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SUB-PICOMOL CHEMILUMINESCENCE DETECTION OF Dns-AMINO ACIDS SEPARATED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH GRADIENT ELUTION

K. MIYAGUCHI, K. HONDA and K. IMAI*
Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo (Japan)
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SUMMARY

A post-column detection system using the bis(2,4,6-trichlorophenyl) oxalate–hydrogen peroxide chemiluminescence reaction is described for the analysis of sixteen Dns-amino acids. The amino acid derivatives were separated on a reversed-phase column (TSK ODS-120A) eluted with imidazole–nitrate buffer and acetonitrile. The detection limit for each amino acid was 2–5 fmol.

INTRODUCTION

Conventional photo-induced fluorescence detection, widely used in high-performance liquid chromatography (HPLC), suffers from fluctuation and stray radiation from the light source. This affects the fluorescence intensity and consequently the sensitivity of detection. A high-performance liquid chromatography–chemiluminescence (HPLC–CL) reaction detection system appeared to overcome this problem[1] and fluorescent compounds were detected at the fmol level[1–4]. However, these systems used only isocratic elution for the separation of fluorescent compounds.

This paper describes a gradient elution system for the separation and sensitive detection of sixteen Dns-amino acids. The post-column chemiluminescence (CL) reaction takes place with bis(2,4,6-trichlorophenyl) oxalate (TCPO) and hydrogen peroxide (H_2O_2) in the presence of fluorescent compounds, as shown in Fig. 1.

EXPERIMENTAL

Materials and reagents

Dns-Amino acids were purchased from Sigma (St. Louis, MO, U.S.A.), imidazole from Merck (Darmstadt, F.R.G.), distilled water, acetonitrile and methanol (fluorescence analysis grade), acetone (HPLC grade) and ethyl acetate (spectroanalysis grade) from Kanto (Tokyo, Japan) and nitric acid (ultrapure grade) and TCPO from Wako (Osaka, Japan). The concentration of H_2O_2 (Mitsubishi Gas Kagaku Co., Tokyo, Japan) was measured by the sodium thiosulphate method using ammonium molybdate as a catalyst, according to Kingzett[5]. All the other chemicals were of reagent grade.

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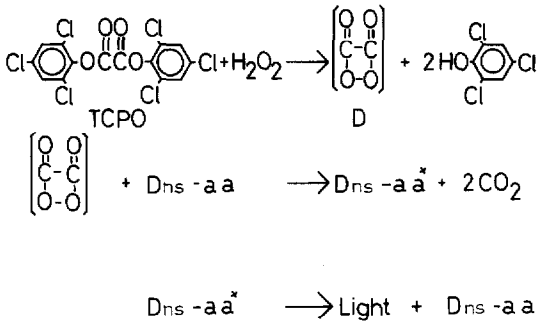

Fig. 1. Chemiluminescence reaction scheme. D = 1,2-Dioxetanedione; Dns-aa* = excited state of Dns-amino acid.

The stock solution of Dns-amino acids (5 m*M* in methanol) was diluted in the eluent solution [0.1 *M* imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (90:10, v/v)] to a concentration of 5 n*M*, and 20-μl aliquots (100 fmol of each Dns-amino acid) were subjected to HPLC analysis.

Apparatus

The flow diagram of the apparatus is shown in Fig. 2. A TRI ROTAR SR-2 elution pump, (P_1) with a gradient system GP-A30 (Japan Spectroscopic Co., Tokyo, Japan) was used for the eluent. This pump gave low baseline noise. However, a constant-flow syringe-type pump might be more effective for the present system[1]. In the sense, Melbin[6] succeeded to regulate the flow using a syringe-type by pump helium pressure. The reagent solution was delivered by a LC-3A reagent pump (P_2) (Shimadzu Seisakusho, Tokyo, Japan). A Model 7120 syringe-loading sample injector (Rheodyne) was used for sample injection. The column was a TSK-GEL Type ODS-120A (150 × 4.6 mm I.D., 5 μm; Toyo Soda Manufacturing Co., Tokyo, Japan). A recently designed 25-μl mixing device (MD) was used for thorough mixing of the eluent solution and the reagent solution[7]. To achieve a certain reaction time, a delay coil (MC) made of PTFE tubing (600 × 0.25 mm I.D.) was set between the mixing device (MD) and the detector (D). The CL detector (D) was from Atto Corp. (Tokyo, Japan) and has a sensitive photomultiplier tube (Type 6199; Hamamatsu Photonics Co., Tokyo, Japan) placed 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.

Chromatography

The gradient mixtures were: A, 0.1 *M* imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (90:10, v/v); B, 0.1 *M* imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (55:45, v/v). After washing with eluent B for 30 min, the column was preconditioned with eluent A for 3 h or 30 min before sample loading. Then, a linear gradient from 30% B (70% A) to 99% B (1% A) over 256 min was performed. The column eluate was mixed with reagent solution with the aid of the mixing device. The reagent solution comprised 0.25 m*M* TCPO and 75 m*M* H_2O_2 in ethyl acetate–acetone (1:3, v/v). The respective flow-rates of the eluent and reagent solutions were 0.3 and 1.8 ml/min. The reaction time was 0.8 sec.

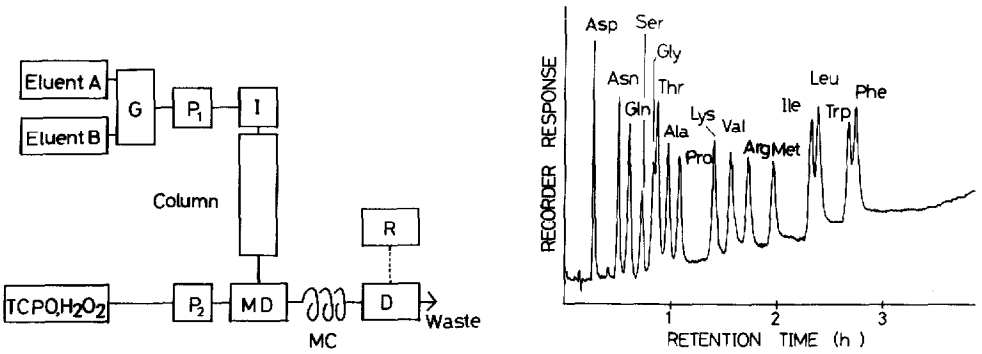

Fig. 2. Flow diagram of apparatus. Eluent A = 0.1 *M* imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (90:10, v/v); eluent B = 0.1 *M* imidazole buffer (pH 7.0, NO_3^-)–acetonitrile (55:45, v/v); flow-rate 0.3 ml/min; G = gradient programmer; P_1 = pump for eluent, TRI ROTAR SR-2; P_2 = pump for reagent, LC-3A; reagent solution is a mixture of 0.25 m*M* TCPO and 75 m*M* H_2O_2 in ethyl acetate–acetone (1:3, v/v), flow-rate 1.8 ml/min; I = injector; column = TSK-GEL Type ODS-120A; MD = mixing device; MC = mixing coil (reaction time 0.8 sec); D = chemiluminescence monitor; R = recorder.

Fig. 3. HPLC separation and chemiluminescence reaction detection of Dns-amino acid standards. Asp = asparatic acid; Asn = asparagine; Gln = glutamine; Ser = serine; Gly = glycine; Thr = threonine; Ala = alanine; Pro = proline; Lys = lysine; Val = valine; Arg = arginine; Met = methionine; Ile = isoleucine; Leu = leucine; Trp = tryptophan; Phe = phenylalanine. Conditions: gradient linear program, from 30% B (70% A) to 99% B (1% A) in 256 min. Each peak corresponds to 100 fmol Dns-amino acid injected.

RESULTS AND DISCUSSION

In the course of our studies on the sensitive detection of fluorescent compounds, we successfully applied a chemiluminescence reaction detection system to the detection of Dns-amino acids and fluorescamine-labelled catecholamines in HPLC column eluates[1,2] and achieved detection limits of 10 and 25 fmol, respectively. Modification of the system by using only one pump for the reagent solution together with the purification of the eluent and the use of a spral type flow cell reduced the detection limits of Dns-amino acids to around 5 fmol[3]. Sigvardson and Birks[4] also demonstrated recently the feasibility of employing HPLC–CL to the separation of eighteen aromatic hydrocarbons such as phenanthrene and perylene with detection limits ranging from 400 pmol to 3 fmol. However, no attempt has been made to extend this technique to gradient elution HPLC.

In this work, we applied the system to the determination of sixteen Dns-amino acids separated by gradient elution, hoping to widen the practical applicability of the system. For simplicity, we modified the gradient elution described by Grego and Hearn[8]. Dns-Glu is therefore not separable from Dns-Asp.

It had been thought that in the gradient elution CL system the baseline would gradually drift with increasing amount of organic solvent in the eluent[1]. When the present system was operated without the column no baseline drift was observed. This indicated that the variation of the organic solvent from 89 to 92% in the final reaction medium may have no effect on the CL intensity. However, when the system was operated with the column preconditioned with eluent A for 3 h, baseline drift was observed during gradient elution. We therefore shortened the period of column pre-

conditioning from 3 h to 30 min, whereupon the baseline drift was markedly reduced. A probable explanation is that impurities in the gradient mixture A were adsorbed on the head of the column during preconditioning and were gradually eluted, thus causing drift of the baseline. The amount of impurities may be small, but in this highly sensitive detection system is enough to affect the detection. It should be stressed that purification of the mobile phase is also of prime importance to increase further the sensitivity of the present method as well as in isocratic elution[3].

The sixteen Dns-amino acids were separated by linear gradient elution and were detected by the CL reaction detection system (Fig. 3). The average deviation of retention time for these Dns-amino acid standards was 3.1% (n = 5). The amount of each amino acid injected was 100 fmol. A detection limit as low as 2–5 fmol (signal-to-noise ratio = 2) has been achieved. For further sensitive detection suppression of baseline drift by purifying the eluent A was essential.

Another way of improving the sensitivity is to adopt better and more suitable fluorescence-labelling reagents for chemiluminescence detection, such as those having polycyclic aromatic hydrocarbon skeletons which are effectively excited[3]. The search for such labelling reagents is the next stage of our approach.

We are currently investigating the applicability of this system to semi-micro column HPLC where compounds are much more concentrated and give higher peaks.

In conclusion, the CL reaction detection system proved to be applicable to HPLC using gradient elution for the simultaneous sensitive detection of many homologous compounds.

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