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An electrochemical biosensor for clenbuterol detection and pharmacokinetics investigation

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ABSTRACT

Clenbuterol is a member of β2 adrenergic agonists, which is widely used not only as a food additive for livestocks, but also a kind of stimulant for athletes; however, the abuse of clenbuterol may pose a significant negative impact on human health. Since it is highly required to develop fast, sensitive and cost-effective method to determine clenbuterol level in the suspected urine or blood, we herein have fabricated an electrochemical biosensor for the determination of clenbuterol. Measurement of the species with the proposed biosensor can also have the advantages of simplicity, high sensitivity and selectivity. Moreover, the sensor can be directly used for clenbuterol determination in rat urine. We have further studied the pharmacokinetics of clenbuterol by using this proposed electrochemical biosensor, so a new tool to investigate pharmacokinetic is developed in this work.

1. Introduction

Clenbuterol is a member of β2 adrenergic agonists, which has been known as a thermogenic drug; however, this kind of β2 adrenergic has also been popularly abused as a food additive for livestocks and stimulant for athletes, due to its omnipotent capacity to improve growth rate and reduce carcass fat [1,2]. Nevertheless, even a tiny exposition to the β adrenergic agonist either from athletic stimulants or from animal product (mainly from pork), may pose severe threat to humans, causing acute poisoning with symptoms of muscular tremor, cardiac palpitation, vomiting, nausea, nervousness and chills [3]. Due to the abominable side effects of clenbuterol, this drug is banned in most countries. To eliminate the abuse of clenbuterol, it is of high necessity and great significance to develop fast, sensitive, and cost-effective methods to determine clenbuterol levels in the suspected urine or animal product. In the meantime, better understanding of the pharmacokinetics of clenbuterol is needed, since it may help guide clinical medication safety, prevent adverse reactions, which may further contributes to unequivocal diagnosis of clenbuterol food poisoning.

So far, many methods by using different detection techniques have been proposed for clenbuterol determination, such as liquid chromatography, mass spectrometry (MS), gas chromatography coupled with MS, capillary electrophoresis with amperometric detection, colorimetric assay, immunoassay and surface-enhanced Raman scattering (SERS) [4–11]. However, the complicated pre-purification procedures, high cost and long testing period have limited their wide applications [12]. Therefore, more easily and conveniently operated, cost-and-time effective methods are still highly required to be developed.

Biosensors, especially electrochemical biosensors, with superb advantages of time-effectiveness, simple procedures and high sensitivity, have received significant scientific and industrial interests [13–15]. Especially in recent years, due to the achievements of surface assembly and modification, as well as the use of nanomaterials, more and more functionalized electrochemical biosensors are fabricated. In this work, based on the formation of platinum nanoparticles (PtNPs), which can perform high electrocatalytic activity toward the reduction of H_2O_2 to simplify analytical procedures [16], we have fabricated an electrochemical biosensor

Abbreviations: MS, Mass spectrometry; SERS, Surface-enhanced Raman scattering; PtNPs, Platinum nanoparticles; CTAC, Cetyltrimethylammonium chloride; 1,4-BDT, 1,4-Benzenedithiol; SD Rats, Sprague-Dawley rats; SCE, Saturated calomel reference electrode; DPV, Differential pulse voltammetry; EIS, Electrochemical impedance spectroscopy; CA, Chronoamperometry; SAM, Self-assembled monolayer.

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for the determination of clenbuterol. The fabricated biosensor cannot only be used for clenbuterol determination in rat urine, but also enables us to understand the pharmacokinetics of clenbuterol in rat urine without any signal interruptions [17]. So, the fabricated biosensor for clenbuterol determination proposed in this work may also hold considerable potentials to the development of next-generation strategy for pharmacokinetic study, in addition to the unequivocal diagnosis of food poisoning in the future.

2. Experimental

2.1. Materials and chemicals

Clenbuterol, cetyltrimethylammonium chloride (CTAC, 25% in water), chloramphenicol, ampicillin, kanamycin and glucose were obtained from Sigma-Aldrich. 1,4-Benzenedithiol (1,4-BDT) was purchased from Alfa-Aesar. H_2O_2, $H_2PtCl_6 \cdot 6H_2O$, $Na_2B_4O_7 \cdot 10H_2O$ were ordered from Nanjing Chemical Co., Ltd. Other reagents were of analytical reagent grade and used as received. Unless otherwise noted, all solutions were prepared with double-distilled water that was purified with a Milli-Q purification system (Barnstead, USA) to a specific resistance of >18 MΩ cm. Female Sprague-Dawley rats (SD) of about 6 weeks age were provided by Nanjing Medical University (Nanjing, China).

The buffer solutions employed in this work were as follows: 1,4-BDT buffer (1 mM 1,4-BDT diluted in ethanol), reaction buffer (4.8×10^{-7} M H_2PtCl_6, 2 mM CTAC, 0.01 M $Na_2B_4O_7$, pH 10.0), working buffer (0.1 M PBS, 10 mM H_2O_2, pH 7.4).

2.2. Preparation of rat urine samples

All animal protocols were performed in accordance with institutional and national guidelines and with the approval by the Animal Care Ethics Committee of Nanjing Medical University. Female SD Rats of about 6 weeks age were provided by Experimental Animal Centre of Nanjing Medical University (Nanjing, China). The rats were raised in metabolic cages for 2 weeks in a controlled environment (23 ± 3 °C with the humidity of 40–60%, 12 h light–dark cycle with lighting from 8:00 am to 8:00 pm). Rats were allowed to have free access to rat chow and water. All rats were randomly grouped into two groups (10 rats per group): an experimental group and a control group. A single dose of 5.0 mg/kg clenbuterol (1 mg/mL in double-distilled water) was intragastricly administrated to each of the female rats in the experimental group. The rats were fasted for more than 12 h before drug administration. The rats used as control groups were administrated with double-distilled water instead after 12 h fastness. The urine samples were collected in aseptic tubes by bladder area stimulation. The collection procedure was performed at 2, 5, 6, 8, 9, 12, 24 h after drug administration. The samples were stored in −20 °C before the electrochemical measurements. The whole process was conducted in aseptic environment, and all the materials and apparatuses were germ free [18,19].

2.3. Preparation of 1,4-BDT modified gold electrode

First, the gold electrode (3 mm diameter) was polished carefully on P3000, P5000 sand paper and alumina slurry (1 μm, 0.3 μm, 0.05 μm), respectively. Subsequently, it was thoroughly washed by ultra-sonicating in both ethanol and double-distilled water to remove the residuals, each for about 5 min. Piranha solution (98% H_2SO_4:30% H_2O_2=3:1) was then carefully dropped on the gold electrode surface (*Caution*: *Piranha solution reacts violently with organic solvents and should be handled with great care*!). After that, the electrode was further incubated in HNO_3 for 30 min, followed by thoroughly ultra-sonicating in both ethanol and double-distilled water for about 5 min. Finally, it was electrochemically cleaned with 0.5 M H_2SO_4 to remove any remaining impurities. After being dried with nitrogen, the electrode was immediately used for 1,4-BDT immobilization for 16 h at room temperature.

2.4. Fabrication of PtNPs assembled electrode surface

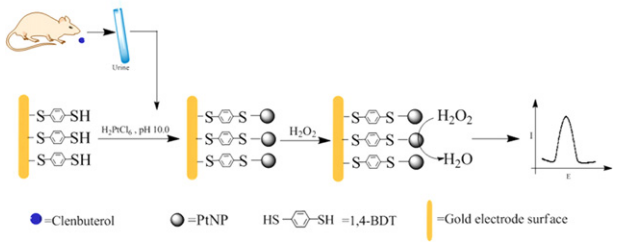
The pre-modified gold electrode was first washed by 5 mL ethanol and water separately in order to remove any non-covalently attached 1,4-BDT. Then it was immersed in the reaction buffer containing target clenbuterol or rat urine for 90 min at 70 °C. PtNPs could be synthesized on the electrode surface in the presence of clenbuterol. After that, the gold electrode was thoroughly washed by double-distilled water.

2.5. Electrochemical measurements

Electrochemical measurements were carried out on an Electrochemical Analyzer (CHI660C, CH Instruments). A three-electrode system was employed, which consisted of the modified gold electrode as the working electrode, a saturated calomel reference electrode (SCE) and a platinum auxiliary electrode. Differential pulse voltammetry (DPV), electrochemical impedance spectroscopy (EIS) and chronoamperometry (CA) were all performed at room temperature. The scan range of DPV was from −0.5 V to 0.1 V, and the initial potential was set to be −0.3 V for CA. For EIS, 5 mM $Fe(CN)_6^{3-/4-}$ with 1 M KCl was employed as the electrolyte. The spectra were recorded by applying a bias potential of 0.259 V vs. SCE and 5 mV amplitude in the frequency range of 0.1–100 kHz. Each measurement was repeated for at least three times.

3. Results and discussion

Scheme 1 may illustrate the principle of the electrochemical biosensor proposed in this work for clenbuterol determination and pharmacokinetics investigation, while urine samples are collected in aseptic tubes every a few hours after drug administration. First, 1,4-benzenedithiol (1,4-BDT), an adapter molecule, is covalently immobilized on the gold electrode surface, contributing to an arranged and compact self-assembled monolayer (SAM) [20], which also leads to a remarkable electron transfer resistance. Subsequently, the reaction between chloroplatinic acid and clenbuterol occurs, involving the oxidation of the amine groups of clenbuterol and the reduction of chloroplatinic acid into atomic platinum [21]. PtNPs are then formed, which has been confirmed by transmission electron microscope (Fig. S1). The formed PtNPs can then make a decrease of the electron transfer resistance (Fig. S2) [22]. Moreover, since PtNPs can electrochemically catalyze the reduction of H_2O_2, notable electrochemical signals can be generated to indicate the existence of clenbuterol in the test



Scheme 1. Schematic illustration of the biosensor for clenbuterol determination and pharmacokinetic investigation in rat urine.

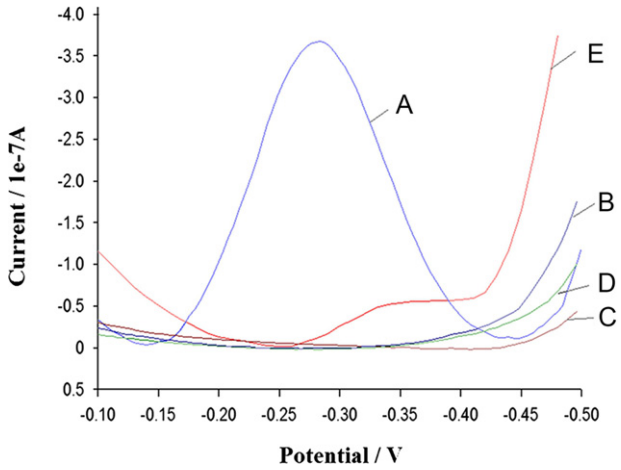


Fig. 1. (A) Differential pulse voltammogram of the electrochemical system for the analysis 0.1 μM clenbuterol. Curves *B–E* for the absence of (B) clenbuterol and chloroplatinic acid, (C) clenbuterol, (D) chloroplatinic acid, (E) H_2O_2.

sample, which have been confirmed by the measurements with differential pulse voltammetry (DPV) (Fig. 1).

As is shown in Fig. 1, only in the presence of clenbuterol, chloroplatinic acid and H_2O_2, notable electrochemical wave in the differential pulse voltammogram at −0.3 V can be observed, contributed to the catalytical reduction of H_2O_2 by the formed PtNPs [23]. On the contrary, electrochemical signal cannot be obtained in the absence of clenbuterol or chloroplatinic acid, while a very tiny signal appears at −0.35 V if there is no H_2O_2 in the test solution although PtNPs have been formed. These experimental results have confirmed that the three chemicals clenbuterol, chloroplatinic acid and H_2O_2 are essential for the achievement of the electrochemical catalysis and the obtained well-defined electrochemical waves. Since the efficiency of the electrocatalyzed reduction of H_2O_2 by PtNPs is very high [16], sensitive detection of clenbuterol can be achieved, which may satisfy the need required by food safety and clinical applications.

To obtain the highest sensitivity in shortest reaction time, we have employed chronoamperometry technique [24] to obtain the most appropriate experimental conditions for PtNPs formation in this study, including the reaction time, temperature and pH value. First, optimum electrochemical signal is obtained at pH 10.0 (Fig. S3). This result is also consistent with the previous report [21]. Second, from time-dependent *i–t* curves, it can be known that the obtained electrochemical signal can reach a saturation value in 90 min (Fig. S4). Therefore, the testing period is 90 min, which is much shorter than the traditional analytical methods for clenbuterol determination.

Fig. 2A shows the differential pulse voltammograms upon analyzing different concentrations of clenbuterol under the optimized experimental conditions. It can be observed that the peak currents increase along with the concentration of clenbuterol. The reason is clear, because more clenbuterol molecules will reduce more chloroplatinic acid, forming larger and more PtNPs, which may contribute to larger electrochemical response by catalyzing more H_2O_2. The calibration plot obtained by the electrochemical measurements has been shown in Fig. 2B. The peak current is linearly correlated to the concentration of clenbuterol in the range from 0.1 to 0.8 μM with a detection limit of 43.96 nM. The linear regression equation is

$$i(1e{-}6A) = 1.124 \times c(1e{-}7M) - 0.931 \quad (1)$$

The correlation coefficient R^2 is 0.9956, indicating a satisfactory precision of the fabricated biosensor. This biosensor can also

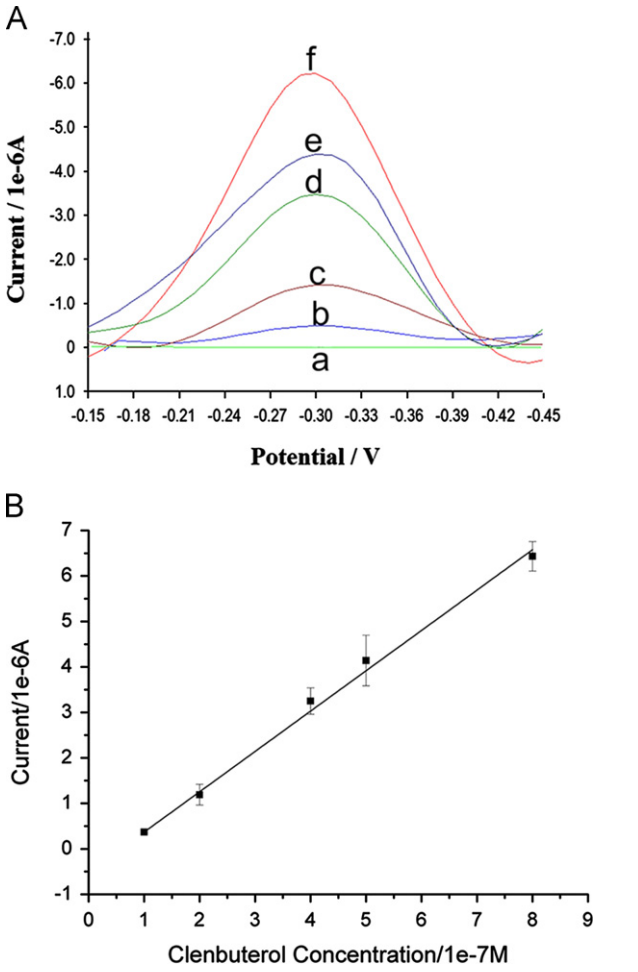


Fig. 2. (A) Differential pulse voltammograms of the electrochemical system for the analysis of (a) 0, (b) 0.1, (c) 0.2, (d) 0.4, (e) 0.5, (f) 0.8 μM clenbuterol. (B) Calibration curve of peak current vs. clenbuterol concentration.

perfectly work in the presence of interference by antibiotics and other substances.

Fig. 3 shows that even when the concentrations of the potential interferents are 400 times higher than clenbuterol, no significant electrochemical signals at −0.3 V are observed. The fine selectivity of this biosensor will be very helpful to guarantee the practical applications in biological real samples such as rat urine. Therefore, after tentative work for the analysis of clenbuterol in purified buffer, we have further challenged the biosensor for the determination of clenbuterol in rat urine. Moreover, since the fabricated biosensor can be directly used for clenbuterol determination in rat urine without any signal interruptions, it has enabled us to understand the pharmacokinetics of clenbuterol in rat urine with a new method. As is shown in Fig. 4, the urine level of clenbuterol decreases as the time for the collection of rat urine samples prolongs. We can then obtain a log regression equation for the experimental curve, which is

$$i(1e{-}7A) = 18.525 \times \exp(-t(h)/7.71) + 1.503 \quad (2)$$

The correlation coefficient ($R^2 = 0.9930$) strongly demonstrates that there is a significant negative correlations between the electrochemical response to clenbuterol residues in rat urine and the time duration. On the other hand, no electrochemical response can be observed for the control group in which clenbuterol is not administrated to the rats (Fig. 4).

Therefore, a precise relation between clenbuterol residues in rat urine and time duration is obtained by coupling Eqs. (1) and (2),

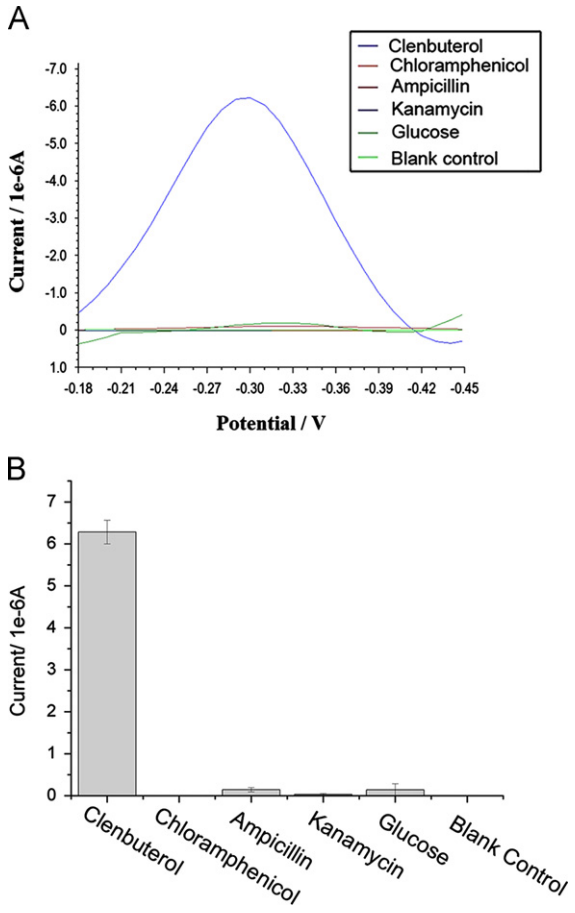


Fig. 3. (A) Differential pulse voltammograms upon the analysis 0.25 μg/mL clenbuterol and 100 μg/mL interfering molecules. From top to bottom: clenbuterol, glucose, ampicillin, kanamycin, chloramphenicol, blank control. (B) Comparison of the peak current of clenbuterol with that of the interfering species.

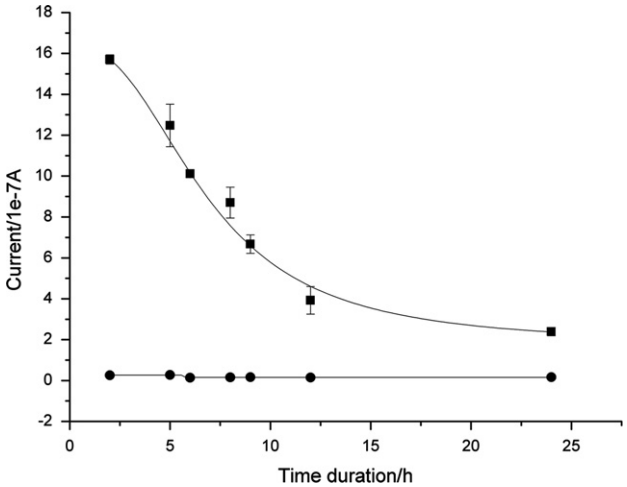


Fig. 4. Time-dependent electrochemical response to clenbuterol concentration in rat urine. (–■–) is the experimental curve, while (–●–) represents the control curve.

the results of which have been shown in Fig. 5.

$$c(1e{-}7M) = 1.65 \times \exp(-t(h)/7.71) + 0.96 \quad (3)$$

The regression results of clenbuterol pharmacokinetics in urine are consistent with those obtained by classic strategies [25], indicating that the tool we have developed in this work for pharmacokinetics investigation is applicable for the study of pharmacokinetics. Moreover, this method may have many advantages over the traditional chromatography method due to the advantages of an electrochemical biosensor.

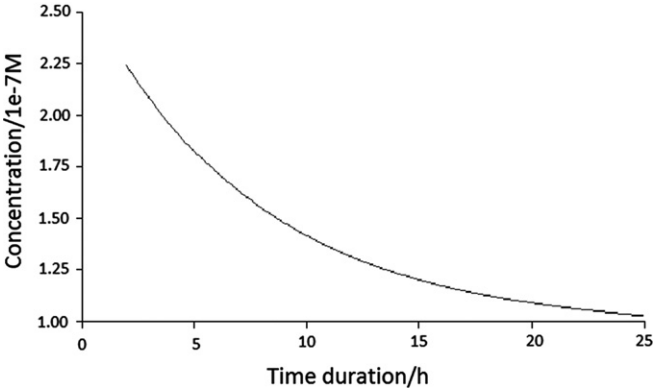


Fig. 5. Clenbuterol pharmacokinetic curve in rat urine.

4. Conclusions

In summary, we have fabricated an electrochemical biosensor for the determination of clenbuterol. The biosensor can be directly used for rat urine sample, and a new tool for the study of pharmacokinetics by coupling both nanotechnology and electrochemical technique is developed in this work. The pharmacokinetics obtained via this novel electrochemical strategy is consistent with that reported with other traditional methods, indicating a satisfactory credibility of this proposed new tool. The biosensor also has the advantages of fast response, low cost, high specificity and sensitivity. So, this study may contribute to the development of a next-generation technique for the study of pharmacokinetics, in addition to a new method for the determination of clenbuterol.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.056.

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