Sensors & Actuators: B. Chemical 273 (2018) 1579–1586

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

High sensitive and selective nano-molecularly imprinted polymer based electrochemical sensor for midazolam drug detection in pharmaceutical formulation and human urine samples

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ARTICLE INFO

Keywords:
Molecularly imprinted polymer
Electrochemical
Sensor
Nanoparticles
Midazolam

ABSTRACT

In this research, molecularly imprinted polymer (MIP) nanoparticles were used as recognition elements for development of a new electrochemical sensor for selective and sensitive determination of benzodiazepine drug, midazolam (MDZ). The MIP nanoparticles synthesized via precipitation polymerization method and then incorporated in composition of carbon paste electrode (CPE) as sensing element. In order to achieve a sensor with maximum performance, the effective parameters on the extraction of MDZ from sample solution and also, electrochemical analysis step were optimized. Under optimal conditions, the fabricated sensor showed good linearity in the ranges of 5.0×10^{-10} to 1.0×10^{-7} M ($R^2 = 0.9920$) and 1.0×10^{-7} to 1.0×10^{-6} M ($R^2 = 0.9939$). Also, the high sensitivity (3.05×10^{8} μA L mol^{-1}) along with low limits of detection (1.77×10^{-10} M) and quantification (5.89×10^{-10} M) was obtained for MDZ determination. This sensor was successfully used to analysis of midazolam drug in pharmaceutical formulation and Human urine samples. The results indicated that the Nano-MIP modified CPE is promising sensor for selective detection of MDZ at trace levels in real samples with complicated matrices.

1. Introduction

Benzodiazepines (BDZ) are the most important group of anxiolytic drugs and although their effect is primarily anxiolytic-sedative, they are also active as hypnotic and anti-convulsive agents [1].

Midazolam (MDZ) is an imidazobenzodiazepine with unique properties comparing to other benzodiazepines. Compared to other benzodiazepines drugs, MDZ has a fast onset of action and relatively high metabolic clearance. Also, MDZ is water soluble in its acidic formulation but, it is highly lipid soluble in vivo. When MDZ administered orally, intramuscularly, or intravenously, produces reliable hypnosis, antianxiety, and amnesia effects. There are many uses for MDZ in the perioperative health care including anesthesia induction, premedication and maintenance, and sedation for diagnostic and therapeutic procedures. MDZ is preferred to diazepam drug in many clinical conditions due to its rapid, non-painful induction and lack of venous irritation [2].

The widespread use of benzodiazepine drugs in recent years makes it necessary the analysis of these drugs not only in pharmaceutical formulation but also in biological fluids, to assure the therapeutic eff ;ectiveness, to identify toxic levels and so prescribing adequate dosage, to minimize the adverse eff ;ects and eventually to gain a better understanding of its action in biological matrices. Thus, there is a growing demand for detection of BDZs at very low concentrations [1,3].

A lot of researches have been done for determination of MDZ and/or its metabolites in pharmaceutical preparation and biological fluids such as: spectrophotometry [4,5] diff ;erent chromatographic techniques (LC, LC/MS, HPLC, HPLC/MS, GC and GC/ MS) [6–12], and various electrochemical techniques (such as polarography, voltammetry and potentiometry) [1,13–19].

The chromatography techniques are accurate, precise and robust, but, due to expensive and sophisticated instrumentation, time-consuming and complicated manipulation steps, require to qualified personnel in some cases and running costs [18], there is a lot of attention to development of a simple, fast, cost effective and user-friendly analytical method to MDZ determination for therapeutic application.

Among the above-mentioned analytical methods, electrochemical techniques are more attractive because of their simplicity, fast response

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https://doi.org/10.1016/j.snb.2018.07.069
Received 9 February 2018; Received in revised form 1 June 2018; Accepted 12 July 2018

time, high sensitivity, relatively low cost and excellent potential for fabrication of miniaturized analytical instrument for portable applications [20].

Voltammetric technique is a high sensitive electrochemical technique which can also provide considerable selectivity to target species by applying an appropriate potential range. However, this way can not a good guarantee for selectivity of the method to the target analyte especially in complex sample matrices with the potential interfering agents in determination of the analyte [21].

The modification of the working electrode with selective adsorbate materials to selectively uptake the target species on the electrode surface is a very good suggestion to further improvement the electrochemical techniques selectivity.

Among the working electrodes that used for fabrication of selective and sensitive chemically modified electrodes(CMEs), carbon-based electrodes especially carbon paste electrodes (CPEs) have received increasing attention due to its relative ease of electrode preparation and modification, low cost, renewable surface, high stability, good electrical conductivity, wide operation potential window, chemical inertness and biocompatibility [3,22].

In recent years, molecularly imprinted polymers (MIPs) are one of the most widely used materials as recognition elements or modifier in construction of modified CPEs for determination of different species especially pharmaceutical compounds [23].

MIPs offer many advantages, such as: ease of preparation, long-term stability, facile integration into transducers, low cost, reusability potential, resistance to microbial damage, and finally, high selectivity comparable with biological receptors for the target substrate [24].

MIPs are synthesized through the polymerizing a mixture of target molecules (template), functional monomers, and an excess amounts of cross-linkers. After the polymerization step and then, the removal of the template from the polymer network, the recognition sites are formed in MIP that are complementary to the analyte molecules in terms of size, shape and orientation of functional groups [25].

In our previous researches [3,20], we developed two electrochemical sensors for determination of the diazepam and chlordiazepoxide (two medication of the benzodiazepine drugs) based on CPE modified by MWCNTs-MIP nanocomposite and MIP nanoparticles which are prepared by precipitation and suspension polymerization methods, respectively.

To the best of our knowledge, there are no previous reports for preparation of imprinted polymer and so electrochemical sensors based on MIP for the midazolam drug. Therefore, in this research, we have attempted to prepare a new Nano-MIP based electrochemical sensor for simple and reliable determination of another benzodiazepine drug, midazolam.

2. Experimental

2.1. Instruments and reagents

Reagents and instruments information can be found in section A1 in Electronic Supplementary Material (ESM).

2.2. Procedures

2.2.1. Preparation of MIP

MIP particles were synthesized by precipitation polymerization method as follow:

The 0.4 mmol of MDZ (template) was dissolved in 50 mL chloroform in a 100 mL glass flask and 2.0 mmol MAA (monomer) was added to the above solution. The mixture was stirred for 60 min to form a pre-complex of monomer-template molecules. Then, 8.0 mmol of EGDMA (cross-linker) and 0.05 g AIBN (initiator) were added into the above solution. In order to removal of dissolved oxygen, the mixture was purged with N_2 for 10 min and then stirred at 60 °C for 24 h in an oil bath. The resulting MIP particles were washed with chloroform to remove the residual reagents. The template molecules were extracted by several times washing of MIP particles with methanol: acetic acid (8: 2, v/v) solution. The particles were further rinsed with methanol to remove the remaining acetic acid and finally dried overnight under vacuum at 60 °C. The non-imprinted polymers (NIPs) were also synthesized by following the same procedure but in the absence of MDZ molecules.

2.2.2. Preparation of the modified electrodes

The instructions for constructing the bare and modified CPEs [22,27], are detailed in the ESM (section A2).

But briefly, to fabrication of modified CPEs, graphite and MIP (NIP) powders were mixed well until a homogeneous blend was obtained. The above composite was added to melted n-eicosane (binder) and mixed well. The resulting paste was compressed into a glass holder. The electrical connection was made simultaneously with the compression of the paste. The prepared electrodes were polished and finally, rinsed with distilled water. The bare CPE, was fabricated in a similar manner but in the absence of a MIP(NIP).

2.2.3. General analytical procedure

The modified CPE was placed into the MDZ solutions with the fixed pH (stabilized by B-R buffer solution) under constant stirring rate for a certain time period (extraction step). The electrode was then inserted into the washing solution to remove any possible physical adsorbates (washing step) and finally, the electrode was inserted into an electrochemical cell containing 10 ml B-R buffer solution with determined pH and then the reduction signal of MDZ was recorded by square wave voltammetry (SWV) technique (analysis step).

2.2.4. Midazolam determination in real samples

The standard addition method based on triplicate analysis for each concentration is used for determination of MDZ in pharmaceutical formulation and Human urine samples by proposed sensor.

2.2.4.1. Midazolam injection ampoules. For this purpose, the ten MDZ injection ampoules (containing 5 mg / mL MDZ in each of ampoule) are mixed to form a homogeneous solution. Then, by considering the amount of MDZ in each ampoule, an appropriate volume of above solution was transferred into a 25 mL volumetric flask and diluted to mark with distilled water for preparing a stock solution of 1.0×10^{-6} M MDZ (solution A). Then, by using the solution A and MDZ standard solutions, four sample solutions were prepared with B-R buffer at pH 6.5 and were subjected to the analysis using the proposed methods.

2.2.4.2. Human urine samples. In order to midazolam assay in Human urine samples, in four centrifuge vials, 1.0 ml of MDZ free-Human urine samples that collected from healthy volunteers (Informed consents were obtained prior to the urine sampling), were spiked with 1.0 mL of MDZ standard solution with appropriate concentrations. The vials were shacked for 5 min and then centrifuged at 14,000 rpm for 5 min. Then, for each sample, the 0.5 mL of the upper clear solution was transferred to a 25 mL volumetric flask and diluted to mark with B-R buff ;er solution (pH = 6.5). Then, the prepared samples were analyzed, according to the proposed procedure.

3. Results and discussion

3.1. Surface characterization

The surface morphology of the MIP particles (MIP_3) were studied with the help of FE-SEM image that shown in Fig. 1. As can be seen, the MIP particles prepared by the precipitation polymerization procedure have a nearly spherical structure and, their dimensions are mainly below 60 nm.

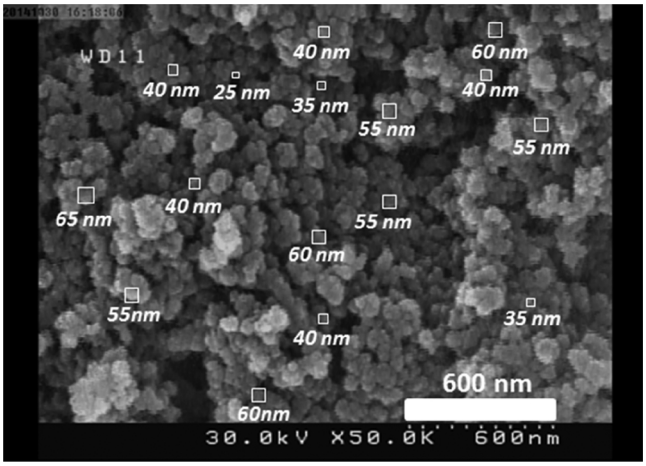

Fig. 1. Scanning electron microscopy image of MIP nanoparticles.

3.2. Electrochemical behaviour of midazolam on the bare and modified CPEs

Generally, cyclic voltammetry (CV) is the first electrochemical test to study the voltammetric behavior of electro-active compounds on the surface of studied electrode. The reviewing of previous studies on MDZ electrochemistry [1,13–16] showed that this compound has an irreversible cathodic peak related to $2e^-$, $2H^+$ reduction of 4,5- azomethine group.

Therefore, at first, the electrochemical behavior of 1.0×10^{-4} M MDZ solution (pH = 3) on the CPE surface during a cathodic sweep in the potential range of 0–1.0 V vs Ag/AgCl with the scanning rate of 100 mV/s was investigated. The CV voltammograms of MDZ and blank (B-R buffer with pH 3.0) solutions are shown in Fig. 2A. As can be seen, similar to previous studies, an irreversible cathodic peak related to reduction of 4, 5- azomethine group (C═N) was observed at the potential of −0.61 V. Therefore, this peak was selected as analytical signal for MDZ determination.

The selection of a suitable electrochemical technique is an important factor to achieve high sensitivity and appropriate limit of detection (LOD) in quantitative electrochemical analysis.

Therefore, in this study, square wave voltammetry (SWV) and differential pulse voltammetry (DPV) techniques were examined for electroanalysis of MDZ. The experiments showed that the magnitude of current signal related to the reduction process of MDZ, obtained via the SWV technique, was greater than that, recorded using DPV, at the same conditions (Fig. 2B). Thus, the SWV technique was selected for MDZ determination in other measurements.

In order to assess the MDZ recognition ability of MIP nanoparticles, the bare CPE and CPEs modified with MIP nanoparticles (Nano–MIP–CP) and NIP nanoparticles (Nano–NIP–CP) were immersed in 5.0×10^{-6} M MDZ solutions (pH = 4.0) for 10 min under stirring at 200 rpm. Following the incubation step, the electrodes were inserted into a washing solution (B-R buffer with pH = 4.0) for 5 s, to eliminate any non specifically adsorbate and then transferred into an electrochemical cell containing 10 mL B-R buffer (pH = 4.0) and the SWV was carried out. The obtained results (Fig. 2C) showed that, the current signal of the Nano–MIP based sensor is higher than those of the Nano-NIP–CP and bare CP electrodes. This performance is due to higher affinity of the imprinted cavities in MIP nanoparticles to MDZ that strongly bonded to this molecule through the hydrogen bonding and not easily removed during washing process. Thus, the MIP nanoparticles were used as selective receptors for fabrication of the proposed electrochemical sensor.

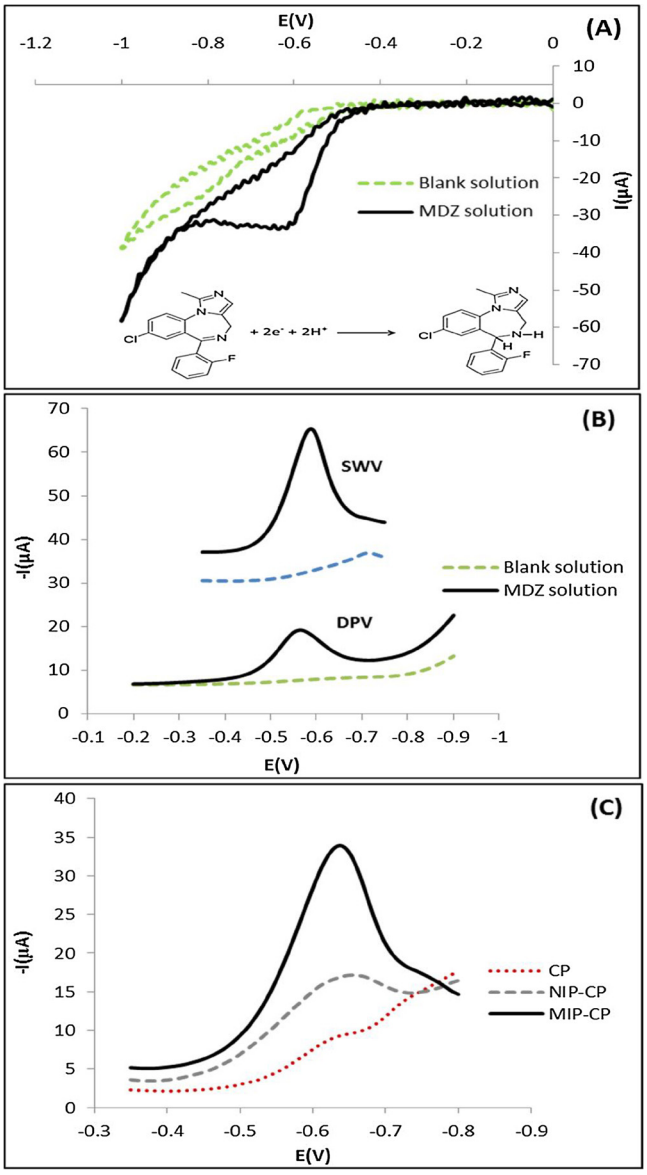

Fig. 2. (A) Cyclic voltammetry behaviour (scan rate: 100 mV s^{-1}) of 1.0×10^{-4} M MDZ solution (pH = 3) on the CPE. (B) Comparison of differential pulse voltammetry(DPV) and square wave voltammetry(SWV) signals of 1.0×10^{-4} M MDZ solution (pH = 3) at the same conditions (C) The SWV signals of bare CP and modified CP electrodes after extraction from 5.0×10^{-6} M MDZ solutions (pH = 4.0). (The SW conditions: ΔE = 20 mV, V_{step} = 6 mV, t_{step} = 0.3 s, Frequency = 30 HZ).

3.3. Optimization of the parameters affecting the sensor response

After assuring the initial response of the proposed MIP based sensor to midazolam, parameters affecting the performance of the proposed sensor such as the ratio between polymerization components in synthesis procedure of MIP nanoparticles as well as important variables involved in extraction, washing and analysis steps, were studied. The extraction solution used for the optimization process is 5.0×10^{-7} M MDZ, and the experiments were repeated three times for each parameter.

3.3.1. Different molar ratios between of template–monomer–cross linker, in the imprinting procedure

In order to avoid the interposition of imprinted cavities with each other or their low density in the polymer network, as well as preventing agglomeration of the polymer particles which leads to access restrictions of template molecules to the recognition sites, determination of the appropriate molar ratio between MIP components including the target molecule, functional monomers, and cross linkers, plays an essential role. In order to achieve a polymer with good molecular identification properties, several MIP were synthesized according to the method mentioned in section 2.2.1, in which the ratio of MDZ: MAA: EGDMA were changed.

The synthesized polymers were used for modification of CPEs and response of these sensors (after extraction and washing steps) was recorded using SWV method. The obtained results(ESM, Fig. A1-I) showed that the best signal was obtained when the molar ratio between MDZ: MAA: EGDMA is 1:8:24(MIP3). Therefore, this ratio was chosen as optimum.

3.3.2. The effect of washing time on the electrode response

In order to find sufficient washing time to remove the weakly and nonspecifically adsorbed MDZ molecules from the electrode surface and improve the repeatability of the sensor response, the duration of washing step after 10 min extraction from 5.0×10^{-7} M MDZ solution (pH = 4), was investigated. As shown in Fig. A1-II (ESM), for MIP based sensor, with increasing washing time up to about 4 s, the sensor response is decreased and afterwards it reaches almost to a constant amount. However, due to weak and non-specific MDZ adsorption on the NIP modified CPE; the sensor response is significantly decreased with increasing of washing time. Accordingly, 6 s was selected as the time taken for the washing step.

*3.3.3. **Effect of the Nano–MIP–CP electrode composition***

The composition of MIP modified carbon paste electrodes is significantly affecting the performance of MIP based CPE sensors. According to the obtained results (section A3 in ESM), the sensor with composition include: 60% graphite, 25% n-eicosane and 15% Nano-MIP has the best responses and selected for other studies.

3.3.4. Optimization of the of extraction and analysis pH

The effect of sample solution pH on the MIP − CPE response was studied in the pH range of 2.5–10.0 for solutions containing 5.0×10^{-7} M MDZ prepared with B-R buffer and the obtained results are shown in Fig. A2-I (ESM). As can be seen, acidic or alkaline media are not suitable for MDZ extraction. By consideration of the pK_a value of MDZ (pK_a = 6.2), and MAA (pK_a = 4.7), in the medium with pH < pKa_{MDZ}, most of the MDZ functional groups are protonated, which tends to reduce the hydrogen bonding with monomers. Also, at pHs < 4, the MDZ molecule is hydrolyzed, during which the azomethine bond in the benzodiazepine ring, is opened [5,11] and thus the resulting structure (ESM, Fig. A3) is no complementary to the recognition sites of MIPs and so, its extraction amount is reduced.

Furthermore, in pHs-higher than pKa of MAA molecules, most of the monomer units exist in anionic form that reduces the possibility of hydrogen interactions between monomers and target molecules. Therefore, the pH range of 6–7 is a suitable range in which most functional groups are present in a suitable form for hydrogen interactions. Accordingly, pH of 6.5 was selected as optimum for MDZ sample solution.

In addition to extraction pH, the pH of supporting electrolyte is an important parameter in MIP based voltammetric sensors. In order to find the best pH for voltammetric measurement of MDZ by MIP–CP sensor, the SWV analysis was carried out in different pH values ranging from 0.7 to 1.0 (prepared with HCl solution) and 2.0 to 7.0 (prepared with B-R buffer).

The diagram of I_{peak} vs pH (ESM, Fig. A2-II) show that the cathodic peak current is strongly depends on the supporting electrolyte pH and decreased with increasing of pH; but at pH < 1.0, the midazolam is unstable and rapidly hydrolyzed prior to electrochemical analysis.

Also, the diagram of peak potential vs pH (inset in Fig. A2-II) showed that the reduction peak is shifted linearly toward more negative potential upon increasing of pH which represents the participation of protons in the reduction mechanism of MDZ and higher pH values are not suitable for its reduction. As well as, the slope of the resulting line ($0.0591\ V\ pH^{-1}$), represents the participation of an equal number of electrons and protons ($2e^-/2H^+$) in the electrode reaction. These results are consistent with previous studies [1,16,17]. Accordingly, the solution with pH ≈ 1.0 (fixed by HCl 0.1 M) was selected as appropriate supporting electrolyte for MDZ electroanalysis.

3.3.5. Optimization of extraction time and stirring speed of sample solution

The effects of extraction time as well as stirring speed of sample solution as two significant factors on the sensor response were investigated. The obtained results showed that increasing of extraction time (ESM, Fig. A4-I) and stirring speed (ESM, Fig. A4-II) up to 7 min and 250 rpm, respectively, lead to considerable increase in amount of adsorbed MDZ on the sensor surface and thus its SWV signal.

Due to occupation most of the active molecular recognition sites on the MIP-CPE surface after 7 min, the longer adsorption time does not have considerable effect on the MDZ extraction. Also, at stirring speeds higher than 250 rpm, the amount of adsorbed MDZ on the Nano-MIP–CPE was decreased. This behavior is probably due to a disturbance in the MDZ adsorption equilibrium on the sensor surface, at stirring speeds above 250 rpm. According to the results, the extraction time of 7 min and the stirring speed of 250 rpm were selected as optimal.

3.3.6. Electrochemical condition optimization

Finally, in order to further increase in the efficiency of the proposed sensor, the dependence of the MDZ reduction signal on two important SWV parameters, including pulse step (V_{step}) and SW frequency (f), was investigated. As can be seen in table A.1 (ESM), the best SWV response was obtained for the pulse step of 8 mV and frequency of 80 Hz.

3.4. Analytical characteristics

The developed sensor was used for determination of MDZ at different concentrations under optimized conditions for plotting of calibration curve. The obtained voltammograms, as well as the diagram of peak current vs MDZ concentration, are showed in Fig. 3. As can be seen, the calibration curve has two linear regions in the concentration ranges of 5.0×10^{-10} to 1.0×10^{-7} M with a regression equation of Ip (μA) = 305.63 C_{MDZ} (μM) + 1.76 (R^2 = 0.992) and 1.0×10^{-7} to 1.0×10^{-6} M with regression equation of Ip (μA) = 25.74 C_{MDZ} (μM) + 29.26 M (R^2 = 0.994).

The difference in the slopes of calibration curve is due to different activity of the modified electrode surface at various concentration of the MDZ. In low MDZ concentration, the numbers of recognition sites on the sensor surface are much higher than the number of the MDZ molecules. This leads to high sensor sensitivity to the target molecules and, consequently, a large slope of the calibration curve in low concentrations of analyte.

But gradually, with increasing MDZ concentration, and the limited number of active detection sites on the electrode surface compared with the number of MDZ molecules, the sensor approaches to the saturation state and its sensitivity to MDZ is decreased [27].

According to the results, the first linear range was selected for

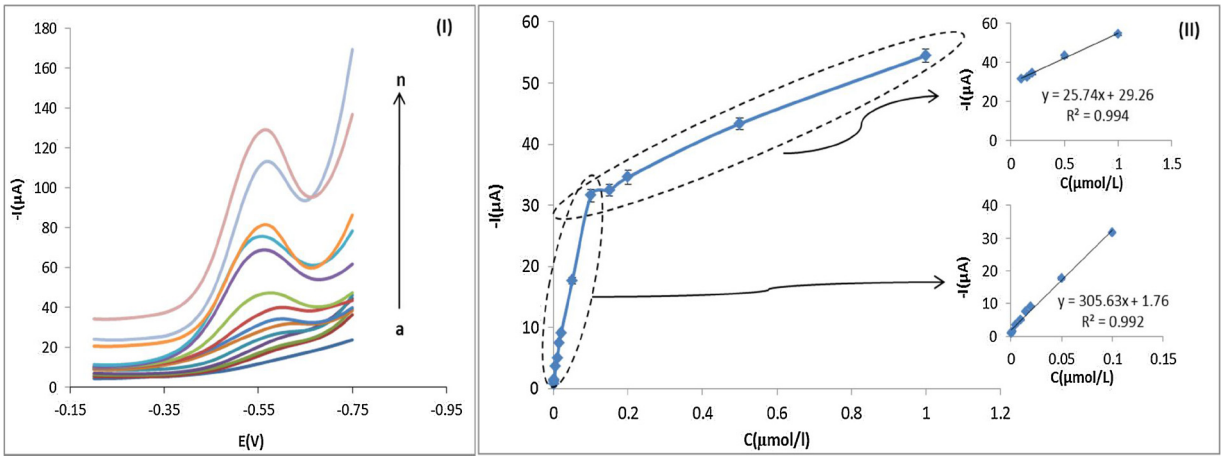

Fig. 3. The SW voltammograms related to MDZ determination at different concentration by Nano-MIP-CP electrode under optimized conditions(I) and the corresponding calibration curve of MDZ(II). The letters a–n correspond to blank solution up to 1.0 μM MDZ.

Table 1
The analytical characteristics of the Nano-MIP modified CPE for MDZ determination.

Results	Parameter
5.0×10^{-10} to 1.0×10^{-7} M & 1.0×10^{-7} to 1.0×10^{-6}	Linear ranges
2.61×10^{-10} M	Limits of detection (LOD)
8.69×10^{-10} M	Limits of quantification (LOQ)
2.93 (%RSD, n = 5)	Repeatability (intra-day)
3.84(%RSD, n = 5)	Reproducibility (intra-day)
8 weeks	Stability

electroanalytical determination of MDZ. The evaluation of other analytical characteristics related to the proposed sensor is described in the ESM (section A3) and the results are summarized in the Table 1.

3.5. The selectivity of proposed sensor

The selective response of the Nano–MIP–CPE to midazolam and its analogues from the benzodiazepines family, including oxazepam, diazepam and alprazolam was investigated.

For this purpose, firstly, the bare CPEs were introduced into 1.0×10^{-5} M (prepared with HCl 0.1 M) individual solutions of these compounds and SWV was performed. As shown in Fig. 4-I_a, all of these compounds have an irreversible reduction peak that is related to the reduction of azomethine group (C═N) in a $2e^-/2H^+$ procedure [15]. Also, the intensity of the signal for these compounds is almost same

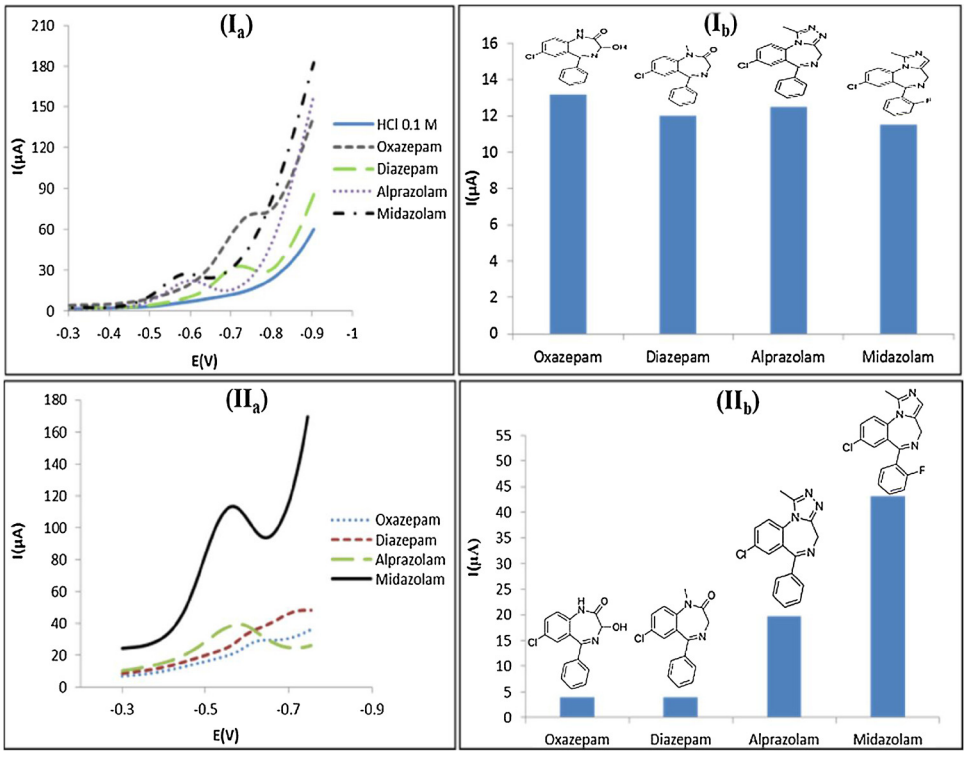

Fig. 4. (I) SWV behavior of the solutions containing 1.0×10^{-5} M oxazepam, diazepam, alprazolam and midazolam (prepared with HCl 0.1 M) on the bare CPEs along with the molecular structure and intensity of the peak current related to each molecule. (II) The SWV signal of mentioned compounds after its extraction from 5.0×10^{-7} M solutions of each of them using the proposed sensor under optimized conditions along with the amount of the peak current related to each molecule.

Table 2
Tolerance limits of some interfering molecules and ions in determination of 2.5×10^{-8} M MDZ solution using Nano–MIP–CPE sensor.

Interferents	Tolerance limit (mol ratio)
Na^+,K^+	2500
Mg^{2+},Ca^{2+}	2000
CO_3^{2-}, HCO_3^-	1500
Cl^-	2500
PO_4^{3-}, SO_4^{2-}	1000
ammonia	1500
Urea	1200
Uric acid	250
creatinine	400

(Fig. 4-I_b).

Following, the Nano–MIP modified CPEs were incubated into the 5.0×10^{-7} M solutions of each mentioned compound for 7 min and after washing the electrodes, they were introduced into HCl 0.1 M and the SW voltammograms were recorded.

As seen in Fig. 4-II_a, in the indirect measurements that conducted according to proposed method, the reduction signal related to each of the studied compounds is also observed, which indicates their adsorption on the surface of Nano–MIP–CPE. In fact, due to the structural similarity between oxazepam, diazepam and alprazolam with MDZ, they have also been able to bonding with the MIP recognition sites at the extraction step.

However, since the imprinted sites in MIP nanoparticles, are complementary to the midazolam molecules, as it is expected, the rebinding of this molecule from the sample solution is much more than the other studied compounds. After that, due to more structural similarity of alprazolam to template molecule (midazolam), the adsorption amount of this molecule on the Nano–MIP–CPE is more than the oxazepam and diazepam molecules (Fig. 4-II_b). These results indicate the high efficiency and effective performance of the molecular imprinting process in selective detection of the print molecule among the other species.

3.6. Interference studies

The interference effects of some ions and molecules on determination of MDZ were evaluated. The tolerance limit was defined as the molar ratio of additive/MDZ that causes a change in the 2.5×10^{-8} M MDZ solution signal, more than ± 5% of its initial value. The results are summarized in Table 2 and show that the existence of interfering species has no considerable effect on the performance of proposed sensor for detection of MDZ. This is due to the presence of molecular recognition sites in Nano–MIP matrix which enhances the selectivity of the Nano–MIP based sensor to MDZ molecules.

Table 3
Results of MDZ determination in real samples (n = 3).

Sample	Added (nM)	Detected (nM)	Recovery (%)	RSD %
Ampoule	–	10.09	102.4	3.91
	10	40.17	101.93	3.18
	20	70.16	101.25	2.90
	40	99.53	100.23	3.51
Urine	–	–	–	–
	5	4.65	93.07	4.13
	20	18.56	92.82	4.66
	60	56.01	93.35	3.97

Table 4
Comparison of the proposed method with UV–vis method for determining the MDZ in injection ampoule samples.

Method	UV-Vis	Developed
Labeled values (mg/mL)	5	5
Found values (mg)[a]	4.96	5.13
RSD (%)	1.25	2.11
Er1 (%)	−0.80	2.60
Er2 (%)	–	3.43
t value[b]	0.75	2.43
F value[c]	1.91	1.91
tPaired value	2.45	2.45

[a] n = 3.
[b] $t_{theoretical} = 4.30$.
[c] $F_{theoretical} = 19$.

3.7. Analysis of real samples

MDZ injection ampoule and MDZ-free Human urine samples were chosen to evaluate the potential application of the proposed MDZ sensor in real samples. These samples were analyzed according to the suggested methods described in section 2.2.4.

The resulting range (Table 3) of recovery values (92.53–100.86) represents the capability of the proposed Nano-MIP modified CPE to assay of MDZ in complex matrices and also shows that the matrices of chosen real samples are no considerable interference in MDZ determination.

Also, in order to verify the accuracy of the proposed method, determination of MDZ in ampoule samples was also done by UV–vis spectroscopy [5] and the obtained results were compared with the results of this research using statistical tests (F-test, *t*-test and paired *t*-test). The formulas used for each statistical test are presented in Table A.2 (ESM) [26].

The results of measurements and the statistical tests are presented in Table 4. As can be seen, for both methods, at 95% confidence level, $t_{calculated} < t_{theoretical}$, which indicates that both methods have no systematic errors. Moreover, the result of F-test at 95% confidence level showed that the precision of the two methods is not significantly different ($F_{calculated} < F_{theoretical}$).

Also, to test whether there is any significant difference between the results obtained by the two methods; the paired *t*-test was used. It was found that, at a confidence level of 95%, $t_{calculated} < t_{theoretical}$, which indicates that there is no significant difference between the results obtained by two methods for determination of MDZ in ampule samples.

3.8. Comparison of the developed sensor with other electrochemical sensors for MDZ determination

Finally, the sensor fabricated in this study was compared with other electrodes used to electrochemical determination of MDZ in terms of detection limits and linear range. As seen in Table 5, the detection limit of Nano-MIP modified CPE is better than the other reported electrodes, and also, the range of its linear response is more or comparable to them.

4. Conclusions

In this research, a new electrochemical sensor based on Nano-MIP modified CPEs for selective and sensitive determination of very low concentrations of midazolam drug was developed.

MIP nanoparticles functioned as both pre-concentrator and high selective recognition elements in the CPE configuration. The presented sensor has long-term stability, good repeatability, ease of preparation and regeneration, suitable response time and low cost along with simplicity and relatively inexpensive apparatus. Also, the presence of

Table 5
Comparison of some characteristics of the developed sensor in this study with other reported electrodes for MDZ determination.

Reference	Real samples	Detection limit (M)	Linear range (M)	Technique	Electrode
13	Ampoule	–	6.10×10^{-8} – 1.90×10^{-6}	DPAdSV[b]	HMDE[a]
14	–	–	9.60×10^{-10} – 8.64×10^{-9}	SWAdSV[d]	SMDE[c]
16	–	6.00×10^{-8}	1.00×10^{-7} – 1.00×10^{-6} 1.00×10^{-6} – 1.00×10^{-5} 1.00×10^{-5} – 1.00×10^{-4} 1.00×10^{-4} – 1.00×10^{-3}	DPP[e]	HMDE
1	Tablet	1.60×10^{-8} 2.00×10^{-9}	1.00×10^{-7} – 1.00×10^{-5} 1.00×10^{-8} – 8.00×10^{-8}	SWV SWCSV[f]	HMDE
17	–	–	4.60×10^{-4} – 2.76×10^{-3}	DPV	GCE[g]
15	Capsule	–	1.00×10^{-6} – 3.00×10^{-5}	AdCSV[h]	HMDE
18	Ampoule Blood serum	6.00×10^{-7}	5.00×10^{-3} – 1.00×10^{-5}	Potentiometry	CPE
19	Tablet	1.00×10^{-5} 5.00×10^{-6}	1.00×10^{-5} – 1.00×10^{-2} 5.00×10^{-6} – 1.00×10^{-2}	Potentiometry	- PVC membrane - Nano-composite modified CPE
This research	Ampoule Urine	1.77×10^{-10}	5.00×10^{-10} – 1.00×10^{-7} 1.00×10^{-7} – 1.00×10^{-6}	SWV	Nano-MIP-CPE

[a] Hanging mercury drop electrode.
[b] Differential pulse cathodic stripping voltammetry.
[c] Static mercury drop electrode.
[d] Square-wave adsorptive stripping voltammetry.
[e] Differential pulse polarography.
[f] Square-wave cathodic stripping voltammetry.
[g] Glassy carbon electrode.
[h] Adsorptive cathodic stripping voltammetry.

interfering species had no significant effect on the analytical response of the sensor.

By considering the successful application of the proposed method for midazolam determination in pharmaceutical formulations and Human urine samples, this method can be used for MDZ analyses in clinical and quality control laboratories.

Acknowledgements

This work was supported by Research Councils in the Birjand University of Medical Sciences, Baqiyatallah University of Medical Sciences and Iran University of Science and Technology.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2018.07.069.

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