J. Flow Injection Anal., Vol. 33, No. 1 (2016) 9–12

Development of Flow Injection Analysis Method for Deoxyribose with Chemiluminescence Detection of Using HNO_3–UV Oxidation Process

Yumeki Tani[1], Megumi Nagai[1], Aiko Kurisaki[1], Shigeo Yamazaki[1,*], and Keiitsu Saito[2]

[1] Chemistry Division, Faculty of Science, Okayama University of Science, 1-1 Ridaicho, Okayama 700-0005, Japan
[2] Division of Natural Environment and Chemistry, Faculty of Human Development, Kobe University, 3-11 Tsurukabuto, Nada, Kobe 657-8501, Japan

Abstract

We report a new flow injection analysis method utilizing an online photochemical reactor for deoxyribose, which is oxidized in the reactor to produce oxalate and other unidentified compounds that reduce $[Ru(III)(bpy)_3]^{3+}$ with chemiluminescence. Deoxyribose injected to dilute HNO_3 as a carrier was delivered to a reaction coil, which was then irradiated with ultra-violet light to promote the oxidation by HNO_3. The process parameters, such as the ultra-violet irradiation time and concentrations of HNO_3 and $[Ru(III)(bpy)_3]^{3+}$, were optimized, resulting in the detection limit of *ca.* 20 pmol. The chemiluminescences of ribose, ATP, deoxyadenosine monophosphate, and DNA from salmon sperm by this method were also observed.

Keywords flow injection analysis, deoxyribose, DNA, chemiluminescence, Ru

1. Introduction

The development of determination methods for deoxyribose is an important undertaking science and engineering because the deoxyribose moiety is included in the structure of DNA. Although there are many analytical methods for deoxyribose determination [1-5], very few involve flow injection analysis (FIA) with determination by chemiluminescence (CL). CL reactions are usually rapid, and the instrumentation used is simple and inexpensive. Furthermore, CL methods have the advantages of wide dynamic range and high sensitivity. Accordingly, a method in which deoxyribose is analyzed based on CL intensity would have many advantages over traditional methods, and would be of enormous benefit to biotechnology.

In this study, we report the development of a method based on CL using tris(2,2'-bipyridine)ruthenium(II) chloride hexahydrate ($[Ru(bpy)_3]Cl_2 \cdot 6H_2O$, hereafter referred to as "Ru-complex"). This method has been previously applied to the detection of oxalate and diketones [6,7], and has been reported to detect oxalate with a sensitivity of *ca.* 0.3 pmol [6,8,9]. Therefore, by developing a flow system in which deoxyribose is converted to oxalate, deoxyribose may be determined using FIA. Consequently, we have attempted to develop a system for deoxyribose analysis combined FIA with Ru-complex-based CL detection (CL-FIA).

Concentrated HNO_3 has been used as an oxidizing agent in many applications, such as saccharide synthesis (e.g., the synthesis of aldaric acid from aldohexose [10]). However, concentrated HNO_3 is harmful to humans and damages the stainless steel components commonly found in laboratory equipment. Consequently, we attempted to develop an oxidation system using dilute HNO_3 as the oxidizing agent, but HNO_3 with a concentration of less than 2 M has almost no oxidation activity [11,12]. Thus, the oxidation by dilute HNO_3 required promotion with UV irradiation. To the best of our knowledge, this is the first report of a HNO_3–UV technique being used in the detection of organic compounds. It is noteworthy that this HNO_3–UV oxidation process could be very useful in the industrial technology.

Herein, we report the design, fabrication, and optimization of the proposed CL-FIA system, and its application to the determination of ribose, adenosine triphosphate (ATP), deoxyadenosine monophosphate (dAMP), and DNA.

*Corresponding author. E-mail: yamazaki@das.ous.ac.jp

2. Experimental

In this study, two methods of FIA were explored. The first employed a photochemical reactor (PR) and cooling coil (CC) (Fig. 1), and the second was a standard FIA system without the PR or CC.

2.1 Chemicals

The Ru-complex, ammonium cerium nitrate (CAN, $(NH_4)_2[Ce(NO_3)_6]$), deoxyribose, and all other compounds were purchased from Wako Pure Chemical Industries, Osaka, Japan, and were of extra pure grade.

2.2 Apparatus

The visible spectra of the Ru-complex and/or CAN solutions were measured using a JASCO V-550 UV/VIS spectrophotometer (Hachioji, Japan). An F-1050 fluorescence spectrophotometer (HITACHI, Tokyo, Japan) was used to obtain the CL spectra.

The FIA system (Fig. 1) used in this study was composed of three pumps, an injector, a reaction coil (PTFE tube, i. d. 0.5 mm) with a UV lamp, a cooling coil, a CL detector (CL-2027, JASCO), and a data processor. A mixed solution of the Ru-complex and CAN was employed as a CL reagent. The solutions of the Ru-complex and CAN in 0.2 M H_2SO_4 were delivered individually, and were mixed passing through the DC.

The PR consisted of a UV lamp coiled with a PTFE tube (i. d. 0.5 mm × 10 m), as reported previously [13], and was also used as a batch system for the oxidation process.

2.3 Procedures

We investigated the conditions for the oxidation of deoxyribose using a batch system photochemical reactor prior to the assembly of the FIA system.

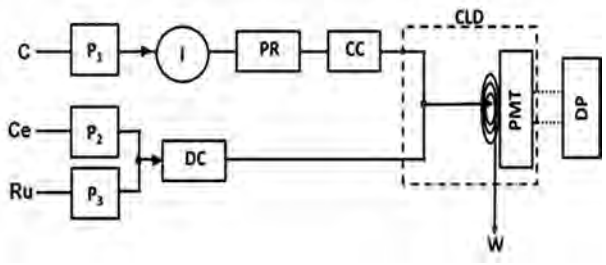


Fig. 1 Schematic of the CL-FIA system. C, carrier, 0.2 M HNO_3; Ce, 0.2 M H_2SO_4 containing 3 mM CAN; Ru, 0.2 M H_2SO_4 containing 1 mM Ru-complex; P_1, P_2, and P_3, pumps (P_1, 0.5 mL/min; P_2 and P_3, 0.25 mL/min); I, injector (10 μL); PR, photochemical reactor (UV 10 W, 2.5 min); CC, cooling coil (50 cm, 0°C); DC, delay coil (5 min); CLD, chemiluminescence detector; DP, data processor; W, waste.

The effect of the concentration of HNO_3 used for oxidizing deoxyribose was investigated using the procedure described above. Various concentrations of HNO_3 were UV-irradiated for 2 min. The reacted solution was injected into an FIA system fabricated by removing the PR and CC from the CL-FIA in Fig. 1.

The effect of irradiation time on the CL intensity was investigated using 0.2 M HNO_3 in the batch system using the procedure described above.

The effect of the CL reagent was investigated in two ways: A) using 1 mM Ru-complex solution containing 0–8 mM CAN; and B) using different concentrations of CL reagent solution while keeping the ratio of [CAN]/[Ru-complex] equal to 3.

The duration of UV exposure to the CL reagent (0.2 M H_2SO_4 containing 1 mM Ru-complex and 3 mM CAN), was investigated based on the visible spectra.

2.4 Production of oxalate

The production of oxalate from the oxidation of deoxyribose was investigated. The identification of compounds produced by the oxidation process was carried out by high performance liquid chromatography (HPLC) combined with the CL detection system, which was prepared by removing the PR and CC from the FIA system in Fig. 1. The reaction solution, comprising 0.1 mM deoxyribose in 0.2 M HNO_3, was UV-irradiated for 2 min in the batch system.

3. Results and Discussion

Recently, CL methods based on the Ru-complex and Ce(IV) (CAN contains Ce(IV)) solutions as CL reagents have been reported for the analysis of various samples, but have not yet been applied for detecting deoxyribose [14-19].

3.1 Proposed detection mechanism for the FIA system

Han *et.al.* have reported a CL method using Ce(IV) and the Ru-complex for nucleic acid using a batch system without the photochemical oxidation process [16]. The mechanism they proposed seems to be different from that proposed by us. Our method was operated at pH < 2, but the CL intensity observed with their method was very weak under these conditions.

A reaction producing an excited Ru(II)-complex from a Ru(III)-complex by a reducing sample causes light emission (CL) [20]. In acidic conditions (pH < 2), mixing the orange colored Ru(II)-complex with CAN leads to an immediate color change to green, which indicates the production of a Ru(III)-complex [21]. It has been reported that the reduction of Ce(IV) by a reducing sample also causes CL [20]. A CL spectrum was obtained to investigate the role of the Ru-complex and/or Ce(IV) in this CL mechanism. This CL spectrum was obtained using the apparatus prepared by removing the PR and CC from the CL-FIA shown in Fig. 1. The spectrophotometer used for the measurement was a fluorescence spectrophotometer without a lamp. The carrier was 0.2 M H_2SO_4 containing 1 mM oxalic acid (0.5 mL/min). The Ce line, Ru line, and delay coil were as described in Fig. 1. The CL spectrum obtained showed a CL band with λ_{max} = 610 nm, which was attributed to the CL of the Ru-complex. Consequently, we suggest that the Ru(III)-complex is generated by the oxidation of the Ru(II)-complex by CAN, and that the CL is emitted from the excited Ru(II)-complex, which forms via the reduction of the Ru(III)-complex by the reducing sample.

3.2 Optimal conditions

Optimal conditions were investigated by the procedures described in section 2.3.

Fig. 2 shows that the plot of CL intensity versus HNO_3 concentration reaches a maximum value at a HNO_3 concentration of ca. 0.2 M. No substantial peak was observed in the FIA without HNO_3. The CL intensity increased with increasing HNO_3 concentration up to 0.2 M, after which it gradually decreased. Therefore, 0.2 M HNO_3 was selected as the oxidizing agent for further experiments.

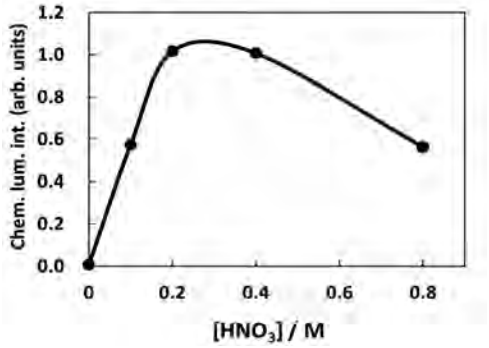


Fig. 2 The effect of HNO_3 concentration on the oxidation using the batch system. Sample, 0.1 mM deoxyribose; oxidation reagent, 0–1 M HNO_3; other conditions are described in the text.

Fig. 3 shows that the plot of CL intensity versus UV-irradiation time reaches its maximum at ca. 3 min. No substantial peak was observed without irradiation. The CL intensity increased with increasing irradiation time to up to 2 min, after which it increased slightly to 4 min, and then gradually decreased. This decrease in CL intensity after 4 min of irradiation suggests that, under excess irradiation, the compounds from deoxyribose that emit light via oxidation by the Ru(III)-complex are decomposed to other compounds without

emitting light. To confirm this, another experiment investigating excess irradiation was performed using oxalate as a reducing sample, and photodecomposition of the oxalate with increasing irradiation time was observed, which supports the explanation above.

In Fig. 3, the maximum CL intensity was observed after 3 min irradiation. UV irradiation time in the FIA system PR was controlled by the length of the PTFE tube exposed to the UV lamp. Therefore, the longer the tube, the wider the peak obtained, and consequently the peak height decreased. Thus, an irradiation time of 2 min was selected for further experiments.

Although Fig. 2 and 3 show that deoxyribose cannot be directly detected by CL-FIA without the oxidation process, after the deoxyribose solution passes through this system it emits light. In other words, the solution itself can act as a reducing sample.

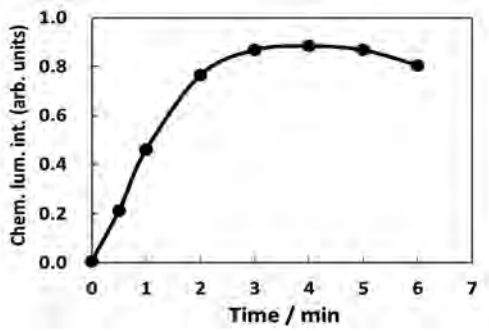
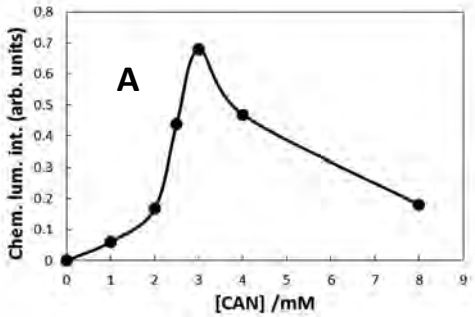


Fig. 3 The effect of UV irradiation time on CL intensity using the batch system. Sample, 0.1 mM deoxyribose; other conditions are described in the text.

He *et.al.* reported the detection of oxalate using the Ru-complex and Ce(IV) using a batch system [9]. In their method, a mixed solution of oxalate and the Ru-complex was prepared preliminary, and then the Ce(IV) solution was injected into the mixed solution. CL was detected from the reaction within 10 sec. However, they did not report any CL when only the Ru-complex and Ce(IV) were mixed. Consequently, we investigated the CL produced from the mixing of the Ru-complex and Ce(IV) solution without oxalate. The Ru-complex and CAN solutions were delivered separately by two pumps, and the CL reagent solution was delivered to the CLD after mixing for 5 min. This was because CL from the mixing of CAN and Ru-complex solution can be clearly observed, and its intensity gradually decreased over 5 min to an insignificant amount. Thus, a 5 min delay coil was employed for experiments on the time progression of the CL intensity from the sample-containing solution in order to eliminate interference from the intrinsic CL of the CAN and Ru-complex.



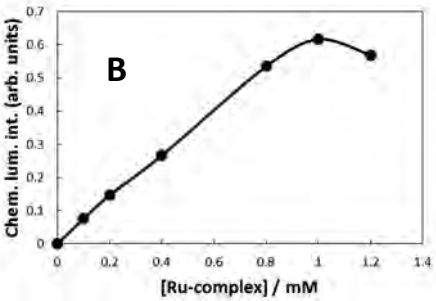


Fig. 4 The effects of CAN and Ru-complex concentration. FIA conditions were as given in Fig. 2, except the CL reagent. A) 0.2 M H_2SO_4 containing 1 mM Ru-complex and different concentrations of CAN, B) various concentrations of CL reagent at [CAN]/[Ru complex] = 3.0.

The effect of the CL reagent is shown Fig. 4 A) and B), where the peak maxima are obtained using 0.2 M H_2SO_4 containing 1 mM Ru-complex and 3 mM CAN as a CL reagent. Consequently this solution was used as a CL reagent for further experiments.

3.3 Production of oxalate

The chromatograph is shown Fig. 5. The efficiency of the sample conversion to oxalate was estimated based on the peak area. Although the ratio was lower than expected, the conversion accounts for *ca.* 12% of the combined peak areas. The compounds that contribute the remaining peak area have not yet been identified. This CL-FIA can also be applied to the determination of compounds such as pyruvate and malonate [22], which have a 1,2- or 1,3-dicarbonyl moiety in their structures. Thus, we speculate that the compounds causing the first large peak may contain 1,2- and/or 1,3-dicarbonyl groups.

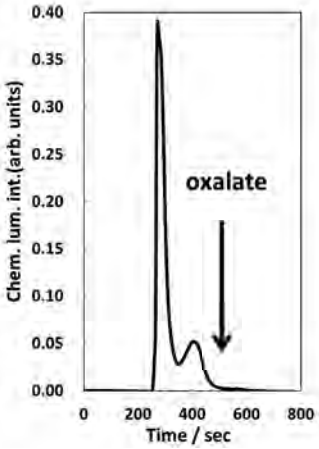


Fig. 5 Chromatogram of reacted solution by HPLC. Eluent, 50 mM CH_3COOH containing 5 mM tetrabutylammmonium hydrogen sulfate and 1% methanol (pH 3.5); flow rate of eluent, 0.5 mL/min; injection volume, 20 μL; column, Chromolith RP-8e(φ4.6 mm×100 mm); another conditions are described in the text.

3.4 Confirmation of 1,2- and/or 1,3-dicarbonyl production

In order to confirm the existence of 1,2- and/or 1,3-dicarbonyl compounds in the first large peak of Fig. 5, the reaction solution was analyzed by HPLC with a minor modification based on the Hantzsch reaction used as a post-column detection system, which was described Kakehi *et.al* [23]. However, the result was inconclusive.

3.5 Calibration curve and detection limit

The calibration curve for deoxyribose was linear from 2 to 100 μM (n = 6). The detection limit with respect to signal to noise ratio was ca. 2 μM.

3.6 Influence of interfering substances

In order to apply the proposed method to the analysis of a real sample, the influence of some possible interfering substances was investigated. The substances and their concentration range are shown in Tab. 1. A mixed solution containing 10^{-4} M deoxyribose and the substance to be examined at a concentration of 10^{-4} or 10^{-3} M was injected into the FIA system. Interference was observed with NaCl and $CaCl_2$, indicating that chloride ions decrease the CL intensity. The additives whose recoveries were larger than 150% can be detected by the proposed method. It should be particularly noted that glucose and fructose exhibit strong CL intensities compared with that of deoxyribose.

Tab. 1 Recovery of deoxyribose (0.1 mM) from various adducts

Adducts	Concentration ratio (adducts to deoxyribose)	Recovery (%)(n = 6)
Glucose	1	242.6
Fructose	1	248.1
Ethanol	1	94.9
	10	167.2
Acetic acid	1	95.5
	10	162.3
Acetone	1	112.6
	10	295.2
Triethylamine	1	106.9
	10	228.8
Dioxane	1	113.7
	10	299.4
NaCl	1	86.9
	10	71.1
$MgSO_4$	1	102.2
	10	96.6
$CaCl_2$	1	87.9
	10	53.4
H_2SO_4	1	90.7
	10	97.9
Na_2HPO_4	1	98.4
	10	99.8

3.7 Other samples

The developed CL-FIA system was applied to the detection of other compounds including or resembling the deoxyribose moiety. ATP includes a ribose moiety, which resembles deoxyribose, and dAMP includes a deoxyribose moiety. Accordingly, it was expected that our CL-FIA method can be applied to the analysis of ribose, ATP, and dAMP. Consequently, CL from each compound was detected, with the CL peak heights for ATP and dAMP being 50% smaller than those of ribose and deoxyribose, respectively. Both ATP and dAMP have an ester bond between the phosphate and ribose or deoxyribose moieties, and this bond is difficult to hydrolyze. This implies that the derivatization of the original ATP and dAMP samples to the reducing samples by UV-irradiation is more difficult due to the ester bond. Thus, the sensitivities for these compounds were lower than those for deoxyribose and ribose, which have no ester bond.

3.8 A real sample

DNA from salmon sperm was employed as a real sample, and CL from the DNA by this method was observed. The sensitivity of the method for the DNA was 10 times higher than that for deoxyribose. Further study is needed to explain the mechanism for this improved sensitivity.

4. Conclusion

Our CL-FIA system is based on the oxidation of saccharides by dilute HNO_3 under UV irradiation. This method possesses several advantages: No reagent for derivatization is necessary; the carrier and CL reagent are both aqueous; deoxyribose and its related compounds can be detected; and a commercially available reagent can be used.

Acknowledgements

The authors thanks to Dr Masunaga at Nichiri Mfg. Co., Ltd., for their critical reading of this article.

References

1. J. M. Gutteridge, B. Halliwell, *Biochem. J.*, **253**, 932, (1988).
2. P. Schmid, C. Schmid, D. C. Brodie, *J. Biol. Chem*., **238**, 1068, (1963).
3. S. Luqmana, R. Kumara, *Int. J. Food Prop.*, **15**, 942, (2012).
4. N. D. Gary, R. E. Klausmeier, *Anal. Chem*., **26**, 1958, (1954).
5. R. Q. Pestell, H. R. Woodaland, *Biochem J.*, **127**, 589, (1972).
6. K. Uchikura, *Bunseki Kagaku*, **39**, 323, (1990).
7. K. Uchikura, *Anal. Sci.*, **15**, 1049, (1999).
8. H. Han, Z. He, X. Li, U. Zeng, *Wuhan Univ. J. Nat. Sci*., **4**, 326, (1999).
9. Z. He, H. Gao, L. Yuan, Q. Luo, Y. Zeng, *Analyst*, **122**, 1343, (1997).
10. T. N. Smith, K. Hash, C. L. Davey, H. Mills, H. Williams, D. E. Kiely, *Carbohydr. Res.*, **350**, 6, (2012).
11. M. Hojo, R. Kato, T. Maeda, Y. Uji-yie, *J. Mol. Liq.*, **163**, 161, (2011).
12. F. A. Cotton, G. Wilkinson, "*Advanced Inorganic Chemistry*", (1980), John Wiley & Sons, Inc., New York.
13. K. Yokota. *Dr. thesis*, Kobe University (2002) [in Japanese].
14. A. M. García-Campaña, W. R. G. Baeyens (eds.), "*Chemiluminescence in Analytical Chemistry*", (2001), Marcel Dekker Inc., New York.
15. J. Chen, Y. Fang, *Sensors*, **7**, 448, (2007).
16. H. Han, X. Li, Z. He, L. Lin, Y. Zeng, *Anal. Sci.*, **15**, 885, (1999).
17. Y. S. Kim, J. H. Park, Y. S. Choi, *Bull. Korean Chem. Soc.*, **25**, 1177, (2004).
18. M. Keyvanfard, A. Mokhtari, I. Emami, *Acta Chem. Slov.*, **58**, 563, (2011).
19. B. Rezaei, A. Mokhtari, *J. Braz. Chem. Soc.*, **22**, 49, (2011).
20. I. Rubinstein A. J. Bard, *J. Am. Chem. Soc.*, **103**, 512, (1981).
21. K. Kalyanasundaram, *Coord. Chem. Rev.*, **46**, 159, (1982).
22. K. Saito, S. Murakami, S. Yamazaki, A. Muromatsu, S. Hirano, T. Takahashi, K. Yokota, T. Nojiri, *Anal. Chim. Acta*, **378**, 43, (1999).
23. K. Kakehi, T. Konishi, I. Sugimoto, S. Honda, *J. Chromatogr.*, **318**, 367, (1985).

(Received March 1, 2016)
(Accepted April 19, 2016)