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# APPLICATION OF A MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–CHEMILUMINESCENCE DETECTION SYSTEM TO THE N-TERMINAL AMINO ACID ANALYSIS OF BRADYKININ

KIMITOSHI MIYAGUCHI, KAZUMASA HONDA, TOSHIMASA TOYO'OKA and KAZUHIRO IMAI*

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

## SUMMARY

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (230 pmol) was dansylated with 5-N,N-dimethylaminonaphthalenesulphonyl chloride (Dns-Cl), followed by isolation in a reversed-phase high-performance liquid chromatography system with fluorescence detection (emission 530 nm, excitation 350 nm). The N-Dns-bradykinin was acid-hydrolysed and 1/500 of the liberated N-Dns-arginine was analysed on Hypersil ODS by gradient elution microbore high-performance liquid chromatography (5 μm, 150 × 2.1 mm I.D.) and postcolumn reaction with bis-(2,4,6-trichlorophenyl) oxalate–hydrogen peroxide. The generated chemiluminescence was detected by a photomultiplier. The technical problems of achieving sensitive detection by this procedure are discussed.

## INTRODUCTION

Application of aryl oxalate chemiluminescence, especially the bis-(2,4,6-trichlorophenyl) oxalate (TCPO)–hydrogen peroxide system, to high-performance liquid chromatography (HPLC) permits the detection of fluorescent compounds with high sensitivity (Fig. 1). Dansyl (Dns) amino acids[1], fluorescamine-labelled catecholamines[2], polyaromatic hydrocarbons[3], and other compounds[4–11] have been sep-

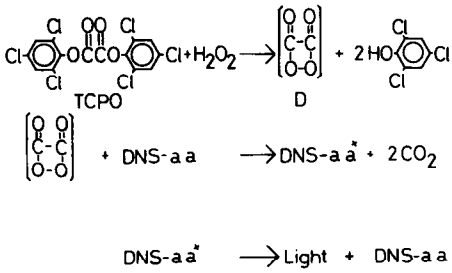


Fig. 1. Chemiluminescence reaction schemes: D = 1,2-dioxetanedione; DNS-aa = Dns-amino acid; Dns-aa* = excited state of Dns-amino acid.

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arated by isocratic elution HPLC and detected at the femtomole level. Dns-amino acids have been separated by gradient elution HPLC and detected at the low femtomole level by the chemiluminescence reaction detection system[12].

This paper investigates the application of microbore HPLC–chemiluminescence to the N-terminal analysis of a peptide. Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) was selected as a model peptide, dansylated with 5-N,N-dimethylaminonaphthalenesulphonyl chloride (Dns-Cl). The Dns-Arg liberated after acid hydrolysis was detected sensitively. Some difficulties encountered in achieving highly sensitive detection by this procedure are discussed.

## EXPERIMENTAL

### *Materials and reagents*

Distilled water (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Nakarai (Tokyo, Japan). Acetone (HPLC grade), methanol, acetonitrile (fluorescence analysis grade), ethyl acetate, diethyl ether (spectroscopic analysis grade), nitric acid (ultrapure grade) and TCPO were from Wako (Tokyo, Japan). Imidazole (Merck, Darmstadt, F.R.G.) was recrystallized from diethyl ether (m.p. 88–89°C). Hydrogen peroxide was from Mitsubishi Gas (Tokyo, Japan). Bradykinin was from Peptide Institute (Osaka, Japan). Dns-Cl and Dns-amino acis were purchased from Sigma (St. Louis, MO, U.S.A.). The stock solutions of Dns-amino acid standards (1–5 m*M* in ethanol) were diluted with the eluent A, described below, to a concentration of 40 n*M*, and 0.5-μl aliquots (20 fmol of each Dns-amino acid) were subjected to HPLC–chemiluminescence analysis. All other chemicals were of reagent grade.

### *Dansylation of bradykinin*

A volume of 6.8 μl (230 pmol) bradykinin in distilled water, 66.6 μl 0.18 *M* borate–sodium hydroxide (pH 9.5) and 33.4 μl 0.1% Dns-Cl in acetone were allowed to react for 60 min at 40°C and stored in a refrigerator until needed for the isolation step.

### *Isolation of the Dns-bradykinin*

A Model 655 liquid chromatograph (Hitachi Seisakusho, Tokyo, Japan) with a 655 proportioning valve and a 655-60 processor was used. In order to separate Dns-bradykinin from the hydrolysate of Dns-Cl (Dns-OH), it was chromatographed on a μBondapack $C_{18}$ column (10 μm, 300 × 3.9 mm I.D., Waters Assoc., Milford, MA, U.S.A.) with the following gradient elution programme: a linear gradient from 100% A (0% B) to 0% A (100% B) in 60 min at a flow-rate of 1.0 ml/min. Eluent A was 0.05% TFA in water and eluent B was 0.05% TFA in acetonitrile. The column eluate was monitored by emission at 530 nm (excitation at 350 nm) with a Model F1000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The N-Dns-bradykinin fraction collected (retention time, 28 min) was of *ca.* 2 ml.

### *Hydrolysis of Dns-bradykinin*

The collected fraction was freeze-dried, hydrolysed with 6 *M* hydrochloric acid at 130°C for 20 h and again freeze-dried. The residue was dissolved in 250 μl of 0.1 *M* imidazole nitrate (pH 7.0)–acetonitrile (90:10).

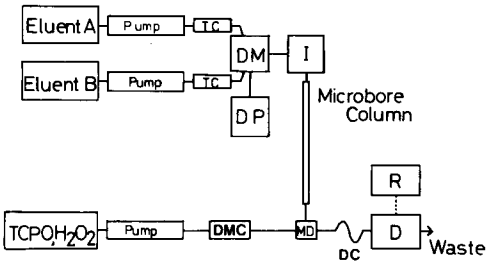


Fig. 2. Flow diagram for gradient elution microbore HPLC–chemiluminescence detection system. TC = Trapping column; DM = dynamic mixer; DP = damper; I = injector; DMC = dummy columns (TSK-gel LS 410 K and TSK-gel ODS-120T, 250 × 4.6 mm I.D., 5 μm, Toyo Soda); MD = mixing device; DC = delayed coil; D = chemiluminescence monitor; R = recorder. See text for details of eluents and reagent.

*Detection of N-terminal amino acid of bradykinin*

A 0.5-μl aliquot of the diluted sample was injected with an injector (Rheodyne) into a gradient-elution microbore HPLC–chemiluminescence system (Hypersil ODS, 5 μm, 150 × 2.1 mm I.D.).

The flow diagram is shown in Fig. 2. Gilson Model 302 and 303 pumps were used for delivering the eluent solution. Eluent A was 0.1 *M* imidazole nitrate (pH 7.0)–acetonitrile (90:10) and eluent B was 0.1 *M* imidazole nitrate (pH 7.0)–acetonitrile (55:45). A linear gradient from 20% A (80% B) to 80% A (20% A) in 100 min was used at a flow-rate of 0.15 ml/min. The gradient system was controlled by a 16-bit microcomputer PC-100 (Nihon Denki, Tokyo, Japan). Columns for trapping impurities in the eluent were inserted between each pump for the eluent solutions and a dynamic mixer. The pressure columns (TSK-gel LS 410 K and TSK-gel ODS-120T, 250 × 4.6 mm I.D., Toyo Soda, Tokyo, Japan) for damping irregular flow of reagent solution were also inserted between the pump for the reagent solution and the mixing device. In order to mix the eluent and the reagent solution effectively, a rotating-flow mixing device was adopted[13]. The reagent solution was 1 m*M* TCPO in ethyl acetate–0.1 *M* hydrogen peroxide in acetone (1:3). The pump for the reagent solution was an LC-3A (Shimadzu Seisakusho, Tokyo, Japan) and the flow-rate was 0.6 ml/min. A delay coil (50 × 0.25 mm I.D.) was connected between the mixing device and the detector. After reaction for *ca.* 2 s, the emitted light was detected with a chemiluminescence monitor from Atto (Tokyo, Japan), which has a photomultiplier tube (Type 6199; Hamamatsu Photonics, Tokyo, Japan), set close to the spiral-type flow-cell (126 μl) for detection.

## RESULTS AND DISCUSSION

In our previous paper[14], a microbore column of 1 mm I.D. was used for the isocratic elution of four Dns-amino acids, which resulted in a detection limit of 200 attomol. Therefore, gradient elution was first applied to the 1 mm I.D. column at a flow-rate of 50 μl/min. However, the baseline noise increased and the signal-to-noise ratio was decreased, compared with isocratic elution. This was caused by the irregularity of the delivery of the eluent solution by the delivery pumps. In gradient elution

at such a low flow-rate (50 μl/min), one pump should deliver the solution at a flow-rate of a few microlitres per minute. At present, a pump suitable for such a flow-rate is not available. Therefore, in this experiment, a column of 2.1 mm I.D. and a rather high flow-rate of 0.15 ml/min were adopted.

The dead-volume after the microbore column should be small in order to suppress the peak broadening and to detect the light signal. Therefore, a mixing device with a capacity of 8.8 μl was used, a smaller volume than mentioned in our previous report[14].

As mentioned previously[12], baseline drift occurred owing to the fluorescent impurities present in the eluent solution, although its character has not been elucidated. It was reported that there was a weak background emission of non-fluorescent compounds in the TCPO–hydrogen peroxide system, the spectrum of which remained the same irrespective of the solvents used, *e.g.* acetone, acetonitrile and ethyl acetate[3]. The conclusion that there was no fluorescent impurity in the reagents was based on a search with a spectrofluorometer. However, since the chemiluminescence detection

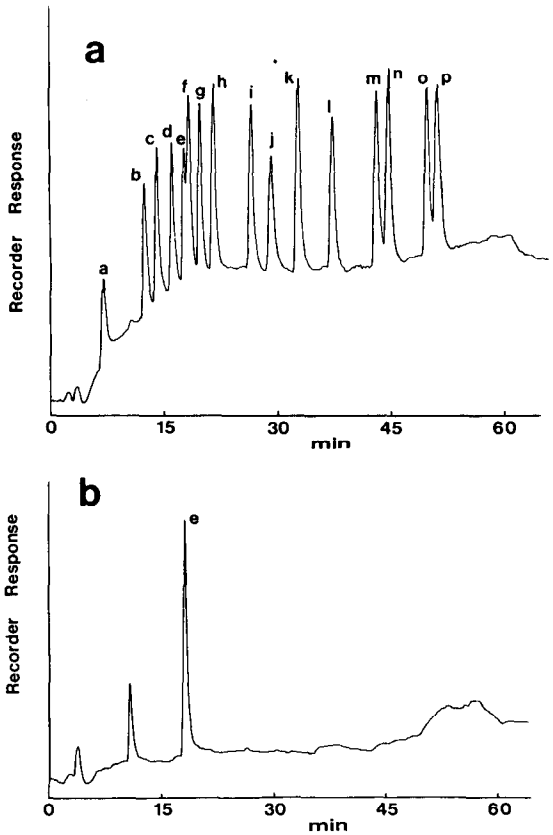


Fig. 3. Chromatograms of (a) Dns-amino acid standards (20 fmol of each amino acid) and (b) the hydrolysate of Dns-bradykinin. A linear gradient from 20% A (80% B) to 80% A (20% B) over 100 min: Peaks: a = Asp; b = Asn; c = Gln; d = Ser; e Arg; f = Thr; g = Gly; h = Ala; i = Pro; j = Lys; k = Val; l = Met; m = Ile; n = Leu; o = Trp; p = Phe.

system is more sensitive than its fluorescence counterpart, the chemiluminescence intensity could be influenced by a lower level of fluorescent impurities not detected with the spectrofluorometer. The impurity in eluent A, adsorbed at the head of the column, was gradually eluted as the concentration of the organic solvent (acetonitrile) in eluent B increased. In order to prevent baseline drift, trapping columns (TC; ODS 50–105 $\mu$m) were inserted between the pump for the eluent solution and a dynamic mixer, (Fig. 2). When these trapping columns were used, the baseline drift became a quarter of the original.

With the improved chemiluminescence detection system, 20 fmol of each of the Dns-amino acids were separated and detected (Fig. 3a). The detection limits were from 0.8 fmol (Val) to 1.7 fmol (Asp).

Dansylated bradykinin (230 pmol) was isolated and hydrolysed in this experiment, and gave the chromatogram shown in Fig. 3b. After a solvent front peak and a Dns-OH peak, the free Dns-Arg appeared. It was confirmed by comparison of its retention time (18 min) with that of standard Dns-Arg. Calculation of the amount of Dns-Arg liberated gave a recovery of Dns-Arg of *ca.* 10% of the starting amount of bradykinin. This low recovery was due mainly to two reasons. First, the bradykinin dansylated with Dns-Cl should have been separated from Dns-OH, which is present in large enough amounts to interfere with the final detection. The isolation of the peak fraction was not complete. Secondly, hydrolysis at 110°C for 20 h may have led to partial degradation of the Dns moiety[15].

Based on a recovery of 10%, 40–80 fmol of the starting materials would be sufficient for the analysis of N-terminal amino acids. However, at present, monitoring of the Dns-peptide peaks by fluorescence enhances the detection limit to a level of a 1 pmol, which would allow us to use the starting materials of *ca.* 1 pmole.

The TCPO–hydrogen peroxide reagent leads to degradation of the reagent (10% loss within 4 h)[16]. A stabilization catalyst should thus be sought, although the peak intensity is compensated for with the internal standard for the quantitative determination.

The chemistry of this chemiluminescence reaction is complex and strongly influenced by the presence of a base catalyst (*e.g.* imidazole) and the reagent degradation product, 2,4,6-trichlorophenol[17]. Further investigation of the reaction mechanism is needed for the sensitive detection of fluorophores[18,19].

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REFERENCES

1 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
2 S. Kobayashi, J. Sekino, K. Honda and K. Imai, *Anal. Biochem.*, 112 (1981) 99.
3 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
4 G. Mellbin, *J. Liq. Chromatogr.*, 6 (1983) 1603.

5 G. Mellbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203.
6 G. J. de Jong, N. Lammers, F. J. Spruit, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 18 (1984) 129.
7 T. Koziol, M. L. Grayeski and R. Weinberger, *J. Chromatogr.*, 317 (1984) 355.
8 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (1984) 1096.
9 M. L. Grayeski and A. J. Weber, *Anal. Lett.*, 17 (1984) 1539.
10 K. W. Sigvardson and J. W. Birks, *J. Chromatogr.*, 316 (1984) 507.
11 R. Weinberger, C. A. Mannan, M. Cerchio and M. L. Grayeski, *J. Chromatogr.*, 288 (1984) 445.
12 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 303 (1984) 173.
13 S. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 1548.
14 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 316 (1984) 501.
15 C. Gros and B. Labouesse, *Eur. J. Biochem.*, 7 (1969) 463.
16 K. Imai, K. Miyaguchi and K. Honda, in K. van Dijke (Editor), *Bioluminescence and Chemiluminescence: Instruments and Applications* Vol. II, CRC Press, FL, U.S.A., 1985, p. 65.
17 K. Honda, J. Sekino and K. Imai, *Anal. Chem.*, 53 (1983) 940.
18 C. L. R. Catherall, T. F. Palmer and R. B. Cundall, *J. Chem. Soc. Faraday Trans.*, 80 (1984) 823.
19 C. L. R. Catherall, T. F. Palmer and R. B. Cundall, *J. Chem. Soc. Faraday Trans.*, 80 (1984) 837.