Flow Chemiluminescent Determination of Catecholamines Based on Permanganate Oxidation

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Synopsis. Luminous oxidation with acidic permanganate was found to be usable for chemiluminescent determination of catecholamines and other polyhydroxybenzenes at picomol levels. The sensitivity is high enough to determine catecholamines in urine. The present CL method is expected to be applicable to the post-column detection of these compounds in high-performance liquid chromatography.

Analytical methods using solution chemiluminescence (CL) as detection means are unique in being capable of low detection limits with simple instrumentation and have been applied to a variety of analytes of clinical and environmental significance.[1-3] The combination with flow injection method[4] and high-performance liquid chromatography[5] have made CL techniques more attractive. However, limited CL reaction system has caused the slow recognition of CL analysis, despite of other advantages including wide linear dynamic range, simplicity, rapidity, low cost per analysis, etc. In order to overcome this shortcoming, for the past several years our effort has been directed toward searching for new CL systems usable for analysis.[6-8] As a part of the investigations, we have reported on the screening of CL systems for determination of some biologically important organic compounds and made it clear that acidic permanganate oxidation may draw intense CL from many of the organic compounds tested, especially catecholamines and other polyhydroxyphenols.[8] Acidic permanganate is a strong oxidizing agent and has been used for CL determination of some drugs.[9]

In this paper, we describe the details of acidic permanganate CL system for the determination of catecholamines and other polyhydroxybenzenes by means of flow injection technique. This has been undertaken for aiming at the post-column detection of these compounds in high performance liquid chromatography.

Experimental

Apparatus. Prior to flow experiments, batch experiments were performed as described previously[10] in order to know luminescent characteristics through the measurements of time course of CL.

A schematic diagram of the flow system is illustrated in Fig. 1. An acidic permanganate solution is delivered through a flow line R_1 and water as a carrier solution through R_2 by a peristaltic pump P, respectively. Catecholamines and other organic compounds are injected by means of a 70-μl rotary value injector S. In order to block stray light, black Teflon tubing (1-mm i.d.) is used for the flow line except pump tubes and a detection flow cell D. For D a spiral flow cell (ca. 450-μl inner volume) made of flexible, transparent Teflon tubing (0.8-mm i.d.) is assembled as before.[11] The distance between S and D is about 4 cm, the minimum distance achieved. The light emitted is detected directly by a photomultiplier tube (Hamamatsu Photonics R376) with no wavelength discrimination. The signal from the photomultiplier tube is fed to picoammeter (Advantest TR-8651) and then recorded on a strip chart recorder. Peak height of the signal recorded is measured as CL signal.

Reagent. Chemicals of reagent grade were used as received. The water used was prepared by deionization of distilled water from a stainless steel apparatus. Each reagent solution was prepared daily from 10^{-1} M (permanganate) or 10^{-2} M (catecholamines and other organic compounds) stock solution (1 M=1 mol dm^{-3}). The permanganate stock solution was standardized with oxalate and stored in a dark room. Each catecholamine stock solution was prepared by dissolving its hydrochloride into and diluted with 10^{-2} M hydrochloric acid.

Results and Discussion

Batch Experiments. CL reaction often proceeds in different ways depending on the mixing order of reagent solution. This may cause considerable retardation of CL reaction and sometimes complete disappear-

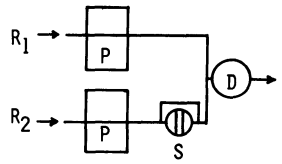

Fig. 1. Schematic diagram of the flow system (for key, see text). Recommended conditions: R_1= 1×10^{-3} M $KMnO_4$/1 M H_2SO_4, 3.2 ml min^{-1}; R_2= water, 4.5 ml min^{-1}.

Table 1. Effect of the Mixing Order of Reagent on the CL Signal for Catecholamines

Mixing order[a]					Relative CL signal[b]		
1	+	2	+	3	Dopamine	Noradrenaline	Adrenaline
$KMnO_4$[c]		H_2SO_4[c]		CA[d]	200	100	400
$KMnO_4$		CA		H_2SO_4	40	130	400
CA		H_2SO_4		$KMnO_4$	220	30	320

a) 0.1 ml of each solution was mixed in that order. b) Normalized with respect to the CL signal (=100) for noradrenaline. c) 10^{-3} M. d) 10^{-4} M catecholamines.

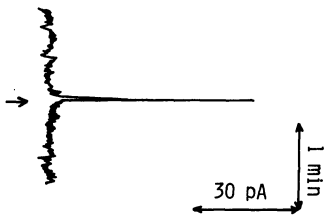

Fig. 2. Time course of CL signal for dopamine obtained by batch method. Arrow indicates the mixing of reagent (10^{-3} M $KMnO_4$/1 M H_2SO_4, 10^{-6} M dopamine).

ance of CL. And also how fast CL reaction occurs is a dominant factor which decides sensitivity in flow CL analysis because transient light emission is monitored. Accordingly, the exploration of the dependency of mixing order is of significant importance for the design of flow system. Thus, time courses of CL were measured on varying mixing orders of the reagent (permanganate, sulfuric acid, catecholamine) by the batch method. The results are shown in Table 1, indicating that the best mixing is in the order, permanganate, sulfuric acid, and catecholamines. Such a mixing order allows us to mix permanganate with sulfuric acid in advance. Consequently, this appears to permit the design of flow system consisting of a single flow line, namely, a sample injection into the stream of acidic permanganate solution. Unfortunately, a single line-flow system may not be assembled owing to a poor matching of the flow system with the speed of CL reaction. That is to say, as can be seen from the time course of CL in Fig. 2, the present CL reaction proceeds very fast and the light emission comes flashingly, resulting in an occurrence of most of light emission before the flow cell. Hence a dual line-flow system was assembled as shown in Fig. 1; the carrier stream of water into which a sample is injected enables the sample to join the stream of acidic permanganate solution just before the flow cell so that the light emission occurs mostly inside the flow cell.

Optimization. In order to determine optimum operating conditions, the CL signal for noradrenaline was taken with respect to reaction variables, i.e., the concentrations of permanganate and sulfuric acid and the flow rates of both streams. As expected, the CL signal was strongly dependent on both reagent concentrations because the oxidizing power of permanganate increases with an increase in the sulfuric acid concentration. The permanganate concentration above the concentration (1×10^{-3} M) providing the highest signal caused a decrease in the signal which was mainly due to the intense color of the permanganate solution, showing absorption maxima at 527 and 546 nm. The higher the sulfuric acid concentration, the higher the CL signal, but the higher the noise level. The highest signal-to-noise ratio was obtained at 1 M sulfuric acid. Other acids such as hydrochloric acid, phosphoric acids ($H_{n+2}P_nO_{3n+1}$, n=1, 2, 4), and monocarboxylic acids (C_1—C_4) were also examined, but none of them surpassed sulfuric acid with regard to the signal-to-noise ratio. The flow rate of the acidic permanganate solution provided a maximum signal at ca. 3 ml min^{-1}, but the increasing flow rate of carrier solution (water) gave an gradually increasing signal under any flow rates of the permanganate solution. For subsequent experiments, the optimum operating conditions were chosen as specified in Fig. 1.

Table 2. CL Signal for Catecholamines and Other Polyhydroxybenzenes

Compound (10^{-5} M)	Relative CL signal[a]
Dopamine	1.3
Noradrenaline	1.0
Adrenaline	1.0
L-Dopa	1.3
3,4-Dihydroxybenzylamine	1.5
Normethanephrine	1.0
Hydroquinone	2.1
Resorcinol	0.5
Catechol	2.1
4-Methylcatechol	1.5
4-*t*-Butylcatechol	5.6
Phloroglucinol	0.8
Pyrogallol	2.9
Gallic acid	0.4
Ethyl gallate	0.4
Tannic acid	0.6

a) Normalized with respect to the CL signal (=1.0) for noradrenaline.

Characteristics of the System. Under the optimum conditions, the background signal is 9×10^{-12} A, comparing with a noise current of 3×10^{-12} A. This high background, and consequently high noise are conceivably based on the luminous oxidation of organic impurities in the water used or from the flow line due to the use of highly acidic and concentrated permanganate solution. In a previous work,[12] the mild conditions (2×10^{-6} M permanganate at pH 2.5) gave much lower background and noise current (10^{-13} A levels), despite the presence of a fluorescent organic compound as sensitizer in the system. Calibration graphs for dopamine, noradrenaline, and adrenaline exhibited straight lines over the concentration ranges of 10^{-7} to 10^{-4} M with a slope of unity and signal ratio of 1.3:1.0:1.0, respectively. The limit of determination (S/N=3) is 7 pmol (70-μl injection of 10^{-7} M solution) for each catecholamine. This sensitivity is high enough to detect catecholamines in urine at 10^{-6} M levels. The relative standard deviation (n=10) for adrenaline (70 pmol) is 2.3%. The good reproducibility is based on an effective mixing caused by the fast flow of solution in the flow cell. The sampling rate can be as high as 500 h^{-1}.

It is known that some polyhydroxybenzenes give rise to light emission by permanganate oxidation under basic conditions.[13] Thus thirteen polyhydroxybenzenes were subjected to test in the present CL system, and CL signals obtained are summarized in Table 2 along with those for catechloamines. It can be roughly said from Table 2 that the unsubstituted phenols with a hydroxyl group at the ortho- or para-position provides higher signals and that the substituted phenols with an electron-donating group provides higher

Table 3. Effect of the Mobile Phase Used for Chromatographic Separation of Catecholamines on the CL Signal for Noradrenaline[a]

Mobile phase[b]	CL signal[c]	S/N[d]
0.2 M sodium formate	460	15
0.05 M citric acid (300 ml)+0.05 M disodium hydrogenphosphate (160 ml)+sodium dodecyl sulfate (15 mg)	140	5
Citric acid (10.5 g)+acetic acid (2.1 ml)+sodium hydroxide (4.8 g)+sodium acetate (8.2 g)	140	4
45% methanol/0.05 M sodium dihydrogenphosphate	120	5
Methanol+0.08 M acetic acid (1:1)	110	3
Water+methanol (28:72)	110	3
0.075 M citric acid	100	1
Ethyl acetate+cyclohexane (3:7)	70	4
0.12 M succinic acid+0.35 M boric acid +0.002 M EDTA (1:1:1)	60	15
0.2 M sodium acetate	50	25
0.15 M sodium dihydrogenphosphate	50	25
0.5 M sodium dihydrogencitrate	50	6
0.17 M acetic acid	40	5
6% acetonitrile/0.03 M sodium dihydrogenphosphate	10	5
0.1 M tris/HCl buffer (pH 8.5)+acetonitrile (87:13)	6	6
Water	50	17

a) 10^{-6} M. b) Flown to R_2 in place of water. c) 10^{-12} A. d) Signal-to-noise ratio.

signals, but those with an electron-withdrawing group lower signals. These trends were not observed in the above basic permanganate system. At any rate, the polyhydroxybenzenes are detected with sensitivity not so much different as those for catecholamines. This implies that these compounds can also be determined by means of the acidic permanganate CL system.

Applicability to Post-Column Detection. It is expected that the present CL system may be applied to the determination of catecholamines and other polyhydroxybenzenes in biological and clinical samples, drugs, foods, beverages, etc. after chromatographic separation. In that case, it should be noted that the constituents of mobile phase might inhibit the CL reaction. Accordingly, various kinds of mobile phases used for the elution of catecholamines were flown to R_2 in place of water, in order to know to what extent the CL signal for noradrenaline was affected. The results are shown in Table 3; the comparison of signal-to-noise ratio indicates that sodium formate, succinic acid/boric acid/EDTA, sodium acetate, and sodium phosphate may be utilized as eluent. The use of mobile phase containing alcohols seems to be undesirable because alcohols increase not only signal itself but also background noise, resulting in a deterioration of the signal-to-noise ratio. This was confirmed by the dependency of organic solvent on the signal for adrenaline. The presence of 20% methanol and ethanol in the carrier water gave about 2 and 18 times higher signal than in the absence of alcohol, but 10 and 100 times increased noise reduced both signal-to-noise ratios to one-sixth, respectively. Acetonitrile and acetone may not also be recommended as the constituent of mobile phase since they reduce signal itself to ca. 1/24 and signal-to-noise ratio to ca. 1/4, respectively.

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