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Short communication

Flow-injection chemiluminescence determination of phentolamine based on its enhancing effect on the luminol–potassium ferricyanide system

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Abstract

It was found that the light emission produced by the oxidation of luminol by potassium ferricyanide in the basic medium was enhanced by phentolamine, a drug recently used to treatment of male and female sexual dysfunction. The optimum conditions for this chemiluminescent reaction were studied in detail by a flow-injection system. A new, simple and rapid method has been developed under the optimum conditions for determination of phentolamine. This method has the advantages of high sensitivity, good reproducibility and low detection limit. On the basis of investigation of chemiluminescent, fluorescent and UV spectra of phentolamine in basic solution containing potassium ferricyanide and luminol, a possible mechanism of this reaction was proposed. In the optimum conditions, CL intensities are proportional to concentrations of the phentolamine in the 0.01–1 μg/mL range. The limit of detection is 3.0 ng/mL for phentolamine. The method has been applied to the determination of phentolamine in the commercial preparations, synthetical samples and biological fluids with satisfactory results.
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Keywords: Chemiluminescence; Flow-injection analysis; Phentolamine; Biological fluids; Pharmaceutical analysis

1. Introduction

Phentolamine is (Fig. 1) an α-adrenergic blocking agent with a slight β stimulating effect. Traditionally, it was used to treat the hypertension and as a valuable research tool in the study of adrenergic receptor-mediated processes. It has been reported as a useful agent for therapy in congestive heart failure, myocardial infarction, arrhythmia, angina pectoris, shock and bronchial asthma [1]. Recently, clinical research has shown that phentolamine is an effective drug in the treatment of male and female sexual dysfunction [2–7]. For example, at oral doses of 40 and 80 mg, respectively, 55 and 59% of men were able to achieve vaginal penetration, with 51 and 53% achieving penetration on 75% of attempts. The correction of erectile dysfunction or improvement to a less severe category of dysfunction was experienced by 53% of men with the 80 mg dose and 40% with the 40 mg dose of phentolamine [4]. However, research also observed the adverse effects, which are consistent with the known pharmacology of phentolamine [7]. Therefore, the safety of using phentolamine to improve the sexual dysfunction has been noticed by medical scientists, and the biological research of phentolamine is very significant in clinical medicine. The development of a rapid, sensitive and simple method for determination of phentolamine in biological samples is especially important. The monitoring of such drug is important for quality assurance in preparations and for obtaining optimum therapeutic concentrations. For its measurement, several methods have been reported, such as spectrophotometry [8], thin-layer chromatography [9,10], spectrofluorimetry [11], gas chromatography (GC) with electron capture detection [12], high-performance liquid chromatography (HPLC) with UV [10,13,14], electrochemical [15,16] or mass spectrometric detection [10,17]. However, most of them have one or more drawbacks. Because of low sensitivity, spectrophotometry could not meet the need of trace analysis. Although the low detection limits are generally provided by electrochemical detection, one serious pitfall of this type of determination arises from the electrode fouling, which causes the instability of signal detection. Also, many methods require

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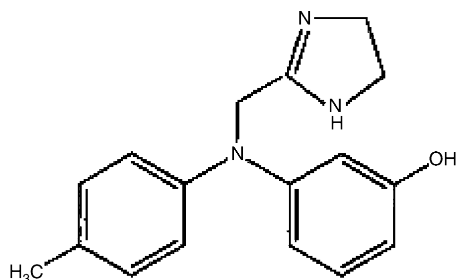


Fig. 1. Molecular structure of phentolamine.

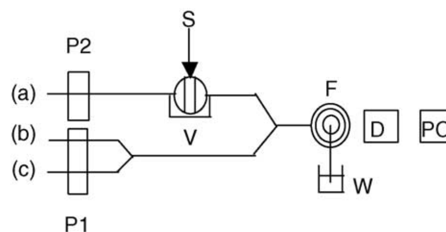


Fig. 2. Schematic diagram of the flow system for the determination of phentolamine: a, H_2O; b, 0.1 mmol/L potassium ferricyanide; c, 50 μmol/L luminol in 0.003 mol/L NaOH; S, sample; P1, P2, peristaltic pump; V, injection valve; F, flow cell; W, waste liquid; D, PMT; PC, personal computer.

time-consuming sample preparation techniques, particularly in the case of using GC and HPLC. Regarding the determination of phentolamine by chemiluminescence, however, there are no literatures dealing with this issue to date. The aim of the present study is to use a flow-injection analysis system with chemiluminescent detection for determination of phentolamine. The chemilumionescent behavior of phentolamine in basic potassium ferricyanide–luminol media has been investigated. It is discovered that a chemiluminescence increases when the phentolamine solution is injecting into mixture of potassium ferricyanide and alkaline luminol. A simple and rapid FIA-CL assay for phentolamine based on the above mentioned discovery was developed and it was applied to the determination of phentolamine in the commercial preparations, synthetical samples and biological fluids with satisfactory results. To our knowledge, this is the first report on the chemiluminescent determination of phentolamine.

2. Experimental

2.1. Reagents

All the reagents were of analytical-reagent grade unless specified otherwise; doubly distilled water was used for the preparation of solutions. Phentolamine (The Shanghai Sixth Pharmaceutical Factory, Shanghai, China) solution (500 μg/mL) was prepared. More diluted solutions were used immediately after preparation. Potassium ferricyanide and sodium hydroxide were obtained from Chongqing Chemical Reagent Company (Chongqing, China). A 0.01 mol/L luminol solution was prepared by dissolving 1.772 g of luminol which from Merck (Darmstadt, Germany) in 1000 mL of 0.001 mol/L NaOH. More diluted solutions were prepared in 0.003 mol/L NaOH and used immediately.

2.2. Apparatus

The flow system employed in this work is shown in Fig. 2, which consisted of two peristaltic pumps P1 and P2 (Longfang Instrument Factory, Wenzhou, China). P1 delivered all reagent streams at a flow rate of 3.6 mL min^{-1} (per tube) and P2 delivered water carrier stream at a flow rate of 3.6 mL min^{-1}. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. The emitted CL was collected with a photomultiplier tube of the Type IFFL-D flow-injection chemiluminescence analyzer (Reike, Xi'an, China). The signal was recorded by using an IBM-compatible computer, equipped with a data acquisition interface. Data-acquisition and treatment were performed with REMAX software running under Windows 98. Chemiluminescence spectrum was monitored by using a RF-540 fluorescence spectrometer (Shimazhu, Japan). An F-4500 spectrofluorimeter (Hitachi, Japan) and a U-2000 spectrophotometer (Hitachi, Japan) were used.

2.3. Procedure

2.3.1. Procedure for calibration

Working standard solutions containing phentolamine in the range of 0.01–1 μg/mL were prepared by dilution of a concentrated fresh standard solution of phentolamine (500 μg/mL). The CL signal was measured by injecting 100 μL of working standard solution into the water carrier stream, which then joined the reagent streams (a mixture of 0.1 mmol/L ferricyanide solution and 50 μmol/L luminol in 0.003 mol/L NaOH solution). The CL emission intensities versus phentolamine concentration were used for the calibration.

2.3.2. Procedure for the pharmaceutical preparations

Injection samples, each with a nominal content of 10 mg of phentolamine in 1 mL (Shanghai Xudong Haipu Pharmaceutical Company, Shanghai), were diluted to 100 mL with doubly distilled water and further diluted to the working range of the determination of phentolamine, then used for an analysis.

2.3.3. Procedure for synthetical sample

A certain mount of phentolamine was put into a flask with the volume of 100 mL, then different foreign substances were added and diluted with doubly distilled water for a quantitative analysis.

2.3.4. Procedure for biological fluids

2.3.4.1. Procedure for spiked plasma. Add an aliquot of standard aqueous solution of phentolamine (500 μg/mL) to

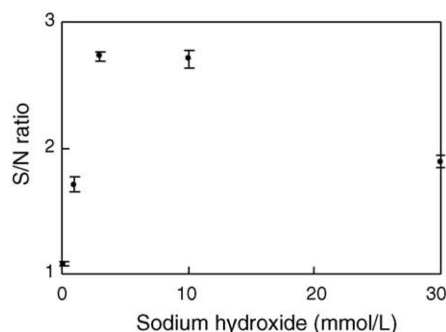


Fig. 3. Effect of sodium hydroxide concentration. Error bars represent one standard deviation for three measurements.

5.0 mL of plasma. Add 1 mL of 6% (w/v) perchloric acid for each mL of the plasma for deproteination. Blend on a vortex mixer and centrifuge at 3000 rpm for 10 min.

Transfer 2.5 mL of the protein-free supernatant into a 50 mL of volumetric flask and dilute to volume with water. Proceed as described above. A blank value was determined by treating phentolamine-free plasma in the same way.

2.3.4.2. Procedure for spiked urine. Add an aliquot of standard aqueous solution of phentolamine (500 μg/mL) to the urine sample. Transfer 1.0 mL of this solution into a 100 mL of volumetric flask and dilute to volume with water. Proceed as described above. A blank value was determined by treating phentolamine-free urine in the same way.

3. Results and discussion

3.1. Effect of sodium hydroxide concentration

Luminol reacts with potassium ferricyanide to produce light emission in basic solution. Therefore, sodium hydroxide was added in a flow line to improve the sensitivity of reaction. The concentration of sodium hydroxide versus signal intensity was studied at different concentrations from 1×10^{-4} to 3×10^{-2} mol/L. It was found that the signal intensity increases with the concentration of sodium hydroxide; however, the background level also increases with the concentration of sodium hydroxide. So the S/N (signal/noise) ratio was used to evaluate the sodium hydroxide effect. As can be seen from Fig. 3, the S/N ratio increases with the concentration of sodium hydroxide up to 3×10^{-3} mol/L, thereafter remains almost constant to 0.01 mol/L sodium hydroxide, above which the S/N ratio decreases. As a compromise between the sensitivity and the background level, finally, 3×10^{-3} mol/L of sodium hydroxide was selected for the present work.

3.2. Effect of potassium ferricyanide concentration

The effect of potassium ferricyanide concentration on the signal intensity was examined ranging from 0.01 to 0.2 mmol/L. It is found that both the signal intensity and the background level increase with the concentration of potassium ferricyanide concentration. However, the S/N ratio remains almost constant over the concentration ranges of interest (Fig. 4). Considering the sensitivity and the background level, finally, 0.1 mmol/L of potassium ferricyanide was used in the coming work.

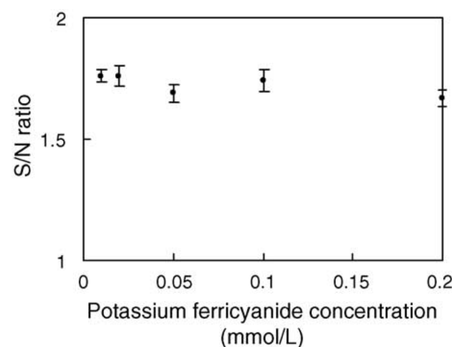


Fig. 4. Effect of potassium ferricyanide concentration. Error bars represent one standard deviation for three measurements.

3.3. Effect of luminol concentration

The effect of luminol concentration on the S/N ratio was investigated ranging from 5 to 100 μmol/L. From Fig. 5, it can be seen that the highest S/N ratio is obtained at 50 μmol/L of luminol concentration. So 50 μmol/L of luminol was selected in the subsequent experiments.

3.4. Performance of the proposed method for phentolamine measurements

Under the selected conditions given above, the calibration graph of emission intensity versus phentolamine concentration was linear in the 0.01–1 μg/mL range ($\Delta I = 866.11$ (phentolamine) (μg/mL) + 27.3; $r = 0.9976$, $n = 8$) with a detection limit (3σ) of 3 ng/mL. Relative standard deviation (R.S.D.) ($n = 11$) was 3.3% for 1 μg/mL phentolamine. Five replicate determinations at three concentration levels were carried out to test the accuracy and precision of the proposed

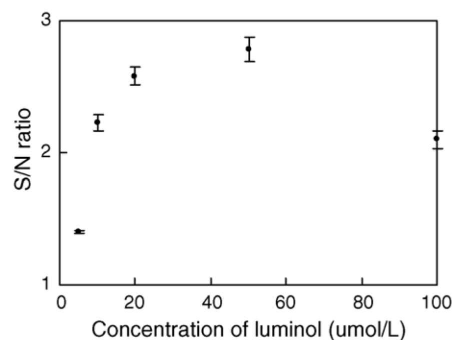


Fig. 5. Effect of luminol concentration. Error bars represent one standard deviation for three measurements.

Table 1
Accuracy and precision data at three concentrations

Concentration added (μg/mL)	Inter-day[a]						Intra-day[a]					
	Plasma			Urine			Plasma			Urine		
	Found (μg/mL)	Error (%)	R.S.D. (%)	Found (μg/mL)	Error (%)	R.S.D. (%)	Found (μg/mL)	Error (%)	R.S.D. (%)	Found (μg/mL)	Error (%)	R.S.D. (%)
0.05	0.048	−4.0	4.5	0.051	2.0	4.8	0.048	−4.0	2.6	0.048	−4.0	4.7
0.1	0.097	−3.0	5.8	0.101	1.0	5.2	0.0997	−0.3	4.0	0.099	−1	4.8
0.5	0.519	3.8	3.2	0.500	0	5.7	0.512	2.4	1.7	0.510	2	3.9

[a] Averages of five determinations. Determinations of precision and accuracy were performed between 5 days and five times in 1 day for intra-day and inter-day, respectively.

Table 2
Results of the determination of phentolamine in injections

Sample	Amount (mg)			
	Label (mg/mL)	Found (mg/mL) (R.S.D.%, $n = 3$)	Added (mg/mL)	Recovery (%) (R.S.D.%, $n = 3$)
Injection 1	10	10.04 ± 0.30	5	99.2 ± 1.13
			10	104.0 ± 1.57
Injection 2	10	9.82 ± 2.01	5	101.6 ± 1.65
			10	95.8 ± 2.62
Injection 3	10	9.56 ± 1.44	5	103.0 ± 0.39
			10	92.0 ± 1.29

Table 3
Results of the determination of phentolamine in synthetical samples

Sample (no.)	Co-existing substances (μg/mL)	Added (μg/mL)	Found (μg/mL)	Recovery (% ± R.S.D.%, $n = 3$)
1	Glucose (5), amylum (5)	0.1	0.10	97.8 ± 3.1
2	Glucose (25), amylum (25)	0.5	0.51	102.5 ± 2.7
3	Glucose (50), amylum (50)	1	1.08	108 ± 4.0
4	Glucose (10), amylum (10), urea (10), starch gum (5)	0.2	0.20	99.4 ± 2.0
5	Amylum (20), EDTA (5), $CaCl_2$ (20)	0.2	0.20	100.6 ± 3.1
6	Glucose (10), amylum (10), KCl (20), $ZnSO_4$ (10)	0.2	0.20	99.0 ± 3.3

method for biological samples and the results listed in Table 1. As can be seen, R.S.D. (precision) of inter-day and intra-day is less than 6%, and accuracy of inter-day and intra-day is satisfactory.

3.5. *Interference study*

The effect of foreign substances was tested by analyzing a standard solution of phentolamine (0.6 μg/mL) to which increasing amounts of interfering substances were added. The tolerable concentration ratios with respect to 0.6 μg/mL phentolamine for interference at 5% level were over 1000 for glucose, $CaCl_2$; 500 for KBr and furosemide (antihypertensive drug); 200 for $NaNO_3$, KNO_3, urea; 100 for zinc sulfate, NaCl, KCl, amylum; 20 for $NaHCO_3$; 10 for Na_2CO_3, $(NH_4)_2SO_4$; 5 for $CuSO_4$, $Al(NO_3)_3$; 3 for sodium nitroferricyanide (antihypertensive drug); 2 for tartrate EDTA; 1 for maltose, $FeCl_3$; 0.1 for Mn^{2+} ($MnSO_4$); 0.05 for ascorbic acid; respectively.

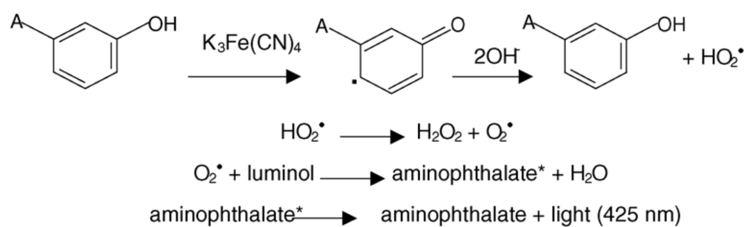
3.6. *Sample analysis*

3.6.1. *Analysis of phentolamine in injection samples and synthetical samples*

Following the procedure described under Section 2, the proposed method was applied to the determination of phen-

Table 4
Results of the determination of phentolamine in spiked urine and plasma

Concentration added (μg/mL)	Found (%) (R.S.D.%, $n = 3$)	
	Urine	Plasma
0.01	94.8 (3.44)	80.92 (2.86)
0.05	92.7 (2.07)	82.1 (2.37)
0.1	92.6 (3.06)	89.1 (3.24)
0.2	93.7 (1.51)	92.5 (1.52)
0.5	91.8 (1.16)	92.5 (1.42)
0.6	105 (1.29)	104 (0.71)
0.8	94.4 (1.70)	97.5 (0.08)
1	96.8 (1.72)	98.2 (0.28)



where A being the remaining parts of phentolamine molecule besides the phenolic group.

Fig. 6. Schematic diagram of possible CL mechanism of reaction.

tolamine in injection samples. Also, a series of synthetical samples were prepared to check the validation of the proposed method. The results are listed in Tables 2 and 3. The recovery tests were carried out on the samples and the obtained recoveries were satisfactory. As illustrated in Tables 2 and 3, the proposed method can be satisfactorily applied to the determination of phentolamine in injection samples and synthetical samples.

3.6.2. *Analysis of phentolamine in spiked urine and plasma samples*

The method presented here has a low detection limit and, therefore, the proposed method allows the determination of phentolamine in biological fluids. The linearity for plasma analysis was in the 0.01–1 μg/mL range ($\Delta I = 873.59$ (phentolamine) (μg/mL) + 23.3; $r = 0.9971$, $n = 9$) with a limit of quantification (LOQ, which is given as the concentration for which the analytical signal is 10 times higher than standard deviation of blank intensity) of 0.06 μg/mL. It was also in the 0.01–1 μg/mL range for urine analysis ($\Delta I = 850.06$ (phentolamine) (μg/mL) + 8.0; $r = 0.9963$, $n = 9$) with a limit of quantification (LOQ) of 0.03 μg/mL. The mean peak serum concentrations of phentolamine after 40 mg dose were about 2.5×10^{-8} g/mL [15]. Thus, the proposed method proved to be satisfactory for the routine estimation of phentolamine in human urine and plasma. For plasma only a deproteination process was carried out using perchloric acid as a sample pretreatment, an extraction procedure was not necessary. Table 4 shows the results of the recovery studies of phentolamine from spiked plasma and urine.

3.7. *Possible mechanism of CL reaction*

The determination of phentolamine proposed in this work is based on its enhancing effect on the light emission produced by the oxidation of luminol by potassium ferricyanide in the basic medium. In order to know more about the characteristics of the reaction, chemiluminescence kinetic characteristics of the CL reaction were studied. It was found that the rate of the CL reaction in solution was very fast; from the reagent mixing to the peak maximum only 1 s was needed and it took about 5 s for the signal to reach baseline again.

The fluorescence spectra of luminol/$K_3Fe(CN)_6$, phentolamine/$K_3Fe(CN)_6$ and of the mixture of luminol/ $K_3Fe(CN)_6$, phentolamine/$K_3Fe(CN)_6$ were studied. The results showed that there was only one fluorescence peak at 425 nm, which was the fluorescence peak of aminophthalate as reported [18]. UV spectra taken from luminol and phentolamine alone and mixed did not reveal the appearance of any extra peak after mixing of both solutions. Hence, no complex is formed between the species. It appears that the reaction product generating CL is to be attributed to aminophthalate ions (the oxidation product of luminol), and not to the phentolamine analyte as such.

In order to study the role of phentolamine, the chemiluminescence spectra of following systems were studied by the RF-540 fluorimetry: (a) luminol/$K_3Fe(CN)_6$/NaOH; (b) luminol/$K_3Fe(CN)_6$/phentolamine. The results show that all the above systems give one peak situating at about 425 nm (same as the maximum emission spectra of aminophthalate), indicating that the role of phentolamine is only an enhancement reagent because there is no new emitter produced in the reaction. Therefore, it can be concluded that phentolamine does not change the mechanism of the chemiluminescent reaction. Phentolamine is a derivative of imidazoline (Fig. 1), which is a strong base. Beside the nitrogen atom in the imidazoline, phentolmaine contains another basic site and a phenolic group, making this drug easily being oxidized in solution. Based on above discussion, the possible enhancement mechanism of phentolamine on the CL reaction between potassium ferricyanide and luminol may be that phentolamine as reducing agent is able to reduce oxidant in an alkaline solution to a superoxide radical [19]. The produced superoxide radical reacts with luminol, yielding an unstable endoperoxide and then leading to an electronically excited aminophthalate. This can be expressed as shown in Fig. 6 although more evidences are not available.

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References

[1] L. Gould, C.V. Ramana Reddy, Am. Heart J. 92 (1976) 397–402.
[2] A.J. Becker, C.G. Stief, S. Machtens, D. Schultheiss, U. Hartmann, M.C. Truss, U. Jonas, J. Urol. 159 (1998) 1214–1216.
[3] M. Mitka, J. Am. Med. Assoc. 280 (1998) 119–120.
[4] I. Goldstein, Int. J. Impot. Res. 12 (2000) S75–S80.
[5] D.G. Hatzichristou, A. Apostolidis, V. Tzortzis, K. Hatzimouratidis, D. Kouvelas, Int. J. Impot. Res. 13 (2001) 303–308.
[6] R.C. Rosen, N.A. Phillips, N.C. Gendrano 3rd., D.M. Ferguson, J. Sex Marital Ther. 25 (1999) 137–144.
[7] E. Rubio-Aurioles, M. Lopez, M. Lipezker, C. Lara, A. Ramirez, C. Rampazzo, M.T. Hurtado de Mendoza, F. Lowrey, L.A. Loehr, P. Lammers, J. Sex Marital Ther. 28 (2002) 205–215.
[8] H. Abdine, A.M. Wahbi, M.A. Korany, J. Pharm. Pharmacol. 24 (1972) 522–524.
[9] S. Goenechea, J. Chromatogr. 36 (1968) 375–377.
[10] M. Eiichi, O. Tsutomu, M. Hiroshi, Forensic Sci. Int. 130 (2002) 140–146.
[11] C. Vannecke, E. van Gyseghem, M.S. Bloomfield, T. Coomber, Y. vander Heyden, D.L. Massart, Anal. Chim. Acta 446 (2001) 413–428.
[12] A. Sioufi, F. Pommier, P. Mangoni, S. Gauron, J.P. Metayer, J. Chromatogr. Biomed. Appl. 11 (1981) 429–435.
[13] F. de Bros, E.M. Wolshin, Anal Chem. 50 (1978) 521–525.
[14] J. Godbillon, G. Carnis, J. Chromatogr. 222 (1981) 461–466.
[15] J. Pérez-Urizar1, P. Aguirre-Bañuelos, G. Castañeda-Hernández, F.J. Flores-Murrieta, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 557–564.
[16] B.D. Kerger, R.C. James, S.M. Roberts, Anal. Biochem. 170 (1988) 145–151.
[17] J.T. Wu, Rapid Commun. Mass Spectrom. 15 (2001) 73–81.
[18] P.B. Shevlin, H.A. Neufeld, J. Org. Chem. 35 (1970) 2178–2182.
[19] H. Kubo, A. Toriba, Anal. Chim. Acta 353 (1997) 345–349.