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Article

Flow-Injection Electrochemiluminescence Detecting Rifampicin Based on Its Sensitizing Effect

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A new flow-injection electrogenerated chemiluminescence (ECL) method for the determination of rifampicin was developed. The method is based on the sensitizing effect of rifampicin on the weak ECL signal of electrochemical oxidation of luminol on the surface of the platinum flake electrode in the medium of 0.02 mol • L^{-1} $Na_2B_4O_7$. This ECL response is in the range of 1.0×10^{-8}—4.0×10^{-6} mol • L^{-1} rifampicin with a detection limit of 8.0×10^{-9} mol • L^{-1}. The relative standard deviation is 1.8%. This method has been applied successfully to the determination of rifampicin in capsule, ocustilla and urine samples.

Keywords electrogenerated chemiluminescence, rifampicin, luminol

Introduction

Rifampicin, a semi synthetic compound, is used primarily in the treatment of tuberculosis, and it is also an excellent amistaphyloco antibiotic when used in combination with other antibiotics, so it is very useful clinically.[1] Therefore, the determination of rifampicin has attracted much attention. From now on, techniques for the determination of rifampicin have been primarily based on spectrophotometry,[2] double-wavelength thin-layer scan,[3] chemiluminescence,[4] high performance liquid chromatography.[5,6] However, these techniques suffer from tedious procedure, low sensitivity or poor selectivity.

In recent years, electrogenerated chemiluminescence (ECL), as a powerful analytical technique, much attention has been paid by the analysts because this technique retains many advantages of chemiluminescence and electrochemical analysis.[7-9] Nowadays, coupled with some new techniques such as: flow-injection analysis (FIA), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), ECL is becoming increasingly important in various fields for its high sensitivity, rapidity, simplicity and feasibility.[10-14] Due to these analytical advantages of the ECL owned, many ECL systems, such as luminol ECL system, lucigenin ECL system and $Ru(bpy)_3^{2+}$ system *etc*., have been developed and used widely in analytical sciences. Of these reported ECL systems, the luminol ECL system was more favorite to analytical applications because it offered the lower oxidizing potential, higher ECL yields, inexpensive reagent price and better solubility in water. Based on these advantages, luminol ECL system has been widely used for the determination of different analytes, such as metal ions,[15,16] hydrogen peroxide[17,18] and some organic compounds.[19] However, up to now, most of the luminol-based ECL systems for detecting organic compounds often need the use of the oxidase enzyme and suffer from the higher experimental fees and the poor stability. Thus, developing the new ECL idea to detect organic compounds without use of the enzyme was more desirable.

In this paper, it was found that in the borax buffer solution, when a 1.20 V (versus Ag/AgCl reference electrode) electrolytic potential was applied to the working electrode, the weak ECL signal, generated from the electrochemical oxidation of luminol, was directly enhanced by rifampicin when it was present in a luminol solution without the use of any incorporation reactions. Based on this finding, a sensitive, selective and rapid flow-injection ECL method for the determination of rifampicin was developed. Compared to the reported methods for rifampicin, the proposed ECL method not only offered the higher sensitivity, but also the rapid analytical procedure as well as the higher selectivity. In addition, the proposed ECL method has been successfully used to determine rifampicin in capsule, ocustilla and human urine samples.

Experimental

Reagents

All solutions were prepared from analytical-reagent grade materials with distilled, de-ionized water. Stock rifampicin solution was obtained by dissolving 50 mg of rifampicin (The Institute of Pharmaceutical and

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Bio-material Authentication, China) in methanol, and transferring this solution into a 50 mL calibrated flask and diluting to volume with methanol. Borax buffer solution: 0.02 mol • L^{-1} and luminol solution: 4.0×10^{-6} mol • L^{-1} were used.

Apparatus

The manifold of the ECL flow system used in this work is shown in Figure 1. Two peristaltic pumps were used to deliver the carrier stream (borax buffer solution), sample solution and luminol solution, and injection of both the mixed solution of sample with luminol and the luminol solution was made using a six-port valve equipped with a 50 μL sample loop. The flow-through electrolytic cell utilized a conventional three-electrode set-up and was arranged as shown in Figure 2. The flow-cell was made of glass tubing (length 3.0 cm, i.d. 3 mm). The working electrode was a platinum foil (length 2.0 cm, width 3 mm). A stainless steel tube and Ag/AgCl wire was used as the counter electrode and reference electrode, respectively. The applied potential for constant potential electrolysis was achieved by a DJS292 potentiostat/galvanostat. The ECL intensity was transformed into an electrical signal by an R456 photomultiplier tube (Hamamatsu), which was operated at 600 V and placed directly in front of the ECL flow-cell. The signal was recorded with an XWT-204 chart-recorder.

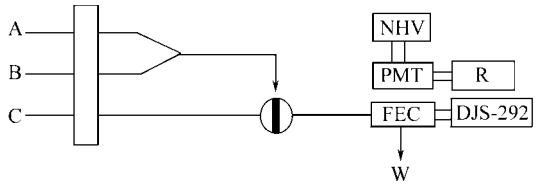

Figure 1 Schematic diagram of the flow-injection ECL system. A: luminol; B: rifampicin sample; C: 0.02 mol/L borax buffer solution; W: waste; NHV: negative high voltage; PMT: photomultiplier tube; FEC: flow-through electrolytic cell; R: recorder; DJS-292: constant potentiostat.

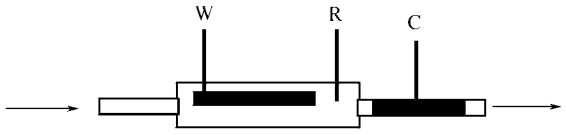

Figure 2 Structure of the flow-through electrolytic cell. W: working electrode; R: reference electrode; C: counter electrode.

Procedures

In order to achieve good mechanical and thermal stability, the instruments were allowed to run for 10 min before the first measurement was made. The carrier stream and sample stream were fed at a flow rate of 2.0 mL • min^{-1}. A 1.20 V potential was applied to the working electrode with the DJS292 potentiostat/galvanostat.

The blank solution, which contained only 4.0×10^{-6} mol • L^{-1} luminol, was injected into the carrier stream by the six-port valve, and a stable blank signal was observed, then the mixed solution of sample or standard rifampicin solution with luminol was injected into the carrier stream. The ECL signal was recorded and the concentration of rifampicin was quantified by the peak height of the enhanced ECL emission intensity obtained by deducting the blank ECL intensity of the luminol solution from that with the sample or standard rifampicin solutions.

Results and discussion

ECL reaction mechanism

The Figure 3 showed the effect of the applied potential on the enhanced ECL signal. The results showed that when the applied potential was $>$0.7 V, the enhanced ECL signal could be observed. The ECL enhanced signal achieved its maximum value at 1.20 V, above 1.20 V, the ECL enhancement decreased slowly with increasing potential. These results revealed that the electrochemically-oxidizing product of rifampicin at 1.20 V was the key species for enhancing the weak ECL signal of luminol.

In addition, it is further find that when all solutions in this ECL system was deoxygenated with pure nitrogen gas, the enhancing function of rifampicin for this weak ECL signal nearly disappeared. This suggested that the dissolved oxygen in the solution was another key species in the proposed ECL system.

For exploring the relationship between these two key species presented above in the proposed enhanced ECL system, an active oxygen removing reagents (ascorbic acid) was added to the solution of rifampicin or luminol. The results showed that both the enhanced ECL signal and unenhanced weak ECL signal nearly disappeared when ascorbic acid was added. This results showed that the "active oxygen", more possible the superoxygen anion radical, may be produced in the proposed ECL reaction. At last, the ECL emission spectrum was obtained with an RF-540 fluorescence spectrophotometer. The result showed that the maximum emission wavelength was 425 nm and indicated that the ECL emitter was excited state 3-aminophthalate.

Based on these results, it is suggested that the ECL reaction mechanism is as follows: initially, both the luminol and rifampicin are oxidized at the surface of the platinum electrode, producing the luminol radical and the electrochemically-oxidizing product of rifampicin, an reducing intermediate, in the diffusion layer of the electrode. Then, because of the stronger reducing ability of this intermediate, it can further reduce dissolved oxygen in the solution to generate the superoxygen anion radical. Finally, the chemical reaction of the superoxygen anion radical with the luminol radical gave the enhanced emission.

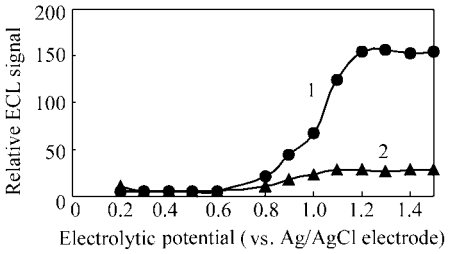

Figure 3 Effect of electrolytic potential on ECL. 1, enhancing ECL signal; 2, blank ECL signal of luminol.

The design of the flow-injection ECL system

For obtaining the strongest enhanced ECL signal arising from rifampicin/luminol, many different designs of flow-injection manifolds were studied. The results showed that when a mixed solution of rifampicin with luminol was injected into the flow-through electrolytic cell, the enhanced ECL signal was the strongest. Thus, such a flow-injection system (as shown in Figure 1) was used in subsequent experiments.

Selecting the carrier stream

In the proposed flow-injection ECL system, the components of the carrier stream, including its pH and the supporting electrolyte, not only provided the medium for the ECL reaction but also was the key factor which affected the reproducibility of this ECL analytical method since the carrier stream was also used as the solution to treat the working electrode to eliminate the problem of electrode fouling by use of a constant potential electrolysis method. Many carrier streams, such as $Na_2B_4O_7$, NaOH, Na_2CO_3, $NaHCO_3$, Na_2CO_3-$NaHCO_3$, $NaHCO_3$-NaOH, KH_2PO_4-Na_2HPO_4 and NH_3-NH_4Cl solution were used for this purpose. We found that when 0.02 mol • L^{-1} borax buffer solution was used as the carrier stream, not only was the maximum ratio of enhanced ECL signal of rifampicin to blank ECL signal of luminol obtained, but the best reproducibility for monitoring rifampicin was achieved. So this buffer solution was selected as the carrier stream for the determination of rifampicin in the proposed ECL system.

Effect of luminol concentration

Initial tests showed that the ratio of the enhanced ECL signal of rifampicin to the blank ECL signal of luminol was dependent upon the luminol concentration. The results showed that the best concentration was 4.0 μmol • L^{-1} luminol, which was selected as the optimum concentration for determining rifampicin.

Analytical performance for rifampicin

Under the selected conditions given above, the response to rifampicin was linear in the range of 1.0×10^{-8} to 4.0×10^{-6} mol • L^{-1}. The regression equation was $I_1=6.0\times10^7c-1.6$ ($c=4.0\times10^{-6}$—4.0×10^{-7} mol • L^{-1}) with the correlation coefficient being 0.9993 and $I_2=5.6\times10^7c+1.9$ ($c=4.0\times10^{-7}$—1.0×10^{-8} mol • L^{-1}) with the correlation coefficient being 0.9996. The detection limit of the method was 8.0×10^{-9} mol • L^{-1} (signal-to-noise=3). The relative standard deviation (RSD) was 1.8% for the determination of 5.0×10^{-7} mol • L^{-1} rifampicin (n=11).

Interference study

The effect of common components in urine samples on the determination of rifampicin was studied. One species was not considered to interfere if it caused a relative error of less than 5% for the determination of 5.0×10^{-7} mol • L^{-1} rifampicin. The tolerable ratios of common components in urine to 5.0×10^{-7} mol • L^{-1} rifampicin are listed in Table 1. It can be seen that ascorbic acid, thiourea, Cu^{2+}, Co^{2+}, Mn^{2+} and Fe^{3+}, *etc.* interfere seriously. Therefore, sample pre-treatments were necessary when the proposed method was applied to the analysis of rifampicin in urine samples.

Documents[1] reported that rifampicin is highly soluble in chloroform whereas ascorbic acid, thiourea and some metal ion such as Cu^{2+}, Co^{2+}, Mn^{2+} and Fe^{3+} are basically water-soluble and almost insoluble in chloroform. Therefore, it is possible that these interferences in human urine samples can be eliminated by extraction with chloroform. Other substances in human urine in the normal concentration range did not interfere with the determination of rifampicin.

In addition, the effect of the methanol, which was used as the solvent for rifampicin, on the enhanced ECL signal was investigated. It was found that the ECL signal was greatly enhanced by appropriate methanol. When 0.5—2.0 mL of methanol was contained in 50 mL of solution, the ECL signal was enhanced the most greatly and it was stable too. Thus, 1.0 mL of it was used.

Table 1 Tolerable concentration ratios with respect to rifampicin for some interfering species

Substance	Tolerable concentration ratios
K^+, NO_3^-, Cl^-	2000
Ca^{2+}, Mg^{2+}, HPO_4^{2-}, starch, urea	1000
EDTA, citric acid	500
AcO^-, NH_4^+, Br^-, glucose, uric acid	200
Oxalic acid, lactic acid	50
Ascorbic acid, benzoic acid, thiourea	1
Fe^{3+}, Al^{3+}	5
Cu^{2+}, Co^{2+}, Mn^{2+}	0.05

Applications

Analysis of rifampicin in capsule and ocustilla

The proposed method was applied to the determination of rifampicin in capsule. Not less than 10 grains of capsules (Chendu Jinhua Pharmaceutical Plant, China) were weighed, ground to fine powder and mixed. A por-

tion of the powder (equivalent to approximately 50 mg of rifampicin) was weighed and dissolved in 50 mL of methanol. After filtering, aliquots of the filtrate were further diluted with water in order that the concentration of rifampicin was in the working range. The method has been further applied to the direct determination of rifampicin in ocustilla (Wuhu Sanyi Pharmaceutical Plant, China). Both of the results are given in Table 2. The recovery obtained by adding a standard solution of rifampicin to each sample ranged between 98% and 102%. The results are given in Table 2 too and agree well with those obtained by an official method.[1]

Table 2 Determination results of rifampicin in capsule and ocustilla [a]

Sample	Claimed Value/g	Official method[1]/g	Present method/g	Added/ g	Recovery/ %
Rifampicin capsule	0.1500	0.1492	0.1486	0.0500	98—101
Rifampicin ocustilla	0.0100	0.0098	0.0094	0.0100	99—102

[a] Average of five measures.

Analysis of urine samples

The proposed method was finally applied to the indirect determination of rifampicin concentration in urine samples of a volunteer. The volunteer was being treated with rifampicin, 600 mg in a single daily dose; Urine samples were collected at appropriate time after taking medicine and were further diluted with water in order that the concentration of rifampicin was in the working range. Interference studies showed that foreign species present in urine such as ascorbic acid, Cu^{2+}, Co^{2+}, Mn^{2+} and Fe^{3+}, *etc*. would interfere with the determination of rifampicin at their normal concentration levels. In order to eliminate these interferences, a prior separation of the analyte by extracting technique with chloroform was employed. The concrete operation process is as follows: put 1.0 mL of diluted urine sample into a filtering funnel, add 9 mL of water and 10 mL of chloroform, shake, put aside for stratification. Collect the organic phase for later use. Add another 10 mL of chloroform into the aqueous phase, shake, put aside for stratification, then discard the aqueous phase. The processed two organic phases were combined and heated to near dryness at 60 ℃ on a water bath and the residue dissolved in 1.0 mL of methanol, and the clear solution was quantitatively transferred into a 50 mL volumetric flask, made up to volume with water and then used to achieve the analysis.

The results of rifampicin determination in urine samples were given in Table 3. In order to evaluate the validity of the proposed method for the determination of rifampicin in urine samples, recovery tests were carried out on samples to which known amounts of rifampicin were added. The recoveries for the different concentration levels varied from 97% to 102% as shown in Table 3, too. As can be seen, the acceptable recovery was obtained by the proposed method.

Table 3 Results of analysis of rifampicin in urine samples [a]

Sample	Amount found [b]/ ($\mu g \cdot mL^{-1}$)	Added / ($\mu g \cdot mL^{-1}$)	Found / ($\mu g \cdot mL^{-1}$)	Recovery/ %	RSD/ %
Urine 1	0	1.00	1.02	102	2.3
Urine 2	40.6	1.00	0.98	98	2.8
Urine 3	178.4	2.00	1.94	97	3.0
Urine 4	58.2	2.00	2.02	101	2.9

[a] Average of three measures; [b] Urines 2, 3, 4: measurement results of rifampicin in urine 2, 6, 8 h after taking medicine, respectively.

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