Flow Injection Analysis for Pyrogallol Using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide as Polyphenol Chemiluminescence Enhancer

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Polyphenols, which have phenolic hydroxy groups, have been reported to have significant antioxidant effects (*e.g.*, antiinflammatory, inhibition of atherogenesis, antiviral and anticancer).¹⁻⁴ Polyphenols have been characterized by ultraviolet,⁵ fluorescence⁶ or electrochemical detection.⁷ These methods are interfered with by various compounds in the analytical samples. Therefore, the exact antioxidant effects and metabolism of the polyphenols have not been clarified. Thus, a selective and sensitive method is needed for the determination of polyphenols.

Recently, chemiluminescence (CL) methods have been reported for the determination of polyphenols. CL is specific for the polyphenols which have a trihydroxyphenol moiety, but the CL is very weak compared to the typical CL compounds, such as luminol and the acridinium ester. Thus, formaldehyde,⁸ *N*-bromosuccinimide⁹ and phenylboronic acid derivatives¹⁰ have been used as enhancers.

We reported that carbodiimide, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) enhances the CL of polyphenol 20–3600-fold.^{11,12} In this study, the effect of EDC enhancement was used to develop a sensitive flow injection analysis (FIA)-CL method for the determination of pyrogallol.

Experimental

Reagents and solutions

Deionized and distilled water purified by the Milli-Q II system (Japan Millipore, Tokyo, Japan) was used. Pyrogallol and EDC were purchased from Nacalai Tesque (Kyoto, Japan). H₂O₂ (31%, v/v) was purchased from Mitsubishi Gas Kagaku (Tokyo, Japan). H₂O₂ and pyrogallol solution were dissolved in water just before use. All other chemicals and solvents used were of analytical reagent grade.

FIA system and FIA conditions

A schematic diagram of the FIA-CL system is shown in Fig. 1. A Tosoh CCPM pump equipped with a Rheodyne 7125 syringe loading sample injector valve (10 μ l loop) was used. Solution A (0.5 M EDC containing 0.1 M NaOH) was pumped at the flow-rate of 1.0 ml min⁻¹. A 10 μ l aliquot of pyrogallol is injected from S (loop injection). The mixture was heated by a coil (50 cm $\times$ 0.5 mm i.d., stainless-steel tube) placed in the Tosoh RE-8010 reaction bath (60°C). Solution B (0.1 M H₂O₂) was introduced into the stream at a flow-rate of 1.0 ml min⁻¹. The pyrogallol CL was measured by a Jasco CL 925 CL detector and the CL signal was monitored by a Hitachi D-2500

integrator.

Results and Discussion

Figure 2 shows the effect of the EDC and the NaOH concentrations in solvent A, and of the H₂O₂ concentration in solvent B. The EDC concentrations up to 1.0 M were examined (Fig. 2 (A)). The maximum peak height was achieved using 1.0 M EDC, but the maximum signal-to-noise ratio (*S/N*) was obtained with 0.5 M EDC; therefore, 0.5 M EDC was selected. The effect of the NaOH concentration over the range of 0.01–1.0 M was then examined (Fig. 2 (B)). A 0.1 M NaOH gave the maximum peak height and *S/N* were obtained. The effect of the H₂O₂ concentration in the range of 0.01–1.0 M was also examined (Fig. 2 (C)). When 0.1 M H₂O₂ was used, the maximum peak height and *S/N* were obtained.

The effect of the flow-rate over the range of 0.5–2.0 ml min⁻¹ was examined (Fig. 3 (A)). The maximum peak height was obtained when 1.0 ml min⁻¹ was used, and the relative standard deviations (R.S.D.) for repeated determinations (*n*=3) was 5.0%.

The effects of the reaction coil length (50–200 cm: 0.5 mm i.d.) and of the reaction temperature (20–90°C) were examined. We postulated that EDC reacts with one of the hydroxy groups of pyrogallol in the reaction coil, and the EDC adduct with pyrogallol accelerates the production of the 3-hydroxycyclohexa-3,5-diene-1,2-diones, which were produced during the initial step of the pyrogallol CL after the addition of H₂O₂.⁹ When a 50-cm coil was used, the maxima peak height and *S/N* value were achieved (Fig. 3 (B)). At 20°C, the maxima peak height and *S/N* value were obtained, but the R.S.D. (*n*=3) was 8.1%. At 60°C, the CL intensity was approximately 70% of that obtained at 20°C, and the R.S.D. was 5.0%. Thus, 60°C was selected.

Under these conditions, the calibration graph plotted of the CL intensities of pyrogallol (0.2, 0.25, 0.5, 1.0, 2.5 and 5.0 μ M) was linear (*n*=3 for each plot) (Fig. 4). The correlation

Fig. 1 Flow system for the determination of pyrogallol.

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Fig. 2 Effect of EDC, NaOH and H₂O₂ concentrations. Conditions: flow-rate, 1.0 ml min⁻¹; coil-length, 50 cm; reaction temperature, 60°C. (A) EDC, (B) NaOH, (C) H₂O₂.

Fig. 3 Effect of flow-rate of solvents, the reaction coil-length and reaction temperature. Conditions: EDC, 0.5 M; NaOH, 0.1 M; H₂O₂, 0.1 M. (A) flow-rate, (B) coil-length, (C) reaction temperature.

Fig. 4 Calibration spectra of pyrogallol. Conditions: EDC, 0.5 M; NaOH, 0.1 M; H₂O₂, 0.1 M; flow rate, 1.0 ml min⁻¹; coil-length, 50 cm; reaction temperature, 60°C. Concentration of pyrogallol (μM): a, 0.2; b, 0.25; c, 0.5; d, 1.0; e, 2.5; f, 5.0.

coefficient for this linear line was 0.998 and the R.S.D. was 1.22 – 6.86% ($n=3$). The limit of detection ($S/N=5$) was 2.6 pmol per 10 μl injection. This FIA-CL method was approximately 4-fold more sensitive than the method using *N*-bromosuccinimide.⁹

In conclusion, the proposed FIA method using the EDC enhancement of polyphenol CL provided a sensitive determination of pyrogallol, and should be applicable to an HPLC-CL system for the sensitive and selective determination of polyphenols.

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References

1. B. H. Kroes, A. J. J. van den Berg, H. G. Quarles van Ufford, H. van Dijk, and R. P. Labadie, *Planta. Med.*, **1992**, 58, 499.
2. B. Fuhrman, A. Lavy, and M. Aviram, *Am. J. Clin. Nutr.*, **1995**, 61, 549.
3. H. Tsuchiya, M. Sato, H. Kato, T. Okubo, L. R. Juneja, and M. Kim, *J. Chromatogr. B*, **1997**, 703, 253.
4. J. Jankun, S. H. Selman, R. Swiercz, and E. Skrzypczak-Jankun, *Nature*, **1997**, 387, 561.
5. S. Shahrzad and I. Bitsch, *J. Chromatogr. B*, **1998**, 705, 87.
6. Y. Ho, Y.-L. Lee, and K.-Y. Hsu, *J. Chromatogr.*, **1995**, 665, 383.
7. M.-J. Lee, Z.-Y. Wang, H. Li, L. Chen, Y. Sun, S. Gobbo, D. A. Balentine, and C. S. Yang, *Cancer Epidemiol. Biomark. Prev.*, **1995**, 4, 393.
8. D. Slawinska and J. Slawinski, *Anal. Chem.*, **1975**, 47, 2101.
9. A. Safavi and M. R. Baezzat, *Anal. Chim. Acta*, **1998**, 368, 113.
10. O. Nozaki, X. Ji, and L. J. Kricka, *J. Biolum. Chemilum.*, **1995**, 10, 151.
11. M. Nakazono, J. Ijichi, Y. Ohba, and K. Zaitzu, *Anal. Sci.*, **1998**, 14, 853.
12. M. Yamashita, M. Nakazono, Y. Ohba, and K. Zaitzu, Report of Center of Advanced Instrumental Analysis, Kyushu University, **1998**, 16, 35.