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Electrogenerated Chemiluminescence Biosensor with Gold Nanoparticles/$Ru(bpy)_3^{2+}$ Multilayer Films on Gold Electrodes for the Determination of Ephedrine Hydrochloride

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The aim of this study presented here is to develop a novel method for the determination of ephedrine hydrochloride with the electrogenerated chemiluminescence (ECL) by using gold nanoparticles/tris(2,2'-bipyridyl)dichlororuthenium(II) (AuNPs/$Ru(bpy)_3^{2+}$) multilayer films modified gold electrode. The ECL active species, $Ru(bpy)_3^{2+}$ was immobilized into the $\{AuNPs/Ru(bpy)_3^{2+}\}_n$ multilayer films by layer-by-layer assembly, which is based on the electrostatic interaction between positively charged $Ru(bpy)_3^{2+}$ and negatively charged AuNPs. The novel multilayer films modified gold electrode was characterized by cyclic voltammetry, ECL and SEM. The ECL biosensor based on the multilayer films containing $Ru(bpy)_3^{2+}$ was used for ECL determination of ephedrine hydrochloride. The ECL intensity was correlated with the ephedrine hydrochloride concentration in the range from 2.0×10^{-7}-1×10^{-4} g/mL (R = 0.9978) and the detection limit was 7.0×10^{-8} g/mL. The relative standard deviation (n = 11) was 3.2 % for detecting 2.0×10^{-6} g/mL ephedrine hydrochloride. This method has been applied successfully to determine ephedrine hydrochloride in pharmaceutical preparations and in ephedra herb. Statistical analysis (Student's *t*-test and variance ratio F-test) of the obtained results showed no significant difference between the proposed method and the reference method.

Key Words: Electrogenerated chemiluminescence, $Ru(bpy)_3^{2+}$, AuNPs, Multilayer films, Ephedrine hydrochloride.

INTRODUCTION

Ephedrine, (1R,2S)-2-methylamino-l-phenylpropan-1-ol (Fig. 1), a sympathomimetic drug widely used as an energizing and stimulating substance and as an additive in various diet foods for athletes designed to excite the central nervous system. It is often used to cure rhinitis, bronchial asthma[1,2]. The extensive use of this chemical concerns the guidelines of international athletes associations and has generated significant interest in the development of reliable methods for its rapid detection in various samples[3]. Up to now, many analytical methods such as HPLC[3-6], gas chromatography (GC)[7,8], LC-MS/MS[9,10], GC-MS[11], electrochemical sensor technique[12,13] *etc*., have been reported. Most of these methods require complicated instrumentation or suffer from poor sensitivity. Thus, a more sensitive and simple and convenient method for ephedrine hydrochloride determination was desirable.

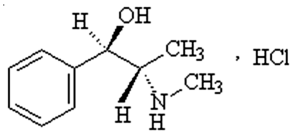

Fig. 1. Structure of ephedrine

Recently, electrogenerated chemiluminescence (ECL) has gained popularity for the detection of selected analytes[14,15]. This is due in part to low detection limits, wide dynamic ranges and instrumental simplicity. $Ru(bpy)_3^{2+}$ has become the most attractive ECL reagent because of its stability, regenerability and excellent luminescence properties[16]. The ECL reaction of $Ru(bpy)_3^{2+}$ allows the detection of a wide range of analytes, such as $C_2O_4^{2-}$ [17,18], DNA hybridization[19], proteins[20], aliphatic amines[21].

The immobilization of $Ru(bpy)_3^{2+}$ on a electrode can reduce the consumption of expensive reagent, simplify experimental design and create a regenerable sensor based on $Ru(bpy)_3^{2+}$ recycled at the electrode surface during the ECL reaction. To date, many methods for effective immobilization of $Ru(bpy)_3^{2+}$ on electrode have been developed, including the immobilization of $Ru(bpy)_3^{2+}$ in polymer layers on electrode surfaces, the direct attachment of $Ru(bpy)_3^{2+}$ to an electrode as a monolayer by a Langmuir-Blodgett technique or by a self-assembly technique and the fabrication of multilayers of $Ru(bpy)_3^{2+}$ on electrode surfaces by layer-by-layer (LBL) technique[22-27].

This paper describes the development of a novel ECL method for the determination of ephedrine hydrochloride based

on the $\{AuNP/Ru(bpy)_3^{2+}\}_n$ multilayer films by LBL assembly on a gold electrode *via* the electrostatic interaction between positively charged $Ru(bpy)_3^{2+}$ and negatively charged AuNPs. The sensitivity of the ECL system for ephedrine hydrochloride at the $\{AuNP/Ru(bpy)_3^{2+}\}_n$ multilayer film-modified gold electrodes was more than 2 orders of magnitude higher than that observed at a pure Nafion films-modified gold electrode. The $\{AuNP/Ru(bpy)_3^{2+}\}_n$ multilayer composite film-modified gold electrode also exhibited long-term stability.

EXPERIMENTAL

Tris(2,2'-bipyridyl)dichlororuthenium (II) ($Ru(bpy)_3^{2+}$) was purchased from Sigma (St. Louis, MO, USA). Nafion (perfluoinated ion-exchange resin, 5 % (w/v) solution in a solution of 90 % aliphatic alcohol 10 % water mixture), $HAuCl_4 \cdot 4H_2O$ was obtained from Guoyao Chemical Company (Guoyao, China). Ephedrine hydrochloride (CAS: 50-98-6) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). And ephedrine hydrochloride injections (30 mg/mL) were purchased from local market made by Tonghua Baishan Pharmaceutical Ltd. Distilled, deionized water was used for the preparation of all aqueous solutions. Unless otherwise stated, all the other chemicals and reagents used in this study were of analytical grade quality.

A 1.0 mg/mL standard aqueous solution of ephedrine hydrochloride was prepared by dissolving ephedrine hydrochloride hydrochloride in 10 mL of water and kept in a brown volumetric flask. Ephedrine hydrochloride working standard solution was prepared daily by serial dilution of the stock standard solution. A $HAuCl_4$ stock solution (2 % $HAuCl_4$, w/w) was prepared by dissolving 1.0 g of $HAuCl_4 \cdot 4H_2O$ (Shanghai Reagent, China) in 412 mL of purified water and stored at 4 ºC.

Unless otherwise stated all chemicals and reagents used in this study were of analytical grade quality. The working electrode was gold electrode coated with $\{AuNP/Ru(bpy)_3^{2+}\}_n$ multilayer films. An Ag/AgCl (saturated KCl) reference electrode was used for all measurements. A platinum wire was used as a counter electrode. All the potentials were measured and reported according to this reference electrode. Cyclic voltammetric (CV) experiments were performed with a CHI660B Electrochemistry Working Station (CH Instruments, Inc., Austin, TX, USA). All experiments were carried out with a conventional three-electrode system. The ECL intensity produced in the electrolytic cell was detected and recorded by a flow injection chemiluminescence analyzer (IFFD, Xi'an Remax Electronic Science Tech. Co. Ltd., Xi'an, China), which was operated by a personal computer. The photomultiplier tube (PMT) used in this analyzer was operated in current mode. And potential supply of the photomultiplier tube was 800V. The ECL cell was placed directly in front of the PMT window and was enclosed in a light-tight box. The experimental set-up was shown in Fig. 2.

The synthesized AuNPs were characterized by a transmission electron microscope (TEM; Hitachi H700, Hitachi, Tokyo, Japan) for the size and morphology. Scanning electron microscope (SEM) images were determined with a Philips FEI Quanta 200 SEM (FEI Company, Einhoven, Netherlands). The gold electrode covered with AuNPs/$Ru(bpy)_3^{2+}$) multilayer film was used for SEM imaging.

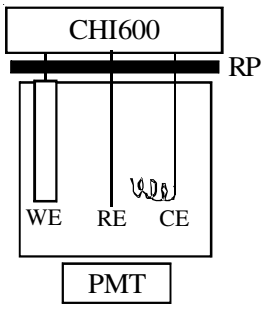

Fig. 2. Schematic diagram of ECL experimental set-up. WE: working electrode; CE: counter electrode; RE: reference electrode; RP: rubber plug

Preparation of AuNPs: 12 nm AuNPs were prepared according to the method reported previously with a slight modification[28]. $HAuCl_4$ and trisodium citrate solutions were filtered through a 0.22 μm microporous membrane filter prior to use and then 3.0 mL of 1 % trisodium citrate was added to 100.0 mL of boiling 0.01 % $HAuCl_4$ solution and stirred for 0.5 h at the boiling point. The final AuNPs prepared by this method have an average diameter of approximately 12 ± 2 nm as measured by TEM. The prepared 12 nm AuNPs were stored in brown glass bottles at 4 ºC.

Preparation of the ECL sensor: Before modification, the gold electrode was polished with a 0.3 and a 0.05 μm aluminum slurry, respectively, rinsed thoroughly with redistilled water and then sonicated in redistilled water for 3 min. A 10 μL aliquot of the AuNPs and $Ru(bpy)_3^{2+}$ aqueous were hand-casted on the surface of a gold electrode by alternate. This sequence was repeated to obtain the desired number of layers. The film was uniform and consistently salmon pink. The film was allowed to dry at room temperature. When not in use, the modified electrode was kept in the dry state at room temperature.

Analytical procedure: Five milliliters blank solution which contained 0.1 mol/L buffer solution was added to the ECL cell and a stable blank ECL signal was recorded when the electrolytic potential was applied to the working electrode. The sample or standard ephedrine hydrochloride solution which contained an appropriate concentration of ephedrine hydrochloride in 0.1 mol/L buffer solution was added to the ECL cell and the ECL signal was recorded. The concentration of ephedrine hydrochloride was quantified *via* the peak height of the ECL emission intensity that was obtained by subtracting the blank ECL emission intensity from that of the sample or standard ephedrine hydrochloride solution.

Sample extraction: The experimental protocols were the same as those described previously[29]. A total of 1 g of powder of ephedrae herba was extracted three times with 3 mL 0.37 % hydrochloric acid (concentrated hydrochloric acid/water = 1/99; v/v) by sonication at ambient temperature for 10 min. After centrifugation (10 min at 3000 rpm), the extracts were combined in a 10 mL volumetric flask and filled up to the final volume with extraction solvent. Prior to use, all samples were filtered through a 0.45 μm nylon membrane filter (Advantec MFS, Inc., USA).

Serum samples preparation: For determination of ephedrine hydrochloride in biological fluids (real serum samples), the serum samples of healthy people collected from volunteers who received a single oral dose of ephedrine hydrochloride table. The volunteers received the drug at 8:30 in the morning. Blood samples were collected in test tubes at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 and 48 h following each dose. The samples were centrifuged and plasma was separated and stored at -20 ºC until analysis.

RESULTS AND DISCUSSION

Voltammetric characterization of immobilized Ru(bpy)$_3^{2+}$: The electrochemical behaviour of Ru(bpy)$_3^{2+}$ immobilized in AuNPs/Ru(bpy)$_3^{2+}$ multilayer film-modified electrode was studied using cyclic voltammetry (CV). Fig. 3 showed CVs of 0.1 mM Ru(bpy)$_3^{2+}$ obtained at a bare gold electrode (a) and a AuNPs/Ru(bpy)$_3^{2+}$ multilayer film-modified electrode (b) in borate buffer (pH 9.5). The oxidation current Ru(bpy)$_3^{2+}$ in (a) was obviously higher than that in (b). The result suggested that the high surface energy of AuNPs contributed more to Ru(bpy)$_3^{2+}$ absorbed in multilayer films resulting in higher oxidation current. CV was used to characterize the increase in quantity of modifiers adsorbed on the multilayer films. With the increasing number of Ru(bpy)$_3^{2+}$ layers, the redox peak currents and the concentration of electroactive Ru(bpy)$_3^{2+}$ in films increase regularly, suggesting Ru(bpy)$_3^{2+}$ was adsorbed onto the multilayer coating in a layer-by-layer fashion.

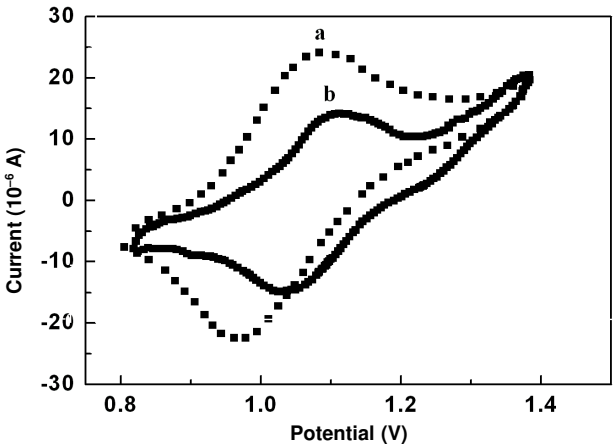

Fig. 3. Cyclic voltammogram of bare gold electrode (a) and {AuNPs/Ru(bpy)$_3^{2+}$}$_2$ films modified gold electrode (b) in pH 9.5 borate buffer solution containing 5×10^{-4} g/mL ephedrine hydrochloride. Scan rate, 100 mV/s

Gold nanoparticles and multilayer film-modified gold electrode characterization: AuNPs were prepared by sodium citrate reduction method. The sodium citrate reduction method yielded uniform AuNPs. The results showed that the particle sizes of nanoparticles were about 12 ± 2 nm. In addition, SEM was also used to characterize the composite film on the gold electrode. As seen from Fig. 4, the composite film is homogenous and the nanoparticles disperse evenly in the film.

Selection of electrochemical parameters: The electrochemical parameters would obviously affect the ECL emission intensity. When linear sweep, constant potential, CV and normal pulse voltammetry electrolytic signal was applied, respectively, ECL signal of ephedrine hydrochloride was observed. It was found that the stronger and stable ECL intensity was obtained when the constant potential mode was employed. The influence of the applied constant potential on the ECL intensity of the biosensor was also examined and the results are shown in Fig. 5. From Fig. 5, it can be seen that I increases with the applied potential increasing from +0.70 to +1.10 V and reaches the maximum at about + 1.10 V, attributed to increasing number of excited-state molecules produced during an electrochemically initiated reaction. With a further increase of the applied potential, however, I decreases. Therefore, a constant potential of 1.10 V was chosen in the following experiments to obtain a high sensitivity and good reproducibility. The corresponding ECL-potential profile is also recorded. The onset of luminescence occurred near 1.0 V and the ECL peak intensity occurred near 1.2 V, where Ru(bpy)$_3^{2+}$ was electrochemically oxidized. The ECL response at the gold electrode modified by the multilayer films was very fast. The phenomena indicated that the oxidation of ephedrine hydrochloride could be *via* a "catalytic route" where electrogenerated Ru(bpy)$_3^{2+}$ reacted with ephedrine hydrochloride.

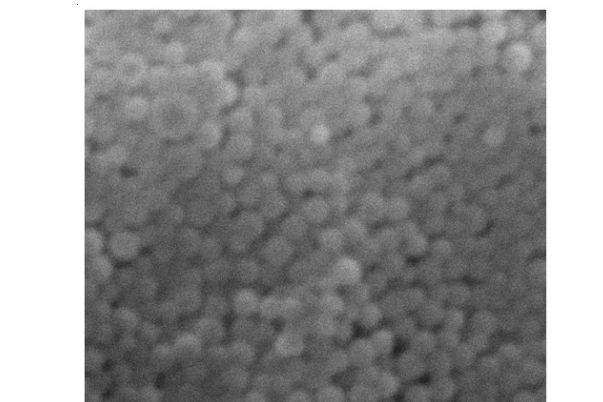

Fig. 4. TEM image of AuNPs (left) (Bar scale is 50 nm) and SEM of the {AuNPs/Ru(bpy)$_3^{2+}$}$_5$ composite film on gold electrode (right)

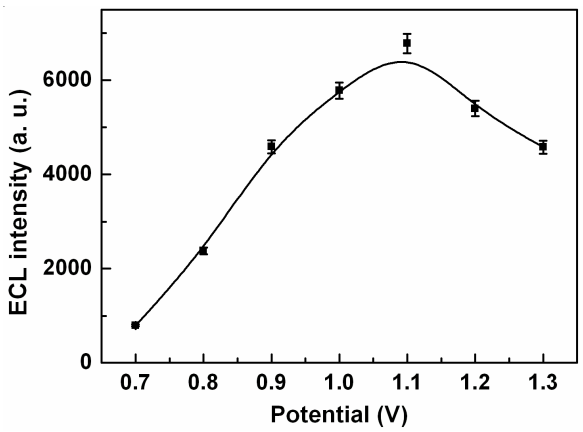

Fig. 5. Effect of applied potential on the ECL intensity. The concentration of ephedrine hydrochloride, 6.0×10^{-5} g/mL, Scan rate, 100 mV/s

Selection of number of multilayer films: The results show a linear change in ECL with increasing number of the bilayers, which was resulted from the contribution of increasing amount of electroactive $Ru(bpy)_3^{2+}$ in multilayer films. ECL emission could be produced *via* the reaction of ephedrine hydrochloride with $Ru(bpy)_3^{2+}$ at the external surface of the films and/or *via* the permeation of the ephedrine hydrochloride into the films and reaction with $Ru(bpy)_3^{2+}$ in multilayer films. But when n becomes higher than 5, ECL begins to decrease (Fig. 6). But the detailed mechanism is not clear at present time. So $\{AuNP/Ru(bpy)_3^{2+}\}_5$ multilayer film-modified electrodes was used for detecting ephedrine hydrochloride in the subsequent research works.

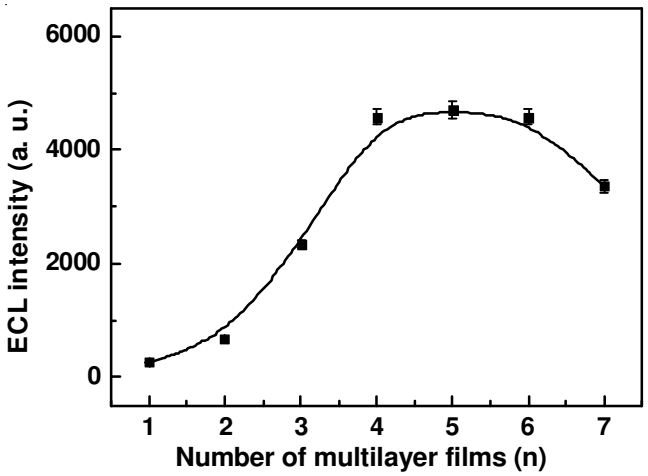

Fig. 6. Effect of the number of layers on the ECL intensity in borate buffer solution containing 5.0×10^{-5} g/mL ephedrine hydrochloride with the scan rate of 100 mV/s

Selection of the ECL reaction medium: The medium of the proposed ECL reaction system not only affected the enhancing ECL effect of ephedrine hydrochloride but also was the key factor that affected the reproducibility of proposed ECL method. In order to obtain better analytical performances, some medium, such as 0.1 mol/L Na_2CO_3, $NaHCO_3$, CH_3COONa, borate and phosphate buffer solutions were investigated. The experimental results suggested that the borate buffer solution offered best ECL sensing performances for ephedrine hydrochloride. Therefore, borate buffer solution was selected as optimum ECL reaction medium for detecting ephedrine hydrochloride in the subsequent research works. The ECL intensity increases gradually with the increasing pH. But when pH becomes higher than 9.5, ECL begins to decrease (Fig. 7). So pH 9.5 was selected.

ECL analytical performances of the proposed ECL sensor for ephedrine hydrochloride: Under the selected conditions, the proposed ECL sensor could linear sense ephedrine hydrochloride in the concentration range of 2.0×10^{-7}-1×10^{-4} g/mL and with a 7.0×10^{-8} g/mL detection limit for ephedrine hydrochloride. The regression equation was I = 30.11 + 0.8947 [ephedrine hydrochloride] (μg/mL) (Fig. 8). The correlation coefficient was 0.9979. The relative standard deviation was 3.2 % for detecting 2.0×10^{-6} g/mL ephedrine hydrochloride (n = 11). A control experiment employing Nafion films-modified gold electrode for the determination of ephedrine hydrochloride

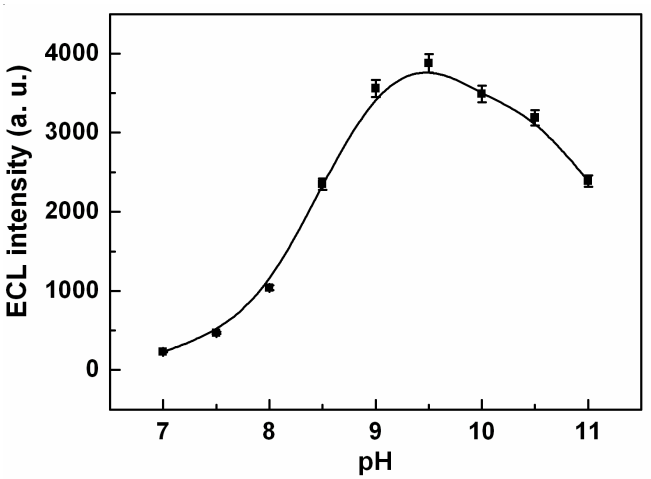

Fig. 7. Effect of pH on the ECL intensity in borate buffer solution containing 4.0×10^{-5} g/mL ephedrine hydrochloride with the scan rate of 100 mV/s

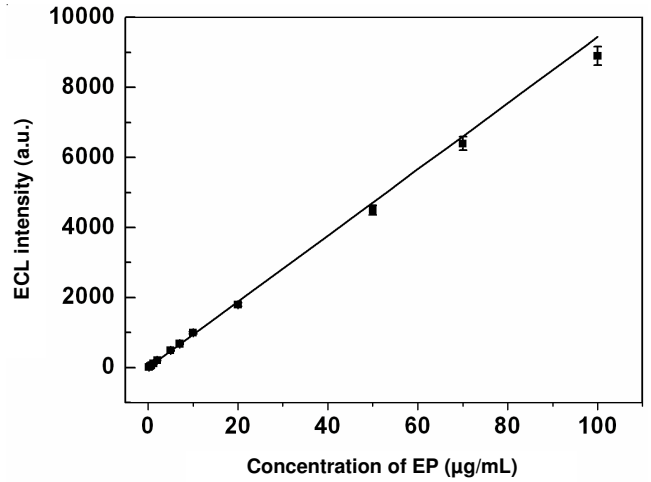

Fig. 8. Standard curve of the ECL intensities for ephedrine hydrochloride

was carried out to further determine the sensitivity of the proposed protein assay strategy. The compared method gives the detection limit of 6.0×10^{-6} g/mL. The results indicated that proposed ECL sensor giving approximately a 100-fold improvement in detection sensitivity compared to Nafion films-modified gold electrode method (we choose the lowest concentration of the linear range as the comparison standard). We also evaluated the intra-assay precision of the method by analyzing the same concentration samples 5 times with multiple replicates and the inter-assay precision by analyzing the same concentration samples on 5 consecutive days. Intra- and inter-assay precision tests indicated good repeatability of our method for ECL intensity (Table-1).

TABLE-1
INTRA-ASSAY AND INTER-ASSAY PRECISION DATA

Concentration (g/mL)	Relative SD (%)[a]	
	Intra-assay	Inter-assay
5.0×10^{-7}	4.9	5.3
5.0×10^{-6}	4.3	3.7
5.0×10^{-5}	2.8	2.4

[a]The average of five determinations.

Interferences study: The effect of foreign substances was tested by analyzing a standard solution of ephedrine hydrochloride (5.0×10^{-6} g/mL) to which increasing amounts of interfering substances were added, as the relative error was not larger than 5 %. The tolerable concentration ratios for interference at the 5 % level were over 1000 for sucrose, glucose, amylum, carbamide, Na^+, K^+, Cl^-, 100 for citrate, Mg^{2+}, Fe^{3+}, Ca^{2+}, NH_4^+, Pb^{2+}, Zn^{2+}, SO_4^{2-}, CO_3^{2-}, dexamethasone and 10 for NO_3^-,Vc, uric acid, 1 for Cu^{2+}, S^{2-}, glycine, respectively.

Stability of ECL sensor: To investigate the storage stability of proposed ECL sensor kept in borate buffer solution, the modified electrode was kept in borate buffer solution for *ca.* 24 h. The following CV measurements were performed by monitoring ECL intensity of this sensor for ephedrine hydrochloride in borate buffer solution (pH 9.5) with intermittent usage (every 2 h). The ECL intensity only decreased 7.2 % compared with the initial steady state value after 24 h of immersion in borate buffer solution. The result suggested that the modified electrode has a good stability.

Analytical application

Application to dosage forms: The ephedrine hydrochloride in its commercial pharmaceutical preparation tablets (with a nominal ephedrine hydrochloride content of 30 mg/tablet) was determined in the optimized conditions by the proposed method. Table-2 shows the results of the determination of ephedrine hydrochloride in pharmaceutical preparations. The accuracy of ephedrine hydrochloride in pharmaceutical preparations was evaluated by determining the recovery of ephedrine hydrochloride by a standard addition method, into which a known quantity of ephedrine hydrochloride was added. The results show that the concentrations obtained by the proposed method are in good agreement with those given by spectrophotometry (λ_{max} = 210 nm) (pharmacopoeia method)[30].

TABLE-2
RESULTS OF DETERMINATION OF EPHEDRINE HYDROCHLORIDE IN PHARMACEUTICAL PREPARATIONS

Sample No.	Claimed (mg/tablet)	Found (mg/tablet)[a]	Reference (mg/tablet)[31]	*t*-Value[b]	F-Value[b]
1	30	30.23 ± 0.82	30.12	2.36	4.79
2	30	29.08 ± 0.45	30.01	2.61	4.85
3	30	30.01 ± 0.53	30.23	2.43	4.23
4	30	28.98 ± 0.69	29.12	2.65	4.02
5	30	29.75 ± 0.70	28.97	2.39	3.99

[a]The average of five determinations (± SD). [b]The theoretical values for *t*- and F-values are equal to 2.78 and 5.05, respectively (p = 0.05).

Application to ephedra herb samples: In order to assess the applicability, the proposed ECL biosensor method was applied for the determination ephedrine hydrochloride in ephedra herb. The quantitative results of ephedrine hydrochloride in these samples are given in Table-3. The results show that the concentrations obtained by the proposed method are in good agreement with those given by spectrophotometry (λ_{max} = 210 nm) (pharmacopoeia method)[30].

Pharmacokinetics study: The concentrations of ephedrine hydrochloride in plasma were measured at different time points for healthy Chinese subjects after oral administration of ephedrine hydrochloride. The mean plasma concentration *versus* time curve is shown in Fig. 9. The main pharmacokinetic parameters are summarized in Table-4.

TABLE-3
DETERMINATION OF EPHEDRINE HYDROCHLORIDE IN HERBA EPHEDRAE

Sample No.	Found (mg/g)[a]	Reference (mg/g)[30]
1	11.33	11.37
2	9.85	9.78
3	12.11	11.97
4	9.95	9.87
5	10.27	10.34
6	8.98	8.98
7	9.02	9.01

[a]The average of five determinations.

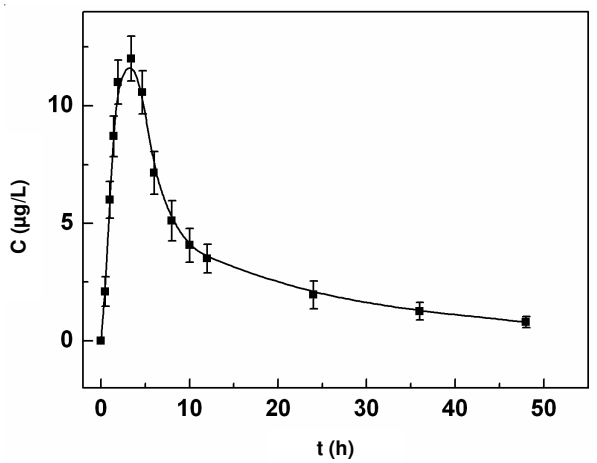

Fig. 9. Mean plasma concentration-time curve after oral administration of 5 mg of ephedrine hydrochloride

TABLE-4
MAIN PHARMACOKINETIC PARAMETERS AFTER ORAL ADMINISTRATION OF EPHEDRINE HYDROCHLORIDE

Parameter	Ephedrine hydrochloride[a]
C_{max} (μg/L)	12.5 ± 3.2
T_{max} (h)	1.8 ± 0.5
$T_{1/2}$ (h)	5.9 ± 1.8
$AUC_{0\text{-}12}$ (μg/h/L)	97 ± 23
$AUC_{0\text{-}\infty}$ (μg/h/L)	103 ± 34

[a]The average of five determinations.

Conclusion

We report a novel approach to ephedrine hydrochloride detection based on the $\{AuNP/Ru(bpy)_3^{2+}\}_n$ multilayer films by layer-by-layer assembly on a gold electrode *via* the electrostatic interaction between positively charged $Ru(bpy)_3^{2+}$ and negatively charged AuNPs. The sensitivity of this ECL sensor is significantly enhanced by encapsulation of thousands of $Ru(bpy)_3^{2+}$ inside the multilayer films on the modified electrode surface. The results also suggest that the proposed ECL sensor has a good stability due to the electrostatic interaction.

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