A STUDY OF SURFACE WATER POLLUTION WITH AZITHROMYCIN IN UKRAINE

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ABSTRACT

INTRODUCTION: Water pollution with antibiotics plays a key role in the formation and spread of antibiotic resistance, which threatens humanity and the environment as a whole.

AIM: The purpose of our work was to develop a method for determining azithromycin in wastewater and surface water using the thin-layer chromatography method.

MATERIALS AND METHODS: The developed technique was tested in the wastewater of Zolochiv district of Kharkiv region.

RESULTS AND DISCUSSION: Using conventional analytical scales and universal chromatography in thin layers of a sorbent, it is possible to identify azithromycin with a water concentration of ≥30 μg/mL without complex and expensive equipment, such as HPLC or LC/MS/MS. The results showed that the concentration of azithromycin is less than 30 μg/mL.

Keywords: *pollutants, antibiotic resistance, thin-layer chromatography, surface water, PEC, PNEC, EMA*

INTRODUCTION

Pharmaceuticals and their derivatives are among the largest pollutants of the environment and in particular water. The excessive and irrational consumption of medicines by people, waste from pharmaceutical plants, as well as wastewater from hospitals, veterinary clinics, pharmaceutical enterprises, and livestock farms are the main pollutants of surface water. Among the large number of drugs that get to water and soil, a significant share is occupied by antibiotics. Most of them are excreted unchanged by the human body, preserving their antibacterial properties; active molecules get to aquatic ecosystems and affect them. In addition, antibiotics cannot be completely removed from wastewater during its treatment at sewage treatment plants. They penetrate surface waters, seep through the soil into underground ones, pollute all natural water storage facilities, and then get to drinking water from there.

The European Medicines Agency (EMA) noted that the impact of active pharmaceutical substances on the environment is an urgent and new environmental problem that requires proper monitoring and urgent solutions (1). There is still no detailed assessment of how different classes of drugs affect the environment, but on the example of antibiotics, there are enough data on their negative direct impact on the environment.

Environmental pollution from pharmaceuticals, such as antibiotics, is another big problem. Antibiotic resistance is recognized as one of the most significant global threats to humanity (2). According to the WHO forecast, as early as 2050, the number of

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deaths due to this problem in the world may reach 10 million people per year, and the annual losses for the global economy will exceed 100 trillion US dollars.

Antibiotic resistance is the development of protective properties to antibacterial drugs by microorganisms. Most often, it occurs due to the irrational and improper use of antibacterial drugs by people; the reason for it is free access to these drugs, their use without a doctor's prescription, non-compliance with the course of treatment (using longer than the prescribed course, use of other people's antibiotics, failure to complete the necessary course of administration), as well as a widespread use of cheaper and not always high-quality genetic antibacterial drugs in developing countries. In Ukraine, there is a state problem with the release of antibiotics without a prescription although they are legally listed as prescription drugs, but we understand the fact that, like most other prescription drugs, they are freely sold in pharmacies.

Especially dangerous is the mass application of antibacterial drugs in animal husbandry for the treatment, control of morbidity, and prevention of infectious diseases, as well as the growth stimulation. According to the EMA, in 2018, 6.500 tons of antibiotics of various classes were sold in 31 EU countries for veterinary needs alone. The same antibiotics as for humans are often used to treat animals. When using animal products, antibiotic substances together with resistant strains get to the human body, and as known, constant exposure to small concentrations of antibiotics leads to resistance of the bacterial flora. In addition, some resistant strains of bacteria have a zoonotic potential, that is, they can spread between animal and human populations. The use of reserve antibiotics, such as polymyxins, cephalosporins of the third and fourth generation, and fluoroquinolones in agriculture is of particular concern since this group of antibiotics is considered critical for medicine, they are drugs of "last hope". Colistin, an antibiotic from the reserve group, has been added to animal feed without control for many years in many countries of the world. For example, in China, the MCR-1 resistance gene to colistin has already been discovered in animals and humans (3). Moreover, some strains of *Klebsiella spp.* (*K. pneumoniae*, *K. oxytoca*, *K. aerogenes*) retain high sensitivity only to colistin (98%) (4). Taking this into account, antibiotics continue to be used on a global scale in animal husbandry in all countries of the world despite the fact that later on infections that are usually easily treated with a course of antibiotics can become fatal for humanity.

A great problem on the part of doctors is the use of empirical antibiotic therapy without taking into account the microbiological identification of pathogens and determination of the sensitivity profile to antibiotics, inadequate prescribing and dosage, the lack of educational work with patients (in particular, the necessity to complete a full course of antibiotic therapy, which causes incomplete eradication of the pathogen).

Wastes from people, animals, pharmaceutical plants, hospitals, and preventive institutions contain antibiotics and their decomposition products; they pollute the environment, getting into water and soil. In many rivers around the world, the concentration of antibiotics is several times, and in the worst cases even 300 times, higher than the permissible norm. It is most often observed in low-income countries in Africa and Asia. In the Kenyan rivers the concentration of antibiotics significantly exceeds the indicator, being 100 times higher than normal. The level of drugs is so high that no river animals can survive (5). The problem lies in the technology of wastewater treatment, which often does not meet all the necessary standards. Countries with cheap production, such as India and China, are part of the global pharmaceutical market, and do not always adhere to the necessary rules for purification and recycling of recoverable resources. They contribute to a large share of environmental pollution with pharmaceutical waste. Due to the presence of antibiotics in water bodies, bacteria that live in water and in river dwellers become resistant to these drugs. When humans and animals come into contact with water and river products, resistant microbes enter their bodies and exchange genetic information about resistance with other bacteria, in particular with pathogens of severe infectious diseases. Thus, antibiotics from water gradually lead to the transformation of ordinary bacteria into resistant pathogens of deadly diseases, such as pneumonia, tuberculosis, syphilis, meningitis, sepsis, etc. As a result, in the near future, humanity risks being left without effective means of fighting infections.

Azithromycin belongs to the checklist of substances to be monitored throughout the European Union in the field of water policy (Directive 2015/495/EC of March 20, 2015) as its content above 90 ng/L in water poses a significant risk to the aquatic environment throughout the European Union (6). Azithromycin is an antibiotic of the macrolide group, it is characterized by a wide spectrum of activity against Gram-positive and Gram-negative bacterial pathogens, used to treat infections, most often those that cause middle ear infections, sore throat, pneumonia, typhoid fever, bronchitis and sinusitis (mild to moderate severity). In recent years, it has been widely used mainly in the treatment of bacterial infections in children and in the treatment of children with weakened immune systems (7). Azithromycin is taken by people more often than other antibiotics. It has a convenient mode of administration (once a day), a short course of treatment (3–5 days) due to its long half-life and a small number of adverse reactions (these are mainly gastrointestinal disorders and hypersensitivity reactions). Indeed, the antibiotic does not have any difficulties in its use despite the fact that it is an important and relatively new chemotherapeutic drug in the range of broad-spectrum antibiotics. Another impetus for its widespread use was the COVID-19 pandemic. Azithromycin is known to have anti-inflammatory and immune regulatory effects, which has led to its prescription to patients with COVID at the beginning of the pandemic when many existing drugs were used off-label. Although the effectiveness of azithromycin when combined with hydroxychloroquine has not been confirmed by research, as evidenced by clinical data, people continue to use azithromycin for viral infections (8). Note that broad-spectrum antibiotics, including azithromycin, are most often subject to polyresistance since they neutralize a large number of bacteria, as opposed to narrow-spectrum ones that affect certain populations. The popularity of azithromycin in recent years and its widespread use during the pandemic was the reason for its choice as the object of our research.

AIM

The purpose of our work was to develop a method for determining azithromycin in water using the thin-layer chromatography (TLC) method. This will allow not to use the complex and expensive equipment, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS/MS), which is not available in every laboratory in our country.

MATERIALS AND METHODS

The study object: azithromycin, a broad-spectrum antibiotic of the macrolide group.

The study subject: development of a method for determining azithromycin in wastewater and surface water.

Research methods: TLC, calculation of predicted effect concentration (PEC) and predicted effect concentration/predicted no-effect concentration (PEC/PNEC) ratio indicators, analysis of scientific sources.

RESULTS AND DISCUSSION

In order to assess the surface water contamination with medicinal products, it is necessary to use indicators such as predicted effect concentration (PEC). According to the Guideline on the environmental risk assessment of medicinal products for human use adopted by the EMA (9), the formula (Fig. 1) is used.

EMA formula

$$PEC_{surfacewarter} = \frac{DOSE_{ai} \cdot F_{pen}}{WASTERW_{inhab} \cdot DILUTION}$$

DOSEai -	the maximum daily dose of the drug consumption in a particular region per capita (mg/person * number of days);
Fpen -	the market penetration share;
WASTEWinhab -	the volume of wastewater per inhabitant per day (L/person*day), regional data;
DILUTION -	the dissolution rate (regional data).

Fig. 1. EMA formula.

This formula allows us to predict the impact of a pharmaceutical substance on the environment and is usually used to assess the environmental profile of new substances.

To determine the PEC of medicines existing at the pharmaceutical market and entering surface water through the sewage system, the following formula is used. Predicted effect concentration is calculated based on the known volume of the drug consumption in a particular region, the volume of wastewater per person, and the degree of the drug elimination (10) (Fig. 2).

$$PEC_{surfacewater} = \frac{M_{ai} \times F_{excr} \times 10^9}{WASRERW_{inhab} \times N \times DILUTION \times 365}$$

where

$PEC_{surfacewater}$ -	predicted effect concentration (PEC) of API in surface waters, (µg/L);
Fexcr -	the degree of API elimination from the body as a result of physiological excretion;
Mai -	sales of the drug calculated with reference to API per year, t;
N -	number of residents of the region, persons;
WASTEWinhab -	the volume of wastewater per inhabitant per day (L/person*day), regional data (by default, it is considered equal to 200 L);
DILUTION -	the dilution ratio of wastewater in surface water (regional data), (by default, it is considered equal to 10).

Fig. 2. Determination PEC of medicines.

If the PEC values obtained are below 0.01 µg/L, it is assumed that the drug will not carry an environmental risk after its intended use by the patient. If the PEC value is equal to or higher than 0.01 µg/L, then the PEC/PNEC ratio is calculated. The predicted concentration that does not have a harmful effect on the environment, PNEC, is reference data and is determined for each substance based on information about the toxic effects of substances on water bodies. If the PEC/PNEC ratio is <1, there is no risk of the negative environmental impact (at the time of determination), and if PEC/PNEC is >1, then the drug is probably found in surface waters in concentrations that are dangerous to the environment (9).

To calculate the predicted environmental concentration, data on the sale of azithromycin in Ukraine in 2020, where 40 trade names were presented, were used. Data on the current population of Ukraine as of December 1, 2020 were taken from the website of the State Statistics Service of Ukraine, 1998–2021 (11). The degree of azithromycin elimination in unchanged form is 50.

The calculation of PEC for azithromycin

$$PEC_{surfacewater} = \frac{M_{ai} \cdot F_{excr} \cdot 10^9}{WASTERW_{inhab} \cdot N \cdot DILUTION \cdot 365}$$

$$= \frac{5546{,}77\,kg \cdot 50\% \cdot 10^9}{200\,l \cdot 41629926\,pers \cdot 10 \cdot 365} = 9{,}13\,mkg/l$$

Fig. 3. The calculation of PEC for azithromycin.

The calculation of PEC for azithromycin is presented on Fig. 3.

The PEC of azithromycin is 9.13 µg/L, which is 36.52 times higher than the norm of the API content in surface waters. The PEC/PNEC ratio is calculated in Table 1 (12).

Table 1. The PEC/PNEC ratio of azithromycin.

PEC, µg/L	PNEC [12], µg/L	PEC/PNEC Ratio
9.13	0.25	36.52

The resulting PEC/PNEC ratio >1 means that the current rate of the azithromycin consumption poses a threat to the environment. Therefore, it is appropriate to develop a method by which it would be possible to determine the permissible limits of the azithromycin concentration in surface and wastewater using the TLC method.

The aim of our study was to develop the method for determining the permissible limits of the azithromycin concentration in surface and wastewater, to select the optimal mobile phase for chromatography and a developer that would clearly detect the chromatographic zones of azithromycin.

The test was performed by TLC. This method has a number of advantages: the use of inexpensive equipment, the ability to detect a compound with high sensitivity and selectivity. In addition, due to its cost-effectiveness, this method has a wide range of applications. Two solvent systems were used. As mobile phases, we used a mixture of methanol-chloroform-pyridine with the ratio of 9:8:1 (System 1) and chloroform-ethanol with the ratio of 1:1. (System 2). Chromatography was performed by the ascending method on Sorbfil plates. The sorbent type was CTX-1BE silica gel, the fraction—8–12 microns, the sorbent layer thickness—100 microns, the substrate type—PETF, silicasol binding agent, additional substance—luminophore, the plate size—10×10 cm. The model and test solutions of azithromycin were applied to the start line using the microcapillary method by fine application. The model solution of azithromycin was prepared by dissolving 0.0030 mg of azithromycin in 100 mL of methanol (azithromycin concentration—30 μg/mL). To prepare the test solution, 1 mL of the model solution was added to a 1 L flask, and diluted with tap water to 1 liter. The test solution was concentrated by evaporation under vacuum at a constant temperature of 35–40°C to the dry residue. The dry residue was diluted with 1 mL of methanol and filtered through a paper filter.

After the chromatography, the plates were dried, and the samples were treated with Dragendorff's reagent (solution of bismuth iodide in potassium iodide and in hydrochloric acid) and UV radiation (the color of the interaction products was determined or the appearance of spots in UV light at a wavelength of 254 nm was observed). It was found that chromatography in both systems proposed was optimal since there was a fairly high mobility of azithromycin in these systems, and Rf was in the range from 0.2 to 0.7 (Rf=0.64 in System 1, Rf=0.66 in System 2). When treating chromatograms with Dragendorff's reagent the spots did not develop clearly. When using UV light (UV lamp 254 nm) as a chromatogram developer, the identical azithromycin spots of the same size were clearly visible; therefore, we suggested that UV light should be preferred as a chromatogram developer (Table 2).

Table 2. The chromatography results of azithromycin.

System	Rf	Detection Results	
		Dragendorf's reagent	UV light (254 nm)
№ 1 (methanol-chloroform-pyridine)	0.64	fuzzy	fluorescence
№ 2 (chloroform-ethanol)	0.66	fuzzy	fluorescence

CONCLUSION

Surface water pollution with pharmaceuticals is a complex and important problem that requires comprehensive approaches in many areas of human life, such as treatment, farming, crop production, pharmaceutical production and drug circulation. Without paying attention and monitoring to at least one of these industries, it is difficult to achieve the effect of reducing the release of pollutants into water and to stop the inevitable development of polyresistance. The problem does exist, and this can be seen from our research. The high predicted ecological concentration of azithromycin in water, which is many times higher than normal, indicates that surface waters of Ukraine are polluted by it. Contaminated surface water is the main source of drinking water that cannot be completely purified from the antibiotic. Hypothetically, we are treated with azithromycin, drink it with water, and eat it with animal and plant foods. It is not difficult to guess what will happen in a few years of such intense and incessant ingestion of it. The irrational use of azithromycin can lead to another loss of an important antibiotic from a number of the necessary chemotherapeutic drugs due to bacterial resistance to it. To confirm the PEC studies, the method that allows us to determine azithromycin in water has been developed using the available TLC method.

The developed technique was tested in the wastewater of Zolochiv district of Kharkiv region. Using conventional analytical scales and universal chromatography in thin layers of a sorbent, it is possible to identify azithromycin with a water concentration of ≥30 μg/mL without complex and expensive equipment, such as HPLC or LC/MS/MS. The results showed that the concentration of azithromycin is less than 30 μg/mL.

REFERENCES

1. Directive 2013/39/eu of the European parliament and of the council. EUR-Lex. 2013. (Position statement). Available from: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32013L0039.
2. Minuhina VV, Zviagintseva TV. Antibiotikorezistentnist mikroorganizmiv: mekhanizmi rozvitku y shlyakhi zapobigannya. 2014:1-16. Ukrainian.
3. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and humans begins in China: a micribiological

and molecular biological study. The Lancet Infectious Diseases. 2016;16(2):131-264. doi: 10.1016/S1473-3099(15)00424-7.

4. Bereznyakov IG. Antibiotikorezistentnist v Ukraini za rezultatami doslidzhennya AURA (ch.1). Zdorovia Ukrainy. Khirurhiia, ortopediia, travmatolohiia, intensyvna terapiia. 2020:5(43). Ukrainian.

5. Natasha Gilbert. World`s rivers «awash with dangerous levels of antibiotics». The Guardian. 2019. Available from: https://www.theguardian.com/society/2019/may/27/worlds-rivers-awash-with-dangerous-levels-of-antibiotics?CMP=aff_1432&utm_content=The+Independent&awc=5795_1559033484_e96fe4384b48a372d4d0acc593a05db7

6. EUR-Lex. Commission implementing decision (EU) 2015/495 of 20 March 2015 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council (Position statement). 2015. Available from: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2015.078.01.0040.01.ENG

7. Gyselinck I, Janssens W, Verhamme P, Vos P. Rationale for azithromycin in COVID-19: an overview of existing evidence. BMJ Open Respir Res. 2021:8(1);e000806. doi: 10.1136/bmjresp-2020-000806.

8. Lamback EB, Oliveira MA, Haddad AF, Vieira AFM, Neto ALF, Maia TDS, et al. Hydroxychloroquine with azithromycin in patients hospitalized for mild and moderate COVID-19. Brazilian J Infect Dis. 2021;25(2):101549. doi: 10.1016/J.BJID.2021.101549.

9. European Medicines Agency. Guideline on the environmental risk assessment of medicinal products for human use (Position statement). 2006. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-environmental-risk-assessment-medicinal-products-human-use-first-version_en.pdf

10. Yevseeva LV, Netesova KYu, Gubin YuI, Zhuravel IA. Otsenka farmatsevticheskikh zagryazniteley poverkhnostnykh vod Ukrainy. Upravlinnia, ekonomika ta zabezpechennia yakosti v farmatsii. 2016;4(48):4-10. Ukrainian.

11. Chyselnist naselennia ta serednia chyselnist za periody roku. 1998-2021. Derzhstat Ukrainy. Available from: http://www.ukrstat.gov.ua/operativ/operativ2020/ds/kn/kn_u/arh_kn2020_u.html. Ukrainian.

12. List of Predicted No-Effect Concentrations (PNECs). 2018. AMR Industry Alliance Antibiotic Discharge Targets. Retrieved from https://www.amrindustryalliance.org/wp-content/uploads/2018/09/AMR_Industry_Alliance_List-of-Predicted-No-Effect-Concentrations-PNECs.pdf