



Determination of itopride hydrochloride by high-performance liquid chromatography with $\text{Ru}(\text{bpy})_3^{2+}$ electrogenerated chemiluminescence detection

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ABSTRACT

In this work, a stable electrogenerated chemiluminescence (ECL) detector was developed. The detector was prepared by packing cation-exchanged resin particles in a glass tube, followed by inserting Pt wires (working electrode) in this tube and sealing. The leakage of $\text{Ru}(\text{bpy})_3^{2+}$ can be compensated by adding a small amount of $\text{Ru}(\text{bpy})_3^{2+}$ into solution phase. Coupled with high-performance liquid chromatography separation, the detector has been used for determination of itopride hydrochloride in human serum. Under the optimal conditions, the ECL intensity has a linear relationship with the concentration of itopride hydrochloride in the range of $1.0 \times 10^{-8} \text{ g mL}^{-1}$ to $1.0 \times 10^{-6} \text{ g mL}^{-1}$ and the detection limit was $3 \times 10^{-9} \text{ g mL}^{-1}$ ($S/N = 3$). The as-prepared ECL detector displayed good sensitivity and stability.

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1. Introduction

Electrogenerated chemiluminescence (ECL) is the process whereby species generated at electrodes undergo high-energy electron-transfer reactions to form excited states that emit light [1]. ECL was widely used in biological, pharmaceutical, chemical, and environmental applications due to its continuance, sensitivity, reproducibility and relative easiness to be automatically controlled [2]. Among numerous ECL reagents, $\text{Ru}(\text{bpy})_3^{2+}$ and its derivatives are the most widely used complexes for analytical investigation and application owing to that it can give strong luminescence, electrochemically be recycled and dissolve in variety of solvents [3–8]. Reactions between $\text{Ru}(\text{bpy})_3^{3+}$ and tertiary amines have led to the development of ECL-based detection devices for a variety of biologically important molecules. According to the mechanism of $\text{Ru}(\text{bpy})_3^{2+}$ ECL, $\text{Ru}(\text{bpy})_3^{2+}$ is oxidized to form $\text{Ru}(\text{bpy})_3^{3+}$, which further reacts with analyte, accompanied by light emission. However, its widespread applications are limited by the requirement to continuously deliver higher concentration of $\text{Ru}(\text{bpy})_3^{2+}$ into the reaction zone because $\text{Ru}(\text{bpy})_3^{2+}$ is consumed, which implies high cost and pollution [9–12]. Since $\text{Ru}(\text{bpy})_3^{2+}$ can be electrochemically recycled, this problem can be overcome by immobilizing $\text{Ru}(\text{bpy})_3^{2+}$ on an electrode surface, which can not only minimize

the consumption of $\text{Ru}(\text{bpy})_3^{2+}$, but also allow simpler instrumentation [13]. Therefore, much efforts have been focused on the immobilization of $\text{Ru}(\text{bpy})_3^{2+}$ on an electrode surface, thus recycling $\text{Ru}(\text{bpy})_3^{2+}$ permanently in theory and making the detection system simpler in ECL-based chemical sensors, biosensors, and detectors in flowing streams. Up to now, a number of different approaches have been tried to immobilize $\text{Ru}(\text{bpy})_3^{2+}$ or its derivatives on a variety of different electrode surfaces, including immobilization of $\text{Ru}(\text{bpy})_3^{2+}$ in Nafion films [14,15], direct attachment to an electrode by a Langmuir–Blodgett techniques [16] or self-assembly [17], immobilization by sol–gel techniques [18–23]. One of the most important factors needs to be considered for construction of a good solid state ECL detector is its stability [24]. A number of different approaches have been tried to improve the stability of $\text{Ru}(\text{bpy})_3^{2+}$ ECL-based sensors or detectors. Covalent attachment of ruthenium complexes to the electrode has been found to give no leaching to solution [25]. Other approach, such as $\text{Ru}(\text{bpy})_3^{2+}$ –poly(sodium 4-styrene sulfonate)–silica composite films [26] and $\text{Ru}(\text{bpy})_3^{2+}$ –titania–Nafion composite films [27], also showed good stability over a long period. Although all of the reported ECL sensors exhibited improved ECL characteristics, especially the characteristic of stability when compared to those based on pure Nafion films, few have however, resulted in the fabrication of stable and reproducible detectors as leaching frequently occurred. Therefore, new approaches are still needed in order to improve the stability in long-term use in a continuous flowing stream.

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Cation-exchanged resin has highly developed structure of pores on its surface, $\text{Ru}(\text{bpy})_3^{2+}$ can be easily incorporated into the material through ion-exchange process and electrostatic adsorption with high exchange capacity. Due to its high tolerability to organic solvent, it could be used as the material for $\text{Ru}(\text{bpy})_3^{2+}$ immobilization in HPLC–ECL or HPLC–CL analysis. Although Lin and Yamada have qualitatively described in their report that the $\text{Ru}(\text{bpy})_3^{2+}$ immobilized on resin could be oxidized by supplying a voltage, it could not be used as the HPLC detector directly for quantitative analysis due to its bad stability, low sensitivity and poor lifetime [28]. The aim of this work was to develop a stable, sensitive and long lifetime $\text{Ru}(\text{bpy})_3^{2+}$ immobilization-based ECL detector for HPLC analysis. The detector was designed based on a modification of Lin's work. Firstly, the ground resin particles and a Pt coil were used in order to increase the resin area directly contacted the electrode. Secondly, the leakage of $\text{Ru}(\text{bpy})_3^{2+}$ from the resin was compensated by adding a small amount of $\text{Ru}(\text{bpy})_3^{2+}$ in mobile phase. The as-prepared ECL detector displayed good sensitivity, stability and obviously smaller dead volume. Due to the quantity of $\text{Ru}(\text{bpy})_3^{2+}$ immobilized on the resin was determined by the quantity of $\text{Ru}(\text{bpy})_3^{2+}$ in mobile phase, the detector could be used for a long time. Application of the proposed detector in HPLC for determination of itopride hydrochloride, a novel gastroprokinetic agent containing a tertiary amine group, proved feasible. Such an approach to stable detection is believed to work in other sensors or detectors based on non-covalent $\text{Ru}(\text{bpy})_3^{2+}$ immobilization.

2. Experimental

2.1. Apparatus and reagents

The analysis were carried out using a LC-6A (Shimadzu, Tokyo, Japan) liquid chromatography equipped with a Rheodyne 7725i syringe loading sample injector valve (Cotati, CA, USA) and an Agilent Zorbax SB-C₁₈ column (I.D.: 250 mm × 4.6 mm, particle size: 5 μm, pore size: 80 Å, Agilent, USA). A dual-electrode electrochemical flow-cell (Fig. 1) was used for the ECL experiments. The constant current was supplied by a JH2C galvanostat (Shanghai Electric Instrument Plant, Shanghai, China). The flow-cell, in which ground resin particles were packed, contains Pt wires working electrode (Ø 0.2 mm) and a stainless steel counter electrode attached to the flow-cell outlet. The flow-cell was placed close to the window of photomultiplier tube. Data collection and processing were performed using an IFFL-D Flow Injection Chemiluminescence Analyzer (Xi'an Remax Electronic Science-Tech Co. Ltd., Xi'an, China).

Tris(2,2'-bipyridyl)dichlororuthenium(II) ($\text{Ru}(\text{bpy})_3\text{Cl}_2 \cdot 6\text{H}_2\text{O}$) was purchased from Sigma (St. Louis, MO, USA). Itopride hydrochloride was obtained from Xiuzheng Pharmaceutical Group (Jilin, China). Acetonitrile was of HPLC grade (Tianjin Kermel Chemical Reagent Co., Ltd., Tianjin, China). All the other reagents were of analytical reagent grade unless specified otherwise. Deionized and doubly distilled water was used throughout.

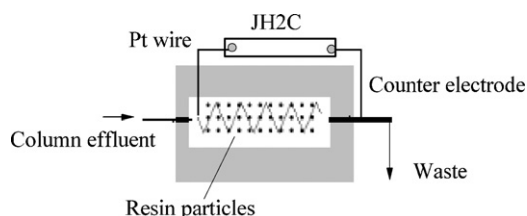


Fig. 1. Side view of the flow-cell. JH2C, galvanostat.

2.2. Stock solutions

Stock solution of itopride hydrochloride (0.5 mg mL^{-1}) was prepared by dissolving 50.0 mg of itopride hydrochloride in 100.0 mL of water and kept in the freezer at -4°C . The diluted solutions were prepared, just before use, by accurate dilution with water. A 1.0 mg mL^{-1} $\text{Ru}(\text{bpy})_3^{2+}$ solution was prepared by dissolving 50.0 mg of $\text{Ru}(\text{bpy})_3\text{Cl}_2 \cdot 6\text{H}_2\text{O}$ in 50.0 mL of water and kept in a brown volumetric flask at -4°C .

2.3. Preparation of serum samples

Blood samples were collected from healthy donors. 0.3 mL 10% HClO_4 was added to 0.5 mL aliquot of serum sample and vortex-mixed for about 30 s. After centrifugation at 8000 rpm for 10 min to remove precipitate, 20 μL aliquot of the supernatant was injected into the HPLC column [29].

2.4. Preparation of the ECL detector

The beads of cation-exchanged resin were ground and the obtained particles were packed into a glass tube, then Pt wires (working electrode) were inserted into this tube. After the tube was sealed, 5 mL 1.0 mg mL^{-1} $\text{Ru}(\text{bpy})_3^{2+}$ solution was pumped through the tube circularly for about 2 h.

2.5. Analytical procedure

The mobile phase of acetonitrile/100 mmol L^{-1} phosphate buffer/1.0 mg mL^{-1} $\text{Ru}(\text{bpy})_3^{2+}$ (25/75/1, V/V, pH 6.8) was pumped at a flow rate of 1.0 mL min^{-1} through the HPLC column and flow-cell successively, then turned on the JH2C galvanostat (current intensity: 30 μA) and equilibrated for at least 30 min. Until stable baseline was achieved, 20 μL standard solution or sample solution was injected into the mobile phase. The quantitative determination was based on the net ECL intensity $I = I_s - I_0$, where I_s is the ECL intensity in the presence of itopride hydrochloride and I_0 is the intensity of blank signal.

3. Results and discussion

3.1. Characterization

An ECL detector was prepared according to the procedure described in Section 2.4 and was evaluated in terms of its stability using itopride hydrochloride as a model. Cation-exchanged resin has highly developed structure of pores on its surface. $\text{Ru}(\text{bpy})_3^{2+}$ can be easily incorporated into the material through ion-exchange process and electrostatic adsorption with high exchange capacity. When the resin was immersed in a solution containing $\text{Ru}(\text{bpy})_3^{2+}$, $\text{Ru}(\text{bpy})_3^{2+}$ was adsorbed into the resin until the balance between adsorption and dissociation was reached. Under the balanced state, the amount of $\text{Ru}(\text{bpy})_3^{2+}$ adsorbed in resin and remained in solution were measured using a conventional $\text{Ru}(\text{bpy})_3^{2+}$ /Tripropylamine ECL method. As shown in Fig. 2(a), the amount of $\text{Ru}(\text{bpy})_3^{2+}$ adsorbed in the resin (expressed as mol L^{-1}) is largely higher than that in the solution phase. This suggested that if a large amount of $\text{Ru}(\text{bpy})_3^{2+}$ has been preadsorbed into the resin, only a small amount of $\text{Ru}(\text{bpy})_3^{2+}$ in solution phase is needed to maintain the adsorption–dissociation equilibration and the constant of $\text{Ru}(\text{bpy})_3^{2+}$ in resin. Namely, the leakage of $\text{Ru}(\text{bpy})_3^{2+}$ from the resin could be compensated by adding a small amount of $\text{Ru}(\text{bpy})_3^{2+}$ in the solution phase. Undoubtedly, the signal obtained by the proposed detector could be divided into two parts. One was from the $\text{Ru}(\text{bpy})_3^{2+}$ in solution phase, another was from the $\text{Ru}(\text{bpy})_3^{2+}$ adsorbed into the resin. The ECL signal obtained by

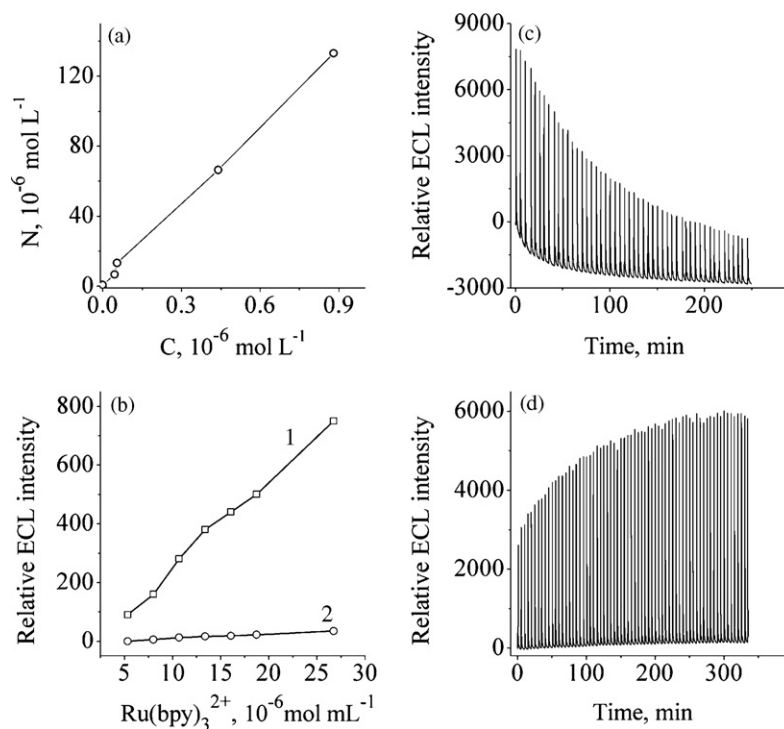


Fig. 2. Characteristics of the proposed detector: adsorption of Ru(bpy)_3^{2+} in the ion-exchanged resin (a), ECL signal with (1) and without (2) the ion-exchanged resin (b) and the stability of the detector in the absence (c) and in the presence (d) of Ru(bpy)_3^{2+} in mobile phase; N and C , concentration of Ru(bpy)_3^{2+} adsorbed in resin and in solution; Current intensity: $30 \mu\text{A}$.

the proposed detector in the presence of $5.35 \times 10^{-6} \text{ mol mL}^{-1}$ to 26.7 mol mL^{-1} Ru(bpy)_3^{2+} was compared with that by a detector containing the same concentration of Ru(bpy)_3^{2+} in the solution only. As can be seen in Fig. 2 (b), the signal obtained by the proposed detector was largely higher than that by a detector containing Ru(bpy)_3^{2+} in the solution only. The results revealed that the signal was mainly from the Ru(bpy)_3^{2+} adsorbed into the resin, but not Ru(bpy)_3^{2+} in solution. Previous investigation on the ECL of Ru(bpy)_3^{2+} immobilized in Nafion film has indicated that ECL could be not generated in any areas within the Nafion film where

Ru(bpy)_3^{2+} did not directly contact the electrode [14]. A similar mechanism was thought to be true in present system. Namely, the ECL signal was mainly from Ru(bpy)_3^{2+} directly contacted the electrode. Further experiments compared the stability of the proposed detector in the absence (Fig. 2, c) and presence (Fig. 2, d) of $1.0 \times 10^{-5} \text{ g mL}^{-1}$ Ru(bpy)_3^{2+} in the flowing stream. Due to the leakage of Ru(bpy)_3^{2+} from the resin into flowing stream, the ECL signal decreased continuously in the absence of Ru(bpy)_3^{2+} . However, if a small amount of Ru(bpy)_3^{2+} ($1.0 \times 10^{-5} \text{ g mL}^{-1}$) was added into the flow stream at this time, the ECL signal increased gradually and retained stable after the balance between the adsorption and dissociation was developed. From above discussions, it suggested that a small amount of Ru(bpy)_3^{2+} in the flowing stream could maintain not only a large amount but also constant concentration of Ru(bpy)_3^{2+} on the surface of the resin particles, and

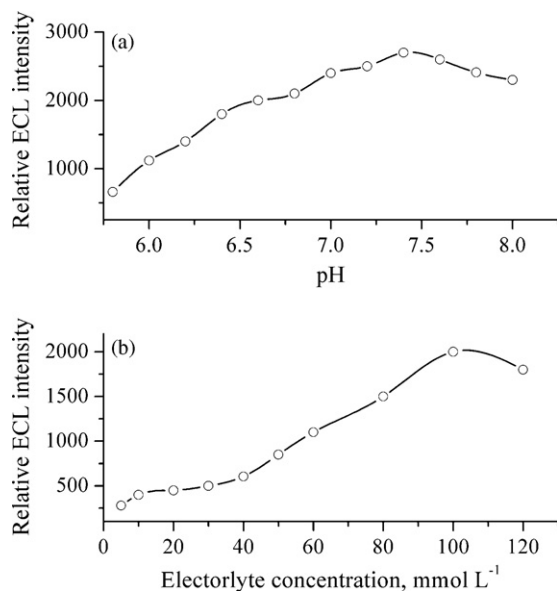


Fig. 3. Effect of pH (a) and electrolyte concentration (b). Current intensity: $30 \mu\text{A}$; Ru(bpy)_3^{2+} concentration: 0.01 mg mL^{-1} .

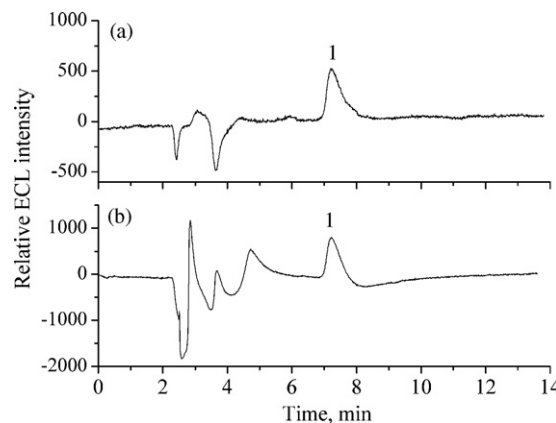


Fig. 4. Typical chromatograms of $5.0 \times 10^{-7} \text{ g mL}^{-1}$ itopride hydrochloride standard solution (a) and a serum sample containing $7.2 \times 10^{-7} \text{ g mL}^{-1}$ itopride hydrochloride (b). Mobile phase: acetonitrile/ 100 mmol L^{-1} phosphate buffer/ 1.0 mg mL^{-1} Ru(bpy)_3^{2+} (25/75/1, V/V, pH 6.8); Flow rate: 1.0 mL min^{-1} . 1: itopride hydrochloride.

Table 1

Determination of itopride hydrochloride in human serum.

Sample	Detected ($\mu\text{g mL}^{-1}$)	Added ($\mu\text{g mL}^{-1}$)	Found ($\mu\text{g mL}^{-1}$)	Recovery (%)	RSD
1	0.048	0.025	0.072	96	4.5
		0.050	0.095	93	3.2
		0.075	0.12	95	3.6
2	0.24	0.25	0.47	93	4.1
		0.50	0.70	92	4.7
		0.75	0.95	94	3.8

therefore constant signal. Due to the quantity of $\text{Ru}(\text{bpy})_3^{2+}$ immobilized on resin was determined by the quantity of $\text{Ru}(\text{bpy})_3^{2+}$ in the flowing stream, the detector could be used for a long time. In this work, the same detector was used for all the experiments and it kept stable during the course of experiments (3 months). Although long-term test was not conducted, theoretically, it can be used for longer time.

3.2. Selection of the ECL reaction medium

The ECL of $\text{Ru}(\text{bpy})_3^{2+}$ /itopride hydrochloride was very sensitive to the solution pH. The effect of solution pH was examined over the range of 5.8–8.0. The results are shown in Fig. 3(a). Maximum ECL intensity was observed at pH 7.4. Considering the tolerance ability of the HPLC column to base, pH 6.8 was selected for the following experiments. The concentration of electrolyte also has important influence on the ECL intensity of $\text{Ru}(\text{bpy})_3^{2+}$ /itopride hydrochloride. The effect was investigated from 5 to 120 mmol L^{-1} using KH_2PO_4 –NaOH buffer as the supporting electrolyte. As shown in Fig. 3(b), the ECL intensity increased with the increase of electrolyte concentration from 5 to 100 mmol L^{-1} and then decreased continuously at each concentration level higher than 100 mmol L^{-1} . This might be due to under these conditions, the rate of $\text{Ru}(\text{bpy})_3^{2+}$ adsorption was lower than that of dissociation and therefore resulted in the continuously decrease of $\text{Ru}(\text{bpy})_3^{2+}$ concentration on the surface of the resin.

3.3. Method performance

HPLC separation of itopride hydrochloride in serum was performed isocratically at a flow rate of 1.0 mL min^{-1} and the eluate from the column was continuously monitored by ECL. Fig. 4 shows the typical chromatograms of a standard itopride hydrochloride solution (a) and human serum sample spiked with itopride hydrochloride (b). As can be seen, itopride was well separated from the impurities. The validation of the proposed method and the recovery test were carried out on spiked samples. The intra-day precision of 7 repeated injections of $5.0 \times 10^{-8} \text{ g mL}^{-1}$ sample solution was 4.3% and the inter-day precision of the proposed method was 7.8% by analyzing the same sample, injected three times every day on three consecutive days. The results were showed in Table 1.

Under the optimum conditions as those described above, the calibration graph of ECL intensity versus itopride hydrochloride concentration was linear in the range of $1.0 \times 10^{-8} \text{ g mL}^{-1}$ to $1.0 \times 10^{-6} \text{ g mL}^{-1}$. The regression equation was $I = 1247.8C + 37.33$ (C : itopride hydrochloride concentration, $10^{-6} \text{ g mL}^{-1}$) with a correlation coefficient of 0.9992. The detection limit was $3 \times 10^{-9} \text{ g mL}^{-1}$ ($S/N = 3$).

4. Conclusion

In this work, an ECL detector with improved stability was developed and used as post-column detector in HPLC for the determination of itopride hydrochloride. The method is based on the immobilization of $\text{Ru}(\text{bpy})_3^{2+}$ in cation-exchanged resin. The results suggest that the leakage of $\text{Ru}(\text{bpy})_3^{2+}$ from the resin into solution phase can be compensated by adding a suitable amount of $\text{Ru}(\text{bpy})_3^{2+}$ in the mobile phase. Compared with the sensor without addition of $\text{Ru}(\text{bpy})_3^{2+}$ in mobile phase, the proposed sensor shows better stability. Application of the detector to analysis of itopride hydrochloride in human serum proved feasible. Theoretically, it can be used for a long-term. Further more, this approach to stable detection is believed to work in other sensors or detectors based on non-covalent $\text{Ru}(\text{bpy})_3^{2+}$ immobilization.

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