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ANALYZING MACROLIDE ANTIBACTERIAL AZITHROMYCIN IN ENVIRONMENTAL SPECIMENS

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ABSTRACT

Recently, the issue of pharmaceutical chemicals being found in wastewater has drawn attention due to its potential effects on human health as well as the environment. An important class of medications used to treat human infections and in veterinary care are antibiotics. Since many of them are not entirely digested by the body, between 30% and 90% of them are excreted and end up in wastewater. Since antibiotics have a direct biological effect on microorganisms and can lead to the development of antimicrobial-resistant bacteria (ARB), they pose a potential risk if released into the environment. A third category of well-known oral antibiotics is the macrolides. By attaching themselves to the bacterial “50S ribosomal subunits” and preventing protein synthesis, macrolides demonstrate their antibacterial effect. The first 15-membered macrolide on the market, azithromycin (AZM), was created in 1980 and proved to be more stable in an acidic environment than erythromycin. With the surge of covid 19 cases worldwide, there are few reports claiming that azithromycin is helpful in improving the conditions of patients along with other medications. However, in further studies it is recommended in Covid patients with concomitant bacterial infections are either suspected or confirmed. The presented paper covers the role of AZM in Covid, analytical methodologies for its determination in environmental samples, sample preparation and extraction procedures. The presented review will be helpful for the researchers in development of further studies in this area.

KEYWORDS: Macrolides, Azithromycin, Environmental samples, Pollutants, Wastewater, Drugs in soil.

INTRODUCTION

A trillion-dollar industry, the pharmaceutical sector produces merchandise that upgrade the existences of billions of individuals. To acquire an upper hand and meet contemporary standards with respect to the safety and ecological effect of its processes and products, the pharmaceutical industry should foster improved production systems.[1] For the first time ever, global pharmaceutical revenues exceeded $1 trillion USD in 2014.The market has been expanding at a rate of 5.8% annually since 2017. Revenue from the global pharmaceutical market was USD 1143 billion in 2017 and is expected to be USD 1462 billion in 2021.[2] World pharmaceutical market is rapidly evolving. Pharmaceutical industry is one of the industries that can significantly contribute to economic development and create added value.[3] In the 19th century, research into the therapeutic properties of animals, minerals, and plants led to the establishment of the pharmaceutical industry. There are several reasons why pharma emerging markets are expanding so quickly. The first is the patent cliff, which affects several well-known drugs that have been on the market for decades. Second, there has been an increase in the availability of biosimilar medicines and a shift toward the use of generic medications in both developed and developing nations. The evolution of disease patterns in developing nations is the third factor. Lastly, a crucial factor in the expansion of the pharmaceutical industry was the significant gap between prices and manufacturing costs.[4]

Pharmaceutical compounds and their metabolites have become major concerns in recent years due to their biological activity in soil and surface water bodies.[5] As drugs contribute significantly to human well-being, it becomes imperative to safeguard not only their intended therapeutic effects but also to assess and manage their impact on the surroundings.[6] Antimicrobial agents stand out among the many kinds of drugs because of the risk they pose to the natural environment. Receiving waters should anticipate the presence of antibiotics given their widespread use.[7-9] Antibiotics are a type of medicine that are used in farming, medicine, and veterinary care. Their presence is cause for concern because they may harm aquatic organisms. For instance, this suggests that they encourage resistance in bacteria.[10]

Among the many types of drugs, anti-microbial agents get specific consideration concerning their gamble to the natural environment. Since antibiotics are frequently prescribed worldwide.[7–9] receiving waters should anticipate their presence. One of the most widely used pharmaceuticals is antibiotics, which are utilized in farming, medicine, and veterinary care.[10] Their presence is cause for concern because they may harm aquatic organisms. They may, for instance, encourage bacterial resistance, as suggested in.[11–14] It is not surprising that recent studies on the presence of environmental micropollutants have included antibiotics. Multiple field campaigns have investigated pharmaceutical concentrations in receiving waters, varying in magnitude based on location and substance. Because the temporal variation of pharmaceutical concentrations is an additional pressure on the preservation of aquatic systems, understanding such variations is a major obstacle in environmental assessment and management.[15]

Azithromycin (AZM, Figure 1) is “(2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-11-[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-2-ethyl-3,4,10-trihydroxy-13-[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-imethyloxan-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one” (IUPAC name) the macrolide class of antimicrobials. By reversibly binding to the “23S rRNA of the 50S ribosomal subunit of the bacterial ribosome of susceptible microorganisms”, AZM prevents the assembly of the 50S ribosomal subunit upon oral administration, thereby inhibiting the translocation step of protein synthesis. Cell death, cell growth inhibition, and protein synthesis inhibition are all affected by this.[16]

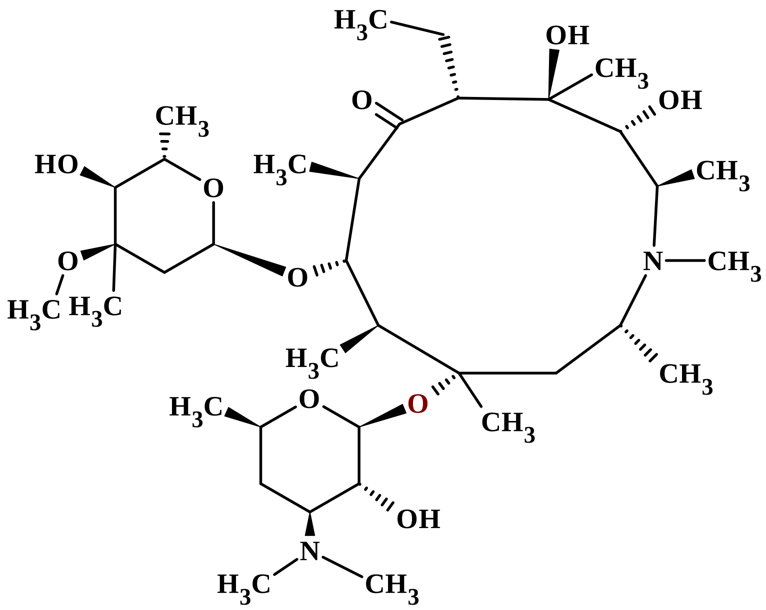


Figure 1: Chemical structure of Azithromycin.

AZM can be taken orally or via parenteral (intravenous) route. AZM's extended-release formulation has been discontinued. For three to five days, the usual dose is 250 mg or 500 mg once daily; in more severe infections, a higher dose is used. Tablets (250 mg or 500 mg), packets (one gram dissolved in one-fourth cup or sixty ml of water), and suspensions for reconstitution (100 mg/5 ml or 200 mg/5 ml) are examples of oral formulations, with or without food. A 500 mg preservative-free solution of intravenous (IV) is available for reconstitution. AZM should not be administered via intramuscular injection or IV bolus; it should be infused over at least 60 minutes. The 1% ophthalmic solution, which is used to treat bacterial conjunctivitis, comes in a 2.5 ml bottle.[17] At gastric pH, AZM is more stable than erythromycin and rapidly and extensively penetrates tissues, reaching high concentrations throughout most of the body. No metabolite is thought to have significant pharmacological activity, and most of an absorbed dose is eliminated unchanged, primarily in the feces.[18]

Erythromycin is the source of AZM; however, it differs chemically from erythromycin.[19] The first 15-membered macrolide (AZM) was synthesized in 1980 by PLIVA Laboratories researchers. It is identified by the addition of a basic -N atom to the macrocyclic ring. In the era of macrolide antibiotics, this represented a breakthrough. Within a short time, AZM established itself as one of the most popular antibiotic brands worldwide. Compared to erythromycin, its minimal inhibitory concentration (MIC) ranged from 16 to 128 g/mL for 90% of *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia* strains. In terms of stability in acidic conditions because of aza-methyl substitution, AZM outperformed erythromycin. The addition of the second -NH_2 group increases half life and tissue penetration compared with erythromycin.[20] AZM distinguishes itself from erythromycin by being better able to inhibit certain gram-negative bacilli, such as *Moraxella catarrhalis* and *Haemophilus influenzae*. AZM is the most effective macrolide against Toxoplasma and has *in vitro* activity against *Crytposporidium* and *Pneumocystis carinii*.[18] It also possesses potent immunomodulatory and anti-inflammatory properties that have been clinically beneficial.[21] After the extensive use of antibiotics during COVID 19 pandemic, it was expected that azithromycin will be present in irrigation water.[22]

Azithromycin in Covid

The published data shows 12,943,741,540 vaccine doses administered till November 15, 2022. If we consider three doses per person means, there are still lot of people yet to receive vaccine.[23] Thus, the practitioners must rely mostly on the available pharmacotherapy. One of the potential micropollutants that has been measured in the environment and WW is pharmaceuticals.[24] Following the COVID 19 pandemic, it is very likely that this antibiotic will be distributed more widely in water bodies due to the significance of increasing prescriptions of AZM.[25]

As a result, macrolides have been suggested as treatments for COVID-19 and other viral respiratory infections with an inflammatory basis.[26] In two distinct phases of the disease, AZM's immunomodulating properties are demonstrated: during the acute phase and after the chronic inflammation has subsided. The ability of AZM to reduce the production of pro-inflammatory cytokines like "IL-8, IL-6, TNF alpha, and MMPs" is clearly demonstrated during the acute phase. Also, macrolide increases oxidative stress and neutrophil apoptosis during the resolution phase.[27]

In response to several reports, the FDA observed a slight increase in cardiovascular deaths and deaths from any reason among AZM 5-day cycle patients in 2012. Arrhythmia risk was hypothesized to rise with AZM's QTc increase. Despite the existence of a few hypotheses, it is currently unknown how AZM might combat SARS-CoV-2. However, there is some *in vitro* evidence that AZM may prevent the replication of other viruses.[28] such as the "human influenza virus H1N13".[29] and the "Zika virus".[30] On March 12, 2013, a communication regarding the safety of AZM for heart rhythms was published by the FDA. In it, the agency warned of the possibility of outcomes that could result in death.
In an in vitro study, Poschet and colleagues[31] discovered that AZM increased the pH of host cells, which may hinder viral entry, replication, and spread. In addition, a distinct furin-like cleavage site in the spike protein—the protein that enables viral entry into host cells—is thought to be present in SARS-CoV-2. Poschet *et al*.[31] discovered that azithromycin inhibits viral entry by lowering levels of the enzyme furin in host cells.

Practitioners had some faith in this theory, but in 2020, the "National Institute for Health and Care Excellence (NICE) in the United Kingdom".[32] issued a guideline stating that antibiotic therapy should only be administered to COVID-19 patients when bacterial co-infection is either suspected or confirmed. Additionally, the most recent findings that have been published.[33] confirm that community patients infected with SARS-CoV-2 do not benefit from AZM treatment, raising concerns regarding the potential dangers associated with its inappropriate use.

Background

Analytical methodologies are important to understand the components of any pharmaceuticals, chemical or environmental samples present in various matrices.[34,35] Several antibiotics, including AZM, were recommended for the treatment of asymptomatic, mild, moderate, and severe COVID-19 with or without complications during the outbreak. AMR is a "hidden" pandemic that threatened healthcare delivery worldwide and claimed 700,000 lives annually prior to the COVID-19 outbreak.[36] Unfortunately, resistance eventually developed to nearly all antibiotics that had been developed.[37,38] In primary care, where viruses cause most infections, antibiotic overprescribing is a particular issue. General practitioners write about 90% of all antibiotic prescriptions, most of which are for RTIs. An infection with bacteria that are resistant to antibiotics may result in severe illness, an increased risk of complications and hospitalization, and higher mortality rates.[39,40] Antibiotic resistance is one of the potential and well known threat in curing patients. One of the reasons is improper disposal of antibiotics in environmental samples. The presented article is regarding highlighting the threat of antibiotic usage, in addition the role of macrolides (Antibiotics) in covid infections and suitable analytical methods for its determination in environmental sample. The presented data will be useful for the further development of more ecofriendly, simple and justified analytical methods in this regard.

Analytical methods

The literature survey of environmental analytical separations of AZM was performed in different scientific databases and world wide web. The summary of all analytical methods is given under Table 1. For determination of AZM in different environmental samples including WW, soil or seavage etc., most of the researchers published chromatographic analysis. In most of these methods, separations were based on MS detector. These detectors are well known for its sensitivity and proven applications for low concentration determinations of analyte. Few spectrophotometry-based methods are also available in the current literature.

Table 1: Summary of analytical methods.

Method	Description	Column	Matrix	LOD	LOQ	References
LC-MS	50:24:2:24 ACN, MeOH, THF, and 0.04 M NH_4OH, 0.3 ml/min	C_{18} (30 × 2.0 mm)	Municipal wastewater	-	4.25 pg/ml	[41]
LC-MS	Gradient A: 10% ACN, B: 90% H_2O + 0.1% HCOOH, 0.2–0.3 mL/min	C_{18} (100 × 2.1 mm,3.5 µm)	Water treatment facilities	-	-	[42]
UHPLC-MS	Gradient: A: ACN, B: H_2O + 0.1% HCHO, 0.5 mL/min.	C_{18} (50×2.1 mm i.d., 1.8 µm)	Hospital, urban influent and effluent WW, river water.	-	-	[43]
LC-MS	, A and B were ACN: H_2O + 0.1% HCOOH	C_8 (150 × 4.6 mm, 3.5 µm)	Surface water analysis	-	-	[44]
LC-MS	MeOH/HPLC water (50:50, v/v), 2 mL/min	C_{18} (100 mm × 4.6 mm, 3.5 µm)	Treated, ground and surface water (9 antibiotics)	-	-	[45]
LC-MS	Gradient elution, A: 0.1% HCHO in water, B: ACN, 0.4 ml/min	C_{18} (150 × 3 mm; 3 µm)	Synthesis intermediates, transformation products in WW effluents and ambient waters (with 3 other macrolides)	-	26, 13, 10 ng/ml, in wastewater, secondary effluents, river water resp.	[46]
HILIC-MS	Gradient elution, A: CH_3COONH_4 buffer (pH = 6.7), B: ACN, 0.6 mL min^{-1}.	150×2.1 mm, 2.7 µm, 50 °C.	WW	0.70 ng/ml	2.20 ng/ml	[47]
FTIR	Spectral range	-	Soil samples	0,76689	2.55630 g/L	[48]

	1744 to 1709 cm^{-1} (Carbonyl's stretching)			g/L		
LC/MS	C_{18} column	150 × 4.6 mm; 5 µm	Hospital WW	-	-	[49]
HPLC-UV	Gradient A: 0.2% HCHO, B: MeOH (+0.2% HCHO), 0.6 ml/min	C_{18} 250×4.6mm, 5µm	WWTP	-	-	[50]
LC-MS/MS	C_{18} column	150 × 3 mm; 3 µm)	Municipal WWTP	-	-	[51]
Differential pulse voltammetry (DPV)	Screen-printed carbon electrode (SPCE), 4-aminobenzoic acid (4-ABA) solution	-	Analysis of tap water and water samples	0.08 µM	0.3 µM	[52]
LC-MS/MS	Gradient: A: H_2O (0.4% HCHO) + 5 mM $HCOONH_4$, B MeOH/ACN 1:1 (v/v)	C_{18} 100 × 2.1 mm, 2.6 µm	WWTP	2.0 ng/L	6.5 ng/L	[53]
UHPLC-MS/MS	Gradient A: 0.1% HCHO in H_2O, B: 0.1% ACN, 0.4 mL/min	C_{18} (50 × 2.1 mm	Surface Water Samples	2 ng L^{-1}	8 ng L^{-1}	[54]
LC-MS/MS	0.1% HCHO & ACN (50:50)	C_{18} (2.1×100 mm, 1.8 µm)	Contamination level of River and Fish Farm	0.017 µg/L	0.05 µg/L	[55]
UHPLC-MS	Gradient, A: 0.1% HCHO in H_2O & B: 0.1% HCHO in ACN	C_{18} (50 mm × 4.6 mm × 3 µm)	WW Effluents and Municipal Dumpsite Leachates	-	-	[56]
HPLC-MS/MS	Gradient, A: HCHO 0.1% in H_2O & B: ACN	C_{18} (3.0 × 75 mm, 3.5 µm)	WWTP	-	2.13 ng/L	[57]
HPLC-PDA	C_{18}	(250×4.6 mm, 5 µm)	WW	-	-	[58]
HPLC-UV	-	-	Adsorption and desorption on/from soils	-	-	[63]
Electrochemical	Ag_2Se/β-cd/rGO modified GCE	-	Industrial water	0.0045 nM	-	[64]

Sample preparation and extraction methods

In the method developed by Koch *et al* [41] the extraction procedures involves usage of K_2CO_3 solution and methyl-*t*-butyl ether by centrifugation process and finally drug was extracted in solvent. In the method proposed by Ferrer *et al*.[42] HLB cartridge was used for extraction of drug using SPE method. For conditioning of column small amount of methanol was used. Similarly, in another method by Gros *et al.*[43] and Ferrer & Thurman.[44] mostly utilizes water for preparation of samples. In their research, methanol in small quantity was utilized for conditioning of SPE cartridges. The Response Surface Methodology (RSM) was firstly used for optimization of extraction process by Mirzaei *et al.*[45] The selected parameters reported were amount of Na_4EDTA, pH and the volume of solvent used in elution. The pka values of AZM (8.74) shows that it they can be recovered in higher values of pH and volume of elution solvent (methanol) was the genuinely critical model term in extraction effectiveness. The study also found decrease in extraction efficiency due to presence of chelating agents like Na_4EDTA may present in the sample matrix and glassware. The method published by Senta *et al.*[46] samples were separated at the pH (7–7.5) using HLB cartridge and limited quantity of methanol. Similar type of SPE method followed by another researchers [47] using ACN in place of methanol before LC-MS analysis. In SPE extraction process performed by Sija *et al.* [48], the cartridges (HLB) were allowed to dry for one day and then eluted with 20 ml acidified methanol and small amount of sodium sulphate (anhydrous) was used as hydrating agent. The proposed method of Senta *et al.*[50] related to analysis in wastewater, the residues obtained after SPE were reconstituted using 0.5 mL of 100 mM $HCOONH_4$/MeOH (1/1, v/v) for instrumental analysis.

Diatomite based removal method of AZM.[51] from aqueous solution is also available. The heavy metals and some organic compounds are adsorbed on the surface of diatomite and after some modification on its surface can also be used to remove drug. In this method, nano diatomite with saponin was used to improve removal of AZM from aqueous solutions. In the further investigation by researchers, the increase in adsorption of drug is due to the presence of large number of saponin's hydroxyl groups on the modified diatomite surface. In published electroanalytical method.[52] an electrochemical molecularly imprinted polymer (MIP) sensor that was electropolymerized by cyclic voltammetry (CV) on a screen-printed carbon electrode (SPCE) with AZM serving as a template molecule in a solution containing 4-aminobenzoic acid (4-ABA), claimed to be "environmentally friendly strategy determination in environmental water".
After filtration for removal of suspension solids from samples, dilutions were prepared in 20% mixture of methanol and acetonitrile acidified with 0.1% formic acid before chromatographic separations.[53] The goal is to use TiO2 in suspension to treat specific antibiotics (including azithromycin) that are present in actual wastewater in a photocatalytic plant. The photocatalytic assays shows removal of AZM from wastewater after 120 min of treatment. The seawater analysis was performed by liquid–liquid extraction (LLE) and addition of methyl tert-butyl ether (MTBE) in small amoun..[54] After separation organic phase was used for chromatographic analysis. The analytical work by Kabir *et al.*[55] is determination of contamination level (antibiotic residues) in river and fish farm water. The LLE method was employed using dichloromethane before chromatographic separations.

The SPE system was also utilized by Ncube *et al.*[56] and final dilution was prepared in 0.1% formic acid in methanol. The method targets analysis of antibiotics in effluents from wastewater treatment plant and leachates from municipal dumpsite.

The UV-assisted removal of nano composite-based separation method from aqueous solution published by Mehrdoost *et al*.[57] the researchers used PAC/Fe/Si/Zn nano composite in which “Fe, Si and Zn” were loaded on the activated carbon powder (PAC).

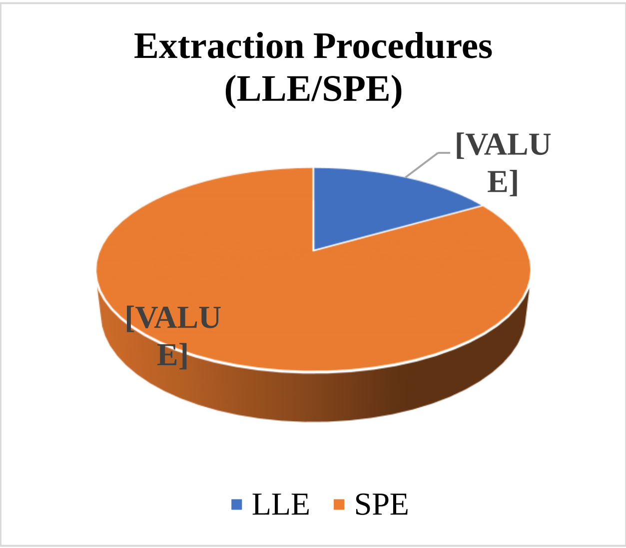
The determination in WWTPs using SPE and HPLC-MS/MS method [58] is for the seasonal changes, occurrence, environmental risk assessments and removal efficiencies of macrolide antibiotics including AZM. In another published method.[59] for removal of AZM in aqueous solutions. Researchers used *Dalbergia sissoo* sawdust to prepare activated carbon (AC) and magnetic activated carbon (MAC). The biosynthesized Hematite nanoparticles (α-HNPs) based separation method for wastewater, developed by Al-Hakkani *et al*.[60] The study extended its application in treatment of Covid-19. The topic is out of context in this paper, hence curious authors are suggested to go through this publication for further details.

A bioreactor, “anaerobic upflow sludge blanket (UASB)” for the breakdown of AZM-contaminated high strength synthetic WW.[61] In this paper, researchers suggested use of a already published colourimetric method ($\lambda = 482$ nm).[62] for the determination of AZM in wastewater and treated wastewater.

The method of analysis of AZM in soil performed by Cela-Dablanca *et al*.[63] using 0.005 M $CaCl_2$ as background electrolyte before chromatographic analysis (HPLC-UV). The metal chalcogenides were Ag_2Se anchored on β-cyclodextrin polymer with reduced graphene oxide (β-cd/rGO) for detection of AZM in environmental samples was performed by Santhan A *et al*.[64] the linear range and detection limit found was 0.023–971.7 µM and 0.0045 nM respectively. The summary of extraction procedures given in Table 2.

Table 2: Summary of extraction procedures.

Reference	Method
Koch *et a*. [41]	LLE
Ferrer *et al*.[42]	SPE
Gros *et al* [43]	SPE
Ferrer & Thurman.[44]	SPE
Mirzaei *et a* [45]	SPE
Senta *et al* [46]	SPE
Sija *et al* [48]	SPE
Senta *et al*[50]	SPE
Kabir *et al*.[55]	LLE
Ncube *et al*[56]	SPE
Mehrdoost *et al*.[57]	SPE
Pan & Yau.[58]	SPE



DISCUSSION

In the study published by Sulis *et al*.[60], COVID-19 pandemic has exacerbated the widespread and frequently inappropriate use of antibiotics, particularly in low- and middle-income countries (LMICs). For instance, in the case of India, AZM sales increased by 34.4% over the previous year's corresponding months, from June to September 2020, but sales decreased after the first epidemic wave's peak. In addition, common infections are becoming increasingly challenging to treat because of rising AMR, particularly antibacterial resistance in bacteria. Thus, this is obvious that suitable methodologies should be there to monitor the presence of antibiotics in environmental samples. This knowledge gap about AZM forms background of this study. During our literature survey (Also consider data given in Table 1), many chromatographic methods were found. The chromatographic methods are known for its better separation efficiency even in the case of trace analysis which is particularly important in the case of analysis in environmental samples. In most of the published methods, SPE methods (Table 2) were used utilizing some quantity of solvents particularly methanol. Also, various mobile phases including solvents were used in these methods. Researchers should approach towards more enviro-friendly and low cost electroanalytical method[49] but limited efficiency in separation restricted application for this cause.

CONCLUSION

The pharmaceutical industries play key role in development and bringing innovative medicines for the improvement of quality of life and health of people world wide. They also have critical role in employment generation and contribution in economical growth. The COVID-19 pandemic had an impact on the global economy, which included the pharmaceutical industry. From conducting research and development on potential treatment options to maintaining a healthy supply chain for medications during times of crisis, the pharmaceutical industry is assisting governments in addressing the COVID-19 unmet needs. Although there is presently no cure for this unique viral disease. There is a surge in the demand of medicines that was initially thought to be effective in this crisis, including AZM. One of the most crucial phases in the analytical process is sample preparation. Since the analytes are often preconcentrated, this procedure can enhance the determination's sensitivity as well as its selectivity by reducing matrix interferences. The summary of all analytical methods for the determination in different environmental matrices are given under Table 1. The presented knowledge can be used by researchers for development of more efficient, economic and environmentally friendly methods for determination of AZM in environment samples. Another intension of present manuscript is to draw attention of scientists, regulatory bodies and health practitioners regarding justified usage of antibiotics. The method development and validation of complicated samples like drug’s environmental samples is tedious task. Thus, the presented article will help analytical scientist in developing suitable sample in the future also.

Conflict of interest: None declared.

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