Total Environment Research Themes 1–2 (2022) 100003

Contents lists available at ScienceDirect

Total Environment Research Themes

journal homepage: www.elsevier.com/locate/totert

Application of micellar liquid chromatographic method for rapid screening of ceftriaxone, metronidazole, amoxicillin, amikacin and ciprofloxacin in hospital wastewater from Sagar District, India

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ARTICLE INFO

Keywords:
Antibiotics
Wastewater
Response surface methodology
Green chromatography

ABSTRACT

The present research work mainly highlights the simultaneous detection of five antibiotics i.e., ceftriaxone (CTX), metronidazole (MTZ), amoxicillin (AMX), amikacin (AMK) and ciprofloxacin (CPFX) in hospital wastewater located in Sagar district (Madhya Pradesh, India). All these antibiotics make their way through drainage systems into the environment in the form of metabolized and unmetabolized compounds. Growing concern about the antibiotic resistance and contamination of wastewater by antibiotics requires fast, sensitive and eco-friendly techniques. Therefore a simple, rapid and eco-friendly chromatographic method has been developed for simultaneous determination of CTX, MTZ, AMX, AMK and CPFX in hospital wastewater samples. Optimization of the method was accomplished using response surface methodology (RSM) with Box-Behnken design (BBD). The optimized mobile phase was 0.15 M SDS-0.01 M NaH_2PO_4-7% (v/v) 1-butanol, pH 3 which provided a chromatographic run time of 11 min. for the simultaneous determination of selected antibiotics. The correlation coefficient (r^2) values were satisfactory between 0.996 and 0.999 over the linear concentration range of 0.04–12 μg/mL. Limits of detection (LODs) and the limits of quantification (LOQs) for the five antibiotics were in the range of 0.02–0.18 μg/mL and 0.04–0.25 μg/mL, respectively. The developed method is simple, rapid, cost-effective and green which could be used for complex matrix (wastewater) without any sample pretreatment other than filtration. The results indicated that the MLC-PDA method proved to be more suitable than reverse phase-high performance liquid chromatography for the simultaneous separation of selected antibiotics.

1. Introduction

In recent times there are a variety of substances in the environment for which no stringent regulations exist and these compounds are together referred as emerging contaminants (ECs) (Hirsch et al., 1999). The harmful effect of these ECs is still not well-established. Pharmaceuticals and personal care products (PCPs) also fall under the category of emerging pollutants (Kronacher and Hogreve, 1936). The presence of pharmaceuticals and PCPs in the aquatic ecosystem is of growing concern due to the fact that most of these compounds are persist in the environment for a long period of time as well as they are toxic (Kümmerer, 2009). The presence of pharmaceuticals in various aquatic segments like surface water, groundwater, river water, drinking water, municipal sewage, hospital effluent, sewage treatment plant effluent etc. are already established (Meffe and de Bustamante, 2014). The pharmaceutical compounds are most commonly used as antibiotics, anti-inflammatory drugs, antiseptics, beta-blockers, analgesics, corticosteroids, sedatives, multivitamins, formulations etc. (Zwiener and Frimmel, 2004). Among the above mentioned groups of pharmaceuticals, antibiotics are the most frequently used drugs. Antibiotics and are commonly known as antibacterial or antimicrobial, are used to treat various bactericidal diseases in humans and animals (domestic or wild). They are also used as growth regulators or promoters in live stocks as well as to prevent infection (prophylaxis) in poultry and aquaculture (Mutua et al., 2020).

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https://doi.org/10.1016/j.totert.2022.100003
Received 5 March 2022; Revised 2 May 2022; Accepted 20 June 2022

According to origin, antibiotics are classified as natural, semisynthetic or synthetic. They generally work either by preventing the growth of bacteria (bacteriostatic) or by killing the bacteria (bactericidal) (Fischer et al., 2012). Since these drugs are designed to be stable and have high water solubility, they are released into the water bodies by various routes i.e., excreted through the body, livestock manures, poultries, disposal of unused drugs from hospitals and wastewater from antibiotic manufacturing industries (Farré et al., 2012; González-Pleiter et al., 2013). These antibiotics may adversely affect the aquatic ecosystem and finally may cause chronic effects on aquatic organisms. Nowadays, excessive use of antibiotics has resulted in developing resistance in living organisms against drugs. Among all the sources contributing to antibiotic pollution in the aquatic ecosystem, hospital effluents account for the highest amount of unused antibiotics released in water bodies (Meffe and de Bustamante, 2014).

Once administered, these antibiotics are generally not fully metabolized either by humans or animals and are directly eliminated from the body. A lot of work on the presence of antibiotics in hospital effluents has been done in developed countries, due to which preventive measures have been taken to reduce the concentration of antibiotics in hospital effluents (Barbosa and Levy, 2000). Whereas in India, very little work has been carried out on the detection of antibiotics in hospital wastewater therefore no proper data on antibiotic residue levels in hospital wastewater is available (Aryal, 1970).

Among different types of antibiotics the most commonly prescribed ones are amoxicillin (AMX), amikacin (AMK), ciprofloxacin (CPFX), ceftriaxone (CTX) and metronidazole (MTZ). All these antibiotics belong to different classes i.e., AMX and CTX are beta-blockers, AMK from aminoglycoside, CPFX from chloroquinoline and MTZ belongs to the imidazole class. These antibiotics are broad-spectrum drugs and can generally be effective against aerobic and anaerobic bacterial diseases (Hutchings et al., 2019; Kümmerer, 2009). Their mechanism of action also differs based on whether they are active on gram-positive or negative bacteria. These drugs are marketed in several forms i.e. capsules, tablets, syrups, suspensions or injectables (Kummerer et al., 2008). After administration, in the system these antibiotics are absorbed, distributed metabolized and finally excreted out of the body via urine and fecal waste which also includes unmetabolized drugs (Kümmerer, 2009). These metabolized and unmetabolized antibiotics enter the environmental water bodies through wastewater effluent which may cause environmental toxicity (Vasoo et al., 2015).

Analysis of antibiotics in environmental samples is not new and based on literature survey, it was found that a number of analytical techniques like UV-spectrophotometry (Gabriela et al., 2020), high performance liquid chromatography (HPLC) (Vosough et al., 2015), ultra-high performance liquid chromatography (UHPLC), tandem mass spectrometry (MS) etc (Xu et al., 2007; Nageswara Rao et al., 2008; Gracia-Lor et al., 2011). were used for the determination of antibiotics in surface water, sewage sludge, ground waters, soil, manure, sediment etc. (Chen et al., 2020; Shaaban and Górecki, 2015). All these techniques have their own advantages and disadvantages and one of the most common disadvantages is per sample analysis cost which makes the high end sensitive technique unaffordable by underdeveloped and developing countries. So there is a need to develop a simple method which is economical, fast and eco-friendly liquid chromatographic technique. In order to overcome this problem micellar liquid chromatography can be a method of choice.

Micellar liquid chromatography is a modified form of reversed-phase liquid chromatography (RP-HPLC) which uses surfactant above the critical micellar concentration as mobile phase. Micellar solutions can solubilize compounds within a wide range of polarities thus avoiding sample pretreatment step (Ibrahim and Nasr, 2014). In addition to solubilization, surfactant monomers form a layer on the outer surface of the stationary phase which modifies its interaction with the analyte. Short-chain alcohols (1-propanol, 1-butanol, 1-pentanol) are generally added into the mobile phase to enhance chromatographic parameters (Rambla-Alegre et al., 2008). The separation in MLC is generally based on a complex interaction of analyte because the analyte is partitioned between three environments (stationary phase, mobile phase and micelles), thus improving the versatility of MLC (Ayad et al., 2021). The technique is able to separate hydrophobic and hydrophilic analytes in isocratic mode simultaneously and the micellar environment also enhances the spectrophotometric activity of the analytes.

In order to reduce the number of injection during chromatographic method optimization, the application of experimental design (chemometrics) is very important. The Box- Behnken Design (BBD) has been applied on the response surface method (RSM) which is one of the useful experimental designs that can be utilized to optimize chromatographic conditions. In the present research work, BBD has been used to separate CTX, MTZ, AMX, AMK and CPFX just by considering SDS concentration, percentage volume of organic modifier and pH of the mobile phase. To the best of our knowledge, no method has been published which reported simultaneous determination of selected antibiotics using MLC method with the aid of BBD design.

For the application part of the developed MLC method wastewater sample from drain were collected from the public and private general hospitals of Sagar. Therefore, the present paper aims to identify and detect the most commonly used antibiotics in hospital wastewater samples in Sagar district (Madhya Pradesh) India by utilizing BBD design of the RSM method. A short survey was also carried out in the study area to know the prescribed antibiotics commonly used in all the hospitals located in this district, their drainage system, number of IPD (inpatient department) admitted, and per day consumption of antibiotics. The aim of the survey was to assess the location of sample collection and the antibiotics to be detected.

2. Experimental

2.1. Standards and reagents

Standard amoxicillin (>98%), amikacin (>98%) were procured from Merck (New Jersey, USA), ciprofloxacin (>99%), ceftriaxone (98%) and metronidazole (>98%) were purchased from Sigma (Saint Louis, USA). Sodium dodecyl sulfate (SDS, 99.0%), sodium dihydrogen phosphate (>98%) were obtained from Himedia Laboratories Private Limited (Mumbai, India). HPLC grade 1-propanol, 1-butanol and 1-pentanol were obtained from Rankem (Delhi, India).

2.2. Instrumentation and chromatographic conditions

The analytes were weighed using analytical balance Mettler-Toledo ME204 (Pocklington, United Kingdom). The pH measurements were performed using a Contech LAB pH meter, Model pH-103 (Mumbai, India). The magnetic stirrer was from PCI Analytics (Mumbai, India). Ultrapure Type-I water used for preparing standards was obtained using Indion LAB Q Ultra system (Mumbai, India).

The liquid chromatographic method development was performed on a Shimadzu Prominence HPLC (Kyoto, Japan) equipped with a diode array detector SPD-M20 A (190–800 nm) and 20 μL loop injector. Chromatographic separation was performed on a Shimadzu C_{18} column with dimensions of 250 × 4.6 mm and particle size of 5 μm (Kyoto, Japan). The data acquisition, analysis and storage were performed with Shimadzu LC solution version 1.22 software. Maximum absorption wavelengths of amoxicillin, amikacin, ciprofloxacin, ceftriaxone and metronidazole were 207, 257, 280, 314 and 261 nm, respectively.

2.3. Survey related to antibiotics use and hospital wastewater treatment

In India, the health care service of the country is implemented and maintained by central (National) and state governments (Regional).

The respective governments are also responsible for public health care systems which is meant to cater the medical need of its urban and rural population. The type and infrastructure of the health care system also depend on geographical area. In rural areas, sub-centres, primary health centres (PHCs), community health centres (CHCs) are established whereas in urban area apart from the PHCs district hospitals or medical colleges are established which basically depends on the population of the region. Apart from the government healthcare system, private and charitable healthcare systems also play a key role in the national healthcare service. Like any other country hospitals in India are divided into different wards like outdoor patient department (OPD), indoor patient department (IPD), surgery, medicine, maternity, daycare unit, emergency care unit etc. and allied departments like pathology, microbiology, research and development etc. The place selected for this study is Sagar, Madhya Pradesh (India). In the state of Madhya Pradesh there are 52 districts and Sagar is one of them. Sagar district is divided into 12 subdivisions (tehsil) for administrative purpose and its geographical area is 12,805 square kilometres (Fig. 1**S**).

As IPD is operative in both urban and rural hospitals therefore information regarding prescription and consumption of antibiotics in IPD was recorded. This information was collected from 35 hospitals of Sagar districts which have a minimum number of 25 beds for IPD patient. Apart from antibiotic consumption information related to hospital wastewater treatment plant and drainage system of each hospitals were also taken into consideration.

2.4. Sample collection

During the survey of the Sagar district, it was observed that most of the patients with mild disease visited PHCs or they went to district hospitals for severe disease and better treatment. Therefore, only those PHCs were selected in the present study, where the patient occupancy was > 50% of the available beds. Those private hospitals that cater to all kinds of indoor patients and have > 50 beds were also selected for the study. Since the tehsil (subdivision) level has less population, only PHCs were selected for the study. In Sagar city 50 bedded hospitals, multiple speciality hospitals under private organizations, Government Medical College, charitable hospitals and district hospitals were automatically selected or they fulfil the abovementioned criteria.

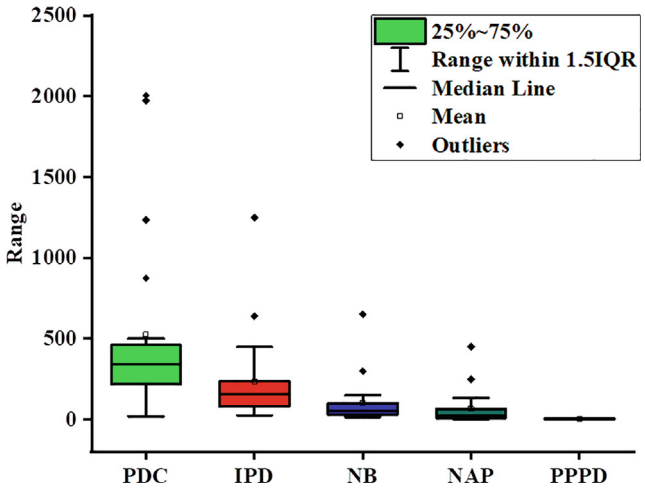

Fig. 1. Graph showing per day antibiotic consumption, number of beds, number of admitted patients in hospitals of Sagar district. *PDC-Per day antibiotic consumption, IPD-Inpatient department antibiotic consumption, NB-Number of bed, NAP- Number of admitted patients, PPPD- per person per day antibiotic consumption.

The sample collection strategy included the collection of one sample from each hospital in autumn as well as in the spring season. Wastewater sample was collected only from near point (the point at which the hospital wastewater was discharged into the sewage/drainage line). During these seasons, the sample collection was performed as the weather was not too hot (vaporization of water) and not rainy (sample dilution). Therefore a total of 25 representative samples (15 from Sagar city and 10 from PHCs) were collected in a 500 mL amber colour glass bottle and transported to the laboratory in ice-box maintaining the temperature at 4C.

2.5. Preparation of the mobile phase, standard and wastewater samples

The micellar mobile phases were prepared by dissolving SDS in ultrapure Type-I water and adjusted to pH 3 using 0.01 M sodium dihydrogen phosphate (NaH_2PO_4) buffer salt. 1-propanol, 1-butanol and 1-pentanol were used in appropriate concentrations as organic modifiers. All the mobile phases were filtered through 0.45 μm nylon membrane filters.

Individual stock solutions of 100 μg/mL of CTX, MTZ, AMX, AMK and CPFX were prepared by dissolving them in ultrapure Type-I water with the help of an ultrasonic bath. Wastewater samples stored at 4C were taken out one hour before analysis (analyzed within 24 h of sample collection). When the samples reached room temperature they were thoroughly shaken and filtered using a 0.45 μm nylon membrane filter for chromatographic analysis.

2.6. Method development using Box-Behnken experimental design

In micellar liquid chromatography, mobile phase optimization with trial-and-error experiments is time-consuming because different sets of mobile phases containing varying concentrations of surfactant, organic modifier and different pH of the mobile phase are tested for method optimization. In order to reduce the number of experiments chemometric approach can be a good choice as it reduces the number of experiments by giving the predicted values for the optimum mobile phase. Nowadays response surface methodology (RSM) is the prevalent method in the field of chromatographic method optimization. The RSM method explains the effect of independent variables, individually or in combination with each other, on the dependent variables. Different Design of Experiments (DoE) methods are available like Taguchi, Central Composite Design, Face-Centred Design, Box-Behnken Design, Doehlert matrix etc. (Aslan and Cebeci, 2007). Based on the number of dependents and independent variables these methods can be effectively applied in chromatographic method optimization.

Box-Behnken designs (BBD) are a class of rotatable second-order designs based on three-level incomplete factorial designs. A comparison between the BBD and other response surface designs (central composite and three-level full factorial design) has demonstrated that BBD is slightly more efficient than the central composite design but much more efficient than the three-level full factorial designs where the efficiency of one experimental design is defined as the number of factors in the estimated model divided by the number of experiments (Ferreira et al., 2007). Therefore Box–Behnken design was used for the optimization of different components of hybrid micellar mobile phase such as SDS concentration (SDS), the volume percentage of organic modifier (OM) and pH of the mobile phase. These components were chosen as low (-1), intermediate (0) and high (+1) levels (**Tables 1**). **Table 2** summarizes the design matrix with 17 experimental runs per the Box–Behnken design and pentaplicate studies at the centre point run. A standard solution concentration of 10 μg/mL was used for all of the experimental chromatographic analysis runs to obtain the retention time for selected antibiotics.

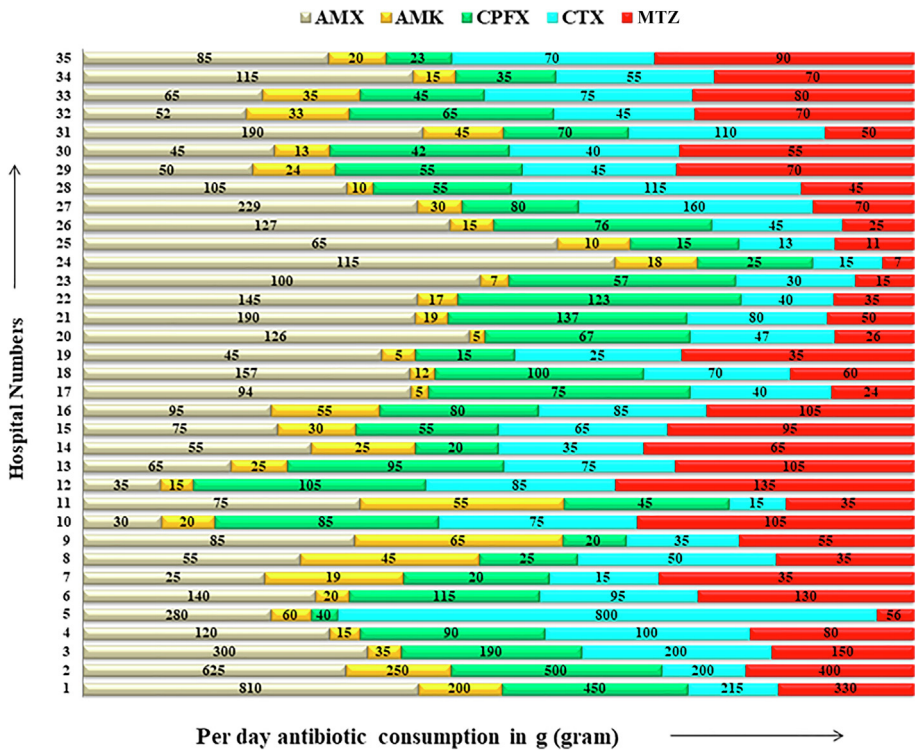

Fig. 2. Types of antibiotics consumed per day in hospitals of Sagar district.

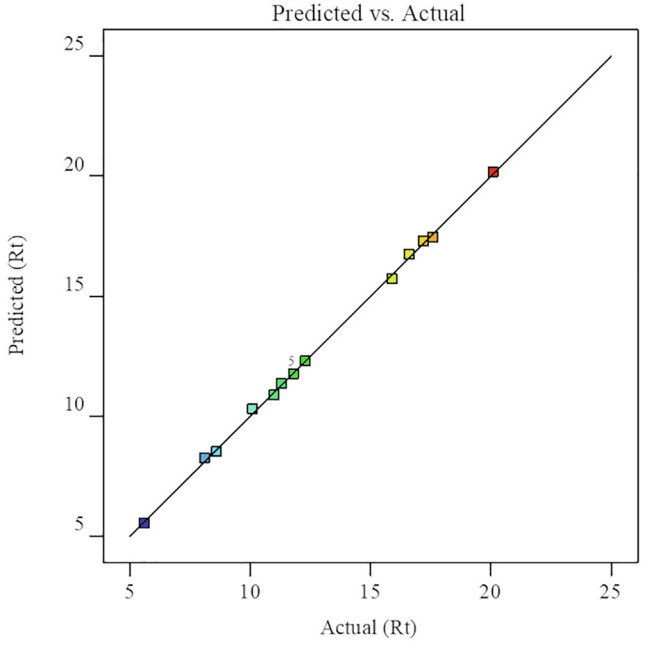

Fig. 3. Graph showing the obtained *vs* predicted elution time of last peak of selected analytes.

2.7. Method validation

The validation was performed as per the United States Environmental Protection Agency (USEPA) 2016.

The MLC method was developed and validated for the analysis of five antibiotics in hospital wastewater. The parameters of selectivity, matrix effect, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness were evaluated for method validation. Tap water was used as an alternative matrix for method validation because it was not possible to obtain a blank effluent wastewater matrix. Tap water was analyzed to ensure that there were no specific interferences during the retention time of the analytes.

The linearity for all the target compounds in tap water was obtained by plotting the peak area against the respective concentration at nine calibration levels ranging from 0.15 to 10 μg/mL except for CPFX which was from 0.04 to 12 μg/mL. The LOQ was defined as the lowest signal distinguished from the baseline noise and the limit of quantification (LOQ) was the minimal concentration which is quantifiable. The LOD and LOQs were taken as the lowest detected and quantified concentration of the selected analytes in tap water, respectively. The upper limit of quantification (ULOQ) was taken as the maximum point of the calibration curve.

Intraday and inter-day accuracy and precision of the method were determined from recovery and % relative standard deviation (RSD) experiments. Tap water was spiked with five antibiotics at the lower level of quantification (LLQ); 0.04–0.20 μg/mL, the middle level of quantification (MLQ); 4–5 μg/mL and upper level of quantification (ULQ); 8–12 μg/mL with five replications on three different days. Recovery results were used to evaluate the accuracy, while relative standard deviations (RSD) were used to evaluate precision. The intraday and interday RSD% were called the repeatability and reproducibility, respectively. Repeatability was evaluated from five replicates spiked samples at each concentration level on the same day, while reproducibility was determined by analyzing five replicates spiked samples at each concentration level on three successive days.

3. Result and discussion

3.1. Survey of antibiotic consumption and wastewater treatment

Twenty-three Government and twelve private hospitals were selected for antibiotics survey in this study. Among the Government hospitals, the Government district hospital and Bundelkhand Medical College had a maximum capacity of 650 and 300 beds. While in private hospitals Sagar Shree, Shukla and Bhagyoday hospitals had a

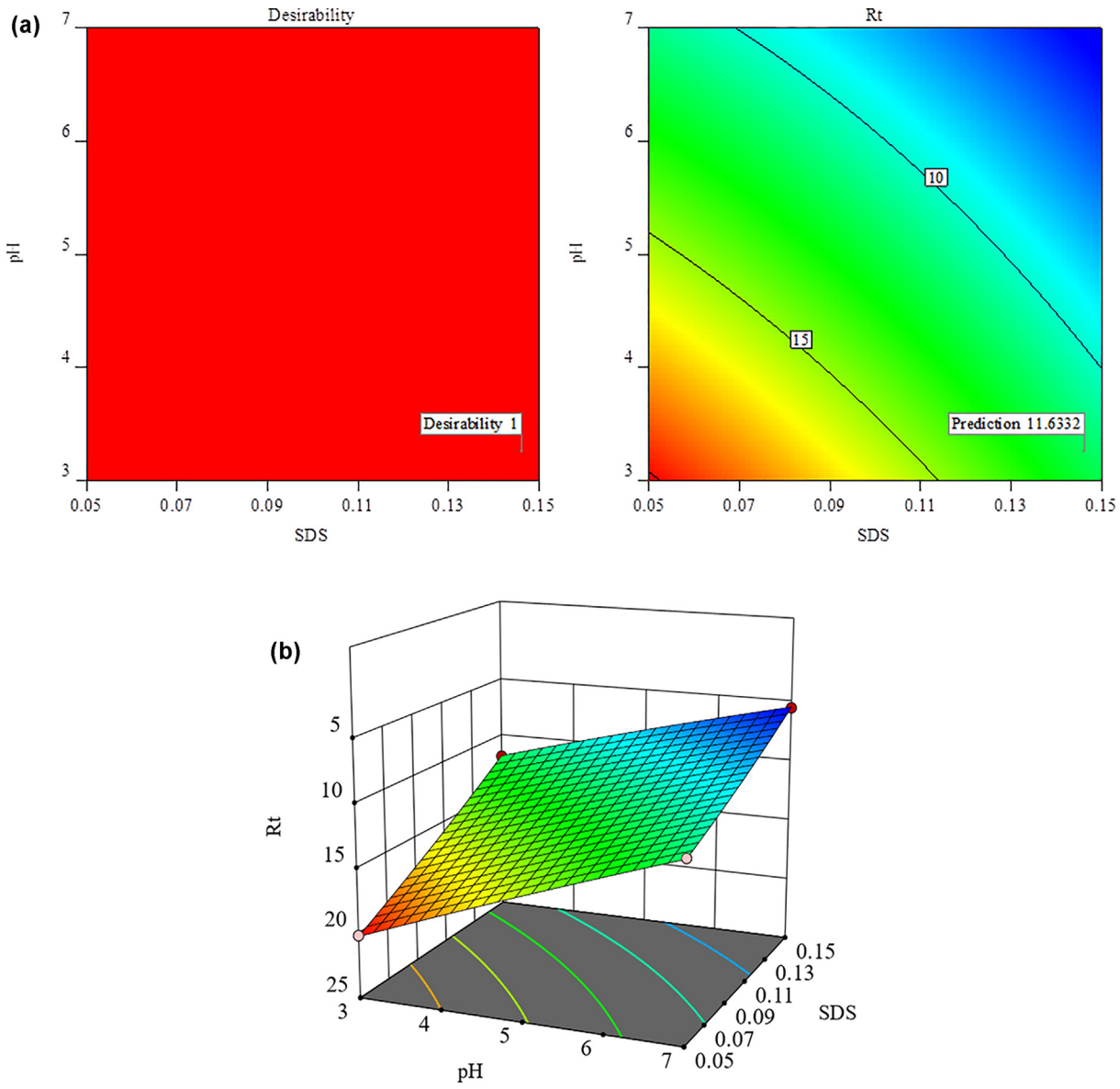

Fig. 4a. -b. Contour plots showing the influence of pH of the mobile phase and SDS concentration on retention time of last eluted analyte.

maximum capacity of 150, 20 and 150 beds, respectively. The Tehsils of Sagar district have very few private hospitals and patients visit mostly PHCs. The data obtained from the hospitals shows that per day antibiotics consumption in these hospitals reached to around 2000 g/day in which the maximum amount was used by IPD is around 1400 g/day. In these hospitals, maximum number of beds was around 650 while the minimum was 25 beds with median value of 200 beds. Apart from this the number of admitted patients also varied from 10 to 500 as shown in Fig. 1. The mean value found for per person per day of antibiotic consumption was around 4.2 g which is much higher than the defined daily dose of antibiotics (AMX; 1 g, CPFX; 1 g).

The data collected from hospitals about per day consumption of AMO, AMK, CPFX, CTX and MTZ has been presented in Fig. 2**.** The data depicted that among all the antibiotics consumed in the selected hospitals, AMX consumption was highest which is also in agreement with the study conducted by WHO from 2016 to 2018 on antibiotic consumption. In contrast, the lowest antibiotic consumption was for AMK. On the other hand, CPFX, CTX and MTZ showed almost similar per day consumption.

The survey confirmed that very few government hospitals treat their wastewater through treatment plants whereas most of the government hospitals in the district, especially those which are located in rural areas discharge their water into pit tanks within the campus of the hospital. Although private general hospitals have wastewater treatment plants but they just bypass it and discharge hospital wastewater directly into the domestic drainage system.

3.2. QbD-based method development as per the experimental design

In order to determine the effect of different chromatographic parameters Box–Behnken design was used. All experiments were performed in random order to minimize the effects of variables which can cause bias in the measurements.

A standard concentration of 10 μg/mL of selected antibiotics was used to obtain retention time. The analysis was performed using response surface methodology (RSM) to assess the relationship between the dependent (SDS, OM, pH) and independent separation variables (chromatographic run time). Various combinations of mobile

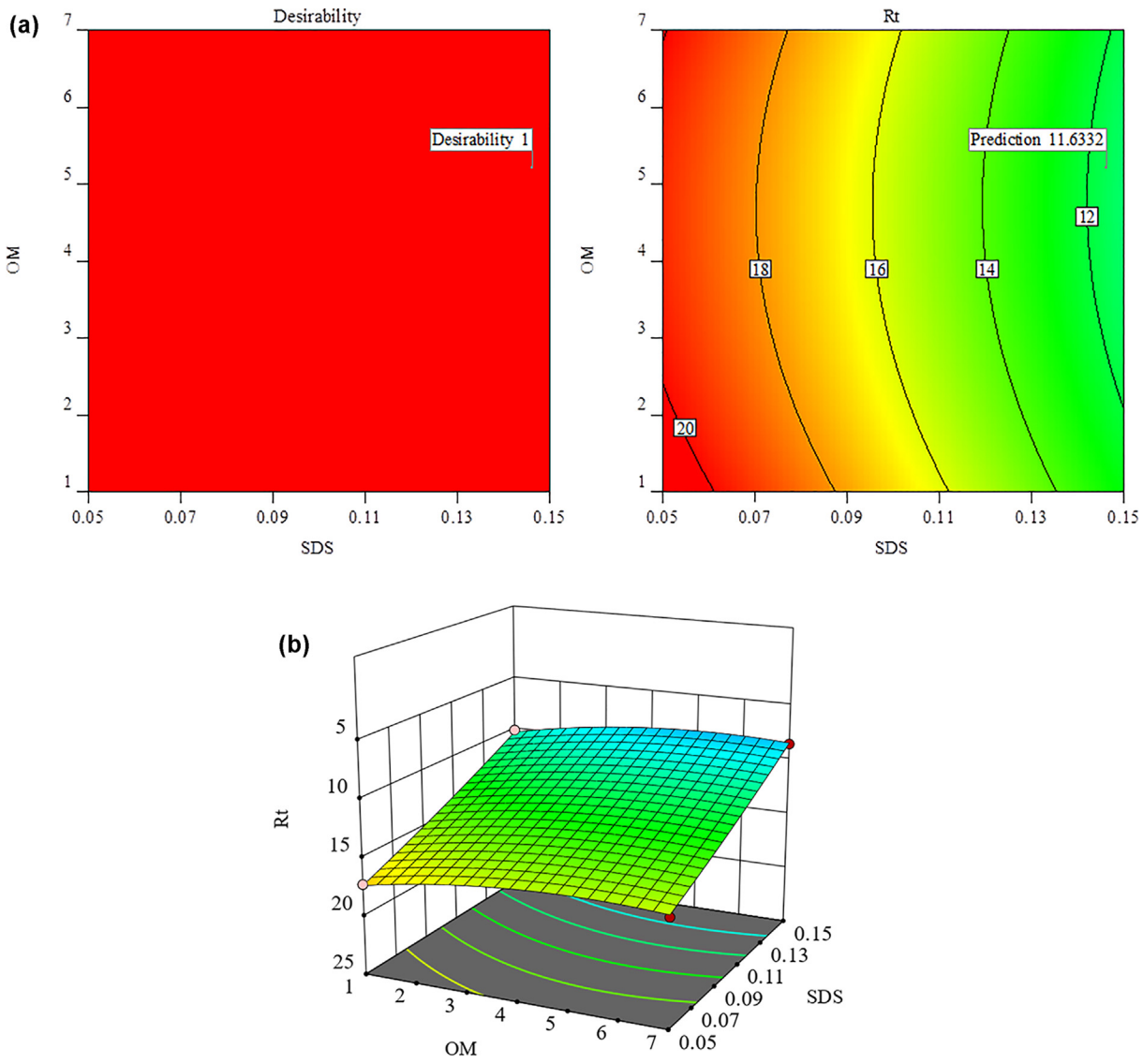

Fig. 5a. -b. Contour plots showing the influence of volume percentage of OM and SDS concentration on retention time of last eluted analyte.

phases were tested to obtain the optimum mobile phase in which all the compounds will resolve with minimum chromatographic run time.

The experimental chromatographic runs suggested as per the design were carried out and analyzed. The linear model was adopted for estimating main effects based on the first-order polynomial equations generated for each response variable, where β1–β7 represent the coefficients of model terms and β_0 the intercept:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 \quad (1)$$

The experimental runs were analyzed as per the design and mathematical modeling was carried out to fit the data to the second-order quadratic polynomial model with model terms for the dependent and independent variables. The model evaluation was carried out by ANOVA, which showed significant p-value and R^2 values (Table 3).

The correlation coefficient (R^2) varies when a regression variable is eliminated from a regression model. In statistical modelling, the adjusted R^2 which takes the number of regression variables into account is usually selected. The model p-value less than 0.05 reveals that independent variables effects are significant on a dependent variable (elution time of last peak). To predict total chromatographic run time the equation in terms of coded factors can be used for given levels of each variable. However, during experimental design, by default + 1 is coded for the high levels of variable, whereas −1 is used for the low level of variable. The coded equation is helpful for identifying the relative effects of dependent and independent variables. The predicted versus actual values for elution time of last peak is presented in Fig. 3 to better understand the effect of variables.

The **Predicted R^2** of 0.987 is in reasonable agreement with the **Adjusted R^2** of 0.998; i.e. the difference is less than 0.2. The **Model F-value** of 1018.47 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. **P-values** less than 0.05 indicate model terms are significant. SDS, pH, OM, SDS × pH, pH × OM were the significant model terms in this study. All other terms were not included because they possess p values > 0.1 which means that the model terms are not significant **(Table 3).** The final equation in terms of actual components and factors which can be used to make predictions about the response (chromatographic run time) for given levels of each factor are as follow:

$$Rt = +19.50 - 80.75\,X_1 + 1.75\,X_2 + 0.28\,X_3 + 2.0\,X_1X_2 - 0.50\,X_1X_3 - 0.008X_2X_3 + 95.0X_{12} - 0.34X_{22} - 0.04X_{32} \quad (2)$$

Response surface analysis was carried out using 2-D (Two-dimensional) contour plots (a-c) and 3D (Three-dimensional) surface plot along with desirability features which help to determine minute scientific details and depicted the relationship between a dependent (SDS, OM, pH) and independent variables (elution time of last peak).

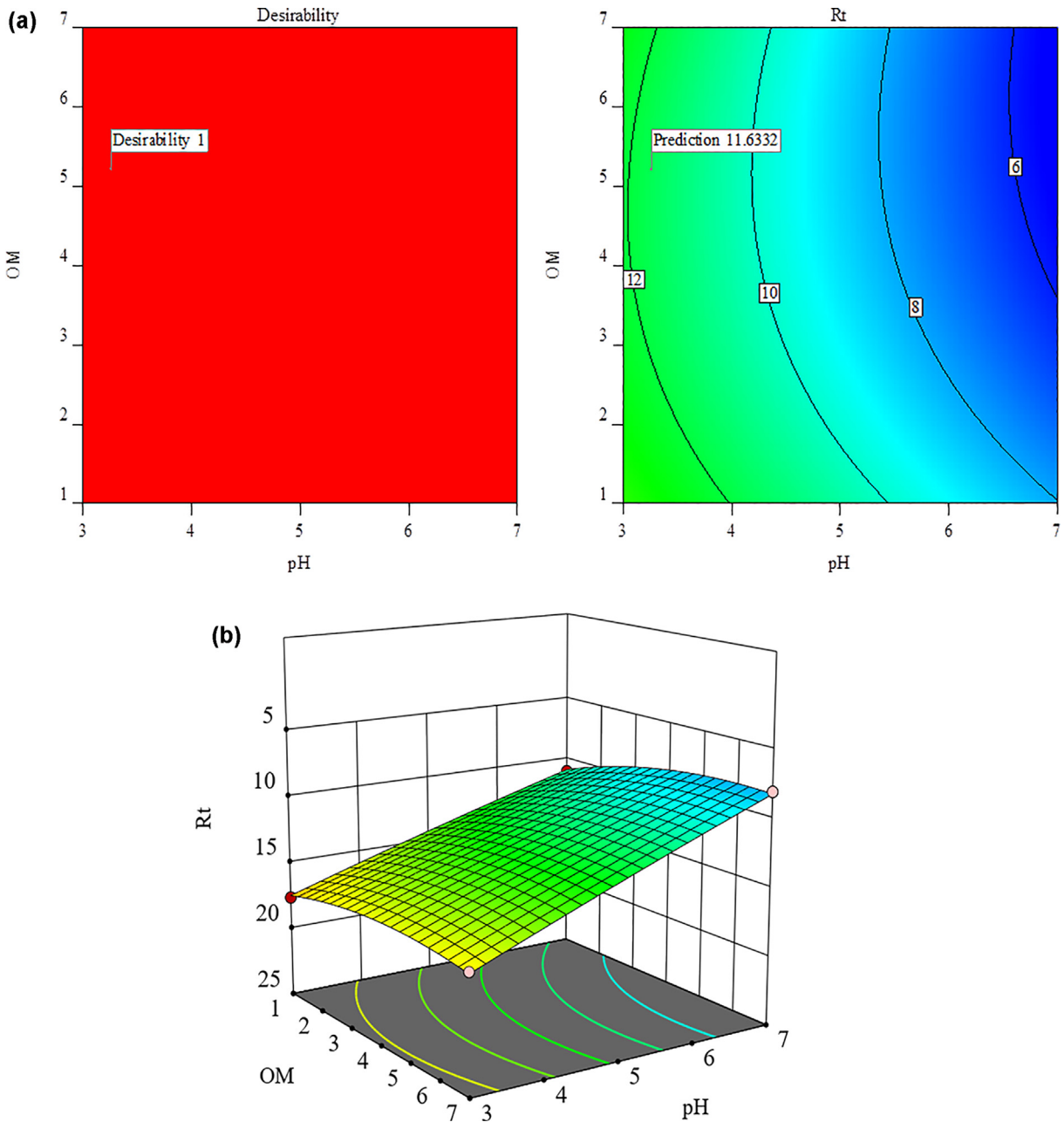

Fig. 6a. -b. Contour plots showing the influence of pH of the mobile phase and volume percentage of OM on retention time of last eluted analyte.

2-D and 3D response surface contour plots are shown in Figs. 4a-b to Figs. 6a-b providing a visual relationship between independent (SDS, OM, pH) and dependent variable (Rt) and the type of interactions between two test variables. The chromatographic run time of the last eluted peak is affected by pH of the mobile phase **(**Figs. 4a-b**)**, SDS concentration **(**Figs. 5a-b**)** and volume percentage of OM **(**Figs. 6a-b**).**

In this regard, the effect of SDS concentration and OM on elution time of last peak was investigated by desirability function contour maps (Fig. 4a**,** Fig. 5a **and** Fig. 6a) and 3D graphs (**Figure 4b, Figure 5b and Figure 6b**). The 3D graph inclined towards the highest concentration of SDS and organic modifier. The contour graphs revealed that increasing the concentration of SDS and OM decreases the elution time of analytes (**Figure 4b**).

pH also plays a very important role in the optimization of the chromatographic method. The pH working range of the C_{18} column is 2.5–7.5. The protonated and deprotonated state of analytes is based on their pKa values (AMX; pKa = 3.2, AMK; pKa = 2.3, MTZ; pKa = 6.7, CPFX; pKa = 6.0, CTX; pKa = 3.3), which play a very important role in the simultaneous separation of analytes using the chromatographic method. In this study, all the selected antibiotics were protonated in the working pH range of column i.e. 3–7. The 3D graph between pH and SDS inclined towards lower values of pH and higher values of SDS, contour plots clearly show that at lower pH (pH 3) the elution time of last peak was optimum (11.6 min.) with the resolution of all the analytes effectively. While at higher pH (pH 7), it was reduced to 6 min., due to which compounds overlapped with

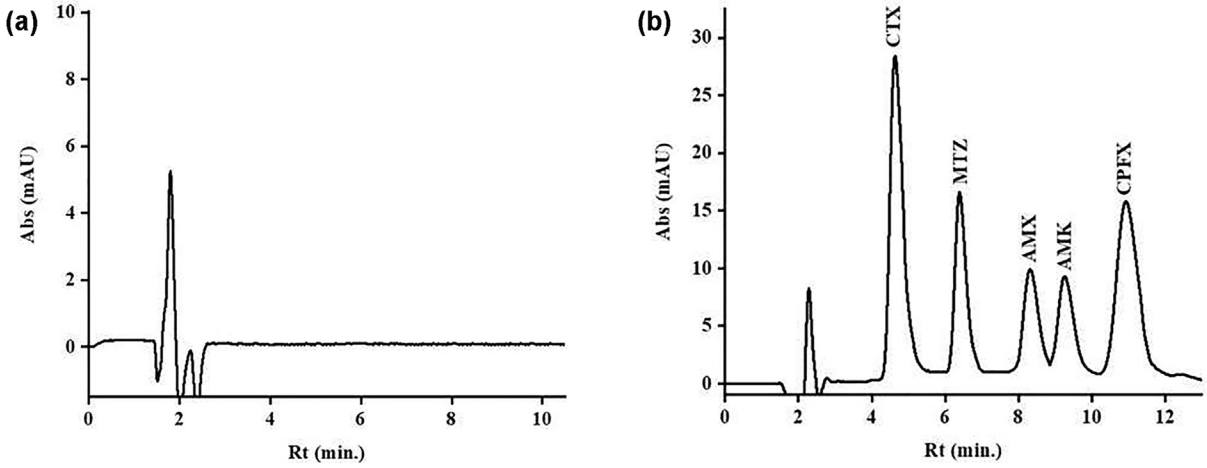

Fig. 7. Chromatogram of blank (a) spiked (b) sample in the optimized mobile phase.

each other and this clearly indicates the influence of pH on the mobile phase. The contour map shows that 5% concentration of organic modifier was sufficient to elute the last peak of selected antibiotics in a reasonable time. In Figs. 4a-c, 5a-c, 6a-c the influence of pH and SDS were evaluated on the elution time of last peak of the analytes. All the compounds were resolved well at pH 3.2 and SDS concentration of 0.14 M.

In all contour plots, the green region shows the optimum condition of the mobile phase along with their predicted values. During the method optimization, 0.14 M SDS-5.2% 1-butanol at pH 3.2 revealed the desirability of 1. However, to improve the efficiency of chromatographic peaks, minor variations were made in the values of all three variables. The optimum selected mobile phase was 0.15 M SDS-7% 1-butanol, pH 3. All the selected analytes were separated with good resolution within 11.6 min. of chromatographic run time. The optimized mobile phase was utilized to validate and analyse real samples.

3.3. Method validation

The validation was performed as per the United States Environmental Protection Agency (USEPA) 2016.

3.3.1. Specificity

Peaks that correspond to endogenous compounds in wastewater eluted within 2.5 min. and no peaks were detected in the time window of analytes. The chromatograms corresponding to the analysis of tap water (Fig. 7a) and spiked samples (Fig. 7b) of selected analytes and presented. The spiked chromatograms were compared with those which were obtained by analysis of the standard solution of selected antibiotics in the optimum mobile phase.

3.3.2. Linearity, limit of detection (LODs) and limit of quantification (LOQs)

Linearity, LOD and LOQ were obtained by preparing calibration curves in tap water within the lower, middle and upper concentration levels ranging from 0.15 to 10 μg/mL except for CPFX which was from 0.04 to 12 μg/mL or selected antibiotics. The calibration curves showed good linearity with correlation coefficients ($R^2 \geq 0.997$).

The value of slope and intercept are shown in **Table 4**. The LODs for the five antibiotics in tap water were estimated in the range of 0.02–0.18 μg/mL. The LOQs for the target compounds were in the range of 0.04–0.32 μg/mL. The relative standard deviation for all the analyzed samples in tap water was lower than 3.64% (**Table 4**).

3.3.3. Precision and accuracy

Precision and accuracy studies were performed by spiking tap water at LLQ, MLQ and ULQ for each analyte and five replicates were analyzed at each concentration on three different days. The RSD% was determined by comparing the standard deviation of the recovery percentages of the spiked samples. As shown in **Table 5**, the mean recoveries of five antibiotics in tap water were in the range of 96.2–104.6%, and the RSD was 0.7–6.7%. The obtained values for accuracy and precision are presented in **Table 5**. The RSD values were less than 6.7 which is acceptable according to European Guidelines for method validation (less than15%).

4. Analysis of wastewater sample

The developed method has been utilized to detect and quantify antibiotics (amoxicillin, amikacin, ciprofloxacin, ceftriaxone and metronidazole) in hospital wastewater from Sagar district, Madhya Pradesh (India). While talking about the study area, Sagar city has Government, charitable, private, medical and district hospitals. As already discussed, wastewater was analyzed from all hospitals which comes under the set criteria for sample collection for the present study (sec.*2.4)*, excluding maternity hospitals. All the hospitals of Sagar city, whether private or Government, have installed wastewater treatment plants, but none of them was working at the time of sample collection. Therefore wastewater from fifteen (15) hospitals located in Sagar city was collected to know the presence of antibiotics in sewage/sewer mainstream.

Hospital No. 15 reported maximum concentration (0.59 μg/mL) while hospital no. 3 had the lowest concentration of 0.05 μg/mL **(**Fig. 8**a)** of CTX. AMK was detected in only one hospital (hospital no. 4) at a concentration of 0.1 μg/mL **(**Fig. 8**b)**. CPFX was detected in 5 (five) hospital wastewater samples with the lowest (1.2 μg/mL), median (1.7 μg/mL) and high (2.1 μg/mL) (Fig. 8**c**) concentrations in charitable (hospital no. 13), medical (hospital no. 5) and multispecialty hospitals (hospital no. 3), respectively. As shown in Fig. 8**d** in all 15 analyzed samples from Sagar city, AMX was measured only in two samples with the concentration of 0.27 μg/mL (hospital no. 10) and 1.0 μg/mL (hospital no. 1). The concentrations which were detected for MTZ were > 1.2 μg/mL. It was detected in 5 (five) samples having the highest concentration of 2.2 μg/mL. The lowest concentration was found from hospital no. 8 (1.2 μg/mL) (Fig. 8**e**). The presence of all the selected antibiotics in sewage indicates that the WWTP was installed in all the monitored hospitals but still none of them was working effectively for removing the antibiotic from the wastewater. The hospital wastewater sample collected from Sagar city

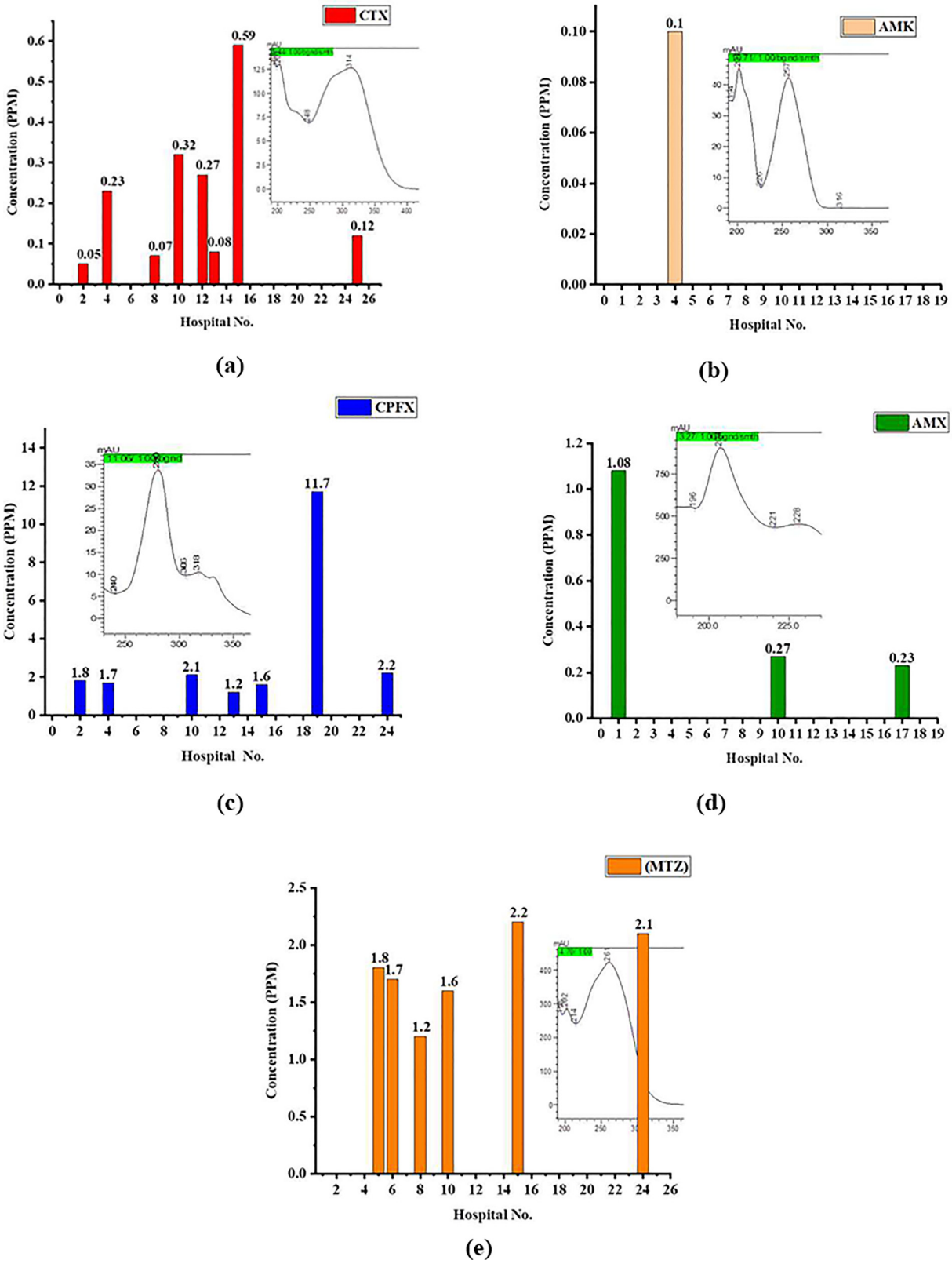

Fig. 8. The found concentration of selected antibiotics from hospital wastewater along with their UV–Vis. spectra (a), (b), (c), (d) and (e).

tested positive for antibiotics. The detected concentration range for different antibiotics was 0.05–0.59 μg/mL.

During the survey of the hospitals of Sagar district, it was found that PHCs had no WWTP. The hospitals generally use pit tanks or septic tanks to dispose of the wastewater from hospitals. These PHCs were 20 bedded where primary treatment was given to all the patients who visited from Tehsil or nearby villages. The hospital wastewater that drained into septic tanks slowly leached into nearby water bodies

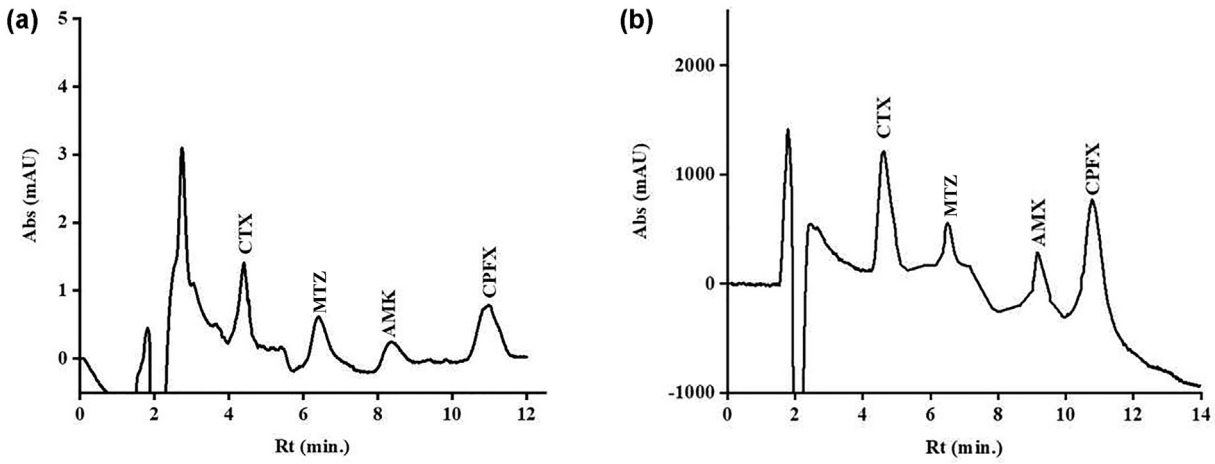

Fig. 9. a-b. Chromatogram of real sample; (a) Hospital no.3 (b) Hospital no.17 in optimized chromatographic condition.

from where it may reach the human body either through vegetation or drinking water of the nearby area. It has been reported in several research studies that high doses of antibiotics may cause adverse effects to humans and the flora fauna of those areas. It means that to treat any infection higher concentration of antibiotics will be required other than the prescribed dose. Consumption of vegetables and water containing antibiotics develops resistance in humans and animals and thus they need a higher dose of antibiotics for the treatment of different infections and diseases. In all the PHCs hospitals, selected antibiotics were detected in one or two hospitals except for AMK which was not detected in any of the samples collected from PHCs hospitals. CPFX was detected in two hospitals with the concentration of 2.2 μg/mL and 11.7 μg/mL all other antibiotics were detected in single hospitals. Hospital no. 17, 25, 24 **(**Fig. 8**a-e)** have shown the presence of AMX (0.23 μg/mL), CTX (0.12 μg/mL) and MTZ (2.1 μg/mL) respectively **(**Fig. 9**a)**. The representative chromatograms of detected antibiotics have been shown in Fig. 9**a and b.**

During analysis of wastewater samples, it was found that there was a variation in the found concentration of antibiotics from hospitals of Sagar city and hospitals of PHCs that clearly indicated the difference in use from urban to rural areas. Out of 10 hospitals of PHCs, only 4 of them have been shown the presence of selected antibiotics in comparison to hospitals located in Sagar city as 9 hospital wastewater found positive for selected antibiotics out of 15 hospitals. At the time of sample collection, there was low use of antibiotics in particular hospitals or the samples got diluted with the mainstream of water.

5. Conclusion

Like pesticides antibiotics now labelled as micropollutants will be a cause of concern in time to come. At present it is one of the emerging pollutants but if stern steps were not taken will turn out a pollutant of concern. The present work is carried out at Sagar, Madhya Pradesh (India), one of the underdeveloped middle-sized towns located in the centre of the country. In this work it is highlighted that developed country do have measures in line to treat wastewater containing pharmaceutical compound but in developing and underdeveloped countries, such wastewater treatment is either not available or are not functional. A small survey was carried out in the selected town to know the trend of antibiotic prescription to the hospital. Based on the survey the most commonly prescribed antibiotics selected for study were CTX, MTZ, AMX, AMK and CPFX in hospital wastewater.

The chromatographic method developed herein is simple, rapid and eco-friendly method for the simultaneous determination of CTX, MTZ, AMX, AMK and CPFX within 11.6 min. using direct injection technique. The developed method validated as per the United States Environmental Protection Agency (USEPA) 2016 guideline with satisfactory results in terms of specificity, linearity, LODs and LOQs, precision, accuracy and robustness. Out of 25 hospitals located in the district 13 hospitals have shown the presence of antibiotics, either single or in combinations in the range of 0.05 to 11.7 μg/mL.

The developed method was then applied to the hospital wastewater collected from different hospitals located at urban and rural Sagar. The developed method can also be routinely used for the simultaneous detection of antibiotics by the quality control division of pharmaceutical companies or drug management systems of hospitals or any other analytical laboratories working on antibiotics.

CRediT authorship contribution statement

Girraj Sharma: Validation, Formal analysis, Data curation. **Priyanka Pahade:** Investigation. **Abhilasha Durgbanshi:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Samuel Carda-Broch:** Conceptualization, Project administration, Funding acquisition. **Juan Peris-Vicente:** Validation, Formal analysis. **Devasish Bose:** Conceptualization, Methodology, Software, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are thankful to DST-FIST and DST-PURSE (Department of Science and Technology; Government of India) for supporting with FIST and PURSE scheme (Sl.No.64 Dated 31-05-2016). We also thank the Universitat Jaume I for its support and Girraj Sharma and Priyanka Pahade grateful to University Grant Commission (UGC) for providing a Senior Research Fellowship.

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