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Modified carbon-paste electrodes as sensors for the determination of 1,4-benzodiazepines: Application to the determination of diazepam and oxazepam in biological fluids

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Abstract

The determination of benzodiazepines in biological fluids is object of great interest for many scientists. In the daily medical practice, it is necessary to perform drug analytical monitorization in order to adequate the dose of these substances to the necessities of the patients, and to avoid toxic effects. In forensic toxicology, benzodiazepines are often found in fatal cases of drug intoxication, as well as in the blood of drivers involved in traffic accidents. These reasons justify the necessity of developing new analytical methods for their determination. The main objective of this paper consists of the development of an electrochemical procedure to analyse benzodiazepines and/or their metabolites in a sensible, versatile and economic way, in biological fluids, such as plasma and urine. A whole electrochemical study of diazepam, temazepam and oxazepam using modified carbon-paste electrodes is reported. Using the optimal conditions for the voltammetric measurements of these pharmaceuticals, diazepam was determined in plasma and oxazepam in urine. Due to the complexity of these biological matrices, a previous solid-phase extraction (SPE) procedure was necessary to separate the active principles before their determination. The best results were obtained for the determination of oxazepam in urine.

Keywords: 1,4-Benzodiazepines; Diazepam; Oxazepam; Temazepam; Voltammetry; Modified carbon-paste electrodes

1. Introduction

Benzodiazepines are pharmaceuticals with important clinical applications, thanks their sedative, anticonvulsant and hypnotic properties [1,2]. As a consequence of this, they are prescribed worldwide for the therapy of anxiety, sleep disorders and convulsive attacks. Amongst the 1,4-benzodiazepines, oxazepam (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiapin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiapin-2-one) and temazepam (7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiapin-2-one) may be underlined. Diazepam is metabolised in the organism to temazepam, amongst other compounds, and this one, in its turn, is metabolised to oxazepam (see Fig. 1). As all hydroxylated metabolites coming from diazepam, oxazepam is excreted in bile and urine in the form of glucuronide conjugates. These glucuronides are, in part, reabsorbed by intestinal via, establishing enterohepatic circulation, which is one of the factors that affects more the duration of the effect of these pharmaceuticals. In short, oxazepam constitutes the form of elimination from the organism of many 1,4-benzodiazepines, such as diazepam and temazepam.

In the up-on-date daily medical practice, 1,4-benzodiazepines must be object of an exhaustive and periodical analytical control. This control gives important information about their plasmatic concentration in order to adequate the dose of these substances to the needs of the patients, as well as to avoid the toxic effects that some of these medicines presents at not very high doses, very close to the usual ones (narrow therapeutic range drugs). That is what they call drugs analytical monitorization.

On one hand, the peculiar pharmacokinetic characteristics [3], tolerance and addiction [4] in particular of 1,4-benzodiazepines, turn them into pharmaceuticals used abu-

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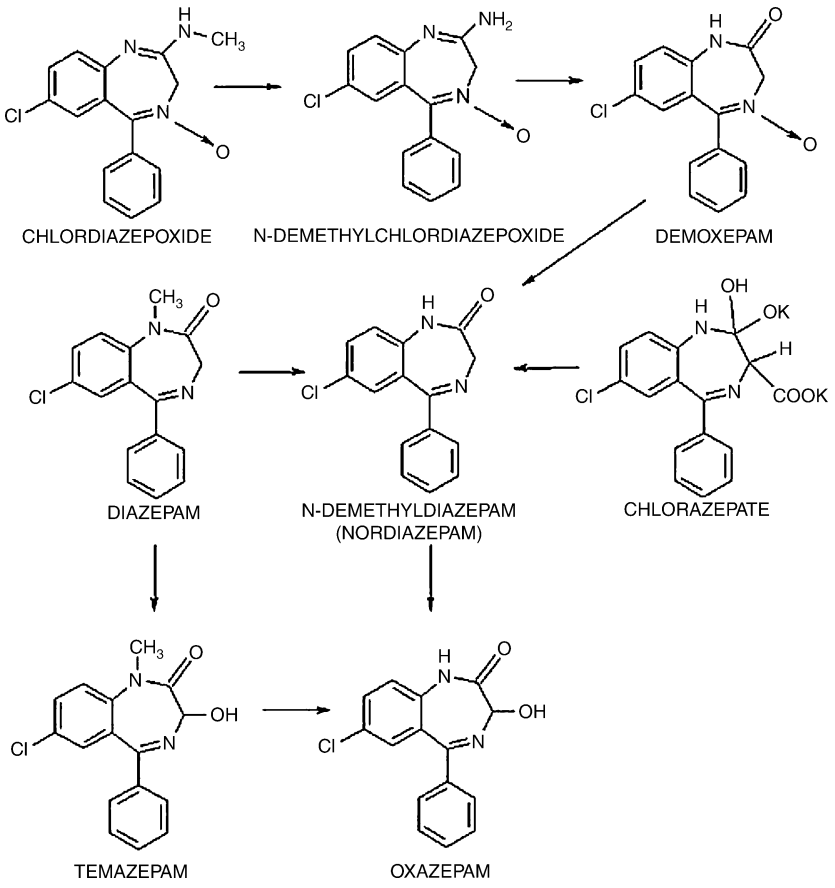

Fig. 1. Metabolic route of different benzodiazepines in the human organism.

sively. So, in forensic toxicology [5], they are often found in fatal cases of drug intoxication, as well as in the blood of drivers involved in traffic accidents. All this justifies the high interest in developing new analytical methods for their determination.

On the other hand, the extensive biotransformation and/or distribution of these substances in the tissues gives place to find either the pharmaceuticals or their metabolites in trace amounts [6,7] in biological fluids.

The determination of benzodiazepines in biological fluids has been a field of interest for many researchers since the early 1960s, when this type of pharmaceuticals came on to the market. Different instrumental techniques to analyse directly benzodiazepines and their metabolites, or their hydrolysed products in biological materials, have been used: spectrophotometry [8], spectrofluorimetry [9], chromatrography [10–12], immunoassays techniques (radioimmunoassay [13], enzyme immunoassay [14,15], and fluoroimmunoassay [16] analysis), capillary electrophoresis [8,17], HPLC [18–20], and electrochemical techniques [21–23].

The most common employed methods for the drugs analytical monitorization are enzymatic and immune. Taking into account the relatively high costs of enzymes and antibodies, it exists a great interest yet in developing new analytical methodologies to allow the determination of pharmaceuticals and/or their metabolites in biological fluids in a simple and economic way, ensuring good sensitivity and selectivity of the procedures at the same time.

In the present paper, an electrochemical study of diazepam, temazepam and oxazepam using modified carbon-paste electrodes is reported. Cyclic voltammetry and differential pulse voltammetry are used to study the nature of the reactions. Because of their low residual current and noise, their ease to prepare and replace, and their wide anodic and cathodic potential ranges, carbon-paste electrodes were employed to perform the electrochemical measurements. Their usefulness to determine different active principles or organic compounds has been shown yet [23–27].

The amperometric sensors utilized in this work have adequate range of application, reproducibility and detection limits for all the analytes studied here. Besides, the application to the determination of oxazepam and diazepam in biological fluids: plasma and urine, both collected from a volunteer, is also shown. Diazepam was determined in plasma and oxazepam in urine. Due to the complexity of these biological matrices, it was necessary to separate the active principles before their determination. The separation processes, established differently for both plasma and urine, were carried out using the solid-phase extraction (SPE) technique [6,20,28].

2. Experimental section

2.1. Instrumentation

The electrochemical measurements, excepting for cyclic voltammetry, were carried out at a VA 646 Processor, coupled to a VA 647 Stand with a stirring system, both of them purchased from Metrohm (Herisau, Switzerland). The studies of cyclic voltammetry were performed with an AutoLab®/PGSTAT20 (Ecochemie, Utrecht, The Netherlands) potentiostat/galvanostat, interfaced with a personal computer, and coupled to a Metrohm VA 663 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. The counter electrode was a platinum wire, and a silver/silver chloride, 3 M potassium chloride electrode was used as the reference. All these elements were also purchased from Metrohm. Carbon-paste electrodes (with a surface area of 7 mm^2), were used as the working electrode.

The active principles of the pharmaceuticals were extracted with high performance solid-phase extraction disk cartridges (Empore 3 M, Zug, Switzerland) in a previous step of pretreatment.

Differential pulse voltammetry (DPV) and cyclic voltammery (CV) were the electroanalytical techniques applied to carry out the electrochemical study of diazepam, temazepam and oxazepam. The initial instrumental parameters for DPV were as follows: initial potential = 0.0 V; end potential = −1.5 V; purge time (N_2) = 10 min; scan rate = 30 mV s^{-1}; pulse amplitude = −50 mV; pulse repetition time = 0.2 s. A preconcentration step at 0 V for 10 min was performed when necessary.

CV scans were made at different scan rate values, between 1 and 50 mV s^{-1}, in the range from 0 to −1.5 V, at the step potential = 0.01 V.

The results obtained by the electrochemical method were compared with those obtained by an HPLC method. A *JASCO* HPLC system with UV detection and a Gemini C_{18} (250 mm × 3.0 mm) 5 μm particle size column was used to carry out the measurements. The analytical flow rate was 0.8 mL min^{-1}. A gradient elution generated by mixing of two solvents: Solvent A: water–1% formic acid; Solvent B: acetonitrile–1% formic acid, was used. Solvent gradient condition changed linearly from 40% B to 60% B in 15 min. Detection was set at λ = 228 nm.

2.2. Reagents and materials

Oxazepam, diazepam and temazepam were from Acofarma (Barcelona, Spain). Boric acid, orthophosphoric acid, acetic acid and sodium hydroxide for the Britton–Robinson buffer solution were from Merck (Darmstad, Germany). Ethanol and methanol were also purchased from Merck. Formic acid was from Sigma-Aldrich (St. Louis, USA). Acetonitrile HPLC-gradient grade was from Panreac (Barcelona, Spain). All reagents were of analytical grade or higher and used as received without further purification.

Graphite powder (spectroscopic grade RBW) was from SGL Carbon (Ringsdorff, Germany). Mineral oil (spectroscopic grade), zeolite and bentonite, reagents used in the electrode preparation procedure, were from Sigma-Aldrich (St. Louis, USA). Nanopure water was obtained by passing twice-distilled water through a Milli-Q system (18 MΩ cm, Millipore, Bedford, MA).

The enzymes used in the solid-phase extraction procedure to separate the pharmaceuticals from the plasmatic proteins, proteinase K from *Tritachium album* (15 units mg^{-1}) and β-glucuronidase from *Helix pomatia* (143 000 units mL^{-1}), were from Sigma-Aldrich, as well as the Protein Assay Kit (Sigma Diagnostics, procedure no. P5656). Tetrahydrofurane (THF), employed as solvent to elute the medicines, was from Probus (Barcelona, Spain).

Carbon-paste electrodes were prepared as described previously [24–27]: 5 g of graphite were mixed with 1.8 mL of mineral oil and the required amount of the modifier to obtain the desired mass proportion. The resulting paste was packed into the electrode and the surface was smoothed. After each measurement, the paste was removed and the cavity of the electrode was cleaned with water and dried with a tissue.

2.3. Preparation of standards and buffer solutions

Diazepam and temazepam (1 g L^{-1}) were solved in ethanol and/or in mixtures of ethanol:H_2O (3:2 and 4:1 v/v, respectively). Alcoholic solutions were more unstable than hydroalcoholic solutions. Oxazepam solutions (0.5 g L^{-1}) were prepared in methanol and/or in mixtures of methanol:H_2O (2:3 v/v). All these solutions were maintained under refrigerated conditions in absence of light. Solutions of less concentration (1–10 μg L^{-1}) were obtained from these ones when carrying out the voltammetric analysis.

The Britton–Robinson buffer solution (I = 0.1, optimal conditions to study benzodiazepines), was employed as support electrolyte (pH 1.5–12) in the electrochemical measurements.

2.4. Origins of the biological samples: plasma and urine

To tackle the voltammetric determination of diazepam and oxazepam, in plasma and in urine, respectively, a healthy volunteer ingested 10 mg of diazepam in only one dose. The volunteer did not suffer from hepatic or renal problems, that could influence the normal metabolism of the pharmaceutical, and was not ingesting other medicines either, which could interfere with benzodiazepines. Finally, the volunteer consumed no sort of addictive drugs or alcoholic drinks, but was a smoker.

From this starting point, the following sampling was accomplished:

- Plasma: several blood samples were collected between 2 and 25 h after the consumption of the diazepam; plasma was obtained from this samples.

- Urine: the whole volume of urine excreted for 11 days was collected, in order to get practically all the metabolised medicine, because of the high average time of elimination of diazepam.

2.5. Plasma extraction procedure for diazepam determination

In a first step, 1 mL of plasma was incubated in a bath at 56 °C for 2–3 h with 15 μL of proteinase K from *Tritachium album* (15 units mL^{-1}) after homogenization; thus, the liberation of the pharmaceutical from the plasmatic proteins is achieved.

In the second step, the enzymatic digestion was transferred to an SPE cartridge, which was previously preconditioned with 0.5 mL of ethanol and 0.5 mL of Britton–Robinson buffer solution (pH 10.0). After washing the cartridge with 0.5 mL of Milli-Q water and 0.5 mL of Britton–Robinson buffer solution (pH 10.0), the medicine was eluted twice with 0.5–1 mL of methanol In this point, the Protein Assay Kit (P5656) was used in order to know the effectiveness of the solid-phase extraction procedure, i.e., whether the proteins contained in the digested samples had been retained in the cartridge or, on the contrary, had passed to the eluates. The tests confirmed the absence of proteins in the eluates, what served us to accept the use of solid-phase extraction cartridges as a deproteinization and preparation system of the plasmatic samples.

Once the extracted has been obtained, and after mixing well the two fractions to their perfect homogenization, the third and last step, consisting of the voltammetric determination of diazepam, begins.

2.6. Urine extraction procedure for oxazepam determination

In the first step, 3 mL of urine were incubated in a bath at 56 °C for 2–3 h after homogenizing with 0.9 mL of Britton–Robinson buffer solution (pH 4.6) and 0.1 mL of β-glucuronidase from *Helix pomatia* (143 000 units mL^{-1}). Thus, the 99% of the medicines are liberated from their glucuronides [29], the main form of excreting these compounds. The previous hydrolysis of these glucuronides allows to obtain more reliable results, independently of the technique of determination: immune, CG, HPLC, and CCF.

In the second step, 2 mL of the enzymatic hydrolysate were transferred to an SPE cartridge, which was previously preconditioned in this case with 0.5 mL of methanol and 0.5 mL of Britton–Robinson buffer solution (pH 2.5). After washing the cartridge with 0.5 mL of Milli-Q water and 0.5 mL of Britton–Robinson buffer solution, the pharmaceuticals were eluted twice with 0.5–1 mL of methanol (for calibration and reproducibility studies) or methanol:THF (1:1 v/v) solution.

The last step, the voltammetric determination of oxazepam, is described in next section.

2.7. Voltammetric procedure to determine diazepam and oxazepam in biological fluids

A volume of 25 mL of Britton–Robinson buffer solution (pH 10.0 for diazepam and pH 2.5 for oxazepam; $I = 0.1$ in both cases) were placed in the electrochemical cell. After deaeration, purging with N_2 for 10 min, 0.5–1 mL of the extract was added. Using a 5% modified carbon-paste electrode, a sweep in the range from −0.25 to −1.5 V was carried out. The optimal values of the electrochemical parameters to perform the measurements are summarized as follows: pulse amplitude = −125 mV; pulse repetition time = 0.7 s; step potential = −6 mV; electrode rotation rate = 1980 rpm. Once the voltammogram was obtained, 50 μL of the analytes solutions (125 mg L^{-1}) were added to the cell and a new scan was performed. The concentration of diazepam and oxazepam in the original biological samples, were calculated from the peak intensity values obtained in the potential ranges from: (a) −1050 to −1200 mV for diazepam, and (b) −800 to −950 mV for oxazepam.

3. Results and discussion

3.1. Optimisation of parameters

3.1.1. Influence of modifier

The fact of mixing the carbon paste with some sort of adsorptive specie (modifier) increases notably the sensitivity of the carbon-paste electrodes. In the present study, several modifiers such as zeolite and bentonite were tested, thanks to their good adsorptive properties [24–27].

Peak intensity (I_p) and the peak potential (E_p) values using unmodified carbon-paste electrode, zeolite-modified carbon paste (5%) and bentonite-modified carbon paste (5%) were studied. The best modifier should give the highest I_p value and resolution and the longest distance from the discharging current of the background. The I_p and the E_p values are shown in Table 1.

As it can be observed, the 5% bentonite-modified carbon-paste electrode gives the highest peak intensity values for all the pharmaceuticals. Although unmodified carbon paste shows good signals in all cases, these ones are lower than when using bentonite. Zeolite-modified carbon paste (5%) always presents the worst signals with respect to unmodified carbon paste.

The modifier also influences the E_p values, although the shifts caused in the peaks of the analytes are not significantly relevant.

Table 1
Comparative study of the paste composition and its influence on potential and intensity values of the voltammetric peaks for the three benzodiazepines

Analyte	Paste composition	Peak potential, E_p (mV)	Peak intensity[a], I_p (nA)
Diazepam	Unmodified CP[b]	−1286	561
	CP + 5% zeolite	−1320	388
	CP + 5% bentonite	−1270	903
Temazepam	Unmodified CP[b]	−882	866
	CP + 5% zeolite	−912	608
	CP + 5% bentonite	−870	1021
Oxazepam	Unmodified CP[b]	−892	857
	CP + 5% zeolite	−914	597
	CP + 5% bentonite	−870	1122

[a] Intensities are expressed in absolute value.
[b] Carbon paste.

Taking into account the previous considerations, bentonite was selected as the best modifier to determine the three benzodiazepines in later experiments, while zeolite-modified carbon paste (5%) was rejected.

3.1.2. Influence of pH

Britton–Robinson buffer solution was selected as the support electrolyte to find the optimal pH values for every analyte. The range of pH investigated was from 2.0 to 12.0. Values outside of this interval did not give either oxidation or reduction waves. Moreover, in some cases, signals were very close to the discharging current of the background, being very difficult the quantification.

The pH affects both E_p and I_p values. With respect to the first parameter, E_p values for the three pharmaceuticals decrease (or increase in absolute value) with pH, being more negative. Fig. 2 shows the dependence of I_p with respect to pH for diazepam, oxazepam and temazepam. From the curves, the optimal pH values for every one of them can be deduced: pH 9.0–10.0 for diazepam; pH 2.5–4.0 for oxazepam; and pH 3.0 for temazepam.

A comparative study of the electrochemical behaviour of diazepam, oxazepam and temazepam was carried out at the limit values of pH, i.e., 2.5 and 10.0. This study was focused in order to find particular zones of potential for every compound that allowed the sequential determination of the three pharmaceuticals in a unique biological sample.

As it can be concluded from Fig. 2, diazepam is the only analyte that gives a significant signal at pH 10.0. As a consequence of this, we are able to know the fraction of the ingested dose that has not been metabolised yet. In this way, it is possible to avoid intoxications in patients, since diazepam has very high average life and is transformed to many active metabolites in the organism. At pH 2.5 it happens the other way round: oxazepam and temazepam gives their best signals and diazepam not. Nevertheless, selectivity is not as marked here as in the previous case, because two analytes offer responses at the same pH value and their peak potentials are really close to each other. However, this should not be a problem, since in clinical analysis the results are given as the global content of benzodiazepines (drugs total index) in urine samples.

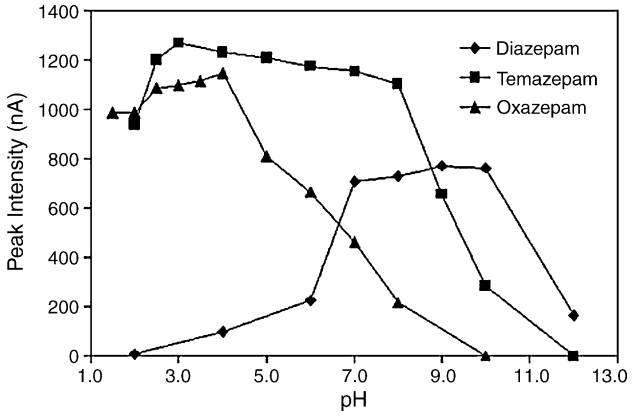

Fig. 2. Influence of pH on the peak intensity values for the three benzodiazepines: (♦) diazepam; (■) temazepam; (▲) oxazepam.

3.1.3. Influence of buffer solution and ionic strength

Several buffer solutions at different ionic strengths if possible, were tested at the pH values selected as optimal.

The results of this study appear in Table 2. There, the E_p and the I_p values of every compound when using several buffer solutions at different ionic strengths are shown. The concentration

Table 2
Comparative study of the influence of the buffer solution and its ionic strength on potential and intensity values of the voltammetric peaks

Analyte[a]	Buffer solution	Ionic strength	Peak potential, E_p (mV)	Peak intensity[b], I_p (nA)
Diazepam	Britton–Robinson	0.01	−1278	867
		0.05	−1272	1226
		0.1	−1270	1364
	Borax	–	−1258	1034
Temazepam	Britton–Robinson	0.01	−853	858
		0.05	−866	1137
		0.1	−865	1490
	HCl/KCl	0.05	−860	916
		0.1	−854	1021
		0.2	−848	1218
	Glicina/HCl	0.05	−854	952
		0.1	−863	1157
		0.2	−854	1191
Oxazepam	Britton–Robinson	0.01	−879	1012
		0.05	−871	1196
		0.1	−858	1820
	HCl/KCl	0.05	−873	1042
		0.1	−872	1124
		0.2	−867	1102
	Glicina/HCl	0.05	−885	826
		0.1	−873	1034

[a] Concentration of the analytes = 0.4 μg mL^{-1}; pH of the buffer solution = 10.0 for diazepam, and 2.5 for temazepam and oxazepam.
[b] Intensities are expressed in absolute value.

of the medicines was 0.4 μg mL^{-1} at their respective optimal pH values.

As it can be observed, independently of the buffer solution, the higher the ionic strength, the better the signal obtained, i.e., the I_p values increase with the ionic strength in all cases. With respect to the E_p values, when increasing the ionic strength, the peak potential suffers no significant shift, depending on the buffer solution. From the results, it was found that the Britton—Robinson buffer solution at the highest ionic strength ($I = 0.1$), gave the best results of peak intensity for the three pharmaceuticals. These conditions were chosen for the next studies.

3.1.4. Influence of percentage of bentonite

Proportions up to 10% were tested and higher values were not used because the mixtures obtained were less compact and fall off the electrode on rotation. The percentages investigated were 5, 7.5 and 10%. Proportions lesser than 5% gave no significant improvement of the signals with respect to the voltammograms obtained with an unmodified carbon paste.

Firstly, for the three analytes, the 10% bentonite-modified carbon-paste electrode gave the worst results. Moreover, in the oxazepam determination, it shows even no signal. Secondly, the immediately lower percentage (7.5%) offered the highest I_p values, but distorting appreciably the symmetry of the peak obtained. Finally, the remaining percentage (5%) showed peaks with the best characteristics of height, shape, and symmetry. Thus, bentonite-modified carbon paste (5%) was selected in latter experiments.

3.1.5. Effect of pulse repetition time and pulse amplitude

The values tested here were 0.2, 0.5, 0.7 and 1.0 s. From this study it can concluded that the I_p values increase progressively with the pulse repetition time up to 0.7 s; for upper values of pulse repetition time, peak intensity decreases drastically. As a consequence of this, 0.7 s was selected as the optimal value.

The other parameter, pulse amplitude (ΔE), is also a typical variable of the electrochemical technique employed. In this paper, ΔE ranged from −50 to −150 mV (with increments of 25 mV in absolute value). For the three analytes, (a) diazepam, (b) oxazepam, and (c) temazepam, a good linear relationship between I_p and ΔE was found in the range investigated: (a) $r^2 = 0.988$; (b) $r^2 = 0.990$; (c) $r^2 = 0.992$. At the same time that an improvement in peak intensity is achieved when increasing ΔE, a distortion in the shape and symmetry of the signal is found. Thus, in order to obtain a compromise between peak intensity and distortion, the value of −125 mV was chosen as optimal.

3.2. Study of the nature of the reaction

Reductions of diazepam, oxazepam and temazepam at the modified carbon-paste electrode were studied using cyclic voltammetry. The scan rate (ν) took values between 1 and 50 mV s^{-1}.

In all cases and from studying the cyclic voltammograms, a similar behaviour was found: at the concentrations used, the behaviour of the three benzodiazepines was clearly irreversible, since the reduction signal corresponding to the direct sweep appeared, but the inverse sweep did not showed the oxidation wave. The rest of the parameters analysed confirmed the irreversibility of the processes [30]: the variation of the peak potential and the peak intensity with the scan rate was studied. With respect to the first case, E_p suffers a slight shift towards more negative potentials with the scan rate. This variation agrees with an irreversible behaviour, although it is also compatible with an adsorption phenomenon of the reagents on the electrode surface, as seen in Fig. 3. A linear relationship was found between I_p and the square root of the scan rate ($\nu^{1/2}$) in the whole range. In the case of diazepam and oxazepam, the higher the scan rate, the worst the linear fitting, i.e., the lower the r^2 values. Finally, a linear relationship between I_p and ν was found as well, particularly when the highest scan rate values were excluded. The correlation coefficient for the three benzodiazepines was 0.99 in all cases, although in different ranges of scan rate: until 1 mV s^{-1} for diazepam and temazepam, and until 5 mV s^{-1} for oxazepam. The deviation from the linearity at higher scan rate values could indicates adsorptive phenomena of the analytes on the electrode surface.

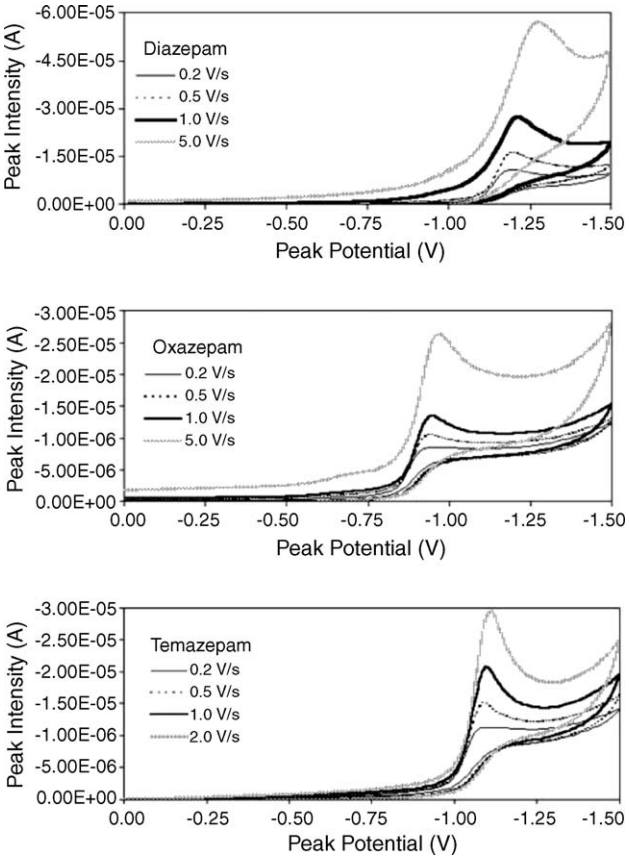

Fig. 3. Cyclic voltammograms at different values of scan rate for the three benzodiazepines.

Besides, as it has been stated in the previous section, good correlation coefficients between the peak height and the pulse amplitude were obtained for the three medicines when performing DPASV. There was also an increase in the peak potential

Table 3
Linear relationships and detection limits existing for the analytes at the concentration ranges in which these analytes are usually found in plasma

Method	Analyte		
	Diazepam	Temazepam	Oxazepam
Electrochemical			
r^2	0.9986	0.9936	0.9986
Concentration ranges ($\mu g\,mL^{-1}$)	0.025–3.0	0.025–0.8	0.025–1.0
Detection limit ($\mu g\,mL^{-1}$)	0.021	0.021	0.012
HPLC			
r^2	0.9975	0.9910	0.9939
Concentration ranges ($\mu g\,mL^{-1}$)	0.025–3.0	0.025–0.8	0.025–1.0
Detection limit ($\mu g\,mL^{-1}$)	0.015	0.013	0.018

Results for electrochemical and HPLC methods are shown.

with the pulse amplitude [31], which agrees with practically irreversible behaviour.

From this discussion, the irreversible nature of the reactions in which the three pharmaceuticals are implied, can be affirmed. Moreover, reasonable clues as to the existence of adsorptive phenomena of the analytes on the electrode surface exist. This corroborates the study of these compounds using electrodes modified with adsorptive species.

3.3. Calibration graphs and reproducibility studies

It seemed to be necessary to obtain the calibration graphs in the most real conditions as possible. In order to get this, solutions in plasma (previously treated as described in Section 2.5) at different concentrations of Valium® were used to determine diazepam. The calibration graphs for the other two analytes were obtained in the same way, but the solutions were prepared in urine (see Section 2.6).

Linear relationships were found for the analytes at different concentration ranges, as it can be seen in Table 3. The detection limits in the linear range for every pharmaceutical, calculated using a statistical procedure [32], as the signal of the blank plus three times its standard deviation, are also reported. The narrowest concentration range was for temazepam. In this table the results obtained by the HPLC comparison method are also shown; in all cases the responses for each analyte were similar for both methods.

A study of reproducibility with the subsequent statistical treatment for six determinations of the analytes was carried out. For diazepam and temazepam, the concentration tested was $0.2\,\mu g\,mL^{-1}$, since it is the average value of their therapeutic concentration ranges in plasma. For oxazepam, the concentrations tested were 0.25 and $0.5\,\mu g\,mL^{-1}$, as they are the highest levels of their therapeutic concentrations in plasma and urine, respectively. The relative standard deviation values were obtained in all cases: ±1.2% for diazepam and ±0.25% for temazepam, both at $0.2\,\mu g\,mL^{-1}$; ±1.4 and ±1.8% for oxazepam at 0.25 and $0.5\,\mu g\,mL^{-1}$, respectively. As seen, good relative standard deviation values were obtained, lesser than 2% in all cases.

3.4. Voltammetric determination of diazepam in plasma

Diazepam was determined voltammetrically in plasma. It always appears in this location since it is not metabolised yet. A previous extraction procedure of the pharmaceutical was carried out, as described in Section 2.5.

Peak intensity values obtained were not the expected either, not even for the samples at the times in which the diazepam reached the highest level of plasmatic concentration. Nevertheless, after the addition of known amounts of diazepam, correspondences between the concentrations added and found were obtained. Thus, an incomplete extraction of diazepam adsorbed in the cartridges could be a plausible answer to the decrease in the peak intensity observed in the samples, perhaps because the elution conditions were not optimal.

In order to investigate the problem of elution with other solvents it was necessary to dispose of a considerable volume of the sample. However, it is difficult to obtain high amounts of plasma, but the study can be performed in urine by means of the determination of oxazepam, the medicine that appears more commonly in this biological fluid.

3.5. Voltammetric determination of oxazepam in urine

As said previously, the voltammetric determination of oxazepam was carried out in urine since, as a metabolite of diazepam, it is normally excreted via urinary system and its highest concentration values are found there. This biological fluid is more available than other biological samples, such as plasma, and higher volumes are possible to obtain. Oxazepam concentrations values are generally higher than those obtained for this pharmaceutical in other kind of biological samples, and its monitorization in urine is possible even several days after the ingestion (the average lifetime of this analyte in plasma is only of 2–3 days). It has to be stated that after a unique dose of diazepam, oxazepam begins to appear in urine from 10 to 12 h after the ingestion; then, its level of concentration increases and stabilizes, maintaining a relatively high value until a week later. Afterwards, the concentration decreases, becoming undetectable from 12 to 15 days after the ingestion.

Urine samples also needed a previous extraction procedure of the pharmaceutical, as described in Section 2.6.

The study of the eluent used in the extraction procedure of oxazepam was carried out. Two solvents were selected: THF, and

Table 4
Comparative study of the eluents proportions (v/v) in order to improve the extraction procedure of oxazepam

Proportion methanol:THF	0:1	1:3	1:2	1:1	2:1	3:1	1:0
Peak intensity[a], I_p (nA)	75.48	61.54	48.32	73.01	34.04	39.23	19.52

[a] Intensities are expressed in absolute value.

methanol. THF was chosen as a possible solvent because it had given greater percentages of recuperation in previous studies. The comparative study of the eluents proportions is shown, in Table 4.

The most complete elution of the analyte was obtained when using only THF (proportion 0:1 v/v methanol:THF), although the mixture of solvents 1:1 v/v methanol:THF also showed good results, since similar peak intensity values for oxazepam were found in both cases. Besides, the concentration values calculated belonged to the usual range of concentrations in which oxazepam appears in urine: from 0.05 to 0.5 $\mu g\,mL^{-1}$. The mixture of methanol:THF 1:1 v/v was selected as optimal.

After this, the recommended procedure to determine oxazepam in urine was tested. The first sample corresponds to the sample collected 10–12 h after the ingestion and the last sample was collected 11 days after the consumption of the pharmaceutical. In all the samples, the recuperated concentration values, calculated by means of the standard addition method, belonged to the concentration range from 0.05 to 0.5 $\mu g\,mL^{-1}$, according to data appearing in bibliography. Besides, they were in the linear zone of the calibration graph.

The recuperated total amount of oxazepam was 8.5 mg, i.e., the 85% of the ingested dose (10 mg of diazepam). This result was quite good, since the theoretically expected amounts had to be in the range from 85 to 90%. Related to this, Norman et al. [33] have studied the pharmacokinetic of nordiazepam and its capability to shorten the average lifetime of elimination of oxazepam, as well as a decrease in the peak height obtained when analysing its plasmatic concentrations in smoker volunteers with respect to non-smokers. In our case, the volunteer is a regular smoker, so it would not be strange to think that this habit could have affected the right metabolization of the pharmaceutical. Moreover, Klotz and Reimann [34] have shown that nordiazepam can be an inhibitor of the elimination of diazepam. As a direct consequence of these factors, the proximity of the recuperated value to the inferior limit theoretically expected could be explained.

4. Conclusions

A whole electrochemical study of diazepam, temazepam and oxazepam has been carried out using modified carbon-paste electrodes. These pharmaceuticals are 1,4-benzodiazepines, showing a sequential metabolic route in human organism. Their determination in biological fluids, such as urine and plasma, both collected from a volunteer that had ingested one dose of diazepam by oral via, is object of great interest.

In order to do this, a sensible, versatile and economic methodology has been developed. This procedure has three sequential steps: (1) liberation of the medicines: in which the active principles of the pharmaceuticals are separated from the plasmatic proteins (in the case of diazepam) or its glucuronides (in the case of oxazepam); (2) extraction process: the analytes are eluted using a mixture of solvents in the correct proportions; (3) voltammetric determination, after establishing the optimal conditions for the measurements: type of modifier and its percentage, pH of the support electrolyte, buffer solution and ionic strength, pulse amplitude and pulse repetition time. Differential pulse voltammetry was the electrochemical technique employed.

Thus, diazepam was determined in plasma and oxazepam in urine. The concentration values found for the first analyte were lower than the theoretical expected ones. However, in the case of oxazepam, the results were quite good, since a recuperation percentage of 85% was achieved, being in the expected range from 85 to 90%, reported in bibliography. Several factors, such as the habit of smoking, and other pharmacokinetic aspects could have avoided higher recuperation percentages of this benzodiazepine.

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