this study, an investigation of 8 sulfonamide antibiotics was carried out in surface waters of lakes, rivers and reservoirs in northern China.

2. Experimental

2.1. Materials and reagents
Symmetry C18 cartridges (150 mm×2.1 mm i.d., 3.5 μm, Waters, USA) and Oasis HLB (500 mg/6 mL, Waters, USA) were used in the study. HPLC grade methanol and formic acid were from J.T. Baker Ltd, USA. Sulfacetamide (SAAM), sulfadiazine (SDZ, 99.5%), sulfamethoxaole (SMZ, 99.5%), sulfathiazole (STZ, 99.5%), sulfamerazine (SMR, 99.2%), sulfisoxazole (SIX, 99.7%), sulfamethazine (SMT, 99.0%) and sulfachloropytidazine (SCP, 99.0%) were obtained from Dr. Ehrenstorfer GbH, Germany. Ultra-pure water was prepared by a Milli-Q pure water system. Stock solutions of all sulfonamide antibiotics were prepared by dissolving each compound in methanol at a concentration of 100 mg/L. Working solutions of different concentrations were prepared by diluting and mixing the stock solution and stored at 4°C in the dark.

2.2. Sample location and preparation
Haihe River Basin is located in north of China including Beijing, Tianjin, Hebei Province, Shanxi Province, Shandong Province, Henan Province, Liaoning Province and Inner Mongolia Autonomous Region. Samples in this study were from lakes, rivers (province boundary) and drinking water sources (Panjiakou reservoir and Yuecheng reservior) in Haihe river basin, as shown in figure 1.

![Figure 1](image_url)

**Figure 1.** Location for the sampling section in Haihe River basin.
Notes: 1, 2, 3-Baiyang Lake. 4-Cetian reservoir. 5-Guanting reservoir. 6-Huliuhe reservoir. 7-province boundary (Gubeikou). 8-province boundary (Xiabao). 9, 10, 11-Panjiakou reservoir. 12, 13-Yuecheng reservoir.

All water samples were filtered through 0.45 μm glass fiber filters. Five hundred mL of the water samples were acidified to pH=4 by adding formic acid. Oasis HLB cartridges were preconditioned
with 5.0 ml of methanol and 5 ml ultra-pure water. Acidified water samples were then passed through Oasis HLB columns at a flow rate of approximately 5 mL/min. The Oasis HLB columns were then rinsed with 5 mL ultra-pure water and dried under vacuum. After drying, sulfonamides were eluted from each cartridge using 2 mL of methanol (3X). Extracts were collected in a glass vial, reduced to a minimum volume, and then dissolved in 20% methanol to a final volume of 1.0 mL.

2.3. Liquid chromatography and mass spectrometry
An Agilent 1260 liquid chromatography system connected to 6410B triplequadrupole mass spectrometer was used in this study. The system was equipped with a vacuum degasser, a binary pump, an autosampler and a temperature controlling system. A Symmetry C18 chromatograph column (Waters, USA, 150 mm×2.1 mm i.d., 3.5 μm) was employed and was operated at 30°C.

The mobile phase consisted of deionized water containing 0.1% formic acid (A) and methanol (B). The gradient was set up as follows: 0-8 min, 20%-80% B; 8-10 min, 80% B; 10-12 min, 80%-20% B; 12-25 min, 20% B. The injection volume was 10 μL and the flow rate was 0.2 mL/min.

The QQQ was operated using an eletrospray ionization (ESI) source in positive mode. High purity liquid nitrogen was used as the nebulizing and drying gas at 350°C with a flow of 10.0 L/min.

3. Results and discussion

3.1. Chromatography
In this study, we compared two chromatography columns. The results indicate that a Symmetry C18 chromatography column (Waters, USA, 150 mm×2.1 mm i.d., 3.5 μm) achieves better separation of the various sulfonamides than a Zorbax SB C18 chromatograph column (Agilent, USA, 50 mm×2.1 mm). The latter column might be too short to separate the eight sulfonamide antibiotics examined in this study. In addition, it was determined that the solvent used for liquid chromatography is very important. The chromatographic separation results were poor when the sample was resolved in pure methanol. The base line was not flat and there was an obvious solvent peak at 2 min. Also, the peak was not sharp. However, chromatographic separation results were good when the initial mobile phase used was 20% methanol as shown in figure 2.

![Figure 2. Total ion chromatogram of 8 sulfonamide antibiotics by HPLC-MS/MS with MRM mode. Notes: 1. SAAM. 2. SDZ. 3. STZ. 4. SMR. 5. SMT. 6.SCP. 7. SMZ. 8. SIX.](image)

3.2. MS/MS-MRM parameters
The mixture of 8 sulfonamide antibiotics was injected for study the initial experiments. The full scan spectra show the presence for protonated molecular ions (mother ions). The mode of multiple reaction monitoring (MRM) was used for detection of all sulfonamide antibiotics. The parameters were set up by following procedures. High purity nitrogen gas was used for
collision-induced dissociation. The fragment ions were formed through the collision with the proper energy. The transition parameters from mother ions to daughter ion were optimized by varying the collision energy. The collision energy (CE) for quantitative and qualitative ions were selected for each compound. Table 1 shows mother ions, daughter ions, optimum fragment voltage and collision energy.

Table 1. MRM Parameters for eight sulphonamides.

| Compound                  | Precursor ion m/z | Quantitative ion m/z | Qualitative ion m/z | Fragment U/V | Collision energy U/V |
|---------------------------|-------------------|----------------------|---------------------|--------------|----------------------|
| Sulfacetamide (SAAM)      | 215.0             | 156.1                | 108.1               | 95           | 10                   |
| Sulfadiazine (SDZ)        | 251.0             | 108.1                | 156.1               | 105          | 23                   |
| Sulfamethoxazole (SMZ)    | 254.0             | 108.1                | 156.1               | 105          | 25                   |
| Sulfathiazole (STZ)       | 256.0             | 156.1                | 108.1               | 95           | 10                   |
| Sulfamerazine (SMR)       | 265.0             | 156.1                | 108.1               | 115          | 15                   |
| Sulfisoxazole (SIX)       | 268.0             | 156.1                | 108.1               | 95           | 10                   |
| Sulfamethazine (SMT)      | 279.0             | 186.1                | 124.1               | 115          | 15                   |
| Sulfachloropyridazine (SCP)| 284.9             | 156.1                | 108.1               | 95           | 10                   |

3.3. Recovery
Rao et al [12] reported that the recovery of antibiotics is related to pH and sample volume. Our results also indicate that recovery of the 8 sulfonamide antibiotics is greater at pH 4 than at pH 7. Recovery of 8 sulfonamide antibiotics from the HLB cartridges was measured with 500 mL of tap water spiked at 20 ng/L and 200 ng/L at pH 4. The results indicate that the recoveries for both spiked concentration levels were greater than 80%. The percent recovered was slightly greater at the higher concentrations (200 ng/L) than the lower concentrations (20 ng/L) as shown in Table 2.

Table 2. Recoveries for 8 sulfonamide and antibiotics at different spike concentrations.

| Compounds                  | 20 ng/L |               | 200 ng/L |               |
|----------------------------|---------|--------------|----------|--------------|
|                            | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| Sulfacetamide (SAAM)       | 91.5    | 93.6         | 95.4     | 2.1          | 93.5 | 95.1 | 96.8 | 1.7 |
| Sulfadiazine (SDZ)         | 95.4    | 94.8         | 91.5     | 2.2          | 99.0 | 97.9 | 95.2 | 2.0 |
| Sulfamethoxazole (SMZ)     | 86.0    | 91.2         | 93.9     | 4.4          | 99.1 | 93.3 | 96.2 | 3.0 |
| Sulfathiazole (STZ)        | 92.5    | 97.6         | 94.5     | 2.7          | 92.2 | 93.6 | 97.6 | 3.0 |
| Sulfamerazine (SMR)        | 89.3    | 90.6         | 94.7     | 3.1          | 99.7 | 96.6 | 93.6 | 3.6 |
| Sulfisoxazole (SIX)        | 83.0    | 83.8         | 89.9     | 4.4          | 98.2 | 99.6 | 96.3 | 1.7 |
| Sulfamethazine (SMT)       | 96.4    | 95.1         | 92.8     | 1.9          | 99.0 | 97.6 | 95.9 | 1.6 |
| Sulfachloropyridazine (SCP)| 94.5    | 98.1         | 98.5     | 2.3          | 97.5 | 99.7 | 98.9 | 1.1 |
3.4. Calibration, limit of detection and limit of quantification

The method of external calibration was used for the sulfonamide antibiotics with the concentration range of 1-100 μg/L. All sulfonamide antibiotics show good correlation with coefficients R² of more than 0.9991. Reproducibility of the method was determined by analyzing six replicates of spiked samples at 100 μg/L. The relative standard deviation (RSD) was lower than ±4%. The limits of detection (LOD) and the limits of quantification (LOQ) were calculated based on signal-to-noise ratio of 3 and 10. The result indicates that, LOD and LOQ are 0.2-0.5 μg/L and 0.5-1.5 μg/L, respectively. Considering that the sample volume was 500 ml, the limits of detection (LOD) and the limits of quantification (LOQ) are in the range of 0.4-1.0 ng/L and 1.0-3.0 ng/L under the assumption that the recoveries would be 100%. Results are shown in table 3.

| Compounds | LOD (μg/L) | LOQ (μg/L) | Linear range (μg/L) | Regression equation | R² |
|-----------|------------|------------|---------------------|---------------------|----|
| Sulfacetamide (SAAM) | 0.2 | 0.5 | 1-100 | Y=282.51X+82.740 | 0.9998 |
| Sulfadiazine(SDZ) | 0.2 | 0.7 | 1-100 | Y=57.743X-20.188 | 0.9995 |
| Sulfamethoxazole(SMZ) | 0.3 | 1.1 | 1-100 | Y=70.531X+48.930 | 0.9994 |
| Sulfathiazole(STZ) | 0.5 | 1.5 | 1-100 | Y=107.776X+34.305 | 0.9999 |
| Sulfamerazine(SMR) | 0.2 | 0.7 | 1-100 | Y=42.019X+18.862 | 0.9991 |
| Sulfisoxazole(SIX) | 0.2 | 0.5 | 1-100 | Y=153.921X+91.560 | 0.9994 |
| Sulfamethazine(SMT) | 0.3 | 1.1 | 1-100 | Y=137.969X-26.050 | 0.9993 |
| Sulfachloropyridazine(SCP) | 0.2 | 0.7 | 1-100 | Y=97.017X-56.451 | 0.9991 |

**Table 3.** Regression analysis of calibration curves, LOD and LOQ of antibiotics.

| Compounds | Concentration of antibiotics in different locations (ng/L) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|-----------|---------------------------------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sulfacetamide (SAAM) | 6.15 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Sulfadiazine (SDZ) | 85.35 | 36.2 | 51.32 | 6.4 | 2.81 | ND | ND | ND | 32.6 | ND | ND | ND | ND | ND |
| Sulfamethoxazole (SMZ) | 103.8 | 75.2 | 107.5 | 6.8 | 29.3 | 4.5 | 7.6 | 67.7 | 10.2 | 32.6 | 10.3 | 3.1 | 3.0 | 3.0 |
| Sulfathiazole (STZ) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Sulfamerazine (SMR) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Sulfisoxazole (SIX) | ND | ND | ND | ND | 0.91 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Sulfamethazine (SMT) | 5.88 | 3.16 | 4.54 | 1.7 | 0.70 | ND | 1.0 | 1.19 | ND | ND | ND | ND | ND | ND |
| Sulfachloropyridazine (SCP) | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

Notes: 1, 2, 3-Baiyang Lake. 4-Cetian reservoir. 5-Guanting reservoir. 6-Huliuhe reservoir. 7- province boundary (Gubeikou). 8-province boundary (Xiabao). 9,10,11-Panjiaokou reservoir. 12, 13-Yuecheng reservoir. ND-not detected.

3.5. Analysis of surface water
In this study, an investigation of 8 sulfonamide antibiotics was carried out in surface waters of lakes, rivers and reservoirs in northern China. Thirteen surface water samples were analyzed using the method above as shown in table 4. The results showed that sulfamethoxazole was detected in these surface waters with the maximum concentrations 107.59 ng/L. These results are similar to the previously published research of Cahill et al [4]. Sulfadiazine was also frequently detected, at concentrations ranging from 2.81 ng/L to 85.35 ng/L. The other sulfonamide antibiotics were not found in most water samples, especially for those samples from drinking water resources. Our data indicate that the concentration of sulfonamide antibiotics in drinking water resources is low. However, there is an increasing concern about the potential environmental risk.

4. Conclusions
In the study, a method was developed and validated for measuring 8 sulfonamide antibiotics using solid-phase extraction HPLC-MS/MS. The results indicate that it is a simple, rapid and effective method for measuring these antibiotic pollutants in environmental water samples. In the study, sulfamethoxazole was found in every surface water sample with a maximal concentration of 107.59 ng/L. Sulfacetamide, Sulfadiazine and Sulfamethazine were detected in some water samples and other antibiotics were not detected. Though the concentration of most antibiotic is not high, it is a big concern for human health. Therefore, the relationship between antibiotic levels in surface waters and human health risks should be studied further.

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