Flow-injection Chemiluminescence Determination of Reserpine in Medicine and Biological Fluids with Controlled-Reagent-Release Technology

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A sensitive and rapid chemiluminescence (CL) flow injection with controlled-reagent-release technology for the determination of reserpine was proposed. The CL reagents, luminol and dichromate, used in this sensor, were all immobilized on anion-exchange resin. Through injection of 100 μL of water, the reagents on the anion-exchange resin column were eluted and in the presence of reserpine, the CL intensity was decreased, by which reserpine could be sensed. Reserpine was quantified by measuring the decrement of CL intensity, which was observed linear with the logarithm of reserpine concentration in the range of 1.0—500.0 ng/mL, and the limit of detection was 0.4 ng/mL (3σ) with a relative standard deviation of less than 3.0%. The proposed procedure was applied in the assay of reserpine in pharmaceutical preparation and biological fluids without any pre-treatment process and with sampling frequencies of 72 times per hour.

Keywords reserpine, flow injection, chemiluminescence, medicine, urine

Introduction

Reserpine [methyl 1,2-didehydro-2,7-dihydro-11,17α-dimethoxy-3β,20α-yohimbane-16β-carboxylate, 18β-trimethoxybenzoate ester], a white to yellowish powder, was isolated in 1952 from the roots of the plant *Rauwolfia serpentina* by Mueller *et al.*[1], and was synthesized by Woodward *et al.*[2] in 1956. Its biological action is to inhibit the storage of dopamine in the synaptic vesicles, thereupon generating evacuation of catecholamines of the sympathetic and central nervous system. It works by controlling nerve impulses along certain nerve pathways and therefore can help to widen arteries and veins, so blood flows better. But the reserpine's toxic effects (including sleepiness, depression, galactorrhoea, ulcer and diarrhoea) were also often reported[3,4] and hence its quantitative determination becomes very important. Many methods for the determination of reserpine, including densitometry,[5] polarography,[6] radioimmunoassay,[7] were reported in the literature. To achieve a selective detection, the techniques of capillary zone electrophoresis,[8] chromatography,[9-15] and spectrofluorimetry[16-24] were applied to the assay of reserpine.

Progress in flow-injection (FI) chemiluminescence (CL) analysis has received much attention in various fields for its high sensitivity, rapidity and simplicity. Pimetsis *et al.*[25] proposed a CL method using a potassium permanganate-polyphosphoric acid CL system to determine reserpine from 0.05 to 3.0 μg/mL, with a detection limit of 0.4 ng/mL. However, a limitation to the application of this method is the requirement to prepare large quantities of analytical reagents and continuously deliver them into the reaction zones. This is undesirable not only for operational convenience and for the simplicity of detection device, but also for the cost, environment and resource considerations.[26] We have recently reported an isoniazid, rutin and berberine sensor, respectively, based on the luminol-Fe$(CN)_3^{3-}$ CL system using Na_3PO_4 as eluant, and the determination of analyte could be performed in 120 s, giving a throughput of about 30 times per hour.[27-29]

In this work, it was found that luminol reacted with dichromate in alkaline medium producing strong CL, and the CL intensity was decreased in the presence of reserpine. The CL reagents, luminol and dichromate, used in this sensor, were all immobilized on anion-exchange resin. Through injection of 100 μL of water in stead of electrolytic solution, the reagents on the anion-exchange column were eluted and the CL intensity was decreased in the presence of reserpine, by which reserpine could be sensed. The concentration of reserpine was quantified via the peak height of the decreased CL intensity which is linear with the logarithm of reserpine concentration in the range of 1.0—500.0 ng/mL with a relative standard deviation less than 3.0%. The method was applied successfully to the determination of reserpine in pharmaceutical preparations and in human urine without any pre-treatment and with sampling frequencies of 72 times per hour.

Experimental

Reagents

All chemicals used were of analytical-reagent grade.

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Doubly distilled water was used throughout. Luminol (Fluka, biochemika) was obtained from Xi'an Medicine Purchasing and Supplying Station, China. Potassium dichromate was purchased from Xi'an Chemical Reagent Plant. The stock solution of reserpine (100 μg/mL) for calibration was prepared by dissolving the reserpine (Shaanxi Institute for Drug Control) in 0.2 mol/L acetate acid and stored at 4 ℃. Luminol was used as supplied to prepare a 0.25 mol/L stock standard solution in 0.5 mol/L NaOH in a 1000-mL calibrated flask. A 0.04 mol/L stock standard solution of $K_2Cr_2O_7$ was made by dissolving the solid in distilled water and diluting to 250 mL in a calibrated flask.

Apparatus of flow injection system

The flow injection system used in this work is shown in Fig. 1. A peristaltic pump (Shanghai Meter Electromotor Plant, Model ND-15, 15 r/min) was used to generate the flows. PTFE tubing (1 mm I.D.) was used in the flow system. The anion-exchange resins containing immobilized luminol (0.05 g) and potassium dichromate (0.10 g) were mixed together and packed into a glass column (I.D. 3 mm and total volume of about 0.5 mL) and plugged with glass wool at both ends to prevent the resins from leaking. A six-way valve injected 100 μL of eluant. Before reaching the flow cell, the streams of luminol, potassium dichromate, sodium hydroxide and analyte were combined in a mixing tube (50 mm in length). The CL emission cell is a twisty glass tube (1.0 mm I.D., 15 cm length) in order to produce a large surface area exposed to the adjacent photomultiplier tube (PMT) (Hamamatsu, Model IP28). The CL signal produced in flow was detected without wavelength discrimination, and the PMT output was amplified and quantified by a luminosity meter (Xi'an Remax Electronic Science-Tech. Co., Ltd., Model GD-1) connected to a recorder (Shanghai Dahua Instrument and Meter Plant, Model XWT-206).

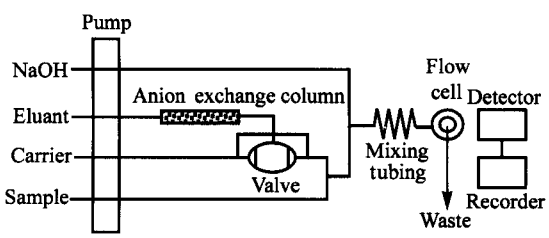

Fig. 1 Schematic diagram of the flow-injection system for reserpine determination.

Preparation of resin with immobilized reagents

Amberlyst (from Rohm and Haas Co.) A-27 (2.0 g) was shaken with 50 mL of 0.25 mol/L luminol or 0.01 mol/L potassium dichromate for 12 h, and then the resin was filtered, washed with doubly distilled water and dry-stored. The most convenient method to determine the amounts of luminol and potassium dichromate immobilized was to measure the losses of these reagents from the immobilization solutions. The concentration was detected at 360 nm for luminol and at 352 nm for potassium dichromate by UV-vis. In the proposed method, the amounts of luminol and potassium dichromate immobilized were (1.99 ± 0.02) (n = 3) mmol/g and (2.40 ± 0.01) (n = 3) mmol/g resin, respectively.

Procedures for determination of reserpine

The carrier water and the solutions (NaOH, sample and eluant) were propelled at a constant flow rate on each flow line. The pump was started to wash the whole flow system until a stable baseline was recorded. Then 100 μL of eluant solution (water) was injected into the carrier stream, luminol and dichromate were eluted quantitatively, which was then mixed with the reserpine stream. The mixed solution was delivered to the CL cell, and the peak height of the CL signal was detected with the PMT and the luminometer. The concentration of reserpine was quantified by decreased CL intensity, $\Delta I = I_0 - I_s$, where I_0 and I_s are CL signals in the absence and in the presence of reserpine, respectively.

Procedure for reserpine injections

The contents (with a nominal content of 1.0 mg of reserpine in 1 mL) of twenty injection samples were mixed. An accurately measured volume equivalent to 1.0 mg of reserpine was transferred into a 100-mL volumetric flask and dilute to the volume with doubly distilled water. A suitable volume as described above was analyzed.

Procedure for spiked human urine

Fresh urine samples were collected and mixed with an aliquot of reserpine solution, and then the mixture was diluted by distilled water directly. Urinary reserpine was determined relatively simply by FI-CL without any pre-treatment procedures.

Results and discussion

The CL intensity-time profile

Before carrying out the flow injection method, the batch method for the CL profiles was used. Without any special eluant, the mixture of luminol and dichromate rinsed by water gave out an evident CL signal. As Fig. 2 showed, the CL intensity reached a maximum 12 s after injection, and then died within 50 s. On addition of the sample into the above mixing solution, a decreased CL signal was recorded. The peak heights of the CL emission were proportional to the logarithm of reserpine concentration.

Designation for the FI-CL system

The assay could be carried out by a continuous-flow mode in two different manifolds. Through injection of 100 μL of eluant (5.0×10^{-5} mol/L of Na_3PO_4), the reagents on the

anion-exchange resin column were eluted and in the presence of reserpine, the CL intensity was decreased, and the decrease of CL intensity was recorded. It was found that when the column with immobilized reagents was put in front of or behind the valve, two significantly different results were observed. As illustrated by the results in Fig. 3, the whole analysis process, including sampling and washing, could be accomplished in 50 s when the column was put in front of the valve *viz*. Fig. 1 manifold, whereas it must take more than 2.0 min when the column was put behind the valve. Therefore, the manifold depicted in Fig. 1 was chosen for subsequent work.

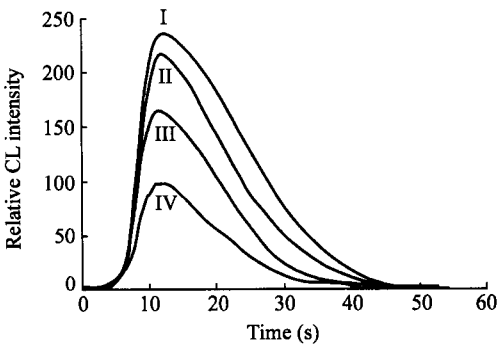

Fig. 2 CL time profiles in the batch system. I: CL intensity in the absence of reserpine; II: CL intensity in the presence of reserpine (5 ng/mL); III: CL intensity in the presence of reserpine (50 ng/mL); IV: CL intensity in the presence of reserpine (500 ng/mL).

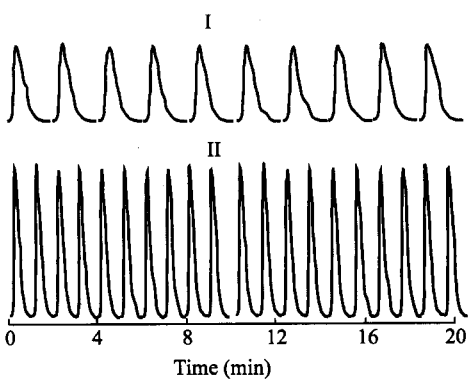

Fig. 3 CL signals in two manifolds. I: the column set behind the injector; II: the column set in front of the injector.

Selection of eluant

Different eluants with volume of 100 μL were injected through the resin column releasing different amounts of luminol and dichromate, thus producing the CL emission. The results are shown in Table 1. It was found that sodium sulfate gave a maximum CL emission. Nevertheless, it was observed that a continuous flow of this eluant through the column resulted in a rather short lifetime of sensor down to only a few hours. It was shown that the immobilized luminol and dichromate anions on the anion exchange resin underwent dissociation with water, thus released trace amounts of luminol and dichromate from the column, and the decrease of reserpine CL signal could be easily observed. In this case, the column could be used over 120 h. As a compromise between higher CL intensity and longer lifetime of the column, water was used as eluant in subsequent work.

Table 1 Character of eluants for reserpine determination[a]

Type of CL intensity	Relative CL intensity				
	H_2O	NaCl	Na_2CO_3	Na_2SO_4	Na_3PO_4
I	308	416	384	568	346
II	230	348	286	324	266
III	78	68	98	244	80

[a] The concentration of each solution was 1.0×10^{-4} mol/L. I: CL intensity in the absence of reserpine; II: CL intensity in the presence of 50 ng/mL reserpine; III: the decrease of CL intensity.

Effect of pH *on* CL *and sensor lifetime*

The best pH of eluant (water) on the performance of the system was evaluated. It was found that along with the increase of pH in eluant, the CL intensity decreased while the lifetime of sensor decreased considerably (Fig. 4). This phenomenon is probably due to that the quantities of hydroxide ions in eluant were increasing. pH 6.5 was then chosen as a compromise between lifetime and a sufficient CL intensity. In this case, the column with immobilized CL reagents could be used more than 120 h in continuous-injection system.

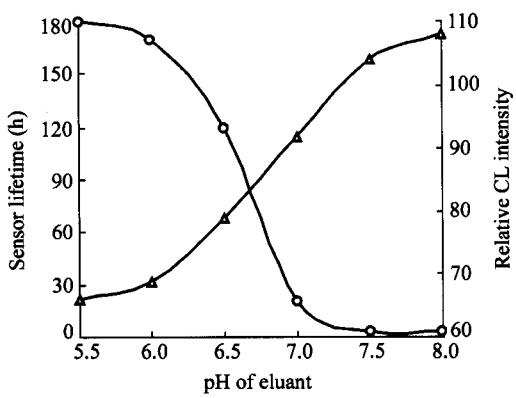

Fig. 4 Effect of eluant pH on sensor lifetime (—○—) and CL intensity (—△—).

Effect of molar ratio of immobilized luminol to dichromate

To examine the influence of the mixing ratio, resins (0.15 g) with different mixing ratios were packed into column with the same internal diameter and volume. By the injection of water at a fixed volume of 100 μL, different amounts of luminol and dichromate were eluted from the resins and emitted CL signals with different intensity. As Fig. 5 shows, the CL intensity dropped drastically from beginning to the next day, then it went down slowly like glacis. The most stable CL signal was found with a molar ratio of 1:2 (luminol to dichromate) and a middling CL intensity was in favor of measuring an inhibitory effect of reserpine on CL reaction.

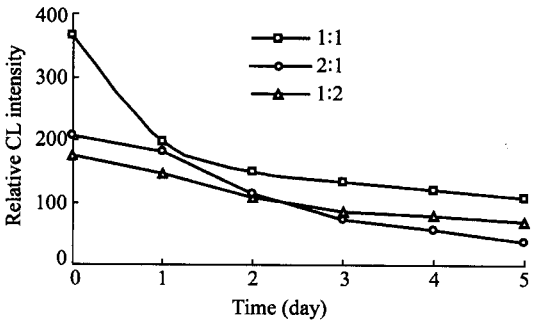

Fig. 5 Effect of molar ratio of luminol to dichromate on CL intensity and sensor lifetime.

Effect of NaOH *concentration*

It was found that luminol reacts with dichromate and emits CL signal only in an alkaline medium. As Fig. 6 shows, a NaOH concentration less than 0.05 mol/L leads to an apparent decrease in ΔI. The maximum intensity was found with 0.05 mol/L NaOH. When concentration of NaOH is higher than 0.1 mol/L, there is a scattering effect in flow cell due to the discrepancy between refractive index of various components. Thus 0.05 mol/L NaOH was selected as an optimal condition.

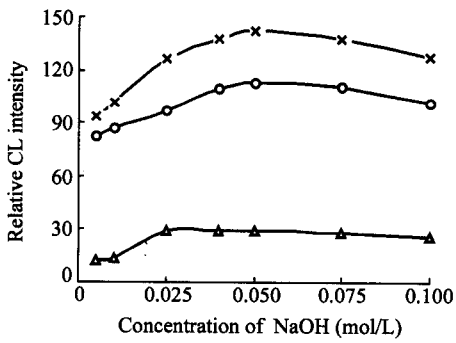

Fig. 6 Effect of concentration of NaOH on CL intensity. —○— CL intensity in the presence of reserpine (I_s); —×— CL intensity in the absence of reserpine (I_0); —△— the decrease of CL intensity (ΔI).

Effect of flow rate and the length of mixing tubing

The CL signal was also dependent on the flow rate of carrier and eluant. The signal-to-noise rate decreased at a higher flow rate because the higher flow rate would impact the rate of contact of sample molecules with the ion-exchange resin. The lower flow rate caused broadening of the peak and slowing down of the sampling rates. Nevertheless, the high flow rate could lead to an unstable baseline and shortening of the sensor lifetime. A rate of 2.0 mL/min was then chosen as a compromise between good precision and lower reagent consumption.

The length of the mixing tubing was also adjusted to yield maximum light emission in the cell. It was found that a 5.0 cm of mixing tubing afforded the best results as regards sensitivity and reproducibility.

Performance of the sensor for reserpine measurements

Under the above optimum conditions, the linearity of reserpine was tested by determining a series of standard solutions with the flow sensor. The inhibited CL intensity was found to be proportional with the logarithm of reserpine concentration. The linear range is from 1.0 ng/mL to 500.0 ng/mL and the regression equation is $\Delta I = 9.6604 \ln C_{\text{reserpine}} + 14.353$, and $R^2 = 0.9994$. The relative standard deviation of five determinations was 2.35%, 1.47% and 1.06% with reserpine concentrations of 1.0, 10.0 and 100.0 ng/mL, respectively, and the limit of detection was 0.4 ng/mL (3σ). At a flow rate of 2.0 mL/min, the determination of analyte could be performed in 50 s, including sampling and washing, giving a throughput of about 60 times per hour.

Interference studies

The effect of foreign ions was tested by analyzing a standard solution of reserpine to which increasing amounts of interfering ions were added. The tolerable concentration ratios with respect to 5.0 ng/mL reserpine for interference at 5% level were over 2000 for Cl^-, NO_3^-, I^-, SO_4^{2-}, PO_4^{3-}, oxalate, amylum, sucrose, barley sugar, glucose, dextrin, magnesium stearate, methanol, ethanol and acetone, and 1000 for Ca^{2+}, Zn^{2+}, Pb^{2+}, CO_3^{2-} and borate, and 100 for Ba^{2+}, Mn^{2+}, citric acid, benzoic acid, EDTA and 8-hydroxyquinoline, and 5 for Cu^{2+}, Ac^-, Fe^{3+} and salicylic acid, respectively.

Operational stability of the flow sensor

100 μL of eluant (water) was flow-injected through the system in the presence of 10 ng/mL reserpine solution and the ΔI ($I_0 - I_s$), was recorded to test the operational stability of the sensor. The experiment lasted for 12 days and the flow system was regularly used over 8 h per day. Fig. 7 shows the stability of the flow sensor, and the average of ΔI was calcu

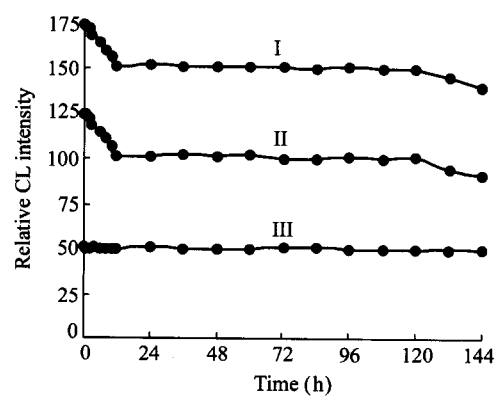

Fig. 7 Stability of the flow sensor. I: CL intensity in absence of reserpine (I_0); II: CL intensity in presence of 10 ng/mL reserpine (I_s); III: the decrease of CL intensity ($\Delta I = I_0 - I_s$).

Table 2 Results for the determination of reserpine in injections[a]

Sample No.	Results by the proposed method[a]					Results by HPLC
	Reserpine supplement (ng/mL)	Mean* (ng/mL)	Recovery (%)	RSD (%) ($n=5$)	Content (mg/mL)	Content (mg/mL)
1	0	9.9	95.4	2.7	0.99	0.97
	5.0	14.7		2.9		
2	0	10.2	103.0	2.5	1.01	0.99
	10.0	20.5		2.4		
3	0	10.1	97.3	2.1	1.00	0.98
	15.0	24.7		1.8		

[a] The average of five determinations.

lated in ten spot check determinations with RSD less than 3.0%. The flow sensor showed remarkable stability and could be easily reused over 120 h.

Determination of reserpine injections

Following the procedure described in Experimental section, the proposed method was applied to the determination of reserpine in injection purchased from the local market. The measured reserpine contents are listed in Table 2. The recovery studies were performed on each of the analyzed samples by adding a known amount of reserpine to the sample before the recommended treatment and the experimental results were also verified by HPLC. The results obtained by the proposed method were in good agreement with those obtained by HPLC.

Determination of reserpine in spiked urine

This proposed method was also utilized in the determination of the studied drugs in human urine without any pretreatment, the results of trial determinations are listed in Table 3.

Table 3 Results for the determination of reserpine in spiked urine samples[a]

Sample	Added (ng/mL)	Found (ng/mL)	t-test $t_{0.05,4}=2.78$	RSD(%) ($n=5$)	Recovery (%)
1	0	9.8	1.38	1.73	99.4
	5.0	14.8		0.75	
2	0	10.2	0.86	0.73	103.8
	10.0	20.6		0.95	
3	0	10.5	1.67	1.19	101.5
	15.0	25.7		1.06	
4	0	10.7	0.93	1.15	100.4
	20.0	30.8		1.68	

[a] The average of five determinations.

Possible mechanism

In the present work, chemiluminescence kinetic characteristics of the CL reaction of luminol-dichromate-reserpine were studied in detail. The reaction process was followed by UV detection at 254 nm in flow system and the results are listed in Table 4. It was obvious that the absorption intensity of reserpine decreased quickly in the presence of dichromate. It was also found that the product of reaction between dichromate and reserpine could not oxidize luminol chemiluminescently. Hence, the mechanism of the inhibition effect of reserpine on luminol-dichromate CL system could be presented as below:

reserpine (Red state) + dichromate ⟶ reserpine (Ox state)

dichromate + luminol ⟶ chromium(III) + $h\nu$ (λ_{max} 425 nm).

Table 4 Results of detecting dichromate-reserpine by UV at 254 nm

Species[a]	A^b
Dichromate	0.179
Reserpine	0.943
Dichromate + Reserpine	0.409

[a] The same concentration and injection volume (10^{-4} mol/mL, 25 μL). [b] The average of five determinations.

Conclusion

The proposed method establishes a new chemiluminescence flow-injection system with reagents immobilized technique for the determination of reserpine and improves general reproducibility and sensitivity of reserpine assay, as well as simplicity of apparatus. Compared with other methods, the proposed one makes possible a rapid and accurate determination of reserpine by means of FI-CL chemosensor that is stable in a wide range of time and has a lower limit of detection. The analytical performance in assay of reserpine in pharmaceutical preparations and biological fluids demonstrates that the sensor can be utilized in routine determination of reserpine and clinical application.

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