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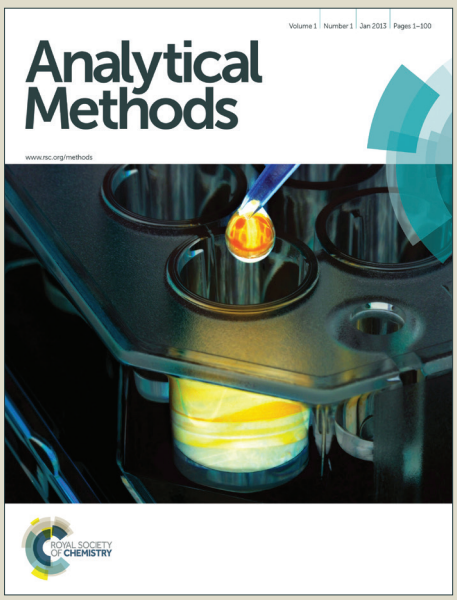

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ELECTROANALYTICAL DETERMINATION OF BUMETANIDE EMPLOYING A BIOMIMETIC SENSOR FOR DETECTION OF DOPING IN SPORT

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Abstract

This paper describes the development and application of a simple, cheap, and clean method for the quantification of bumetanide in urine samples from athletes and in pharmaceutical formulations to detect doping, using biomimetic sensor based on a carbon paste modified with Copper (II) 1, 2, 3, 4, 8, 9, 10, 11,15,16,17,18,22,23,24,25 hexadecafluoro-*29–H,31-H*–phthalocyanine (a biomimetic catalyst of the P450 enzyme). The sensor was evaluated using cyclic voltammetry and square wavevoltammetry, for electrochemical characterization and quantification purposes, respectively. Square wave voltammetry analyses were carried out vs. Ag/AgCl (KClsat), using a 0.15 mol L−1 Britton-Robinson buffer solution at pH 7.0 as the support electrolyte. This method was optimized using a chemometric experimental design. Under these optimized analytical conditions, the sensor showed a linear response between 9.9×10^{-7} and 8.3×10^{-6} mol L^{-1} (R=0.996) and limits of detection and quantification 2.7×10^{-7} and 9.0×10^{-7} mol L−1, respectively. The proposed method was successfully applied in the analysis of bumetanide in spiked urine, demonstrating that it is a reliable alternative method for the detection of bumetanide doping in sport.

Keywords: Biomimetic sensor, P450 enzyme, bumetanide, doping, copper phthalocyanine.

1. Introduction

A diuretic is a kind of drug widely used in clinical practices for the treatment of edemas, hypertension, congestive heart failure and prophylaxis of renal failure [1]. Bumetanide [BMT, 3-butylamino-4-phenoxy-5-sulfamoylbenzoic acid] is one of the most potent diuretics of the sulfamoyl category which produces rapid onset and short duration of action [2,3]. The bumetanide is about 40 times more potent than furosemide, having a half-life from 0.3 to 1.5 hours. Bumetanide is 81% eliminated from the body via renally, in which about 65% are in the unchanged form and 35% is metabolized [1,4]. That mean that in a commercial formulation contained 1 mg of bumetanide the expected amount in urine is 7.2×10^{-6} mol L^{-1}. However, diuretics are on the list of prohibited substances published by the World Anti-Doping Agency (WADA) [5], and is expected that no amount of this compounds will be found in athletes. Nonetheless, some athletes misuse diuretics in competition sports in order to achieve acute weight loss before competition in sports where weight categories are involved, or to mask the use of other doping agents by diluting their concentration in the urine [6]. Thus, it is essential to develop a sensitive, rapid and convenient analytical method for the determination of illicit diuretics in human urine samples, especially in the urine samples of athletes.

Several analytical methods have been reported for the determination of bumetanide in pharmaceutical formulations or in biological fluids such as capillary electrophoresis [7,8], high performance liquid chromatography with fluorescence detection [9], gas chromatographic with mass spectrometry detection [10], flow injection coupled to fluorescence spectrophotometry [11], liquid chromatography with tandem mass spectrometry [12], in-situ analysis used an optical fiber sensor [13]. Unfortunately, some of these methods lack adequate detectability (require large

amounts of sample), some are time-consuming or costly, and others just have low sensitivity and long run times which are not suitable in all conditions. In addition, in despite of their various advantages, literature reports only few electrochemical methods for this analyte.

Among the electrochemical methods that enabling sensitive and selective quantification of drugs, are the sensors and biosensors with voltammetric transduction. Advantages of these devices include high sensitivity, selectivity, versatility, low cost and portability [14-16]. However, in general, biosensors are not robust and have lifetimes that are limited by denaturation of the biological material present on the electrode surface. One way of overcoming these disadvantages of biosensors is to construct sensors using biomimetic catalysts that mimic the active site of redox enzymes.

All the P450 enzymes contain a common active site, iron protoporphyrin IX, which catalyzes numerous chemical reactions in organisms, usually producing metabolites that are physiologically essential or beneficial [17]. These enzymes catalyze a wide range of chemical reactions in organisms, producing metabolites that are physiologically essential or beneficial to them [18]. In addition, the degradation of xenobiotics such as drugs, pesticides and endocrine disruptors is possible through hydroxylation, oxidation or reduction reactions [18,19]. Materials derived from the phthalocyanines and porphyrins of iron and other metals have been successfully used in the construction of biomimetic chemical sensors for analytical use [20–24], since these compounds mimic the chemical structure of the P450 active site (iron protoporphyrin IX).

On these bases, this paper describes the development and application of a simple, portable and environmentally friendly method for the rapid determination of

bumetanide in urine and commercial tablets. The proposed method is based on square wave voltammetry sensor modified with a copper (II) phthalocyanine [CuPc] complex, a potential biomimetic catalyst of the P450 enzyme, in the direct, selective and sensitive detection of bumetanide. Experimental design methodologies were used to optimize the measurement conditions.

2. Experimental

2.1. Instruments

The electrochemical measurements were conducted at room temperature in a conventional three-electrode cell, with a modified carbon paste electrode used as the working electrode. An Ag/AgCl(KClsat) electrode and a platinum wire were used as the reference and counter electrodes, respectively. The measurements were performed using a potenciostat model micro-Autolab type III of Autolab/Eco Chemie®, which was interfaced with a microcomputer running GPES software 4.9 version, for control of potential and acquisition of data.

The electroanalytical techniques used in this work were cyclic voltammetry and square wave voltammetry. Cyclic voltammetry was first used to investigate the electrochemical behavior of bumetanide on the sensor surface. Measurements were subsequently carried out at a suitable potential for the catalytic process, based on the results of the voltammetric experiments. The square wave voltammetry technique was used in the optimization studies and for quantification of bumetanide.

2.2. Reagents and solutions

All the chemicals used were analytical or HPLC grade. Copper (II) 1, 2, 3, 4, 8, 9, 10, 11,15,16,17,18,22,23,24,25 hexadecafluoro 29-H, 31-H phthalocyanine [CuPc], bumetanide, mineral oil and graphite powder were purchased from Sigma–Aldrich.

NaH_2PO_4, H_3BO_3, NaOH, ethanol, acetic acid were purchased from Synth-Brazil. Methanol was obtaines from J.T.Baker® and phosphoric acid from Mallinckrodt®. The bumetanide and buffer solutions were prepared with water purified using a Milli-Q (Direct – 0.3) system.

2.3. Biomimetic sensor construction

The modified paste was prepared by homogenizing 15 mg of CuPc with 85 mg of graphite powder and 1.0 mL of 0.1 mol L^{-1} phosphate buffer solution (at pH 7.0).The material obtained was dried at room temperature, and then mixed with mineral oil to obtain a homogenous paste. The paste was placed into the cavity of a glass tube (4 mm internal diameter, 1 mm depth), and a platinum slide was inserted for electrical contact with the paste.

2.4. HPLC analyses

Chromatographic analyses were performed using a Shimadzu Model 20A liquid chromatography, coupled to an SPD-20A UV/Vis detector, a SIL-20A autosampler and a DGU-20A5 degasser. The chromatography system was controlled by a microcomputer. A C18 column (250 mm × 4.6 mm, Shimadzu Shim – Pack CLC - ODS) was positioned inside a Shimadzu CTO-10AS oven in order to maintain a constant temperature. The mobile phase was a mixture of acetonitrile and 2.5 x 10^{-2} mol L^{-1} phosphate buffer at pH 2.5, in a volume ratio of 80:20, passing through the column

at a flow rate of 1 mL min-1. The sample injection volume was 10 μL. The absorbance of bumetanide was monitored at 230 nm [25].

2.5. Study of selectivity

The selectivity of the sensor was investigated by means of square wave voltammetry response to 13 drugs. For this, 1.0 $\times 10^{-3}$ mol L^{-1} stock solutions of all drugs were dissolved in the water/ethanol (9:1, v/v) solution.

2.6. Preparation of real samples

Three commercial samples of pharmaceutical formulations (tablets) containing 1 mg of BMT from different batches and trademarks were purchased in local drugstores in Araraquara city (Brazil) and analyzed by the proposed method. Ten tablets of each commercial trademark were weighed exactly and ground to a fine powder. A portion of this powder was accurately weighed and dissolved with 10.0 mL of ethanol (proposed method) or methanol (comparative method). This solution was filtered and an aliquot of 2.5 mL of the filtrate was transferred to a 10.0 mL volumetric flask and the volume was completed with water or methanol, respectively.

Urine samples were collected from six volunteers and all experiments were performed in compliance with the relevant laws and institutional guidelines (Brazil Platform the national and unified database of records for research involving human, process number 27946014.8.0000.5426). Samples A to F were from healthy people aged between 20 and 60 years. Sample F was from a volunteer who consumed two doses of diuretic amiloride daily, for control of arterial pressure. Each sample was enriched with bumetanide. For this, 10 mL of the sample were centrifuged for 10 min at

2000 rpm. The supernatant diluted 2-times with water and the solution was transferred into the voltammetric cell to be analyzed without any further pretreatment. Standard addition method was used for the determination of BMT in real samples, and results were compared with a chromatographic method described in literature [25].

3. Results

Cyclic voltammetry experiments were performed in the absence and presence of bumetanide using the biomimetic sensor in a potential range of 0.2 – 1.0 V, with a scan rate of 50 mV s^{-1}. It can be seen (Fig. 1) that response to bumetanide was tested using an unmodified carbon paste, a small variation in the anodic current was observed. On the other hand, the sensor based on carbon paste modified with the copper (II) complex showed an increase in the anodic current from a potential of 0.7 V vs. Ag/AgCl (KClsat).This indicated that the presence of the copper (II) 1, 2, 3, 4, 8, 9, 10,11,15,16,17,18,22,23,24,25-hexadecafluoro-*29-H*,*31-H*-phthalocyanine complex is important to oxidation of analyte (Chart 1).

FIGURE 1

CHART 1

Then square wave voltammetry measurements were carried out in order to optimize the analytical parameters influencing the sensor response, with the values

selected being based on the highest sensitivity as indicated by the respective analytical curves.

3.1. Optimization of variables

Due to the fact that more than one variable is potentially important, and that it would be difficult to optimize the conditions through a unit-variant optimization procedure, the experimental conditions were obtained using a chemiometric experimental design. The optimization was developed by two kinds of designs: (*i*) the factorial design to evaluate which of the variables were significant factors in the sensitivity of the proposed sensor; and (*ii*) the central composite design, to obtain the response surface from which the optimal factors that give a maximum response can be obtained.

Initially, a $2^{(7-3)}$ fractional factorial design was carried out, which allowed simultaneously studying seven factors that could have an important effect on the current obtained with proposed sensor. The factors of interest were the amount of complex in the paste (%) (w/w), pH, buffer, buffer concentration (mol L^{-1}), step potential, frequency and amplitude. In this design, the variables were studied at two levels: low (–1) and high (+1). For this design sixteen experiments were necessary, which were realized in triplicate and randomized to eliminate any environmental variation. The highest and lowest values of each variable were defined based on preliminary experiments. As a result of the fractional factorial design, a Pareto chart was drawn in order to visualize the estimated effect of the main variables. It can be observed in Fig. 2 that three variables (pH, frequency and step potential) were considered be significant.

FIGURE 2

Then similarly, $2^{(3)}$ full factorial design was carried out with the mentioned variables, can be observed that two variables (frequency and step potential) were considered be significant. Afterwards, the above-mentioned variables were optimized by the response surface methodology. These variables were studied at five levels, including four central points for statistical validity within the range of –1.41 to +1.41, which corresponds to frequency with a range of 40–80 (Hz) and step potential with a range from 0.001 up to 0.050 V. Fig. 3 shows the response surface estimated as a function of frequency and step potential.

FIGURE 3

It can be observed in the surface shape that the optimal region was found and also that the maximum response were achieved when frequency 60 (Hz) and step potential 0.006 V. The quadratic regression model could be described by:

$$z = - 0.19381 + 0{,}00826x - 0.00009x^2 + 73{,}589y - 8181{,}2y^2 + 0{,}42501xy \quad \text{(eq. 1)}$$

where z is the response factor corresponding to the sensitivity value, x is the frequency, and y is the step potential. The responses of the model, the R^2 values, were greater than 95%. Implying that the model was well-fitted by the data at 95% confidence level for the sensitivity of the sensor. Table 1 gives the optimum values of the variables studied.

TABLE 1

3.2. Electrochemical characterization

The electrocatalytic oxidation of bumetanide was carried out through experiments varying the sweep rate of cyclic voltammetry, allowing to evaluate the electrochemical characteristics of the sensor based CuPc. Analyzing the anodic peak current in function of the square root of the scan rate, it can be visualize a linear dependence (data not shown) in the range between 5 and 200 mV s^{-1} indicating that the mass transport of bumetanide on the sensor surface is controlled by diffusion.

The existence of an electrocatalytic process was confirmed by plotting the graph of the scan rate-normalized current (i $v^{-1/2}$) against the scan rate (v) (Fig. 4), whose profile suggests that the process of the bumetanide oxidation is the electrocatalytic type CE (chemical/electrochemical catalytic), which is characterized through of step chemical coupled to an electrochemical process [26].

FIGURE 4

Based on the results described above, it was possible to propose a mechanism for the biomimetic sensor. Initially the chemical reaction (chemical step) between the bumetanide and molecule of reduzed [CuPc], promote the oxidation of bumetanide and redution of copper in the phthalocyanine. In the electrochemical step coupled to this chemical oxidation at the electrode surface the re-oxidation of metal (Cu^{+} to Cu^{2+}) in the phthalocyanine leads to obtaining the anodic current observed due to the presence of bumetanide in the measurement cell.

FIGURE 5

3.3. Evaluation of the biomimetic characteristics of the sensor

The graphic obtaining plotted the current of the oxidation peak of square wave voltammograms *versus* concentration including saturated amounts of analyte should be similar to that of an enzymatic biosensor, since the complex used had a structure similar to that of the P450 heme-enzymes. In the case of biosensor based on redox enzymes, the current signal initially increases linearly with the concentration of the substrate ([S]), and then, as [S] is further increased, the enzyme becomes saturated, and the current signal reaches a maximum value [23,27] producing a graph with hyperbolic profile. A biomimetic sensor, just as our, should therefore produce the same graphic, that means the sensor response follows a pseudo-Michaelis–Menten kinetics, which is expected, since our catalyst is based on the structure of the active site of a redox enzyme. In addition, is possible to estimate the apparent Michaelis–Menten constant (K_{MM}^{app}) from the hyperbolic curve (graph not shown) using the double reciprocal plot (Fig. 6),

obtained in this case the value of 2.68×10^{-4} mol L^{-1}, which shows that the analyte had a good affinity with the sensing phase complex. This value is in agreement with the expected and reported values for biomimetic sensors and biosensors [27].

FIGURE 6

A useful biomimetic sensor must show a high level of selectivity. In order to evaluate the selectivity of the proposed sensor, its response was tested with the following drugs in the concentration of 1.0×10^{-3} mol L^{-1}: hydrochlorothiazide, furosemide, methyldopa, captopril, ketoprofen, ciprofloxacin, aminophylline, nifedipine, urea, uric acid, ascorbic acid and xanthine. In this study was observed that ascorbic acid presented an oxidation peak at 0.25 V, and in this sense it compound is not an interference in the application of the proposed sensor. Since, that the urine

analysis will be carry out in fasting. On the other hand, hydrochlorotiazide caused a small interference in the analysis from molar ratio of 1:10 (analyte/interfering).

3.4. Determination of the figures of merit

The proposed sensor was validated used SWV (Fig. 7) by considering the linear dynamic range, repeatability, limit of detection (LOD), limit of quantification (LOQ), precision, lifetime of the sensor, interferences, and recovery. The analytical curve shows in the inset in Fig. 7, was linear in the range 9.9 x 10^{-7} to 8.3 x10^{-6} mol L^{-1}. The regression equation obtained was:

$$Ip = 0.2122 (\pm 0.1087) + 640120 (\pm 20678) [BMT] \quad (R = 0.996) \quad (eq. 2)$$

The LOD and LOQ were 2.7×10^{-7} mol L^{-1} and 9.0×10^{-7} mol L^{-1}, respectively. Precision is expressed as the relative standard deviation (RSD) of the analytical response. To a method to be considered precise, the RSD should be less than 4.0%. Here, the precision of the method was assessed by repetition of experiments to obtained analytical curves at different times on one day (intra-day), and on different days (interday). The coefficients of variation obtained were 2.7% and 3.5%, respectively. Finally, the lifetime of the sensor was estimated to be around 5 days (n= 105 analysis).

In order to evaluate the complex influence in sensor response, the successive addition of bumetanide were made used an unmodified carbon paste electrode (CPE) under the same conditions previously optimized for the sensor the results obtained are shown in Supplementary data.

3.5. Application

The new technique was applied using three samples of drugs and six samples of human urine which were spiked with bumetanide. The results obtained in the recovery experiments carried out, showed recoveries of around 100%, indicating that there were no matrix effects for this type of sample. The results obtained by the proposed method were compared statistically (using t-tests at a 95% confidence level) with those obtained using the comparative method [25], and showed good agreement (Table 2 and 3). The calculated t-values did not exceed the critical values, indicating that there was no significant difference between the two methods in terms of precision and accuracy.

4. Conclusions

This study demonstrates the feasibility of employing a simple system with voltammetric detection in the control of sports doping, using a biomimetic sensor modified with a CuPc complex. The developed method represents an advantageous alternative to other traditional methods for detection of bumetanide in urine because it is inexpensive, simple, portable, precise, and accurate, allows rapid determination at low operating costs and requires minimum amounts of samples and reagents/solvents, and thus can be considered an environmentally friendly analytical method.

Acknowledgements

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References

[1] J. G. Hardman and L. E. Limbird, *Goodman and Gilman's Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, 9th edn,1996.

[2] M. J. Asbury, P. B. B. Gatenby, S. O'Sullivan, E. Bourke, Bumetanide: potent new loop diuretic, Br. Med. J. 1 (1972) 211–213.

[3] A. Ward, R.C. Heel, Bumetanide: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic use, Drugs 28 (1984) 426–464.

[4] S. C. Halladay, D. E. Carter, I. G. Sipes, B. B. Brodie, R. Bressler; Evidence for the metabolism of bumetanide in man, Life Sciences v . 17 (1975), 1003-1010.

[5] Available at: www.wada-ama.org/en. Accessed on November 26, 2010.

[6] M. L. Riekkola and J. H. Jumppanen, Capillary electrophoresis of diuretics J. Chromatogr. *A*, **735**, (1996), 151–164.

[7] X. Y. Zheng, M. H. Lu, L. Zhang, Y. W. Chi, L. H. Zheng, G. N. Chen; An online field-amplification sample stacking method for the determination of diuretics in urine by capillary electrophoresis amperometric detection. Talanta 76 (2008) 15–20.

[8] L. H. Zheng, X. Y. Zheng, P. Tong, G. N Chen, L. Zhang; Study on the interaction between two kinds of diuretics and bovine serum albumin by capillary electrophoresis-amperometric detection. Chin. J. Anal. Chem. 37 (2009) 115–118.

[9] K. Tang; Content determination of the principal agent in bumetanide tablets by HPLC—fluorimetry. Chin Pharm 6 (2005) 1493–1494.

[10] V. Morra, P. Davit, P. Capra, M. Vincenti, A. D. Stilo, F. Botr'e; Fast gas chromatographic/mass spectrometric determination of diuretics and masking agents in human urine. Development and validation of a productive screening protocol for antidoping analysis. J Chromatogr A, 1135 (2006) 219–229.

[11] P. Solich, K. P. Christoforos, A. K. Michael, E. E. Constantinos , Automated flow injection fluorimetric determination and dissolution studies of bumetanide in pharmaceuticals. Anal Chim Acta 438 (2001) 131–136.

[12] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J. L. Veuthey; Fast analysis of doping agents in urine by ultra-high-pressure liquid

chromatography–quadrupole time-offlight mass spectrometry I Screening analysis. J Chromatogr. A, 1216 (2009) 4423–4433.

[13] Y. Li, L. Li, Q. Z. Zhang, In-situ analysis of bumetanide injection by optic fiber sensor. J Xinjiang Med Univ 32 (2009) 18–20.

[14] N.S. Lawrence, E.L. Beckett, J. Davis, R.G. Compton, Advances in the voltammetric analysis of small biologically relevant compounds, Analytical Biochemistry 303 (2002) 1–15.

[15] M.I. Prodromidis, M.I. Karayannis, Enzyme based amperometric biosensors for food analysis, Electroanalysis 14 (2002) 241–261.

[16] H. Suzuki, Advances in the microfabrication of electrochemical sensors and systems, Electroanalysis 12 (2000) 703–715.

[17] P.B. Danielson, The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans, Curr. Drug Metab. 3 (2002) 561-597.

[18] M. Sono, M.P. Roach, E.D. Coulter, J.H. Dawson. Heme-Containing Oxygenases. Chem. Rev. 96 (1996) 2841-2887.

[19] S. Kalgutkar, R.S. Obach, T.S. Maurer, Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions, Curr. Drug Metab. 8 (2007) 407-447.

[20] W.J.R. Santos, A.L. Souza, M.D.P.T Sotomayor, F.S. Damos, S.M.C.N. Tanaka, L.T. Kubota, A.A. Tanaka, Mangnese Phthalocyanine as a Biomimetic Electrocatalyst for Phenols in the Development of an Amperometric Sensor, J. Braz. Chem. Soc. 20 (2009) 1180-1187.

[21] A. Wong, M.R.V. Lanza, M.D.P.T. Sotomayor, Development and Application of a Highly Selective Biomimetic Sensor for Detection of Captopril, an Important

Ally in Hypertension Control., Comb. Chem. High Throughput Screening 13 (2010) 666-674.

[22] V. Batista, M.R.V. Lanza, I.L.T. Dias, S.M.C.N. Tanaka, A.A. Tanaka, M. D.P.T. Sotomayor, Electrochemical sensor highly selective for estradiol valerate determination based on a modified carbon paste with iron tetrapyridinoporphyrazine, Analyst 133 (2008) 1692-1699.

[23] A.C. Boni, M.D.P.T. Sotomayor, M.R.V. Lanza, S.M.C.N. Tanaka, A.A. Tanaka, Application of a Biomimetic Sensor Based on Iron Phthalocyanine Chloride: 4-Methylbenzylidene-Camphor Detection, J. Braz. Chem. Soc. 21 (2010) 1377-1383.

[24] A. Wong, M.D.P.T. Sotomayor, Biomimetic sensor based on 5,10,15,20-tetrakis(pentafluorophenyl)-*21H,23H*-porphyrin iron (III) chloride and MWCNT for selective detection of 2,4-D, Sensors and Actuators B: Chemical (2013), 181, 332–339.

[25] Z. Zhang, D. Wang, L. Zhang, M. Du, G. Chen, Determination of diuretics in human urine by hollow fiber-based liquid-liquid-liquid microextraction coupled to high performance liquid chromatography, Analyst (2008), 133(9), 1187-1194.

[26] A.J. Bard, R.D. Faulkner, Electrochemical methods-fundamental and applications. 2nd ed. NewYork: Wiley, 2001.

[27] M.D.P.T. Sotomayor, A.A. Tanaka, R.S. Freire, L.T. Kubota, Amperometric sensors based on biomimetic catalysts, in: C.A. Grimes, E.C. Dickey, M.V. Pishko (Eds.), Encyclopedia of Sensors, American Scientific Publishers, California, 2006.

CHART

Chart 1. Chemical structure to catalyst used in this work.

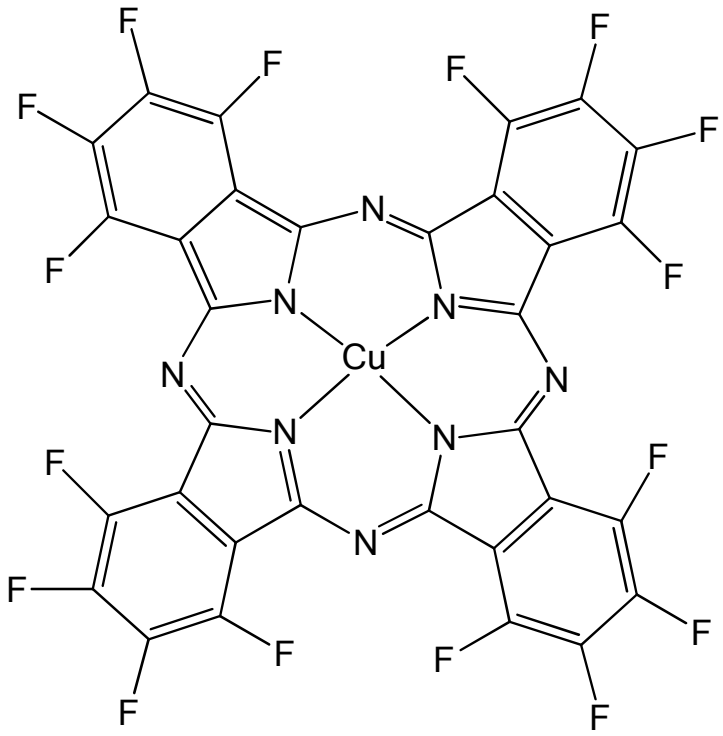


FIGURE CAPTIONS

Figure 1: Electrochemical profiles obtained by cyclic voltammetry in the absence (A) and presence (B) of complex CuPC with (---) adition of 4.8 x 10^{-5} mol L^{-1}. Measurements performed using a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) and scan rate of 50 mV s^{-1}.

Figure 2: Pareto diagram for visualizing the effects of the potenciostat and chemical variables on the square wave voltammetry measurements using a $2^{(7-3)}$ factorial design.

Figure 3: Central composite design response surface obtained for sensitivity values as a function of frequency and step potential.

Figure 4: Plot of the scan rate-normalized current density ($\Delta i\ v^{-1/2}$) *versus* the scan rate (v). [BMT]= 1.0×10^{-4} mol L^{-1} in 0.1 mol L^{-1} B.R. buffer solution.

Figure 5: Schematic representation of the possible mechanism response to the proposed biomimetic sensor.

Figure 6: Lineweaver-Burk plot for the BMT oxidation catalyzed by the CuPc-based sensor.

Figure 7: Typical square wave voltamogram obtained for successive additions of bumetanide. Inset the analytical curve. Measurements performed under optimized conditions.

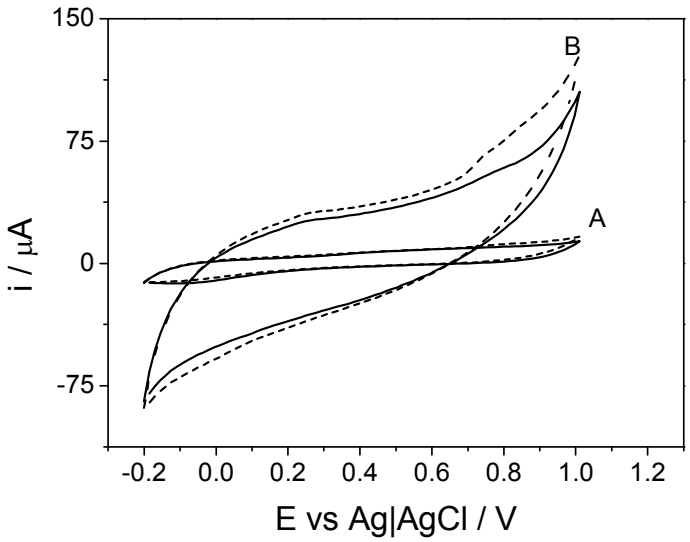


FIGURE 1

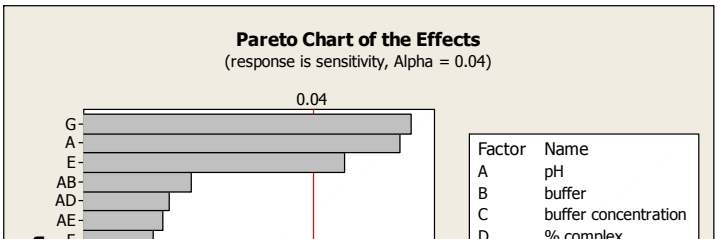


FIGURE 2

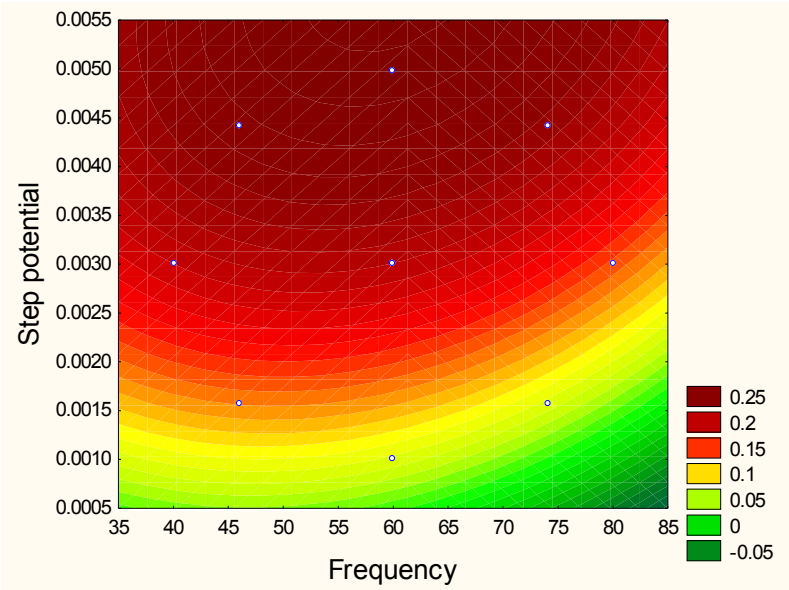


FIGURE 3

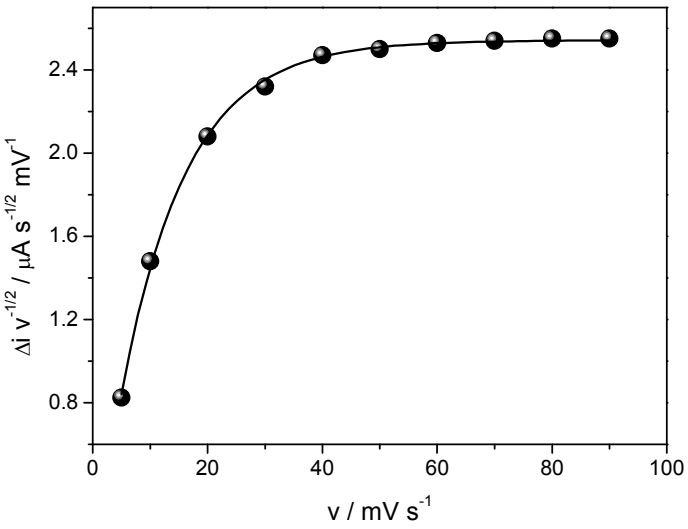


FIGURE 4

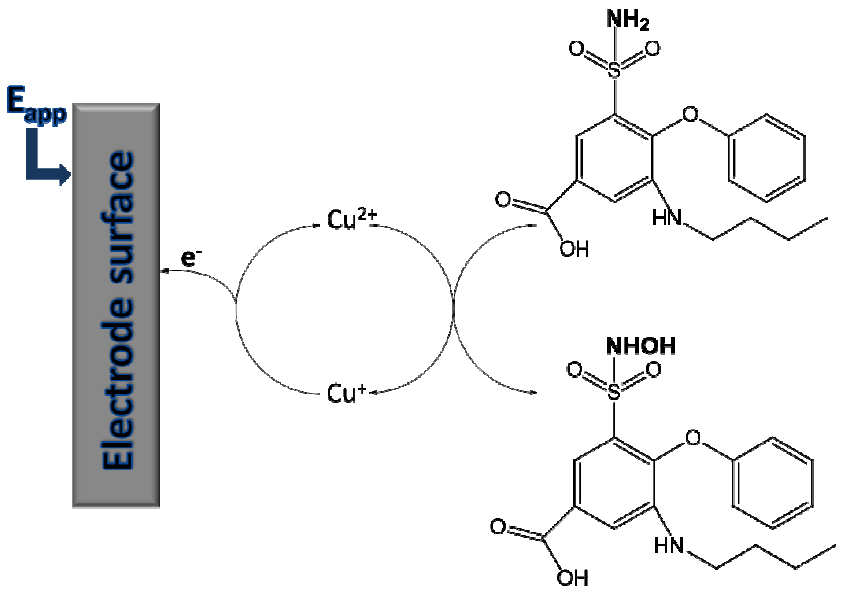


FIGURE 5

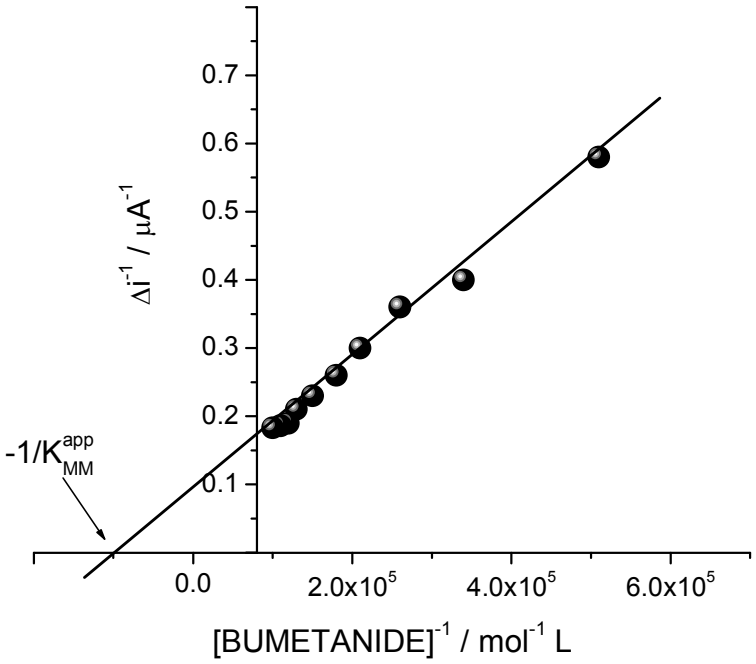


FIGURE 6

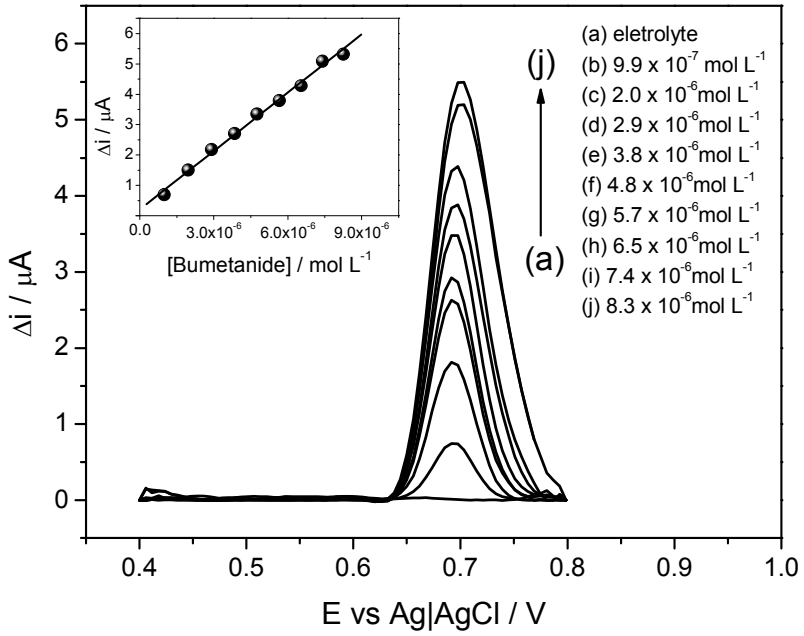


FIGURE 7

Table 1. Parameters optimized used multivariate calibration for the proposed biomimetic sensor for bumetanide quantification.

Variables	Parameters optimized
Amount of complex in the paste (% w/w)	15
pH	7.0
buffer concentration (mol L^{-1})	0.15
Buffer	Britton Robinson
Amplitude (V)	0.1
Frequency (Hz)	60
Step potential (V)	0.006

Table 2. Determination of bumetanide in pharmaceutical formulations.

Sample[a]	Proposed method [BMT] x 10^{-4} (mol L^{-1})	Comparative method [BMT] x 10^{-4} (mol L^{-1})	*t*-Test[c]
	Found[b]	Found[b]	
A	0.961 ± 0.028	1.0020 ± 0.0002	2.54
B	0.957 ± 0.030	1.0140 ± 0.0001	3.29
C	0.989 ± 0.024	1.0320 ± 0.0002	3.10

[a] Declared value: 1 mg bumetanide / tablet
[b] Standard deviation of three replicates.
[c] Critical values of t at 95% confidence level, t_t = 4.303. Values obtained considering the value suppliedby the comparative method as the true.

Table 3. Recoveries of bumetanide added to urine samples

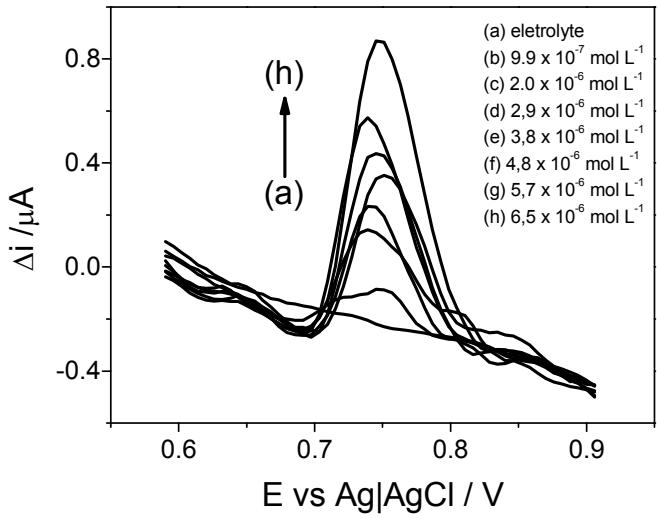
Sample[a]	*Proposed method* [BMT] x 10^{-4} (mol L^{-1})		*Comparative method* [BMT] x 10^{-4} (mol L^{-1})		
	Found[b]	*Recovery (%)*	*Found*[b]	*Recovery (%)*	*t-Test*[c]
A	*0.961 ± 0.023*	*96*	*1.00300 ± 0.00001*	*100*	*3.16*
B	*0.962 ± 0.026*	*96*	*1.00200 ± 0.0001*	*100*	*2.66*
C	*0.952 ± 0.031*	*95*	*1.0290 ± 0.0002*	*103*	*4.30*
D	*0.963 ± 0.035*	*96*	*1.0150 ± 0.0001*	*101*	*2.57*
E	*0.950 ± 0.035*	*95*	*1.0090 ± 0.0001*	*101*	*2.92*
F	*0.971 ± 0.038*	*97*	*1.0490 ± 0.0002*	*105*	*3.56*

[a] Added value: 1.0 x 10^{-4} mol L^{-1}
[b] Standard deviation of three replicates.
[c] Critical values of t at 95% confidence level, t_t = 4.303. Values obtained considering the value supplied by the comparative method as the true.

Supplementary Data

Influence of the copper complex in the sensor response

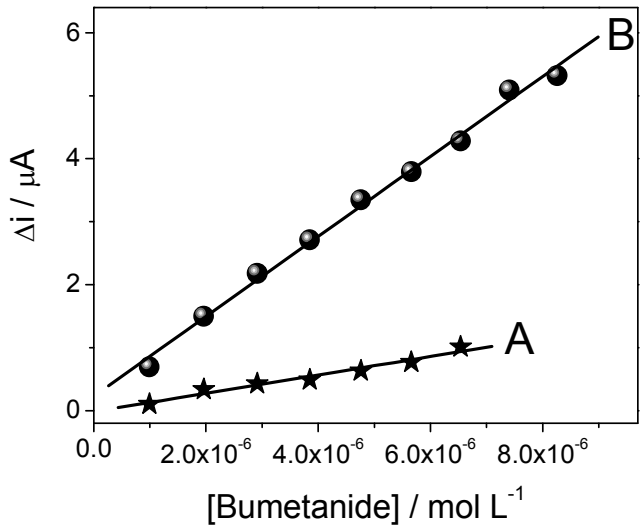
When compared the CPE response with Figure 7 (sensor response), is observed that the unmodified paste shows not quantitative currents and occurs adsorption of the oxidation product on the electrode surface, since the E_p change to each addition of analyte, differently those that is observed in the proposed sensor using the copper complex as modifier, in which the E_p appears at lower potential (700 mV vs Ag|AgCl). Thus, these results shows the importance of the biomimetic catalyst in the sensor construction and quantification of bumetanide.



S1. Typical square wave voltammograms obtained for successive additions of bumetanide using an unmodified carbon paste electrode (CPE).

The analytical curves for the CPE and proposed sensor (based on copper (II) 1, 2, 3, 4, 8, 9, 10, 11,15,16,17,18,22,23,24,25 hexadecafluoro-*29*–*H,31-H*–phthalocyanine) are shown in S2. For CPE the linear response was in the range from 9.9 x 10^{-7} to 6.5 x10^{-6} mol L^{-1}, adjusted by the mathematical equation (1):

I_p = -0.016 (± 0.052) + 145,815 (± 12,293) [BMT] (R =0.983) (eq. 1)



S2. Analytical curves in the absence (A) and presence (B) of complex CuPc in carbon paste. Measurements performed under optimized conditions.

In addition, for quantification of bumetanide, the modified carbon paste electrode (sensor) showed a sensitivity of about 4.4 times larger than the unmodified graphite.

FIGURE CAPTIONS

Figure 1: Electrochemical profiles obtained by cyclic voltammetry in the absence (A) and presence (B) of complex CuPC with (---) adition of 4.8 x 10^{-5} mol L^{-1}. Measurements performed using a 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) and scan rate of 50 mV s^{-1}.

Figure 2: Pareto diagram for visualizing the effects of the potenciostat and chemical variables on the square wave voltammetry measurements using a $2^{(7-3)}$ factorial design.

Figure 3: Central composite design response surface obtained for sensitivity values as a function of frequency and step potential.

Figure 4: Plot of the scan rate-normalized current density ($\Delta i\ v^{-1/2}$) *versus* the scan rate (v). [BMT]= 1.0×10^{-4} mol L^{-1} in 0.1 mol L^{-1} B.R. buffer solution.

Figure 5: Schematic representation of the possible mechanism response to the proposed biomimetic sensor.

Figure 6: Lineweaver-Burk plot for the BMT oxidation catalyzed by the CuPc-based sensor.

Figure 7: Typical square wave voltamogram obtained for successive additions of bumetanide. Inset the analytical curve. Measurements performed under optimized conditions.

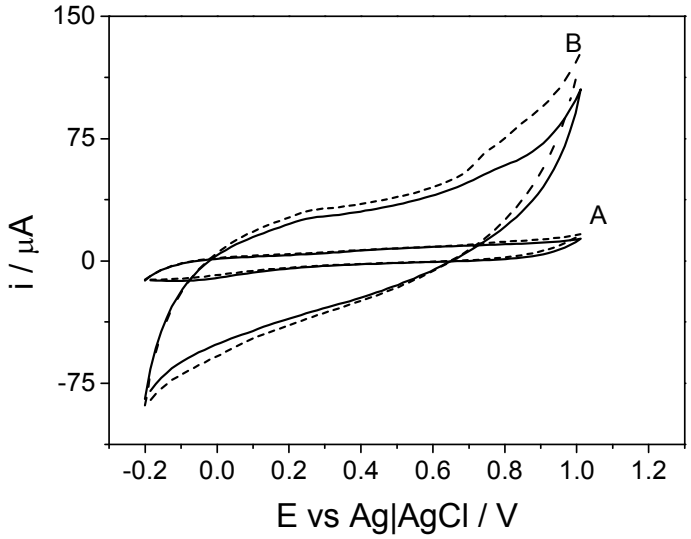


FIGURE 1

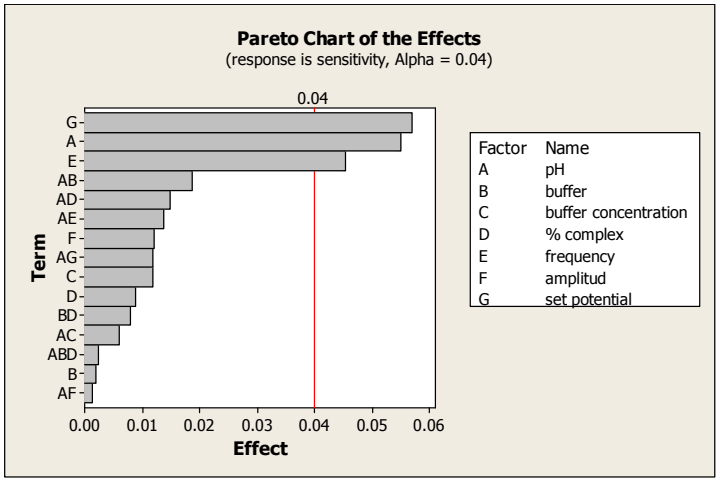


FIGURE 2

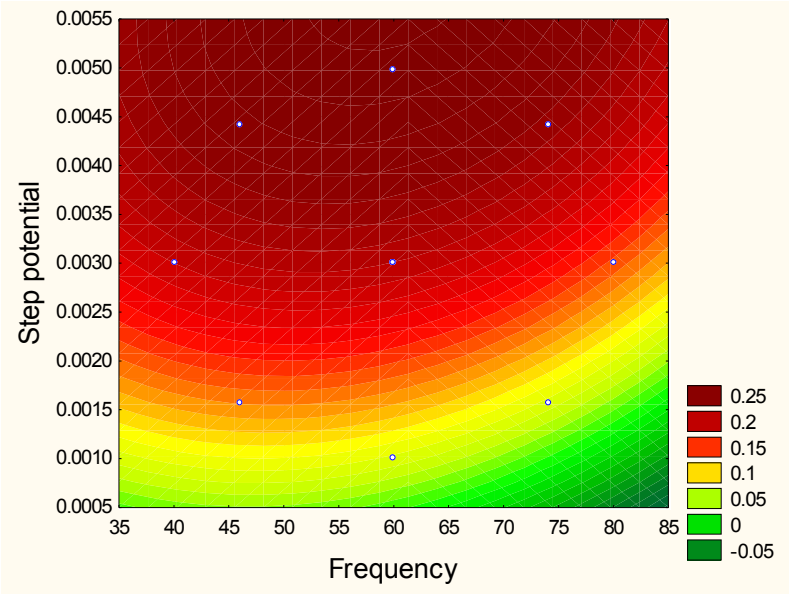


FIGURE 3

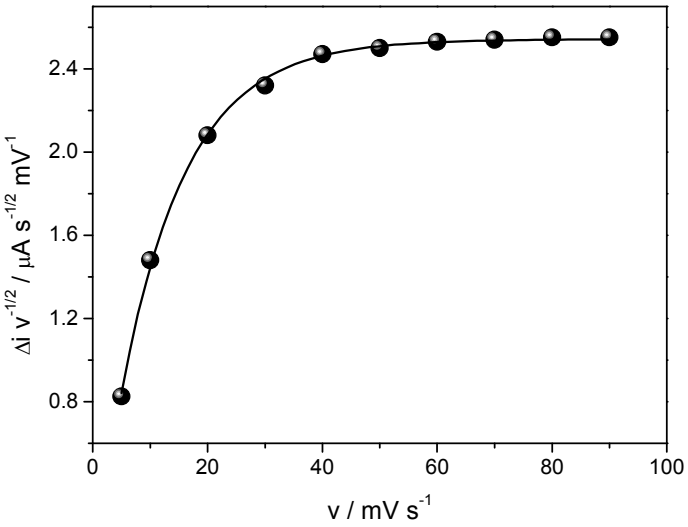


FIGURE 4

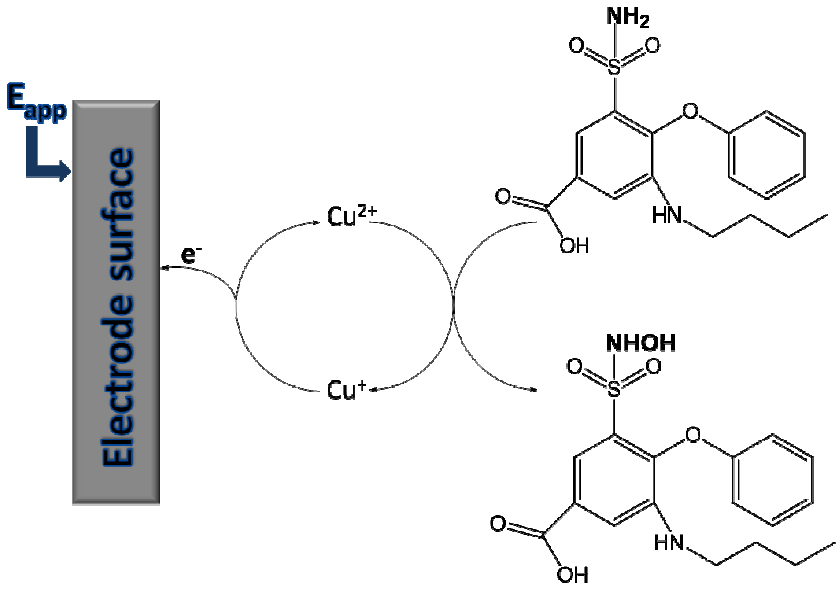


FIGURE 5

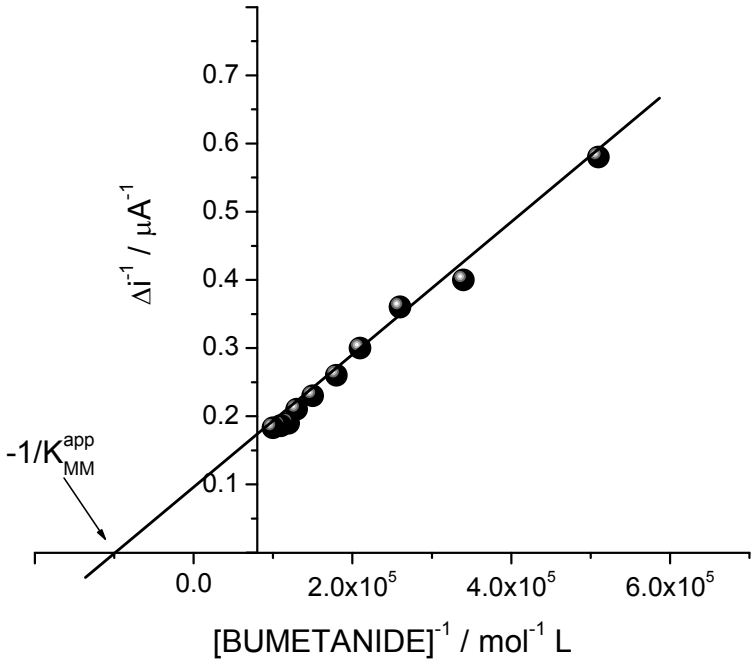


FIGURE 6

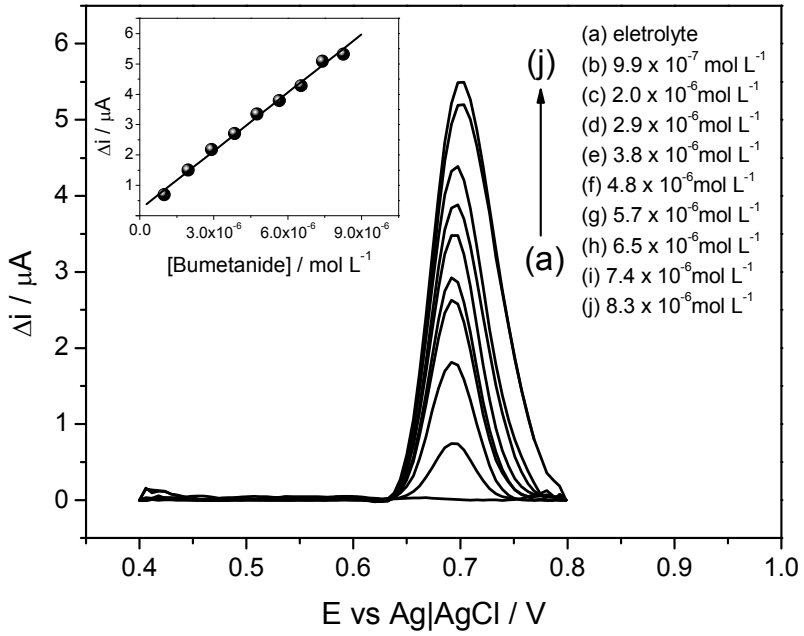


FIGURE 7

Table 1. Parameters optimized used multivariate calibration for the proposed biomimetic sensor for bumetanide quantification.

Variables	**Parameters optimized**
Amount of complex in the paste (% w/w)	15
pH	7.0
buffer concentration (mol L^{-1})	0.15
Buffer	Britton Robinson
Amplitude (V)	0.1
Frequency (Hz)	60
Step potential (V)	0.006

Table 2. Determination of bumetanide in pharmaceutical formulations.

Sample[a]	Proposed method [BMT] x 10^{-4} (mol L^{-1})	Comparative method [BMT] x 10^{-4} (mol L^{-1})	*t*-Test[c]
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