Comparison of Directly Suspended Drop Microextraction with Dispersive Liquid-liquid Microextraction Method for Extraction of Doxepin in Water and Biological Samples Prior to UV-Vis Spectrophotometer

Khani, Samaneh; Mofazzeli, Farideh*[+]

Departent of Chemistry, Quchan Branch, Islamic Azad University, Quchan, I.R. IRAN

ABSTRACT *The present research describes the use of directly suspended drop microextraction and air-assisted dispersive liquid-liquid microextraction method coupled with UV-Vis spectrophotometer for the analysis of doxepin in water and biological samples. Several important parameters which influence the extraction efficiencies of these two microextraction methods, such as the kind of extracting solvent, volume of the aqueous sample solution (donor phase), volume of the organic extraction solvent (acceptor phase), pH of the donor phase, salt effect, extraction time, stirring rate and number of the air injection were investigated. Under the optimal conditions the enrichment factors were above than 30. The linearity of the methods has been investigated between the ranges of 0.005-1.5 and 0.003-1 µg/mL. The precisions of two methods which are based on the average relative standard deviations are lower than 6, for three different concentrations of the analyte. Finally, the proposed methods were applied for the determination of doxepin in real samples including environmental water, urine and human plasma samples under the optimal conditions and the reasonable relative recoveries were obtained.*

KEYWORDS *Directly Suspended Drop MicroExtraction (DSDME); Air-Assisted Dispersive Liquid-Liquid MicroExtraction (AADLLME); Doxepin; Plasma; Urine; Water analysis; UV-Vis spectrophotometer.*

INTRODUCTION

Depression or depressive disorder is a widespread and common mental disorder in our society that affects about 350 million people around the world [1]. Depression is one of the serious global economic problems because the patients often lose the ability of working. In addition, depression may ultimately result in suicide [2]. The depression treatment includes various forms of psychotherapy as well as pharmacotherapy with antidepressant drugs [3]. Tricyclic antidepressants (TCAs) are a group of important drugs that are widely used for the treatment of this psychiatric disorder. Sometimes, they are encountered for emergency toxicology screening, forensic medical examinations and drug-abuse testing [4-8]. The function of these drugs is to block the reuptake

** To whom correspondence should be addressed.*
+ E-mail:f_mofazzeli@yahoo.com ; drmofazeli@iauq.ac.ir
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of the neurotransmitters norepinephrine (as in the case of desipramine, nortriptyline and protriptyline secondary amines) and serotonin (as in the case of amitriptyline, imipramine, clomipramine and doxepin tertiary amines) in the central nervous system [9]. Therapeutic drug measurement for antidepressant agents in biofluids is important for quality assurance in preparations and for obtaining optimum therapeutic concentrations, while minimizing the risk of overdose and adverse effects. On the other hand, TCAs are used extensively by the psychiatric patients suffering from clinical depression, especially in the developed countries and they can enter the aquatic environment mainly through human excretion. Many of these drugs cannot be completely removed or degraded during the sewage treatment process and therefore, it is important to develop methods for monitoring their presence in order to determine their concentrations in the environmental samples. Due to complexity of the real samples matrices and trace amounts of the various pharmaceutical such as TCAs, the development of sensitive and reliable analytical methods for detection of them in the real samples is therefore very important [10-12].

Sample preparation is still the most important challenge for the analysis of different compounds from various complex matrices, especially real samples. Conventional Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) have been usually applied as useful sample preparation methods for the determination of drugs for many decades [13,14]. However, both methods have certain drawbacks. LLE is a time consuming and tedious procedure and needs very large amounts of the high-purity, expensive and also hazardous organic solvents. In SPE methods often the artifacts introduce into the sample solution; therefore, the limitation for pH ranges of this sample solution is very important factor. On the other hand, they may require lengthy processing such as washing, conditioning, eluting and solvent evaporation [15]. During the last decade, the research activities have been oriented toward the development of efficient and miniaturized sample preparation methods. Solid-Phase MicroExtraction (SPME) [16], Liquid-Phase MicroExtraction (LPME) [17] and stir-bar sorptive Extraction (SBSE) [18-20] have been introduced according to this attitude. Although, SPME is a solvent-free extraction technique; but, the used SPME fibers in this extraction method are expensive and fragile. Also, the limited life-time and the sample carry-over can be great problems [16]. LPME can be divided into three broad categories: single drop microextraction (SDME), Hollow Fiber based Liquid Phase MicroExtraction (HF-LPME) and Dispersive Liquid–Liquid MicroExtraction (DLLME) [21-28].

Single drop microextraction is a mode of LPME that provides analyte extraction in a few microliters of an organic solvent [21]. SDME avoids some problems of the solid phase microextraction (SPME) method such as sample carry-over and fiber degradation. It is also quick, inexpensive and uses very simple equipment. In the SDME technique, a microdrop of an organic solvent is immersed in a stirred aqueous sample solution [21]. In recent years, *Lu Y.C. et al.* developed directly suspended droplet microextraction (DSDME) as a new sampling method of SDME [22]. In this method, a stirring bar is placed at the bottom of a vial containing an aqueous sample and rotated at a speed required to cause a gentle vortex. If a small volume of an immiscible organic solvent is added to the surface of the aqueous solution, the vortex results in the formation of a single droplet at or near the center of rotation. The drop itself may also rotate on the surface of the aqueous phase, increasing mass transfer. Compared with the other LPME techniques based on drop systems (e.g., SDME), it provides more flexibility in the choice of the operational parameters, especially the amount of the organic solvent and the stirring frequency. The possibility of applying larger volumes of the organic solvents in this method also makes it a useful technique to match with HPLC and UV-Vis spectrophotometer [22].

DLLME is based on the rapid injection of a mixture of a water-immiscible organic solvent (extraction solvent) and a water-miscible organic solvent (disperser solvent) into a sample solution with a syringe, in order to form a cloudy solution containing tiny droplets of the extraction solvent which is fully dispersed in the aqueous phase [26]. This phenomenon is due to the difference in solubility among the ternary solvents, water sample solution, extraction solvent and disperser solvent. After the formation of tiny droplets, the surface area between the extraction solvent (acceptor phase) and the aqueous sample solution (donor phase) becomes infinitely large and the equilibrium is achieved quickly due to the rapid transition of the analyte from the aqueous phase

to the extraction solvent. Subsequent, this cloudy solution is centrifuged to cause the accumulation of the extraction phase at the bottom of the conical test tube. The advantages of DLLME are the simplicity of the operation, rapidity or short extraction time, the high Enrichment Factor (EF) and low cost. Of course, like the other analytical methods, DLLME has some drawbacks, which result mainly from requirements related to the organic extraction and disperser solvents. To overcome these drawbacks, researchers have recently attempted to perform this microextraction method by using low density organic solvents [29] and also, in the absence of a disperser solvent [30].

In the present work, we used DSDME and AADLME methods which are combined whit UV-Vis spectrophotometer for the extraction and preconcentration of the target compound (doxepin) from environmental water and biological samples. Also, in this research, for reducing the used organic solvent volume as much as possible, an innovative method was applied in both microextraction methods [Fig. 1]. In this manner, a fixed volume of deionized water (90 µL) was placed by a 100 µL HPLC microsyringe in the bottom of the quartz microcell (where itis not in the optical path) [Fig. 1 (A)] and after then, the fixed volume of the organic extraction solvent was inserted into the microcell by the syringe, above deionized water, exactly in the optical path [Fig. 1 (B)].

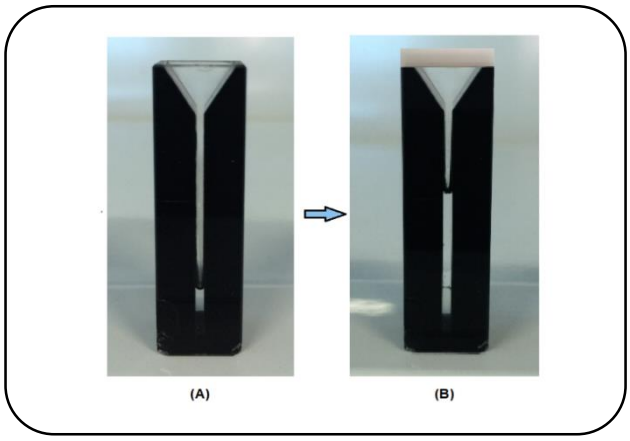

Fig. 1: An innovative method for reduction of the organic extractant volume in the quartz microcell.

EXPERIMENTAL SECTION

Chemicals and reagents

Doxepin with purity of > 99 % were kindly supplied from an Iranian pharmaceutical company, Darou Pakhsh Holding Co. (Tehran, Iran). Analytical reagents grade such as: 1-octanol, *n*-hexane, *n*-heptane and methanol (high purity) were purchased from Merck (Darmstadt, Germany). All the other used chemicals, acetone, NaCl, HCl and NaOH were purchased from Merck (Darmstadt, Germany) and used without further purification

Apparatus

Spectrophotometric measurements were carried out using a UV-Vis Unicode 2100 spectrophotometer (USA) equipped with quartz microcell of appropriate path length with internal volume of 300 µL.

The IKA heating magnetic stirrer (50–2500 rpm, Germany) was used for agitation of the sample solutions in the DSDME and AADLLME procedures.

The Hettich Centrifuge (model EBA 200, UNIVERSAL, USA) was used for the phase separations from cloudy solutions in the AADLLME procedure.

Preparation of samples

Stock solution of doxepin (1000 µg/mL) was prepared by dissolving calculated amount of the drug in the methanol and stored protected from the light in the refrigerator at 4 °C. Fresh working solutions in the various concentrations were prepared daily by diluting the appropriate amount of the stock solution in distilled water and the solution pH was adjusted.

Directly suspended drop microextraction method

The experimental procedure of DSDME was done according to the following steps. At first, a 15 mL cylindrical glass sample cell with a PTFE coated stirring bar was placed on a heating-magnetic stirrer. Then, a volume of 9 mL aqueous sample solution containing 1 µg/mL of doxepin was transferred into the vial as donor phase. The magnetic stirrer was turned on and adjusted to a desired stirring speed to form a conical vortex. A volume of 1-octanol (160 µL, acceptor phase) was delivered at the end of the aqueous sample solution vortex carefully with a 500 µL HPLC microsyringe. So, it forms a self-stable single microdrop system, easy to operate and control. After agitating of the solution for 15 min at 1300 rpm, the acceptor phase was retracted into the microsyringe and transferred into the 400 µL quartz microcell and introduced into the spectrophotometer for measuring the absorbance at 279 nm.

Air-assisted dispersive liquid-liquid microextraction method

The extraction was performed according to the following procedure. 5 ml of the aqueous sample solution (deionized water spiked with 1 µg/mL of doxepin) was transferred into a 10 mL screw cap glass centrifuge tube with flat bottom. Extraction solvent (1-octanol, 160 µL) was withdrawn into a 500 µL HPLC microsyringe and then, rapidly injected into the glass tube via syringe needle in order to form a mixture of the aqueous sample solution and the organic solvent. Then, the mixture was repeatedly aspirated from the tube and infused into it several times. This caused the solution to become more and more turbid. This operation results in the extraction of the target analyte from the sample solution into the fine droplets of 1-octanol. After performing predetermined number of aspiration–infusion cycles (eleven times), the mixture was centrifuged for 3 min at4000 rpm. This made the dispersed fine droplets of the low density extractant to gather above the aqueous sample solution.

After this extraction step, a PTFE coated stirring bar was inserted very carefully at the flat bottom of the glass tube and the set up was placed above a magnetic stirrer. By turning on the stirrer and adjusting to a desired stirring speed, the thin film of the organic extraction solvent convert to a conical vortex with an enough volume for collecting by the needle syringe. Finally, the extractant was withdrawn back by the HPLC microsyringe and transferred into the quartz microcell for measuring the absorbance in the UV-Vis spectrophotometer. The varius steps of this microextraction method are illustrated in our previous work in Fig. 1 [31]).

RESULTS AND DISCUSSIONS

Optimization method

In order to obtain the optimal extraction conditions for the best efficiency, various parameters were investigated and optimized such as: kind of the organic solvent, extraction times and stirring speed (in DSDME), different volumes of the donor and acceptor phases, pH of the donor phase, salting effect and the number of air injection (in AADLLME).

Organic solvent selection

In two phases LPME methods such as DSDME and DLLME, the type of the organic solvent is an essential factor for achieving the efficient analyte preconcentration. For example, in DSDME method, the appropriate organic solvent should have lower density than water to be suspended easily in the aqueous sample solution, it should be immiscible with water to avoid dissolution in the aqueous sample solution and when a UV-Vis spectrometer is used as the analytical instrument, the organic extractant should have no absorption in the maximum wavelength of the analyte.

In the conventional DLLME method, generally high density organic solvents are used as extractants and all of them are hazardous chlorinated solvents (chlorobenzene, carbontetrachloride). Therefore, the choice of the extraction solvent becomes crucial; because, the number of these solvents available is limited. Several studies reported the use of low density solvents in DLLME to broaden its applicability [29]. In the present work, we used low-density extraction solvents without any special device and complicated operation which causes to permit the use of a wider range of the organic extraction solvents.

According to the above explanations, 1-octanol, *n*-hexane and *n*-heptane have been tested during this experiment to investigate their effects on the extraction efficiency. In Fig. 2, a comparison between the measured absorbance of the extraction solvents in two microextraction methods is shown and 1-octanol is the most effective extraction solvent giving the highest extraction efficiency and was selected as the extraction solvent for both microextraction methods.

Effect of the extraction time and stirring speed in DSDME method

Like the other techniques of microextraction, DSDME is a type of equilibrium extraction. Maximum efficiency is obtained at the equilibrium, and usually it takes too long and a further increase in extraction time does not affect the amount of the extracted analyte. Therefore, the extraction time is expected to be an important factor in the extraction efficiency of the process. The effect of the extraction time was examined by using the different times for stirring between 5 to 20 min with stirring speed of 1500 rpm. Experiments showed that the best extraction time was 15 min (Fig. 3). With the shorter extraction times (5 and 10 min), the absorbances were lower due to the incomplete mass transfer of the target compound which occurred at the equilibrium, and with the longer time (20 min),

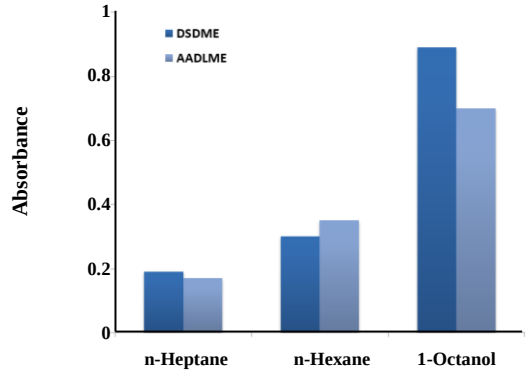

Fig. 2: Effect of the kind of organic solvent on the analyte absorbance of two microextraction methods.

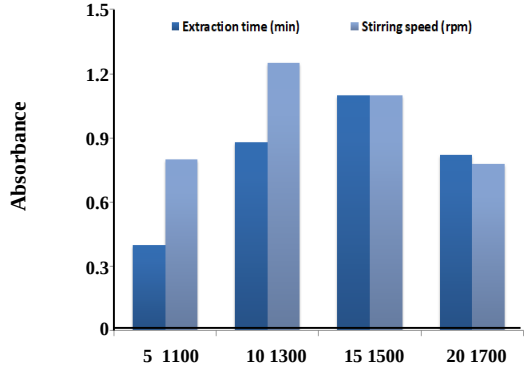

Fig. 3: Effect of the extraction time and stirring speed on the extraction efficiency of DSDME method.

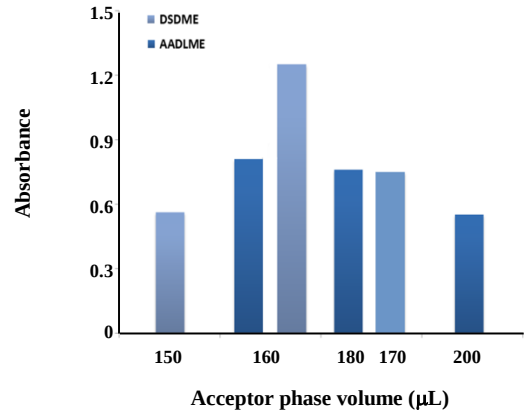

Fig. 4: Effect of the organic extraction solvent (acceptor phase) volume on the extraction efficiencies of two methods.

the absorbance nearly decreased; therefore, the extraction time was set at 15 min.

On the other hand, increasing of the stirring speed caused an increasing in the mass transfer and the extraction kinetics. In DSDME procedure, the stirring speed has a direct influence on both of the drop shape and the mass transfer characteristic in the aqueous sample. In DSDME, the procedure adopts a symmetrical rotated flow field created by a stirring bar, placed at the bottom of the cylindrical sample cell and the organic single drop is delivered at the end of the aqueous sample solution vortex which was created by agitation. Thus, it forms a self-stable single microdrop system, easy to operate and control. Furthermore, the rotation of the drop around a symmetrical axis may cause an internal recycling and intensify the mass transfer process inside the drop. Therefore, the stirring speed was also optimized for better extraction. As the stirring rate increases, the drop will collect towards the rotation axis and stretch along it. But exorbitant speed may make the drop break up and disperse into the aqueous phase. Besides, higher stirring speeds are often followed by a more unstable fluid field, which is unfavorable for the operation. In general, a proper stirring speed should be convenient for the operation and intensify the mass transfer effectively. With these goals in mind, we investigated the extraction process at various stirring speeds (with extraction time of 15 min), and the results are shown in Fig. 3.

Volume ratio of the donor to the acceptor phases

In the various microextraction procedures, the volume of the extraction solvent is a critical parameter with a significant effect on the analytical signals. Generally, the extraction solvent volume is selected as small enough to achieve the higher EFs and the least toxicity hazards. On the other hand, the extractant volume should be taken as large enough to extract maximum possible amount of the analyte. In this way, different volumes of 1-octanol (150, 160 and 170 µL) for DSDME and (160, 180 and 200 µL) for AADLLME methods were tested and the results was shown in Fig. 4. The best volumes were selected 160 µL for both of the microextraction methods.

Furthermore, the phase ratio of the donor and acceptor phases was examined by changing the volume of the donor phase whilst the volume of the acceptor phase was kept constant at 160 µL (Fig. 5 A, 5 B). In LPME methods,

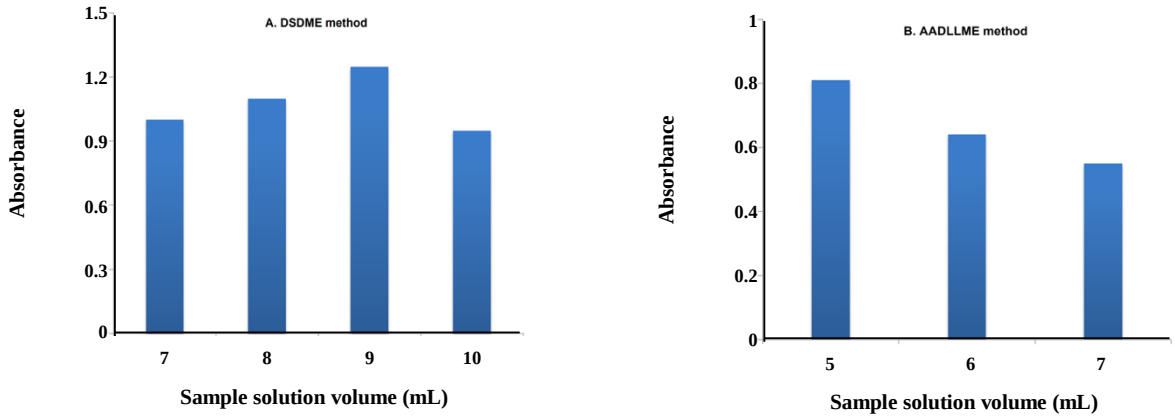

Fig. 5: Effect of the sample solution (donor phase) volume on the extraction efficiencies of two methods.

the enrichment factor can be improved by increasing the volume ratio of the donor and acceptor phases. Therefore, when the volume of the organic extraction solvent was 160 µL, the results indicate that the best extraction efficiencies were obtained when 9 mL of the sample solution was extracted in the DSDME and the largest analytical response was obtained when 5 mL of the donor phase was used in AADLLME.

Number of air-injections in AADLLME method

In the AADLME method, the number of air-injection was defined as the numbers of repeatedly sucking the mixture of extraction solvent and sample solution into the microsyringe and then rapidly injecting this mixture along with the air into the glass centrifuge tube. Partly similar to the multiple batch extraction, it is predictable that by increasing the number of air-injection, the enrichment factor should be increased, too. Therefore, to reach the equilibrium state, various number of the air-injection was studied and the results were shown in Fig. 6. By increasing the number of air-injection (up to 11 times), the analytical signals increased whereas, by further injection (15 times), the absorbance nearly remained constant. Therefore, the 11 air-injections times were used in further steps of AADLLME procedure.

Addition of a salt into the donor phase

Addition of a salt can often improve the extraction efficiency when liquid extraction methods are used. When a salt is added to the sample solution, two different behaviors are observed. For this purpose, sodium chloride is normally used [32-34]. By the salt addition the extraction efficiency may be enhanced due to the *salting out* effect; whereby, water molecules form hydration spheres around the ionic salt molecules. These hydration spheres reduce the amount of water available to dissolve the analyte molecules in water; thus, it is expected that the target compounds will drive into the organic solvent. On the other hand, with increasing of the salt concentration and ionic strength, *salting in* effect occurs that polar molecules may participate in electrostatic interactions with the salt ions in the solution and as a result, the mass transfer is reduced. Moreover, increasing of the solution viscosity decreases the mass transfer of the analyte to the organic extraction solvent, too. To evaluate the salt effect for two microextraction methods, sodium chloride (2–6%, *w/v*) was added to the aqueous phases spiked with the target analyte along with keeping all other optimized experimental conditions constant. The results of the NaCl addition showed negative effects on the extraction efficiencies (*salting in* effect). Therefore, all the experiments were performed without any salt addition in both of the microextraction methods.

Effect of the aqueous sample solution pH

The correct adjustment of pH in the sample solutions can enhance the extraction efficiencies of the acidic and basic analytes; so, it is an important key to achieve high distribution ratio and enrichment factor by adjusting the sample pH in the donor phase. This can lead to neutralization of the analyte and reduce its solubility in the sample solution. Because doxepin

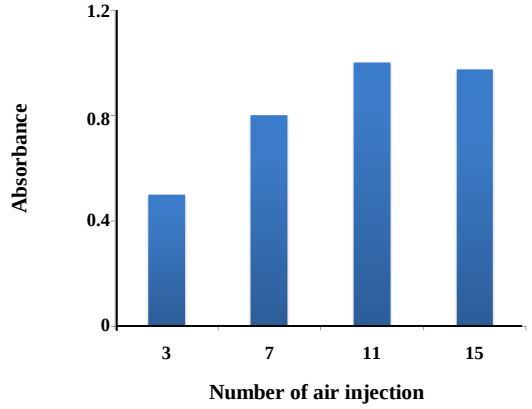

Fig. 6: Effect of the number of air-injection on the measured absorbance in AADLME method.

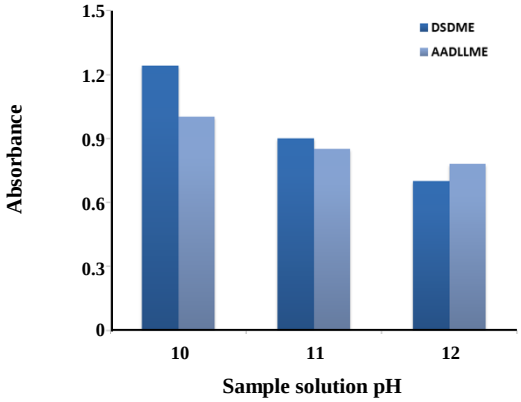

Fig. 7: Effect of the aqueous sample solution pH on the extraction efficiencies of two methods.

is weak basic compound, the pH of the donor solution was adjusted in the proper basic range to neutralize the compound and reduce its solubility in the sample solution. Thus, the aqueous solution was made alkaline with NaOH in various concentrations (10^{-4}-10^{-2} mol/L) or three different pH values of the aqueous sample solutions were investigated: 10, 11 and 12. According to the obtained results which are shown in Fig. 7, the pH 10 would be sufficiently basic to neutralize the analyte and the absorbance of the target compound decrease when the pH of the aqueous sample solution (donor phase) increases. For pH higher than 10, addition of NaOH causes *salting in* effect due to the simultaneous production of NaCl in the solution. Therefore, the aqueous sample solution pH was selected 10 for future studies.

Quantitative consideration

The evaluation of the practical applicability of two proposed methods, repeatability (RSD), Linear Range (LR), Limit Of Detection (LOD), Limit Of Quantification (LOQ) and e nrichment f actor (EF) were investigated under the optimal extraction conditions by utilizing the standard solutions of doxepin in water. The LOD and LOQ of two methods were calculated theoretically. The calibration curves for the target compound were obtained by plotting absorbance vs. the various analyte concentrations by using two proposed microextraction methods. The analytical data for two methods are summarized in Table 1.

Applications of two microextraction methods in real samples

To evaluate the application of the proposed methods to various real samples and owing to the importance of the analysis of drugs in the biological samples, two microextraction methods were applied for separation and determination of doxepin in various real samples, including environmental water, human plasma and urine samples.

Environmental water samples

Two real environmental water samples including spring and river water samples were collected from Quchan County. These water samples were collected using pre-cleaned glass bottles and kept in the dark at 4°C until use. No pretreatment were conducted on them.

The results show that the contents of doxepin in two real samples are all under the detection limits for two proposed methods. Therefore, separate samples were spiked with 0.05 and 0.1 µg/mL of the target compound for extraction under the optimal conditions by DSDME and AADLLME methods, respectively and the relative recoveries were calculated and listed in Table 2.

Human plasma sample

The human plasma samples were obtained from Bagherololoom medical laboratory (Mashhad, Iran) from healthy volunteers. These samples were stored at −20 °C, thawed, and shaken before extraction. TCAs drugs such as doxepin are extensively bounded to plasma proteins

Table 1: Analytical performance of the proposed microextraction procedures for the extraction of doxepin.

Methods	RSD % (n=5)	Correlation Coefficient (r)	LR (µg/mL)	LOD (µg/mL)	EF
SDME	3	0.9984	0.005-1.5	0.0015	54
AADLME	6	0.9925	0.003-1	0.001	31

Table 2: The relative recoveries of doxepin in real environmental water samples (n=3).

Methods	DSDME[a]	AADLLME[b]
Samples	RR ± RSD	RR ± RSD
Spring water	86 ± 3	94 ± 10
River water	89 ± 4	88 ± 8

(91–97 %) and should be liberated prior to microextraction procedure [35]. So, the spiked plasma samples were prepared as follow: 0.5 mL of the plasma sample was transferred into a test tube and in order to decrease protein bonding and protein precipitation, 1.00 mL of methanol was added to it. The mixed solution was centrifuged for 15 min at 1000 rpm. A volume of 0.5 mL of the supernatant was transferred to a volumetric flask and diluted with water to 10 mL and the solution pH was adjusted. At first, this real sample was extracted at optimal conditions by DSDME procedure. The obtained results showed that there was no analyte in the human plasma; therefore, the plasma sample was spiked with 0.05 µg/mL of doxepin as the target analyte and the extraction procedures were performed (n=3) under the optimal conditions and the relative recovery (% RR) of 88 was subsequently calculated with relative standard deviation (% RSD) of 4 (88 ± 4).

Urine sample

Drug-free urine samples were supplied by healthy volunteers. The urine samples were separately collected and stored in −20 °C until analysis. The hydrolysis of the frozen urine samples after defrosting at room temperature was carried out as follows. First, 2 mL of 10 M KOH was added to 10 mL of urine sample and then it was hold in 60 °C for 10 min. Then, the mixture was centrifuged for 5 min at 4000 rpm. The supernatant was transferred to a clean glass beaker and set under a 50 W ultrasound for 5 min. The resulting solution was filtered through a 0.4 µm filter. After then, 5 mL of this sample solution was transferred to a clean glass volumetric balloon and adjusted to pH 10 with addition of HCl solution and finally, subjected to the AADLLME process. Forasmuch as, we used the drug free urine samples, these samples were spiked with 0.1 µg/mL of doxepin and the extraction procedure was performed under the optimal conditions and the relative recovery of 99 was subsequently calculated with relative standard deviation of 8 (% RR ± % RSD = 99 ± 8).

CONCLUSIONS

The present work describes the possibility of using two LPME methods in the extraction and preconcentration of doxepin from environmental water and biological samples prior to UV-Vis spectrophotometer. In DSDME method, contrary to the ordinary single drop liquid phase microextraction technique, an organic large drop is freely suspended without using a microsyringe as supporting device. This large drop causes an increasing in mass transfer process and decreasing in equilibrium time. In AADLLME method, differently from the conventional dispersive liquid-liquid microextraction technique, the organic solvents lighter than water were used as extraction solvents and also, the disperser solvent was eliminated. Moreover, compared to the most conventional extraction procedures, these extraction methods are really fast, easy and simple. The analyte can be extracted from real water and biological samples quantitatively with reasonable relative recoveries.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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