Talanta 89 (2012) 448–454

Contents lists available at SciVerse ScienceDirect

Talanta

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

Use of a Sonogel-Carbon electrode modified with bentonite for the determination of diazepam and chlordiazepoxide hydrochloride in tablets and their metabolite oxazepam in urine

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ARTICLE INFO

Article history:
Received 15 September 2011
Received in revised form
18 December 2011
Accepted 22 December 2011
Available online 28 December 2011

Keywords:
Square Wave Voltammetry
Diazepam
Oxazepam
Chlordiazepoxide hydrochloride
Sonogel Carbon electrodes
Bentonite
Tablets
Human urine samples

ABSTRACT

Sonogel-Carbon electrode (SngCE) modified with bentonite (BENT) shows an interesting alternative electrode to be used in the determination of 1,4-benzodiazepines by square wave adsorptive cathodic stripping voltammetry (SWAdCSV). Diazepam (DZ) and chlordiazepoxide hydrochloride (CPZ), were determined using SngCE modified by 5% BENT. An electrochemical study of different parameters (such as pH, buffer type, ionic strength, accumulation potential, scan rate, and accumulation time) which affect the determination of DZ and CPZ is reported. Linear concentration ranges of 0.028–0.256 $\mu g\,mL^{-1}$ DZ ($r = 0.9997$) and 0.034–0.302 $\mu g\,mL^{-1}$ CPZ ($r = 0.9997$) are successfully obtained after an accumulation time of 60 s. The quantification and detection limits were calculated to be 14.0 and 4.0 $ng\,mL^{-1}$ for DZ, and 16.0 and 5.0 $ng\,mL^{-1}$ for CPZ, respectively.

The surface of the proposed electrode was characterized by scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDAX).

The developed method was applied to the analysis of commercially available tablets and human urine real samples. Analysis was performed with better precision, very low detection limits, and faster than previously reported voltammetric techniques.

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1. Introduction

Benzodiazepines are a group of drugs with important clinical applications. They are prescribed worldwide as anxiolytic-sedative, hypnotics, anticonvulsive, and sleep regulator agents. The major impact of these compounds operates widely in the brain, affecting emotional reactions, memory, thinking, muscle tone and coordination. In addition, and because of these "delicate" uses, these drugs have major risks to be subject of an excessive utilization or abuse especially by drug addicts [1]. Consequently, it is essential for a forensic laboratory to develop a rapid method for the analysis of these drugs in pharmaceutical preparations and biological fluids, and a growing demand to develop analytical methods able to sense very low concentrations has been evident.

Typical benzodiazepines compounds as Diazepam (DZ) (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one) and chlordiazepoxide hydrochloride (CPZ) (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide hydrochloride) are metabolized by liver cytochrome P450 s to *N*-desmethyldiazepam which in turn converts to the major excreted metabolite oxazepam (OXZ) (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one). OXZ is usually excreted in urine, sweating, saliva, and breast milk in the form of glucuronide conjugates [2]. These benzodiazepines are characterized by the presence of a phenyl ring fused to partially saturated seven member ring with nitrogen at positions 1 and 4 (Scheme 1). For this reason, adsorption process on electrode surface can be considered advantageous to develop polarographic or adsorptive stripping voltammetric procedures for the determination of benzodiazepines at HMDE or modified carbon paste electrodes.

Conventional analytical methods, such as spectrophotometric [3–8], spectrofluorimetric [9–11], chromatographic [12–15], enzymatic and immunoassays [16,17], and electrochemical [18–25] can be found in the literature. Electrochemical approach, thanks its simplicity, low cost and adaptability for in situ analysis, has attracted special attempt. It can be illustrated by notable use of: hanging mercury dropping electrode (HMDE) in both polarography and stripping voltammetry techniques [20–23], carbon paste electrode (CPE) in stripping voltammetry as sensing systems [18,19], ion selective electrodes in potentiometric sensing

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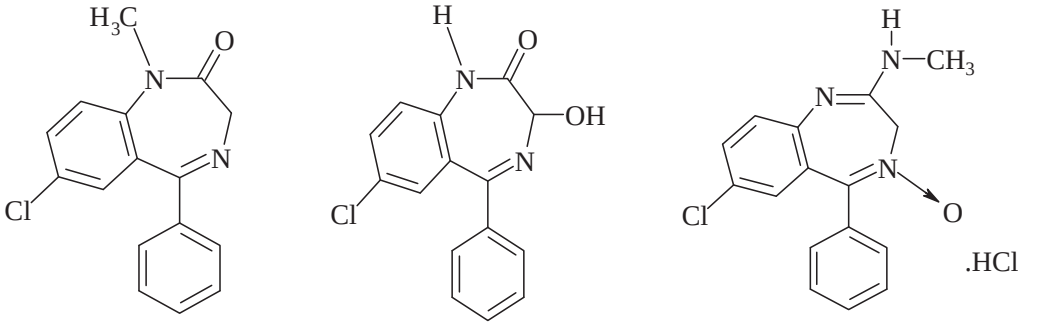
doi:10.1016/j.talanta.2011.12.061



Scheme 1. Structures for DZ, CPZ and OXZ.

mode [24,25] and/or hanging galinstan drop electrode (HGDE) [26].

Nevertheless, from ecological point of view, mercury is highly toxic, potent contaminant and its sustainable disposal is very difficult and expensive. CPE has the crucial disadvantages of ageing effects which limit seriously its life time [27]. Also, HGDE have a short life time because of the passivation of the electrode surface due to the formation of oxide layer [28]. Thus, search of alternative electrodes has been encouraged. Currently, various solid electrodes are available and their bulk or surface modification to improve their sensitivity and selectivity constitutes a principal issue for the majority of electroanalysts worldwide. Ceramic Carbon Electrodes (CCEs), based on synergic advantages of sol–gel as inert binding materials and carbon grains as high conductive, are considered as attractive route to make alternative electrodes amenable to modification, conductive, and mechanically stable [29,30]. Sonogel-Carbon electrode (SngCE), a new device of CCEs developed by our group [31–33], encloses these general characteristics and can be easily modifiable with organic–inorganic receptor and biological recognizing species to enhance its sensitivity and selectivity toward different analytes. In this work we describe possible modifications of this electrode to achieve an electrochemical sensor able to detect and determine trace concentrations of DZ, CPZ and OXZ.

Several modifiers such as C18, polyethylene glycol (PEG), and bentonite (BENT, $Al_2O_3{\cdot}4SiO_2{\cdot}H_2O$), were mixed with Sonogel-Carbon and responses of obtained electrodes toward DZ and CPZ were compared; BENT, in a percentage of 5% in the Sonogel-Carbon mixture, was selected as the best of these modifiers. Operational conditions were optimized and electrochemical behavior of DZ and CPZ was investigated. Finally, calibration, validation in pharmaceutical tablets and real urine samples and comparison of the proposed method as alternative to other found in the bibliography were explored.

2. Experimental

2.1. Instrumentation

The electrochemical measurements, square wave and cyclic voltammetry, were carried out with an AutoLab®/PGSTAT20 (Ecochemie, Utrecht, Netherlands) potentiostat/galvanostat, interfaced with a personal computer, and coupled to a MetrohmVA663 Stand.

The AutoLab software GPES (General Purpose Electrochemical System) was used for waveform generation and data acquisition and elaboration. The experiments were carried out in a single-compartment three-electrode cell, at room temperature ($25 \pm 1\,^{\circ}C$), under nitrogen atmosphere. Ag/AgCl, 3 M KCl and a platinum wire were used as reference and auxiliary electrode, respectively. All these elements were also purchased from Metrohm. Glass capillary tube, i.d. 1.15 mm, filled with Sonogel-Carbon modified material was used as the working electrode.

The synthesis of the sol–gel matrices was carried out by sonicating with a high power ultrasonic generator, SONICATOR 3000, from MISONIX (MISONIX, Inc. Farmingdale, NY, USA) equipped with a 13-mm titanium tip, which provides a maximum power of 600 W.

Scanning electron microscopy (SEM) studies were carried out on a QUANTA 200 (FEI Company, Hillsboro, OR, USA) operating at 20 keV and equipped with a Microanalyzer (EDAX) to perform energy dispersive spectroscopy (EDS).

2.2. Reagents and materials

Graphite powder spectroscopic grade RW-B was from SGL Carbon (Ringsdorff, Germany). Methyltrimethoxysilane (MTMOS) was from Merck (Darmstad, Germany) and HCl was from Panreac (Barcelona, Spain). Bentonite (BENT) and polyethyleneglycol (PEG) were from Sigma–Aldrich (St. Louis, USA). C18 was from Strata (Phenomenex, Torrance, CA).

DZ and CPZ and their metabolite OXZ were from Acofarma (Barcelona, Spain). Stock standard aqueous solutions (1×10^{-2} M) were prepared in absolute ethanol. Working standard solutions were prepared by dilution of these solutions. Both stock and working solutions were kept in refrigerator to avoid the decomposition of analyte.

Pharmaceutical formulations: Valium® tablet formulated to contain 5 mg DZ per tablet, and Omnalio tablet formulated to contain 10 mg CPZ per tablet were from ROCHE FARMA, S. A. (Madrid, Spain) and Laboratorio Estedi S. L. (Barcelona, Spain), respectively.

Other chemicals were analytical grade or higher (Merck, Fluka, Sigma or Panreac), and they were used as received without further purification. Aqueous solutions and electrolytes were freshly prepared using high pure water obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm^{-1}, Millipore, Bedford, MA).

2.3. Procedure

2.3.1. Preparation of working electrodes

To prepare the Sonogel-Carbon, 500 μL of MTMOS and 100 μL of 0.2 M HCl were mixed and then insonated during 5 s; with the high-power ultrasonic processor, the mixture is subjected to the phenomenon of ultrasonic cavitation, by which the sol–gel process begins, and we avoid the use of alcoholic solvent and reduce drastically the time needed to get an unique phase. To do the insonation in a right way, the tip of the probe must be just contacting the superior surface of the mixture (only about 1 mm of length must be introduced). After insonation, 1 g of graphite powder and the adequate amount of modifier were added and homogeneously dispersed in the sonosol obtained. The whole procedure for the fabrication of

unmodified Sonogel-Carbon electrodes has been described previously [31].

In this work three modifiers were tested: clay in the form of BENT, PEG and C18. Modified electrode containing BENT was prepared at different percentages with graphite; so electrodes containing 2.5, 5.0, 7.5, and 10% of BENT (w/w of graphite) were prepared. PEG and C18 modified electrodes were prepared adding 12.5 μL of 5:2 (w/w) H_2O:PEG solution to the sonosol solution and mixing the produced solution with graphite, and mixing 0.1 g of C18 and 0.9 g of graphite with sonosol solution, respectively. The resulting material was incorporated in a glass capillary tube (i.d. 1.15 mm) and let it to solidify for up 8 h. After this, the electrodes were smoothed against emery paper No. 1200 to remove extra composite material, wiped gently with weighing paper, and then washed with Mili-Q water.

2.3.2. Measurements and samples preparation

For electrochemical measurements the cleaned electrode was immersed in 25 mL of selected buffer also used as electrolyte support, oxygen was removed by purging with pure N_2 for 10 min, then the solution was maintained under this inert gas flow, the electrode–solution interface was equilibrated by applying a potential of −0.4 V during 5 s and cyclic or square wave polarization was applied to the electrode to record the background signal. Then, a few microliters of a solution of the analyte was injected in the cell, which is also deaerated for about 2 min, and reduction signal was recorded in the potential range from −0.4 to −1.4 V. To produce reproducible electrode surface, an electrochemical cleaning procedure, which consist of a polarization of the electrode at −1.2 V during 120 s in blank solution, was applied after each measurement.

To determine DZ and CPZ in pharmaceutical formulations (tablets), the samples were powdered in mortar and a specific weight of grounded tablets was dissolved in 100 mL of absolute ethanol. This solution was filtered, and the filtrate was ten times diluted in ethanol to constitute the stock solution; usually 25 μL of the diluted was injected in 25 mL buffer solution for measurement.

The proposed electrode was used to determine DZ and CPZ in biological fluids. Real urine samples were collected from healthy non-smoking volunteers during 24 h (2 collections at 12 and 24 h after single dose oral administration). Both DZ and CPZ were determined in real urine sample as its mainly documented metabolite OXZ. But before the electrochemical determination of OXZ in real urine sample, it is necessary to separate the active principle from the biological matrices. Extraction of OXZ was achieved in two steps: The first step is the liberation of medicines from their glucuronides [34], which consists in an incubation, in bath at 56 °C for 2–3 h, of 3 mL of urine homogenized with 0.9 mL of Britton–Robinson buffer (pH 4.6) and 0.1 mL of β-glucuronidase (143,000 U mL^{-1}). The second step is solid phase extraction (SPE) of the analyte from this enzymatic solution. Thus, the cartridge for SPE was conditioned with 0.5 mL of methanol and same quantity of Britton–Robinson (B.R.) buffer at pH 2.5. Then, 2.0 mL of the produced enzymatic solution were passed through the SPE cartridge and the metabolites were eluted twice with 0.5–1.0 mL of methanol, and extracted from the solid phase by a mixture of methanol–THF (1:1, v/v). The extract constitutes the analytical solution and 50 μL of it was directly injected in the electrochemical cell containing the buffer solution.

3. Result and discussion

3.1. Choice and optimization of modifier

As it was mentioned above, the electrochemistry of benzodiazepines may effectively exploit considerable adsorption capabilities of these compounds. Thus the choice of adequate modifier can increase notably the sensitivity of electrochemical sensor. In this article, the effect of different modifiers on determination of DZ and CPZ was studied. BENT, C18 and PEG were selected for this study. The first and the second were selected thank to their demonstrated good adsorptive properties [35–40]; whereas the last was selected to increase the concentration of hydroxyl groups in the surface of the SngCE and to study its effect on the adsorption of analyte. Voltammetric signals of unmodified SngCE, and SngCEs modified with BENT, C18, and PEG were compared in presence of various concentrations of DZ. With respect to unmodified SngCE, the responses of the three modifiers can be arranged as follows: BENT-SngCE > SngCE > C18-SngCE > PEG-SngCE. The experimental results show that BENT modification increases notably the sensitivity of SngCE toward DZ in approximately 25% with great repeatability, whereas modification of this electrode with extended carbon chain compounds such as C18 and PEG decrease drastically the produced signal and have not significant effect on its repeatability.

It seems reasonable to expect an improvement in the sensitivity of modified SngCE by increasing the concentration of silicate sites in the electrodes surface as principal adsorption sites. So, BENT with four silicate molecules in its formula, demonstrates a favorable effect. On the contrary, C18 and PEG, which can bond to silicate atoms in MTMOS avoiding a normal polycondensation of the sol–gel and decreasing the concentration of silicate in the surface of the electrode, show an inverse effect. This can be proved by a negative shift of peaks potential for the two last modifiers compared to that of the unmodified and modified BENT (E_p = −0.895, −0.910, −0.946 and −0.966 V for SngCE, BENT-SngCE, C18-SngCE and PEG-SngCE, respectively). To investigate the effect of modification on the surface of the electrode, morphological and elemental chemical analysis studies were carried out using SEM and X-ray diffraction spectroscopy. Comparing surface topographies of BENT, C18 and PEG modified SGCEs, BENT and PEG modifications show compact and homogenous surfaces whereas C18 shows a surface with granular and non homogeneous aspect. Also EDAX analysis performed on the same equipment and in several magnitudes and spots proved an increase in silicon atomic percentage in the surface of BENT modified electrode in contrast to those modified with C18 or PEG. This fact can prove the turn of sensitivity of the electrodes and concentration of silicate in its surface.

Taking into account the favorable effect of BENT, different percentages of BENT were studied to optimize its concentration. The responses of several electrodes modified with 2.5, 5.0, 7.5 and 10.0% BENT were compared in presence of DZ and CPZ in similar conditions. Between the studied concentrations of BENT, the response obtained for 5% modification was the best one. Lower percentages of BENT caused also favorable effect increasing the sensitivity. However, percentages higher than 5.0% manifested a hard sonocondensation, getting less compacted electrodes, and producing electrochemical signals with large background and low repeatability. Consequently, a 5% modification of BENT into the SngCE was adopted for all subsequent studies.

3.2. Optimum condition for electrochemical analysis

It is well reported that electro-reduction of benzodiazepine compounds depends deeply on the reduction of azomethine group in positions 4 and 5 in diazepine ring at very low potential. The electro-active behavior of these compounds may be influenced by several impediments, such as high irreversible adsorption of these molecules on the electrode surface, low accessibility due to steric effect, presence of several phenol rings, and the low polarity of these molecules. Thus, detailed studies must be carried out with the aim to optimize operational conditions.

Table 1
Optimized conditions for the determination of DZ and CPZ at BENT-SngCE using SWAdCSV method.

	Parameters	Optimum values
Instrumental	Electrochemical cleaning	−1.2 V for 120 s
	Initial potential	−0.40 V
	End potential	−1.40 V
	Frequency	100 Hz
	Scan rate	1 V s^{-1}
	Accumulation time	60 s
	Accumulation potential	−0.4 V
Chemical	pH	4.6
	Buffer type	Formic acid–NaOH
	Ionic strength	I = 0.05 M

Several parameters were investigated in detail to see their effects on the reduction signal of DZ and CPZ. The approach consisted in taking into account the sensitivity and signal reproducibility as criteria to select each condition.

3.2.1. Instrumental parameters

Waveform, accumulation potential and its duration, scan rate, frequency, and an electrochemical cleaning of the electrode surface were selected as instrumental variables able to perform electrochemical measurements and the results of optimum conditions are summarized in Table 1. To select the best wave form, the reduction of both DZ and CPZ were studied by comparing Differential Pulse Voltammetry (DPV) and Square Wave Voltammetry (SWV). The data show that signal obtained for reduction of DZ and CPZ using SWV is about 3.3 times higher than that of DPV, this is in accordance with literature which reports an increase oscillating from 3.3 to 4 times depending of electrochemical behavior of the analyte [41,42].

The possibility of an electrochemical adsorption process was also explored polarizing the electrode during 60 s at a potential range from +0.1 to −0.5 V. Comparison of obtained signals showed no obvious effect of accumulation potential on the peak potential whereas maximum currents were showed at potential of −0.4 V. Both DZ and CPZ undergo an adsorptive preconcentration at the BENT-SngCE before its reduction process. This fact was confirmed by studying the effect of accumulation time at open circuit. SWAdCV (Square Wave Adsorptive Cathodic Striping Voltammetry) signals for several concentrations of DZ and CPZ were registered after various accumulation times from 0 to 900 s. Stripping signals increased when increasing accumulation time up to 300 s. Whereas, at longer time periods, the peak current decreased with a low reproducibility, possibly due to an excess coverage of the electrode surface by the drugs. However, for five experiments repeated for 0.285 μg mL^{-1} DZ and 0.336 μg mL^{-1} CPZ at 60 s accumulation time, relative standard deviations were found to be 2.8% and 2.5% for DZ and CPZ, respectively.

Other instrumental variables were also optimized. Increases in scan rate and frequency from 0.5 to 5 V s^{-1} and 50 to 250 Hz, respectively, demonstrated a favorable effect on peak current but with a bordering of its shape at highest scan rate. Thus, scan rate of 1.0 V s^{-1} and frequency of 100 Hz were selected as optimum.

Adsorption of benzodiazepines at electrode surface is irreversible and signal of adsorbed analyte persists even after careful washing of the electrode. Liquid electrodes as mercury have not this limitation thank to the possibility of removing the drop, whereas solid electrodes can be seriously affected. Regeneration of the surface of solid electrode was required after each measurement. Several procedures were used in this work to reduce this limitation; the first one was mechanical cleaning, the second one, similar to that used in classical works in the issue, consisted in chemical cleaning in a agitated nitric solution 0.5 M for several times [36], and the third one consisted in total electrolysis of the adsorbed analyte. Applying the first two methods several changes in surface electrochemical signal were observed which indicate serious damages of the electrode, whereas applying the third method a reproducible signal was achieved. This method was carefully optimized: various potentials over reduction peak potential (i.e. from −1.0 to −1.4) were applied during 120 s in stirred solution without analyte; the best electrode regeneration was optimized at −1.2 V which induces to complete absence of residual signal. This procedure was applied after each measurement for both DZ and CPZ.

3.2.2. Chemical parameters

Chemical parameters can affect remarkably SWAdCSV signals of benzodiazepines compounds at BENT-SngCE. In this sense, the influence of the solution pH, buffer type, and ionic strength on the electrochemical responses for DZ and CPZ were studied and a summary of optimum condition is shown in Table 1.

The pH effect was studied using 0.05 M B.R. buffer at pH values ranging from 2.5 to 10.5. The obtained results of this study show an increase in DZ and CPZ responses with pH in the range up to 4.6, and a decrease from 4.6 up to 10.5. Thus, the optimum pH value for further studies was set at pH 4.6. Also, the peak potential was found to be shifted in the negative direction with increasing pH. This is in accordance with early studies in reduction behaviors of some benzodiazepines compounds at HMDE, and can be attributed to the fact that the reduction of these compounds involves a proton-coupled electron transfer at the electrode surface.

The second chemical parameter tested was the buffer type solution. In this order, the responses of DZ and CPZ were registered in four different buffers (B.R., HAc–NaAc, formic acid–NaOH, and $KHC_8H_4O_4$–NaOH) at a concentration of 0.05 M, and indicated that formic acid–NaOH buffer gives the best response. So this buffer was used in further measurements. Finally, and also taking into account minimal background current, the best curve and the highest sensitivity, the effect of ionic strength, I, of formic acid–NaOH buffer medium was studied. And it revealed that the best concentration of formic acid–NaOH buffer to detect both DZ and CPZ corresponds to I = 0.05 M.

3.3. Electrochemical behaviors of DZ and CPZ at BENT-SngCE

Since a method to detect DZ and CPZ was optimized, it will be interesting if we describe the electrochemical behavior of DZ and CPZ at BENT-SngCE. Hence, the effect of pH on the peak potential and peak current of 28.47 μg mL^{-1} DZ and 33.68 μg mL^{-1} CPZ was investigated by recording cyclic voltammogram in different values of pH ranging from 1.75 to 9.25 and varying scan rate from 0.1 to 0.75 V s^{-1}.

DZ shows only one well-defined reduction peak in the forward sweep, with potential ranging from −0.8 to −1.3 depending on pH values. In the reverse sweep, no oxidation peak is observed, which confirms the nature of total irreversibility of electrochemical reaction of DZ over the whole studied pH range. Also, it was noticed that with increasing the value of pH, the peak potential was shift toward more negative values which indicates that hydrogen ions are consumed in the electrochemical reduction of DZ. The plot of peak potential against pH (Fig. 1) shows three linear zones with two breaks at 3.25 and 6.25. The pK_a of this drug was found to be near 3.58 associated with N-4 protonation which is near the first break. The three linear sections have slopes of −0.102, −0.055 and −0.07 V pH^{-1}, respectively. The recorded peak current was dependent on pH, showing an increase in peak current up to pH value of 4.6, and decreasing at higher values of pH. This result was in accordance with that observed for SWAdCSV studies.

In the case of CPZ, over the pH range ≤4.0, three peaks were recorded in the forward sweep at −0.7, −0.9 and −1.4 V; in the

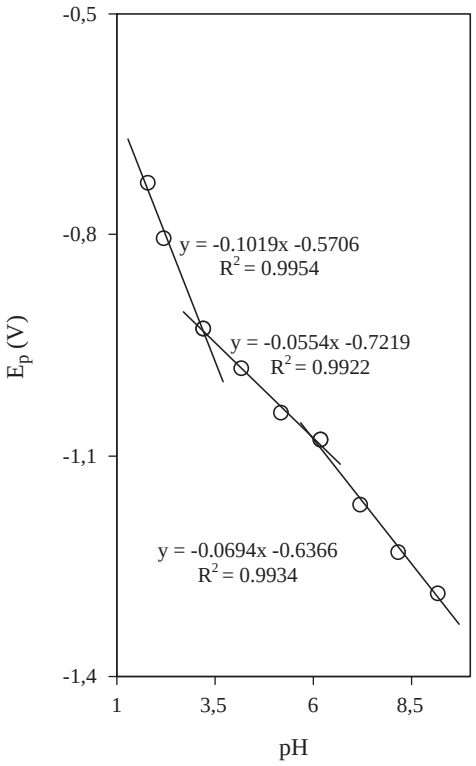


Fig. 1. Influence of pH (B.R. buffer) on peak potential values for 28.47 μg mL^{-1} DZ using cyclic voltammetry.

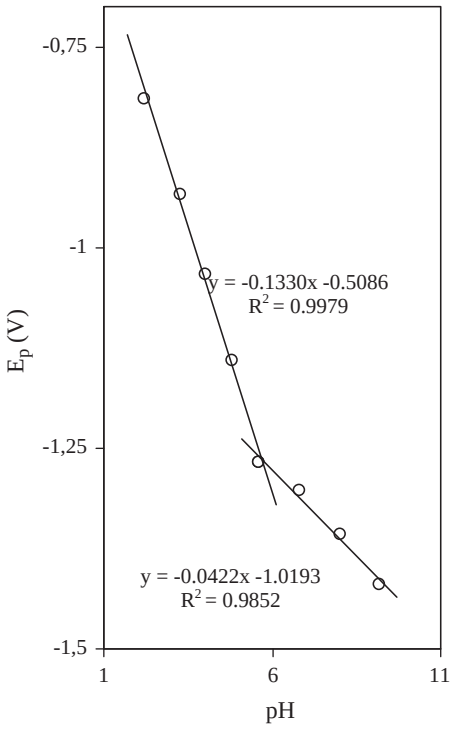


Fig. 2. Influence of pH (B.R. buffer) on peak potential values for 33.68 μg mL^{-1} CPZ using cyclic voltammetry.

reverse direction small waves signals were recorded especially at highest scan rate. This is in accordance with literature reporting three separated peaks linearly correlated to the concentration of CPZ in sulphuric acid solution [43,44]. The first peak has been attributed to reduction of N-oxide group in position 4, the second one was related to reduction of 4,5-azomethine group, and the third one to reduction of C=N in positions 1 and 2 of diazepine ring. In our case the first two peaks were well defined in spite of the low intensity of the first one, while the third peak was like a wave. At pH values in the region (4.0 > pH < 5.80) the third peak is completely disappeared, while the first peak appears as a small shoulder signal of the second one, which become more defined manifesting a resolved highest intensity. With increasing pH values (pH ≥ 5.80) only one peak appears and the oxidative wave persists with a well defined signal, so it can justify the attribution of this signal to the oxidation of the reduced azomethine group.

The second signal is the most important from an analytical point of view, so the study of its evolution can be interesting. The plot of this peak potential against pH (Fig. 2) shows two linear portions with only one break at 5.6, which is a little higher than pK_a of CPZ molecule determined by potentiometry, and found to be equal to 4.79. The slopes of the first and second portions were −0.133 and −0.042 V pH^{-1}, respectively. Also and just like DZ, there is an agreement in the results obtained from square wave and cyclic voltammetric studies of the effect of pH on this peak current.

Electrochemical behavior of all 1,4-benzodiazepines depends essentially on availability of >C=N— double bond in positions 4 and 5 of diazepine ring to be reduced at electrode surface under consumption of 2e$^-$ and 2H$^+$. If the studied molecule contains, additionally, other reducible groups, the electrode process becomes more complicated. It is the case of CPZ which contains a methylamine group in position 2. In acidic medium this compound shows three electrochemical signals, the second of them has the position near to the signal showed for DZ. So this signal can be attributed clearly to reduction of 4,5-azomethine group. Also this assignment can be confirmed by the accordance existing between the variations in the slopes of peaks potential against pH. The mechanism of electrochemical reduction of CPZ has been postulated as can be seen in Scheme 2. It was reported that the first step is very fast. This fact can explain our result, so at high scan rate we can assume that our signal is in reality the mixture of the two peaks.

3.4. Analytical performances

3.4.1. Effect of possible interferences

The selectivity of the proposed analytical method was conducted by comparing the response of BENT-SngCE to 0.285 μg mL^{-1} DZ or 0.336 μg mL^{-1} CPZ registered in optimal conditions but in presence of several substances which frequently accompany theses drugs in pharmaceutical formulas or biological fluids. It was found that up to 100-fold of ascorbic acid, glucose and sucrose had no effect on the determination of the studied drugs. These substances have no significance effect on standard signal of DZ and CPZ (without interferences) in the potential range from −0.4 to −1.4 V. Also, under the obtained experimental conditions, up to 100-fold of inorganic ions such as Cu^{2+}, Pb^{2+}, Ni^{2+} and Cd^{2+} did not interfere with the determination of DZ and CPZ. Thus, it seems demonstrated that the proposed procedure was able to sense DZ and CPZ in the presence of several possible interferences with high selectivity.

3.4.2. Calibration curves

Under optimized conditions, at 60 s accumulation time, the proposed sensor shows a linear response toward DZ and CPZ in the concentration range from 0.028 to 0.256 and from 0.034 to 0.302 μg mL^{-1}, respectively. These linear relationships can be expressed according to the following equations:

$$I\,(\mu A) = -0.010(\pm 0.002) + 1.136(\pm 0.010)[DZ]\,(\mu g\ mL^{-1}),\quad (r = 0.9997,\ n = 5)$$

$$I\,(\mu A) = 0.024(\pm 0.001) + 0.849(\pm 0.007)\,[CPZ]\,(\mu g\,mL^{-1}),\quad (r = 0.9996,\ n = 5)$$

Table 2
Results for the determination and recovery test of DZ and CPZ in dosage forms.

Sample	Found[a] (mg)	Recovery (%)	Average recovery (%)
Valium®	25.50[b]	102.0	99.92
(Labeled contain	24.50[b]	98.0	
5 mg DZ per tablet)	25.00[b]	100.0	
	25.00[b]	100.0	
	24.90[b]	99.6	
Omnalio	20.50[c]	102.5	102.00
(Labeled	20.00[c]	100.0	
contain 10 mg	21.00[c]	105.0	
CPZ per tablet)	19.50[c]	97.5	
	21.00[c]	105.0	

[a] Compared with statistically calculated value ($\mu = \bar{x} \pm (ts/\sqrt{n})$), 24.98 ± 0.44 (for DZ) and 20.40 ± 0.84 (for CPZ).
[b] Calculated for 0.8657 g of 5 grounded Valium® tablets (content 25 mg DZ).
[c] Calculated for 0.6051 g of 2 grounded Omnalio tablets (content 20 mg CPZ).

Detection and quantification limits were statistically estimated as $3\sigma/b$ and $10\sigma/b$, respectively, where b is the slope and σ is blank standard deviation [45]. The proposed method shows detection and quantification limits of 4.0 and 14.0 ng mL^{-1} (for DZ), and 5.0 and 16.0 ng mL^{-1} (for CPZ), respectively.

3.4.3. Pharmaceutical preparations

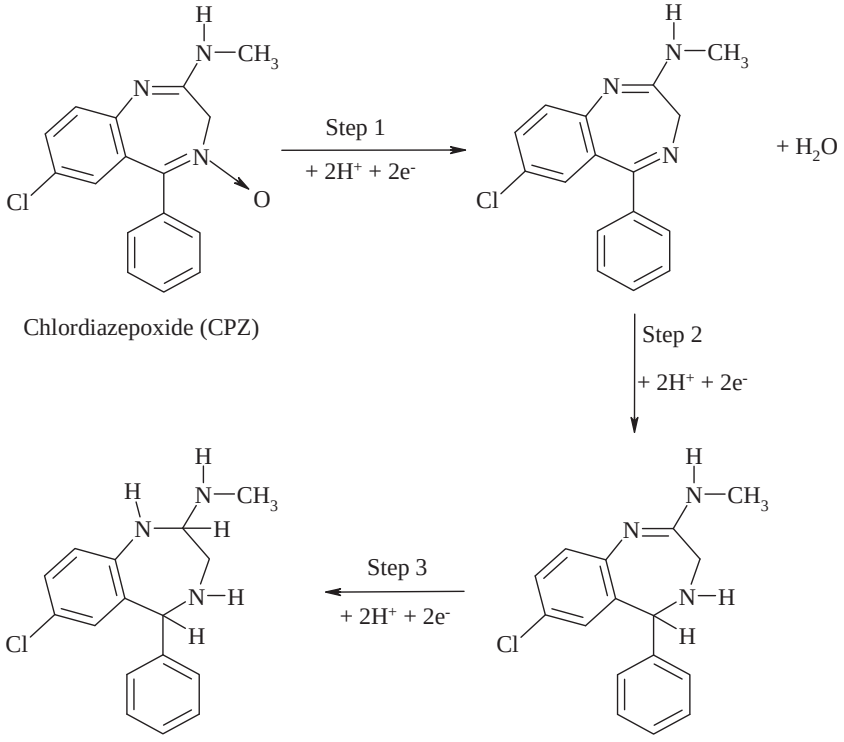
The proposed method was applied to the determination of DZ and CPZ in Valium® and Omnalio tablets, respectively. The content of these commercial samples was determined using standard addition method. In both cases, for DZ or CPZ, only one peak was observed as a result of addition of an aliquot volume of sample solution to the electrochemical cell. This peak increased linearly with successive additions of pure drug (DZ in case of Valium® and CPZ in case of Omnalio). Based on five replicates measurements, the proposed method shows a good accuracy and precision when compared with the data mentioned on the drug packaging (Table 2).

The proposed method did not require any time consuming extraction or evaporation steps prior to assay the samples, and comparing the obtained data to those obtained by reported spectrophotometric and chromatographic methods, our method can be considered as alternative to classical procedures.

On the other hand, applying the proposed method for the analysis of DZ and CPZ in dosage forms validated the accuracy of the suggested procedure. The analysis of DZ and CPZ in tablets exhibited correlation coefficients of 0.9994 and 0.9986, respectively, with relative standard deviation (RSD) of 2.70 and 2.30, respectively.

3.4.4. Real urine sample

The major impact of benzodiazepines compounds operates widely in the central nervous system, affecting emotional reactions, memory, thinking, muscle tone and coordination. In addition, and because of its "delicate" uses, these drugs have major risks to be subject of an excessive utilization or abuse especially by drug addicts [1]. Thus, it will be useful if we can determine these compounds in biological fluids in which these drugs will be excreted. After ingestion, both DZ and CPZ are metabolized into OXZ, which in turn will be excreted in sweating, saliva, and urine and breast milk in the form of glucuronide conjugates [2]. In this article, we preferred to use real human urine samples, which are more available than other biological samples and higher volumes are possible to obtain. But as OXZ is excreted in the form of glucuronide conjugates, urine samples require a previous extraction procedure to avoid any interference from urine components. Two samples were collected and investigated after a single dose oral administration of Valium® tablets. The first sample corresponds to the sample collected 12 h after, while the second sample was collected 24 h after oral administration of the medicine. In all the samples, the recovered concentration values, calculated by means of the standard addition method, belonged to the concentration range from 0.05 to 0.5 μg mL^{-1}, in concordance with data appearing in bibliography. In our case, the volunteer was a healthy donor, did not smoke and did not drink alcohol, so it was expected that the normal metabolization of pharmaceuticals would not be affected. Fig. 3 shows the voltammograms obtained with standard addition method, after



Scheme 2. Electrochemical reduction of CPZ at electrode surface.

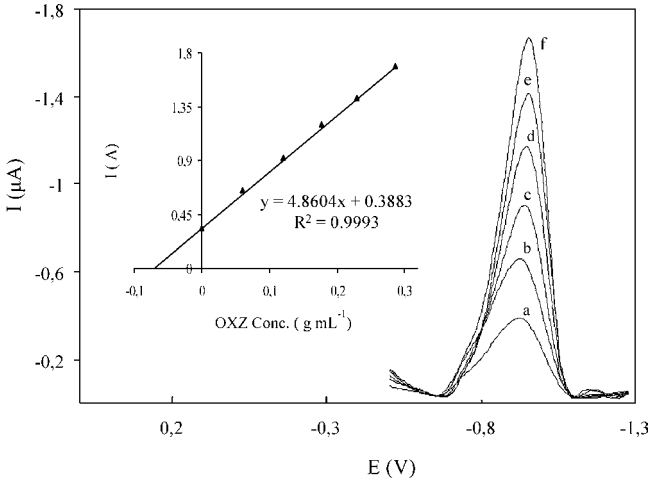


Fig. 3. (a) SWAdSV voltammogram for 60 s preconcentration time of 50 μL of urine real sample after SPE treatment, followed by successive additions of known concentrations of OXZ: (b) 0.06 μg mL^{-1}, (c) 0.11 μg mL^{-1}, (d) 0.17 μg mL^{-1}, (e) 0.23 μg mL^{-1}, and (f) 0.28 μg mL^{-1}. Inset, calibration graph for direct determination of OXZ in urine sample using standard addition method.

60 s pre-concentration time for blank solution containing an aliquot of extracted real urine sample solution (curve a) followed by successive additions of pure solutions of OXZ (curves b–f). The obtained linear relationship can be expressed as follows:

$$I(\mu A) = 0.388(\pm 0.01) + 4.860(\pm 0.058)[OXZ](\mu g\ mL^{-1}), \quad (r = 0.9993, n = 5)$$

Considering OXZ is a normal result of metabolization process of DZ and CPZ, both detection and quantification limits were calculated as 6.0 and 19.5 ng mL^{-1}, respectively.

The reproducibility of the results was evaluated for the collected samples containing 0.075 μg mL^{-1} OXZ, resulting in a relative standard deviation of 1.9%.

4. Conclusion

In this article we introduce a new, sensitive, selective, low cost and time saving method for the quantification of DZ and CPZ. In formic acid–NaOH buffer (pH 4.6), DZ and CPZ can be determined using BENT modified SNGC electrode. The proposed method is applied for the determination of DZ and CPZ in the concentration range of 0.028–0.256 and 0.034–0.302 μg mL^{-1}, respectively. Using standard addition method, DZ and CPZ can be detected at 4.0 and 5.0 ng mL^{-1}, and quantified at 14.0 and 16.0 ng mL^{-1}, respectively. Under the selected optimum conditions the electrode is successfully used for the determination of DZ and CPZ in Valium® and Omnalio tablets, respectively. As the benzodiazepines have important risks to be subject of an excessive or abusive utilization especially by drug addicts, and because there are different uses in which they can affect central nervous system, we applied successfully the developed electrode to the determination of OXZ (the major metabolite in human body of both DZ and CPZ) in human urine real samples. In comparison with spectrophotometric and chromatographic methods, and with other electrodes used in voltammetric determination of 1,4-benzodiazepines, use of Sonogel-Carbon electrode modified with 5% BENT can be considered as a sensitive, selective, low cost and time saving procedure.

Acknowledgments

We thank the Cultural Counselor of the Embassy of the A.R. of Egypt and the members of the Egyptian Institute of Islamic Studies in Spain for a grant to Ahmed Hosny Naggar. We would like to express our gratitude to the Junta de Andalucía/FEDER (FQM249/P08-FQM-04006) and Ministerio de Ciencia e Innovación of Spain/FEDER (CTQ2007-67563/BQU) for their financial support.

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