# Trace Extraction of Metoprolol from Plasma, Urine and EBC samples Using Modified Magnetic Nanoparticles Followed by Spectrofluorimetric Determination for Therapeutic Drug Monitoring Purposes

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**Abstract:** ***Background*****:** Metoprolol is a selective $\beta_1$-adrenergic receptor antagonists (β -blockers). It is widely used for the treatment of hypertension and other related diseases. Metoprolol can be used as doping agent in sport, thus has been added to the list of forbidden drugs. In Iran, therapeutic drug monitoring (TDM) of beta-blockers is an applied procedure in some cases. Therapeutic regimen could be easily managed by determination of drug levels in biological fluids which is relatively costly process and requires high skilled technical staff. Using a simple and low-cost analytical procedure may help to use TDM in routine clinical practice.

***Method*****:** A real biological sample was prepared and adjusted to pH 3-4, then metoprolol was quickly extracted using iron oxide magnetic nanoparticles (MNPs) modified by the surfactant sodium dodecyl sulfate (SDS) and determined applying spectrofluorimetry at 340 ± 3 nm after excitation at 283 ± 3 nm.

***Results*****:** The extraction and determination conditions including, the amount of NPs and SDS, pH of solution, standing time and desorption solvent type and volume were investigated and adjusted and optimized. Calibration curves were linear over the concentration range 6–100 ng/ml for plasma and 5–100 ng/ml for water, urine and exhaled breath condensate (EBC) samples, respectively. Intra and inter-day precision values for metoprolol in different samples were less than 5.6 and 6%, respectively, and accuracy (as relative

error) was better than 5%. Standard addition recovery tests were carried out, and the analytical recoveries ranged from 86% to 113%. The limits of detection (LOD) and quantification (LOQ) of metoprolol were found to be in the range of 2.1-3.4 and 6.3-10.2 ng/ml, respectively.

***Conclusion*:** The developed method was successfully applied to one volunteer with hypertension who had been given an oral tablet of 50 mg metoprolol.

**Keywords:** Metoprolol, iron oxide magnetic nanoparticles, plasma, urine, exhaled breath condensate, solid phase extraction, spectrofluorimetry.

## 1. INTRODUCTION

Metoprolol, [2-propanol, 1-[4-(2-methoxyethyl) phenoxy]-3-[(1-methylethyl) amino]-,(±)-,[R-(R*,R*)]-2,3-dihydroxybutanedioate], is a selective $\beta_1$-adrenergic receptor antagonists (β -blockers) [1]. It is widely used for the treatment of hypertension, angina, myocardial infarction, arrhythmia, hyperthyroidism and other related diseases [1, 2]. Metoprolol could be formulated as its tartrate salt for immediate release requiring multiple daily dose or as its succinate salt for an extended release formulation for a single daily dose administration [3]. Metoprolol can be used as doping agent in sport, thus has been added to the list of forbidden drugs by the International Olympic Committee [4]. Therapeutic drug monitoring (TDM) of beta-blockers is available in the United States to detect medication non-adherence in patients with resistant hypertension [5]. In Iran, TDM of beta-blockers is an applied procedure in some cases. In a brief meta-analysis of 16 non-adherent patients in the United States, memory loss (in 2 cases), debilitating fatigue (in 3 cases) and drug cost (in 5 cases) were recognized as the major barriers to non-adherence [6]. In addition to the mentioned patient-related factor of non-adherence, one should consider clinician-related factors such as non-appropriate drug dose or therapeutic inertia [7]. Therapeutic regimen could be easily managed by determination of drug levels in biological fluids which is relatively costly process and requires high skilled technical staff.

Using a simple and low-cost analytical procedure may help to use TDM in routine clinical practice.

Up to now, various analytical methods have been developed for metoprolol quantification in different biological samples, including high performance liquid chromatography (HPLC) [8-14], HPLC-mass spectrometry (HPLC-MS) [9, 15-18], capillary electrophoresis (CE) [19, 20], gas chromatography-MS (GC-MS) [2], electrochemical [1, 21-23] and spectrofluorimetry [24]. Different extraction methods such as SPE [9, 12, 16, 18, 20], SPE with microextraction column [19], liquid-liquid extraction (LLE) [2, 8, 13, 15, 16], dispersive liquid-liquid microextraction (DLLME) [10], ultrasound-promoted dispersive micro-SPE (US-D-μSPE) [14] and salting-out assisted LLE extraction (SA-LLE) [11] have been used for the extraction of metoprolol from biological samples before its determination with mentioned methods.

Sample preparation procedures of β-blockers and their metabolites, in most cases, include either LLE, or SPE. However, these procedures suffered from major drawbacks including consumption of large amounts of expensive and toxic organic solvents, loss of analyte, the need for costly and complex equipment, being tedious and time-consuming and large production of disposable cartridges [10, 11, 14]. Miniaturized sample preparation techniques such as DLLME and SA-LLE still use remarkable amounts of

toxic organic solvents. In addition DLLME suffers from low partition of the analytes into the extraction solvent and the lack of sample clean-up [10]. Also, US-D-μSPE use complex equipment such as ultrasonic bath and centrifuge. A micellar liquid chromatographic method was developed for simultaneous quantification of metoprolol with furosemide and verapamil. The method could be used for a direct injection of the filtered plasma into the chromatographic system [25].

Recently, novel sample preparation techniques based on MNPs, especially magnetic iron oxide nanoparticles (MIONPs), were used in sample preparation techniques. These MNPs have high dispersibility and can be readily isolated from sample solution by using of an external magnet. Moreover, their surfaces can be easily modified especially by the aid of ionic surfactants such as cetyltrimethylammonium bromide (CTAB) or SDS. Covering the surfaces of MNPs with these surfactants can improve the adsorption capacity and extraction efficiency of target analytes, since nonpolar components can be easily interact with the hydrophobic layers on the surface of MNPs [26-28]. This MSPE method based on mixed-hemimicelles assemblies has a number of advantages including high extraction yields due to high adsorption capacity of analytes, high breakthrough volumes, easy elution of analytes, easy regeneration of adsorbent and reducing the analysis time through the rapid isolation of MNPs with a strong magnet [26]. With these remarkable benefits,

it has been extensively used for the extraction and preconcentration of a variety of organic [26-38] and inorganic [39-47] compounds from various matrices.

In the present work, SDS coated MNPs were synthesized and employed as sorbent in NP based SPE of metoprolol from human urine, plasma and EBC samples. Urine and plasma are the most routinely used biological samples in the areas of pharmaceutical and biomedical analyses and EBC attracted more attentions in recent years [48, 49] and is a promising non-invasive sample. However, due to high dilution of very low concentrations of drugs in EBC, its analysis requires more sophisticated analytical methods. Fluorescence spectrometry was used for the detection of extracted analyte due its great sensitivity and selectivity as well as its relative low cost.

## 2. EXPERIMENTAL

### 2.1.Apparatus

A Shimadzu RF–5301 PC spectrofluorophotometer equipped with a 150 W Xenon lamp and 1.00 cm quartz cells was used for all spectrofluorimetric measurements. Instrument excitation and emission slits both were adjusted to 5 nm. The pH measurements were done using a pH–meter model M120 (Halstead, Essex, England CO9 2DX) supplied with a glass combined electrode. Shacking of the mixtures was performed using a Unimax 1010 Shaker–Inkubator (Heidolph, Germany).

## 2.2.Materials

The ionic surfactants, i.e. CTAB and SDS were obtained from Sigma-Aldrich (St. Louis, MO) and E. Merck (Darmstadt, Germany), respectively. All other chemicals including iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), sodium chloride (NaCl), hydrochloric acid (HCl) and sodium hydroxide (NaOH) and also solvents including ethanol (EtOH), methanol (MeOH), acetone (Ac) and acetonitrile (ACN) were obtained from E. Merck (Darmstadt, Germany).

A stock solution of metoprolol Tartrate (gifted by Sobhan Darou Co. (Tehran, Iran) at a concentration of 500 μg $mL^{-1}$ was prepared by dissolving appropriate amount of drug powder in MeOH and was kept away from the light in a refrigerator at approximately 4°C. Successive dilution of this stock solution was used for preparing other working standard solutions. A solution of 0.025 M of SDS was prepared by dissolving appropriate amount of SDS in deionized water and diluting up to the mark. All other reagents were of analytical reagent grade or higher. Ultrapure water (Milli–Q Advantage A 10 system, Millipore) was used throughout the work.

## 2.3.Preparation and Characterization of MNPs

MIONPs were synthesized by the co–precipitation of ferrous and ferric chlorides in sodium hydroxide solution and under vigorous stirring

with $N_2$ passing continuously through the solution during the reaction. Slight modifications was performed according to our previous works [30, 39]. The obtained MIONPs were separated from the reaction medium by a strong magnet, washed with 200 mL of deionized water four times, and then re–suspended in 250 mL of deionized water. The concentration of prepared MNPs was found to be 1% m/v. The average particle size of obtained MNPs was less than 42 nm by using a scanning electron microscopy (SEM). The FTIR spectra were used for the proving of adsorption of the surfactant to MIONPs [30].

### 2.4.Procedure for Biological Samples

All sample donors signed a consent form for the collection of biological samples. The consent form was confirmed by ethics committee of Tabriz University of Medical Sciences (with ethical code number of 381). The samples were collected and treated based on reported methods with some modification [30, 50, 51]. A volunteer was received an oral tablet of 50 mg of metoprolol (Metoral, Alborz Darou). Blood sample (10 mL) was collected into heparinized tube 2 h after oral administration. Blood samples were centrifuged at 4000 rpm (i.e., 0.8 kg $dm^{-3}$) for 45 min and the plasma was separated and kept frozen at −20 ºC until analysis. A frozen plasma sample was thawed at room temperature, then an aliquot of 2.5 mL

transferred into a 15 mL centrifuge tube and spiked with metoprolol at proper concentration range. For precipitation of plasma proteins, a volume of 2.5 mL of Ac was added. The contents were mixed and centrifuged at 4000 rpm for 15 min. An aliquot of 2 mL of clear supernatant solution was subjected to MSPE as described in below section. Drug–free plasma samples were obtained from the Blood Transfusion Organization (Tabriz, Iran) and stored at -20 °C until they were analyzed. This sample named as quality control (QC) samples.

Urine samples were also collected at the following time intervals: 0 – 2, 2 – 4, 4 – 6, 6 – 8 and 8 – 12 h, after oral administration and the samples were stored at −20 ºC until analysis. The samples were centrifuged for 15 min at 4000 rpm. Afterwards, 1.0 mL aliquots of the supernatant solutions was diluted up to 100 mL and 2 mL of diluted urine sample were spiked with metoprolol at proper concentration range and subjected to the general procedure described below.

EBC samples were obtained from healthy volunteers using a lab-made setup based on a cooling trap system patented in the national patent office [51]. It works simply by trapping the frozen droplets of lung lining fluid and also the dissolved analytes in the fluid using a cooling trap. Patient's breathing was collected in rest condition for a period of 20 min using a nose clip and frozen directly in the collecting cup cooled down up to

-25 °C. These samples stored until analysis time. A frozen EBC sample was thawed at room temperature then an aliquot of 2 mL of each sample was subjected to the metoprolol determination as described below.

### 2.5.Procedure for MSPE

For the batch adsorption experiments, an aliquot of prepared biological samples were placed in a 25 mL Erlenmeyer flask and spiked with metoprolol to give a concentration in the ranges specified in Table 3. Then, 2 mL of 1.0% m/v NPs solution and 2 mL of 0.025 M SDS solution were added into the flask and the volume was diluted approximately up to 10 mL. The pH was adjusted to 3-4 by drop wise addition of 0.1 M HCl solution, then the volume was made up to 10 mL with ultra-pure water. The flask was shaked at 200 rpm and allowed to complete the extraction process for 10 min. Then, the SDS–coated MIONPs were collected in the bottom of the beaker by applying an external magnet and supernatant was removed. The adsorbed analyte was desorbed from the MNPs by addition of 2 mL of ACN with the aid of shaking at 200 rpm for 10 min. After desorption, the eluent was separated by magnetic decantation and fluorescence intensity of eluted drug was measured at 340 ± 3 nm with the excitation wavelength set at 283 ± 3 nm.

## 3. RESULTS AND DISCUSSION

For the MSPE of metoprolol from biological samples, the influences of different factors on the extraction and elution steps were studied and optimized. Fig. **1** shows the excitation and emission spectra for metoprolol extracted from aqueous or biological samples using optimized MSPE conditions that were established in this work.

**Fig. 1**

### 3.1.Effect of pH

For the assessment of pH effect on the extraction of metoprolol, the pH of sample solution was adjusted in the range 1.0 – 8.0 by adding the appropriate values of HCl or NaOH to the sample solution. The results were presented in Fig. **2**, indicated that, by increasing of the pH from 1.0 up to 3.0, the analytical response and hence extraction was enhanced remarkably, so the maximum adsorption was achieved at pH between 3.0 to 4.0, then decreased with a steeper slope at higher pH values. It can be interpreted such that by adjusting the solution pH before MSPE, the surface of NPs becomes charged, and enables the adsorption of surfactant charged oppositely. So at pH's lower than $pH_{zpc}$ (e.g. pH zero point charge) of MIONPs (≈6.5) [35, 37], the surface of NPs was positive and the positively charged MNPs was favorable for the adsorption of anionic surfactants and thus targeted analyte.

When the pH value was higher than $pH_{zpc}$, the positive charge density of the MIONPs surface was decreased. Thus the electrostatic attraction between the surface of MIONPs and SDS was not strong enough to produce hemi micelles [26, 28, 30, 32], which disfavored the great adsorption of metoprolol. According to these explanations, pH range of 3.0 – 4.0 was chosen for all subsequent experiments and 100 µL of 0.1 M HCl was used for the pH adjustment in this range.

**Fig. 2**

### 3.2.The Amounts of MIONPs and Sample Volume

Due to high surface area of MNPs, it can be assumed that by using fewer amounts of NP sorbents the adsorption capacity of analytes can be improved. Moreover, due to strong magnetism of MNPs the analysis time can be reduced through the rapid isolation of MNPs from large volumes of the sample solution by using of a strong magnet [26, 28, 30].

The effect of MNPs amount on the extraction of metoprolol was studied by using different volumes of 1% m/v $Fe_3O_4$ suspension, ranging from 0.5–4.0 mL. The experimental results (shown in Fig. **3**) indicated that the fluorescence intensities was enlarged with increasing amount of magnetic sorbent and reached to its maximum value at 2 mL of $Fe_3O_4$ suspension; that could be due to the increase in the surface area and thus active sites for the

analytes on the adsorbent [32], and then kept approximately invariant in more quantities. Therefore, 2.0 mL of 1% $Fe_3O_4$ suspension (eq. to final concentration of 0.2%) was selected as optimum amount of MNPs in the following studies.

**Fig. 3**

### 3.3.Effect of Surfactant Type and Amount

As mentioned earlier covering the surfaces of MNPs with ionic surfactants can improve the adsorption capacity and efficiency of the extraction. In mixed hemimicelles phase the outer surface of hemimicelles is hydrophobic whereas that of admicelles is ionic, so both hydrophobic interactions and electrostatic attraction can be used for the adsorption of target analyte onto surfaces of MNPs [26, 30, 39]. For investigating the type of ionic surfactant the effect of cationic CTAB and anionic SDS surfactant was examined and the results showed that a significant higher extraction efficiency was obtained when SDS was used for modifying the surface of MIONPs. Also, in the absence of SDS the fluorescence signal and thus the extraction efficiency of metoprolol was negligible. This may be explained by the fact that the surface of MIONPs was hydrophilic without any modification and hence had low adsorption affinity for the studied analyte [26, 30].

The influence of SDS concentration on the extraction of metoprolol was studied when SDS was added to the solution at concentrations ranging from 0.5 to 12 mM to modify the surface of MIONPs. From Fig. **4**, it can be concluded that the fluorescence intensities increased with the increasing amount of SDS and reached to its maximum value when SDS amounts was in the range of 5-6 mM. Then decreased gradually when SDS amount was above this range and reached approximately to zero above 8 mM. This may be described by the fact that SDS molecules formed micelles at concentrations higher than its CMC (e.g. 6-8 mM) [33, 37], so the analytes was lost in magnetic isolation step due to redistribution into the bulk aqueous solution [26, 30, 37]. Given these findings, SDS in the final concentration of 5-6 mM was selected for further studies.

**Fig. 4**

### 3.4.Extraction and Desorption Time

In MSPE process the adsorption of analyte onto MIONPs and its desorption were time dependent, thus the effect of extraction and desorption times were examined by changing the time from 1 to 20 min and under the optimal conditions mentioned above. The results in Fig. **5** indicated that the optimum fluorescence intensities were obtained when the adsorption and desorption time were 10 and 5 min, respectively. Further increase at these times had no

significant effect on the analytical signals, therefore these times were selected as the best adsorption and desorption times for further assays. Homogeneous dispersion of high surface area MNPs in sample solution along with its super paramagnetic properties resulted in such a fast extraction times [26, 28, 30, 37].

**Fig. 5**

### 3.5.Desorption Condition

Organic solvents are well known to disrupt rapidly and completely the mixed hemimicelles and admicelles, therefore making elution of the analytes from the surface of MNPs [26, 30, 32, 33]. A variety of elution solvents including MeOH, EtOH, acidified MeOH or EtOH, Ac and ACN, were tested to evaluate the complete disruption of hemimicelles and admicelles in the desorption step. The results in Fig. **6** indicated that ACN as desorption solvent offers the best performance for elution of metoprolol from modified MIONPs. Additionally, the effect of desorption solvent volume on the elution of metoprolol was studied in the range of 1.0-5.0 mL. The results revealed that 2.0 mL of ACN was sufficient for the complete desorption of the retained analyte; by further increasing the volume of ACN the analytical signal was decreased because of the dilution effect.

**Fig. 6**

### 3.6.Reusability and Stability of the Sorbent

The reusability and stability of solid adsorbents is of great importance for both economic and environmental standpoints [28]. To examine the re-applicability of the MNPs, the sorbent which used in the general MSPE procedure was rinsed with EtOH and deionized water, respectively, and then dried at 50 °C each time prior to reuse. Then, metoprolol was extracted in two concentration levels by these MNPs. The experimental results showed in Table **1** revealed that the adsorbent can be reused at least up to four times without significant decrease in the analytical recoveries which were still above 80%.

On the other hand, the stability of MNPs by the storing at the time was examined by storing the prepared MNPs at the time period over nine months and after each month an aliquot portion of prepared MNPs was used for the MSPE of fixed concentration of metoprolol (i.e. 100 ng $ml^{-1}$). The results in Table **2** showed that no significant degradation was observed in the sorbent performance, and the analytical recoveries were still above ap. 80% after five months storage.

**Table 1**

**Table 2**

### 3.7.Method Validation

The method was validated considering the linearity, sensitivity, precision, accuracy and matrix effect (ME) and according to guidelines set by the FDA [52] in order to demonstrate the performance of the proposed method. The calibration curves were constructed from samples of waters, human urine, plasma and EBC spiked with standard solutions of metoprolol. The regression plots showed a linear dependence of spectrofluorimetric signals on drug concentration over the ranges cited in Table **3**. The limit of detection (LOD) and quantification (LOQ) were defined by considering the three and ten times the standard deviation of the blank signals ($S_b$) and based on $3S_b/m$ and $10S_b/m$ equations, respectively, where m is the slope of the calibration line. The LOQ was defined as the lowest concentration on the calibration curve which can be quantified reliably. The analytical figures of merit of the constructed method have presented in Table **3**.

**Table 3**

The precision was expressed as relative standard deviation (RSD,%) whereas accuracy was expressed as relative error (RE,%) from the nominal value. The precision and accuracy were assessed under the optimized conditions for both intra- and inter- days and according to guidelines set by the FDA [52]. In order to do this, quality control (QC) samples were prepared at three concentration levels (*e.g.* low, medium and high) and intra-day and inter-day precision and accuracy were determined at these different QC

concentrations in replicate (n = 5) over three consecutive days. The precision defined as RSD% must be lower than 20% for low QC and 15% for the other QC samples. Similarly the accuracy defined as the RE% must be within ±20% for low QC and ±15% for other QC samples [9, 52]. Table **4** presents the obtained precision and accuracy data, where the intra- and inter-day precision values were lower than 5% and 6%, respectively. Similarly the intra- and inter-day accuracies were better than 4.5% and 5%, respectively. The results indicated in this Table proved that the method met the requirements of a bioassay set by regulatory guidelines [8, 9, 13, 52].

**Table 4**

A comparison between the MSPE-spectrofluorimetry performance in the extraction of metoprolol and the other extraction/spectrometric approaches reported in the literature is given in Table **5**. The most significant feature of the proposed method is that the achieved LOD's using the proposed method are comparable to those using very sensitive methods such as HPLC, HPLC-MS and GC-MS. It is also evident that the proposed procedure provides reasonable recoveries, proper dynamic linear range and repeatabilities when compared with other cited methods in this Table. It should be noted that no report was found on the analysis of metoprolol in EBC samples and the proposed method is the first report of metoprolol analysis in EBC.

**Table 5**

### 3.8.The Recovery Experiment and Interference Study

To further show the accuracy of the proposed method, the spiked-recovery approach was adopted. For this purpose, the QC samples were spiked with different concentrations of metoprolol at 7, 30 and 90 ng $ml^{-1}$ concentration level, and three repeated determinations were made for each concentration level. The obtained recoveries were listed in Table **6**, which ranged from 86.4% to 113.4% and proved the accuracy of the proposed method.

**Table 6**

The matrix effect on the analytical response of metoprolol was evaluated by comparing the slopes of calibration graph obtained with spiked biological samples with those obtained with solvent-based standards. For this purpose the matrix-to-solvent slope ratio was calculated and found to be 0.86, 0.81 and 0.95 in the case of urine, plasma and EBC samples, respectively. This result showed that the matrix does not significantly influence the extraction efficiency.

Also, typical excitation and emission spectra for standard solution of metoprolol, blank of each biological sample, a biological sample obtained from one volunteer, and the last spiked with standard solution of metoprolol were prepared and shown in Fig. **1**. No additional peaks, caused by interfering

compounds, were observed at the emission wavelength used in this work. Therefore, the similarities in the excitation and emission spectra for each sample, along with reasonable recoveries indicated that there wasn't no important matrix interferences for the samples analyzed by the proposed methods.

### 3.9.Stability

The freeze–thaw stability of biological samples was determined by the following three freeze–thaw cycles. The spiked samples at concentration of 60 ng $ml^{-1}$ of metoprolol were frozen at -20 °C for 24 h and thawed at room temperature. After completely being thawed, the samples were refrozen and this cycle was repeated three times. For the short-term stability, spiked biological samples were kept at room temperature for a time periods of 2, 4 and 6 h and then analyzed consecutively after each time. For the long-term stability, spiked biological samples were analyzed on three consecutively days. The results in Table **7** indicated that the recoveries were still above 80%, thus no significant degradation was observed when samples of metoprolol were taken and analyzed different experimental conditions.

**Table 7**

### 3.10. The Application of the Method

The applicability of the proposed procedure for the proper extraction and purification of three biological matrices including human plasma, urine and EBC samples was studied under the optimal mentioned conditions. The biological samples for this purpose were collected after administration of a single oral dose of 50 mg metoprolol to one volunteer and treated as described in the "Procedure for biological samples" section. Maximum plasma concentration of metoprolol concentration was found to be 61 ng $ml^{-1}$. This finding is in agreement with the levels reported for patients who donated plasma samples and metoprolol levels were determined using LC-MS/MS method [55]. Also, urine samples were collected between 0 to 12 h time intervals and the urinary volumes were recorded. The results of trial determinations of metoprolol in these samples were summarized in Table **8** which showed the urinary excretion of metoprolol. A total metoprolol excreted through urine was 4.2% of administered drug that taken dose in a total volume of 0.65 L urine [2, 35]. On the other hand, the concentration of metoprolol in EBC samples was below the LOQ of the proposed method. Although no reliable concentration of metoprolol was found in EBC real samples due to less sensitivity of the proposed method, the proposed method is capable of detecting metoprolol in higher EBC concentrations, e.g. in toxicological analyses.

**Table 8**

## CONCLUSIONS

In this work, MSPE based on MIONPs was successfully developed for the extraction and spectrofluorimetric determination of metoprolol in real biological samples. There are some advantages of the method, including:

1. The sample preparation time is dramatically decreased by the fact that the dispersed MNPs in the bulk solution, can be simply separated by using an external magnet so, the extraction achieved very quickly and there is no need to use a centrifuge for phase separation.

2. Compared with HPLC, HPLC-MS, GC-MS and CE methods used for the determination of metoprolol in biological fluids, the proposed method does not require high levels of financial investment or involve high instrument maintenance costs.

3. The proposed method represents a promising approach in the area of pharmaceutical monitoring with low operation cost, simplicity of instrumentation and low or non-polluting respect.

4. Method validation using spiked real samples demonstrated that the method is capable of detecting trace metoprolol with adequate accuracy and precision. Also, sensitivity of the method is enough for the determination of metoprolol in biological fluids.

All these results indicated that using MSPE combined with spectrofluorimetric detection is a very simple, safe, sensitive, rapid and inexpensive method for the extraction and determination of metoprolol.

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**Caption for figures**

**Fig. 1:** Excitation and emission spectra after MSPE: (a, A) reagent's blank, (b, B) EBC blank, (c, C) Urine blank, (d, D) Plasma blank, (e, E) Plasma containing spiked drug (100 ng $ml^{-1}$), (f, F) Urine containing spiked drug (100 ng $ml^{-1}$), (g, G) EBC containing spiked drug (100 ng $ml^{-1}$), (h, H) standard solution of metoprolol (100 ng $ml^{-1}$). Conditions: pH 3-4, NP (0.2%), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

**Fig. 2:** The effect of pH on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text**.**

**Fig. 3:** The effect of NP amount on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

**Fig. 4:** The effect of CTAB amount on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

**Fig. 5:** The effect of extraction time on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

**Fig. 6:** The effect of the elution solvents on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of each solvent, other conditions have been mentioned in the text.

**Caption for Tables**

**Table 1.** The effect of reusability on the analytical efficiency of 50 & 100 ng $ml^{-1}$ of metoprolol.

**Table 2.** The effect of stability of synthesized MNPs on the analytical efficiency of 100 ng $ml^{-1}$ of metoprolol.

**Table 3.** Analytical characteristics of the proposed method.

**Table 4.** Intra– and inter–day precisions and accuracies for determination of metoprolol.

**Table 5.** Analytical characteristics of different methods used for extraction and determination of metoprolol.

**Table 6.** Recoveries of metoprolol from spiked plasma samples

**Table 7.** Freeze–thaw stability results.

**Table 8.** The results of trial determinations of metoprolol in collected urine samples.

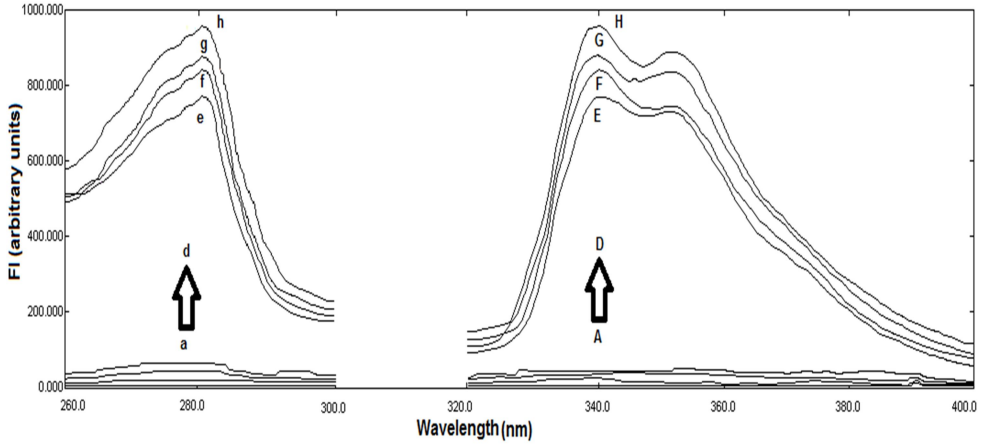


**Fig. (1).** Excitation and emission spectra after MSPE: (a, A) reagent's blank, (b, B) EBC blank, (c, C) Urine blank, (d, D) Plasma blank, (e, E) Plasma containing spiked drug (100 ng $ml^{-1}$), (f, F) Urine containing spiked drug (100 ng $ml^{-1}$), (g, G) EBC containing spiked drug (100 ng $ml^{-1}$), (h, H) standard solution of metoprolol (100 ng $ml^{-1}$). Conditions: pH 3-4, NP (0.2%), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

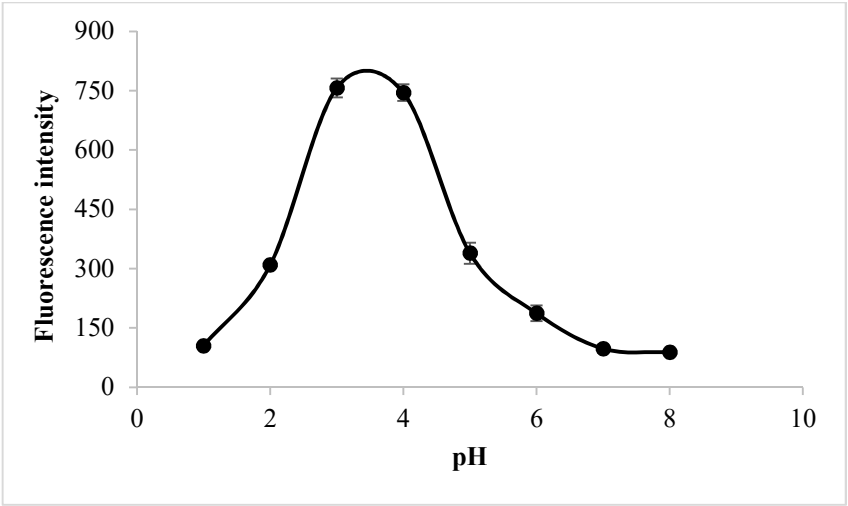


**Fig. (2).** The effect of pH on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

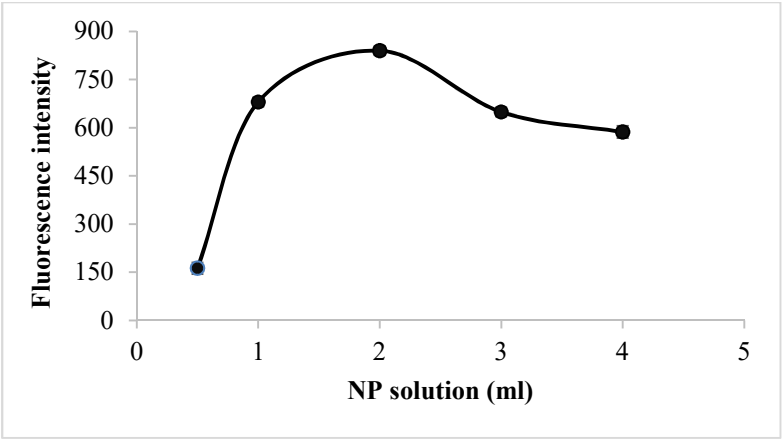


**Fig. (3).** The effect of NP amount on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

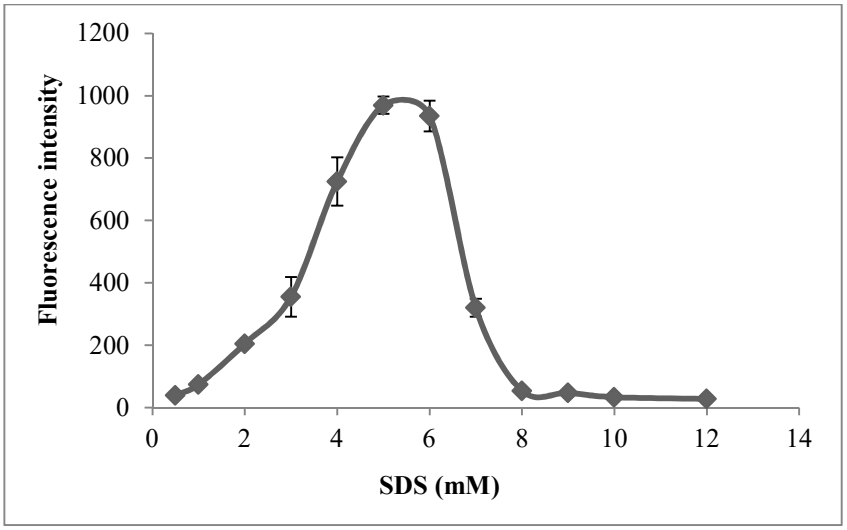


**Fig. (4).** The effect of CTAB amount on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

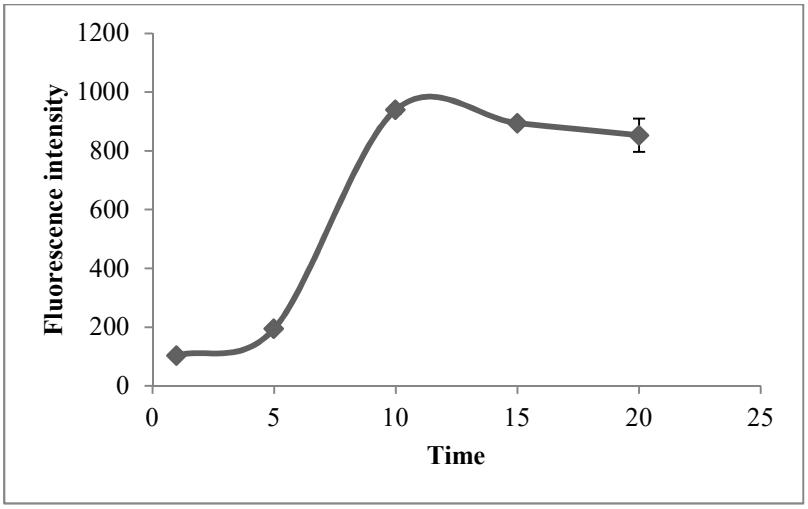


**Fig. (5).** The effect of extraction time on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of ACN, other conditions have been mentioned in the text.

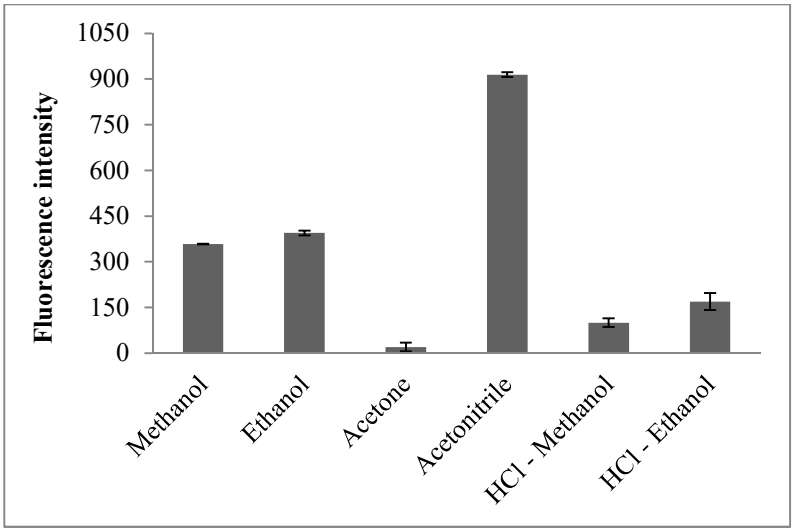


**Fig. (6).** The effect of the elution solvents on the analytical signal for 100 ng $ml^{-1}$ metoprolol. Conditions: NP (0.2), SDS (5 mM), desorption with 2 mL of each solvent, other conditions have been mentioned in the text.

**Table 1.** The effect of reusability on the analytical efficiency of 50 & 100 ng $ml^{-1}$ of metoprolol.

| Reusability | Added Concentration (ng $ml^{-1}$) | Found ± SD (n = 3) (ng $ml^{-1}$) | Recovery (%) | Added Concentration (ng $ml^{-1}$) | Found ± SD (n = 3) (ng $ml^{-1}$) | Recovery (%) |
|---|---|---|---|---|---|---|
| New sorbent | 100 | 99.5 ± 0.54 | 99.5 | 50 | 50.3 ± 1.05 | 101.0 |
| 1st | 100 | 94.5 ± 1.02 | 94.5 | 50 | 48.9 ± 1.89 | 97.8 |
| 2nd | 100 | 89.1 ± 2.03 | 89.1 | 50 | 47.0 ± 2.07 | 94.0 |
| 3rd | 100 | 84.9 ± 1.07 | 84.9 | 50 | 45.3 ± 1.20 | 90.6 |
| 4th | 100 | 81.3 ± 2.50 | 81.3 | 50 | 42.6 ± 2.10 | 85.2 |
| 5th | 100 | 75.1 ± 1.02 | 75.1 | 50 | 40.6 ± 1.44 | 81.2 |
| 6th | 100 | 51.1 ± 2.15 | 51.1 | 50 | 31.3 ± 1.77 | 62.6 |
| 7th | 100 | 33.2 ± 1.77 | 33.2 | 50 | 15.3 ± 2.02 | 30.6 |
| 8th | 100 | 21.5 ± 1.45 | 21.5 | 50 | 9.6 ± 0.83 | 19.2 |
| 9th | 100 | 9.9 ± 2.23 | 9.9 | 50 | 2.50 ± 1.44 | 5.0 |

**Table 2.** The effect of stability of synthesized MNPs on the analytical efficiency of 60 ng $ml^{-1}$ of metoprolol.

| Month | Added Concentration (ng $ml^{-1}$) | Found ± SD (n = 3) (ng $ml^{-1}$) | Recovery (%) |
|---|---|---|---|
| New sorbent | 100 | 99.4 ± 1.46 | 99.4 |
| 1st | 100 | 98.9 ± 1.20 | 98.9 |
| 2nd | 100 | 98.1 ± 1.37 | 98.1 |
| 3rd | 100 | 99.7 ± 1.45 | 99.7 |
| 4th | 100 | 99.7 ±1.32 | 99.7 |
| 5th | 100 | 100.6 ± 0.89 | 101.4 |
| 6th | 100 | 97.55 ± 1.36 | 100.6 |
| 7th | 100 | 98.1 ± 1.98 | 98.1 |
| 8th | 100 | 96.5 ± 1.56 | 96.5 |
| 9th | 100 | 95.4 ± 1.13 | 95.4 |

**Table 3:** Analytical characteristics of the proposed method

| Sample | Concentration range (ng $ml^{-1}$) | Regression equation (n=3) | $R^2$ | LOD (ng $ml^{-1}$) | LOQ (ng $ml^{-1}$) |
|---|---|---|---|---|---|
| Water | 5 - 100 | 9.1381 c + 33.551 | 0.9998 | 2.11 | 6.33 |
| Urine | 5 - 100 | 7.8453 c + 15.978 | 0.9997 | 2.39 | 7.17 |
| Plasma | 6 - 100 | 7.4377 c +2.2817 | 0.9995 | 3.41 | 10.23 |
| EBC | 5 - 100 | 8.7027 c + 9.046 | 0.9998 | 2.29 | 6.87 |

C = metoprolol concentration (in ng $ml^{-1}$)

**Table 4:** Intra– and inter–day precisions and accuracies for determination of metoprolol

| Sample | Nominal C* (ng $ml^{-1}$) | Precision (RSD%) (n = 6) | | Accuracy (R.E%) | |
|---|---|---|---|---|---|
| | | Intra–day | Inter–day | Intra–day | Inter–day |
| Urine | | | | | |
| | 8.0 | 4.93 | 4.94 | -4.00 | +3.62 |
| | 40.0 | 2.49 | 2.02 | -3.27 | -5.00 |
| | 80.0 | 1.68 | 1.62 | -2.25 | +3.87 |
| Plasma | | | | | |
| | 8.0 | 3.80 | 4.81 | +2.94 | +4.01 |
| | 40.0 | 3.04 | 1.49 | -4.35 | -3.85 |
| | 80.0 | 3.32 | 2.62 | -4.23 | -0.89 |
| EBC | | | | | |
| | 8.0 | 4.55 | 5.00 | -3.88 | -2.49 |
| | 40.0 | 2.17 | 2.08 | +2.26 | -4.06 |
| | 80.0 | 2.31 | 2.65 | -3.05 | -2.48 |

*C = concentration**,** R.E = relative error

**Table 5:** Analytical characteristics of different methods used for extraction and determination of metoprolol

| Ex./determination Method | Sample | Concentration range (ng $ml^{-1}$) | $R^2$ | RSD% | LOD (ng $ml^{-1}$) | Mean recovery (%) | Ref. |
|---|---|---|---|---|---|---|---|
| GC–MS | P | 15-500 | 0.9960 | 1.0-3.4 | 5.0 | 88.6-95.1 | 2 |
| Ultrafiltration/CE | P | 2-500 | 0.9800 | 2.3-4.1 | 0.8 | 104.3-105.0 | 4 |
| HPLC | P & U | 5-600 | 0.9982-0.9986 | 2.0-4.6 | 2.5 | 86.9-99.5 | 8 |
| SPE/HPLC & HPLC–MS/MS | P & U | 25-2000 & 10-1000 | 0.9940< & 0.9920< | ≤5.4 & <10 | 25.0 & 10.0 (LOQ) | 68.0 & 72.6–102 | 9 |
| Tandem | P & PF | 3-2000 | 0.9900< | 5.7 | 1.0 | 91.0-103.0 | 10 |
| DLLME/HPLC HPLC | U | 0.2-8.0 | 0.9930≤ | <2.0 | 0.2 (LOQ) | 94.0< | 11 |
| LLE/HPLC | P & U | 3 – 200 and 5 – 300 | 0.9980 & 0.9960 | <2.0 & <2.2 | 1.0 & 1.5 | 95.2-97.3 | 13 |
| US-dispersive μSPE/HPLC | P & U | 5-5000 | 0.9900 | 4.6 | 1.5 | 90.0 | 14 |
| LLE/UPLC–MS/MS | P | 1-500 | 0.9900< | 1.9-7.0 | 1.0 (LOQ) | 89.8-111.1 | 15 |
| μSPE/CE | U | 5-1000 | 0.9910 | 6.4-8.1 | 0.75 | 103.0-106.0 | 19 |
| F | P | 0-500 | 0.9820 | <3.0 | 1.5 | 86.0-96.0 | 24 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| GC & GC-MS | - | 1.25-100 | 0.9996 | <4.5 | 1.5 (ng) (IDL) | 78.0-103.0 | 53 |
| DLLME/HPLC | P | 20-1000 | 0.9970 | 5.3-15.0 | 2.0 | 33.0 | 54 |
| MSPE/F | P & U & EBC | 6-100 & 5-100 | 0.9995-0.9998 | 1.6-5.6 | 2.1-3.4 | 86.4-113.4 | This work |

LLE = liquid-liquid extraction; SPE = solid phase extraction; DLLME = dispersive LL microextraction; US-dispersive μSPE = Ultrasound dispersive micro SPE; CE = capillary electrophoresis; UPLC = ultra-performance liquid chromatography; F = spectrofluorimetry; P= plasma; U = urine; EBC = exhaled breath condensate; IDL= instrumental detection limits

**Table 6:** Recoveries of metoprolol from spiked plasma samples

| Sample | Added ($ng\ ml^{-1}$) | Found ± SD (n = 3) ($ng\ ml^{-1}$) | Recovery (%) |
|---|---|---|---|
| Urine | | | |
| | 7 | 6.05 ± 0.3 | 86.4 |
| | 30 | 26.7 ± 0.9 | 89.0 |
| | 90 | 83.8 ± 1.5 | 93.1 |
| Plasma | | | |
| | 7 | 7.94 ± 0.4 | 113.4 |
| | 30 | 33.2 ± 1.1 | 110.7 |
| | 90 | 78.1 ± 1.7 | 86.8 |
| EBC | | | |
| | 7 | 6.32 ± 0.3 | 90.3 |
| | 30 | 27.0 ± 0.9 | 90.0 |
| | 90 | 96.4 ± 0.3 | 107.1 |

**Table 7.** Stability of metoprolol in different biological samples on different conditions.

| Sample | *Short-term stability | R% | *Long-term stability | R% | *Freeze–thaw stability | R% |
|---|---|---|---|---|---|---|
| Urine | | | | | | |
| | 53.2 ± 1.83 | 88.7 | 53.2 ± 1.82 | 88.7 | 53.1 ± 1.95 | 88.5 |
| Plasma | | | | | | |
| | 49.7 ± 2.00 | 82.8 | 49.5 ± 2.01 | 82.5 | 49.7 ± 2.23 | 82.8 |
| EBC | | | | | | |
| | 56.5 ± 2.28 | 94.2 | 55.8 ± 2.22 | 93.0 | 55.5 ± 1.89 | 92.5 |

*60 ng $ml^{-1}$ of metoprolol spiked to each sample and results are stated as Found ± SD (n = 3).

**Table 8.** The results of trial determinations of metoprolol in collected urine samples.

| Time (h) | 0 - 2 | 2 - 4 | 4 - 6 | 6 - 8 | 8 - 12 |
|---|---|---|---|---|---|
| Volume of urine (mL) | 97 | 82 | 71 | 56 | 79 |
| Excreted drug (mg) | 1.00 | 0.59 | 0.277 | 0.16 | 0.073 |