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Method validation for simultaneous detection of ciprofloxacin and enrofloxacin residues in water samples by salting-out assisted liquid–liquid extraction and liquid chromatography

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Abstract
Now a days the antibiotics become an emerging pollutant for the aquatic ecosystems and presence of antibiotics in the any environmental compartment have a great concern because it can provoke the development of resistance in the bacterial community. In fluoroquinolone antibiotic group ciprofloxacin and enrofloxacin antibiotics are used for the treatment of human and animal infection because of their wide antibacterial spectrum. The rampant and extralable use of these antibiotics could lead to contamination of environment. This study describes a simple salting-out liquid–liquid extraction technique for simultaneously detection of ciprofloxacin and enrofloxacin residues in water samples. The high-performance liquid chromatographic photodiode array detection containing C18 X-bridge analytical column was used for the determination of these antibiotics in water samples. The developed method has linear range of 50-500ppb with the linearity coefficient of 0.99. The limit of detection and limit of quantification of the method for ciprofloxacin was 17.4 and 52.8 µg/L, respectively and for enrofloxacin was 15.9 and 48.3 µg/L, respectively. The recoveries for the studied antibiotics ranged from 64.5-81.4% with relative standard deviations between 5.1 and 8.6%. The method was applied for the analysis of antibiotic residues in 15 water samples samples collected from Durg, Chhattisgarh, India and 2 sample found contaminated with antibiotic residues. The method has advantages of simplicity, easy operation and consumption of low volume of the less hazardous organic solvent.

Keywords: Antibiotic residues, HPLC, limit of detection, limit of quantification, recovery

Introduction
The discovery of antibiotics is considered the greatest scientific and medical milestone of the 20th century. Their development and use in human and veterinary medicine resulted in the significant reduction of the mortality and morbidity rates of important bacterial diseases and communicable diseases of childhood. Consequently, the remarkable efficacy and efficiency of the antibiotics retains a sense of the miraculous but now this miracle is under threat due to development of antibiotic resistance [1]. During the last decades, the presence of antibiotics in the environment has aroused an increasingly concern worldwide they are now consider as emerging pollutants. They are regarded as "pseudo persistent" contaminants due to their continual input into the ecosystem. Therefore, the occurrence of antibiotics in the environment has received considerable attention.

Antibiotics have been detected in various compartments of the aquatic environment, e.g. wastewaters, surface and ground water and in drinking water as well [2, 3, 4]. They are released to the aquatic environment in different pathways. After the administration to humans and animal, they are excreted as metabolites but also a considerable amount is eliminated in unchanged form as parent compounds via urine and faeces into the sewage. Many researches have shown the incomplete removal of pharmaceuticals including antibiotics during wastewater treatment processes [5, 6]. Wastewater treatment plants (WWTPs) are considered to be major contributors of presence of antibiotics in the environment. Pharmaceuticals along with their metabolites have been found in the effluents from WWTPs [7, 8, 9]. Therefore they can reach the surface and groundwater. Hospitals are also one of the most important contributors of the occurrence of the antibiotics into the aquatic environment [10, 11]. Aquaculture continues to be the fastest growing animal food-producing sector and they also work as a sink of different type of antibiotics and pharmacological active substance to tackle disease related

problem and to achieve better growth and these added antibiotics ultimately goes into water ecosystem.

Antibiotic pollution in the environment compartment leads to growing interest about their presence, persistence and fate in the environment because low levels of antibiotics can favor the proliferation of antibiotic resistant bacteria. The use of antibiotics in animal agriculture has been linked to the increased emergence of resistant strains of pathogenic bacteria that have potential to impact human health as well as non-target aquatic flora and fauna due to the reason of ecotoxicology of these compounds. Resistance genes and/or antibiotic resistant bacteria can be transferred from animals to humans. In addition, bacteria can develop cross-resistance between antibiotics used in veterinary medicine with those of similar structures used exclusively in human medicine [12, 13].

Fluoroquinolone antibiotics (FQs) are derived from quinolones and are widely used in human and veterinary medicine due to their broad activity spectrum against Gram negative bacteria through the inhibition of DNA gyrase and good oral absorption. In veterinary medicine, FQs are commonly and inappropriately used in food-producing animals for the treatment and prevention of diseases and as feed additives to increase animal mass. Ciprofloxacin is approved for human use and it's a first choice drug to treat gestro intestinal infection [14]. Ciprofloxacin is the most consumed antibiotic drug and as a consequence, fluoroquinolone resistance has been reported in both humans and animals in India [15].

It is very important to have information on the physical and chemical properties of an analyte (e.g., log K_{ow}, pK_a) because that may help to determine whether a compound is likely to concentrate in some specific conditions. Log K_{ow} is an indicator of the lipophilicity of the compound, high log K_{ow} is typical for hydrophobic compounds, whereas a low K_{ow} signifies a compound soluble in water. Most pharmaceuticals have acidic and/or basic functionalities; their ionization rate depends on acidic dissociation constants (i.e. pK_a values) and is controlled by solution pH. FQs have two relevant ionisable functional groups, 3-carboxyl group and the N-4 of the piperazine substituent. Therefore, FQs have two pK_a values and their acid-base behaviour is significantly affected by the physicochemical properties of the solvent. The intermediate form of FQs is a zwitterion pH plays an important role in the any extraction method because it affects the ionization status as well as the solubility of the analytes [16].

Various chromatographic detection techniques have been developed for the determination of FQ residues such as HPLC–UV [17], HPLC–DAD [18] and LC–MS/MS [19, 20]. LC/MS/MS has best technique because of its high sensitivity and selectivity, the direct injection of diluted solvent extracted from samples can provide a fast and reliable way to determine target antibiotics. However, MS is still quite expensive technique and till date not available in the most of the laboratories. HPLC methods are widely applied because of their high selectivity, sensitivity and simple sample treatment using different detection systems. Therefore, HPLC/PDA was chosen for the determination of FQs in this study. As the residues of antibiotics are usually present at very low concentrations in the environmental water, a sample preparation and pre-concentration step are necessary before analysis [21]. Several extraction procedures have been previously developed for the pre-concentration of antibiotics from water matrices including solid phase extraction (SPE) [22], liquid–liquid extraction (LLE) [23], and salting-out assisted liquid–liquid extraction for different pharmaceutical drugs [24]. Each of these methods has its own advantages and disadvantages.

Salting-out assisted liquid–liquid extraction (SALLE) is based on the phase separation of water–miscible organic solvents from the aqueous solutions in the presence of high concentration of salts. It uses water–miscible organic solvents which generally have low toxicity as the extractants, and the use of salts causes almost no pollution to the environment [25]. It is a cost effective technique and also take less time for sample preparation. Having such benefit's, SALLE was selected to extract ciprofloxacin and enrofloxacin from water sample in the present study. The objective of this study was the optimization of analytical parameters for the extraction by SALLE and determination of ciprofloxacin and enrofloxacin antibiotic residues in water samples using HPLC–PDA.

Materials and Methods

Equipment

HPLC measurements were carried out using a quaternary gradient chromatographic system from Waters, Inc. (USA), model Alliance ® – e2695, coupled to a photodiode array detector, Waters®2998. Data acquisitions were performed by Empower™ 3 Chromatography Software. Other equipment such as Sartorius electronic weighing balance, refrigerated centrifuge (Thermofisher ™, USA), pH meter (pHTutor ® Digital pH Meter), vacuum concentrator (Vacufuge® plus, Eppendorf™ AG, Germany) and Vortex Shaker (Spinix ™ Tarson Instruments, India, Pvt. Ltd.) were also used in the present study.

Standards and Reagents

Ciprofloxacin (CIP) (98–99%) and enrofloxacin (ENRO) (98–99%), analytical standards were purchased from Sigma-Aldrich (Fluka and Vetranal), Co, USA. Different analytical grade salts used were magnesium sulfate (Himedia Industries, Mumbai), sodium chloride (ThermoFisher, 99.5%). Hydrochloric acid, sodium hydroxide and HPLC grade methanol and acetonitrile were purchased from Merck (Germany). HPLC grade water was obtained from Milli-Q water purification system from Millipore (USA).

Preparation of Standard Solutions

Individual Standard stock solutions of Ciprofloxacin (CIP) and enrofloxacin (ENRO) were prepared at a concentration of 0.1 mg/mL, by dissolving an accurately weighed quantity of each compound in 1 mL acetic acid and adjusting to volume with methanol. The standard solutions were stored in dark glass bottles at -20°C and were stable for a period of 3 months. Working solutions were prepared daily by appropriate dilution of aliquots of the standard stock solutions in HPLC grade water. The working solutions were used for preparation of calibration curves of concentration 50, 100, 200, 300 and 500 µg/L.

Sample Extraction procedure

CIP and ENRO residues from water samples were extracted as per the method described by Gezahegn *et al.* (2019) [26] with some modification. The 10 mL water sample was first centrifuged at 4000 RPM for 10 min, filtered through 0.45 µm filter and pH was adjusted to 3 using 0.1N HCL solution. Sample was then spiked with a predetermined volume of the standard solution containing the target analyze and quantitatively transferred the 50 mL screw-capped

polyethylene test tubes. Next, 5 mL acetonitrile was added and vortexed for 1 min. and then 4 g $MgSO_4$ were added to mixture. Thereafter, the solution was vortexed at high speed for 6 min to ensure complete dissolution of the salt. This was followed by centrifugation of the solution at 4000 rpm for 5 min which resulted in phase separation. The upper organic phase was carefully withdrawn using micro-pipet and the extract was collected in a clean beaker and concentrated to dryness at 60 °C in vacuum concentrator. The residues were dissolved in 1 ml methanol: water (20:80) and filtered through a 0.22-µm syringe filter and stored at -20°C for further analysis. An injection volume of 15 µL was finally injected into the HPLC system for detection of the residue in water sample.

HPLC Analysis

CIP and ENRO were separated on a Waters XBridge C18 column (4.6 × 150 mm, 3.5 µm, (Milford, MA, USA) at 40°C with a flow rate of 0.82 mL min^{-1}. The injection volume was 15 µL and the detection was on PAD at 278, 280 nm and 300 nm. However, detection of the FQs was optimum at 280 nm and therefore this wavelength was selected in this study. The mobile phase used for this study was proposed by Moema *et al.* (2012) [27] which consisted of A (acetonitrile) and B (0.1% formic acid in water) at pH 2.74. Base-line resolution was achieved using the following gradient elution mode: initially A was at 13% and it was gradually increased to 15% in 7 min, decreased to 10% in 7 min and finally increased to 13% in 1 min. then it was maintained for 2 min. for next run thus total run time required for good separation of CIP and ENRO was 17 min.

Method Validation

The proposed method was validated for different performance criteria; *viz.* linearity, intraday assay and interday assay, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). The linearity response was examined by triplicate analysis of standard solution with ENRO and CLP at five levels (50, 100, 200, 300 and 500 µg/L). The standard calibration curves were obtained by plotting concentrations (µg/L) against peak area.

LOD and LOQ were calculated from the standard deviation (σ) of y-intercepts of regression analysis and the slope of calibration curve (m) using equations: 3σ/m and 10 σ/m, respectively. The precision of the method consists of intraday assay precision and interday assay precision and expressed as % RSD of peak area measurements. The intraday assay precision was determined by spiking three Mili Q water samples at a single concentration level of 200 µg/L and evaluation was done through the results obtained with the method operating over 1 day under the same conditions. The inter-assay precision was determined at three fortification levels, 50, 100 and 200 µg/L and the analyses were performed over the period of three consecutive days.

The accuracy of the method expressed as recovery % was determined by triplicate analysis of spiked MiliQ water samples at three fortification levels (50, 100 and 200 µg/L). The recoveries were calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

Results and Discussion

Optimization of HPLC conditions

The mobile phase composition described by Moema *et al.* (2012) [27] for simultaneous detection of fluoroquinolones antibiotics in chicken meat was used for optimization of chromatographic separation in the present study .Gradient elution of Acetonitrile (ACN): 0.1% formic acid in water at pH 2.74 was used. In this study, we used X-Bridge C18 column (4.5 × 150 mm, 3.5 µm, (Milford, MA, USA) which is a different from the column used by the Moema *et al.* (2012) [27] they used Waters XTerra MS C18 column (3.0 × 150 mm, 3.5 µm, (Milford, MA, USA) so all sepration related parameter like injection volume, flow rate, column temperature and maximum absorption wavelength were optimized to obtain good separation of both the studied compound . Thus, by using a 15µL injection volume, adjusting the flow rate to 0.82mL/min and column temperature to 40 °C, excellent separation for targeted antibiotics standard was achieved on X-Bridge column using photodiode array detector. Thus, the optimized method resulted in effective separation of ciprofloxacin and enrofloxacin antibiotics belonging to fluroquinolone group of antibiotics in a single run with adequate resolution. The maximum absorption wavelength for each compound was optimized to attain maximum sensitivity. Thus, the detection wavelength of 280 nm gave maximum absorption for these compounds.

Extraction of ciprofloxacin and enrofloxacin from water

SALLE is an environmental-friendly technique that generally uses organic solvents with low toxicity and salts that almost are not pollutants for the environment. But the development of unique sample preparation in SALLE requires extraction solvent, type of the salt, solution pH. For extraction studies, the MiliQ water samples fortified with antibiotic standard solutions (200 µg/L) were employed.

Several water-miscible organic solvents such as acetonitrile, acetone, methanol, ethanol, and ethyl acetate can be used in the SALLE. Acetonitrile is the most conventional solvent used in SALLE due to being chemically inert with organic analytes, and its wide use as the mobile phase in liquid chromatography. Various inorganic and organic salts such as sodium chloride (NaCl, magnesium sulfate ($MgSO_4$), sodium acetate ($CH3COONa$), ammonium sulfate ($[NH4]_2SO_4$), and ammonium acetate ($CH3COONH_4$) can provide the salting-out effect and enhance the transfer of hydrophilic analytes to the organic solvent. It is necessary that the selected salt needs to be soluble in the aqueous sample and have partial solubility in the organic solvent [28, 25]. The pH value is important as it affects the ionization status as well as the solubility of the analytes [29, 30]. Development of efficient extraction methods for ionizable and relatively polar compounds, pH of the sample solution plays a decisive role. The sample solution pH should be lower than the pKa of the analytes to obtain the target analytes in their unionized forms so that they have a higher tendency to partition into the organic phase [31, 32, 33].

For extraction of different pharmaceuticals and antibiotics from water, various extraction solvents and salts described in the literatures such as methanol, acetone, acetonitrile and salts like NaCl, $MgSO_4$ at solution pH 3, 4 and 7 were evaluated with best results obtained from acetonitrile and $MgSO_4$ at pH 3 that resulted in simultaneous extraction of ciprofloxacin and enrofloxacin antibiotics from water with good recoveries. The acetonitrile as a extraction solvent and $MgSO_4$ as a extracting salt has been used by many researchers for the extraction of various pharmaceutical including antibiotics from water with good results [34, 32, 26, 35].

Method evaluation

The HPLC-DAD method was validated for the determination of ciprofloxacin and enrofloxacin by assessment of following parameters: linearity,specificity, intraday assay and interday assay precision, accuracy, LOD and LOQ. The results are presented in Table 1.The calibration graph was constructed by plotting the peak area versus the corresponding concentrations with 5 levels in the range of 50 µg/L to 500 µg/L. The linearity was characterized with a good determination coefficient (R^2) of 0.9998 for both the target compound. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation (s) of y-intercepts of regression analysis and the slope of calibration curve (m) using equations 3s/m and 10 s/m, respectively. As presented in Table 1, the LOD for the studied antimicrobials ranged from 15.9 to 17.4 µg/L. Recovery experiments were carried out by spiking , MiliQ water with working standard solutions of ciprofloxacin and enrofloxacin at three fortification levels: 50,100 and 300 µg/L with three replicates for each level. Repeatability of the method was calculated in terms of the percent relative standard deviation (% RSD).

Table 1: Method validation parameters for HPLC-DAD method optimized for the determination of CIP and ENRO, in water sample

Method Validation Parameters	CIP	ENRO
Linear range (µg/L)	50–500	50–500
Linearity (R^2)	0.99	0.99
Linear regression (Equation)	y = 129.18x - 536.18	y = 113.87x + 46.348
LOD (µg/L)	17.4	15.9
LOQ (µg/L)	52.8	48.3
Intraday assay precision, n = 3 (%RSD) 200 µg/L	3.2	4.1
Inter day assay precision, n = 3 (%RSD)		
50 µg/L	6.0	5.4
100 µg/L	8.6	5.1
200 µg/L	5.6	6.5
Recovery %		
50 µg/L	69.1	73.6
100 µg/L	71.0	74.5
200 µg/L	72.9	75.7

Environmental water sample analysis

The procedure was applied to the analysis of ciprofloxacin and enrofloxaicn in the ponds, fish pond and hand pump water 15 samples, located in adjoining villages of Durg city, Chhattisgarh, India, were analyzed for the presence of targeted antibiotics. Analyses showed that the 2 water samples were positive for ciprofloxacin and enrofloxacin residues with levels below the LOQ of the method for these antibiotics. The purpose of conducting analysis of the water samples is to corroborate the performance of the method and not to perform quality control testing.

Conclusion

A SALLE method coupled with HPLC–PDA was presented for the simultaneous extraction, identification and quantification of ciprofloxacin and enrofloxacin in water samples, and successfully utilized for the determination of ciprofloxacin and enrofloxacin residue in environmental water. The above method offered a number of features including good liniarity, high recovery, and short analysis time, simple operation process, cost effective, and environmentally friendly. The method has advantages of simplicity, easy operation and consumption of low volume of the less hazardous organic solvent, acetonitrile. Therefore, the developed method can be utilized as an attractive method for the determination of antibiotic ciprofloxacin and enrofloxacin in environmental water matrices.

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