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RESEARCH PAPER

Flow injection tyrosinase biosensor for direct determination of acetaminophen in human urine

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Abstract

An amperometric biosensor compatible with a flow injection analysis (FIA) for highly selective determination of acetaminophen (APAP) in a sample of human urine was developed. This biosensor is also suitable for use in the routine pharmaceutical practice. To prove this statement, two different commercially available pharmaceutical formulations were analyzed. This nano-(bio)electroanalytical device was made from a commercially available screen-printed carbon electrode covered by a thin layer of non-functionalized graphene (NFG) as amperometric transducer. A biorecognition layer was prepared from mushroom (*Agaricus bisporus*) tyrosinase (EC 1.14.18.1) cross-linked using glutaraldehyde, where resulting aggregates were covered by Nafion®, a known ion exchange membrane. Owing to the use of tyrosinase and presence of NFG, the developed analytical instrument is able to measure even at potentials of 0 V. Linear ranges differ according to choice of detection potential, namely up to 130 μmol L^{-1} at 0 V, up to 90 μmol L^{-1} at −0.1 V, and up to 70 μmol L^{-1} at −0.15 V. The first mentioned linear range is described by the equation I_p [μA] = 0.236 − 0.1984c [μmol L^{-1}] and correlation coefficient r = 0.9987; this equation was used to quantify the content of APAP in each sample. The limit of detection of APAP was estimated to be 1.1 μmol L^{-1}. A recovery of 96.8% (c = 25 μmol L^{-1}, n = 5 measurements) was calculated. The obtained results show that FIA is a very selective method for APAP determination, being comparable to the chosen reference method of reversed-phase high-performance liquid chromatography.

Keywords Acetaminophen · Amperometry · Biosensor · Flow injection analysis · Tyrosinase · Human urine

Introduction

Acetaminophen (APAP), known also as paracetamol, is a *p*-aminophenol derivative (*N*-acetyl-*p*-aminophenol) with analgesic and antipyretic activities. APAP has widely replaced acetylsalicylic acid use in medicine [1]. In many countries, APAP is usually used as an alternative to aspirin and phenacetin [2]. The usual dose for adults is one or two 500 mg tablets up to four times in 24 hours. The maximum recommended single dose for children is 500 mg usually over 8 hours, but this interval can be shortened [3]. Despite the recommended dosage, there is a serious risk of poisoning which causes up to 42% of acute liver failure and requires up to 300,000 hospitalizations per year [4]. The direct cost of APAP overdose has been estimated at $87 million annually in the USA [5].

APAP is metabolized in the liver by conjugation to glucuronide, sulfate, and 4–15% is oxidized by cytochrome P450 (CYP2E1, CYP1A2, CYP3A4) to the reactive *N*-acetyl-*p*-benzoquinone (NAPQI) metabolite which is conjugated with glutathione as a donor of SH groups. Most of the active substance is excreted from the body in the form of non-toxic metabolites in urine (40–60% as glucuronate, 30% as sulfates), and 2% in unchanged form [3]. In overdose, the production of the reactive metabolite NAPQI increases and unfortunately it can irreversibly bind to an SH group of at least 23 proteins (of which at least six are mitochondrial). Histological sections clearly indicate necrotic cell death by centrilobular hepatic necrosis [4, 6]. Thus, it is clear that

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selective determination of APAP in urine is of great importance in clinical analysis.

Flow injection analysis (FIA) utilizing screen-printed carbon electrodes (SPCE) or their modifications as amperometric detection systems is often used in analytical instruments in the pharmaceutical [7] and clinical analysis [8, 9]. APAP is usually directly detected at a constant positive voltage (oxidized) to form NAPQI with participation of $2e^-$ and $2H^+$ [10] or indirectly using catalytic biosensors [11–16]. Fundamentally, APAP is enzymatically oxidized to electroactive products which can be subsequently amperometrically detected. The former type is suitable for analysis of pharmaceuticals because interference from the accompanying substances is not expected. In contrast, different enzymes have been used in the construction of biosensors for the quantitative analysis of APAP. Tyrosinase biosensors represent an attractive alternative for APAP determination, given their better selectivity.

An aryl-acylamidase (EC 3.5.1.13) amperometric biosensor can be considered as the first type of these bioelectroanalytical devices. However, this biosensor is not suitable for use in the urine analysis because it is based on electrochemical oxidation of produced *p*-aminophenol whose determination is significantly interfered with in the presence of gentisic, ascorbic, and uric acids [11, 12]. Other biosensors utilized the polyphenol oxidase enzymes (EC 1.10.3.1) naturally occurring in avocado (*Persea americana*) [13] and eggplant (*Solanum melongena*) [14] tissues. As a result of the low catalytic activity of these enzymes, appropriate biosensors do not exhibit high enough sensitivity to be applied in clinical analysis.

The last type is represented by horseradish peroxidase (EC 1.11.1.7) amperometric biosensors. These biosensors are based on electrochemical reduction of produced NAPQI at −0.2 V [15–17]. Although these biosensors are very sensitive analytical tools, they are not suitable for urine analysis because the matrix of urine provides a false positive signal. For that reason, they are preferred for pharmaceutical analysis. If the working potential is set to more negative values, the risk of interference by accompanying substances (products of oxidative metabolism) will increase because they will be reduced as well.

In 2003, a kinetic study of APAP oxidation by tyrosinase enzyme (EC 1.14.18.1) was performed [18]. In the first step, the APAP is hydroxylated to 4-acetamido-1,2-catechol (hydroxylase). Then, this intermediate product is oxidized to 4-acetamido-*o*-benzoquinone (4-AOBQ) in the second step (catecholase activity) [19, 20]. The resulting 4-AOBQ can be electrochemically reduced at a constant voltage with participation of $2e^-$ and $2H^+$ [21]. For application of tyrosinase biosensors in urine analysis it is very important that the reduction of 4-AOBQ begins at a potential closer to 0 V, unlike in cases of previously mentioned enzymatic sensors based on electrochemical reduction of NAPQI. At this detection potential, any interference of substances contained in the sample matrix is not expected. The most common interfering substances (ascorbic acid and uric acid) are not subject to electrochemical reactions.

It should be noted that a tyrosinase biosensor for FIA has been already developed for real-time monitoring of the photocatalytic degradation of APAP [21]. An optimal potential of −0.05 V vs Ag/AgCl was found. Setting of this potential allowed the sensor to achieve good sensitivity while potential interferences were minimalized. However, this biosensor was not tested in the analysis of biological samples [21].

A completely new amperometric tyrosinase biosensor based on commercial screen-printed carbon electrode covered by a thin layer of graphene and Nafion® [22, 23] film embedded with tyrosinase enzyme cross-linked by glutaraldehyde [24] is presented. In this contribution, it is possible to understand why graphene [25] was preferred over popular carbon nanotubes [26], how the tyrosinase enzyme was anchored in the order to not be eluted during measurements, how amounts of cross-linker and polymer used for the tyrosinase encapsulation were chosen, and how other steps of time-consuming optimization were solved. Finally, the developed biosensor was tested in analysis of commercial drugs and spiked human urine from a healthy volunteer. The results obtained showed that flow injection analysis combined with the biosensor is a very selective analytical method for APAP determination, being comparable to reversed-phase high-performance liquid chromatography (RP-HPLC) with spectrophotometric detection, which was used as the reference method [27, 28]. It has to be also noted that a short lifetime was observed. This phenomenon is a typical disadvantage of tyrosinase-based biosensors [21, 29]. However, a solution to this problem was proposed.

Materials and methods

Materials and reagents

Acetaminophen, *N,N*-dimethylformamide (DMF), ≥99.9% HPLC grade methanol, 25% glutaraldehyde (GTA), 5% Nafion® in 55% ethanol, hyaluronic acid sodium salt (30–50 kDa) from *Streptococcus equi*, chitosan (~50 kDa), and lyophilized powder of mushroom (*Agaricus bisporus*) tyrosinase (EC 1.14.18.1) (119.5 kDa using electrophoresis) were purchased from Sigma-Aldrich (Prague, Czech Republic). A single layer of non-functionalized graphene (NFG) (resistivity ≤0.30 Ω cm; specific surface area 400–1000 $m^2\ g^{-1}$) was obtained from ACS Material, LLC (Medford, USA). Multi-walled carbon nanotubes (MWCNTs) of diameter 10–30 nm, length 5–15 μm, specific surface area 40–300 $m^2\ g^{-1}$, and single-walled carbon nanotubes (SWCNTs) of diameter < 2 nm, length 5–15 μm, specific surface area > 400 $m^2\ g^{-1}$ both from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China) were also tested as suitable surface modifiers. Highly purified

water (resistivity > 18 MΩ cm) was prepared using purification system Milli-Q from Merck Millipore (Burlington, USA). Chemicals needed for preparation of 0.1 mol L^{-1} phosphate buffer solution (PB) were from Lach-Ner s.r.o. (Neratovice, Czech Republic).

Preparation of biosensor

A simple preparation of the SPCE/NFG amperometric transducer consisted of pipetting 20 μL single layer graphene dispersion in DMF (2 mg mL^{-1}) onto a commercial screen-printed carbon electrode (DRP-150 from DropSens, Llanera, Spain) surface. The graphene dispersion in a small vial was carried out in an ultrasonic bath for 20 min. The resultant dispersed drop was left to dry under laboratory conditions for 24 h. Immobilization of tyrosinase enzyme on the transducer surface involved three separate steps. First, 5 μL of enzyme solution (2.0 mg mL^{-1}) in PB was applied and allowed to dry under laboratory conditions for 30 min. After direct enzyme adsorption onto the thin graphene layer, individual enzyme molecules were cross-linked by addition of 1% glutaraldehyde (3 μL) for 20 min to avoid excessive elution of the enzyme from the polymer used. Finally, 10 μL fluoropolymer-copolymer solution (1% Nafion® neutralized with 8% ammonia solution [30]) was applied directly and allowed to dry under laboratory conditions for 30 min. When not in use, the biosensor was stored in a refrigerator at 5 °C.

Apparatus

Electrochemical experiments were performed using cyclic voltammetry in a conventional three-electrode arrangement consisting always of one of following electrodes: bare SPCE, SPCE/NFG, or SPCE/NFG/tyrosinase-GTA/Nafion® (proposed biosensor). All amperometric experiments were carried out using an FIA setup consisting of a multichannel peristaltic pump MINIPULS 3 from Gilson, Inc. (Middleton, USA), Rheodyne automatic six-stage dosing valve from IDEX Health & Science GmbH (Wertheim, Germany), the developed tyrosinase biosensor inserted into the wall-jet flow cell from DropSens (Llanera, Spain), and a computer. The different types of SPCEs were connected to a potentiostat/galvanostat AUTOLAB model PGSTAT101 operated via NOVA 1.11 software from Metrohm (Prague, Czech Republic).

Procedure

As supporting electrolyte, non-deaerated 0.1 mol L^{-1} phosphate buffer of pH 7.0 was chosen for each electrochemical measurement owing to optimum tyrosinase biocatalytic activity in the neutral pH range. Characterization of the developed biosensor was accomplished using cyclic voltammetry of 500 μmol L^{-1} APAP at a potential window from −0.2 V to +0.8 V, initial potential 0 V, potential step 5 mV, and san rate 10 mV s^{-1}. Amperometric detection in the wall-jet configuration was usually performed at 0 V vs pseudo-reference silver electrode at a flow rate of 0.6 mL min^{-1}. Otherwise, any change in the working conditions is described in the legends of the corresponding figures.

Reference analysis by liquid chromatography

Chromatographic analysis was performed using a HPLC system equipped with a LC-20ADXR binary gradient pump, a DGU-20 degassing unit, a SPD-M30A DAD, a SIL-20 AC XR autosampler (all Shimadzu, Kyoto, Japan), and a LCO 102 column thermostat (Ecom, Prague). The optimum separation was achieved with an Ascentis Express C18 column (150 mm × 3.0 mm, 2.7 μm). The detection wavelength was set to 243 nm. The separation was performed with mobile phase consisting of 0.3% formic acid in water (A) and methanol (B) using a gradient program from 20% to 40% B for 10 min at constant temperature 30 °C. The flow rate was 0.5 mL min^{-1} and injection volume 5 μL.

Sample preparation

Pills of Paralen® 500 and Tylex® 750 obtained from a Czech pharmacy were dissolved in a 250-mL volumetric flask using PB (amperometry) or 80% methanol (HPLC). The resulting solutions were filtrated through a polytetrafluoroethylene syringe filter with pore size 0.45 μm and diluted 20-fold using deionized water.

Commercially available pooled normal human urine (50 mL) was purchased from Innovative Research, Inc. (Novi, USA) distributed by Divbio Science Europe (Ulvenhout, the Netherlands). Additionally, a sample of urine was obtained from a healthy volunteer who signed information consent. The obtained sample of human urine was anonymized before the study. Both urine samples were only artificially enriched with a defined amount of analyte and served only as a complex matrix. Thus, all experiments with urine samples were done in accordance with the WMA Declaration of Helsinki, June 1964.

A volume (250 μL) of stock solution (0.01 mol L^{-1} APAP in water) was pipetted into a 50-mL volumetric flask and then was filled to the mark with the human urine. Depending on the person's acid–base status, the pH of urine may range from 4.5 to 8. As a result of the very broad normal pH range and high salinity, the spiked urine sample had to be diluted a minimum of fivefold using PB and then was filtered through the 0.45-μm polytetrafluoroethylene syringe filter before bioanalysis.

For HPLC analysis, the spiked urine sample was prepared according to the reported procedure [27, 28] with only minor modifications, because during optimization, it was found that acetonitrile can be fully replaced with methanol. The sample

of human urine (0.5 mL) with 4 mL of pure methanol was placed into a 10-mL volumetric flask. Several drops of 1.0 mol L^{-1} NaOH were added to the obtained solution to achieve the desired pH ~8 and the solution was completed to the mark by adding ultrapure water. The resulting mixture was centrifuged at 5000 rpm for 3 min, filtered through the 0.45-μm polytetrafluoroethylene syringe filter, and then injected into the liquid chromatograph.

All analyses of selected samples were always repeated five times and the final results are presented as confidence intervals $\bar{x} \pm st_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t distribution (2.015) for a given number of determinations at a significance level α of 0.05 (95% probability).

Results and discussion

Biosensor design

The main objective of this study was to develop a highly sensitive and selective biosensor that could be used universally in the analysis of various pharmaceutical and clinical samples. In general, several necessary conditions must be met. Biosensors should be able to detect very low content of target analyte and provide a stable response for a long time duration (i). Substances other than analyte must not significantly affect the measurable signal (ii). If possible, they should be made from cost-effective materials (iii).

For amperometric sensors, it is necessary to have the biorecognition element (tyrosinase enzyme) as close as possible to the electrochemical transducer (electrode) which is often modified by conductive materials with high active surface area and catalytic effect. As mentioned above, the electrode surface should be as large as possible [31, 32]. Surfaces of SPCEs were modified by different types of non-functionalized carbon nanomaterials (CNs) to obtain the optimum amperometric transducer. Cyclic voltammetry (CV) showed that APAP provides significantly better electrochemical behavior at SPCE/NFG (see Electronic Supplementary Material (ESM) Table S1) than in the case of carbon nanotubes (CNTs). An explanation for this difference can be found by comparing the homogeneity of each surface structure which depends on the arrangement of CNs used. Immobilized CNTs create irregular skeins of various sizes and are mutually interconnected. Consequently, the active surface area of CNTs cannot be fully utilized unlike in the case of NFG which creates a structure of coarse cloth probably due to mutual interaction of graphene nanosheets having a typical planar configuration [33, 34]. Overall, the surface is characterized by higher homogeneity than in the previous case of CNTs. Thus, it is not surprising that more satisfactory repeatability of current response during amperometric detection can be predicted.

For effective immobilization of tyrosinase enzyme, three commonly used polymeric materials were tested, namely chitosan, hyaluronic acid sodium salt, and Nafion®. The presence of chitosan or hyaluronic acid caused many times higher increase in background current than in the case of Nafion®. For that reason, Nafion® was chosen as the optimum polymeric material for tyrosinase incorporation. As for glutaraldehyde used for preparation of cross-linked enzyme aggregates (CLEAs) [35], it was observed that addition of more than 3 μL of 1% solution causes a dramatically increased background current (baseline signal) and decreases the current response to APAP.

In order to investigate the suitable working electrode (see ESM Fig. S1a), APAP electrochemical behavior at different transducers was studied using cyclic voltammetry. No evident difference between using MWCNTs and SWCNTs was observed (see ESM Table S1). Figure 1 shows typical cyclic voltammograms obtained at bare SPCE, SPCE/NFG, and the biosensor itself. It was confirmed that APAP is reversibly oxidized to *N*-acetyl-*p*-benzoquinone-imine (NAPBQI) with participation of two electrons and two protons, as shown in ESM Fig. S1b. However, APAP undergoes hydroxylation reaction at the free *ortho* position and subsequent oxidation to form 4-acetamido-*o*-benzoquinone (4-AOBQ) in basic aqueous solutions [10]. This is a typical electrochemical oxidation pathway for *para*-substituted phenols at commonly used carbon-based electrodes [36]. The aforementioned hydroxylation reaction and subsequent oxidation of resulting 4-acetamido-1,2-catechol (3-OH-APAP) is dominant in the presence of tyrosinase enzyme (EC 1.14.18.1) from mushroom *Agaricus bisporus* which catalyzes these oxidation processes by dissolved oxygen due to cresolase and catecholase activity (see ESM Fig. S2), when the final products are 4-AOBQ and water.

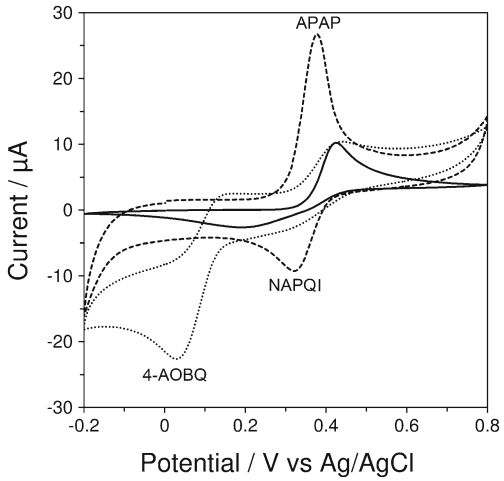

Fig. 1 Cyclic voltammetry of 0.5 mmol L^{-1} APAP at bare SPCE (solid line), SPCE/NFG (dashed), and tyrosinase biosensor (dotted line) performed in 0.1 mol L^{-1} PB pH 7 at 10 mV s^{-1}

Because of biosensor selectivity, it was important that cathodic reduction of 4-AOBQ starts at relatively high voltage (+0.146 V) with maximum current signal at +0.04 V vs pseudo-reference Ag/AgCl electrode (see Fig. 1). Consequently, amperometric detection at a constant voltage of 0 V can be selected for the detection of 4-AOBQ.

Effect of pH

The influence pH on the current response was studied in the pH range from 6 to 8. This interval was assumed to afford the highest catalytic activity of the enzyme used. The results showed that the highest sensitivity was obtained at pH 7.0, which is in agreement with the data reported previously [37–39].

Effect of flow rate

Flow rate represents one of the most important parameters in the FIA system because it defines the duration over which APAP is in the column where enzymatic reactions occur. At the set potential of −0.1 V, flow rate was investigated from 0.2 to 2.0 mL min^{-1}; for demonstration, the resultant flow-injection amperograms obtained for 50 μmol L^{-1} APAP at 0.2 (red), 0.6 (blue), 1.0 (green), and 1.8 mL min^{-1} (orange line) are shown in ESM Fig. S3. It was found that peak height significantly increased up to 0.6 mL min^{-1}. Above this flow rate, a slight decrease in peak current was observed. Therefore, a flow rate of 0.6 mL min^{-1} was chosen as optimum.

Effect of applied detection potential

Since the detection potential must be kept constant during the analysis, it plays the most important role in the application of amperometric biosensors [40]. This crucial parameter affects the sensitivity and the selectivity of the developed method [41]. The effect of applying different potentials from 0 to −0.3 V on peak current height was studied. Figure 2 shows that values lower than −0.15 V did not cause any statistically significant increase in peak current. Moreover, it was found that background current increased at potentials lower than −0.2 V. Hence, it can be assumed that the value of −0.15 V is the optimum detection potential. This value may be used in APAP determination in pharmaceuticals because they only contain non-interfering accompanying substances such as preswollen corn starch, Povidone K-30 (polyvinylpyrrolidone, PVP), croscarmellose sodium, and stearic acid. However, this statement does not apply if human urine is analyzed, because the urine is considered to be a very complex matrix.

A sample of human urine spiked with 50 μmol L^{-1} APAP was used to find the optimum detection potential (dashed line). This concentration has not been selected randomly because the APAP is marketed as Paralen in 500 mg pills. Assuming that 2% APAP is excreted unchanged, a minimum detected concentration of ~50 μmol per liter of urine might be expected [3]. The urine itself provided a false positive current response corresponding to the electrochemical reduction of naturally occurring electroactive metabolites (solid line). The intensity of this parasitic signal increased with decrease in detection potentials to more negative values as is shown in Fig. 3. Only at potential of 0 V was the current response of pure urine not higher than the standard deviation of five repeated injections of spiked urine (see ESM Fig. S4). Therefore, a detection potential of 0 V was chosen as the optimum.

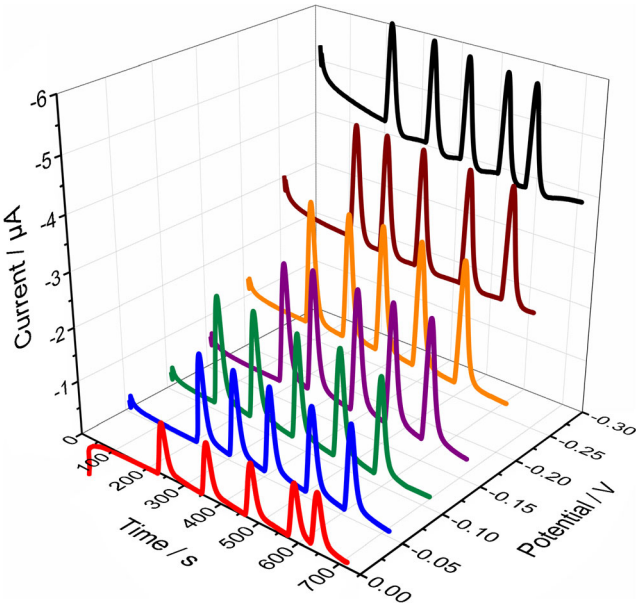

Fig. 2 Effect of detection potential of tyrosinase biosensor current response. Hydrodynamic amperograms obtained for five additions of 50 μmol L^{-1} APAP. Supporting electrolyte, non-deaerated 0.1 mol L^{-1} PB of pH 7.0; injection volume, 100 μL; flow rate, 0.6 mL min^{-1} and room temperature

Performance of proposed system

The performance of the proposed system for FIA was studied at the optimum working conditions. Limit of detection (LOD) and quantification (LOQ) were calculated according to the equations LOD = 3 *s*/*k* and LOQ = 10*s*/k, respectively, where *s* is the standard deviation of five repetitions ($n = 5$) of chosen concentrations (5 μM) of APAP and *k* represents the slope of the calibration curve (linear range). Typical recorded peaks obtained during FIA of APAP at −0.15 V are shown in ESM Fig. S5. Linear ranges vary depending on the selected detection potential, namely 4.0–130 μmol L^{-1} with LOD = 1.1 μmol L^{-1} at 0 V, 3.0–90 μmol L^{-1} with LOD = 0.8 μmol L^{-1} at −0.1 V, and 1.5–70 μmol L^{-1} with LOD = 0.5 μmol L^{-1} at −0.15 V. The first mentioned linear range described by the equation $I_p = -0.0083c + 0.0101$ with a correlation coefficient $r = 0.9987$ was used for APAP determination in a model sample, two types of pills and spiked human urine (Fig. 4).

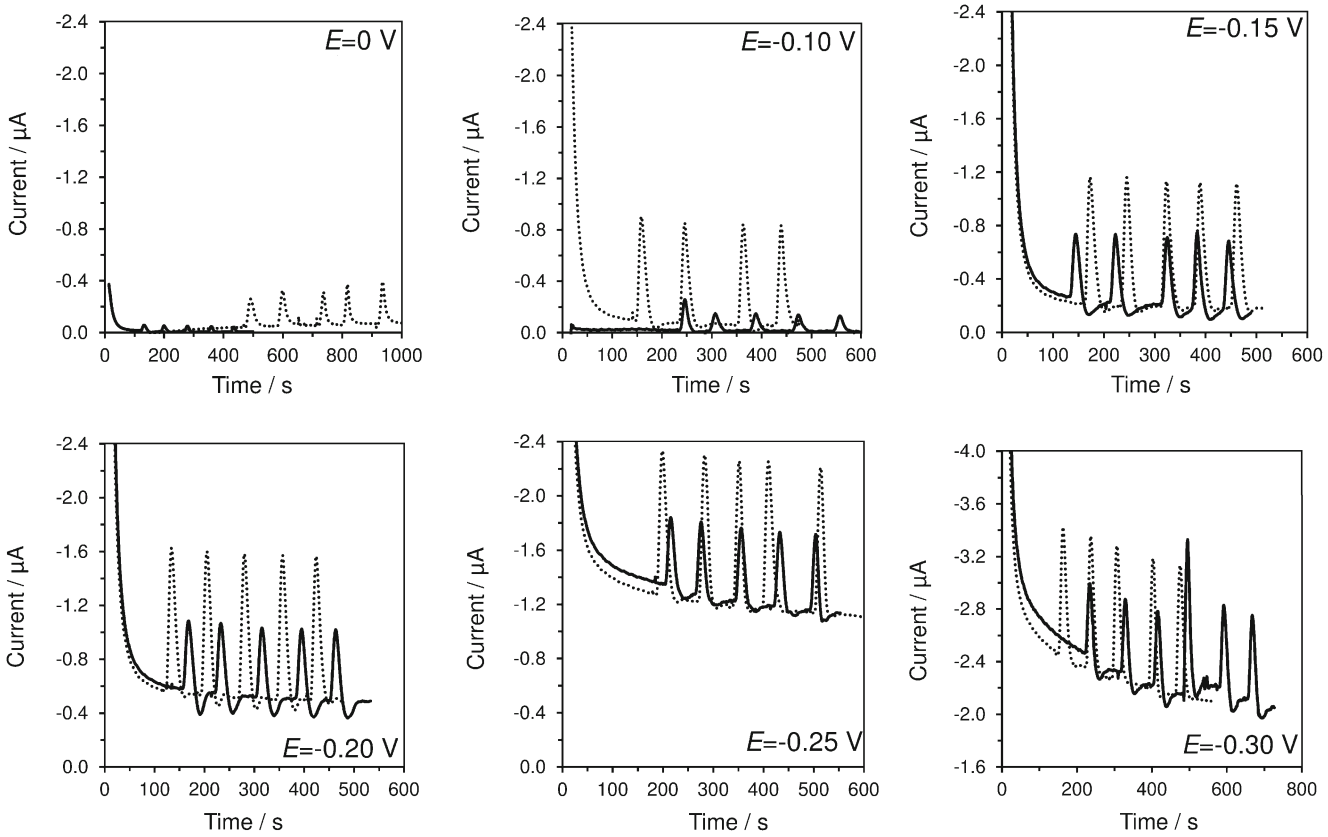

Fig. 3 Typical FIA amperograms obtained after five injections of fivefold diluted human urine (solid) and that containing 50 µmol L^{-1} APAP (dashed line) at different detection potentials. Supporting electrolyte, non-deaerated 0.1 mol L^{-1} PB of pH 7.0; injection volume, 100 µL; flow rate, 0.6 mL min^{-1} and room temperature

In comparison with previously developed enzymatic sensors (see Table 1), it should be emphasized that substantial progress in the sensitivity has been obtained at detection potential −0.15 V. Existing biosensors based on different enzymes do not provide such selectivity to be used in human urine analysis. The previously reported enzymatic biosensors have been used in the determination of APAP in pharmaceutical formulations only [11–16].

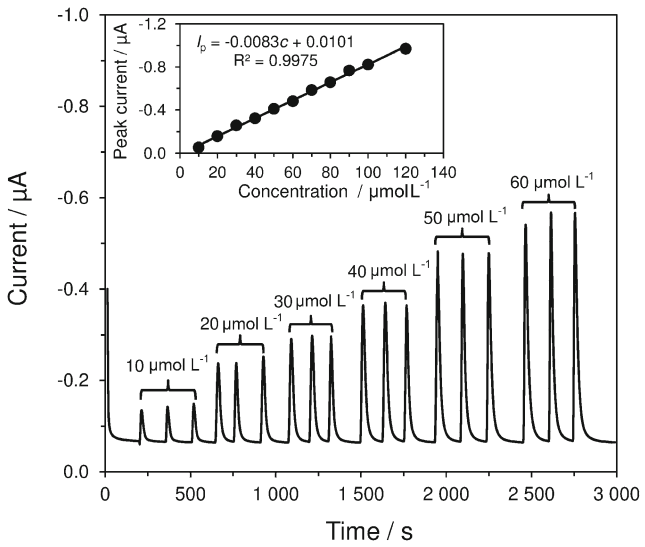

Fig. 4 Typical amperograms of calibration measurement obtained at detection potential 0 V. Supporting electrolyte, non deaerated 0.1 mol L^{-1} PB of pH 7.0; injection volume, 100 µL; flow rate, 0.6 mL min^{-1} and room temperature

Freshly prepared biosensor provided unstable behavior due to elution of incompletely entrenched enzyme from the Nafion® membrane. A constant peak current with relative standard deviation (RSD) less than 2% was obtained after a half hour of washing with phosphate buffer (ESM Fig. S6). After this time, no significant decrease in the current response due to enzyme elution was observed, as shown in Fig. 5. Satisfactory repeatability, defined as a statistical measure of the consistency of repeated measurements, was achieved, namely about 1.9% RSD.

Another important criterion is response time which is usually shorter for amperometry in a batch configuration than in the case of FIA. It depends on the distance of the dosing six-way valve from the flow cell where the biosensor is located. From this distance and flow rate, it is possible to calculate the response time after contact of the analyte with the biosensor which was shorter than 5 s.

The relatively short lifetime is a typical drawback of tyrosinase biosensors [21, 29]. Hence, a basic study of lifetime was

Table 1 An overview of recently reported amperometric biosensors for acetaminophen determination

Material/enzyme	LOD (μmol L^{-1})	Linear range (μmol L^{-1})	*r*	Reference
SPCE/aryl-acylamidase	–	0–2800	0.9990	[11]
CA-SPCE/aryl-acylamidase	–	0–2000	0.9990	[12]
CPE/avocado tissue	88	120–5800	0.9927	[13]
CPE/eggplant tissue	5.0	20–200	0.9985	[14]
GCE/HRP/PAM microgels	3.1	10–500	0.9997	[15]
GCE/HRP/Romanian clay-PEI	0.6	5.3–50	0.9980	[16]
GCE/PEI/SWCNT-HRP	1.36	10–79	0.9633	[17]
SPE-CoPC/tyrosinase	0.5	1.6–40	–	[21]
[a]SPCE/NFG/tyrosinase-GTA/Nafion®	0.5	1.4–70	0.9989	This work
[b]SPCE/NFG/tyrosinase-GTA/Nafion®	1.1	3.8–130	0.9987	This work

CA cellulose acetate membrane, *CO* cobalt phthalocyanine, *HRP* horseradish peroxidase, *GTA* glutaraldehyde, *NFG* non-functionalized graphene, *PEI* polyethyleneimine, *PAM* polyacrylamide

Data obtained at −0.15 V[a] and 0 V[b]

carried out to find out whether the developed biosensor could be utilized in routine pharmaceutical and clinical analysis. Generally, it can be stated that a freshly prepared biosensor may be used for analysis during a whole day. The main problem remains the storage of the biosensor. It leads to a significant decrease in sensitivity.

The stability of the system was evaluated by daily injection of 50 μmol L^{-1} APAP ($n = 5$). During the first 6 days, the amperometric signal of the enzymatic product dramatically decreased. After that, it decreased slightly and finally on the 12th day it became stable (ESM Fig. S7). It has to be clear that the sensitivity substantially decreases with the aging of the biosensor. From a practical point of view, there is not any problem to use an already prepared transducer, and before each analysis, freshly immobilize the tyrosinase enzyme. Moreover, the existence of commercially available SPCEs modified with NFG should be mentioned. They could probably be used to guarantee the desired reproducibility between different biosensors. However, the reproducibility between different home-made biosensors is lower and depends on individual steps in the production process. A value less than 10% RSD was calculated for five simultaneously prepared tyrosinase biosensors. Therefore, it is recommended to always perform a calibration before the analysis.

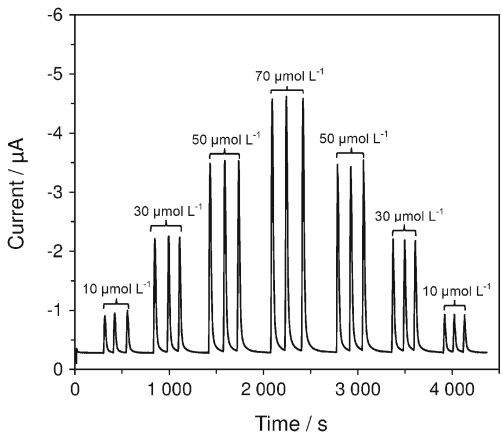

Fig. 5 FIA record obtained after injections of supporting electrolyte solutions containing different APAP concentrations at detection potential −0.15 V. Supporting electrolyte, non-deaerated 0.1 mol L^{-1} phosphate buffer of pH 7.0; injection volume, 100 μL; flow rate, 0.6 mL min^{-1} and room temperature

Interferences

In the patient information leaflets, it was found that the analyzed pharmaceutical formulations usually contain corn starch, PVP, croscarmellose sodium, and stearic acid. These compounds cannot be classified as potential interferences because they are not subjected to any electrochemical reactions

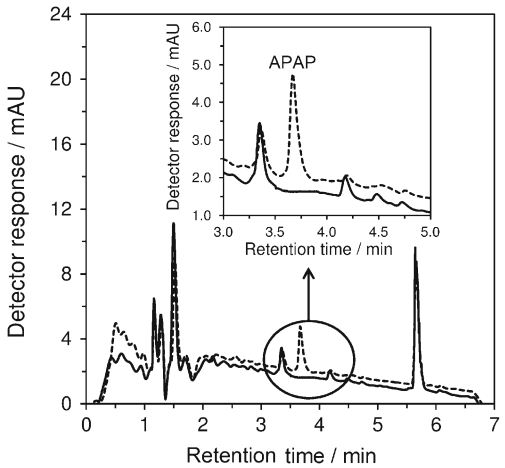

Fig. 6 HPLC analyses of sample of pure human urine (solid) and that containing 50 μmol L^{-1} APAP (dashed line). Ascentis Express C18 column (150 × 3 mm, 2.7 μm); mobile phase, 0.3% formic acid in water (A) and methanol (B); gradient program from 20% to 40% B for 10 min; flow rate, 0.5 mL min^{-1}; sample volume, 5 μL; temperature, 30 °C; detection at 243 nm

Table 2 Comparison of FIA with HPLC in analysis of pharmaceuticals and spiked human urine

Sample	FIA	HPLC	Declared
Model	242 ± 19 μmol L^{-1}	244 ± 15 μmol L^{-1}	250 μmol L^{-1}
Paralen® 500	490 ± 28 mg per pill	491 ± 9 mg per pill	500 mg per pill
Tylex® 750	725 ± 52 mg per pill	745 ± 16 mg per pill	750 mg per pill
Human urine (spiked)	52 ± 4 μmol L^{-1}	51 ± 3 μmol L^{-1}	50 μmol L^{-1}

Values given as confidence intervals $\bar{x} \pm st_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation, and $t_{1-\alpha}$ the critical value (2.015) of Student's t distribution for 5 repetitions of each analysis at $\alpha = 0.05$

at the applied working potentials. Thus, numerous direct voltammetric methods for APAP determination in the pharmaceutical formulations have been developed [42–44].

In the case of urine analysis, measurements shown in Fig. 3 and ESM Fig. S4 should be considered as sufficient interference studies because the matrix of urine did not have any statistically significant interference effect at 0 V. Current responses of pure urine were lower than standard deviation (n = 5) of 50 μmol L^{-1} APAP (2% unmetabolized form of 500 mg dose in 2 L of urine) [3].

Analysis of pharmaceutical preparations and human urine

Before the analysis of real samples, it was necessary to determine the accuracy of the proposed system. The accuracy represents the accordance between the real concentration of analyte and that found by an analytical method used. This analytical parameter is often verified using analysis of a model sample (recovery), declared amount, or by comparison with a reference method based on another physicochemical principle. Both mentioned approaches were used in the accuracy validation. A recovery of 3.8% RSD was calculated from five repetitions for FIA of a model sample containing 250 μmol L^{-1} APAP. Before the analysis, the model sample had to be tenfold diluted using PB.

Although this work is dedicated to the analysis of human urine, it was expedient to first test the biosensor in the analysis of pharmaceutical preparations, especially pills. A previously described protocol for urine analysis using HPLC was slightly modified by changing the gradient elution parameters to prevent elution of accompanying substances existing in the sample matrix at the same retention time as analyte (Fig. 6).

The calibration solutions (3.0–50 mg L^{-1}) were prepared by diluting of APAP in methanol. The calibration curve of the analytical method was characterized by the following equation: A [mAU] = 17,610.82 + 35,118.84c [mg L^{-1}] with a correlation coefficient r = 0.9993. As shown in Table 2, the proposed tyrosinase biosensor provides results comparable to the chosen reference HPLC method characterized by recovery of 97.4% (n = 5).

Conclusion

A sensitive and selective amperometric tyrosinase biosensor for acetaminophen determination in the pharmaceutical formulas and human urine was successfully developed. It is based on the commercial screen-printed carbon electrode covered by a thin layer of graphene and Nafion® film embedded with tyrosinase enzyme cross-linked by glutaraldehyde. This type of immobilization permits the construction of a relatively simple biosensor (without elution of biorecognition element after certain time) which exhibits excellent selectivity owing to amperometric detection at a potential of 0 V. Nevertheless, it should be reminded that the sensitivity significantly decreased with the aging of the biosensor. It can be assumed that the storage conditions will have a significant effect on the biosensor lifetime. Optimization of storage conditions will be an important stage in tyrosinase-based biosensor development. From a practical point of view, simple immobilization of tyrosinase onto the surface of the designed transducer or on commercially available single-layer graphene-modified SPCE (type DRP-110GPH) allows one to always use the freshly prepared biosensor because the whole process does not take more than half an hour.

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Compliance with ethical standards

Informed consent The study was not aiming to investigate functions/diseases of the human body or a process of medical treatment; therefore, the administration of acetaminophen by the participant was not performed and the drug was used only for artificial spiking of commercial and the participant's own urine sample. A healthy volunteer received a complete description of the study and gave written informed consent before providing the urine samples. The obtained sample of human urine was anonymized before the study. The ethical principles for medical research of the components of human beings have not been violated because no compounds other than acetaminophen (artificially enriched urine) were determined. Therefore, all experiments with human urine samples were done in accordance with the WMA Declaration of Helsinki, June 1964.

Conflict of interest The authors declare that they have no conflict of interest.

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