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Short Communication

Improvement of peroxyoxalate chemiluminescence detection in liquid chromatography with gradient elution and a long reaction time

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ABSTRACT

In peroxyoxalate chemiluminescence (PO-CL) measurement using bis(2,4,6-trichlorophenyl) oxalate, background emission occurs much faster than that from analytes. By using a long reaction time of more than 15 s in most instances the background level can be reduced while maintaining sufficient analyte intensity. PO-CL detection with a reaction time of 20 s was applied in measurements of dansylated amino acids by liquid chromatography with gradient elution. The background was very low and baseline drift did not influence the detection of femtomole amounts of dansylated amino acids. The detection limits were 2–4 fmol (signal-to-noise ratio = 2).

INTRODUCTION

Since its first application with high-performance liquid chromatography (HPLC) [1], peroxyoxalate chemiluminescence (PO-CL) has become widely acknowledged as a highly sensitive detection method for HPLC, flow-injection analysis (FIA) and various other analytical techniques. One advantage of this method is that the background signal is much lower than that in the conventional fluorescence method owing to the absence of a light source and accounts in large part for the improved sensitivity of PO-CL detection. However, a background signal higher than that of the photomultiplier tube (PMT) dark current is still observed when using a PO-CL detector in an HPLC system [2–4]. There is therefore light emission in the final mixture of column effluent and PO-CL reagent solution without the addition of fluorescent analytes and the detection capacity of the PO-CL detector is limited by fluctuations in the background light intensity.

The existence of background emission also limits the application of the PO-CL method to HPLC with gradient elution. The composition of the final solution changes as the gradient proceeds with a consequent effect on the background intensity, eventually causing baseline drift of the chromatogram. This drift is not negligible particularly in cases of highly sensitive detection and sometimes makes the measurement of analytes impossible.

Little research has been reported on gradient HPLC analysis with PO-CL detection. Weinberger [5] measured dansylated (Dns) steroids by this technique, but the amounts of sample injected were in

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the range of 5–15 pmol and the measurement was not very sensitive. Miyaguchi *et al.* [6] measured 100 fmol of Dns-amino acids with the flow-rate of the eluent set low (0.3 ml/min) and that of the PO-CL reagent six times higher (1.8 ml/min). Change in the water content in the final solution was therefore small, which resulted in a small baseline drift but a long measurement time (*ca.* 3 h).

The kinetic rates of PO-CL emission were recently found to differ considerably between the analytes and the background when using bis(2,4,6-trichlorophenyl) oxalate (TCPO) [7]. Assessment was made of the influence of various factors on the intensity and kinetic rate of background emission. Based on the data obtaiend, measurement conditions for Dns-amino acids by isocratic HPLC with CL detection were optimized using a long reaction time (t_1) and, as a result, the background level was reduced more than tenfold.

In this study, the application of gradient elution to the optimized HPLC–CL system was investigated. Gradient conditions for the separation of Dns-amino acids were determined by HPLC with UV detection and thirteen Dns-amino acids were measured under these conditions and with PO-CL detection. It was considered that a reduced background level would resulted in a smaller baseline drift and highly sensitive PO-CL detection applicable to HPLC with gradient elution.

EXPERIMENTAL

Reagents

Dns-Amino acids were purchased from Sigma (St. Louis, MO, USA) and TCPO from Tokyo Kasei (Tokyo, Japan). Water was purified with a Yamato WG-25 system. All other chemicals were of analytical-reagent grade.

Apparatus

The gradient HPLC system with a PO-CL detector is shown in Fig. 1. It consisted of Shimadzu Type LC-9A HPLC pumps (P), a DGU-4A degasser (G), an FCV-9AL low-pressure gradient flow

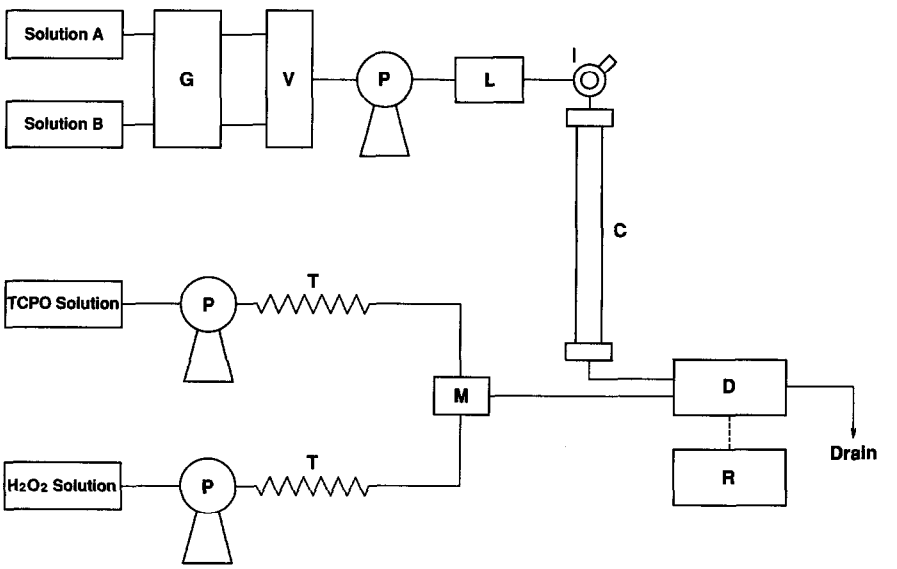

Fig. 1. Schematic diagram of the gradient HPLC system with PO-CL detection. P = pump; G = degasser; V = low-pressure gradient flow control valve; L = mixer for low-pressure gradient; I = injector, C = LC column; M = mixer; D = detector; R = Chromatopac. A stainless-steel tube (T) (2 m × 0.1 mm I.D.) was connected to the output of each pump for the PO-CL reagent. For all other flow lines, stainless-steel tubing (0.5 mm I.D.) was used. Solution A, 1.8 mmol of imidazole dissolved in 1000 ml of water [adjusted to pH 7.0 with nitric acid (pH 7.0, NO_3^-)]; solution B, 1.8 mmol of imidazole in a mixture of 400 ml of water and 600 ml of acetonitrile (pH 7.0, NO_3^-); flow-rate, 0.8 ml/min; TCPO solution, 0.5 mmol of TCPO in 1000 ml of acetonitrile, flow-rate 0.5 ml/min; hydrogen peroxide solution, 40 mmol of hydrogen peroxide in 1000 ml of acetonitrile, flow-rate 1.2 ml/min; voltage applied to photomultiplier tube, −0.8 kV; temperature of detector, 30°C.

control valve (V), a mixer for the low-pressure gradient (L), a Rheodyne Model 7125 injector (I) with a 20-μl sample loop, a Shimadzu Shim-Pak CLC ODS column (C) (150 × 4.6 mm I.D.) and a premixer (M) for a Shimadzu LC-6A pump. The gradient flow control valve was operated by the pump for the eluent according to the manufacturer's instruction. D was the same CL detector as that in the previous study [7], using a PTFE tube (4 m × 0.5 mm I.D.) for the reaction line of the effluent and CL reagents. Output signals were recorded with a Shimadzu Chromatopac C-R6A recorder (R). For the determination of the gradient conditions for Dns-amino acid separation, a Shimadzu SPD-10A UV detector was used instead of the CL detector. A stainless-steel tube (T) (2 m × 0.1 mm I.D.) was connected to the outlet of each reagent pump and stainless-steel tubing (0.5 mm I.D.) was used in all other flow lines.

Determination of gradient conditions

The wavelength for detection was 340 nm [7]. Solution A consisted of 1.8 mmol of imidazole dissolved in 1000 ml of water (pH 7.0, NO_3^-) and solution B was 1.8 mmol of imidazole in a mixture of 400 ml of water and 600 ml of acetonitrile (pH 7.0, NO_3^-). The flow-rate was 0.8 ml/min. A mixture of thirteen amino acids (10 ng each) (see below) dissolved in 20 μl of solution A was injected. All measurements were made at room temperature (*ca.* 23°C).

HPLC–CL measurement of Dns-amino acids

The sample was a mixture of thirteen Dns-amino acids dissolved in 20 μl of solution A. The amounts of the amino acids were as follows: 82 fmol of aspartic acid (Asp), 85 fmol of glutamic acid (Glu), 77 fmol of asparagine (Asn), 62 fmol of glutamine (Gln), 92 fmol of serine (Ser), 80 fmol of threonine (Thr), 75 fmol of alanine (Ala), 67 fmol of proline (Pro), 80 fmol of valine (Val), 22 fmol of lysine (Lys), 42 fmol of isoleucine (Ile), 75 fmol of leucine (Leu) and 63 fmol of phenylalanine (Phe). The column was washed with solution B for 30 min and preconditioned with a mixture of solitions A–solution B (75:25) for 15 min before sample loading. A linear gradient from 75% to 30% A over 35 min was then performed. The column temperature was room temperature (*ca.* 23°C).

The TCPO solution consisted of 0.5 mmol of TCPO in 1000 ml of acetonitrile. The flow-rate was 0.5 ml/min. The hydrogen peroxide solution was 40 mmol of hydrogen peroxide in 1000 ml of acetonitrile and its flow-rate was 1.2 ml/min. The voltage applied to the photomultiplier tube, temperature and response were −0.8 V, 30°C and 3 s, respectively.

RESULTS AND DISCUSSION

Gradient conditions for Dns-amino acid analysis

For gradient measurements of the thirteen Dns-amino acids, the flow-rate of the eluent, concentration of imidazole and solution pH were the same as those optimized in the previous study [7]. Only the water content in the eluent was varied by changing the ratio of solutions A and B. Good separation and minimum time for measurement were achieved under the conditions specified in the Experimental section.

HPLC–CL measurement of Dns-amino acids

For the detection of Dns-amino acids, the following parameters were optimized in the previous study [7]: t_1 of the effluent with PO-CL reagents, the concentration of each reagent, the flow-rate of each reagent solution and the temperature of the detector. As a result, the background level was reduced by as much as twentyfold and the signal-to-noise ratio (S/N) was enhanced more than tenfold. The reaction line of the detector in this study was as long as that used for the optimized t_1 as indicated in ref. 7 and the same detection conditions including t_1 (20 s) were used for HPLC measurements of Dns-amino acids with the optimized gradient elution.

A typical chromatogram obtained is shown in Fig. 2A. When the nonoptimized conditions used in the previous study (t_1 = 5 s, concentration of hydrogen peroxide = 10 mM, temperature of detector = 23°C and imidazole concentration = 2 mM) were applied to this gradient system, the background was high (65 nA at the start of measurement) and the baseline drift was more than ten times that shown in Fig. 2. Quantitative analysis of a sample was virtually impossible. Under the optimum conditions, the background level decreased to 2.7 nA and the ratio of the baseline drift to peak height of Dns-amino acids was almost the same as

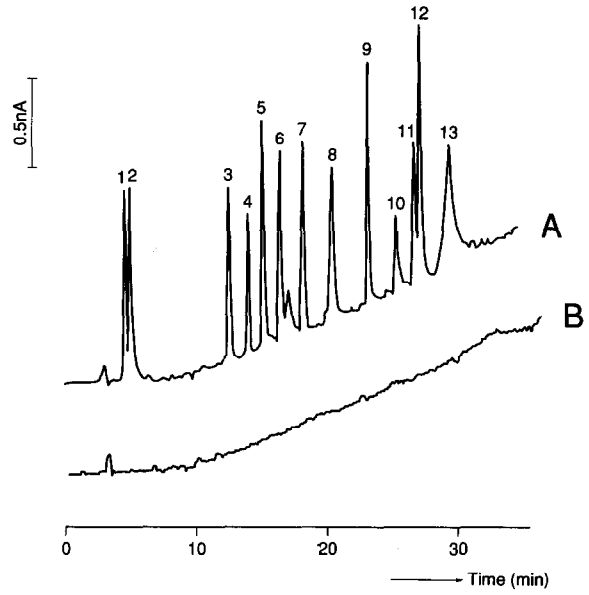

Fig. 2. (A) Chromatogram of Dns-amino acids. Peaks: 1 = 82 fmol of Asp; 2 = 85 fmol of Glu; 3 = 77 fmol of Asn; 4 = 62 fmol of Gln; 5 = 92 fmol of Ser; 6 = 80 fmol of Thr; 7 = 75 fmol of Ala; 8 = 67 fmol of Pro; 9 = 80 fmol of Val; 10 = 22 fmol of Lys; 11 = 42 fmol of Ile; 12 = 75 fmol of Leu; 13 = 63 fmol of Phe. Conditions: linear gradient from 75% to 30% solution A in 35 min. (B) Baseline measured under the same gradient conditions as those in (A) without an LC column.

that for the chromatogram of Miyaguchi *et al.* [6]. It should be noted that the changes in the water content of the eluent (26.2% during measurement) and the final solution (8.4% in the same period) were 1.5 and 3.4 times greater, respectively, and the measurement time was as short as 35 min.

Baseline drift may possibly result from elution of impurities from the LC column. If fluorescent impurities trapped in a column elute gradually with increase in the amount of organic solvent, the background level will increase during the course of measurement. For the evaluation of this effect, the baseline was measured by FIA after replacing the column with a stainless-steel tube (2 m × 0.3 mm I.D.). The baseline obtained is shown in Fig. 2B. In either instance the amount of drift was the same and was concluded not to be generated by changes in the amounts of impurities eluted from the LC column. This agrees well with the previous study in which the PO-CL background may not have been the emission from impurities but reaction intermediates and its intensity and the kinetic rate were influenced by certain factors such as water content [7].

Nevertheless, small, irregular peaks appeared on the chromatograms during measurements and good cleaning and conditioning of the column were found to be essential for their prevention and to obtain reproducible results. The column was washed and preconditioned before each measurement as described in the Experimental section. As a result, the detection limits of Dns-amino acids were 2–4 fmol (S/N = 2) and the R.S.D. of the peak height was less than 3%.

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