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Pre-concentration and Sensitive Determination of Propranolol and Metoprolol Using Dispersive Solid-Phase Microextraction and High-Performance Liquid Chromatography in Biological, Wastewater, and Pharmaceutical Samples

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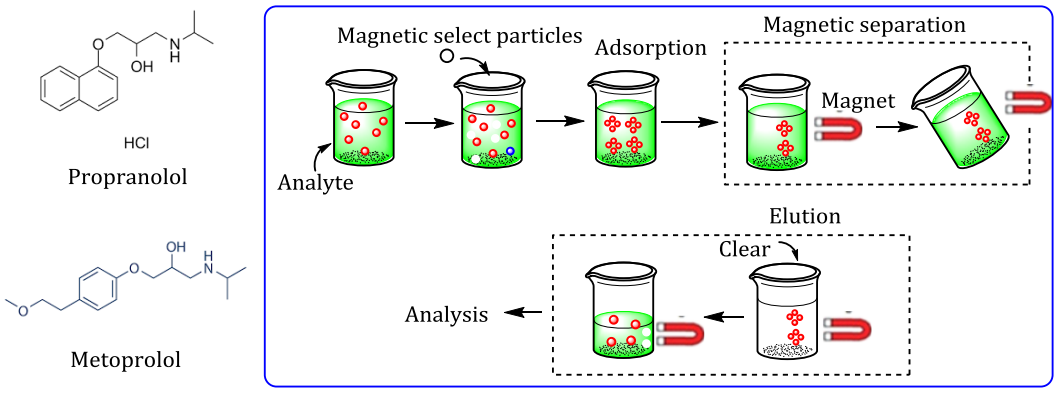
ABSTRACT

Determination of drugs at environmental and biological samples is a critical issue for controlling the effects of a drug on human health and environmental pollution. In the present study, a dispersive solid phase microextraction mode was developed for the preconcentration and cleanup of low amounts of propranolol and metoprolol before their determination with a high-performance liquid chromatography diode array detector. Carboxyl functionalized single-walled carbon nanotubes supported by magnetic nanoparticles (Fe_3O_4@SWCNT-COOH) were synthesized as a sorbent using a chemical procedure. Various factors affecting the microextraction method such as pH, donor phase volume, extraction desorption time, sorbent quantity solvent type and volume, and stirring rate were evaluated along with improving efficiency. The dynamic linear range of the method for the propranolol and metoprolol determination was 0.1-234 and 0.2-187 mg L^{-1} with a determination correlation coefficient (R^2) of 0.9989 and 0.9984, respectively. Besides, the enrichment factor for the determination of propranolol and metoprolol were 283.1 and 278.7, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were turn down by 0.06 and 0.2 for the analyte determination, correspondingly. Intraday and inter-day RSD% were determined for five times to determine propranolol and metoprolol at a concentration of 2.0 ng.mL^{-1} and were lower than 3.26 and 4.29%, respectively. Determination of propranolol and metoprolol in hospital wastewater, human urine and plasma samples indicated that this proposed method can be employed to analyze the actual samples without the significant matrix effects with a recovery range of 91.0-97.2% and an RSD of less than 5.49%.

GRAPHICAL ABSTRACT



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Introduction

Propranolol (PROP) and metoprolol (MET) are important drugs with a heterogeneous group in the chemical structure which are in a systematic fashion exhibited as beta-adrenergic receptor blockers [1]. Propranolol and metoprolol are generally recommended for medical care for hypertension, angina, cardiac arrhythmias, and hypertrophic aortic stenosis [2-4]. Also, PROP is occasionally utilized as a narcotics agent in frolic [5]. PROP was mainly metabolized in the liver to *N*-desisopropylpropranolol and 4-hydroxypropanolol and excreted in urine through the kidney [6,7]. MET was also metabolized to O-demethylmetoprolol and α-hydroxymetoprolol [8]. PROP, MET and their metabolites can enter water sources and lead to pollution in the environment. Hence, the determination of these compound in wastewater and biological samples are essentialy to restraint their effects on the human health and environment to investigate doping in athletes.

In recent years, various analytical methods have been developed to determine PROP or MET in different real samples such as the voltammetric [9, 10] and electrochemical method [11-13], the spectrophotometric method [14-16], the high-performance liquid chromatographic method [17-19]. However, due to the high complexity of the matrix of hospital wastewater samples and biological samples and the low concentration of PROP and MET in these samples, the need for new methods with high sensitivity, accuracy, selectivity, and low cost is always considered. Besides, a sample preparation method is essential to present the sample to an analytical instrument, and reduce the matrix effect of real samples, and preconcentration of PROP and MET in real samples before the analyte determination [20]. Microextraction methods are a status of sample act of getting ready methods that are widely utilized due to their unique characteristics such as organic solvent and low sample consuming, excellent clean up, high preconcentration factor and purification [20, 21].

Among them, solid-phase microextraction (SPME) was improved by minimizing the solid phase extraction method, in which a suitable SPME fiber was prepared by depositing a suitable sorbent on the surface of a proper core [22,23]. The preparation of an SPME fiber is a critical step in SPME which greatly affects the reproducibility of the method. Although commercial fibers are commercially available, it is very difficult to provide exactly the same SPME fibers for specific applications in the method, which can be considered as a limitation of the method. One development procedure of SPME is dispersing solid-phase microextraction (DSPME) in which the attractive is dispersed directly into the specimen solution for analyte extraction and no fiber is required in this method [20]. In DSPME, analyte extraction efficiency is highly influenced by the dispersion efficiency of the sorbent in the specimen solution and the segregation of the sorbent from the solution after the extraction process. The use of magnetic sorbents in this method assists in easily separating the sorbent in the presence of an exterior magnetic domain, reducing the separation time, and eliminating the centrifuge step [24].

The sorbent plays an essential position in the choosy extraction of an target compound in the DSPME method through proper interaction with the analyte [24]. Nanomaterials play an important role in science [25-30]. This type of nanomaterials improves the quality of different branches of science [31-40]. Nanomaterial and nanocomposites are applied as unique sorbents in the DSPME procedure due to their excellent quality such as elevated surface to volume relationship, easy preparation, suitable thermal and chemical stability, and high adsorption efficiency [41,42]. However, these compounds are generally functionalized with permission functional groups that increase the selectivity and efficiency of adsorbent extraction compared with analytes [43]. Metal nanoparticles can be used as sorbent and modifiers in molecular and ion imprinted polymer technique in the measurement of drugs with electrochemical methods [41,42]. The advantages of the DMSPE method as a new trend in DSPME include convenience of preparation and surface modification of the adsorbent, excellent dispersibility of the adsorbent in media, high

selectivity, and fast separation process. Sample preparation has been investigated by LLE or SPE. In the proposed method, no memory effect is observed and also the nano magnetic adsorbent can be synthesized by a simple and cheap chemical reaction. Also, there is no demand for centrifuging or filtration of the samples before analysis, which is the advantage of the magnetic nano adsorbent.

In the present study, a commoner and sensitive DSPME was improved to preconcentrate and cleanup propranolol and metoprolol as beta-adrenergic receptor blocker drugs prior to determination with a HPLC with diode array detector. Single-walled carbon nanotubes were firstly functionalized with carboxyl function groups (SWCNTs-COOH) using nitric acid and a hydrothermal procedure. Then, Fe_3O_4 nanoparticle was synthesized in presence of SWCNTs-COOH to form a magnetic sorbent (Fe_3O_4@SWCNTs-COOH) by a chemical Co-precipitation method in alkaline media. New synthesized magnetic composites showed suitable extraction efficiencies toward PROP and MET and reduction in time of the sorbent segregation from the target analyte solution.

Materials and Methods

Apparatus and instruments

To simultaneously measure PROP and MET in standard and samples, a Knauer Associates HPLC system including a port injection valve with a 20-µL injection, photodiode array detector (K-2600), and an EZ-Chrom Elite software was applied. A Eurospher 100/5C18 analytical column (4.6 mm × 250 mm, 5 µm) was performed at room temperature (22±0.5 °C) to obtain the chromatographic separation. The mobile phase included a mixture of phosphate buffer (100 mM, pH 3.14) and acetonitrile with a ratio of 60:40 and a flow rate was 1.2 mL min^{-1}. The DAD detector was set at wavelengths of 289 and 224 nm for the PROP and MET determination, respectively [44]. A pH meter (Metrohm 780, Switzerland) was used to measure the pH of sample solutions. The sorbent was dispersed into the desorption solvent and the sample solution by an ultrasonic probe (Hielscher, Germany).

Reagents

Single-walled carbon nanotubes with a purity of 99.8% were purchased from Iran Research Center for Oil Industry. Propanolol and metoprolol were purchased from Fluka (Switzerland). Stock working solution (1000 µg mL^{-1}) was uniquely arranged by dissipating 0.100 mg PROP and MET in methanol with a final volume of 100.0 mL. Newly solutions were made ready every day by diluting the solution in double distilled water. Other materials such as nitric acid, $FeCl_3{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$, phosphoric acid, NaH_2PO_4, methanol (HPLC grade) borax and hydrazine, were purchased from Merck company with grade analytical (Germany).

Synthesis of Fe_3O_4@carboxyl functionalized single-walled carbon nanotubes

Single walled carbon nanotubes (SWCNTs) were functionalized with carboxyl function group in acid media based on a previous paper [45]. Briefly, SWCNTs (0.5 g) was poured 15.0 mL of nitric acid (30% v/v) and was ultrasonically dispersed for 20 min at room temperature. The obtained termination was forwarded to a flask and refluxed at 140 °C for 24 h. The resulting precipitation (SWCNTs-COOH) was centrifuged at 5000 rpm for 12 min and precipitation was separated. SWCNTs-COOH was washed several times with distilled water until its pH reached about 6. SWCNTs-COOH was eventually having moisture removed at 60 °C for 18 h in an oven.

A mixture restraint of 0.1 g of SWCNTs-COOH, 0.12 gr of $FeCl_3.\ 6H_2O$, and 0.05 gr of $FeCl_2.\ 4H_2O$ was poured into 45.0 mL of distilled water and sonicated for 20 min at room temperature. Nitrogen gas flow with a flow rate of 2.0 mL min^{-1} was passed through the suspension and an ammonium hydroxide solution (28%) was supplemented in drops to the mixture with vigorous stirring up to the time when the pH of the suspension obtained was about 10. The obtained precipitate (Fe_3O_4@SWCNTs-COOH) was separated using a strong magnet and washed three times with ethanol and dried at 60 °C for 18 h in a vacuum oven [46].

Microextraction procedure

PROP and MET aqueous solution (12.5 mL) were made flow in a vial and its pH was adapted to 5.0 using a phosphate buffer. The sorbent Fe_3O_4@ SWCNTs-COOH nanocomposites (13 mg) was combined to the solution and the blend was sonicated for 15 min at room temperature. The sorbent was dispersed well into the target solution to extract PROP and MET. The magnetic sorbent was withdrawn from the sample solution using a magnet. The sorbent was transferred to a new vial and 100 µL of methanol attributed the desorption solvent was made flow into it. The mixture was sonicated for 7 min and the methanol was separated. The methanol phase (20 µL) was injected into the HPLC column to simultaneously determining PROP and MET.

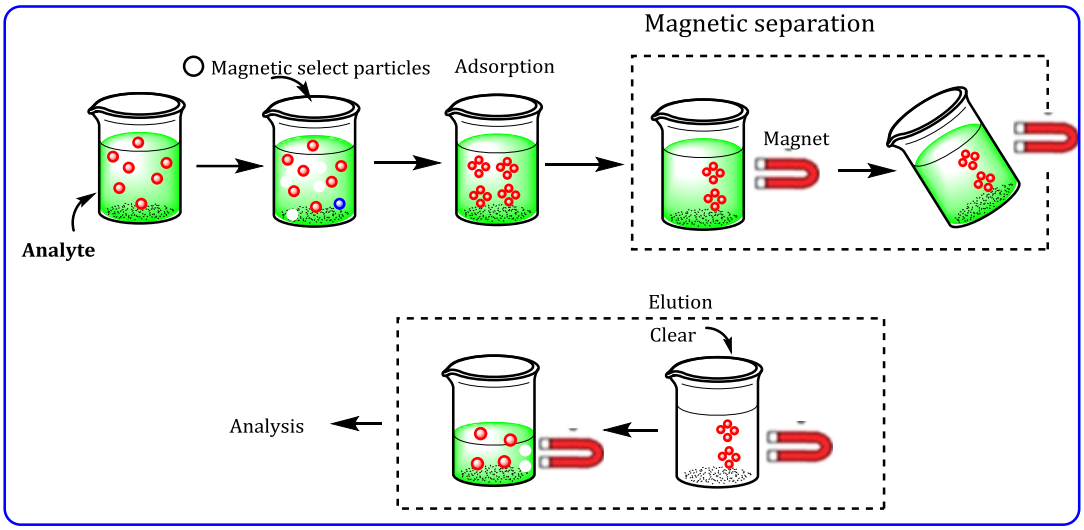

Figure 1: Schematic of the proposed method for the determination PROP and MET

Results and Discussion

Effect of pH study

The pH of the donor phase is one of the important factors affected in the adsorption of PROP and MET on the sorbent and can ultimately increase the extraction efficiency of PROP and MET. Therefore, the pH effects on the PROP and MET extraction were investigated in the range of 3-7. The obtained results are shown in Figure 2, indicating the extraction recoveries were increased with increasing pH up to 5 and decreased afterward for both analytes. It may be due to an electrostatic interaction between a negative charge on the sorbent surface and a positive favorable charge at the analytes at pH 5. The pKa of PROP and MET were 9.4 and 9.7, respectively, indicating a positive charge was formed on the analyte surface due to adsorb hydronium ion on the analyte surface at pH 5. Besides, pH_{pzc} of the carboxy functionalized single-walled carbon nanotubes as the sorbent was 3.1 and a positive charge was carried out on the sorbent surface at pH 5. Therefore, the elevated extraction recovery was observed at pH 5 and this pH was chosen as the best Condition and subsequent measurements were done at this pH.

Effect of salt addition

The addition of salt can increase the recovery of an analyte through the salting-out effect. Adding salt to a solution of aqueous sample may have several effects on extraction, which usually depends on solubility target analytes. Adding salt to the sample increases its extraction of more polar analytes. It was assumed that apart from the salting-out effect, salt addition causes a second effect named, salting-in effect. This phenomenon leads to changes in the physical properties of the Nernst diffusion film. Therefore, the execute of the salt (NaCl) in the PROP and MET extraction were studied in the range of 1.0 to 11.0%. According to ESM, the extraction recoveries of PROP and MET did not show a significant change with increasing salt. Therefore,

no salt was added to the sample solution in further extractions.

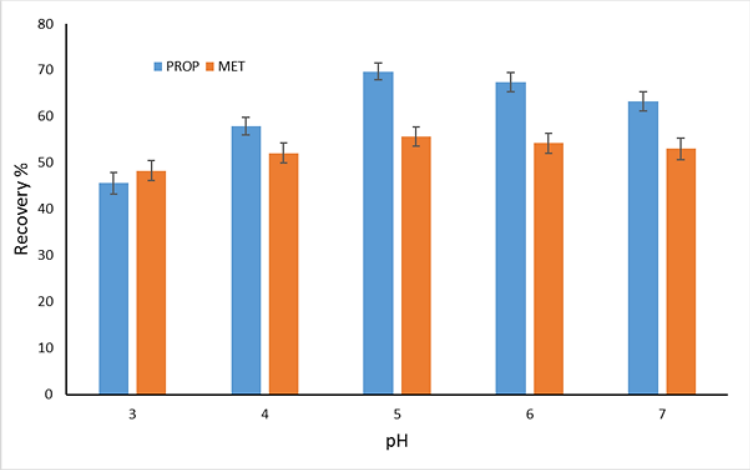

Figure 2: Effect of pH on the PROP and MET extraction. **Condition:** Concentration of PROP and MET (10 n**g** mL^{-1}), salt addition (0.0%), donor phase volume (10.0 mL), extraction time (10 min), sorbent amount (10 mg), desorption solvent (methanol), desorption solvent volume (200 µL), desorption time (5 min)

Effect of extraction time

Extraction efficiency in equilibrium-based techniques like MSPE depends on the time it takes the interaction to reach equilibrium. Inward the dispersive solid-phase microextraction, analyte extraction is an equilibrium procedure in which the analyte is allocated between the phases of the sample solution and the sorbent surface [21]. Therefore, this procedure is time-dependent and demands least possible amount extraction time to achieve this equilibrium. The effects of the extraction time on the extraction of PROP and MET were evaluated in the time in range of 5-25 min though other factors were steadfast. The other consequences are presented in Figure 3, showing that the process recoveries of PROP and MET were elevated with elevating the extraction time in the range of 5-15 min and remain almost constant at greater values of extraction time. Thus, a time of 15 min was selected like the extraction time for the PROP and MET determination.

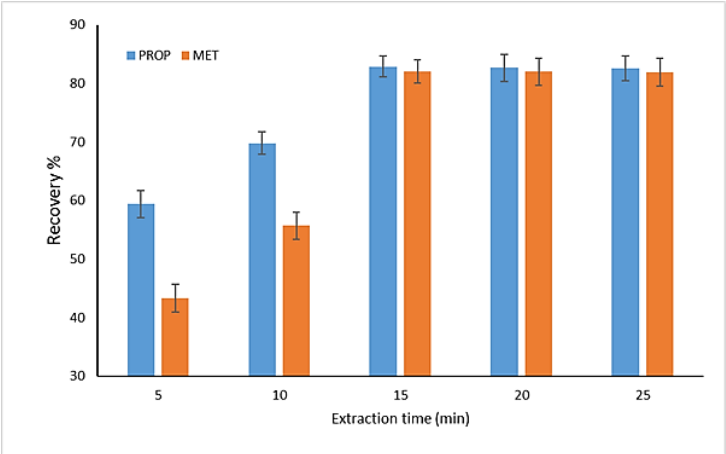

Figure 3: Effect of extraction time on the PROP and MET extraction. **Condition:** Concentration of PROP and MET (10 n**g** mL^{-1}), pH (5), donor phase volume (10.0 mL), salt addition (0.0%), sorbent amount (10 mg), desorption solvent (methanol), desorption solvent volume (200 µL), desorption time (5 min)

Effect of the donor phase volume

The aqueous phase volume has an important role with a high effect on the preconcentration factor. On the other hand, the preconcentration factor is proportional to the proportional relation of the amount of the donor phase to the amount of the desorption solvent. Thus, the preconcentration factor was increased by increasing the donor phase volume to a constant volume of desorption

solvent. However, the preconcentration factor becomes free of the amount of the aqueous sample in its very large volume. The effects of the donor phase amount for the PROP and MET extraction were surveyed in the confine of 5.0-20.0 mL. The highest recoveries of the analytes were obtained for a donor phase volume of 15.0 mL and remains constant afterword. Therefore, an amount of 15.0 mL was selected as the best donor phase amount for further studies.

Sorbent amount

The sorbent plays a critical role in the PROP and MET extraction thorough form suitable interaction with the analytes. However, these interactions occur in the functioning groups present in the sorbent surface; therefore, the number of functional groups at the sorbent surface can be an important factor in the analyte extraction. As the sorbent amount is increased, the number of functional groups available for the analyte extraction is increased, leading to an increase in the analyte extraction efficiency. The effect of sorbent amount was investigated in the range of 4-20 mg, showing that the PROP and MET recoveries were increased with increasing sorbent amount up to 13 mg and remain constant for a further amount of the sorbent (Figure 4). So, 13 mg sorbent was selected as the optimum amount of the sorbent.

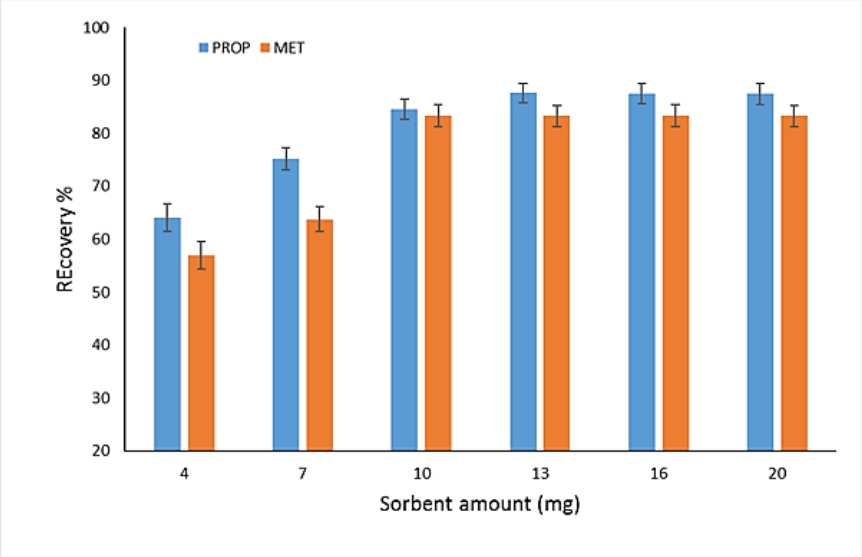

Figure4: Effect of sorbent amount on the PROP and MET extraction. **Condition:** Concentration of PROP and MET (10 n**g** mL^{-1}), pH (5), donor phase volume (12.5 mL), salt addition (0.0%), extraction time (15 min), desorption solvent (methanol), desorption solvent volume (200 µL), desorption time (5 min)

Effect of desorption solvent type

In the study, four organic solvents, including chloroform, acetonitrile, ethanol, and methanol were chosen as solvent and their effects on the PROP and MET extraction were investigated. The consequences are exhibited in ESM Figure 4. The maximum recoveries were acquired by using methanol for the extraction of the analytes. The hydrogen bonds intermediate **to** the target analytes and the desorption solvent may be responsible for the removing the analyte from the sorbent surface and the transfer into the organic solvent. The strength of this hydrogen bond is greater in methanol than in ethanol because the polarity of methanol is loftier than that of ethanol. Therefore, methanol was chosen as the best desorption solvent for further studies.

Effect of desorption time

Like the extraction process, the desorption process is a time-dependent equilibrium process. Therefore, optimization of desorption time is necessary to achieve the desorption equilibrium of the target analytes intermediate to the sorbent surface and desorption solvent phases. In this investigate, the time was changed between 3-15 min and its effects on the recoveries of PROP and MET were investigated. The results exhibited that a set a time of 7 min is sufficient to reach the

desorption equilibrium and no significant changes in the recoveries of the analytes are observed with a further increase in the desorption time. Therefore, a set of time of 7 min was chosen as the best desorption time for future studies.

Effect of desorption solvent volume

Another parameter with a high sensation in the extraction of PROP and MET is the desorption solvent volume. Obviously, the analyte adsorbed in the sorbent surface must be desorbed to present into HPLC for the determination of the analytes. An optimal amount of the solvent is necessary for the success of the target analytes desorption process from the sorbent surface because the desorbed analyte is diluted with a large volume of desorption solvent and the extraction recovery is reduced. Besides, if the volume of solvent and desorption is low, complete desorption of the analyte from the sorbent surface is not performed, leading to a reduction in the recovery of analytes. Thus, the effects of the desorption solvent volume were investigated and optimized. The consequence revealed that the recoveries of PROP and MET were enlarged with enlarging desorption solvent amount to 100 µL and then decreased afterward due to the effect of the dilution of the desorbed PROP and MET. Therefore, a volume of 100 µL was selected as the optimum desorption solvent amount for further studies.

Figures of merit

Several analytical parameters, including linear range, LOD, LOQ, RSD, and enrichment factor (EF) were investigated under the optimum condition to evaluate the performance of the DSPME-HPLC method for the determination of PORP and MET. The DSPME-HPLC procedure was linear in the range of 0.1- 234 and 0.2-187 ng mL^{-1} with the determination coefficients (R^2) of 0.9989 and 0.9984 for the determination of PROP and MET, respectively. LOD and LOQ were calculated based on the following equation 1 and 2:

$$LOD=3S_b/m \quad (1)$$

$$LOQ=10S_b/m \quad (2)$$

Where S_b and m are the standard deviation of the blank sample and slope of the calibration curve of the analytes, respectively. LOD and LOQ for the determination of PROP and MET were lower than 0.06 and 0.2 ng mL^{-1}, respectively. The relative standard deviation (RSD%) were determined for five times the measurement of PROP and MET with a concentration of 2.0 ng mL^{-1} in one day (intraday RSD) or three sequential days (inter-day RSD). The intraday and inter-day RSD for the determination of PROP and MET were lower than 3.26 and 4.29%, respectively. EF was obtained from the proportional relation of target analytes concentration after the microextraction process to its concentration in the absence performing the microextraction process and was equal to 283.1 and 278.7 for determining PROP and MET, respectively. All acquired results are tabulated in Table 1.

Table 1: Figures of merit of the DSPME-HPLC method in the simultaneous determination of the PROP and MET in aqueous samples

Analyte	Linear range (ng mL^{-1})	R^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	RSD (n=5)		EF
					Intraday	**Interday**	
PROP	0.1-234	0.9989	0.03	0.1	3.26	4.17	283.1
MET	0. 2-187	0.9984	0.06	0.2	3.19	4.29	278.7

Real sample analysis

To appraise the efficiency and capability of the method, several real samples including biological, hospital wastewater, and pharmaceutical samples were analyzed to determine the concentration of PROP and MET. The wastewater samples were acquired from Sina Hospital (Mashhad, Iran). The urine and plasma specimen were obtained from healthy volunteers with an age in the range of 35-45 years. The volunteers had not taken any drugs in one month before sampling. The concentrations of PROP and MET

in the initial analysis of real samples were not detectable. Therefore, the real specimens were spiked accompanied by the standard of PROP and MET at two concentrations of 1.0 and 5.0 ng ml^{-1} and analyzed by the DSPME-HPLC method without any other preparation step. The consequences are exhibited in Table 2, indicating the relative recoveries of PROP and MET in the real samples are in the ranges of 91.0-97.2% with RSDs between 4.12-5.49% for occurring at the same time determination of PROP and MET. The consequence exhibited that the DSPME-HPLC method is an appropriate protocol for the determination of drugs in wastewater and biological samples without significant matrix effects.

Table 2: Analysis of real samples for the simultaneous determination of the PROP and MET with the DSPME-HPLC method

Sample	Spiked (ng mL^{-1})	PROP			MET		
		Found[1]	Recovery%	RSD%	Found	Recovery%	RSD%
Wastewater	0	ND[2]	---	---	ND	---	---
	1.0	0.92±0.04	92.0	5.43	0.91±0.05	91.0	5.49
	5.0	4.71±0.19	94.2	4. 30	4.73±0.20	94.6	4.23
Plasma	0	ND	---	---	ND	---	---
	0.1	0.90±0.04	90.0	4.44	0.92±0.05	92.0	5.43
	0.5	4.69±0.20	93.8	4.26	4.75±0.21	95.0	4.42
Urine	0	ND	---	---	ND	---	---
	0.1	0.94±0.05	94.0	5.31	0.93±0.04	93.0	4.30
	0.5	4.83±0.20	96.6	4.14	4.86±0.20	97.2	4.12

1ng mL^{-1}, [2] Not detected

Also, the DSPME-HPLC procedure was useable to determine PROP and MET in Tablet. A tablet of PROP (10 mg) and a tablet of MET (50 mg) were obtained from Tehran Darou CO. (Tehran, Iran). Both tablets were ground in a mill and the powder (0.1 g) was conveyed to an Erlenmeyer and 25.0 ml of double distilled water was supplemented to it. The resulting mixture was sonicated for 20 min and centrifuged at 5000 rpm at 10 min. The supernatant was then segregated and made fainter to 100.0 ml with double distilled water. The solution was finally analyzed by the DSPME-HPLC method. The amount of PROP and MET in the tablets was 9.94 and 49.77 mg.

Conclusion

For the first time, a new disposable sorbent Fe_3O_4 @SWCNTs-COOH nanocomposites was demonstrated to extraction and simultaneous determination propranolol and metoprolol in the environment and urine samples with high-performance liquid chromatography -diode array detector.

The validation of the proposed method was carried out under optimum extraction conditions of 15 mL of donor phase volume, 13 mg of Fe_3O_4 @SWCNTs-COOH nanocomposites as an adsorbent, 100 µL of methanol as an desorption solvent, 15 min of extraction time and 7 min of desorption time, urine pH adjusted in 5 and applying no salt addition.

A simple synthesis was applied to prepare the DSPME sorbent based on carboxyl functionalized single-walled carbon nanotubes supported by magnetic nanoparticles (Fe_3O_4@SWCNT-COOH). The method showed a suitable linear range of 0.1-234 and 0.2-187 mg L^{-1} to a determination coefficient (R^2) of 0.9989 and 0.9984 for the propranolol and metoprolol determination, respectively. Besides, a high enrichment factor of 283.1 and 278.7, and low LOD of 0.03 and 0.06 ng mL^{-1} were obtained to measure propranolol and metoprolol, respectively. Intraday and inter-day RSD% were determined for five times the determination of propranolol and metoprolol with a concentration of 2.0 ng mL^{-1} and were lower than 3.26 and 4.29%, respectively. Determination of propranolol and metoprolol in

hospital wastewater, human urine and plasma samples indicated that the proposed method can be used to analyze the real samples without the significant matrix effects with a recovery range of 91.0-97.2% and a RSD of less than 5.49%. This method can be used successfully for the analysis of biological analytes in pharmaceutical and biological samples.

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Authors' contributions
All authors contributed toward data analysis, drafting and revising the paper and agreed to responsible for all the aspects of this work.

Conflict of Interest
The authors proclaim no disagree of interest.
The human urine samples were gathered by free choice donors with their informed approval.

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