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Cytochrome P450 2D6 based electrochemical sensor for the determination of codeine

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ABSTRACT

Considering the enzymatic activity of the cytochrome P450 2D6 on substrates such as codeine, the current paper includes the development of an enzymatic biosensor for detection of this drug. Home-made screen-printed electrodes were used as electrochemical transducers of the biosensor, in which the enzyme was covalently attached to the carbon surface of the working electrode, this type of modification being the most suitable for the immobilization of the biological element. Chronoamperometric measurements were carried out under optimum conditions of pH and working potential, pH 7 and +200 mV vs. screen-printed Ag/AgCl electrode, giving a reduction signal related to the concentration of codeine in solution. Consecutive additions of a solution of codeine were performed to obtain calibration curves in order to validate the electrochemical method in terms of precision and calculate its capability of detection. These biosensors were used for the determination of codeine in urine and commercial pharmaceutical samples.

1. Introduction

Codeine is widely used in medical treatment of light to moderate pain, cough, constipation, etc. Although it belongs to the same opioid family as morphine, codeine produces less analgesia, sedation and respiratory depression than the latter.

Codeine is primarily metabolized in the liver. Opioid metabolism involves demethylation and glucuronidation stages, with the latter being the main route of metabolism [1]. The demethylation pathway is performed by two cytochrome P450 isoforms, which are part of a wide group of heme-enzymes characterized by their Soret band at 450 nm when interacting with carbon monoxide [2]. On one hand, cytochrome P450 3A4 is responsible for the transformation of codeine into norcodeine through the N-demethylation of the tertiary amine group. On the other hand, the isoform 2D6 (CYP2D6) carries out the O-demethylation of the methoxy group in the third position, giving morphine as a product of the biotransformation [3]. Unfortunately, not all the population has functional CYP2D6 enzymes, due to a genetic polymorphism, that is, not everybody is capable of transforming codeine to morphine for pain relief [4].

Although codeine is commonly used as a pharmaceutical drug, it is sometimes improperly consumed due to the subsequent euphoric effects. As drug abuse is a world-wide problem, the development of methods for determining and quantifying these substances is essential. Different analytical techniques have been reported for its determination such as chromatographic [5,6], chemiluminiscent [7], electrophoretic [8] and electrochemical methods [9–23]. Good results in the determination of codeine have been obtained by electrochemical methods, but chronoamperometric analyses require the application of high potentials that can provoke the simultaneous oxidation of codeine and other interferences [10,14]. Nevertheless, this inconvenience has been solved by the incorporation of different enzymes to the electrochemical system, using mediators such as phenazine methosulphate and tetratiafulvalente (TTF) [24,25]. In this work, the selective determination of codeine has been attempted using a non-mediated electrochemical biosensor based on the enzyme CYP2D6 [26]. The well-known mechanism of the cytochrome P450 involves the combination of oxygen with codeine to produce morphine and formaldehyde, being the electron delivery provided by the SPCE in this case (Fig. 1) [27–32]. Consequently, the recorded reduction current can be related to the concentration of codeine in the electrochemical cell.

Home-made screen-printed carbon electrodes (SPCE) have been modified with this enzyme through three different types of immobilization, namely covalent attachment, crosslinking immobilization and simple adsorption on the working electrode surface. The best conditions of pH and working potential to carry out chronoamperometric measurements have been optimized using an experimental design methodology. Furthermore, the developed method has been characterized in terms of precision

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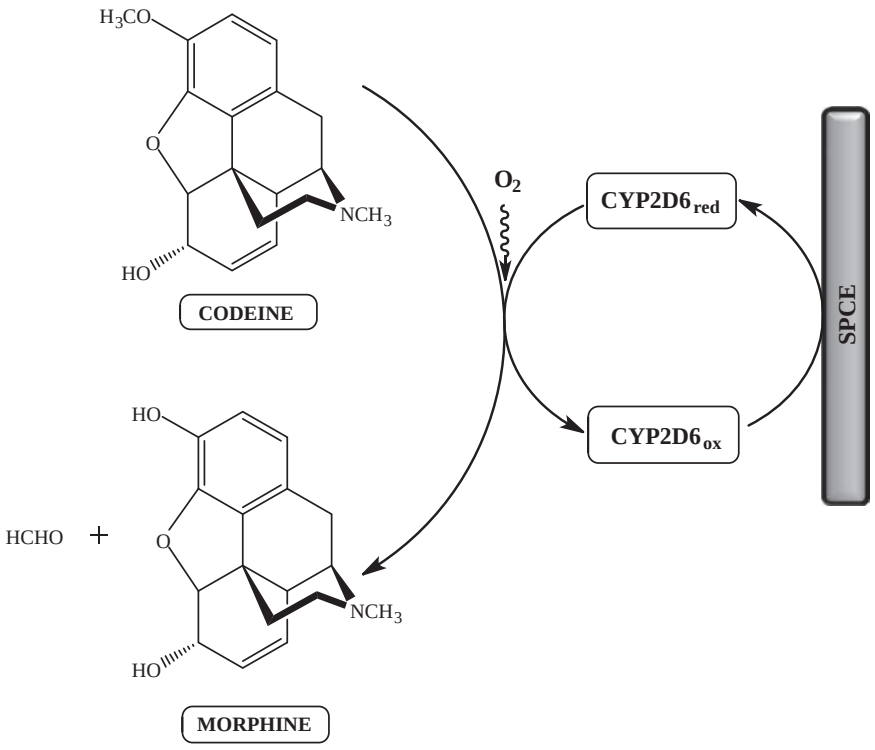

Fig. 1. Biotransformation of codeine into morphine catalyzed by the enzyme cytochrome P450 2D6.

and capability of detection and applied for the determination of codeine in pharmaceutical drugs and urine.

2. Experimental

2.1. Reagents

Home-made screen-printed devices were fabricated using the following commercial inks: carbon ink C10903P14 (Gwent Group, Torfaen, UK), silver ink Electrodag 418 and Ag/AgCl ink Electrodag 6037 SS (Acheson Colloiden, Scheemda, The Netherlands), and dielectric ink 242-SB (ESL Europe, Agmet Limited, Reading, UK).

Analytical grade reagents including CYP450 2D6 (C9095-15K2, US Biological, Swampscott, MA, USA, $\sim$1 mg mL^{-1}), glutaraldehyde (GA) and bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) were used as received. All solutions were prepared with Milli-Q water.

A solution of 3 mM of 4-nitrobenzenediazonium tetrafluoroborate ($N_2C_6H_4NO_2$ BF_4, Sigma-Aldrich, Steinheim, Germany) was prepared in acetonitrile, containing 100 mM of tetrabutylammonium tetrafluoroborate (NBu_4BF_4, Sigma-Aldrich, Steinheim, Germany). Additionally, a solution of water:ethanol (9:1 v/v) (VWR Prolabo, Fontenay-sous-Bois, France) containing 100 mM of KCl (Merck, Darmstadt, Germany) was prepared for the reduction of the diazonium salt.

Solutions of 40 mM N-hydroxysuccinimide (NHS) (Sigma-Aldrich, Steinheim, Germany) and 80 mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (Fluka, Steinheim, Germany) were prepared in 10 mM phosphate buffer pH 4.

Stock standard solutions of codeine were daily prepared by dissolving the adequate amount of codeine hydrochloride (Alcaliber S.A., Madrid, Spain) in 100 mM phosphate buffer pH 7.

In our work, 100 mM phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$, Merck, Darmstadt, Germany) solution was used as a supporting electrolyte and adjusted to the desired pH value with 1 M NaOH (J.T. Baker, Deventer, The Netherlands) solution.

2.2. Apparatus

A DEK 248 printing device (DEK, Weymouth, UK) was used for the fabrication of the screen-printed transducers.

Electrochemical measurements were made with a PalmSens Sensor Interface (PalmSens BV, Utrecht, The Netherlands) with PalmSensPC software.

The pH of the solutions was measured with a pH meter HANNA instruments Model HI221 (USA).

3. Methods

3.1. Home-made SPCEs fabrication

SPCEs were manufactured by sequential deposition of different commercial inks, followed by their curing process. Four polyester meshes with different patterns were used to print each of the parts of the electrochemical setup on the surface of a polyester substrate.

The electrochemical system consisted of three silver conductive tracks, a carbon counter electrode, a carbon working electrode (an area of 12.6 mm^2), a Ag/AgCl reference electrode and an insulating film utilized to cover the silver tracks and to define the final geometry of the three electrodes.

3.2. Functionalization of SPCEs

A drop of 50 μL of a 3 mM solution of $N_2C_6H_4NO_2$ BF_4 was placed on the electrochemical system. This salt was successfully linked to the surface of the working electrode by sweeping the potential between +800 mV and −400 mV vs. screen-printed

Ag/AgCl electrode (Ag/AgCl SPE) at a scan rate of 200 mV s^{-1} [31]. Two sequential scans were performed.

Then, a drop of 100 μL of a solution of water:ethanol (9:1 v/v), containing 100 mM of KCl, was placed on top of the aryl diazonium salt-modified SPCE. Two scans were recorded between 0 mV and −1700 mV vs. Ag/AgCl SPE at a scan rate of 200 mV s^{-1} [31].

CYP450 was covalently attached to the working electrode using the procedure described elsewhere [30]. Briefly, once the working electrode was washed using 10 mM phosphate buffer pH 4, 0.5 μL of a CYP2D6 solution was applied, followed by 2 μL of a 40 mM NHS solution and 2 μL of an 80 mM EDC solution. After 90 min at 4 °C, the biosensor was washed to eliminate the enzyme non-covalently attached and stored at 4 °C until its use.

3.3. Electrochemical measurements

Chronoamperometric measurements were carried out at room temperature under constant magnetic stirring in a cell containing 5 mL of a 100 mM phosphate buffer solution of the selected pH. A potential of +200 mV vs. Ag/AgCl SPE was applied, except for the experimental variables optimization process.

3.4. Real-life sample preparation

A commercial pharmaceutical drug, named CODEISAN®, was used to test the suitability of the developed biosensor in complex matrices. According to the patient leaflet, each of the tablets contains 28.7 mg of the active ingredient. Two tablets were homogenously crushed and an aliquot from each of them was dissolved in the supporting electrolyte solution. Then, the suspension was centrifuged at 10 °C for 1 min at 13,000 rpm and the supernatant was transferred to a vial, where it was kept until its use.

Additionally, codeine was determined in spiked diluted urine samples. The urine sample was centrifuged to eliminate solids in suspension and spiked with codeine to a final concentration of 10 mM.

4. Results and discussion

4.1. Optimization of the experimental variables that influence the chronoamperometric response of codeine by the CYP2D6-SPCE

Chronoamperometric measurements are influenced by different factors that mainly depend on the enzyme used as biorecognition element of the biosensor. Two main variables known to influence the electrochemical response are pH of the buffer solution and working potential. These two variables were studied through a 2^2 central composite design, taking as response variable the chronoamperometric reduction current of a 0.2 mM solution of codeine [33,34]. According to the experimental design, a total of 11 experiences were performed with the covalently-immobilized CYP2D6-biosensor and then, the results were evaluated using the STATGRAPHICS PLUS software package [35]. The values of pH 7 and +200 mV vs. Ag/AgCl SPE were obtained as the best conditions to achieve the maximum response (Fig. 2).

In order to ascertain that the recorded chronoamperometric current was due to the interaction codeine-CYP2D6, control experiments were performed under the optimum conditions using bare and aryl diazonium salt-functionalised electrodes without the enzyme. No electrochemical signal was obtained with these control electrodes.

Additionally, different kinds of immobilization were tested to evaluate the quality of the chronoamperometric reduction signal under these optimum conditions. Adsorption immobilization was carried out by casting 0.5 μL of enzyme on the surface of the working electrode and letting it dry at 4 °C. On the other hand, crosslinking immobilization was accomplished by the sequential deposition onto the carbon working electrode of 0.5 μL CYP2D6, followed by 2 μL 6% w/v BSA solution in 10 mM phosphate pH 6 and 1 μL 2.5% v/v GA solution in water. This mixture was left to dry at 4 °C.

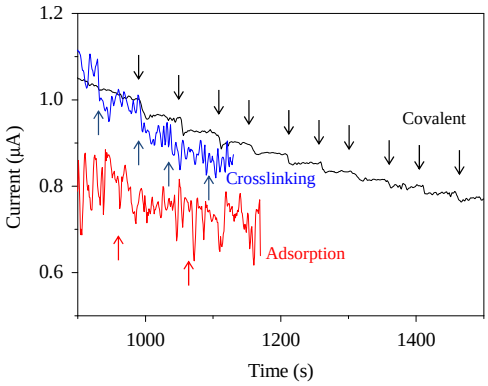

Fig. 2. Chronoamperograms recorded by successive additions of 50 μL of a 0.5 mM solution of codeine, pointed by arrows, in 5 mL supporting electrolyte under the optimum conditions using CYP2D6 based biosensors built by covalent, adsorption and cross-linking procedures.

Fig. 2 presents the comparison of the chronoamperometric signal of the three biosensors under the optimum pH and working potential conditions. As it can be seen, the signal corresponding to the covalently-modified biosensor was better than the other biosensors: a better definition of the registered current for each concentration of codeine was observed, since a lower background current was recorded. This fact can be attributed to the strong link of the enzyme onto the electrode, which prevents its leakage in the stirred solution, leading to more reproducible devices.

4.2. Precision and capability of detection

The developed analytical procedure was characterized by establishing its precision in terms of reproducibility and repeatability. Additionally, the capability of detection was calculated to get information about the sensitivity of the method.

In order to estimate the reproducibility of the biosensor built through the covalent immobilization procedure, calibration curves with different CYP2D6-SPCEs were performed under the optimum conditions in the calibration range from 4.9 to 45.4 μM. Furthermore, several calibration curves were recorded using a single CYP2D6-SPCE, under the same conditions as for the reproducibility, to obtain the repeatability of the electrochemical system.

The calibration parameters and the standard deviation of all calibration curves were properly evaluated using PROGRESS [36]. This program evaluates the presence of anomalous points using a least median square regression and, after the elimination of these points from the calibration set, an ordinary least square regression is built with the remaining points. Both reproducibility and repeatability were calculated in terms of relative standard deviation (RSD) associated with the slopes of the different calibration curves. The reproducibility of the developed CYP2D6-SPCEs was estimated to be 8.9% ($n=6$). It was also observed that the biosensor kept approximately the 30% of sensitivity after the third calibration curve using the same CYP2D6-SPCE.

Furthermore, storage conditions of the developed CYP2D6-SPCEs were evaluated. Four CYP2D6-SPCEs were built at the same time and kept at 4 °C prior to use. Calibration curves were then recorded, under the optimum conditions, during 3 consecutive

weeks (Fig. 3). The activity of the biosensor did not change after the first week, but a decrease is observed after the second one, keeping the 50% after the third week.

The capability of detection of the biosensor was calculated for a given probability of false positive (α) and negative (β) [37,38], using the validated calibration parameters of calibration curves recorded under the optimum experimental conditions. The minimum detectable concentration was lower than the concentration of the first standard used to build the calibration curves, 4.9 μM. Consequently, from an analytical point of view, the latter has been taken as the capability of detection of this method [39]. This value highlights the great affinity of CYP2D6-SPCEs to codeine, working under the optimum conditions, compared to the previously reported amperometric procedures (Table 1).

4.3. Application in pharmaceutical drugs

Once the performance of the developed biosensor was evaluated, it was used for the determination of codeine in complex matrixes. Codeisan®, a commercial pharmaceutical drug whose active ingredient is codeine phosphate 1/2 H_2O, and urine samples were chosen for this study, using the standard addition methodology to minimize possible matrix effects.

Different calibration curves were performed under the optimum chronoamperometric conditions. After obtaining a stable baseline, a volume of 50 μL of the studied solution was added into the electrochemical cell and then, successive additions of 50 μL of a 0.5 mM codeine solution were made. The average concentration of codeine found in the pharmaceutical drug was [30 ± 2] mg/tablet (n=5, α=0.05). This value agrees not only with the patient leaflet, 28.7 mg, but also with the provided by using an acetylcholinesterase based biosensor [29 ± 3] mg/tablet (n=5, α=0.05) [25].

The same methodology was successfully carried out to quantify codeine in spiked urine samples to a final concentration of 10 mM. After the addition of the studied urine solution, consecutive additions of 50 μL of a 10 mM codeine solution were performed. The average codeine concentration found in the spiked urine sample was [10.8 ± 1.3] mM. Additionally, urea and ascorbic acid, which might be present in urine samples, were tested under the optimum conditions. It was observed that they were not electroactive using the developed methodology.

The analysis of these complex matrices showed the great selectivity of the developed biosensor towards codeine.

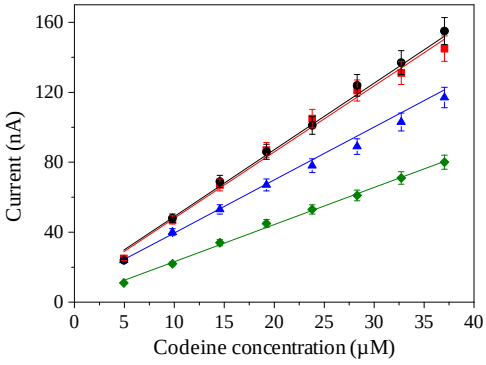

Fig. 3. Evaluation of the effect of the storage time on the sensitivity of CYP2D6-SPCEs. Calibration curves recorded in the codeine concentration range from 5 to 37 μM, under the optimum conditions, using four different CYP2D6-SPCEs. (●) Calibration curve recorded the same day that the biosensor was built, (■) calibration curve recorded one week after, (▲) calibration curve recorded 2 weeks after, (♦) calibration curve recorded 3 weeks after.

5. Conclusions

The development of a biosensor based on the covalent immobilization of the enzyme CYP2D6 on the surface of a carbon working electrode of a three screen-printed electrode system allows the chronoamperometric determination of codeine. Although this enzyme was also immobilized by crosslinking and adsorption onto the electrode, covalent binding led to the best chronoamperometric signals. The chronoamperometric reduction current obtained under the optimum conditions, buffer solution pH 7 and working potential of +200 mV vs. screen-printed Ag/AgCl electrode, can be related to the concentration of codeine in solution. In this way, reproducible CYP2D6-SPCEs (RSD=8.9%, n=6) were obtained in a codeine concentration range from 4.9 to 45.4 μM, with a capability of detection of 4.9 μM for a probability of false positive and negative of 0.05. The biosensor can be successfully applied to the determination of codeine in pharmaceutical drugs and urine samples.

Table 1
Electrochemical biosensors for codeine determination.

Technique	Electrode	Bioelement	Mediator	Supporting electrolyte pH	Working potential (V)	Capability of detection (μM)	Real sample	Ref
Amperometry	Pt	Morphine dehydrogenase and NADPH	Phenazine methosulphate	8	+0.1[a]	9	–	[24]
Amperometry	Clark	Morphine dehydrogenase, salicylate hydroxylase and NADPH	–	7.5	−0.6[a]	2.7	–	[40]
CV	Au	RNA-Aptamer	–	7	–	–	–	[41]
DPSV	PDDA-MWCNT-Pencil graphite electrode	dsDNA in solution	–	7	–	0.1	Blood serum, urine samples and pharmaceutical formulations	[42]
Amperometry	$SPC_{TTF}E$-	Acetylcholinesterase	TTF	7	+0.25[b]	20	Pharmaceutical formulations & urine	[25]
Amperometry	SPCE	CYP2D6	–	7	+0.20[b]	4.9	Pharmaceutical formulations & urine	This work

PDDA: Poly(diallyldimethylammonium chloride), MWCNT: multiwall carbon nanotubes, $SPC_{TTF}E$, screen-printed electrode modified with tetrathiafulvalene.

[a] vs. Ag/AgCl,

[b] vs. Ag/AgCl SPE.

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