A SELECTIVE DISPERSIVE LIQUID-LIQUID MICRO-EXTRACTION TECHNIQUE FOR TRACE LEVEL POLLUTANTS ENRICHMENT OF PHARMACEUTICAL RESIDUES FROM HOSPITAL WASTEWATERS FOLLOWED BY LIQUID CHROMATOGRAPHIC ANALYSIS

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ABSTRACT. In this study a dispersive liquid-liquid micro-extraction (DLLME) method was utilized for extraction of trace pharmaceuticals in wastewater samples. Factors influencing the extraction performances were tested and accordingly 900 µL dichloroethane as the extraction solvent, 1400 µL acetonitirle as dispersive solvent, 10 min extraction time at 4000 rpm centrifugation, and pH 5 were found optimum. Acetonitrile and 0.2% formic acid in water were used as eluent. The column temperature was maintained at 25 °C and the optimum detection wavelengths of 273 nm for sulfamethoxazole and 280 nm for ciprofloxacin were used. For both the analytes, the coefficients of determinations (r^2) were found to vary from 0.9989 to 0.9997, confirming good linearity in the concentration range of 0.005-100 µg mL^{-1} for sulfamethoxazole and 0.01-100 µg mL^{-1} for ciprofloxacin. The LOD and LOQ were in the range of 0.78-1.58 ng mL^{-1} and 2.24-5.28 ng mL^{-1}, respectively; the RSDs were 0.41-3.21% for intra-day precision and 0.37-6.44% for inter-day precision. The concentrations of the three pharmaceuticals determined ranged from 0.76-1.53 µg mL^{-1} in the wastewater samples collected from Black Lion Hospital. However, ciprofloxacin and doxycycline were not detected and only low concentration of sulfamethoxazole was detected in the wastewater samples collected from Menelik II Hospital.

KEY WORDS: Antibiotics, DLLME, Dispersive solvent, Hospital wastewater

INTRODUCTION

Pharmaceuticals are a class of emerging chemical contaminants in aquatic environments that are integral to human and veterinary medicine, where they are applied to diagnose, treat, or prevent disease [1]. By design, each pharmaceutical has a specific mode of action, which enables the compound to be divided into subgroups, including but not limited to analgesics, anti-inflammatory drugs, antibiotics, contraceptives, beta blockers, lipid regulators, and neuroactive compounds [2].

Antibiotics are types of antimicrobial that particularly treat or prevent infections caused by pathogenic bacteria or fungi in human and animal which specifies them from disinfectants or other antimicrobials. In the past, the term antibiotic only referred to natural compounds generated by bacteria or fungi such as tetracyclines, but today they also include synthetic or semisynthetic compounds such as sulfonamides. Antibiotics can kill (thus bactericidal) or suppress the growth of bacteria or fungi [3].

The first antibiotic, penicillin, was introduced by Alexander Fleming in 1928 [4]. To date, several kinds of antibiotics have been developed and extensively used in medicine, and around 250 different antibiotics have been known to be used as human and veterinary pharmaceuticals. Due to the increased volumes of the varied types of these chemical substances produced, there has been a great research interest to understand the extent to which their occurrences and thus accumulation negatively affect the environmental compartment. This is primarily because the quantities of pharmaceutical production, extent of consumption and their ultimate discharge into the environmental is steadily increasing [5].

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Furthermore, different pharmaceuticals are supplied to the consumers at high levels to ensure prompt biological responses; however, large proportions of consumed pharmaceuticals are excreted from the body and enter the environment through wastewater effluents. Although this is the most common way pharmaceuticals entering the environment, they could also directly be released into wastewater systems from the manufacturers [6, 7]. Human actions, termed as 'involuntarily' and 'purposefully', are primarily responsible for the release of pharmaceuticals into the environment. Involuntary actions include pharmaceutical excretion through the body or washing of topical medicines down the drain. When compared to other aquatic pollutants such as pesticide residues, the entry of pharmaceuticals into the environment depends on a number of integral factors [8]. These factors include the overall pharmaceutical consumption rate, the pharmacological fate of the drug within the body, the behavior of the drug during the wastewater treatment process and the ability of the receiving water to provide adequate dilution. Human pharmaceuticals are excreted into the sewage system as a mixture of the parent compound and metabolites, comprising mostly of transformation products and conjugated glucuronides [9]. In contrast, purposeful actions include the disposal of unused or out-of-date medicines down the drain or into the waste [8, 10]. The antibiotics found in the aquatic ecosystem, come from domestic, hospitals, the pharmaceutical industry, aquaculture, and agricultural activity.

The presence of antibiotics in the aquatic environment is a serious concern because it may accelerate the proliferation of antibiotic-resistant pathogens, through genetic mutations and resistance vectors with high transfer rates between pathogens, thus lowering the therapeutic effect of antibiotics. According to the World Health Organization [11], antimicrobial resistance is a significant challenge to global human and animal health, food safety, and development today, with the perspective of aggravation in the upcoming years, unless adequate measures are not taken. The toxicity of antibiotics on aquatic organisms has been evaluated, and found that these compounds may have harmful effects on growth, development, reproduction for time of life [12]. A wide range of antibiotics such as macrolides and sulfonamides showed negative effects on the development and growth of algae. Antibiotics can also damage the photosystems of plant cells and can reduce the rate of carbon dioxide transformation [12]. The residues of antibiotics in the aquatic environment can be spread widely due to the lack of proper wastewater treatment systems. The antibiotics present in water may enter the soil system affecting the function of native biota that plays essential role in the biogeochemical cycling of elements [13].

The chronic exposure to antibiotics by eating food or water provokes human health risks, for that it has been suggested to ban the use of antibiotics in the production of animals for food [14]. Consequently, the more pharmaceuticals consumed, the greater the concentrations that will be discharged into the environment, thereby elevating the extent of their occurrence. Since there are numerous species of pharmaceuticals in the environment at trace levels, possessing a wide range of physicochemical properties, the development of reliable analytical techniques to better quantify these compounds is imperative [15].

Sample preparation techniques are important in sample analysis procedure to remove the interfering substances by concentrating the trace residues, for enabling amenable instrumental measurements [16]. Interfering matrices in measurements could also be removed by cleaning up the extracts, while rendering them in a form that is compatible with the analytical system [17]. To date, quite a number of sample handling techniques have been developed mainly by reducing the scale of analytical operations as well as that of the extraction devices, i.e., miniaturization. Several novel miniaturized approaches have enabled workers to overcome the disadvantages of conventional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [18]. Some of the most well-recognized procedures include hollow fiber protected liquid-phase microextraction (HF-LPME) [19], dispersive liquid-liquid microextraction (DLLME) [20], solid phase microextraction (SPME) [21], and magnetic solid-phase extraction (MSPE) [22]. In the present study, DLLME has been considered and employed to efficiently enrich traces of the residues of the analytes under study. The advantages of DLLME, including the easy operation, rapidity and

high recovery, as well as other variables that can be regulated to improve extraction efficiency, makes this technique exceedingly suitable for pharmaceutical analysis [23, 24].

Dissemination of antimicrobial resistance bacteria in the environment is a major problem in developing countries, mainly due to improper antibiotic usage, ineffective infection control program and lack of better management of hospital wastewater. In Ethiopia, rapid urbanization and industrialization with improper environmental planning often lead to the discharge of industrial and hospital sewage effluent directly to the environment [25]. The purpose of this study is thus to develop the DLLME method for determination of selected pharmaceutical trace residues in environmental wastewaters and applying it to wastewater sample analysis around the localities where the residues are contaminating the different environmental resources from selected hospitals in Addis Ababa.

EXPERIMENTAL

Chemicals and reagents

All the solvents used in this study are of HPLC grade, and the chemicals and reagents are also of analytical grade. HPLC grade methanol (Carlo Erba, Rodano, Italy > 99.9%), acetonitrile (Sigma-Aldrich, for HPLC, UV and GC, > 99%), formic acid (Sigma-Aldrich, 85%), and ethanol (Fisher Scientific, UK, 99.9%) were used as received. The dispersive solvents and extraction solvents were obtained from different sources: dichloroethane was purchased from Sigma–Aldrich, (Germany); chloroform from Sigma Aldrich (Seelze, Germany); dichloromethane (≥99.6%) was from Sigma–Aldrich,(Germany); carbon tetrachloride from BDH Chemicals Ltd. (Poole, England), acetone from Scharlau (Barcelona, Spain) and extra pure sodium chloride was received from Oxford laboratory (Mumbai, India). Sodium hydroxide pellet was from BDH Laboratory Supplies (Poole, England) and hydrochloric acid from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Deionized water used as reagent water throughout this study was obtained by purifying with deionizer (EASYPure LF, Dubuque).

All the standards of antibiotic compounds (Table 1); *viz.,* ciprofloxacin (CIP), sulfamethoxazole (SMX) and doxycycline (DC), were of analytical reagent grade and were the kind donation from Ethiopian Public Health Institute (EPHI).
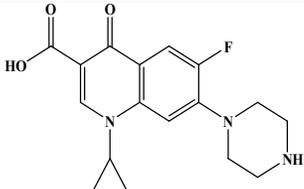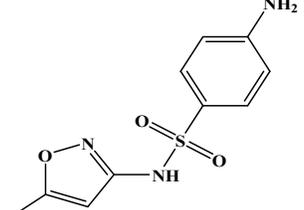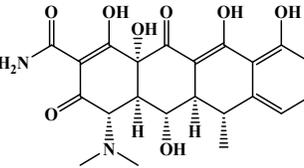
Instrumentation

The HPLC system used for sample analysis was Agilent 1260 infinity with Quaternary Pump, Agilent 1260 Series Vacuum Degasser, Agilent 1260 Series Autosampler and Agilent 1260 Series Diode Array Detector Purchased from Agilent Technologies (Hewlett-Packard Strasse Waldbronn, Germany). Data acquisition and processing were accomplished with LC Chemstation software (Agilent Technologies). Adwa pH meter (AD1020 pH/mV/ISE/Temperature, Hungary) was used for sample and extract pH adjustment and A 800 model centrifuge (China) was used to speed up the phase separation. Electronic balance (Adam Equipment Company, UK) was utilized for weighing different chemical substances. Chromatographic separation was achieved with an OmniSpher C_{18} reversed phase column (4.6 mm x 250 mm x 5 μm), and the injection volume was 10 μL.

Preparation of standard solutions

The stock solutions of CIP, SMX and DC were prepared by dissolving 10 mg of the target analytes in chromatographic grade methanol and deionized water of 1:1 *v/v* ratio, in order to obtain a concentration of 200 μg mL^{-1} of each analyte in 50 mL volumetric flask. All the standard solutions

were stored at 4 ºC in the refrigerator. Working standard solutions were prepared by diluting the stock solutions with equal volume of methanol and deionized water. All solutions were filtered through 0.22 mm nylon membrane syringe before injection into the chromatographic system.

Table 1. Physicochemical properties and chemical structure of the selected antibiotics [26].

Class	Compound	Molecular Formula	Mol. weight, $g\ mol^{-1}$	Chemical Structure	Solubility $mg\ mL^{-1}$	Log K_{ow}	pK_a
Fluoroq-uinolone	Ciprofloxa-cin (CIP)	$C_{17}H_{18}FN_3O_3$	331.3		36	0.4	3.01, 6.38, 8.70
Sulfon-amide	Sulfameth-oxazole (SMX)	$C_{10}H_{11}N_3O_3S$	253.3		0.61	0.89, 0.48	1.85, 5.6
Tetra-cycline	Doxycycline (DC)	$C_{22}H_{24}N_2O_8$	444.43		630	—	3.0, 7.9, 9.2

Sample collection and handling

The wastewater samples were collected from Black Lion Hospital; located at 9˚01'13"N latitude and 38˚44'59"E longitude with elevation of 2388 m above sea level and Menelik II Hospital; at a latitude of 9˚02'20"N and longitude of 38˚46'30.4"E with elevation of 2352 m above sea level; both found within Addis Ababa; the capital of Ethiopia. The wastewater samples were collected in clean amber glass bottles, rinsed at least three times with the samples. About 1.0 L wastewater sample was collected from each sampling site and transported to the Analytical Chemistry Laboratory of the Addis Ababa University (AAU). Upon arrival to the laboratory, all the wastewater samples collected were immediately filtered, through 0.45 μm filter paper, and stored in amber bottles at 4°C in the refrigerator.

Chromatographic conditions

Separation in liquid chromatography is highly affected by the mobile phase; both solvent type and composition. The mobile phase was selected based on the physicochemical properties of the pharmaceutical drugs in order to provide good separation [27]. The optimum mobile phase composition utilized throughout the chromatographic analysis was acetonitrile (B) and 0.2%

formic acid in water (D) at a flow rate of 1 mL min^{-1}, in a gradient elution mode. The column temperature was maintained at 25°C and the detector was adjusted at the optimum detection wavelength of 273 nm for SMX and DC, and 280 nm for CIP. Aliquot of 10 μL of the extracted sample was injected into the HPLC system and eluted for 18 min run time and 2 min post run time. The mobile phase was delivered using the following gradient elution program: 0 min (15% B); 10 min (40 % B); 18 min (100% B); 20 min (15% B); at a flow rate of 1 mL min^{-1}. Finally, the peak area was utilized as the instrumental response and the analysis was obtained under the aforementioned chromatographic conditions.

Dispersive liquid-liquid micro-extraction (DLLME) procedure

A 5 mL wastewater sample with pH 5, which was adjusted using 0.1 mol L^{-1} HCl/NaOH was placed in a 10 mL glass centrifuge tube. Then, 1400 μL acetonitrile (dispersive solvent), containing 900 μL of dichloroethane (extraction solvent) was rapidly injected into the sample solution by micropipette. The injection of the extraction mixture led to the formation of cloudy sample solution which was hand shaken for 3 min. The phase separation was performed by rapid centrifugation at 4000 rpm for 10 min. The lower organic phase was taken by a microsyringe and transferred to a vial. The content was then evaporated in an oven at 40°C, and the residue was reconstituted with 400 μL acetonitrile/deionized water (1:1, *v/v*). Finally, 10 μL enriched extract was injected into the HPLC-DAD system.

RESULTS AND DISCUSSION

Optimization of the DLLME conditions

To optimize extraction conditions and extraction efficiency, several factors including the type and volume of extraction and dispersive solvents, extraction time, sample pH and salting out effect were studied and optimized. The peak area of the analyte was the signal used to evaluate the influence of various parameters on the performance of the DLLME. It is to be noted that during analytical method development and optimization, only the following two compounds; Ciprofloxacin (CIP) and Sulfamethoxazole (SMX) were utilized. Doxycycline (DC) was not well extracted and the peak was found to be at trace level. However, its occurrence was confirmed by standard addition to the sample extract; this has further been discussed under application section.

Selection of extraction solvent

Selection of the organic extraction solvent for DLLME is based on its appropriateness in fulfilling certain requirements. These include (1) the good affinity the solvent should possess for the target compounds, (2) it should have a low solubility in water, (3) it should have a higher density than water, and (4) the solvent should have no interferences with the analyte peaks when directly injected for chromatographic analysis [28]. Depending on these requirements, the following extraction solvents; namely, dichloroethane (C$_2$H$_4$Cl$_2$, density: 1.26 g cm^{-1}), chloroform (CHCl$_3$, density: 1.49 g cm^{-1}), dichloromethane (CH$_2$Cl$_2$, density: 1.33 g cm^{-1}), and carbon tetrachloride (CCl$_4$, density: 1.58 g cm^{-1}); all with density higher than water and different polarities were tested for their extraction efficiency. The effects of these solvents on extraction performance are given in Figure 1. As can be seen, dichloroethane has relatively higher extraction efficiency for both the analytes compared to the rest three solvents. This may be attributed to the higher polarity of dichloroethane compared to the other solvents, and this property could have contributed towards better solubility of the target analytes; which are containing polar groups such as carboxyl, hydroxyl, or amino groups. Therefore, dichloroethane was selected as the extraction solvent of choice in this study.
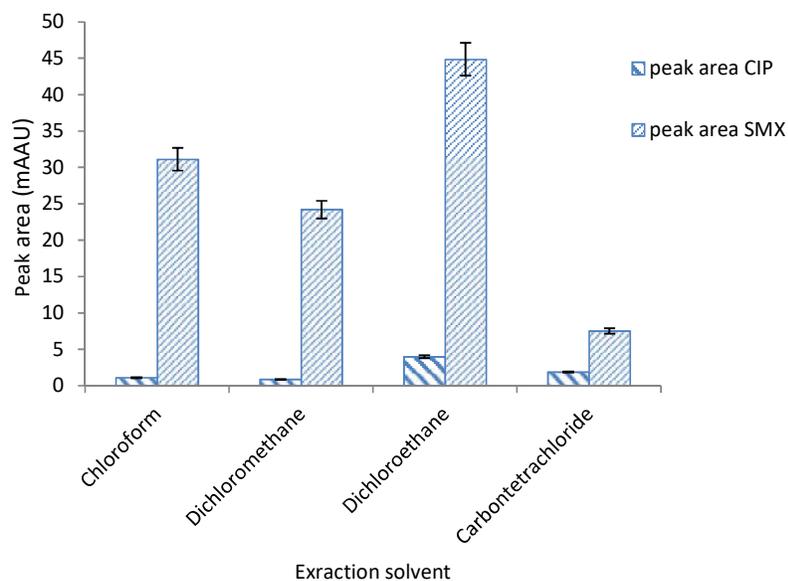
Figure 1. Effect of the extraction solvent on extraction of antibiotics in samples spiked at 25 mg L^{-1}: DLLME conditions: sample volume, 5 mL; sample pH, 7; dispersive solvent, 600 μL methanol; extraction time, 10 min; stirring rate, 4000 rpm.
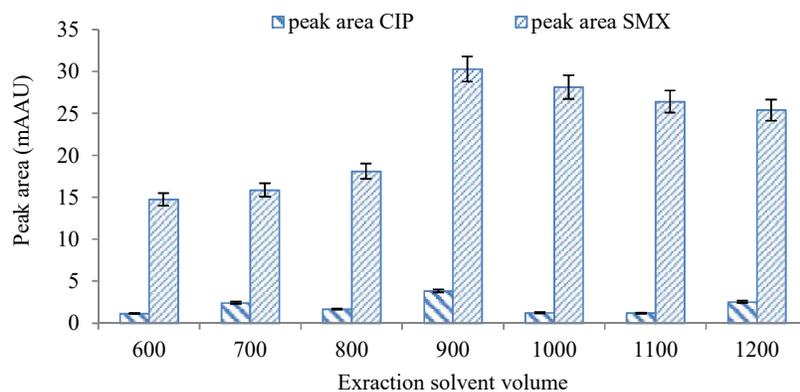

Figure 2. Effect of the extraction solvent volume on the extraction efficiency of the antibiotics in the samples spiked in 25 mg L^{-1}. Other experimental conditions are same as those indicated in Figure 1.

Effect of extraction solvent volume

The volume of extraction solvent, dichloroethane, required to exhibit optimum efficiency was evaluated. To this end, the sample solutions containing different volumes of dichloroethane, ranging from 600 to 1200 μL were tested. Extractions were conducted from 5 mL aliquots of the

sample solutions. Experimental results obtained are shown in Figure 2, and based on the obtained results, maximum instrumental responses were obtained when the volume was 900 μL for both analytes. It has been noted that with a lower volume of extraction solvent, dispersion of the analytes into the solvent may not be effective, while with the higher volumes of the solvent than the optimum value, the extracts would get diluted thus causing reduced extraction efficiency [29]. Hence, 900 μL dichloroethane was finally selected as optimized volume for the subsequent experiments.

Selection of dispersive solvent

One of the most important parameters affecting selection of dispersive solvent is its relative miscibility with the extraction solvent and aqueous phase. Appropriate dispersive solvent can disperse the extraction solvent to fine droplets in aqueous sample and increases the surface area for transferring the target compounds from sample matrix to the extraction solvent [30]. Several solvents such as methanol, ethanol, acetonitrile and acetone were tested for use as dispersive solvents. One of the major characteristics properties the dispersive solvent should possess include the capacity of effective dispersion with the extraction solvent in the aqueous phase for efficient cloud formation of the entire contents [30]. As a result, solubility of the analytes in dichloroethane would increase; leading to increased recoveries of the analytes. Therefore, acetonitrile, fulfilling these characteristic properties exhibited better results, Figure 3. Thus, it was selected as the dispersive solvent of choice for subsequent analyses.
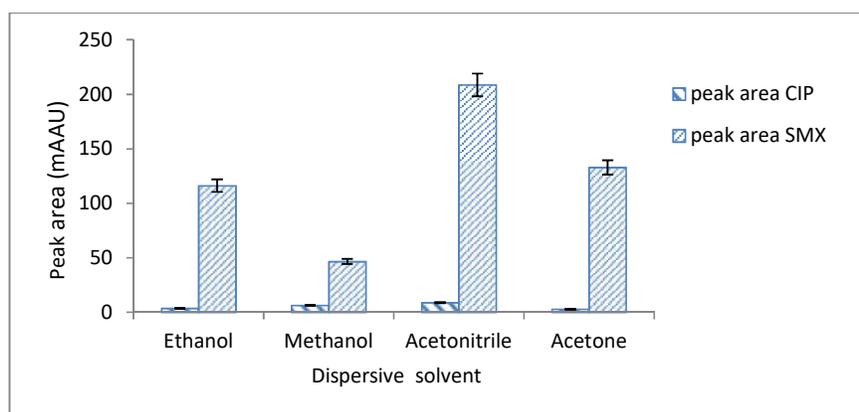

Figure 3. Effect of the dispersive solvent on extraction of antibiotics in the samples spiked at 25 mg L^{-1}. Extraction solvent volume, 900 μL dichloroethane; other experimental conditions are same as those indicated in Figure 1.

Effect of dispersive solvent volume

The volume of dispersive solvent has also crucial effect on the analyte extraction efficiency. Commonly, at low dispersive solvent volume, the tiny droplet of extraction solvent may not be formed effectively, thereby lowering the extraction efficiency. On the other hand, at higher volumes of the dispersive solvent solubility of the analytes in sample solution increased, which may further lower the partitioning of the analytes into the droplets of extraction solvent leading to decreased extraction efficiency [31]. Therefore, different volumes of the dispersive solvent (ranging from 600 to 1900 μL) were tested. The results in Figure 4 indicated that the extraction

efficiency increased with increasing acetonitrile volume from 600 μL to 1400 μL and then slightly decreased. At the same time, the volume of organic phases decreased with increasing of acetonitrile. No phase separation was observed when the volume of acetonitrile was higher than 1600 μL, which could be due to the higher solubility of dichloroethane in sample dispersive solution. Based on the observed results, 1400 μL acetonitrile was chosen for further experimental works.
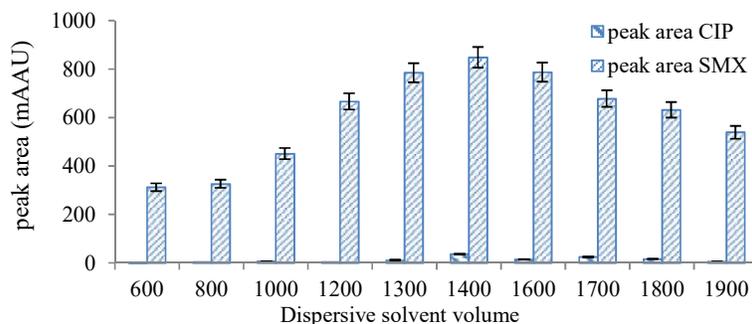

Figure 4. Effect of the dispersive solvent volume on extraction of antibiotics in the samples spiked at 25 mg L^{-1}. Other experimental conditions are same as those indicated in Figure 3.

Effect of the sample solution pH

The pH of the sample solution is a crucial parameter for DLLME. It is the major contributor involved in the extraction efficiency, especially for acidic/basic analytes. With regard to pK$_a$ of the analytes, the transport of the analytes from the sample solution to the extraction solvent is highly affected by variation of pH of the sample solution [32]. The extraction efficiency for an organic compound can be changed by adjusting the pH of the aqueous solution, because the existing form of the analyte is dependent on it. Only when the sample solution was adjusted to a desired pH, where the analytes were uncharged that the analytes could be extracted effectively with organic extractants. Therefore, the pH of the solution was varied from 3 to 6 by using 0.1 mol L^{-1} HCl/NaOH solutions. Sample with pH 5 provided satisfactory recoveries for the tested target analytes, and thus pH 5 was selected for the subsequent studies.

Effect of ionic strength

The extra salt added to the extraction sample solution may greatly change the physical properties of the extraction film and thus reduces the diffusion rate of the analytes into the organic phase. Moreover, target analytes could also participate in electrostatic interactions with the salt ions in solution, thereby decreasing the tendency for their movement into the organic phase [33]. In order to evaluate this phenomena, varied amounts of sodium chloride, in the range of 0–25% (w/v), at 5% interval, were added to investigate the influence of ionic strength on extraction performances. The presence of sodium chloride increased the ionic strength of the sample solution and decreased the solubility of extraction solvent in water, which increased the volume of organic phase. However, there was no significant variation on the extraction efficiency for any of the target analytes studied. Therefore, salt was not introduced into the sample solution in any further DLLME procedure.

Effect of extraction time

In DLLME, extraction time was defined as the time interval between the formation of homogeneous cloudy solution and phase separation [34]. In order to study this effect, the time of centrifugation was varied and it was observed that extraction efficiency increased in the range of 5–10 min. When the extraction time was further increased, beyond the observed optimum, the extraction efficiency was exhibited a decreasing tendency. Due to the large contact surface between extraction solvent and aqueous phase in the emulsion system, the extraction equilibrium can easily be achieved within a short period of time. However, the emulsion solution was unstable and it would delaminate in the course of over-extension of extraction time, which could break the equilibrium and lead to lower extraction efficiency. Consequently, 10 min was chosen as the optimized extraction time.

Method validation

The proposed method was validated according to ICH guideline for accuracy, precision, limit of detection, limit of quantification and linearity parameters [35]. To evaluate the DLLME method, important parameters confirming the performance characteristics such as precision, sensitivity and linearity were determined by extracting 25 μg mL^{-1} spiked standard solution from deionized water under the optimized conditions. The optimized conditions were 900 μL of dichloroethane as the extraction solvent, 1400 μL acetonitrile as dispersive solvent, 10 min extraction time at 4000 rpm centrifugation, and pH 5. Calibration curves were constructed using linear regression analysis, and the obtained linearity was very satisfactory where correlation coefficients (r^2) were higher than 0.990. Further discussions under each of these performance characteristics of the analytical method are given in the following subsections.

Precision study

Precision of the analytical method expresses the degree of scatter in the results obtained from multiple analyses of the homogeneous sample and the results are calculated as the relative standard deviation (RSD) [36]. The precision of the method, expressed as RSD, was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) studies. The intra-day and inter-day precision of the method were determined under optimal conditions by successive three time analysis of a 5 μg mL^{-1}, 10 μg mL^{-1} and 25 μg mL^{-1} spiked wastewater sample and the RSDs were 0.41–3.21% for intra-day precision and 0.37–6.44% for inter-day precision. The results obtained from both studies demonstrated acceptable precision for the studied target analytes, as has also been indicated in Table 2.

Table 2. The intra-day and inter-day precision of the method determined under optimal conditions with different concentration levels of the spiked standard solution.

Antibiotics	Spiked standard conc. (μg mL^{-1})	Precision in % RSD	
		Intra-day precision, RSD (%), n = 3	Inter-day precision, RSD (%), n = 3
SMX	5	1.14	2.56
	10	0.48	0.37
	25	0.41	1.48
CIP	5	1.78	2.73
	10	2.45	1.02
	25	3.21	6.44

Relative recovery

Experiment on recovery was conducted for determining accuracy of the method, which was evaluated by performing determination of the influent wastewater samples spiked at 10 mg L^{-1} and 25 mg L^{-1} concentration levels. For this purpose, wastewater samples spiked at the two concentration levels were prepared and analyzed under the optimum conditions. The relative recovery was used to evaluate the matrix effect on the selective isolation and quantitative determination of trace levels of the target analytes by the developed analytical method, Table 3. It is defined as the ratio of the peak area of the spiked wastewater sample extract to the peak area of spiked reagent water extract [37] at the same concentration levels [38]. Calculation of the relative recovery was performed based on the following equation:

$$\%RR = \frac{\text{Peak area of spiked wastewater extract}}{\text{Peak area of spiked reagent water extract}} x100$$

Table 3. Relative recovery (%RR) of the target analytes at the optimized conditions.

Antibiotics	Spiked standard conc. (µg mL^{-1})	Relative Recovery (%RR)	
		Black Lion hospital	Menelik II hospital
SMX	10	91.18 (0.8)	111.55 (1.1)
	25	92.56 (3.4)	79.13 (0.3)
CIP	10	105.7 (0.4)	120.43 (2.2)
	25	118.42 (1.7)	109.73 (2.6)

Sensitivity

The sensitivity of the method is usually expressed in terms of the limit of detection (LOD) and limit of quantification (LOQ). The LOD is the lowest analyte concentration that can be detected but not necessarily quantified. The LOQ, on the other hand, is the lowest level or signal of the analyte in sample that can accurately and precisely be measured [39]. LOD and LOQ were determined as;

$$LOD = \frac{3xSD}{m} \quad \text{and} \quad LOQ = \frac{10xSD}{m}$$

where, 'SD' is the standard deviation of six blank measurements, and 'm' is the slope of the calibration curve with preconcentration. The LOD and LOQ determined in this study were found to be in the range of 0.78–1.58 ng mL^{-1} and 2.24-5.28 ng mL^{-1}, respectively, Table 4.

Table 4. Analytical performances characteristics of the proposed analytical method.

Antibiotics	Linearity (µg mL^{-1})	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	%, Recovery		Precision in % RSD (n =3)	
					Black Lion Hospital	Menelik Hospital	Intra-day	Inter-day
SMX	0.005-100	0.9989	0.7813	2.242	92.57	79.13	0.41	1.48
CIP	0.01-100	0.9997	1.583	5.277	118.42	109.73	3.21	6.44

Linearity

The linearity of the analytical method is the ability to achieve test results that correspond directly to the concentration of the analyte in the samples within the range of the standard curve. For the purpose of quantitative analysis, calibration of the chromatographic system was carried out for each analyte in the linear range from the LOD to the highest probable concentration ranging over five orders of magnitudes [40]. For all analytes, the coefficients of determinations (r^2) of the calibration curves were 0.9989-0.9997, confirming good linearity in the concentration range of 0.005–100 µg mL^{-1} for SMX and 0.01–100 µg mL^{-1} for CIP and DC.

Application to hospital wastewater samples

The DLLME–HPLC–DAD analytical method developed in this study was experimentally tested for its application for analysis of antibiotics in wastewater samples, collected from two hospitals in the city of Addis Ababa, *viz.*; Black Lion hospital and Menelik II hospital. Trace quantities of some antibiotics were identified in the extracts of the wastewaters. Besides, one more peak was observed in the wastewater extract of Black Lion hospital. This unidentified peak was suspected to be trace of doxycycline (DC) since the peaks eluted at the retention time of DC. Then, small drop of the DC standard solution was spiked into the extract (standard addition), obtained from Black Lion hospital. In the resulting chromatogram, the peak of the unknown was increased in its height and area, exactly at the same retention time of DC. Further addition of drops of the standard solution further increased both the peak height and peak area at the retention time of DC. Then, it was learnt that the experimental exercise confirmed the unknown peak to be DC or it was occurring in the sample of the wastewater collected from the effluent discharged from Black Lion hospital. Chromatogram of the standard solution containing the contaminants along with the standard of DC spiked is shown in Figure 5a.
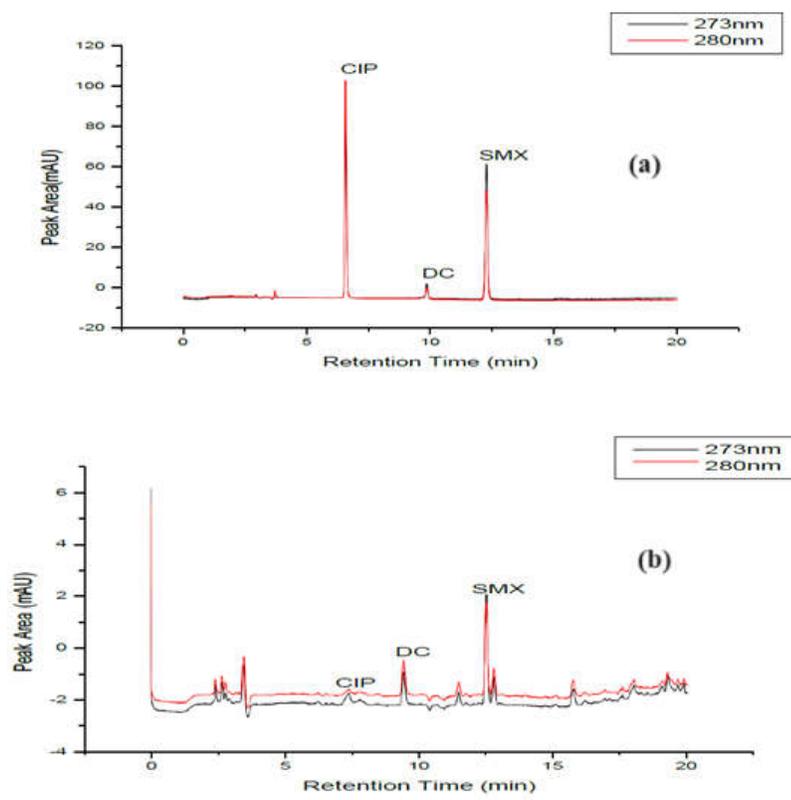

Figure 5. Chromatograms of (a) the standard solution of the antibiotic samples, and (b) the non-spiked wastewater sample from Black lion hospital using the optimized DLLME–HPLC-DAD.

In the similar manner, wastewater extracts from Menelik II hospital was also analyzed under the same separation conditions. The signals observed in the chromatogram indicated the presence of trace quantities of sulfamethoxazole (SMX); with estimated quantity of 0.75 µg mL^{-1}. However, ciprofloxacin (CIP) and the trace quantities of DC appeared in the extract of Black Lion hospital wastewater were not observed in the wastewater from Menelik II hospital.

The amounts of trace pharmaceutical residues determined in the wastewater from Black Lion hospital were varied in the range of 0.76-1.53 µg mL^{-1}. As has also been indicated in Figure 5b, ciprofloxacin, doxycycline and Sulfamethoxazole were determined in these extracts and found to be 0.76, 0.80 and 1.53 µg mL^{-1}, respectively. Comparing the accumulation levels of the contaminants, it was observed that wastewater samples from Black Lion hospital were more contaminated than the wastewaters from Menelik II hospital which could mainly be due to the larger number of patients treated in Black Lion hospital, which require higher administration of the antibiotics.

Comparison of the proposed DLLME with other modes of DLLME techniques

The analytical performance of the proposed DLLME-HPLC-DAD method has been compared with the results of the literature reports, for multi-residue analysis of trace level pharmaceuticals. All the methods considered for comparison, followed common analytical DLLME procedure for mixtures of analytes possessing similar physicochemical properties [41–43]. In most of the studies, the matrix chosen by various workers were also similar in composition, i.e., wastewater, except for one study that was based on the raw milk sample, Table 5 [42]. The findings showed that the DLLME method currently developed, is comparable or in some cases demonstrated better results; for example, in terms of precision, recovery and the quantity of the solvents used for extraction.

Table 5. Comparison of DLLME-HPLC-DAD with other reported methods.

Methods	Analytes studied	Matrix	LOD (ng mL^{-1})	Precision (RSD%)	Recovery (%)	Ref.
DLLME-UHPLC/DAD	ciprofloxacin, sulfamethoxazole	Mineral water; Run-off water	0.35–10.5	1–20	78–117	[41]
DLLME-UPLC-MS/MS	Ciprofloxacin	Raw cow milk	0.1–2.0	0.1–9.3	72.3–104.4	[42]
SPE-DLLME-UHPLCMS/ MS	ciprofloxacin, doxycycline, sulfamethoxazole	Drinking water; Running water; effluent wastewater; River water	0.08–1.67	2.0–9.6	64.16–99.80	[43]
DLLME–HPLC-DAD	Sulfamethoxazole, ciprofloxacin	wastewater	0.78-2.24	0.37-9.22	69.83 - 120.43	This work

CONCLUSION

The work presented in this study was to determine trace pharmaceuticals in environmental wastewaters the DLLME-HPLC–DAD. The advantages of DLLME, such as easy operation, rapidity and high recovery, miniaturization, etc the technique exceedingly suitable for pharmaceutical analysis. The extraction parameters in DLLME which were optimized along with corresponding values include: 900 µL dichloroethane as the extraction solvent, 1400 µL acetonitrile as dispersive solvent, 10 min extraction time at the stirring rate of 4000 rpm, and sample pH 3. Important analytical parameters confirming suitability of the performance characteristics such as precision, sensitivity and linearity were determined by extracting 25 µg mL^{-1} spiked standard solution under the

optimized conditions. For both analytes the coefficients of determinations (r^2) were 0.9989-0.9997, confirming good linearity in the concentration range of 0.005-100 µg mL^{-1} for SMX and 0.01-100 µg mL^{-1} for CIP. The LOD and LOQ results were found to vary from 0.78–1.58 ng mL^{-1} and 2.24–5.28 ng mL^{-1}, respectively; and the RSDs were below 3.21% for intraday precision and below 6.44% for interday precision signifying good precisions. The concentrations of the trace pharmaceuticals determined ranged from 0.76-1.53 µg mL^{-1} in the wastewaters collected from both hospitals (Black Lion and Minilik hospitals)

Traces of DC was found in the signal of the chromatogram for the wastewater sample from Black Lion hospital; initially unknown but by standard addition technique, it was identified. It is thus to be noted that wastewaters from Black Lion hospital need careful treatment, since the waste discharges are more contaminated with antibiotics than that of the wastewater from Minilik hospital. The high contaminant doses could be due to the extensive use and continuous release, and thus series of waste treatment actions could possibly minimized the undesired environmental and human health risks.

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