

Techniques and Methods: Detection of antibiotics in environmental samples

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Use of antimicrobial substances had been extensive since the 20th century which revolutionized medicine along with vaccination against infection. The existence and detection of antibiotics in environment have recently drawn attention with the advent of sophisticated analytical chemistry methodologies [1]. The occurrence, source and adverse effect of antibiotic especially in water bodies had been an important issue in order to assess the environmental risk they may pose. The advancement of high performance liquid chromatography coupled mass spectrometry and recent shift from gas to liquid chromatography has remarkably improved the detection and quantification of antibiotics and other pharmaceutical compounds in environmental sample including surface- and groundwater; and soil samples of veterinary firm house etc. [2,3]. The reproducibility, precision and reliability of result for the detection of extremely low concentration of antibiotics has dramatically improved with modern mass spectrometry methods [4]. Antimicrobials such as triclosans and biphenyls were detected in trace amount from urban wastewater and marine sediments using advanced chromatographic and mass spectrometric methods [5]. The present chapter reviews the advancement of technologies for detection, purification, identification and characterization of antimicrobial agents from environmental sources.

Keywords: Environmental samples; Antibiotics; Chromatography; Mass spectrometry

1. Introduction

The goal of the modern analytical chemistry involves detection of exotic contaminants at micro level to access the risk associated with it as well to estimate its adverse effect in environment. Pharmaceuticals, pesticides, hydrocarbons, plastic and polymers, refinery waste, waste from compost plant, slaughterhouse, and milk industry etc. release enormous amount of contaminants in soil, water and streams. Pharmaceuticals, most importantly the antibiotics present in the environment, are highly disadvantageous for the target and non-target organisms. Presence of pharmaceuticals, including antibiotics for the use in veterinary and release in soil or surface water runoff after application of manure has been described [6]. Substantial amounts of antibiotics were detected in municipal sewage, hospital effluents, influents and effluents of sewage treatment plants (STPs), surface water and groundwater [7]. Today, the fate and effect of these compounds in environment and their transformation products is an important topic of discussion due to the widespread usage of antibiotics in humans and livestock and their detection in environmental matrices, such as water and soil. The modern techniques and analytical methods enables concentration of many litres of water extract into few microliters and thereafter separation, purification and identification of compound by sophisticated chromatography and mass spectrometry methods. The method of identification of antibiotics from the environment such as extremely complex matrices including wastewater, soil, sediment, air particulate matter, biota, and human serum has three major components: extraction of compounds from the environmental samples, chromatographic separation and finally identification by mass spectrometry.

The present chapter describes the current methodologies and development in the field of detection of antibiotic in the environment along with a comprehensive description of the reports of identification of antibiotics present in nature.

2. Antibiotics in Environment: Case Studies

A sharp rise of antibiotic usage has been a recent concern worldwide. Currently, estimated 4.5 million pounds of antibiotics are being administered for medical treatment and 21.7 million pounds are used in farm and companion animals within US itself [8]. For the sake of weight gain and prevent losses due to diseases in livestock, significant rise of use of antibiotic has been documented [9, 10, 11]. Therefore, the phenomenal rise of antibiotic use on livestock is one of the sole cause of antibiotic contamination in the environment. Another report suggests that estimated 24.6 million pounds of antimicrobials are administered on livestock each year, in which 10.3 million pounds on hogs, 10.5 million pounds on poultry, and 3.7 million pounds on cattle non-therapeutically [12]. Antibiotics are also prescribed for prevention of disease and promotion of growth in swine with for more than 90% of starter feeds, 75% of grower feeds, 50% of finishing feeds, and 20% of sow feeds [13]. Therefore, the release of antibiotic in environment especially in soil and water bodies are predominantly from urine and faeces of livestock, unused fodder and waste from livestock being

utilized as fertilizer etc. [14]. In another environmental monitoring study, detection of antibiotic suggested large amount of Erythromycin and Tetracycline in sediment samples with concentration range between 82 - 128 $\mu\text{g kg}^{-1}$ [15]. The contamination of antibiotics in soil and sediments were also described by Kim and Carlson, 2007 [16]. The study suggested presence of tetracycline antibiotics and macrolides having concentrations ranging between 2.1 – 24.3 $\mu\text{g kg}^{-1}$. Hu and Coats, 2009 [17] reported strong sorption of antibiotic tylosin in soil with significantly high sorption distribution coefficients between 42 - 65 ml g^{-1} in soils with 0.5 to 4% organic matter. The presence of antibiotics in liquid manure samples has been documented where 20 mg kg^{-1} for sulfonamides, 11 mg kg^{-1} for salinomycin, and 43 mg kg^{-1} for tiamulin were found [18]. Thus, the potential source of antibiotic contamination in water could be through drainage systems or runoff from agricultural fields. More than 18 antibiotic substances were detected in water sample from sewage treatment plant which included erythromycin degradation products such as roxithromycin, and sulfamethoxazole at concentrations up to 6 $\mu\text{g L}^{-1}$ [19]. The most frequently detected antibiotics include tetracyclines, sulfonamides, and macrolides consist of tylosin and roxithromycin. Another report suggests dehydratoerythromycin was the most prevalent macrolide antibiotic detected in water sample where the concentrations was above 50 ng L^{-1} and reached a peak concentrations of 130 ng L^{-1} and 300 ng L^{-1} occasionally [20]. An interesting survey conducted by the United States Geological Survey in 2002 concluded that antibiotics were found in 48% of 139 streams tested which was alarming and needed [21]. The study showed that the highest concentrations of three antibiotics detected were sulfamethoxazole at 1.9 $\mu\text{g L}^{-1}$, erythromycin at 1.7 $\mu\text{g L}^{-1}$ and lincomycin at 0.73 $\mu\text{g L}^{-1}$. Kim and Carlson, 2007 [22] found six compounds close to tetracycline class with concentrations between 0.02 – 0.18 $\mu\text{g L}^{-1}$, while the sulfonamide and sulfamethoxazole were the most frequently detected of this class with concentration nearly 0.11 $\mu\text{g L}^{-1}$. The report presented the data wherein maximum numbers of cases dehydrated form of erythromycin was most abundant macrolide. The highest concentration detected in water was at 0.45 $\mu\text{g L}^{-1}$ with an average concentration of 0.12 $\mu\text{g L}^{-1}$. The other similar reference suggested mixtures of antibiotics detected in 13% of water samples examined [23]. The yearlong effort of various research groups on antibiotics in the environment has provided substantial information on sorption and mobility of a few classes of antibiotics, however, little is known yet on mobility, degradation and fate of antibiotics in the environment [24].

2.1 Source of Contamination of Antibiotic in Environment

The major source of contamination of antibiotics in the environment differs from country to country as per the usage of the antimicrobial substance for various purposes. The hospital effluent and inappropriate disposal of antibiotic is the major cause of contamination of drugs in the environment (Fig. 1). Thomas et al., 2007 [25] showed that less than 10% of certain analysed antibiotics were from hospitals. In Germany, about 75% of antimicrobials substances are administered within the community and 25% in hospitals [26]. However, another study describes that pharmaceutical in municipal sewage water is solely not caused by hospitals [27, 28]. De Wirth et al. 2004 found that 5–20% of the total contaminated antibiotics were from the European hospitals [29]. Whereas, such antibiotic contamination due to community use was reported to be nearly 70% in the United Kingdom [30] and 75% in the United States [31]. Interestingly, the rise of antibiotic in the environment has been correlated with the intake or use of antibiotics without prescription which markedly vary from country to country [32]. The other major source of contamination is by the use of antibiotic in veterinary and fisheries (Fig. 1). Antibiotics are often used to promote the growth of animals in some countries [33]. The modern animal breeding and fattening methods involves use of drugs in low dose for improvement of quality of product, percentage of protein and fat content in meat [34]. It is also administered in fisheries for therapeutic purposes as prophylactic agents and thereby increasing the chances of contamination in water bodies. Such antimicrobial drugs include oxytetracycline, florfenicol, premix, sarafloxacin, and ormethoprim [35]. Grave et al., 2008 reported the usage of antimicrobial drugs in farmed fish in Norwegian aquaculture between the periods of 2000–2005 [36]. Other studies showed that compared to other pharmaceutical products the concentrations of antibiotics in sewage and surface water remains within the same range measured in different countries [37, 38, 39, 40, 41, 42]. In general, concentrations ($\mu\text{g litre}^{-1}$) of various antibiotics were found higher range in hospital effluent, comparatively lower $\mu\text{g litre}^{-1}$ range in municipal wastewater, and in between range in case of different surface-waters, ground-water and sea-water in a harbour [43].

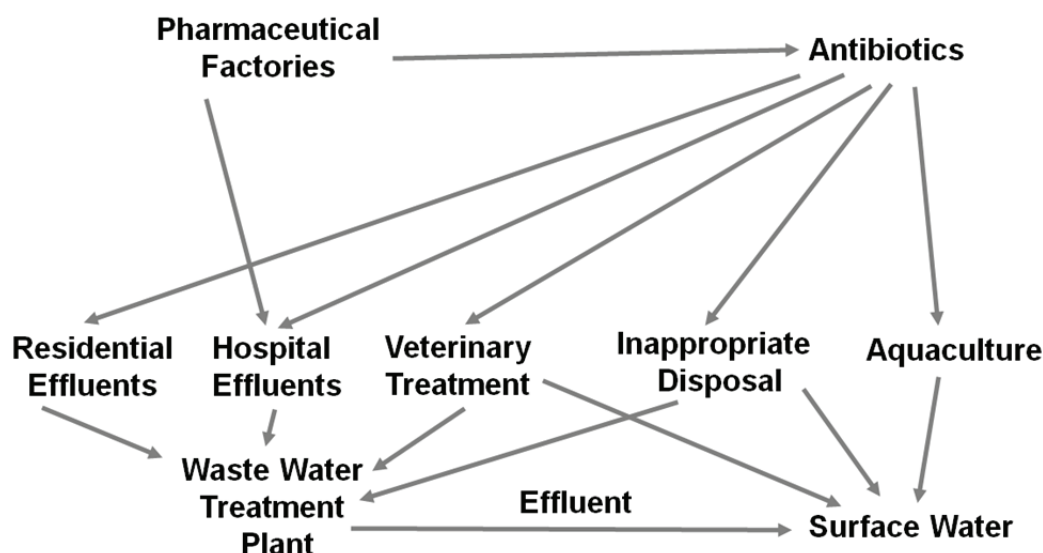


Fig. 1 Major sources of antibiotics contamination in the environment.

3. Methods for Detection of Antibiotics in Environment

The method for detection of pharmaceutically active compounds in the aquatic environment was first developed by LC–MS–MS in the mid-1990s by Ternes group at the Institute for Water Research and Water Technology, Wiesbaden, Germany [44]. Several antibiotics, beta-blockers, X-ray contrast media, and neutral drugs in different water matrices were identified by using GC–MS and LC–MS–MS. However, the research on detection of pharmaceuticals in the environment began in the USA in the late 1970s [45, 46]. Since then an increasing concern for detection of antibiotics and other pharmaceuticals were documented with world-wide interest for high-profile environmental contaminants such as chlorinated dibenzo-*p*-dioxins and dibenzofurans [44]. The first report on fully automated solid phase microextraction (SPME) coupled with LC–MS–MS revealed the presence of numerous pharmaceuticals in the aquatic environment [47].

3.1 Sampling and extraction of antibiotics from environmental sources

The major setback for sampling and extraction of any antibiotic as a natural product from plant, bacteria, *Actinomyces* or as contaminant from various sources remains in its availability in low concentrations in various natural sources. Solid-phase extraction method (SPEM) is the most common method for sampling prior to LC–MS–MS analysis. Most SPE procedures are in general used offline, however, three independent groups have reported the development and use of automated online, miniaturized sample preparation followed by LC–MS–MS analysis. Sixteen antibiotics and five fluoroquinolones were reported to be detected by SPEM approach coupled liquid chromatography and mass spectrometry [47, 48]. The newly developed tube approach coupled with LC-MS-MS enables rapid and high throughput analysis of target analytes online. Stoob et al, 2005 described the use of an on-line SPE–LC–MS–MS system which includes three-directional autosampler, three LC pumps with large volume dispenser, two six-port valves [49]. An important aspect of method validation and procuring consistent result, precision and detection limits are determined by batch to batch performance of methods [50, 51, 52, 53, 54, 55]. Use of method blank and/or method spiked samples ensures maintenance of method performance for a specific batch of samples, and the reagent blank of quality control samples determines no carry over from previous run of analytical standard or samples.

Other sample preparation strategies includes freeze drying and liquid-phase microextraction (LPME), routinely used for pre-concentration of pharmaceuticals. The method relies on immobilization of organic solvents at the tip of a syringe [56] or inside pores of porous hollow fibers [57] for enriching the sample. The method was more selective, however, the precision of the LPME approach must still needs to be improved. In case of SPME, first the water samples are filtered to remove suspended materials to avoid clogging of the cartridges. Then depending on the nature of antibiotics the targets are subdivided into acidic, neutral or basic according to their pKa and to be extracted under corresponding pH conditions. Like every other method, the SPE-based sample preparation is labor-intensive and requires extensive training to the technical staffs for achieving desired proficiency. The reproducibility of these on-line SPE and SPME approaches seemed to be good and the data demonstrate the potential of using automated sample preparation to improve operational efficiency and data quality [47, 48, 49].

3.2 Separation of antibiotics by liquid chromatography (LC)

The separation of organic compounds for identification and purification had a major breakthrough with the advancement of Reversed-phase liquid chromatography (RPLC) technology. In case of reverse phase liquid chromatography, water, methanol, and acetonitrile are the most commonly used solvents of choice for the complete separation of compound mixture present in extracted samples. The right column of choice is another crucial factor to attain successful separation of compound in mixture. In general, standard C8–C18 columns with 5- μm silica, 4.6-mm internal diameter (i.d.), 150 mm long LC columns were widely used for this purpose. The flow rates are set at 750–1,000 $\mu\text{L min}^{-1}$ for analysis and purification. Recently a simple method was developed with 0.3 mm i.d. Hypersil ODS capillary column for determination of diazepam content [58]. Detection of 0.1 and 0.4 $\mu\text{g L}^{-1}$ was achieved using a sample volume of 990 μL . However, the reverse phase columns are suitable for compounds with hydrophobic interaction only. Very recently, another method was developed with hydrophilic interaction for compounds such as spectinomycin and lincomycin (HILIC) [59]. In this case, water and acetonitrile (containing 0.1% formic acid) were used as mobile phases in silica-based Altima HP column. For increasing the efficiency for both LC separation and ionization, organic ion-pairing reagents are often used for example in case of analysis of the aminoglycoside antibiotic gentamicin [60]. Here heptafluorobutyric acid (HFBA) was used as ion pairing agent that formed HFBA–gentamicin pair and thereby reduced the polarity of gentamicin and intern increasing its retention on a C18 column.

3.3 Detection of antibiotics by Mass spectrometry (MS)

With the advent of Mass spectrometry based method for identification and quantification of organic compounds, detection of antibiotics in environment had become much easier and cost effective. The method is an inevitable tool for detection of trace amount of organic moieties and providing unambiguous identification by library search as well as quantification with reference to standard. The combination of liquid chromatography and mass spectrometry in late nineteen-eighties with highly selective and sensitive tandem mass spectrometry (MS–MS) has made LC–MS and/or LC–MS–MS as the best tool for identification and quantification of antibiotics. The greatest achievement with mass spectrometry over the other spectrometry such as NMR lies with its requirement of minute quantity of sample and the requirement of derivatization step for the analysis made the GC–MS method more time consuming and labour intensive.

The most important aspect of mass spectrometry based detection depends on ionization of compounds present in complex mixture and separated by chromatography. The compounds could be from acidic to basic to neutral. Tributylamine (TBA) was first used to enhance the ionization efficiency of the positive molecular ions by sodium displacement mechanism [61]. The antibiotics which are acidic in nature are generally analysed by the use of negative-ionization mode, whereas neutral and basic antibiotics are analyzed in positive-ionization mode. The use of appropriate mobile phase additives is required sometimes to improve ionization efficiency. The great advantage of Electrospray Ionisation Mass Spectrometry (ESI-MS) lies for switching from positive to negative mode for simultaneous determination of antibiotics such as atorvastatin, novobiocin, and roxithromycin in a single run [62].

Sometime different fragments of ion of same target are observed due to variations in the design and experimental conditions as well as for different nature of instruments used. Both 3D ion trap (IT) mass spectrometers and triple-stage quadrupole (TSQ) have been used in LC–MS–MS analysis for detection of antibiotics. For example, while detecting sulfamethoxazole, m/z 92 and 108 was observed using a Sciex TSQ [63], whereas major fragment ion at m/z 156 was observed when Micromass TSQ or Thermo Finnigan IT [64, 65] was used. Thus, selection of instrument in MS analysis is crucial for successful detection of antibiotics. In general, for MS detection, ammonium acetate, aqueous ammonia, acetic acid, formic acid, and trialkylamine are added as electrolytes to the mobile phases for enhancing the efficiency of ionization in the ESI interface. However, the major disadvantage of the electrospray ionization remains with the likelihood of matrix effects [66]. Atmospheric pressure chemical ionization (APCI) can reduce matrix effects by 3 to 10 folds [67]. The use of APCI could be effective only in case of certain group of compounds as the ionisation is comparatively less in case of APCI compared to ESI. To minimize this drawback, use of internal standards, especially isotope-labeled internal standards (ILIS) is very effective alternative. The internal standard can be used for correcting the matrix effects, but on the other hand, the same standard may not be available or could be very expensive [66].

Two types of commercial mass spectrometer instruments are being used for several identification studies namely — the Waters (Micromass) Q-TOF system, and the Sciex QSTAR system. For example, the exact mass and fragmentation pattern of the compound Carbamazepine was identified by a method developed using Q-TOF from a mixture of unknown contaminants in surface water [68]. High resolutions of hybrid quadrupole/time-of-flight (q-TOF) instruments are often being used for unknown or metabolite identification by furnishing accurate mass information from individual compounds. Positive confirmation can also be achieved by the use of high-resolution mass spectrometry [68, 69, 70, 71]. For example, recently Phenazine 1,6-di-carboxylic acid (PDC) with antimicrobial and cytotoxic activity was isolated from culture supernatant of *Pseudomonas* grown in presence of crude oil as carbon source and identified by mass spectrometry based method [72]. Despite of the high specificity of LC–MS–MS, many times the compounds can still be mistakenly identified. This is solely because of interference from matrix components and metabolites. Thus, running blank is very much essential for unambiguous interpretation. To further improve data quality and avoid any false positive results, European Commission Decision 2002/657/ECD has framed guidelines. Thus, to confirm positive

finding for the detection of low concentration of any compound in complex mixture, different transitions must be calculated and compared with those of a standard. For that the ion ratios and retention time deviations must meet with the Decision [71] because compounds at very low concentrations bring less satisfactory responses.

4. Conclusion

Antibiotics are indispensable therapeutic agents for microbial infectious diseases and being administered as orally as tablets or syrups, and as injections after surgery for post operational care. Inappropriate disposal of these drugs not only contaminate the environment, but also brings chances for development of antibiotic resistance strains within our surrounding. The cause of this contamination offers direct and indirect effect on target and non-target organisms present in the environment. The other major sources of contamination of antimicrobial agents are through its veterinary use in soil, surface water runoff after application of manure, fodder etc. which has taken a global concern [73, 6]. The detection of antibiotics is therefore an important aspect by which the threshold concentration of drugs in environment can be estimated and necessary measures can be taken to combat the spread of pharmaceuticals in the environment. So far, the data suggest that concentrations of antibiotics in municipal sewage, hospital effluents, influents and effluents of STPs, surface water and groundwater remained in the same range, respectively during the last decade.

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