Sensitive Chemiluminescent Determination of a Wide Variety of Nucleosides and Their Derivatives by Flow Injection Method

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Chemiluminescence (CL) observed in a reaction between OH radicals and nucleosides or their derivatives is applied to their sensitive determinations by a flow injection method. The OH radicals are produced by the reaction with hydrogen peroxide and cobalt(II)-1,10-phenanthroline complex in alkaline solution. The proposed flow system is comprised of three flow lines for cobalt(II), 1,10-phenanthroline and hydrogen peroxide. The proposed CL is specific to nucleosides and their related compounds. Their detection limits ranged from 68 pmol (uridine-5'-diphosphoglucose; UDP-Glu) to 89 pmol (guanosine) in a 10 μ l injection volume. The relative standard deviation is 1.2 % in 5 repeated runs for UDP-Glu of 178 pmol in a 10 μ l injection volume. Analytical time required is *ca.* 2 s.

Keywords Chemiluminescence, flow injection analysis, nucleoside, OH radical, cobalt(II)-1,10-phenanthroline complex

Since nucleosides are components of mononucleotides or polynucleotides (DNA or RNA), a new analytical technique to determine some nucleosides with high sensitivity and/or selectivity becomes a significant tool for solving problems related to various kinds of nucleotides.

Currently, there are several techniques for the determination of certain nucleosides¹⁻⁷, *i.e.*, radioenzymatic technique¹, chromatography with photodiode array detection² or radioimmunological technique³, fast atom bombardment and tandem mass spectrometry⁴ and analytical chemiluminescence (CL) technique.⁵⁻⁷ However, these techniques contain several problems to be solved; simplification of the analytical process, development of new analytical techniques applicable to the determination of a wide variety of nucleosides, improvement of sensitivity and/or selectivity, *etc.*

CL analysis is characterized by its high sensitivity and ease of instrumentation and operation, but only a few researchers have applied this technique to the determination of nucleosides and their derivatives.⁵⁻⁷ The reasons may be that the selectivities are poor and that there are only few CL reaction systems applicable to the determination of nucleosides. The current CL methods⁵⁻⁷ are well applicable to the highly sensitive detection of a limited kind of nucleosides and their derivatives, but could not be applied to a wide variety of their compounds, including adenine-5'-monophosphoglucose (AMP-Glu), uridine-5'-diphosphoglucose (UDP-Glu) or uridine-5'-diphosphomannose (UDP-

Mann). Therefore, emphasis should be placed to the intense studies for the development of new analytical techniques applicable to the highly sensitive and/or selective determination of a wide variety of nucleosides and their derivatives.

We extended analytical uses of weak CL.⁸⁻¹² Recently, we have found a new CL reaction system for nucleosides and the derivatives, which consists of 1,10-phenanthroline (Phen), cobalt(II), hydrogen peroxide, sodium hydroxide, surfactant and nucleosides. This CL was observed in a reaction between nucleosides and OH radicals and specific to these compounds.

This paper deals with a new flow injection method (FIA) with the CL detection for highly sensitive determination of a wide variety of nucleosides and their derivatives including UDP-Glu and UDP-Mann undetectable by the currently proposed CL methods.⁵⁻⁷

Experimental

Flow system and its operation

The FIA/CL flow system (Fig. 1) is essentially the same as that described previously⁹⁻¹², except that a three channel peristaltic pump (SJ-1220, Atto, Tokyo) was used instead of two other pumps. Also, the proposed flow system was operated under the optimal operating conditions.

Fig. 1 FIA/CL flow system and the optimal operating conditions. L₁, 0.2 mM 1,10-phenanthroline, 4 mM hexadecylethyldimethylammonium bromide and 4 mM sodium hydroxide at the flow rate of 3.5 ml/min; L₂, 20 μ M cobalt(II) chloride at the flow rate of 3.5 ml/min; L₃, 1.2% hydrogen peroxide at the flow rate of 3.5 ml/min; P, three channels peristaltic pump (Atto, SJ-1220); M, 1 m long mixing coil; S, 10 μ l sample injector (Rheodyne, 7120 type); D, detector equipped with spiral polyvinylchloride flow cell and photomultiplier tube (Hamamatsu Photonics, R376).

Fig. 2 Typical chemiluminescence profile. A: background chemiluminescence signal from 1,10-phenanthroline; B: chemiluminescence signal of *ca.* 0.5 mM uridine-5'-diphosphoglucose (UDP-Glu) by the 10 μ l injection. Conditions: same as for Fig. 1, except for the concentration of cobalt(II)chloride, 0.1 mM.

Reagents and solutions

Nucleosides and the derivatives were purchased from Sigma Chemicals (St. Louis, MO, USA). Other chemicals were analytical grade of Kanto Kagaku (Tokyo) or Wako Chemicals (Osaka). The CL reagents were prepared according to the previous reports.⁹⁻¹²

Distilled and deionized water obtained with the water purifier (RO-10C type, Tokyo Rikakikai, Tokyo) was used.

The standard solutions (0.1 M) of nucleosides and their derivatives were prepared in water as stock solutions; the solutions were stable for at least one month at 4 °C, and were diluted with water before use.

Results and Discussion

CL profile and CL reaction scheme

The CL profiles for nucleosides and their derivatives were similar to each other. The typical CL profile for UDP-Glu is illustrated in Fig. 2. The BG-CL (line A in Fig. 2) is produced from Phen itself.⁹ The CL signal for UDP-Glu which appeared through the reaction of OH radicals with UDP-Glu is indicated by peak B. Since the CL signals were recorded within *ca.* 2 s after sample introduction, the proposed flow system can be used for their rapid determinations.

The CL reaction scheme for UDP-Glu was estimated by the following experimental results. 1) When the equimolar solution of UDP-Glu (1 mM) was mixed with Nitroblue Tetrazorium (trapper of superoxide radical anion), the CL intensity with UDP-Glu decreased slightly but the BG-CL intensity decreased down to *ca.* 1/3 of the case of UDP-Glu alone. This implies that the CL response for UDP-Glu is not dependent on the coexistence of superoxide radical anion. 2) When the sample was prepared by adding 0.5 ml of 99.5% ethanol (trapper of OH radicals) to 5 ml of *ca.* 1 mM UDP-Glu,

the CL intensity of UDP-Glu decreased down to *ca.* 1/2 of the case of UDP-Glu alone, but the BG-CL response was not reduced. 3) In the case of acetonitrile, the result was reverse to that of ethanol: The CL response of UDP-Glu was almost unchanged. This indicates that acetonitrile is not effective for trapping the OH radicals. These two results, 2) and 3), show that the CL response of UDP-Glu is based on the OH radicals and that the reactivity of the OH radicals is in the order of ethanol > nucleosides > hexadecylethyldimethylammonium bromide (HDEDAB) in the proposed CL reaction system. Also, ESR studies on a reaction between OH radicals and nucleosides concluded that "whenever a substructure in nucleosides, either sugars like glucose or bases like adenine, was attacked by OH radicals, either reaction of the hydrogen removal from the sugars or the radical addition to the base could occur".¹³⁻¹⁵ Since this reaction produced the nucleoside radicals, the radicals could successfully contribute to the CL reaction and the OH radical played a key role for the CL reaction of nucleosides and related compounds: the CL of UDP-Glu occurred according to the following reaction process, although the photon emitter for the CL reaction has not yet been identified.

When the CL peak signal is used for the determination, the *S/N* ratio should be evaluated because it affects the sensitivity. Since the *S/N* ratio is equivalent to the intensity ratio of the nucleoside CL (Nu-CL) to the background CL (BG-CL), the Nu/BG value, the value was used as the *S/N* ratio in this study.

Selection of surfactants and metallic ions

For the selection of surfactants for the CL enhance-

Table 1 Chemiluminescence intensities obtained with some metallic ions

Metal species	Nu-CL ^a /pA	BG-CL/pA	Nu/BG
Cu(II)	2.5×10 ³	3.3×10 ³	0.76
Pb(II)	3.0×10 ²	4.3×10 ²	0.70
Co(II)	310	185	1.68
Ni(II)	8.9	8.4	1.1
Fe(II)	7.7	18.7	0.41
Zn(II)	7.2	5.8	1.2
Fe(III)	6.3	5.4	1.2
Sn(II)	6.4	5.1	1.2
Hg(II)	5.7	4.7	1.2
Cr(III)	5.4	3.6	1.5
Mn(II)	5.5	3.5	1.6
Cd(II)	6.2	3.1	2.0
None	6.0	5.0	1.2

Each metallic ion (10 mM) was introduced through line L₂ in the system.

a. Chemiluminescence intensity of 0.1 mg/ml UDP-Glu by the 10 µl injection.

ment, HDEDAB, cationic surfactant, was employed according to our previous report.⁹

The CL intensity of UDP-Glu obtained with various metallic ions in the HDEDAB solution is listed in Table 1.

The CL intensity of UDP-Glu with cobalt(II) and HDEDAB was *ca.* 50 times larger than that with HDEDAB alone; cobalt(II) gave fairly intense CL of UDP-Glu and high Nu/BG value; thus it was employed in this CL system.

The BG-CL intensities obtained with copper(II) and lead(II) were higher than those with other ions, because the two ions easily form the complexes with Phen. Cadmium(II), manganese(II) or chromium(III) showed weak quenching responses on the BG-CL. This is because their metallic ions interfere with the complex formation between Phen and small amounts of metallic ions (for example, copper(II) in the water or in the reagents), which causes the BG-CL.

Selectivity of the CL reaction

The CL intensities obtained with some chemical species introduced into the proposed system are given in Table 2 in terms of relative molar CL intensity. Most of the nucleosides and their derivatives gave strong CL responses. The CL intensity for other nucleosides and their derivatives ranged 40–80% of that of UDP-Glu, although the differences among them were small. The CL intensities for copper(II) and lead(II) were 0.02% and almost zero level in comparison with the CL intensity of UDP-Glu. This is due to the entire consumption of Phen required for the complex formation with cobalt(II). These results show the proposed CL reaction system is applicable to the selective detection of nucleoside groups.

Optimal operating conditions

The sensitivity depends on the Nu/BG value which

Table 2 Selectivity of the proposed chemiluminescence system

Species (0.1 mM)	Relative molar CL intensity, %
UDP-glucose	100
UDP-mannose	60
UDP-xylose	59
UDP-galactose	44
UMP ^a	80
UDP ^b	45
UTP ^c	75
Uridine	65
Cytidine	56
Adenosine	51
Guanosine	40
Cu(NO ₃) ₂	0.02
Pb(NO ₃) ₂	0.00
Other metal compounds ^d	0
Others ^e	0
None (water)	0

a. Uridine-5'-monophosphate.

b. Uridine-5'-diphosphate.

c. Uridine-5'-triphosphate.

d. 0.1 mM solutions of AlCl₃, SnCl₂, Ni(NO₃)₂, FeCl₃, ZnCl₂, HgCl₂, Co(NO₃)₂, K₂Cr₂O₇, K₂Cr₂O₄, FeSO₄·(NH₄)₂SO₄, K₃[Fe(CN)₆], (NH₄)₆Mo₇O₂₄, and CdCl₂.

e. 0.1 mM solutions of L-ascorbic acid, HCl, NaI, CH₃COOH, KCN, NH₄OH, EDTA, Na₂CO₃, NaNO₂, Na₂C₂O₄, KSCN, Na₂S₂O₃, C₆H₅Na₃O₇·2H₂O, Na₂HPO₄, Na₂S, NaBr, NaF, Na₂SO₃, MgCl₂, KClO₃, Na₂SO₄, and NaCl.

Conditions: same as for Fig. 1.

corresponds to the usual *S/N* ratio. The optimal operating conditions were investigated in order to obtain the largest Nu/BG value.

The concentrations of Phen, sodium hydroxide, hydrogen peroxide, HDEDAB and cobalt(II) were examined in the ranges of (1 µM–10 mM), (10 µM–5 mM), (0.1–10)%, (0.1 mM–20 mM) and (0.1 µM–1 mM), respectively.

The result for Phen concentration is shown in Fig. 3. The CL intensity of UDP-Glu gave a rising upper-convex curve to 0.15 mM of Phen concentration, was constant in the range (0.15–0.3) mM, and decreased beyond 0.3 mM. The BG-CL intensity increased with Phen concentrations ranging from 1 µM to 0.1 mM; it was constant from 0.1 mM to 0.46 mM and decreased thereafter. The concentration of 0.2 mM Phen was adopted after consideration of the Nu/BG value.

Similar CL intensity changes for Phen concentration were observed for sodium hydroxide, hydrogen peroxide, HDEDAB and cobalt(II). Since the largest Nu/BG value was obtained using 4 mM sodium hydroxide, 1.2% hydrogen peroxide, 4 mM HDEDAB and 20 µM cobalt(II), those concentrations were employed for the following experiments.

Next, the flow rate was investigated in the range of (0.5–6.0) ml/min in order to obtain the largest Nu/BG value. At the flow rate of (0.5–0.8) ml/min, the CL

Fig. 3 Effect of 1,10-phenanthroline concentration on chemiluminescence intensity of nucleosides and background chemiluminescence intensity. Curves: A, chemiluminescence signal of UDP-Glu; B, background chemiluminescence signal. Conditions: 25 mM hexadecylethyldimethylammonium bromide and 50 mM sodium hydroxide at the flow rate of 2.5 ml/min for the line L₁; 10 μ M cobalt(II)chloride at the flow rate of 2.5 ml/min for the line L₂; 3% hydrogen peroxide at the flow rate of 2.5 ml/min for the line L₃; 10 μ l injection of ca. 1 mM UDP-Glu.

response of UDP-Glu could be obtained, but the BG-CL response was not observed. At the higher flow rate, both of the responses showed tendencies similar to that of Phen, and both of them were maximum and constant at the flow rate of (3.0–4.0) ml/min. Therefore, the flow rate of 3.5 ml/min was employed in this study.

Analytical characteristics

For the calibration graphs of various nucleosides and related compounds, the linear and 45 degree lines were obtained between the molar concentrations and the CL intensities on the log-log plane. A typical calibration graph of UDP-Glu is shown in Fig. 4.

The lower detection limit ($Nu/BG=1.5$) was 68 pmol in a 10 μ l injection volume. The dynamic range for the determination was 2 orders of magnitude. As for other nucleosides and their derivatives listed in Table 2, their lower detection limits ranged from 71 pmol (UMP) to 89 pmol (guanosine) in a 10 μ l injection volume.

The relative standard deviation in 5 repeated runs by 10 μ l injections of 0.178 mM UDP-Glu was 1.2%. This value was about two times smaller than that of the current methods.⁴⁻⁷ The analytical time required was ca. 2 s.

Effect of various compounds on the UDP-glucose CL intensity

The effect of some species on the CL intensity of 50 μ M UDP-Glu was investigated under the coexistence of UDP-Glu and the tested species. The evaluation was based on the degree of attenuation of the CL response for the mixed sample from that of UDP-Glu alone. The

Fig. 4 Calibration graph for uridine-5'-diphosphoglucose. Conditions: same as for Fig. 1.

Table 3 Effect of various compounds on the CL intensity of UDP-glucose

Species (0.1 mM)	Relative error, %	
	0.1 mM	1.0 mM
UDP-glucose	—	—
UDP-mannose	80	149
UDP-xylose	78	137
UDP-galactose	59	103
UMP ^a	103	183
UDP ^b	51	96
UTP ^c	97	172
Uridine	78	143
Cytidine	70	126
Adenosine	62	113
Guanosine	45	85
Cu(NO ₃) ₂	0	0.3
Pb(NO ₃) ₂	0	0
Other metal compounds ^d	0	0
Others ^e	0	0
None (water)	0	0

a. Uridine-5'-monophosphate.

b. Uridine-5'-diphosphate.

c. Uridine-5'-triphosphate.

d. AlCl₃, SnCl₂, Ni(NO₃)₂, FeCl₃, ZnCl₂, HgCl₂, Co(NO₃)₂, K₂Cr₂O₇, K₂Cr₂O₄, CdCl₂, FeSO₄·(NH₄)₂SO₄, K₃[Fe(CN)₆], and (NH₄)₆Mo₇O₂₄.

e. HCl, L-ascorbic acid, NaI, CH₃COOH, KCN, NH₄OH, EDTA, Na₂CO₃, NaNO₂, Na₂C₂O₄, KSCN, Na₂S₂O₃, C₆H₅Na₃O₇·2H₂O, Na₂HPO₄, Na₂S, NaBr, NaF, Na₂SO₃, MgCl₂, KClO₃, Na₂SO₄, and NaCl.

Conditions: same as for Fig. 1.

results are listed in Table 3. A significant CL increase was observed for all nucleosides tested. It is because those compounds have the nucleoside structure. However, no interference was observed for the rest of species.

Recovery tests

In order to test the reliability of the proposed system,

Table 4 Recovery tests of UDP-glucose from the uridine and uridine-5'-triphosphate matrix by the proposed method

Matrix		UDP-glucose ^b		Recovery, %
UTP ^a / mM	Uridine/ mM	Taken/ mM	Found/ mM	
0.1	0.1	0.0152	0.0151	99.3
0.5	0.1	0.0505	0.0512	101
0.5	0.02	0.0887	0.0886	99.9
0.03	0.05	0.220	0.221	100
1.0	0.08	0.0186	0.0187	101
0.1	1.0	0.765	0.764	99.9
0.5	0.1	1.86	1.85	99.5

a. Uridine-5'-triphosphate.

b. Uridine-5'-diphosphoglucose.

Conditions: same as for Fig. 1.

the recovery tests of UDP-Glu from the UTP and Uridine matrix were made using the standard addition method. Seven recovery test samples for UDP-Glu were prepared and analyzed. The analytical results are given in Table 4. The recoveries were almost 100%.

A highly sensitive determination method for a wide variety of nucleosides and their derivatives was developed; the proposed method is detectable down to 68 pmol/10 µl of UDP-Glu, for which the current CL methods can not be adaptable enough. The proposed method is applicable to various fields where detection of a wide variety of nucleosides is required. Further, the proposed system is specific to nucleosides and related compounds, but in order to determine the individual compounds selectively, the support of other analytical techniques such as chromatography and electrophoresis

is indispensable. Such a system is under development.

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