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Quantitative analysis of β-blockers in water samples by HPLC using directly suspended droplet microextraction

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Original Research

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Abstract:

The aim of this study is using Directly Suspended Droplet Microextraction (DSDME) as the sensitive drug assays in environmental waters. This work explains the success of this liquid-phase microextraction technique used as sample pre-concentration technique. This attractive method, which is selective and enable substantial pre-concentration, has been used for determination of three β-blockers; Atenolol, Metoprolol and Propranolol as the model compounds. The effective parameters such as organic solvent, pH of donor and acceptor phases, phase volumes, extraction and back-extraction time, stirring rate and addition of salt are discussed. The extracted β-blockers were analyzed at room temperature by high performance liquid chromatography (HPLC), equipped an Agilent Eclipse XDB-C18 column with particle size of 5 μm (250 mm$\times$4.6 mm i.d.) and programmed wavelength fluorescence detector. The mobile phase was 0.01 mol/L NaH_2PO_4 (adjusted to pH 3.0 with phosphoric acid)-methanol-acetonitril (45:45:10, v:v:v). The flow rate was 1.0 mL/min. The separated β-blockers were detected using fluorometric detection. Results showed that practical pre-concentration factors varied from 34.8 to 171.6.

Keywords: β-blockers; Directly suspended droplet microextraction (DSDME); Fluorescence tetection; HPLC

1. Introduction

β-blockers are clinically important drugs and are used in the treatment of disorders such as hypertension, angina pectoris and arrhythmia [1, 2]. They are very toxic and most have only a narrow therapeutic range. β-blockers have been forbidden by the medical commission of the International Olympic Committee (IOC) for typical concentration of 500 μg/L in urine and prohibits the use of these drugs because they reduce heart rate and muscular tremor in archery, billiards and rifle competitions [3, 4]. Therefore, screening and determination of β-blockers in biological samples are required in many circumstances such as clinical control for diagnosis and treatment of diseases, doping control, forensic analysis and toxicology. Beside, another new research that scientists have decided to fund in recent decades is Pharma identification and reduction of environmental risk caused by the use of human pharmaceuticals. These researches have been focused onto the study of the effects of pharmaceutical residues in the environment. Some works are also reported in wastewater treatment to remove undesired pharmaceutical residues [5].

Most of the common β-blockers are weakly basic compounds (pKa 8.7-9.7) and structurally have one secondary amino group and one hydroxyl group situated on adjacent carbon atoms (Fig. 1). These similarities suggest the possibility of simultaneous analysis of these compounds. The most widely used technique for the determination of these highly polar and basic compounds is HPLC with UV detection [6, 7], fluorimetric [8, 9], electrochemical [10, 11] and Mass Spectrometry (MS) detection [12, 13]. Furthermore, most of the methods require laborious cleanup of the biological and environmental samples because β-blockers are generally present at low concentration in these complex matrixes. To achieve a more efficient, practical, and reliable method for the analysis of β-blockers, sample preparation is a very important step.

Sample pre-treatment is usually necessary in order to extract, isolate, and concentrate the analytes of the interest from complicated matrices to obtain samples compatible for instrumental analysis. The extracted and enriched analytes of interest from the sample matrix are often accomplished

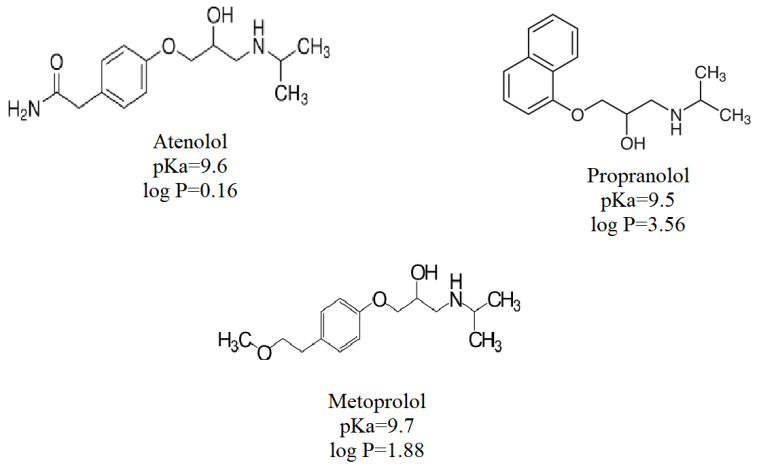

Figure 1. Structures of three studied β-blockers.

by procedures such as Liquid–Liquid Extraction (LLE) and Solid-Phase Extraction (SPE) [14, 15]. LLE offering high reproducibility and high sample capacity, but it is more suitable for the bulk extraction of large quantities of analytes and not for trace analysis, also it has some drawbacks, e.g; large amounts of expensive and toxic solvents consumption, tedious and time-consuming procedure, analyte loss and analysis errors due to multi-step methodology. To address these problems, recent research is oriented towards the development of miniaturized sample pretreatment technologies. The invention of Solid-Phase Microextraction (SPME) by Pawliszyn and co-worker basically initiated the interest for microextraction techniques in analytical chemistry [16]. SPME satisfies most of the requirements of a good sample preparation technique, including simplicity of use, automation, and low consumption of materials. SPME technique has some disadvantages such as limited lifetime, fragility of fibre and possibility of sample carry-over. In addition, it is very difficult to extract some highly polar compounds without derivatisation. Furthermore, when SPME is coupled to HPLC, a special SPME–HPLC interface device has to be used for solvent desorption to recover all absorbed analytes and to avoid carry-over. Because of these problems, an alternative miniaturized sample preparation approach, i.e. Liquid-Phase Microextraction (LPME), was invented in the late 1990s [17, 18]. LPME utilizes only a small amount of solvent for concentrating analytes from aqueous samples. It is simply a miniaturized format of LLE and overcomes many of its disadvantages as well as some of those of SPME. The applications of LPME in environmental and biological analysis have been described in several papers [19, 20].

Hollow Fiber LPME (HF-LPME) is a simple and inexpensive method with the added benefit of the fiber being disposable after use. The three-phase HF-LPME involves pH adjustment of the sample solution (donor phase) to a pH where the analytes are uncharged. The analytes are extracted through the organic phase immobilized in the pores of the hollow fiber and into the aqueous acceptor phase, that has a pH where the analytes are charged preventing them from back diffusion into the organic solvent. Hydrophobic analytes are easily extracted into organic solvents from the donor aqueous phase, but hydrophilic and polar analytes have low solubility in the water immiscible organic solvents. Therefore, these analytes are difficult to extract by three-phase LPME [21, 22].

Newly in the field of liquid phase microextraction, Yangcheng et al. developed a new sampling method termed Directly Suspended Droplet Microextraction (DSDME) [23]. Recently, Sarafraz-Yazdi et al. have developed this method for the determination of diclofenac in environmental water samples [24]. In three-phase DSDME, a droplet of an aqueous solvent is suspended freely in the surface-center of an immiscible organic solvent, which has been laid on the surface of the aqueous sample while being agitated by a stir bar. Agitation of the sample causes a weak gentle vortex. If a small volume of an aqueous droplet is added to the surface of the organic solvent, the vortex results in the formation of a single microdroplet at or near the center of rotation. The droplet itself also rotates on the surface of the organic solvent, so mass transfer is increased. Sarafraz-Yazdi et al. also used DSDME with GC with Flame Ionization Detection (GC-FID) for the determination of amitriptyline and nortriptyline and BTEX compounds [25, 26]. Very recently a critical review on LPME methods is reported which covers most of the related works in this field [27].

Several pretreatment methods such as SPME [28–30] and LPME [31–35] have been used for cleanup of the β-blockers. In this work we used DSDME for analysis of β-blockers in water samples. Different aspects of the extraction procedure such as organic solvent, pH of donor and acceptor phases, phase volumes, extraction and back-extraction time, stirring rate and addition of salt were investigated.

2. Experimental

2.1 Reagents and chemicals

Atenolol, Metoprolol tartrate and Propranolol hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A). Methanol and acetonitril (HPLC grade) were purchased from Fluka (Buchs SG, Switzerland). These compounds

Figure 2. Illustration of the used apparatus for DSDME: (a) addition of the organic solvent to the aqueous sample solution, magnetic stirrer is off; (b) magnetic stirrer on, extraction occurring (T1); (c) addition of the acceptor phase droplet into the organic solvent, stirrer off; (d) magnetic stirrer on, back extraction occurs (T2) after the back extraction, droplet is withdrawn with the microsyringe.

were all of analytical grade. The deionized water and other solvents were filtered by a Milli-Q filtering system (Millipore).

2.2 Stock and working solutions

Stock standard solutions of Atenolol, Metoprolol and Propranolol (1000 mg/L) were prepared in methanol and stored at 4 ^{o}C. Working solutions for optimization experiments and calibration curves were prepared by appropriate dilution of the stock standard solutions with ultrapure water.

2.3 Apparatus

The apparatus used for the HPLC analysis was an Agilent 1100 series (Agilent Technologies, USA) with a fluorescence detector. Data acquisition and analysis were performed using the Chem Station Rev. A 10.01. The mobile phase was 0.01 mol/L NaH_2PO_4 (adjusted to pH 3.0 with phosphoric acid)- methanol - acetonitril (45:45:10, v:v:v). The flow rate was 1.0 mL/min. The separated β-blockers were detected by fluorometric detector (excitation and emission wavelength programming). Separation was carried out at room temperature by an Agilent Eclipse XDB-C18 column with particle size of 5 μm (250 mm L $\times$4.6 mm I.D.).

2.4 Fluorescence Detection

A programmed wavelength fluorescence method was used for the measurement of the individual compounds. Three different detection conditions were used corresponding to the elution of the different compounds. The optimal excitation and emission wavelengths were 228, 225, 229 and 307, 307, 341 nm for Atenolol, Metoprolol and Propranolol respectively. For the first period, the optimal detection conditions of Atenolol were used, while in the second and the third time period, the excitation and emission were adjusted to those optimal for Metoprolol and Propranolol.

2.5 Quantitative Aspects

The practical pre-concentration factors, linear ranges, Relative Standard Deviations (RSD%) and Limit of Detections (LODs) have been calculated under optimum experimental conditions. The calibration curves for the target compounds were obtained by plotting peak areas vs. the sample concentrations. LODs were calculated as the minimum concentrations providing chromatography signals three times higher than background noise. Limit of Quantifications (LOQs) were estimated as the minimum concentrations preparing chromatographic signals ten times higher than background noise. Practical pre-concentration factors were calculated as the proportion peak areas after extraction to before that. Also, repeatability (R.S.D%) evaluated with three replicated experiments. LPME is not an exhaustive extraction method, so relative recovery was determined as the ratio of the concentrations found in natural and distilled water samples spiked with the same amount of analytes, under the optimized conditions.

2.6 Extraction Procedures

Extraction was performed according to the following procedure. The 4.0 mL sample solution (0.01 mol/L, NaOH containing 1000 μg/L Atenolol, 500 μg/L Metoprolol and 100 μg/L Propranolol) was placed in a glass vial (6 mL cylindrical sample cell). A stirring bar (7 mm$\times$3 mm) was used to facilitate the mass transfer process. The sample vial was placed above the heating-magnetic stirrer for stirring the extraction mixture.
250 μL organic solvent was then added to the sample solution by a 500 μL Knauer syringe (Gaithersburg, MD, USA). An aluminum foil was used to cover the lid of the vial during extraction to prevent the evaporation of the organic phase. Then the mixture was agitated vigorously for 80 s at 1250 rpm and a cloudy mixture of the sample solution and the tiny drops of the organic solvent were obtained. After then, the mixture was allowed to be quiescent for few seconds to gather the drops of the organic solvent together up to the aqueous sample solution and therefore, the organic layer which was enriched by the analytes created again above the donor phase. Afterward, the acceptor phase (6 μL 0.1 mol/L HCl) was delivered with a 10 μL flat-cut Hamilton HPLC microsyringe at the top-center position of the immiscible organic solvent. After stirring the mixture in the rate of 700 rpm for 30 min, the microdroplet was withdrawn-back by the HPLC microsyringe and then was injected into the HPLC system. The experimental microextraction setup is shown in Fig. 2.

3. Results and Discussion

3.1 Optimization of analytical procedure

In order to obtaining high pre-concentration and extraction efficiency of the analytes, the main parameters affecting the

Table 1. Characteristics of organic solvents [36].

Solvent	Density (gm/L)	Solubility in water (g/L)	Surface tension (dyne/cm)	Boiling point °c)	log(Po/w)
Benzene	0.878	1.87	28.22	80.1	2.13
Cyclohexane	0.779	Insoluble	24.99	81	3.44
Ethyl acetate	0.897	0.016	23.6	77.1	0.73
Ethyl benzene	0.866	0.150	29.2	136.2	3.15
Toluene	0.867	0.50	28.5	110.6	2.69
n-Heptane	0.683	0.003	20.21	98.4	4.66
n-Hexane	0.659	0.013	18.4	69	3.94
1-Octanol	0.824	Insoluble	27.50	195	3.0
Orthoxylene	0.880	0.20	29.76	144	3.12

extraction including organic solvent, pH of donor and acceptor phases, phase volumes, extraction and back-extraction time, stirring rate and addition of salt were evaluated.

3.1.1 Selection of organic solvent

Selection of organic solvent is very important for achieving efficient analyte pre-concentration. There are several requirements for obtaining the selected organic solvent. (a) The appropriate organic solvents in this work should have lower density than the water to float on the top of the aqueous sample solution, (b) The organic phase must therefore be immiscible with both the acceptor and donor phase to avoid dissolution in these phases, because it serves as a barrier between them, (c) The organic solvent should have appropriate viscosity to hold the microdroplet at its top-center position (Fig. 3) without using a microsyringe as supporting device, (d) The solubility of the analytes should be higher in the organic phase than the donor phase to promote the extraction of the analytes. On the other hand, the solubility of the analytes should be lower in the organic phase compared to the acceptor phase, in order to achieve a high degree of recovery of analytes in the acceptor phase, (e) The solvent should have a high boiling point avoid evaporation during experiment.

Several organic solvents (1-Octanol, Toluene, O-xylene, n-Hexane and n-Heptane) with different polarities were used to study their effects on extraction efficiency. The characterizations of these solvents are shown in Table 1. Toluene and 1-Octanol have the best conditions for the extraction and showed the higher analyte pre-concentration factors than the others. Among them, Toluene showed the highest analytes pre-concentration. But, we were faced with a practically problem due to the instability of microdroplet inside it, which was solved by mixing a small amount of 1-Octanol with Toluene. 1-Octanol helped to increase the lifetime of the microdroplet inside the organic phase. Therefore binary mixture of Toluene/1-Octanol was used as the optimal organic solvent system. Toluene; having high pre-concentration factor, and 1-Octanol; having high viscosity for holding the microdroplet, were selected as the extractants. The best extraction efficiency has been obtained in the binary mixture of Toluene /1-Octanol, 90:10 (v/v). Hence, this mixture was chosen as our organic solvent for our subsequent studies.

3.1.2 The pH of donor and acceptor phases

As was expressed earlier, the pH value of both aqueous donor and acceptor phases plays an essential role in the extraction processes. Therefore, the pH of the sample solution (donor phase) and the aqueous microdroplet (acceptor phase) was optimized. For basic drugs, the donor phase should be strongly alkaline to effectively deionized the analytes and consequently reduce their solubility within the sample, while the acceptor phase should be acidified in order to promote dissolution of the basic analytes. 0.05 mol/L NaOH was used as donor media and 0.5 mol/L HCl was

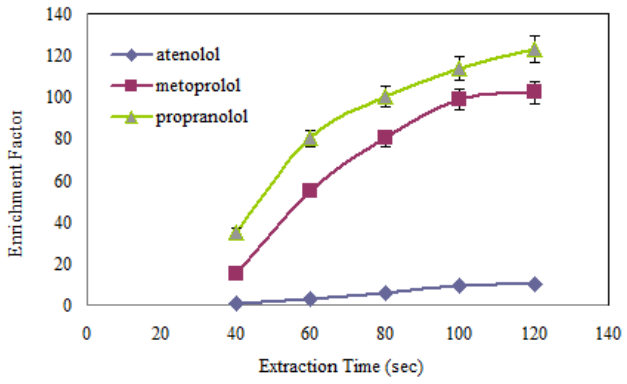

Figure 3. The effect of extraction time (T1) on the DSDME efficiency.

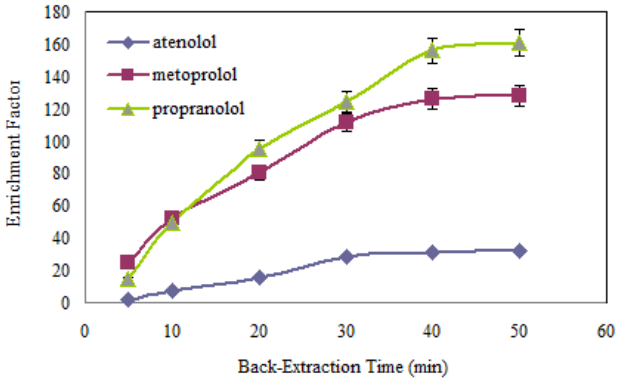

Figure 4. The effect of back-extraction time (T2) on the DSDME efficiency.

used as acceptor phase.

3.1.3 Phase volumes

In the present work, the phase volume of donor phase, acceptor solution and organic solvent was optimized. With attention to selected glass vial shape and volume, 3.5 mL volume for aqueous sample solution was better than any other (between 2.5, 3, 3.5 and 4.0 mL donor phase volumes). On the other hand, the volume of the organic phase is too important, due to the special design of the extraction device and must be carefully optimized. In this setup, the organic solvent in addition to be an extractant, it acts as the holder of the acceptor phase and its volume affects the life time of the droplet. The organic solvent keeps the aqueous droplet at the top of its surface. The volume of the organic layer will affect the lifetime of the aqueous droplet and the extraction efficiency. The best volume of the organic solvent was found to be 200 μL. Smaller volumes of the organic solvent tend to cause instability of the aqueous droplet during agitation, whereas the extraction efficiency is reduced if a larger volume of organic phase is used. Consequently, a 200 μL volume of the organic solvent was chosen for the subsequent extractions.

The volume of the acceptor phase was changed while the volume of the donor phase was kept constant at 3.5 mL. The volumes of the acceptor phase were changed from 5 to 8 μL, and with a 6 μL droplet the best pre-concentration factor was obtained. Very large droplet causes a decrease in the pre-concentration factor due to the dilution of the analytes in these large droplets. On the other hand, these large droplets are not very stable especially at the high stirring rates.

3.1.4 Extraction time (T1)

The main goal in microextraction techniques is to achieve sufficiently high extraction efficiency within a relatively short period of time. In this work, we used the organic solvents which are insoluble in water and have lower density than that of water. Thus, before addition of the suspended microdrop, the aqueous sample solution and the organic phase (Toluene/1-Octanol, 90:10 v/v) was agitated at 1250 rpm and mixed together vigorously for a defined time (T1). Afterwards, a cloudy mixture of the sample solution and the tiny droplets of the organic solvent was obtained. Due to the high intersections between the donor solution and these tiny droplets of the organic solvent, the mass transfer occurred very fast. As shown in Fig. 3, the pre-concentration factors were enhanced with the increase in exposure time up to 100 s and remained constant afterwards. Thus, the extraction time (T1) for further experiments was chosen as 100 s.

3.1.5 Back-extraction time (T2)

As was mentioned, three-phase suspended droplet is not an exhaustive extraction technique. Although the maximum efficiency is attained at equilibrium, a complete equilibrium is not necessary because of increasing the analysis time. Droplet lifetime cannot be too long due to drop dissolution, loss or fall. We have tested different back-extraction times from 5 min to 50 min. The experimental results, which were shown in Fig. 4, indicate that for all of the three analytes, the pre-concentration factors reach the highest at the extraction time of 40 min and then, there is no significant increase with further increasing of the back-extraction time. On this basis, 40 min was selected as optimal back-extraction time

Table 2. Analytical performance of DSDME.

Compound	Atenolol	Metoprolol	Propranolol
Correlation coefficient (r)	0.9998	0.9996	0.9997
Intra-day deviation RSD (%) (n=5)	6.2	5.6	5.8
LOD (μg/L) (n=7)	10	5.0	0.1
LOQ (μg/L) (n=5)	30	16	0.4
Linear range (μg/L)	30-5000	16-1000	0.4-500
Practical Pre-concentration Factor (n=3)	34.8±0.48	138.6±3.8	171.6±5.3

Table 3. Relative Recovery of β-blockers in real water samples by use of DSDME –HPLC.

Analyte	Concentration*	DSDME		
		Relative Recovery (%)		
	(μg/L)	Tap water	Clinical waste water	Industrial waste water
Atenolol	50	94.5	90.1	90.2
Metoprolol	25	96.7	93.2	92.7
Propranolol	0.5	97.2	96.8	90.7

*Spiked amount of analytes.

for the experiment.

3.1.6 Effect of stirring rate

Agitation of the sample solution is generally applied to facilitate the mass transfer process and reduces the time required to reach thermodynamic equilibrium. Increasing the stirring rate of the donor phase enhances the diffusion of analyte through the organic phase and improves the repeatability of the extraction. Increasing the stirring rate can decrease the thickness of the diffusion film in the aqueous phase and improve the repeatability the extraction method. In the present work, the procedure adopts a symmetrical rotated flow field created by a stirring bar, placed at the bottom of the cylindrical sample cell and the droplet is delivered at the top-center position of the organic solvent. Thus, it forms a self-stable single microdroplet system, easy to operate and control. Furthermore, the rotation of the microdroplet around a symmetrical axis may cause an internal recycling and intensify the mass transfer process inside the droplet. Therefore, the stirring rate was also optimized for better extraction, while the back-extraction was performed. In this work, extraction was performed at the maximum magnetic stirrer performance, 1250 rpm.
In back extraction phase, different stirring rates, i.e. 500, 600, 700 and 800 rpm were checked. Higher rate of agitation increased extraction efficiency but the aqueous microdroplet (acceptor phase) become unstable at high rate of the magnetic stirrer and falls down in the vortex, which is created in the organic solvent by agitation. Moreover, establishing the extraction equilibrium in the interfacial layer of both phases is difficult. Agitation of donor phase induces convection in the organic membrane. Consequently, the stirring rate was selected at 700 rpm for further analysis.

3.1.7 Salt addition effect

Addition of a salt can often improve extraction efficiency when the extraction methods are used. To evaluate the effect of salt in this work, NaCl was added into the donor sample solution and the effect was studied from 0% to saturation. The results indicate an initial increase in the pre-concentration factors with increasing salt concentration, along with a maximum being reached at 15%, followed by a decrease in EFs with further increase in salt concentration to saturation. After determining the optimal conditions, method was evaluated and the results were listed in Table 2. Extraction conditions: donor phase = 0.05 mol/L NaOH solution containing different concentrations of Atenolol, Metoprolol and Propranolol; donor phase volume = 3.5 mL, organic solvent = Toluene /1-Octanol 90:10 (v/v); organic solvent volume = 200 μL; acceptor phase = 0.5 mol/L HCl; micro-droplet volume = 6 μL, stirring rate for extraction = 1250 rpm, stirring rate for back-extraction = 700 rpm; extraction time = 100 s; back-extraction time = 40 min; NaCl % = 15 % w/v, all extractions were performed in

Table 4. Comparison of some methods which were used for determination of β-blockers.

No.	Instrument	Detection	Matrix method	Extraction	Analytes	LOD (μg/L)	LR (μg/L)	Pre-concentration factor	Ref.
1	CE	UV	Urine water	LPME +stacking	Atenolol Pindolol	500 80	1000-10000 500-10000	110 72	[31]
2	HPLC	MS	Plasma	CM-LPME	Atenolol Other drugs	15 -	25-1500 -	R%=18 -	[33]
3	GC	MS	Urine water	HF-LPME (in situ derivatization)	Metoprolol Propranolol Other drugs	0.08 0.05 -	0.25-400 0.25-400 -	R%=96.6-104.5 R%=96.4-105.5 -	[34]
4	HPLC	UV	Urine	CM-LPME	Propranolol Other drugs	5.0 -	50-8000 -	182 -	[35]
5	HPLC	FL	Water	HF-SLPME	Atenolol Metoprolol Propranolol	15 10 1.0	50-9000 30-9000 4-1500	25.2 392 822	[8]
6	HPLC	FL	Water	DSDME	Atenolol Metoprolol Propranolol	10 0.5 0.1	30-5000 16-1000 0.4-500	34.8 138.6 171.6	This work

*MS:mass spectrometry, CM-LPME:carrier-mediated liquid phase microextraction, R%:recovery percent

triplicate.

3.2 Application to real samples

To demonstrate the practical applicability of the mentioned techniques, real water samples were analyzed using this method. Drinking water from the Mashhad water-supply network, a clinical waste water sample which was obtained from one of the central hospitals in Mashhad, Iran and an industrial waste water from Toos Industrial Zone were spiked with 50.0, 25.0 and 0.5 μg/L of Atenolol, Metoprolol and Propranolol respectively, and extracted under optimal conditions.

The relative recovery of the analytes from these real water samples were higher than 90% compared with that of spiked pure water. This indicates that the matrix effect does not have any significant effect on the extraction efficiency of both methods. The relative recoveries for three types of water samples, are presented in Table 3.

Although DSDME is a simple, low-cost, rapid extraction technique, it requires careful and complex manual operations that lead to problems such as droplet instability and time-consuming and tedious steps. When DSDME is used for extraction of complex matrixes, extra filtration of the sample is necessary. The results obtained, indicates that this method is a good alternative extraction technique for hydrophilic drugs and offers highly interesting advantages from an analytical point of view, such as possibility of extracting and pre-concentrating the analytes of different polarities. Moreover, this procedure offers several advantages over traditional extraction techniques such as; a reduction in extraction time (typically 20–45 min), also this method is economical and easy to use. The linearity of the calibration plots constructed after analysis of spiked samples was good, with correlation coefficients always greater than 0.996. The review of some methods which were used for the determination of β-blockers in the environmental and biological samples, including this research, is demonstrated in Table 4.

4. Conclusions

Three-phase DSDME is rapid, effective, inexpensive, virtually solvent free method with high selectivity and pre-concentration. DSDME technique requires very little sample solution and little expensive and toxic organic solvents. The method has a high pre-concentration factor and excellent selective clean up of samples. Good linearity and reasonable relative recovery were also obtained. This technique is compatible with a broad range of analyses, in biological and environmental samples, and when used with HPLC, may provide a strong platform for analytical microextraction in the future. We used this method to isolate β-blockers from natural water samples successfully.

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Ethical approval:
This manuscript does not report on or involve the use of any animal or human data or tissue. So the ethical approval does not applicable.

Authors Contributions:
The paper was prepared by the corresponding author.

Availability of data and materials:
The data that support the findings of this study are available on request from the corresponding author.

Conflict of Interests:
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.

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