

Electrochemical Behavior of Propranolol and its Major Metabolites, 4'-Hydroxypropranolol and 4'-Hydroxypropranolol Sulfate, on Glassy Carbon Electrode

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Foi estudado o comportamento eletroquímico do propranolol (PRO) e seus principais metabólitos. Medidas voltamétricas foram realizadas em eletrodo de carbono vítreo (GCE). Todos os compostos examinados sofrem oxidação via eletroquímica. Em todos os casos foi observada uma separação dos picos de oxidação, sugerindo a possibilidade de eletroanálise simultânea de propranolol e seus metabólitos. A linearidade das curvas de calibração foi obtida para concentrações entre $4,22 \times 10^{-6}$ - $1,35 \times 10^{-4}$ mol L⁻¹ para PRO, $4,00 \times 10^{-6}$ - $4,81 \times 10^{-5}$ mol L⁻¹ para 4'-hidroxipropranolol (4'OH PH) e $3,52 \times 10^{-6}$ - $4,22 \times 10^{-5}$ mol L⁻¹ para o sulfato de 4'-hidroxipropranolol (4'OH PS). O método descrito é rápido e simples, e pode ser aplicado para determinar os compostos mencionados acima em amostras biológicas. Os resultados obtidos foram verificados por cromatografia líquida de ultra-alta eficiência (ultra HPLC).

The electrochemical behavior of propranolol (PRO) and its major metabolites was studied. Voltammetric measurements were made on glassy carbon electrode (GCE). All examined compounds were electrochemically oxidized. In all cases, a separation of oxidation peaks was observed, suggesting the possibility of simultaneous electroanalysis of propranolol and its metabolites. The linearity of the calibration curves was obtained for concentrations between 4.22×10^{-6} - 1.35×10^{-4} mol L⁻¹ for PRO, 4.00×10^{-6} - 4.81×10^{-5} mol L⁻¹ for 4'-hydroxypropranolol (4'OH PH) and 3.52×10^{-6} - 4.22×10^{-5} mol L⁻¹ for 4'-hydroxypropranolol sulfate (4'OH PS). The described method is rapid and simple, and could be applied to determine the above mentioned compounds in biological samples. The obtained results were verified by ultra-high performance liquid chromatography (Ultra HPLC) method.

Keywords: propranolol, 4'-hydroxypropranolol, 4'-hydroxypropranolol sulfate, voltammetry, glassy carbon electrode

Introduction

Propranolol (PRO), 1-(isopropylamino)-3-(1-naphthoxy)-2-propanol, is a widely used β -adrenergic drug, which has been administered to treat hypertension, cardiac arrhythmias and prophylaxis of secondary myocardial infarction.

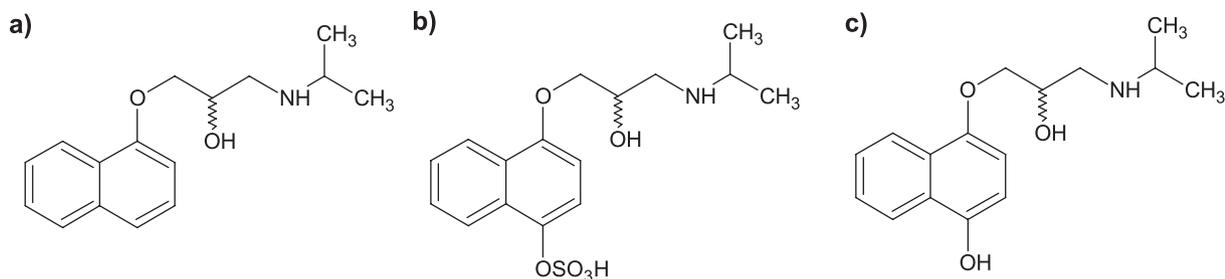
PRO is consumed in sport and other stressful activities, behaving as a doping agent. The drug slows down the heart rate of animals, leading to its use in the horse racing industry. PRO is rapidly metabolized after oral administration, and even traces of the drug may be difficult to detect in biological fluids some time after administration.

The detection of the metabolites of the drug will provide proof of its consumption.

Ingested PRO is excreted mainly as the sulfate conjugate, 4'-hydroxypropranolol sulfate (4'OH PS) (Scheme 1). The determination of the presence of this metabolite could yield useful information about PRO consumption. The second interesting metabolite of PRO is 4'-hydroxypropranolol (4'OH PH), which displays β -blocking properties.

High performance liquid chromatography (HPLC) methods have been commonly used to determine the presence of PRO.¹⁻⁴ Simultaneous determination of PRO and 4'OH PH has already been reported.⁵⁻⁷ PRO and 4'OH PH exhibit natural fluorescence properties, permitting the use of HPLC methods with fluorescence detectors for both compounds. Other methods that have been reported include capillary

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Scheme 1. Structure of PRO (a), 4'OH PS (b) and 4'OH PH (c).

electrophoresis,⁸⁻¹⁰ spectrofluorimetric^{11,12} and electrochemical methods.¹³⁻¹⁵ A polarographic method was used to determine the quantity of PRO in pharmaceutical formulations. PRO was combined with nitric acid to yield nitropropranolol, which was then measured in Britton-Robinson buffer on a static mercury drop electrode.¹³ Adsorptive stripping differential pulse voltammetry was used to quantify PRO in a tablet dosage form on a carbon paste electrode.¹⁴ Another electrochemical method used a stabilized lipid film biosensor for rapid screening of PRO in pharmaceutical pills.¹⁵

Electrochemical methods have only been applied to the determination of PRO in pharmaceutical formulations, and to the best of our knowledge, no such methods have been established to find the major metabolites of PRO.

The aim of this study was to evaluate the usefulness of cyclic voltammetry (CV) and differential pulse voltammetry (DPV) as simple and rapid methods in the determination of PRO and its metabolites. These methods have been applied to characterize the electrochemical behavior of PRO, 4'OH PS and 4'OH PH on a glassy carbon electrode (GCE). The 4'OH PH has β -adrenergic properties, while 4'OH PS is the metabolite of PRO with the highest concentration in patient urine. Therefore, a new voltammetric method for the simultaneous determination of these compounds is very important. The electrochemical behavior was also studied using multi-wall carbon nanotube (MWCNT)-modified GCE. However, those results were no better than those obtained from a bare GCE, especially for PRO and 4'OH PS, substances that could be present in urine, so the latter unmodified electrode was used. This paper also proves that selected metabolites can be determined simultaneously in the presence of PRO. PRO and its metabolites in urine samples were also determined by ultra high performance liquid chromatography (ultra HPLC) method.

Experimental

Materials

The hydrochloride salt of PRO was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Metabolites

of PRO, 4'-hydroxypropranolol sulfate (4'OH PS) and 4'-hydroxypropranolol (4'OH PH) as its hydrochloride salt, were bought from Toronto Research Chemicals Inc. (North York, Canada). Acetic acid (CH_3COOH), nitric acid (HNO_3), *o*-boric acid (H_3BO_3), *o*-phosphoric acid (H_3PO_4), sodium hydroxide (NaOH), dimethylformamide (DMF), methanol and ethanol, all of analytical grade, were bought from POCH (Gliwice, Poland).

Instrumentation

The voltammetric measurements were conducted using the $\mu\text{AUTOLAB}$ potentiostat, type III (Eco-Chemie, Netherlands). All measurements were carried out in a three-electrode cell with a glassy carbon electrode (GCE), or a multi-wall carbon nanotube (MWCNT)-modified GCE as the working electrode. A saturated silver/silver chloride electrode ($\text{Ag}/\text{AgCl}/\text{KCl}_{(\text{sat.})}$) and platinum wire were used as the reference and auxiliary electrodes, respectively. Voltammetric measurements were carried out in a 3 mL electrochemical glass cell. Nitrogen was used to remove oxygen from the measured solutions. The pH of the buffer solutions was measured using a pH meter of model CP-401 (ELMETRON, Zabrze, Poland).

A reversed phase ultra HPLC system containing a UV detector model L-2400U (Hitachi, Tokyo, Japan), L-2350 column oven (Hitachi, Merck, Tokyo, Japan), two L-2160U pumps, and a reversed phase Chromolith[®] Fast Gradient monolithic $\text{C}_{18\text{e}}$ column (50 mm \times 2 mm) (Merck, Germany) was used. Samples were injected by an L-2200U autosampler (Hitachi, Tokyo, Japan).

Preparation of the modified electrode

The MWCNT (BU-201, Bucky USA, Houston, USA) were purified by stirring in a 2 mol L^{-1} nitric acid solution for 24 h. Next, 2 mg of MWCNT were mixed with 1 mL of DMF. The solution was then sonicated for 3 h. A 2 μL aliquot of the solution was then placed directly onto a polished GCE and allowed to dry. It was rinsed with water before use.

Stock solutions

5.00 mg (0.0141 mmol) of 4'OH PS, 5.00 mg (0.0160 mmol) of 4'OH PH and 11.23 mg of propranolol hydrochloride, correspond to 10.00 mg of PRO (0.0339 mmol) were weighed to make stock solutions of each in 10 mL volumetric flasks, by dissolving the reference substances into mixtures of methanol:water (1:1 v/v). The resulting concentrations of the stock solutions of 4'OH PS, 4'OH PH and PRO were 1.41×10^{-3} mol L⁻¹, 1.60×10^{-3} mol L⁻¹, and 3.39×10^{-3} mol L⁻¹, respectively. The stock solutions were stored at 4 °C and were stable for at least three weeks.

Analytical procedure

The GCE was polished manually to a mirror finish using an alumina slurry. The particle sizes of the alumina used were 1.0, 0.3 and 0.05 µm. Before transferring the electrode to the electrolyte solutions, the GCE was cleaned with water in an ultrasonic bath. A 2 mL portion of the Britton-Robinson buffer (BR), which served as a supporting electrolyte, was transferred into the 3 mL voltammetric glass cell. Oxygen was removed by purging with pure nitrogen for 10 min. The voltammogram of the pure supporting electrolyte was recorded, and an appropriate amount of stock solution was added. The series of voltammograms were recorded at different analyte concentrations. The measurements were performed in triplicate, using fresh sample solutions. The experimental conditions for the voltammetric measurements were: initial potential -0.5 V, final potential 1.5 V, starting potential 0 V, and scan rate in the range of 0.005-0.3 V s⁻¹. The parameters used in DPV measurements were: initial potential -0.3 V, final potential 1.5 V, scan rate 0.025 V s⁻¹, pulse amplitude 50 mV, and pulse width 50 ms. All the experiments were performed at ambient laboratory temperature.

Calibration curves

The calibration curves were evaluated by a least squares linear regression method. The correlation between peak current and concentration was recorded in concentration ranges of 3.39×10^{-6} - 2.03×10^{-4} mol L⁻¹ for PRO, 1.75×10^{-6} - 5.62×10^{-5} mol L⁻¹ for 4'OH PS and 2.00×10^{-6} - 6.41×10^{-5} mol L⁻¹ for 4'OH PH. The peak current was measured from polynomial baseline.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated using the relationship kSD_b/a , where $k = 3$ or $k = 10$, respectively, where the SD_b parameter is the standard deviation of the intercept, and a is the slope of the calibration curve.¹⁶

Determination of PRO, 4'OH PS and 4'OH PH in urine samples

Urine samples were collected 2, 4, 5 and 10 h after administration of PRO (10 mg dose of PRO was administrated), and were filtered through 0.45 µm nylon Bakerbond filters to eliminate the residues. Each 5 mL sample of urine was precipitated adding 5 mL acetonitrile and 5 mL of phosphate buffer (pH 6.88) with stirring. The samples were centrifuged (2500 rpm) for 10 min. The supernatant was filtered through a 0.45 µm nylon Bakerbond filter to remove precipitated proteins from urine samples and transferred to an Oasis® HLB cartridge (500 mg, 6 mL). The solid phase extraction (SPE) procedure consisted of conditioning (6 mL methanol, 6 mL distilled water), sample loading, washing (4 mL distilled water), drying and elution (2×2 mL of methanol). The eluates were evaporated to dryness under a stream of nitrogen. The residues were dissolved in 2 mL BR buffer and transferred to the voltammetric cell.

The recoveries of the determined substances were established for blank human urine samples spiked (before SPE enrichment step) with known amounts of PRO and metabolites. The results obtained for real urine samples were verified by ultra HPLC method.¹⁷

Determination of PRO, 4'OH PS and 4'OH PH by ultra HPLC method

Analyses were carried out at 20 °C with gradient elution.¹⁷ The best gradient program evaluated for the determination using the C₁₈ monolithic column was defined as 0.05% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution program is presented in Table 1. The PRO and its metabolites were monitored by UV detection at $\lambda = 227$ nm.

Table 1. Gradient elution program for ultra-HPLC equipment solvents: A, 0.05% TFA in water; B, Acetonitrile

time / min	Solvent A / (%)	Solvent B / (%)	Flow / (mL min ⁻¹)
0.0	100	0	1.5
2.0	98	2	1.0
4.0	90	10	0.9
5.0	90	10	1.5
10.0	10	90	1.5

The samples were prepared similarly as reported for voltammetric determination by SPE method. The eluates were evaporated to dryness under a stream of nitrogen.

The residues were dissolved in 1 mL of a methanol:water mixture (1:9, v/v) and then 1 mL was introduced to the chromatographic column.

Results and Discussion

The imino and hydroxyl groups in PRO and its metabolites suggest that the compounds may be electrochemically oxidized. The set of voltammograms shown in Figure 1 confirms this finding. As the voltammogram of PRO shows, the only one well-defined oxidation peak occurs on GCE in acidic solution. The electrochemical oxidation of the metabolites 4'OH PS and 4'OH PH results in two well-defined peaks. For each metabolite there are only three peaks appearing in the entire potential range between -0.5 and 1.5 V, two resulting from oxidation processes and one from reduction.

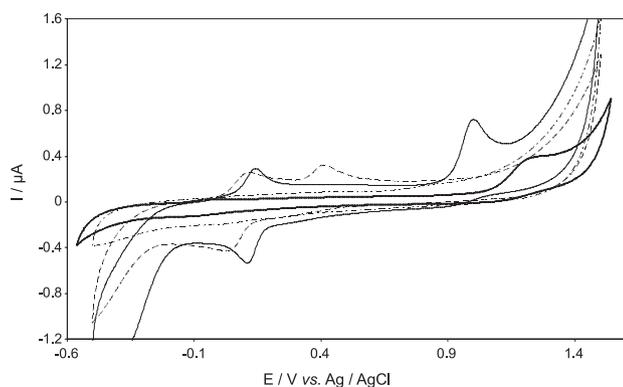


Figure 1. Cyclic voltammograms recorded for 1.35×10^{-4} mol L⁻¹ PRO (heavy line), 4.22×10^{-5} mol L⁻¹ 4'OH PS (solid line) and 4.81×10^{-5} mol L⁻¹ 4'OH PH (dashed line) in BR buffer of pH 3.00 on GCE. For comparison, the background curve obtained in pure BR buffer is shown (dotted line). Initial potential -0.5 V, final potential 1.5 V, starting potential 0 V, scan rate 0.025 V s⁻¹.

The first oxidation peaks for 4'OH PS and 4'OH PH, appear at 0.156 V and 0.114 V respectively and are connected with the reduction peaks at 0.098 V and 0.048 V corresponding to reversible systems, as shown by the distances between reduction and oxidation peaks, close to theoretical values 0.059 V. Those redox couples could be connected with phenol oxidation giving possibly quinone systems. The second oxidation peaks of these two metabolites are related to irreversible processes, thus making the peaks at around 0.4 V and 1.0 V analytically useful. The presence of analyzed compounds in solution does not affect the background CV, except in the region in which these molecules are being reduced or oxidized. That unchanged background suggests a lack of adsorption of the analyzed compounds on GCE surface. This observation is verified in the scan rate dependence studies, described below.

Effect of pH

The pH effect of the electrolyte was examined between pH values of 2.00 – 9.00 for PRO and metabolites. As pH decreases, the oxidation turns more difficult and shifts to more positive potential. The position of the peak potential E_p vs. pH was obtained for all examined compounds. The cyclic voltammograms of the PRO metabolites show more than one peak. Therefore, only the irreversible oxidation peaks were used to evaluate the effects of pH (for 4'OH PS peak at a potential near 1.0 V, for 4'OH PH peak at a potential near 0.4 V). The observed pH effects corroborate the use of acidic buffered solutions as supporting electrolytes. The oxidation peaks of all examined compounds were broader in neutral media, making quantification unreliable. However the highest peaks were observed at pH 3.0 . Therefore this pH value was used for analytical applications.

The E_p vs. pH dependence for 1.35×10^{-4} mol L⁻¹ of PRO at pH < 9 may be expressed by equation:

$$E_p/V = 1.399 - 0.055 \text{ pH} \quad (1)$$

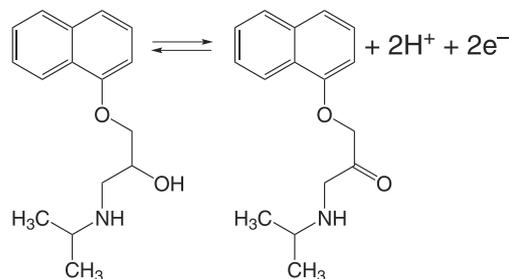
The slope of the equation suggests that the number of electrons and protons transferred in the reaction of PRO are equal. Similar relationships were determined for metabolites of PRO. The adequate relationships are presented below for 4.22×10^{-5} mol L⁻¹ of 4'OH PS (equation 2) and for 4.81×10^{-5} mol L⁻¹ of 4'OH PH (equation 3):

$$E_p/V = 1.292 - 0.056 \text{ pH} \quad (2)$$

$$E_p/V = 0.541 - 0.053 \text{ pH} \quad (3)$$

In both cases, the plots have slopes close to 0.059 , which is characteristic for a reaction in which the number of electrons and protons that are transferred is the same. The plots of peak potentials as a function of pH were inserted in Figures 2, 3 and 4, respectively.

The observed oxidation peak for PRO is probably connected with the oxidation of the secondary alcoholic group in the PRO molecule.¹⁸ The proposed mechanism involves 2 protons and 2 electrons according to:



The irreversible oxidation peak observed for metabolites of PRO could be connected with adequate mechanism of oxidation process.

Effect of scan rate

The peak current was plotted against scan rate in order to determine whether the irreversible oxidation processes of PRO and its metabolites are diffusion- or adsorption-controlled.^{19,20} The linear correlation between peak current and scan rate is characteristic of adsorption-controlled processes, indicating that the electroactive species are being adsorbed onto the electrode surface. The linear relationship between peak current and square root of scan rate is typical for diffusion-controlled systems, meaning that the correlation between peak current and the concentration of analyte could be used as an analytical tool. In this study, the effect of the scan rate was determined in the scan rate range of 0.005-0.25 V s⁻¹. The peak current for PRO solutions was found to be linear to the square root of the scan rate according to the relationship:

$$I_p / \mu\text{A} = 0.069 (v / (\text{mV s}^{-1}))^{1/2} + 0.028 \quad (4)$$

with a correlation coefficient of $R^2 = 0.9983$. The dependence indicates that the process is diffusion-controlled. A similar dependence was determined for PRO metabolites. For 4'OH PS, the dependence was determined on the basis of a peak found at a potential around 1.0 V. The peak current vs. square root of scan rate is described as:

$$I_p / \mu\text{A} = 0.062 (v / (\text{mV s}^{-1}))^{1/2} + 0.080 \quad (5)$$

with a correlation coefficient of $R^2 = 0.9961$. The effect of the potential scan rate on the peak current may be expressed as $\log(I_p)$ vs. $\log(v)$, which has a slope of 1.0 for the species confined to the electrode surface, or 0.5 for the diffusion controlled system. For the 4'OH PS, the dependence of $\log(I_p)$ vs. $\log(v)$ can be expressed by the equation:

$$\log(I_p / \mu\text{A}) = 0.425 \log(v / (\text{mV s}^{-1})) - 0.999 \quad (6)$$

with a correlation coefficient of $R^2 = 0.9957$.

The value of the slope confirms that the process is diffusion-controlled. The effect of the scan rate for 4'OH PH was determined on the basis of measurements of peak current at a potential around 0.4 V. The relationship obtained in this situation also indicates that the process is diffusion-controlled. The peak current vs. square root of scan rate may be expressed as:

$$I_p / \mu\text{A} = 0.035 (v / (\text{mV s}^{-1}))^{1/2} + 0.011 \quad (7)$$

with a correlation coefficient of $R^2 = 0.9976$.

Analogously, as for 4'OH PS, the relationship of $\log(I_p)$ vs. $\log(v)$ was examined. The dependence could be expressed by the equation:

$$\log(I_p / \mu\text{A}) = 0.467 \log(v / (\text{mV s}^{-1})) - 1.379 \quad (8)$$

with a correlation coefficient of $R^2 = 0.9981$.

The value of the slope confirmed that the process is diffusion-controlled.

Calibration curves of PRO, 4'OH PS and 4'OH PH

The DP voltammograms, recorded at a scan rate of 0.025 Vs⁻¹, were used with the calibration curves. This scan rate value was applied because, in all cases, the peaks width increases with the increase of scan rate. This effect could generate interferences, especially in simultaneous determination of PRO and metabolites, when the distance between peaks is smaller. The voltammograms were recorded in BR buffer at pH 3.00.

The peak current vs. concentration dependence for PRO was linear over a concentration range of 4.22×10^{-6} - 1.35×10^{-4} mol L⁻¹. The obtained dependence complies with the equation:

$$I_p / \mu\text{A} = 2.1 \times 10^{-3} C / \mu\text{mol L}^{-1} + 0.07 \quad (9)$$

with a correlation coefficient of $R^2 = 0.9956$ (Figure 2).

The calibration curve for 4'OH PS was obtained (on the basis of peak potential appeared approx. 1.0 V) over a concentration range of 3.52×10^{-6} - 4.22×10^{-5} mol L⁻¹ and is expressed by the equation:

$$I_p / \mu\text{A} = 5.0 \times 10^{-3} C / \mu\text{mol L}^{-1} + 0.21 \quad (10)$$

with a correlation coefficient equals $R^2 = 0.9937$. The appropriate voltammograms are shown in Figure 3.

A similar dependence was determined for 4'OH PH (on the basis of peak potential appeared approx. 0.4 V) over a concentration range of 4.00×10^{-6} - 4.81×10^{-5} mol L⁻¹ (Figure 4). The resulting straight line is expressed by the equation:

$$I_p / \mu\text{A} = 3.1 \times 10^{-3} C / \mu\text{mol L}^{-1} + 0.05 \quad (11)$$

with a correlation coefficient of $R^2 = 0.9944$.

The acquired parameters of the calibration curves and the values of LOD and LOQ for all examined compounds are summarized in Table 2.

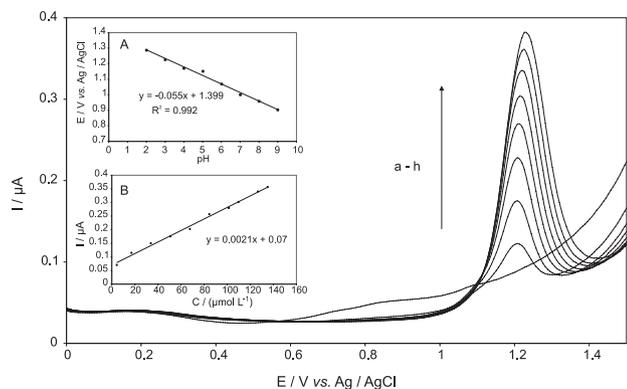


Figure 2. DPV curves recorded for PRO solutions in BR buffer of pH 3.00 on GCE for different concentrations: (a) 4.22×10^{-6} mol L⁻¹, (b) 2.11×10^{-5} mol L⁻¹, (c) 4.22×10^{-5} mol L⁻¹, (d) 6.33×10^{-5} mol L⁻¹, (e) 8.44×10^{-5} mol L⁻¹, (f) 1.06×10^{-4} mol L⁻¹, (g) 1.27×10^{-4} mol L⁻¹, (h) 1.35×10^{-4} mol L⁻¹. Initial potential -0.3 V, final potential 1.5 V, scan rate 0.025 V s⁻¹. Inserts: (A) Plot of peak potentials as a function of pH of buffer solutions. (B) Plot of peak current as a function of PRO concentrations.

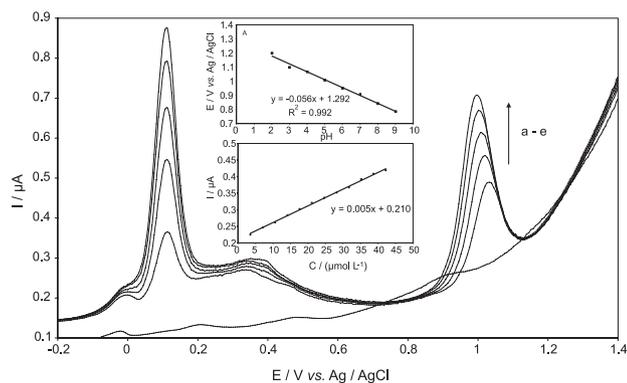


Figure 3. DPV curves recorded for 4'OH PS solutions in BR buffer of pH 3.00 on GCE for different concentrations: (a) 3.52×10^{-6} mol L⁻¹, (b) 1.06×10^{-5} mol L⁻¹, (c) 2.11×10^{-5} mol L⁻¹, (d) 3.17×10^{-5} mol L⁻¹, (e) 4.22×10^{-5} mol L⁻¹. Initial potential -0.3 V, final potential 1.5 V, scan rate 0.025 V s⁻¹. Inserts: (A) Plot of peak potentials as a function of pH of buffer solutions. (B) Plot of peak current as a function of 4'OH PS concentrations.

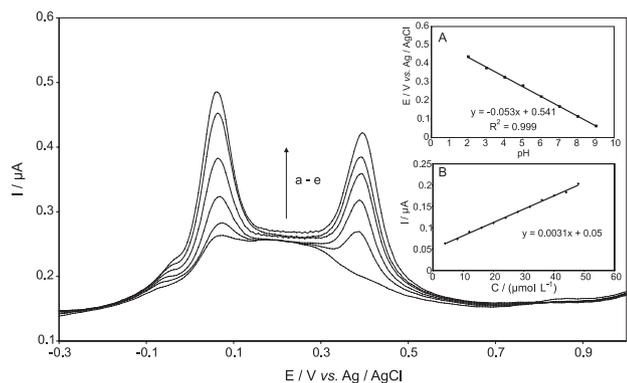


Figure 4. DPV curves recorded for 4'OH PH solutions in BR buffer of pH 3.00 on GCE for different concentrations: (a) 4.00×10^{-6} mol L⁻¹, (b) 1.20×10^{-5} mol L⁻¹, (c) 2.40×10^{-5} mol L⁻¹, (d) 3.61×10^{-5} mol L⁻¹, (e) 4.81×10^{-5} mol L⁻¹. Initial potential -0.3 V, final potential 1.5 V, scan rate 0.025 V s⁻¹. Inserts: (A) Plot of peak potentials as a function of pH of buffer solutions. (B) Plot of peak current as a function of 4'OH PH concentrations.

Determination of PRO, 4'OH PS and 4'OH PH in a mixture

The peak potentials of the compounds examined are sufficiently different to permit the simultaneous determination of PRO and its metabolites. The distances between most peak potentials are more than 0.4 V. Only the distance between the irreversible oxidation peak of 4'OH PS and the peak of PRO is less than 0.2 V, making peak separation unsatisfactory. The distinction between the two was made using the second derivative (Figure 5).

To verify the possibility of simultaneous determination, the DP voltammogram of the buffer solutions at pH 3.00 into which all the compounds were injected was recorded, as shown in Figure 5. The slopes and intercepts of the calibration curves provide the values for the LOD and LOQ, obtained for PRO and its metabolites, as shown in Table 2.

Determination of PRO, 4'OH PS and 4'OH PH on MWCNT-modified GCE

Measurements for many drugs have been more accurate on MWCNT-modified GCE than on bare electrodes, making the modification attractive for our own purposes. Figure 5 shows that the voltammograms recorded on modified electrode are practically identical to those obtained on bare GCE for 4'OH PS. The voltammograms of PRO recorded on MWCNT-modified GCE are worse than adequate voltammograms obtained on bare GCE. Therefore, only the results obtained on unmodified GCE are presented, as that method is faster, simpler and cheaper.

Determination of PRO, 4'OH PS and 4'OH PH in urine samples

The methods described here can be used to determine the presence of the above compounds in urine samples prepared as described in Experimental section. All measurements were performed in triplicate by a standard addition method. Urine samples collected from various patients were examined. The voltammograms of one set of urine sample are shown in Figure 6. The recoveries from urine were measured by spiking drug free urine with known amounts of PRO and metabolites before SPE step. The obtained results are presented in Table 3.

As expected, the level of 4'OH PH concentration was very low, and could not be observed in urine samples 2 h after administration. The peaks characteristic for PRO and 4'OH PS were observed. The matrix signals were observed at potentials lower than 1.0 V and did not have an effect

Table 2. Linear regression equations for calibration plots obtained in the quantitative determination of PRO, 4'OH PS and 4'OH PH (n = 6)

	Slope a / ($\mu\text{A } \mu\text{mol}^{-1} \text{L}$)	Intercept b / μA	Correlation coefficient R ²	Linearity range / ($\mu\text{mol L}^{-1}$)	LOD ^c / ($\mu\text{mol L}^{-1}$)	LOQ ^d / ($\mu\text{mol L}^{-1}$)
PRO	0.0021	0.07	0.9956	4.22-135.0	1.37	4.11
4'OH PS ^a	0.0050	0.21	0.9937	3.52-42.2	1.10	3.31
4'OH PH ^b	0.0031	0.05	0.9944	4.00-48.1	1.29	3.90
PRO in a mixture	0.0020	0.06	0.9951	4.68-135.0	1.43	4.31
4'OH PS ^a in a mixture	0.0048	0.20	0.9989	4.52-42.2	1.45	4.35
4'OH PH ^b in a mixture	0.0030	0.05	0.9937	4.20-48.1	1.37	4.11

^abased on peak potential approximately 1.0 V; ^bbased on peak potential approximately 0.4 V; ^cLOD is the limit of detection; ^d LOQ is the limit of quantification. Regression equation $I_p/\mu\text{A} = a C/\mu\text{mol L}^{-1} + b$, where a is the slope and b the intercept of the calibration plot, C is the concentration of adequate substance.

Table 3. Determination of PRO, 4'OH PH and 4'OH PS in spiked urine samples

	Spiked / (mol L ⁻¹)			Found / (mol L ⁻¹)			Recovery / (%)		
	PRO	4'OH PH	4'OH PS	PRO	4'OH PH	4'OH PS	PRO	4'OH PH	4'OH PS
Sample 1	2.11×10 ⁻⁶	2.00×10 ⁻⁶	1.76×10 ⁻⁶	1.94×10 ⁻⁶	1.87×10 ⁻⁶	1.54×10 ⁻⁶	91.94	93.50	87.50
Sample 2	4.22×10 ⁻⁶	4.00×10 ⁻⁶	3.52×10 ⁻⁶	3.89×10 ⁻⁶	3.75×10 ⁻⁶	3.16×10 ⁻⁶	92.18	93.75	89.77
Sample 3	6.33×10 ⁻⁶	6.00×10 ⁻⁶	5.28×10 ⁻⁶	5.86×10 ⁻⁶	5.63×10 ⁻⁶	4.78×10 ⁻⁶	92.57	93.83	90.53

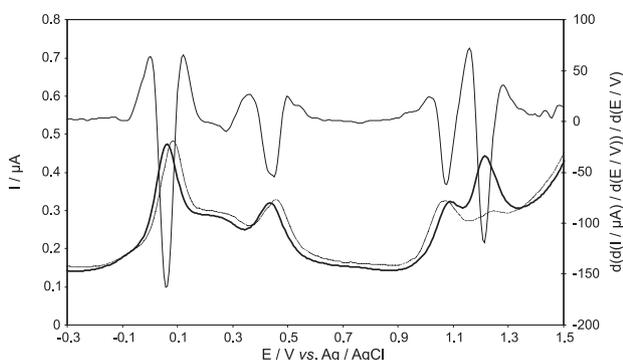


Figure 5. DP voltammogram recorded for a mixture of $4.22 \times 10^{-5} \text{ mol L}^{-1}$ PRO, $5.03 \times 10^{-5} \text{ mol L}^{-1}$ 4'OH PS and $2.40 \times 10^{-5} \text{ mol L}^{-1}$ 4'OH PH in BR buffer at pH 3.00 on GCE (solid line) and MWCNT-modified GCE (dotted line). Gray line is second derivative of DP voltammogram recorded on GCE in the studied potential region. Initial potential -0.3 V , final potential 1.5 V , scan rate 0.025 V s^{-1} .

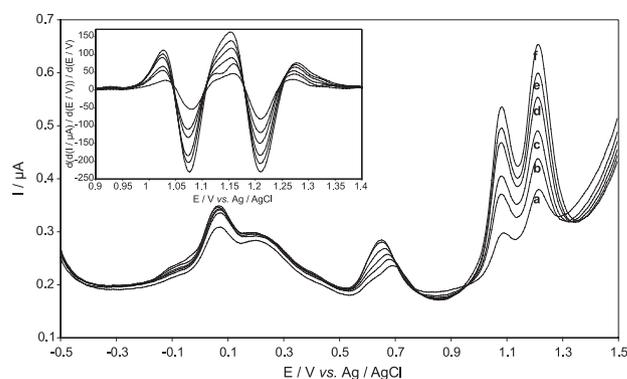


Figure 6. DPV curves recorded for urine sample collected 2 h after drug administration (a) with stock solutions of PRO and 4'OH PS: (b) $8.44 \times 10^{-6} \text{ mol L}^{-1}$ and $3.52 \times 10^{-6} \text{ mol L}^{-1}$, (c) $2.53 \times 10^{-5} \text{ mol L}^{-1}$ and $1.06 \times 10^{-5} \text{ mol L}^{-1}$, (d) $5.06 \times 10^{-5} \text{ mol L}^{-1}$ and $2.11 \times 10^{-5} \text{ mol L}^{-1}$, (e) $7.60 \times 10^{-5} \text{ mol L}^{-1}$ and $3.17 \times 10^{-5} \text{ mol L}^{-1}$ and (f) $1.01 \times 10^{-4} \text{ mol L}^{-1}$ and $4.22 \times 10^{-5} \text{ mol L}^{-1}$ in BR buffer of pH 3.00 on GCE, respectively. The second derivative curves are placed as an insert of the Figure. Initial potential -0.3 V , final potential 1.5 V , scan rate 0.025 V s^{-1} .

on PRO and 4'OH PS determination. The peak observed at potential *ca.* 0.65 V decreases with following scans. The additional experiments indicated that this peak is connected with oxidation reaction of uric acid. The best results were obtained in urine samples collected 2 h after administration, in which the following concentrations were determined: $3.34 \times 10^{-6} \text{ mol L}^{-1}$ of PRO and $5.76 \times 10^{-6} \text{ mol L}^{-1}$ of 4'OH PS. The concentrations of the examined compounds in urine collected from patients were determined to lie in the ranges of 2.15×10^{-6} - $4.52 \times 10^{-6} \text{ mol L}^{-1}$ and 2.04×10^{-6} - $5.76 \times 10^{-6} \text{ mol L}^{-1}$ for PRO and 4'OH PS, respectively. The results are presented in Table 4. The described method could be applied for determination of PRO and metabolites in

urine sample collected from patients treated simultaneously by popular diuretic, spironolactone. The second most popular diuretic, furosemide, could interfere with PRO and it must be eliminated.

The results obtained for urine samples were verified by ultra-HPLC method. At first the ultra-HPLC chromatogram was recorded for mixture of standard solutions and presented in Figure 7.

The chromatogram recorded for urine sample collected 2, 4 and 10 h after PRO administration was presented in Figure 8.

Table 4. Determination of PRO and 4'OH PS in urine samples

	Urine collected after	Found* PRO / (mol L ⁻¹)	SD _{PRO} / (mol L ⁻¹)	Found* 4'OH PS / (mol L ⁻¹)	SD _{4'OH PS} / (mol L ⁻¹)
Patient 1	2 h	3.34×10 ⁻⁶	1.31×10 ⁻⁷	5.76×10 ⁻⁶	1.33×10 ⁻⁷
	4 h	2.52×10 ⁻⁶	1.17×10 ⁻⁷	4.37×10 ⁻⁶	1.27×10 ⁻⁷
	10 h	-	-	2.20×10 ⁻⁶	1.26×10 ⁻⁷
Patient 2	2 h	3.16×10 ⁻⁶	1.21×10 ⁻⁷	5.52×10 ⁻⁶	1.24×10 ⁻⁷
	4 h	2.15×10 ⁻⁶	1.27×10 ⁻⁷	4.16×10 ⁻⁶	1.29×10 ⁻⁷
	10 h	-	-	2.04×10 ⁻⁶	1.34×10 ⁻⁷
Patient 3	2 h	4.52×10 ⁻⁶	1.15×10 ⁻⁷	4.73×10 ⁻⁶	1.01×10 ⁻⁷
	5 h	3.11×10 ⁻⁶	1.20×10 ⁻⁷	3.89×10 ⁻⁶	1.11×10 ⁻⁷
	10 h	-	-	2.11×10 ⁻⁶	1.23×10 ⁻⁷

*Average of three determinations.

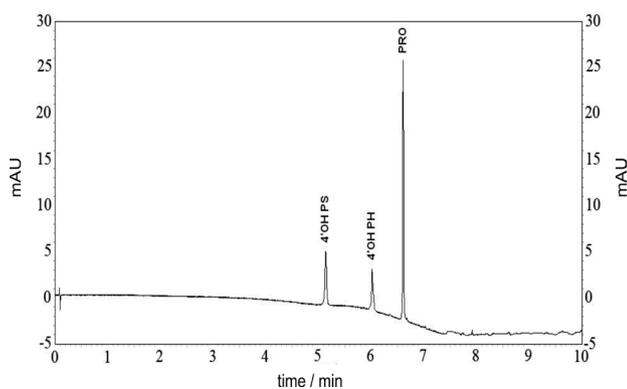


Figure 7. An ultra-HPLC chromatogram obtained for standard solutions of PRO and metabolites with the gradient elution program. The concentrations of PRO, 4'OH PH and 4'OH PS were 1.93×10^{-5} mol L⁻¹, 1.60×10^{-5} mol L⁻¹ and 1.41×10^{-5} mol L⁻¹, respectively. The injection volume was 1 μ L.

The comparison between the DPV and the ultra-HPLC method was performed through the statistical *t*-student test at 0.05 significance level of the mean experimental values obtained in the quantification of PRO and 4'OH PS in urine samples. The results are given in Table 5. The test shows no statistical difference between results obtained by examined methods, neither for PRO nor for 4'OH PS.

The proposed voltammetric method in comparison with chromatographic method¹⁻⁷ is easier and low cost. In the comparison with other electrochemical methods,^{13,15} the described method is not time consuming, do not involve accumulation time and do not require the nitration step. The obtained calibration ranges for PRO determination are wider than that described in literature date.¹³⁻¹⁵ To the best of our knowledge, the electroanalytical method for simultaneous determination of PRO, 4'OH PS and 4'OH PH was not yet reported.

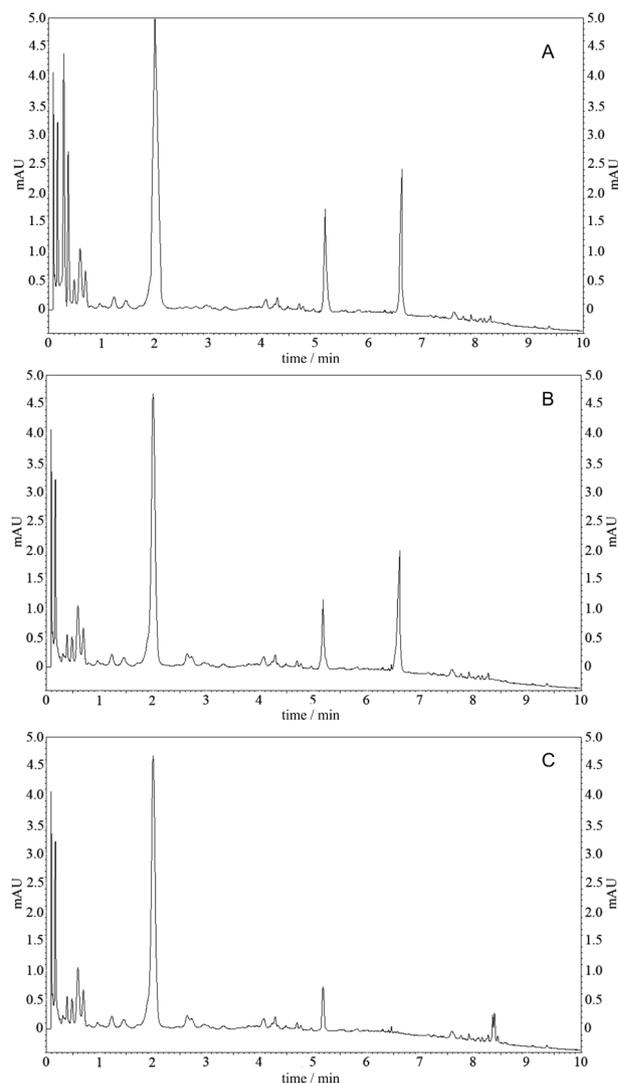


Figure 8. The ultra-HPLC chromatogram recorded for urine sample collected from a patient 2 h (A), 4 h (B) and 10 h (C) after PRO administration

Table 5. Comparison between the obtained results using DPV and ultra-HPLC

Method	PRO / (mol L ⁻¹)	SD _{PRO} / (mol L ⁻¹)	4'OH PS / (mol L ⁻¹)	SD _{4'OH PS} / (mol L ⁻¹)
DPV ^a	2.52×10 ⁻⁶	1.17×10 ⁻⁷	4.37×10 ⁻⁶	1.27×10 ⁻⁷
Ultra-HPLC ^b	2.41×10 ⁻⁶	9.11×10 ⁻⁸	4.23×10 ⁻⁶	9.23×10 ⁻⁸
<i>t</i> -test (<i>t</i> -calculated < <i>t</i> -theoretical)	1.411 < 2.365		1.676 < 2.365	

^afor n = 6; ^bfor n = 3.

Conclusions

A simple and rapid electroanalytical method for the determination of PRO and its metabolites has been described. The metabolites are electrochemically oxidized in two steps in a diffusion-controlled mechanism, and no adsorption effects are observed. In both cases, the first oxidation process is reversible, and the second one is irreversible. The measurements indicate that the existing difference between the peak potential positions makes simultaneous determination of PRO and its metabolites possible. The proposed method could be applied for monitoring PRO and its metabolites in biological fluids.

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