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MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CHEMILUMINESCENCE DETECTION OF Dns-AMINO ACIDS

K. MIYAGUCHI, K. HONDA and K. IMAI*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)

SUMMARY

Application of the chemiluminescence (CL) post-column reaction to microbore column (250 × 1 mm I.D., 10 μm) high-performance liquid chromatography is described for the sensitive detection of Dns-amino acids. Dns-alanine, -valine, -isoleucine and -phenylalanine, separated on a reversed-phase column, were detected with CL, generated by the reaction with bis(2,4,6-trichlorophenyl) oxalate and hydrogen peroxide. The detection limits are of the order of 2×10^{-16} mol (S/N = 3) and the average deviation for the peak height of 10 fmol of each Dns-amino acid was 1.3% ($n = 5$).

INTRODUCTION

Bioluminescence and chemiluminescence (CL) have been gradually accepted as means of detection of minute amounts of biological substances, because of their high sensitivity and wide dynamic range. Also, owing to the fact that they do not need a light source, the detectors are not as expensive and complex.

The firefly luciferin-luciferase system has been used for the detection of ATP and ATP-related enzyme activities, and bacterial luciferin-luciferase has been used to detect minute amounts of substances in enzyme systems requiring NADH and FMN. The CL of luminol, lucigenin and oxalic acid aryl esters has been used for the sensitive detection of hydrogen peroxide. Recent progress in this area of analytical chemistry has been reviewed by Kricka and Thorpe[1].

Previous papers have reported the detection of fluorescent compounds, separated by HPLC (high-performance liquid chromatography) at the fmol (10^{-15} mol) level[2-6] by a combination of TCPO [bis(2,4,6-trichlorophenyl) oxalate] and hydrogen peroxide, as shown in Fig. 1. This method is based on the CL excited by the so-called 1,2-dioxetanedione[7].

For more sensitive detection, microbore column chromatography might be a good choice, since injected samples are not diluted as much as in conventional columns, and a higher peak can be obtained after the CL reaction. In this work, the combination of a microbore column with the TCPO–H_2O_2 CL reaction detection system was investigated to determine Dns-amino acids at higher sensitivity. The final detection limits are of the order $2 \cdot 10^{-16}$ mol.
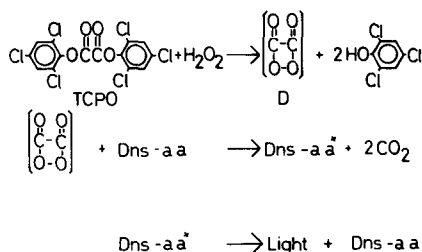
Fig. 1. Chemiluminescence reaction scheme. D = 1,2-dioxetanedione; Dns-aa* = excited state of Dns-amino acid.

EXPERIMENTAL

Solvents and reagents

Distilled water, acetonitrile (fluorescence analysis grade), acetone (HPLC grade), methanol (fluorescence analysis grade) and ethyl acetate (spectroanalysis grade) were obtained from Kanto Chemical (Tokyo, Japan). Imidazole was purchased from Merck (Darmstadt, F.R.G.). Nitric acid (ultrapure grade) and TCPO were from Wako (Osaka, Japan). Dns-amino acid standards were purchased from Sigma (St. Louis, MO, U.S.A.). All the other chemicals were of reagent grade.

The stock solution of Dns-amino acids (5 mM in methanol) was diluted in the eluent solution (0.1 M imidazole buffer [adjusted to pH 7.0 with nitric acid–acetonitrile (7:3, v/v)] to a concentration of 6 nM and a 0.5-μl aliquot (3 fmol of each Dns-amino acid) was subjected to HPLC analysis.

Apparatus

The flow diagram for this system is shown in Fig. 2. The pumps used were: LC-5A (Shimadzu Seisakusho, Tokyo, Japan) for the eluent and FAMILIC-300 (Japan Spectroscopic, Tokyo, Japan) for the reagent. The injection valve (7410, P/N 228-12800) and microbore column (MBC-ODS-2, 250 × 1 mm I.D., 10 μm) were from Shimadzu Seisaksho (Tokyo, Japan). A 25-μl mixing device (MD), which had been used for the wide-bore HPLC column[3,4,8], was used for thorough mixing of the eluent solution and the reagent solution; a 0.1 mm I.D. stainless-steel tube was in-
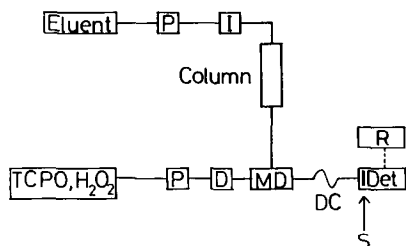

Fig. 2. Flow diagram for microbore HPLC–chemiluminescence detection system. P = Pump; I = injector; D = dummy column (TSK-GEL LS 410K and TSK-GEL ODS-120T, 250 × 4.6 mm I.D., 5 μm; Toyo Soda Manufacturing Co., Tokyo, Japan); MD = mixing device; DC = delay coil; Det = CL monitor; S = spiral-type flow cell; R = recorder. Eluent: 0.1 M imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (7:3, v/v). Reagent: 1 mM TCPO (ethyl acetate)–0.1 M H_2O_2 (acetone) (1:3, v/v).

serted into the cylindrical chamber as shown in Fig. 3. To achieve a certain reaction time, a delay coil (DC), made of PTFE tubing (400 × 0.25 mm I.D.), was interposed between the mixing device (MD) and the detector (Det).

The CL detector (Det), from Atto (Tokyo, Japan), has a sensitive photomultiplier tube (Type 6199; Hamamatsu Photonics, Tokyo, Japan), set close to the flow cell for detection of light emitted by fluorescent compounds. A Technicorder Type 3047 recorder (R) from Yokogawa Denki (Tokyo, Japan) was used.
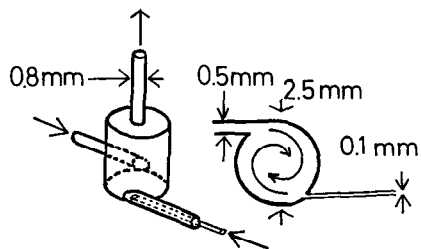

Fig. 3. Mixing device for microbore column.

Chromatographic conditions

The flow-rate of the eluent [0.1 M imidazole buffer (pH 7.0, $NO_3{}^-$)–acetonitrile (7:3, v/v)] was 0.03 ml/min. The reagent was a mixture of 1 mM TCPO in ethyl acetate and 0.1 M H_2O_2 in acetone (1:3, v/v); the flow-rates varied from 0.2 to 1.0 ml/min.

RESULTS AND DISCUSSION

TCPO dissolves well in ethyl acetate and is stable and gives a high CL[2]. Eluents for HPLC usually consist of a water-rich solvent, such as the 70% buffer used in this experiment. Therefore, a third solvent such as acetone is required to mix well with both the column eluate and the reagent solution of ethyl acetate[2]. In view of the easy mixing and the high CL obtainable, the ratios of the flow-rates of the eluent, ethyl acetate and acetone were selected as 1:1:3, as suggested in previously[3].

In this experiment, we adopted the apparatus for the CL reaction detection system used in the conventional HPLC system with a minor modification of the mixing device (25 μl). The stainless-steel tube (0.1 mm I.D.) was directly inserted into the mixing device to prevent band broadening of the eluted peak (Fig. 3).

In the present flow system for microbore column HPLC, the flow-rate of the eluent was fixed at 0.03 ml/min while the reagent, dissolved in the premixed solution of ethyl acetate and acetone (1:3, v/v), was delivered at various faster speeds than that of the eluent, since in microcolumn HPLC peak broadening might be caused by slow flow-rates of the reagent solution. With a 40-cm (0.25 mm I.D.) delay coil, the peak broadening and resolution between two Dns-amino acids (Dns-Ile and -Phe) were measured (Table I). Peak broadening occurred at a reagent flow-rate of less than 0.3 ml/min. At a reagent flow-rate of more than 0.6 ml/min, the resolution of the peaks was better. The peak heights measured are shown in Fig. 4. The highest signal to noise ratio (S/N) was obtained at 0.6 ml/min, when reaction was achieved

TABLE I

PEAK BROADENING AND RESOLUTION AT VARIOUS REAGENT FLOW-RATES

10 fmol each of Dns-Ile and -Phe were tested in the HPLC–CL detection system. The flow-rate of the eluent was 0.03 ml/min. The volume of the mixing device was 25 μl. Tw = Band width of the peaks for Dns-Ile (Tw1) and Dns-Phe (Tw2); R_s = resolution.

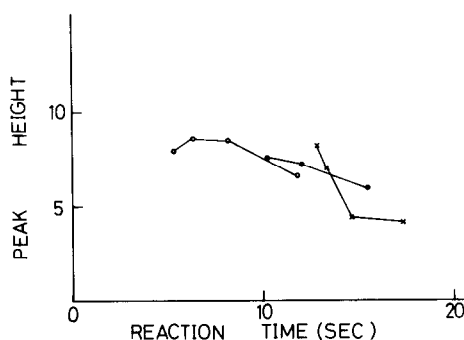| Reagent flow-rate (ml/min)	Reaction time (sec)	Tw1 (min)	Tw2 (min)	R_s
0.2	17	2.66	3.06	2.2
0.3	12	2.54	2.84	2.3
0.6	6.3	1.70	2.04	3.1
1.0	3.9	1.64	2.21	3.1

Fig. 4. Peak height of Dns-Ile, separated and detected by the microbore column–CL detection system. 10 fmol Dns-Ile were injected. The flow-rate of the eluent [0.1 M imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (7:3, v/v)] was 0.03 ml/min. The flow-rate of the reagent solution was 0.2 ($\times$), 0.3 ($\bullet$) or 0.6 ml/min (○). The length of the delayed coils (0.25 mm I.D.) were varied among 5, 9, 40, 79 and 158 cm.

in about 6.5 sec. In order to detect at an earlier stage of the reaction and make the system more sensitive, a smaller volume mixing device and tubing of a narrower diameter (0.1 mm) might be required.

    Four Dns-amino acid standards (Ala, Val, Ile and Phe) were separated on a reversed-phase microbore column and detected in the proposed system, as shown in Fig. 5. The detection limits for Dns-Ala, -Val, -Ile and -Phe were 160, 220, 220 and
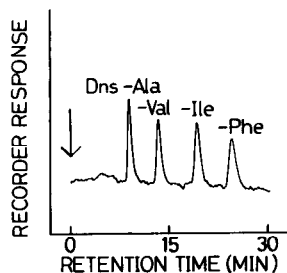

Fig. 5. Chromatogram of Dns-amino acids, obtained with the microbore HPLC–CL detection system. The amount of each amino acid injected was 3 fmol. Eluent: 0.1 M imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (7:3, v/v); flow-rate 0.03 ml/min. Reagent: 1 mM TCPO (ethyl acetate)–0.1 M H_2O_2 (acetone) (1:3, v/v); flow-rate 0.6 ml/min.

280 attomol (10^{-18} mol) (S/N = 3), respectively, and the average deviation for the peak height of 10 fmol of each Dns-amino acid was 1.3% (n = 5).

Few substances are detectable by isocratic elution. Linear or stepwise gradients are necessary to detect a series of biological important substances. For the sensitive detection of sixteen amino acids, gradient HPLC with a wide-bore column was successfully applied to the CL detection system[9]. We are currently applying gradient elution to our microbore column HPLC–CL detection system.

In conclusion, the TCPO–H_2O_2 CL reaction detection system for conventional HPLC proved to be applicable to microbore column HPLC with a minor modification. Almost ten times lower detection limits were obtained compared to those obtained with the wide-bore HPLC–CL detection system. However, to achieve more sensitive detection, a more elaborate arrangement for reducing the dead volume in the reaction system might be required.

ACKNOWLEDGEMENTS

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