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Full Length Research Paper

Detection of oxytetracycline and chloramphenicol in untreated wastewater effluents by reverse phase-high performance liquid chromatography (RP-HPLC) technique

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The occurrence of antibiotics in wastewater effluent has been a concern worldwide for the development of antibiotics resistance and the impact of antibiotic residues on living components in surface water may pose plausible threats on humans. Reverse phase - high performance liquid chromatographic (RP-HPLC) method was used for the detection and estimation of Oxytetracycline (OTC) and Chloramphenicol (CAP) in untreated wastewater effluents in Bangladesh. The flow rate was at 1.0 mL/min with UV detection at 270 and 310 nm. The retention time of OTC was 3.3 ± 0.1 min while CAP was 4.7 ± 0.1 min. The recovery was found to be ≥95% for both antibiotics demonstrating the accuracy of the protocol. Precision of the developed method both inter-day and intra-day were less than the maximum allowable limit (RSD% ≤ 2.0) according to ICH, USP and FDA guidelines. The method showed linear response with correlation coefficient (r^2) value of 0.9996 and 0.9998 for OTC and CAP, respectively. The concentration of OTC was found to be 0.670-1.799 µg/mL and the concentration of CAP was found to be 0.316-0.921 µg/mL. Therefore, the presence of these antibiotics in waste-water poses a potential threat to the surrounding environments as well as its biotic components.

Key words: Antibiotic resistance, Bangladesh, Effluents, Surface water.

INTRODUCTION

Wastewater treatment plant (WWTP) often act as a suitable arsenal of different antibiotic residues and the breeding ground for resistant bacteria to develop and disseminate in the environment (Karkman et al., 2018). In Bangladesh, WWTPs are not available in rural regions and are insufficient in urban areas which result in high loads of antibiotics released from untreated wastewater into surface waters like rivers and surroundings (Shah et al., 2018). Municipal sewage treatment plants are not capacitated enough to completely remove antibiotic residues and this partially treated water may reach in drinking water sources like rivers and lakes (Lundborg and Tamhankar, 2017). In addition, the effects of antibiotics on the biotic environment can be more serious

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(Foroughi et al., 2020).

Even though, several methods were developed to eliminate antibiotics from the sewage system, not all drugs were removed completely, thus there is ample possibility of these being subsequently released into the natural waters (Radmehr et al., 2021). The risk of persistent antibiotics in WWTPs has drawn more attention, since it may contribute to the development of antibiotic-resistant bacteria in nature and in different environments (Singh et al., 2019). Current WWTPs help to remove antibiotics from wastewater to a certain degree. However, even in high income countries, antibiotic residues have been found in effluent from treatment plants, indicating that current technologies do not meet the criteria to completely eliminate antibiotics (Danner et al., 2019).

Due to OTC and CAP's high usage in Bangladesh's livestock and poultry sector, these antibiotics were targeted for this study to detect in untreated wastewater effluents by using RP-HPLC. CAP is a cheap and broad-spectrum antibiotic and easy to manufacture, having inhibitory effect on both Gram-positive and Gram-negative bacteria (Mbodi et al., 2014). It is widely used in animal husbandry to control diseases or as growth promoters (Yuan et al., 2012). OTC is also a broad-spectrum antibiotic of tetracycline group and most widely used as human and veterinary medicines (Wu et al., 2018). OTC is used as both growth promoter as well as prevention and control of diseases in animal husbandry and poultry industry (Ferdous et al., 2020).

The aim was to detect the presence of OTC and CAP residues in Dhaka city's WWTP effluents in Bangladesh. RP-HPLC method was used in this study, which is one of the most recent approaches in analytical techniques to investigate the presence of antibiotic residues in different environmental samples (Narvaez et al., 2012). Therefore, an attempt was taken to investigate the existence of CAP and OTC in untreated wastewater effluents from WWTP.

MATERIALS AND METHODS

Study area

One wastewater representative grab sample of 2 L was collected from the main sewage flow from Dhaka city's wastewater treatment plant (PAGLA wastewater treatment plant) prior to wastewater treatment plant inlets. The sampling areas' GPS location was N23°41.004"/ E90°27.080" with 7 m elevation. Sample was collected after first filtering step without effluent processing in every one-month interval except January and April, 2018, similar 10 samples were collected from the WWTP throughout June 2017 to May 2018 and each sample was promptly transferred to lab and stored at 4°C until further analyses (Supplementary Table 1).

Reagents and chemicals

High-purity reference materials of OTC and CAP were purchased from Sigma-Aldrich (MO, USA). Solvents, such as methanol and acetonitrile used in this study, were all of HPLC grade and were purchased from BMA market in Dhaka City.

Instrumentation

The analyses were performed using the HPLC system (Shimadzu-UFLC Prominence), with an auto sampler (Model- SIL 20AC HT) and UV-Visible detector (Model-SPD 20A). LC-solutions software was operated for data documentation. RP-HPLC method has been adopted from Tanjin et al. (2013) and used for the quantitation of antibiotic residues in water samples. RP-HPLC method is much more sensitive and precise than HPLC to detect non-polar compounds like these antibiotics. RP-HPLC method has non-polar stationary phase that add extra benefit for the detection. This protocol may be applied for the detection and analyses of antibiotic residues in waste-water samples.

Chromatographic conditions

Analytical reversed phase C-18 (ODS column, 250 × 4.6 mm, 5 µm, Phenomenex, Inc) was used for separation. A mixture of buffer (Potassium dihydrogen phosphate and Phosphoric acid, pH 7.80) and acetonitrile in the ratio of 50:50 (v/v) was injected at a flow rate of 1.0 mL/min at 270 nm and 310 nm for UV detection of both antibiotics used for mobile phase. Membrane filter with a pore size of 0.22 µm was used to filter the mobile phase followed by sonicated and degassed prior use at room temperature (~26°C) temperature.

Method development

In order to achieve the optimum separation, mobile phase composed of buffer and acetonitrile with different proportions and pH were observed. Finally, it was concluded that buffer (Potassium dihydrogen phosphate and 85% Phosphoric acid, pH 7.80) and acetonitrile (50: 50 v/v) were found appropriate to develop medium for good resolution and acceptable system suitability parameters.

Standard solutions preparation

Standard OTC and CAP was precisely weighed (10.0 mg) and transferred into a 100 mL clean dry volumetric flask then mixed with diluent and sonicated for 5 min. The calibration curve was obtained by using final concentration of the preparation of 100 µg/mL followed by eleven different solutions having 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, and 2.5 µg/mL concentrations. Each solution of 20 µl was injected into chromatograph prior filtered through 0.45 µm filter membrane and the chromatograms were recorded.

Preparation of sample solutions

Wastewater was filtered through Whatman No. 01 filter paper. Then water sample (pH 3.0) was prepared by using 10% meta-phosphoric acid. The water sample was injected into HPLC vials prior being filtered through 0.45 µm filter and all the HPLC vials were put into the rack of auto-samplers.

Validation of method

Specificity

The standard solution comprises of two different antibiotics, when

Table 1. Accuracy (% recovery) results of OTC and CAP.

Injected (OTC) Conc. (µg/mL)	Recovered Conc. (µg/mL)	% Recovery	Injected (CAP) Conc. (µg/mL)	Recovered Conc. (µg/mL)	% Recovery
10	9.59581	96.0	10	9.49321	95.0
20	19.91523	99.58	20	19.87268	99.4

injected into chromatograph did not interfere, thus the LC method was evaluated for specificity.

Linearity

The average peak areas of 2.5, 10, 30, 60 and 80 µg/mL solutions were plotted by injecting 20 µl from each solution using the auto sampler and monitored at 270 and 310 nm and repeated three times. Coefficient of correlation and intercept values were calculated by using calibration curves and the linearity was evaluated.

Accuracy

The accuracy is calculated by using the percentage recovery (R%) of analyte recovered by the assay. Successive analysis (n=2) for standard OTC and CAP solutions of 10 and 20 µg/mL concentrations were carried out to evaluate the accuracy. % Recovery and % Error were within the expected recovery range (95-105%). Accuracy of the method was demonstrated by recovery values. Recovery values were found to be 96.0% and 99.58% for OTC; 95.0% and 99.4% for CAP, respectively (Table 1).

Precision

Precision of the assay was demonstrated by intra-day and inter-day variation studies with consideration of repeatability and reproducibility. The percentage of relative standard deviation (%RSD) of the values precision studies was found <2% by using the formula as recommended by ICH guidelines:

[RSD (%) = (Standard deviation/Mean) × 100]

Single concentration of 5, 10 and 15 µg/mL of standard solution for both OTC and CAP was used for precision determination for the current method development and validation protocol. Standard solutions were repeated thrice in a day for three consecutive days for this precision studies.

Sample analysis

The antibiotics in wastewater samples were calculated by using the calibration curves with successive analysis (n = 3) by the proposed method and formula:

$y = mx + c$

where y = peak area of the analyzed sample; m = slope of the calibration curve; c = intercept of the calibration curve; x = concentration of the analyzed sample.

RESULTS

RP-HPLC method has been established and validated as per ICH, USP and FDA guidelines by Tanjin et al. (2013). The retention time of OTC was 3.3 ± 0.1 min and CAP was 4.7± 0.1 min and was performed at a flow rate of 1 mL/min (Figure 1).

Calibration curve development

A good correlation coefficient (r^2) was obtained for OTC and CAP (0.9996 and 0.9998, respectively) within the accepted range of guidelines. A good correlation coefficient was found by using average peak areas plotted against different concentration ranges (2.5 to 80 µg/mL). For OTC, the slope (m) and intercept (c) of the calibration curve were found at 58497 and 34568, respectively and for CAP the slope (m) and intercept (c) were found at 47707 and 14211, respectively (Table 2 and Figures 2 and 3).

Quantitative analysis of OTC and CAP residues

Quantitative analyses of OTC and CAP in 10 different wastewater samples was performed. The quantity of OTC and CAP was measured from the peak area of the different samples and calibration curve using the formula:

$X = (y-c)/m$

The concentration of OTC and CAP was measured as 1.799 and 0.921 µg/mL, respectively from the peak area and calibration curve using the aforementioned formula at retention time 3.3 and 4.7 min (Figure 4).

DISCUSSION

One of the most critical public health concerns in the 21st century is the emergence and dissemination of antimicrobial resistance (AMR). AMR has been directly linked to the presence of antimicrobial residues in the environment (Hendriksen et al., 2019). There are several fast and sensitive methods for antibiotic detection already existed such as capillary electrophoresis, different chromatography (gas, liquid) with mass spectrometry, plasma detection, electrochemistry, etc. (Cristea et al., 2017).

However, in recent times HPLC based method has turned out to be one of the most efficient and accurate

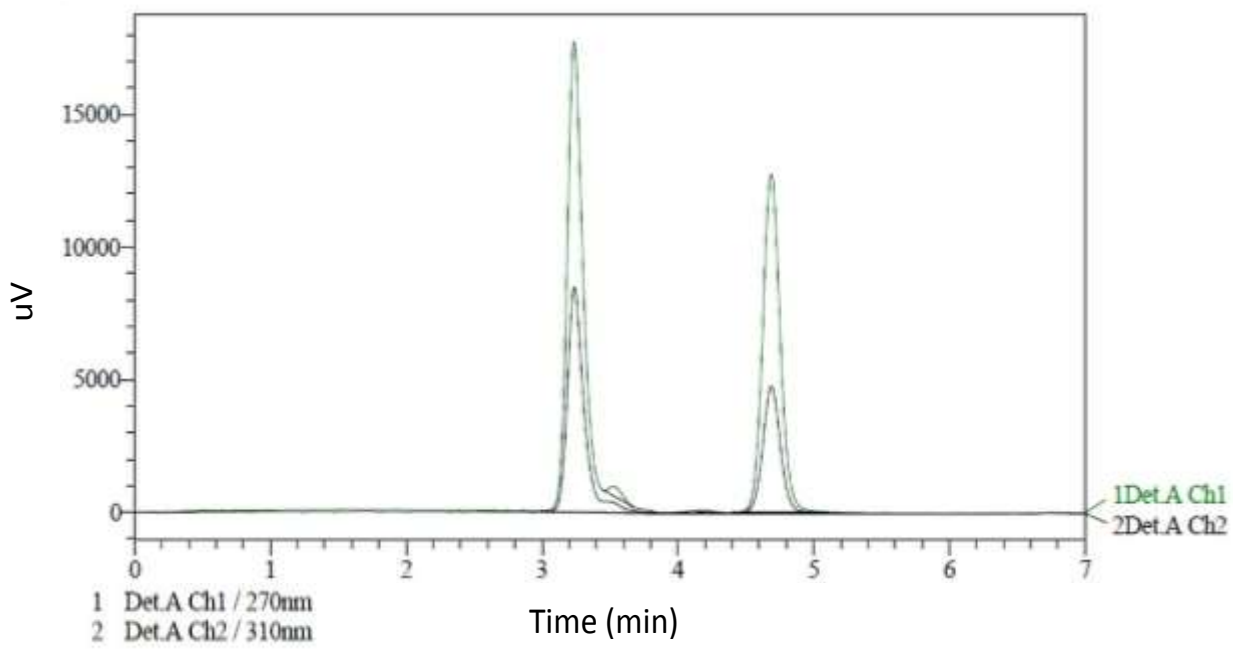

Figure 1. A chromatogram of standard solution of 2.5 µg/mL OTC and CAP at retention time 3.3±0.1 and 4.7±0.1, respectively.

Table 2. Linear regression data for calibration curve.

Parameter	OTC	CAP
Linearity range (µg/mL)	2.5-80	2.5-80
Correlation coefficient (r^2)	0.9996	0.9998
Slope	58497	47707
Intercept	34568	47707

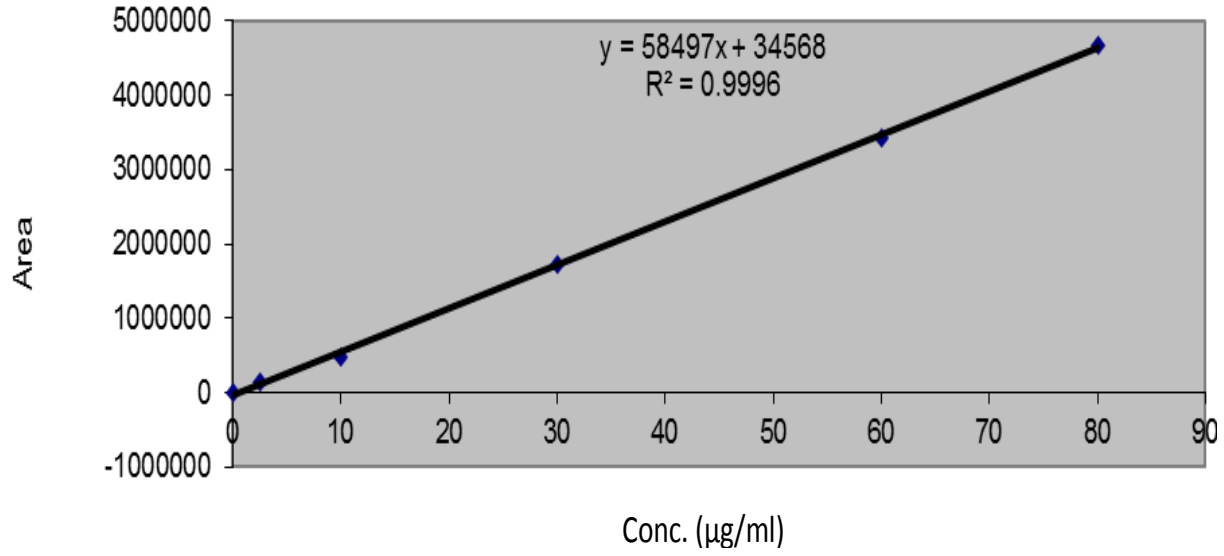

Figure 2. Calibration curve of Oxytetracycline (OTC).

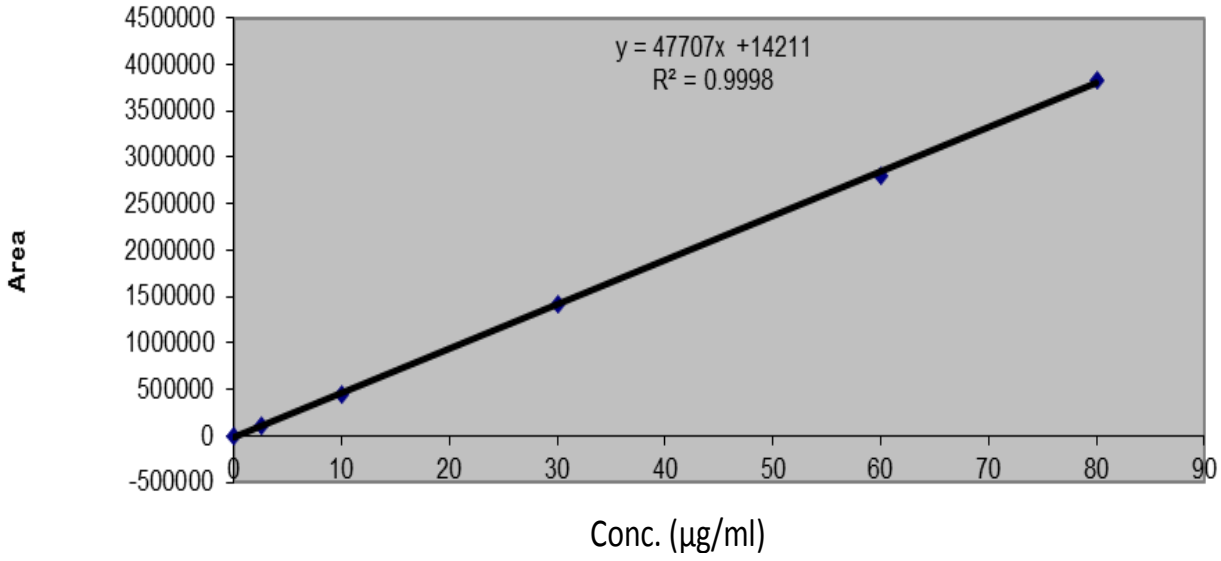

Figure 3. Calibration curve of Chloramphenicol (CAP).

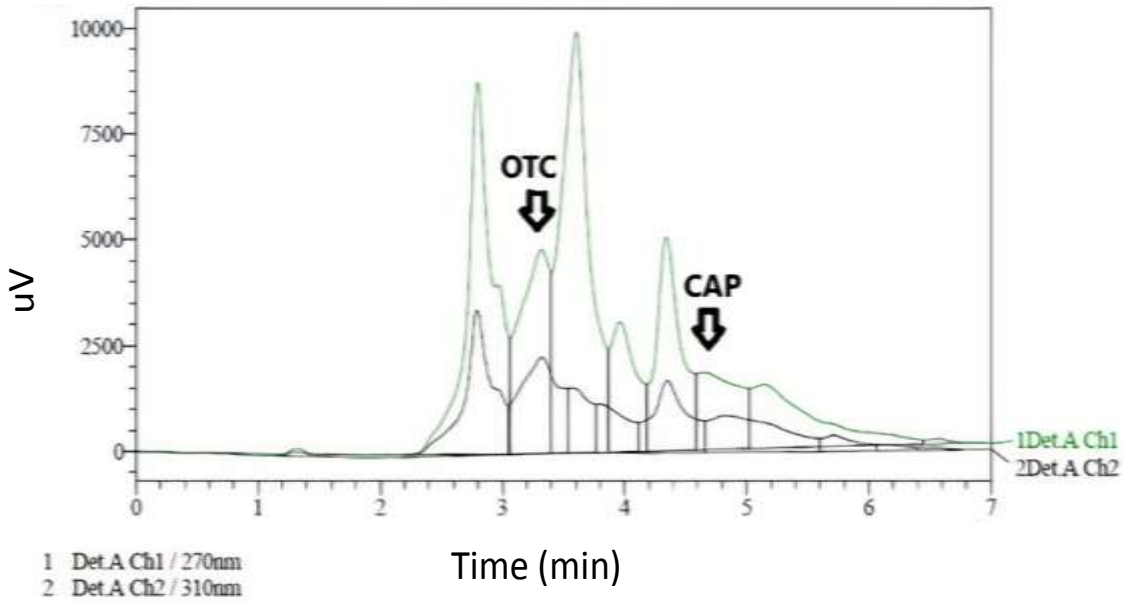

Figure 4. HPLC chromatogram of wastewater sample (N1) showing peak at retention time 3.3 and 4.7 min indicated by arrow represents OTC and CAP, respectively.

approach for the detection of antibiotics in different environmental samples (Zafar et al., 2021).RP-HPLC method was established for the detection of OTC and CAP in 10 different wastewater untreated-effluent samples. The different amounts of OTC and CAP residues were found in these samples (Supplementary Table 2). Each water sample contains both OTC and CAP in µg/mL level except sample N9 and N10 where no OTC and CAP were detected, respectively. The highest concentration (1.799 µg/mL) of OTC was found in sample N1 and lowest concentration (0.670 µg/mL) was found in sample N3. A similar finding was reported in Northern China where OTC was detected with 2.4±0.7 µg/mL (Hou et al., 2016). The highest concentration (0.921 µg/mL) of CAP was found in sample N1 and lowest concentration (0.316 µg/mL) was found in sample N9. CAP was also detected from wastewater in Tunisia; however, the concentration was 1000 fold below compared to this study(Tahrani et al., 2016). Both of these antibiotic residues found in different effluents crossed the predicted

no effect environmental concentrations (PNEC) values. Since, PNEC values for resistance selection have been proposed for OTC 0.5 ng/mL and for CAP 8 ng/mL (Bengtsson-Palme and Larsson, 2016). PNEC values must be kept below the risk levels in order to curb the bacterial resistance development. It may be served as a guideline for environmental monitoring programs and interventions to resist antibiotic resistance. Laboratory studies demonstrated that very low antibiotic concentrations below the minimum inhibitory concentration (<100 fold) can select for and maintain resistance similar to those in the environment (Gullberg et al., 2011).

Urban WWTPs create a suitable playground for resistance spread and development because bacteria are continuously mixed with antibiotics concentrations above the PNEC range. Long-term persistence of antibiotics in environment have gained increased attention, and these phenomena may surge the widespread emergence of antibiotic resistant bacteria and genes in the environment (Hanna et al., 2018).

The detection of antibiotic residues following wastewater treatment was not performed. However, it is expected to further perform analyses to investigate the presence of antibiotics following treatment. However, instrumental precision was done, and recovery regarding spiking of standard solutions/wastewater was not performed.

Conclusion

WWTPs act as one of the major sources of antibiotic resistance dissemination and development in developing countries like Bangladesh. This is the first time in Bangladesh that Oxytetracycline and Chloramphenicol from wastewater were detected. High level of these antibiotics were found in this study and if wastewater samples without proper treatment would be release into the surrounding environments that might accelerate the process of resistance emergence. In order to curb the development of antibiotic resistance, effective treatment method for wastewater must be implemented and irrational use of antibiotics should be minimized.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. Wastewater sampling plan details.

S/N	Name of sampling	Sampling date	pH	Temperature (°C)
1	N1	14.06.2017	9.5	22
2	N2	20.07.2017	9.4	21
3	N3	22.08.2017	9.5	28
4	N4	13.09.2017	7.5	25
5	N5	16.10.2017	7.5	26
6	N6	25.112017	10.0	24
7	N7	18.12.2017	10.0	23
8	N8	12.02.2018	8.5	24
9	N9	24.03.2018	8.5	25
10	N10	15.05.2018	9.0	22

Supplementary Table 2. Determination of Oxytetracycline (OTC) and Chloramphenicol (CAP) in wastewater by HPLC.

Sample name	OTC		CAP	
	Peak area (OTC)	Conc. (µg/mL)	Peak area (CAP)	Conc. (µg/mL)
N1	70687	1.799	29723	0.921
N2	10271	0.767	6847	0.441
N3	4700	0.670	8838	0.483
N4	16279	0.869	1963	0.339
N5	27196	1.056	11761	0.544
N6	21192	0.953	1008	0.319
N7	29036	1.087	18133	0.678
N8	39024	1.258	20209	0.721
N9	0	0	845	0.316
N10	25025	1.019	0	0