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Antibiotic Residues in Waste Water of Rural and Urban Hospitals from South India

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Hospital effluent acts as the store house of pharmaceutical residues, harmful infectious agents such as the pathogens and microorganisms possessing multiple drug resistant genes. The antibiotics detected in hospitals have been shown to possibly exert effects on bacteria that lead to increased resistance. Present study was chosen to quantify the antibiotic residues in water associated with hospitals in South India. The samples were analyzed using high performance liquid chromatography (HPLC). A total of ten samples were analyzed. A total of 45 different antibiotics were identified and quantified among 10 hospital effluents. The quantification was given in method

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detection quantification limit (MDQL) and in trace. Among all the ten samples the maximum MDQL was detected as 17834 ng/ L (trimethoprim). Out of the gallery of antibiotics detected in this study, four antibiotics supported MDQL with more than 10000 ng/ L. The lowest trace value of antibiotic with 46 ng/ L (gatifloxacin) and maximum of 896 ng/ L (cefipime) was detected in sample 10 and 5 respectively. Hospitals and its effluents are one of the high sources for discharge of antibiotics and multidrug resistant strains into the environment and proportionallyexert a serious public health threat through confining the antibiotic pool. Likewise studies are required to figure out the presence of antibiotics in aquatic environment and the development of antimicrobial resistance and its subsequent public health impact.

Keywords: Antibiotics; hospital effluents; HPLC; antibiotic resistance.

1. INTRODUCTION

Antibiotics are the most frequently prescribed drugs in modern medicine for a small health problem to life threatening ones. The global consumption of antibiotics elevated by 65% and is predominant in developing countries [1]. "Presence of antibiotics in surface and treated drinking water is becoming of great concern around the world because of their susceptibility to induce microbial resistance" [2-4]. "The pharmaceutical induced resistance in the environment can be transferred to humans through drinking water and food chain and which results in reducing the effectiveness of current medications" [5]. "The concentration of antibiotics in the environment depends on antibiotic consumption and their patterns of use; therefore vary among areas and countries. Occurrence of antibacterials in aquatic systems is also affected by their chemical stability, partition and sediment characteristics" [6,7].

"The problem of antibiotic resistance is increasing alarmingly and the increasing problem of antibiotic resistance has repeatedly been placed on the global agenda as a threat to functioning health systems" [8,9]. Environmental fate of antibiotics was reported by several studies and it emerged as an important research topic from the past decade. The incident of antibiotics into the environment is happening by human excretion, improper disposal practice and discharge from pharmaceutical industries &from hospitals [10]. "Wastewater treatment plants (WWTPs) are not fully designed to completely remove antibiotics, and consequently they are released into natural waters. Moreover, antibiotics can pass through all natural filtrations and reach ultimately to drinking water due to their high water solubility and often poor degradability" [11]. Presence of antibiotics in the environment, even though in low levels, continuous exposure of microbes to these antibiotics normally or broadly develop resistance and become ineffective in treatment [12,13].

"Hospitals are the place to serve patients, known for the treatment of ill persons, but we are unaware about the adverse effects of waste generated and its impact on environment and humans. A modern hospital is a multidisciplinary system that consumes many products for delivering of medical care, so it is not an unusual thing to have the leftovers. The hospital waste consists of general, pathological, pharmaceutical, infectious, chemical, radiological wastes etc. Studies have shown that the release of wastewater from hospitals was associated with an increase in the prevalence of antibiotic resistance [14] and are a potential health hazard to healthcare workers, public, flora and fauna of the area. Hospital effluent is one of the important contributory sources of antibiotics into environment. Various studies in India reported the presence of antibiotics like quinolones, sulphonamides, macrolides, imidazole derivatives like (metronidazole and tinidazole) in hospital effluents. Ciprofloxacin was found in between 0.7 and 124.5mg/L in hospital effluent and was assumed to be the main source of genotoxic effects" [15]. "Therefore, even if the hospitals are discharging their effluents into sewerage system, it is mixed with the sewage and gets in surface water without proper treatment. If the hospital effluents are not treated, concentrated forms of infectious agents and antibiotic resistant microbes are shed into communities resulting in water borne diseases such as cholera, typhoid fever, dysentery and gastroenteritis" [16].

It was already proved that hospital effluents contain pathogenic microorganisms like *Salmonella, Shigella* and other pathogens that could pose a serious threat to community health [17]. "The samples taken from the Ohio River contained *Escherichia coli* showed resistance to

penicillins, tetracyclines and vancomycin" [18]. "Presences of these pharmaceuticals in waste system have a potential impact due to continuous exposure in wildlife ecosystem, the livelity rate get reduced and endangered. The analysis of various antibiotics in the environment represents a difficult task due to the high complexity of the matrices analyzed and to the usually low concentrations at which target compounds are present in the aquatic environments. This condition leads to the development of very sensitive analytical methods suitable for the monitoring of these analytes in low concentration levels. However, one the most common approach for the analysis of antibiotics in environment includes HPLC and is capable of qualitative and quantitative detection of antibiotics with low detection limits" [19].

Limited data on occurrence of pharmaceuticals especially of antibiotics in surface water and hospital effluents is a draw back in assessing potential human health risks from exposure to trace concentrations. The proper treatment technologies and monitoring programmes are not well established in many countries. Untreated hospital effluent discharge into the environment directly or indirectly must have been adding more problems. Hence, the current study was carried out to explore the presence of antibiotics in the hospital effluents and to quantify their amount due to over spreading of antimicrobial resistance.

2. MATERIALS AND METHODS

2.1 Sample Collection

Ten hospital effluent samples were collected and used for the determination and these effluents were collected from tertiary care teaching hospital, reference government hospitals, district headquarters hospitals, primary health centres and private speciality hospitals. The portal of the samples from, sewage hospital outlet before entering into municipal sewage, sewage hospital outlet hospital after entering into municipal sewage, recycling unit of the hospital, special ward and normal water supply in hospitals.The effluent samples collected from hospitals were designated as RPA01 to RPA10 for easy identification and documentation to minimise the bias.

Two hundred and fifty (250) ml of effluent were collected in amber glass bottle with tight screw cap lids. Prior to sample collection, bottles were disinfected with sodium hypochlorite (0.525%) followed by ethyl alcohol solution and sterile water to avoid the contamination and stored immediately at temperature below $4^{o}C$ and transferred to the analytical laboratory for antibiotic determination. All the collected samples in screw capped amber bottles were wrapped with aluminium foil to prevent photo degradation of some antibiotics including fluoroquinolones and tetracyclines.

2.2 Chemicals, Reagents and Materials for Quantification

The samples were subjected to quantify the antibiotics. Solvents were included and selected based on the antibiotics to be determined; solvents used for different groups of antibiotics were depicted in Table 1. All other chemicals used were of analytical reagent grade where the standard purity was also determined [20]. The working standard solution was prepared by diluting the mixed standard solution with acetonitrile to a series of proper concentrations. All the stock solutions were stored at $4^{o}C$ until use.

2.3 Solid Phase Extraction

Initially 50 ml of each sample was filtered through 0.45 (µm) micron pore sized minipore filter paper (PT-14400314 CN). Further the filtrate was acidified to pH 3.0 by adding sulphuric acid (H2SO4) (MERCK, Mumbai-61752705001046). Then the sample was subjected solid phase extraction with flow rate of SP column at 8 drops/ min. Sample was passed through activated C- 18 cartridge activated with 5 ml of methanol, 5 ml of methanol/ water (50/50- v/v) and 5 ml of water at pH 3.0, then the cartridge was washed further with 5 ml of acidified water at pH 3.0 and then the cartridge was eluted with 5 ml of triethylamine (5% v/v) in methanol. The eluted solution was evaporated to 20 µl using nitrogen gas at $45^{o}C$ finally the sample volume was reconstituted to 1ml by adding water- acetonitrile mixture at the concentration 95:5 (v/v) and the required portion was injected in the HPLC system.

2.4 Preparation of Mobile Phase

In general water based organic solvent or mixtures of 2 solvents are mainly used as mobile phase in HPLC. A buffer solution is often used as the aqueous solvent. Apart from the buffer solution, many other unforeseen factors affect the mobile phase preparation including the method of mixing the solvents.

Table 1. Solvents used in HPLC analysis

Antibiotics	Solvent used
Sulpha group and Macrolides	Water and Acetonitrile
Tetracyclines and Lincosamides	Methanol
Aminoglycosides and Cephalosporins	Dihydrate + dichloromethane + formic acid + HCl + water
Penicillin and Glycosides	Acetonitrile
Trimethoprim	Chloroform + acetone
Fluoroquinolones, Nitroimidazole and Nitrofurans	Acetonitrile + methanol
Quinolones	Ethylacetate + methanol + acetonitrile

The sample size of 10 different hospital effluents initially measured as 250 ml. In the chromatographic analysis the volume of the sample was determined as 15µl. After packing the chromatographic column the gradient program started after loading the mobile phase. The flow rate was 1.0 ml/ min and the injection volume of the sample was 15µl. The chromatographic column used was 250×4.5mm, econosphire- 18 column- 5µm particle sizes. The flow rate was fixed to 1.0 ml/ min whose sample volume of the analyte was 15µl. In this HPLC parameter, the capillary voltage was 3kg, the cone voltage was 50v, the source temperature was 120^{o}C, dissolvation temperature was 450^{o}C and the dissolvation gas flow rate was 800 litre/ hour. In this study nitrogen was used as dissolvation compound and the temperature was fixed to 40^{o}C after loading the sample for determining the antibiotics quantitatively from the effluents.

Each and every chromatography and spectrophotometry has its own wave length to determine the analysis. The range subjected for HPLC study starting from 210nm to 230nm. In this study the wave length of 210nm was fixed.

A liquid phase HPLC system consists of a model 510B pump to deliver the mobile phase and model 481 variable wave length detectors were used (Waldbronn, Germany). This equipment came with a quaternary pump, a degasser an auto sampler and absorbance detection unit. The detection signals include a wave length of 210nm, in sometimes when detecting sulpha group, macrolides, fluoroquinolones, nitroimidazole, nitrofuran groups 300nm was applicable, where 0.05 absorbance units fuel state was fixed for antibiotics determination. The analysis was performed with a HPLC Shimadze stainless steel column (Type VP- ODS 250 litre, filled with grafted silicagel and column C-18 in reversed phase). In this instrument two pumps were used

1. S-N: C 20974009859J2- Model LC- 10 ATVP
2. S-N: C 21014009440CD- Model LC- 10 ATVP

Along with the pump two UV detectors were used

1. SPD- 10Avp- 10AVvp equipped with deuterlium laboratory with 310nm wave length
2. S-N: C 21004001496LP with 190- 350nm

Method detection quantification limit (MDQL) is the minimum concentration of a substance that can be measured and repeated with 99% confidence that the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte (72). The quantification was achieved by comparison of the peak area of the samples with that of external standard. The identical chromatogram was quantified by the peak area of samples with that of standard in same retention time.

The chromatographic conditions of the antibiotics detected were well analyzed by comparing with group of antibiotics supported MDQL value and trace. The term MDL is defined for the phenomena and calibration of the qualitative evaluation (detection), whereas MDQL is the term used for the quantification of the compound present on the samples. The MDQL is determined by the multiplication of the MDC with 3.18. Thus the understanding of the quantification of assay of samples was well depicted using HPLC analysis and results were documented by MDQL value [21,22]. The value of 1000 ng/L is considered as MDQL and the below was determined as trace [20,21].

3. RESULTS AND DISCUSSION

The results of this study include optimal instrumental conditionsfor analysis of subjected antibiotics. By HPLC quantification assay, a

battery of total of 45 different antibiotics was identified, quantified among 10 hospital effluents. These antibiotics were grouped into 13 popular, routinely prescribed and used antibiotics. There were 6 beta lactams (amoxicillin, ampicillin, flucloxacillin, oxacillin, minocycline and penicillin G), 4 macrolides (azithromycin, clarithromycin, erythromycin and roxithromycin), 4 cephalosporins (cefazolin, cefipime, cefotaxime and ceftiofur), 4 tetracyclines (tetracycline, chlortetracycline, doxycycline and oxytetracycline), 10 fluoroquinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, gatifloxacin, ofloxacin, orbifloxacin, morbofloxacin, norfloxacin and sarafloxacin), 2 lincosamides (clindamycin and lincomycin), 3 quinolones (flumiquine, oxolinic acid and nalidixic acid), 2 aminoglycosides (gentamycin and kannamycin), 3 nitro groups (metronidazole, nitrofuratoin and nitrofurazone), 3 sulphonamides (sulfadiazine, sulfamerazine and sulphamethaxazole) and 1 each of dihydrofolate reductase (trimethoprim), glycopeptides (vancomycin), chloramphenicol (chloramphenicol) and carbapenems (imipenem). The sample wise description of antibiotics determination is summarized in Figs. from 1- 10.

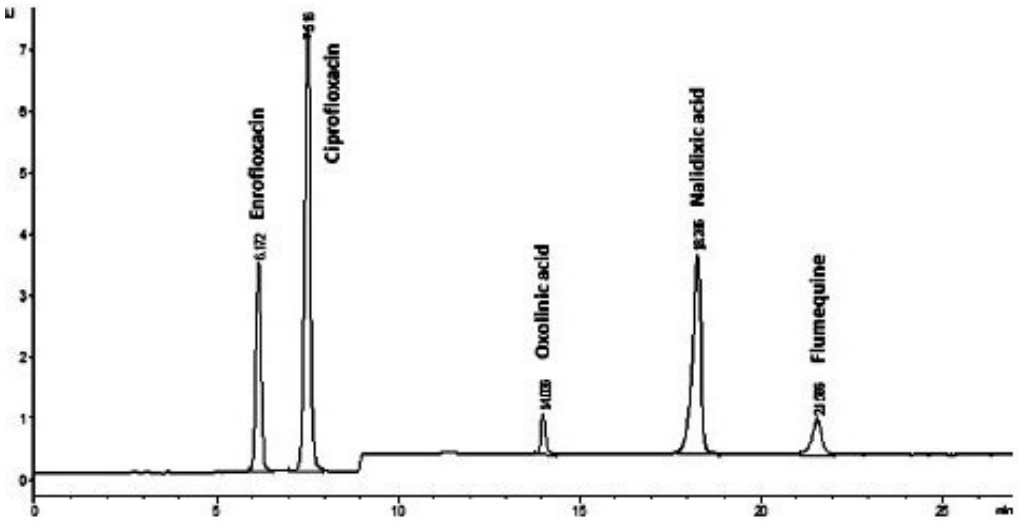

Fig. 1. HPLC chromatogram and data peak report of effluent sample 1

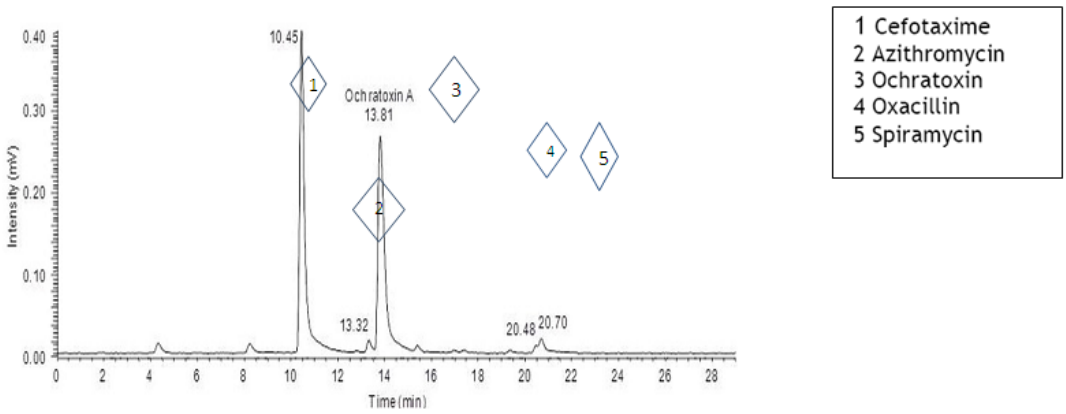

Fig. 2. HPLC chromatogram and data peak report of effluent sample 2

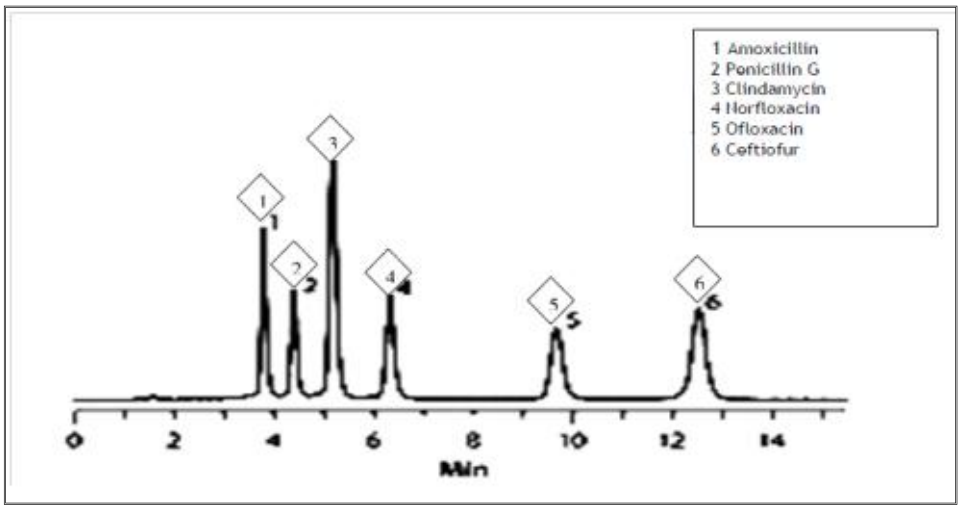

Fig. 3. HPLC chromatogram and data peak report of effluent sample 3

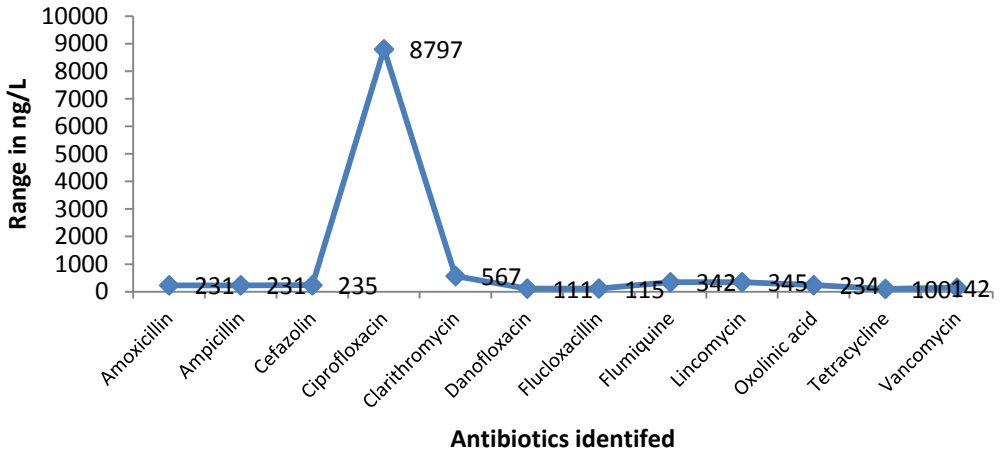

Fig. 4. Chromatographic determination of antibiotic residues in sample 4

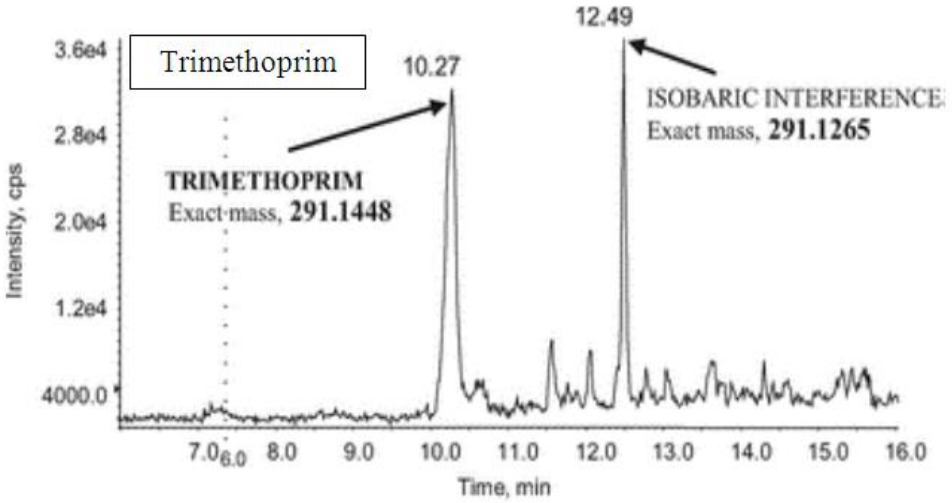

Fig. 5. HPLC chromatogram and data peak report of effluent sample 5

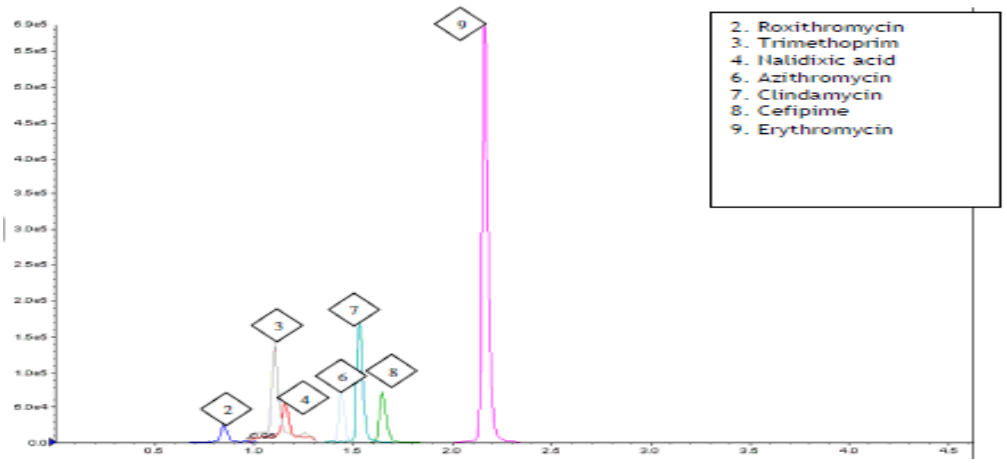

Fig. 6. HPLC chromatogram and data peak report of effluent sample 6

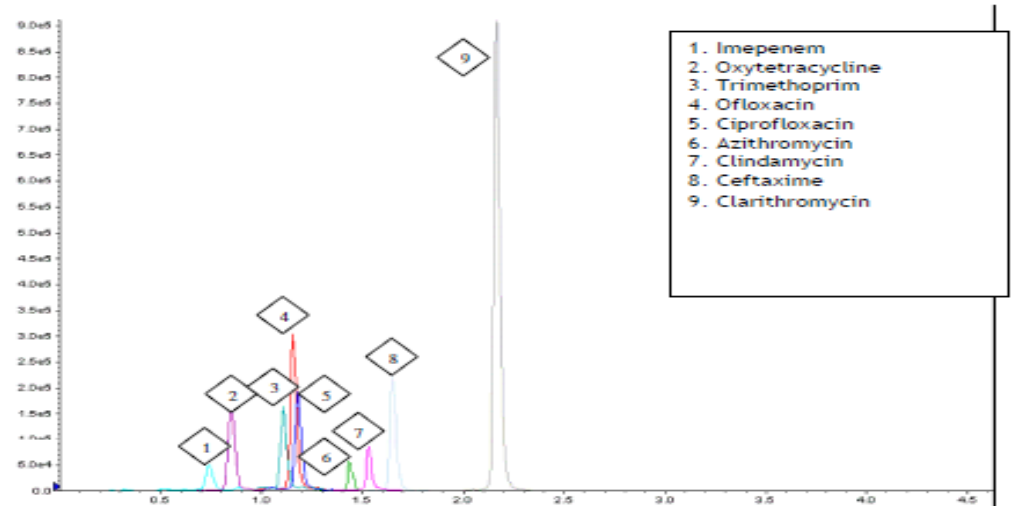

Fig. 7. HPLC chromatogram and data peak report of effluent sample 7

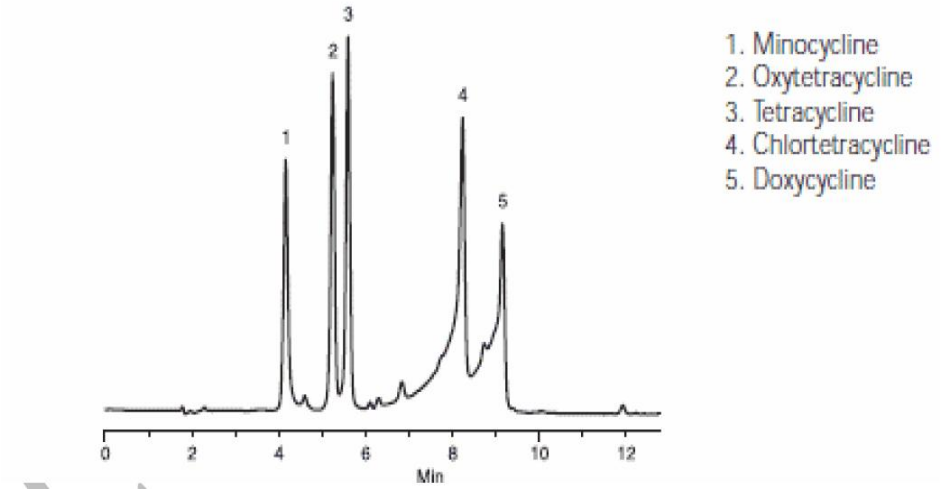

Fig. 8. HPLC chromatogram and data peak report of antibiotics in sample 8

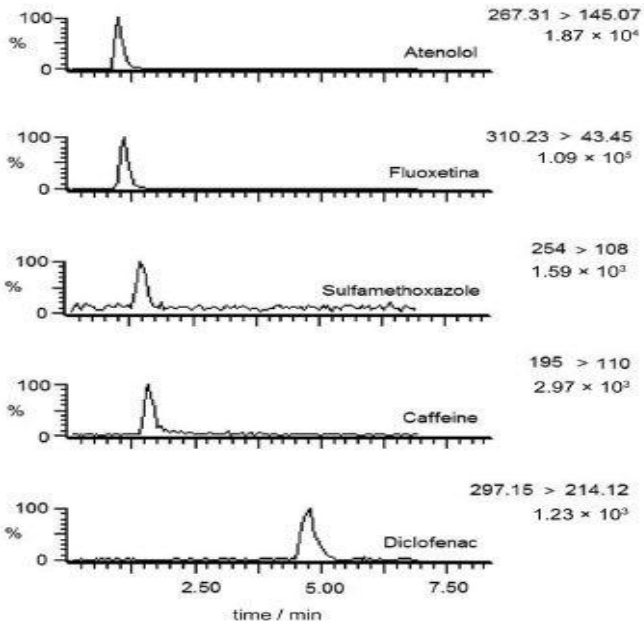

Fig. 9. HPLC chromatogram and data peak report of antibiotics and other drugs in sample 9

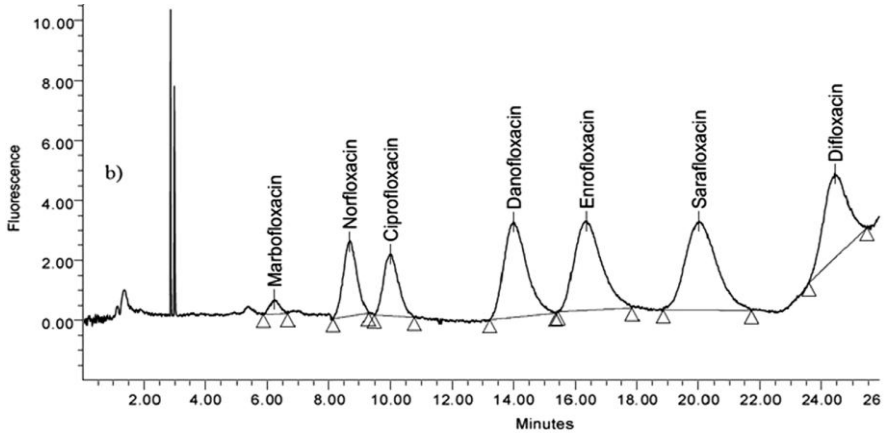

Fig. 10a. HPLC chromatogram and data peak report of antibiotics in sample 10

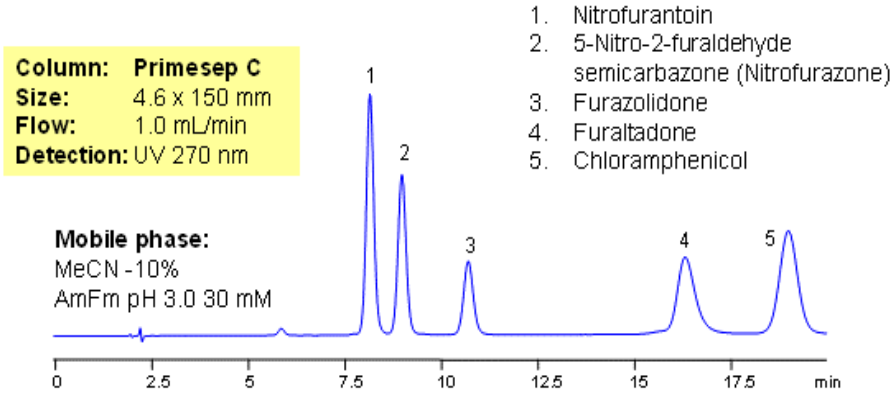

Fig. 10b. HPLC chromatogram and data peak report of antibiotics in sample 10

The overall average concentration of antibiotics obtained at method detection quantification limit were analyzed and descriptively correlated. The maximum concentration of 17834 ng/ L of trimethoprim and minimum concentration of 1256 ng/ L erythromycin identified. Ciprofloxacin, clindamycin and enrofloxacin showed maximum concentration of 14798, 12457 and 15674 ng/ L respectively. Simultaneously, the trace antibiotics were also analyzed there by the maximum concentration identified was cefipime at 896ng/ L, followed by nitrofurazone (878ng/ L), penicillin (768ng/ L), clarithromycin (675ng / L). Further the complete validated analyses of overall appropriate concentration of antibiotics in MDQL and in trace value were tabulated (Table 2).

Table 2. Estimated concentration of antibiotic residues in hospital effluents in ng/ L

Antibiotics	RPA1	RPA2	RPA3	RPA4	RPA5	RPA6	RPA7	RPA8	RPA9	RPA10
Amoxicillin	-	-	5467	231	342	-	-	-	-	-
Ampicillin	-	-	-	231	333	-	-	-	-	-
Azithromycin	-	4536	-	-	-	234	112	-	198	-
Cefazolin	-	-	-	235	234	-	-	-	-	-
Cefepime	-	-	-	-	896	234	-	-	-	-
Cefotaxime	-	2343	-	-	-	-	114	112	-	-
Ceftiofur	-	349	3456	-	111	-	-	-	-	-
Chloramphenicol	-	-	-	-	-	-	-	-	347	-
Chlortetracycline	-	-	-	-	-	-	-	-	126	-
Ciprofloxacin	6547	-	-	8797	-	-	14798	-	-	5673
Clarithromycin	-	-	-	567	-	-	675	-	095	-
Clindamycin	-	-	12457	-	-	564	-	-	-	-
Danofloxacin	-	-	-	111	-	-	-	-	645	1679
Difloxacin	-	-	-	-	-	-	-	-	-	3174
Doxycycline	-	-	-	-	134	-	-	-	-	-
Enrofloxacin	4567	-	-	-	-	-	-	-	-	15674
Erythromycin	116	1256	-	-	-	078	-	-	114	-
Flucloxacillin	-	-	-	115	-	-	-	-	-	-
Flumiquine	7865	-	-	342	-	-	-	-	-	348
Gatifloxacin	-	-	-	-	-	-	-	-	-	046
Gentamycin	-	-	-	-	-	-	-	-	-	178
Imipenem	-	-	-	-	156	-	345	-	456	157
Kanamycin	-	-	3452	-	-	-	-	-	-	-
Lincomycin	-	-	324	345	-	-	-	-	-	-
Ofloxacin	-	-	2349	-	145	-	-	-	-	-
Orbifloxacin	-	-	-	-	-	-	-	-	111	-
Oxacillin	-	3456	-	-	-	-	-	-	134	-
Oxalinic acid	2342	-	-	234	-	-	-	-	-	-
Oxytetracycline	-	-	-	-	-	-	-	3457	-	-
Marbofloxacin	-	-	-	-	-	-	-	-	-	2356
Metronidazole	-	-	113	-	-	-	-	-	-	-
Minocycline	-	-	-	-	134	-	234	235	-	-
Nalidixic acid	-	-	113	-	234	342	566	-	-	-
Nitrofuratoin	-	-	-	-	098	124	-	-	-	167
Nitrofurazone	-	-	-	-	-	876	-	-	-	132
Norfloxacin	-	-	109	-	-	-	-	-	-	2341
Penicillin G	-	-	124	-	-	-	768	605	145	-
Roxithromycin	-	-	-	-	-	2345	234	-	-	-
Sarafloxacin	-	3454	-	-	132	-	443	-	-	157
Sulfadiazine	-	-	-	-	112	-	-	-	-	-
Sulphamethaxazole	-	-	-	-	-	111	232	-	6758	-
Sulpfamerazine	-	-	145	-	-	109	-	-	-	-
Tetracycline	-	-	-	100	-	121	-	2358	-	-
Trimethoprim	-	234	123	-	17834	6758	098	3425	112	-
Vancomycin	-	123	-	142	-	-	124	-	-	-

(Values below 1000 ng/ L is considered as trace)

The usage of antibiotics is been increased in large volume every year. The presence and potential adverse effects of pharmaceuticals in environment have begun to receive increased attention [23].Our primary aim was to identify the occurrence and quantify different antibiotics in the effluents. Awareness towards improper disposal of medicines and its hazardous effects on environment is now considered as a major issue which was been neglected [24]. Further, in general there is a paucity of information on the influence of environmental factors on antibiotics use leads to the development of antibiotic resistance [25].

The analysis of antibiotics and other drugs in the environment represents a difficult task due to the high complexity of the matrices analyzed and low concentration of the target compounds [26]. But in this investigation, the samples were collected from hospital environment thus it is not much difficult to analyze the matrices because of knowing the institutional drug prescription patterns. In the present study a total of 45 different antibiotics were detected and quantified (depicted in Table 1). In order to validate the quantification of antibiotics detected from hospital effluents, it was showed that the maximum MDQL of 17834 ng/ L of trimethoprim followed by 15674 ng/ L of enrofloxacin, the same type of analysis with more efficiency were documented with 9 analytes [27]. Remaining other drugs were quantified with 29 LODs, whereas remaining all detected as trace. The concentrations of certain antibiotics were high in present study in some samples (Table 2). The reason for their high level existence is due to lack of waste water treatment facilities as a result antibiotics find their way into water sources and is a problem of concern in terms of direct and indirect impact on environmental and humans.

Some earlier reports from India highlighted the high concentration of ciprofloxacin (up to 31,000 µg -1) and other antibiotics in the effluents from waste water treatment plant [28]. Studies on the presence of antibiotic residues in the environment matrices from India are limited [29]. However, the present investigation reported extremely high antibiotic residues concentration; such high level poses serious environmental, ecological and human health impacts. The long term exposure of bacteria to sub therapeutic concentration of antibiotics may arouse development of resistance that may lead to the failure of currently used antibiotics [30]. This clearly demonstrating the need of effluents quality monitoring before discharging from hospital sewage system and in-depth research has to be performed in the institutions using composite and periodical sampling.

4. CONCLUSION

A rapid, simple, accurate, precise and selective HPLC detection method was used for the quantification of various antibiotics in the hospital effluents. The present study helped in ranking the antibiotic residues contamination in hospital effluents and prioritization of the authorities seeking highest attention. A research team with good investigation has to be established in all health care institutions for adopting good transparent environmental practice and targeting to zero antibiotic discharge by adopting modern techniques to reduce environmental impacts. The current study eminent the need of monitoring effluent of the hospital sewage system and to adapt necessary sanitary and treatment measures to prevent the exacerbation of antibiotic residues and dissemination of resistant bacteria into the environment and more studies are required to analyse its impact. Hospitals should follow, monitor and regulate proper sanitary measures of generated effluents to forestall the dissemination of multi drug resistant bacteria transfer to the environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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