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Short communication

Indirect time-resolved fluorescence detection of both non-fluorescent and fluorescent compounds separated by high-performance liquid chromatography

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Abstract

Indirect time-resolved fluorescence (ITRF) detection of non-fluorescent phenylthiohydantoin (PTH)-Ala and fluorescent dansyl (DNS)-Ala separated by high-performance liquid chromatography (HPLC) was carried out. Addition of europium (Eu^{3+}) chelates to the eluent makes it possible to detect both non-fluorescent and fluorescent compounds simultaneously. This is because the fluorescence lifetime of Eu^{3+} chelates is extremely long, whereas that of normal fluorescent compounds is short. The HPLC conditions with the Eu^{3+} chelate system were studied. © 1997 Elsevier Science B.V.

Keywords: Indirect time-resolved fluorescence detection; Detection, LC; Derivatization, LC; Phenylthiohydantoin-alanine; Dansyl-alanine; Europium chelates

1. Introduction

Europium (Eu^{3+}) chelates have unique fluorescence properties, such as long Stokes shift, narrow band width of emission, high quantum yields, and long fluorescence lifetimes ranging from tens of microseconds to submilliseconds [1–7]. Using the fluorescence of Eu^{3+} chelates, a flow-injection spectrofluorimetric system has developed [3,6]. In liquid chromatography, a postcolumn reaction system has been developed for the measurement of amino and thio compounds [1] and tetracyclines [3]. Fluorescence enhancement of the Eu^{3+}–tetracycline complex by DNA has been also studied [4]. Dynamic quenching of Eu^{3+} and Tb^{3+} fluorescence by inorganic anions with a detection method such as ion chromatography has also been investigated [2]. On the other hand, the enhancement of the sensitivity of fluorescence detection in liquid chromatography has been reported by applying time-resolved detection with a pulsed xenon lamp and a gated photomultiplier tube by making use of labels with a long fluorescence decay time [7]. Most recently, we constructed a simple, low-cost, time-resolved fluorescence detector (TRFD) for analysis of amino compounds with high-performance liquid chromatography (HPLC) using Eu^{3+} chelates, and have reported the relative performance in comparison to the conventional fluorescence detector (FD) [8]. Under high background conditions, TRFD has been proved to be highly selective and sensitive because fluorescence lifetimes of many co-existing materials are

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short ($<10^{-7}$ s). Because of this, it has been recognized that most fluorescent compounds can be indirectly detected by TRFD in HPLC by the use of an eluent that contains Eu^{3+} chelates. Unlike the conventional indirect method [9–11], both non-fluorescent and fluorescent compounds can be detected at a single run. Signals of both compounds can be detected as a negative peak from a constant, high background level introduced by the eluent containing Eu^{3+} chelates. This indirect detection technique is based on the measurement of the signal intensity while decreasing the enhancing ability of the Eu^{3+} chelate system in the presence of the analytes from HPLC. This kind of indirect time-resolved fluorescence detection (ITRF) detection method or a detector, to our knowledge, has not been proposed in the field of HPLC.

In this paper, a preliminary study is presented for optimizing HPLC conditions. Results of ITRF detection for both non-fluorescent phenylthiohydantoin (PTH)-Ala and fluorescent dansyl (DNS)-Ala are shown.

2. Experimental

2.1. Chemicals

Triton X-100 and europium(III) acetate tetrahydrate ($Eu(CH_3COO)_3 \cdot 4H_2O$, 99.9%) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and pivaloyltrifluoroacetone (PTA) and tri-*n*-octylphosphine oxide (TOPO) from Dojin (Kumamoto, Japan). Dansyl-alanine (DNS-Ala) and phenylthiohydantoin-alanine (PTH-Ala) were manually derivatized from free alanine in our laboratory [12,13]. All other regents were of analytical grade.

2.2. Apparatus

A TRFD system, which consisted of a d.c.-operated xenon lamp, two synchronized mechanical choppers, and spectroscopic filters for excitation and emission sides, was constructed. Details of the system were described in a previous report [8].

Reversed-phase HPLC was performed on a Jasco 900 series HPLC system (Tokyo, Japan) with a Jasco CrestPak C18T-5 column (50 mm×1.5 mm I.D.; particle size, 5 μm). The fluorescence signal of the eluent was monitored by TRFD and a commercial FD (ex 290 nm, em 615 nm, 820-FP, Jasco Co.) Samples were separated using an isocratic acetonitrile–sodium acetate buffer containing Triton X-100, TOPO, PTA and europium acetate at a flow-rate of 0.1 ml/min.

2.3. Procedures

Standard eluent for ITRF detection was prepared by dissolving 500 mg of Triton X-100, 5 ml of 5 m*M* TOPO (dissolved by acetonitrile), 16 ml of 0.25 m*M* PTA (dissolved by acetonitrile) and 50 ml of 200 m*M* sodium acetate buffer in water, diluting the solution with water to 500 ml. The final concentrations were 0.1%, 50 μ*M*, 8 μ*M* and 20 m*M* for Triton X-100, TOPO, PTA and sodium acetate, respectively. Europium acetate were dissolved in the above buffer. Buffers were filtered through 0.22-μm membrane filters and degassed by sonication prior to use. The sample was dissolved in buffer–acetonitrile (1:1, v/v) at concentrations of 0.6 and 0.1 mg/ml for DNS-Ala and PTH-Ala, respectively so that adequate signals could be obtained. Injection volume of the sample was 10 μl.

The column was rinsed with 1 m*M* EDTA and 50% acetonitrile for 30 min each in order to remove metal ions, and then equilibrated with the eluent for 60 min, at a flow-rate of 0.2 ml/min.

3. Results and discussion

In order to enhance the fluorescent intensity, the Eu^{3+} chelate system requires the presence of substances, such as Triton X-100, TOPO, acetate buffer and PTA as β-diketone [1,8]. The eluent containing those components introduces a high, constant background emission. However, other substances for separating analytes in the eluent affect the fluorescence intensity of Eu^{3+} chelates. Therefore, the fundamental conditions for HPLC were studied. The reversed-phase mode was used because it is popular and powerful.

3.1. Effect of pH

In this study, the time-resolved fluorescence (TRF) intensity of the eluent containing 10 n*M* europium acetate was first measured in a stopped-flow manner by using 0.1% Triton X-100, 50 μ*M* TOPO, 8 μ*M* PTA and 20 m*M* sodium acetate. Fig. 1 shows the relationship between the pH of the eluent and the TRF intensity. High intensities were obtained over pH 5.0. As the highest intensity was at pH 6.5, subsequent experiments were performed under pH 6.5. The eluent was stable for 2 weeks at room temperature in the dark.

3.2. Effect of concentration of acetonitrile

The effect of acetonitrile on the TRF intensity of the Eu^{3+} chelates was examined. Acetonitrile is usually used as the eluent in reversed-phase HPLC; which contains 0.1% Triton X-100, 50 μ*M* TOPO, 8 μ*M* PTA, 100 n*M* europium acetate and 20 m*M* sodium acetate (pH 6.5). As shown in Fig. 2, the intensity decreased sharply when the concentration of acetonitrile exceeded 20%. However, the upper limit of the concentration of acetonitrile was increased from 20 to 30% when each concentration of the component in the eluent was increased by 10 times, that is, 1% Triton X-100, 500 μ*M* TOPO, 80 μ*M* PTA, 100 n*M* europium acetate and 200 m*M* sodium acetate (pH 6.5). All the following experiments were carried out with concentration of acetonitrile below 20% in HPLC.

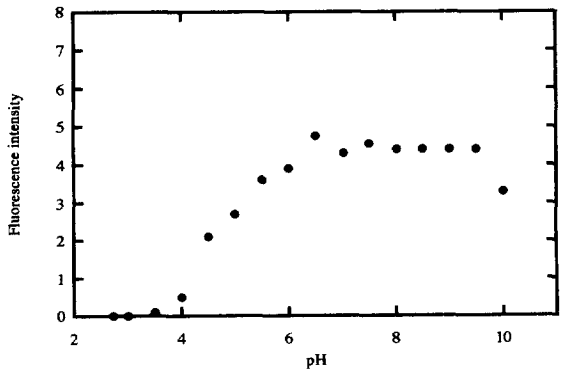

Fig. 1. Effect of eluent pH on time-resolved fluorescence intensity. The fluorescence intensity of the eluent was measured by using 0.1% Triton X-100, 50 μ*M* TOPO, 8 μ*M* PTA, 10 n*M* europium acetate and 20 m*M* sodium acetate (pH 2.7–10.0) in a stopped-flow manner.

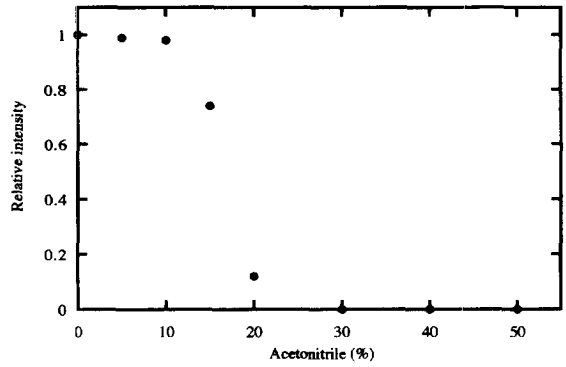

Fig. 2. Effect of concentration of acetonitrile on time-resolved fluorescence intensity. The fluorescence intensity of the eluent was measured by using 0.1% Triton X-100, 50 μ*M* TOPO, 8 μ*M* PTA, 100 n*M* europium acetate, 20 m*M* sodium acetate (pH 6.5) and 0–50% acetonitrile in a stopped-flow manner.

3.3. Optimization of other experimental conditions

We have determined other optimal conditions for HPLC experiments. The parameters to be determined are the individual concentrations of the following; PTA, TOPO, Triton X-100, europium acetate, sodium acetate buffer, etc. The conditions experimentally obtained were as follows: eluent (2.4–240 μ*M* PTA, 15–150 m*M* TOPO, 0.1–0.3% Triton X-100, 10–100 n*M* europium acetate, 60–200 m*M* sodium acetate buffer, pH 6.0–7.0); and flow-rate (0.1–0.2 ml/mim).

When phosphate buffer was used instead of acetate buffer, the intensity was decreased markedly. Further, we found that the enhancing ability of thenoyltrifluoroacetone (TTA), which is a β-diketones, was about 7 times superior to that of PTA, although the ability was lowered to about one tenth within several days.

3.4. Indirect time-resolved fluorescence detection of DNS-Ala and PTH-Ala

In order to demonstrate ITRF detection, we have analyzed a mixture of PTH-Ala (0.1 mg/ml) and DNS-Ala (0.6 mg/ml). The fluorescence lifetimes of DNS-Ala lie in the nanosecond region. On the other

hand, PTH-Ala emits no fluorescence. A chromatogram obtained from FD is shown in Fig. 3a and one from TRFD in Fig. 3b. The eluent was 24 μ*M* PTA, 150 m*M* TOPO, 0.3% Triton X-100, 100 n*M* europium acetate, 60 m*M* sodium acetate (pH 6.5) containing 20% acetonitrile at a flow-rate of 0.1 ml/min. Chromatographic peaks marked 1 and 2 are due to DNS-Ala and PTH-Ala, respectively, and the asterisk (*) represents a solvent peak. FD gave a positive peak for DNS-Ala and a negative one for PTH-Ala. The positive peak means that the fluorescence intensity of DNS-Ala at this concentration was very large in comparison with that of the eluent. The negative peak indicates detection with conventional indirect fluorescence. On the other hand, TRFD gave two negative peaks, which means that ITRF detection was successfully carried out. The detection limit in TRFD was estimated to be 4.8×10^{-9} mol for PTH-Ala ($S/N=3$).

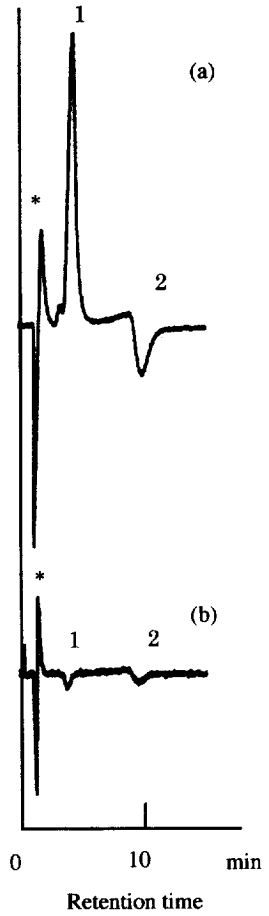

Fig. 3. Chromatograms of DNS-Ala and PTH-Ala obtained by FD (a) and by TRFD (b) using indirect fluorescence detection. Chromatographic conditions: eluent, 24 μ*M* PTA, 150 m*M* TOPO, 0.3% Triton X-100, 100 n*M* europium acetate, 60 m*M* sodium acetate (pH 6.5) containing 20% acetonitrile; flow-rate, 0.1 ml/min; column, Crest C18T-5 (50 mm long×1.5 mm I.D.); column temperature, room temperature. Samples: 1, DNS-Ala (0.6 mg/ml); 2, PTH-Ala (0.1 mg/ml); *, a solvent peak. Injection volume, 10 μl. TRFD gave two negative peaks for both compounds, while FD only gave one for PTH-Ala.

4. Conclusion

ITRF detection of both non-fluorescent and fluorescent compounds separated by HPLC has been successfully carried out using Eu^{3+} chelates. The experiment shows that the pH of an eluent, the concentration of acetonitrile, and other parameters influence the signal intensity. This concept for this separation and detection technique is novel. For practical applications, however, many problems must be solved:

1. in order to enhance both separation efficiency and detection sensitivity at the same time, a simple eluent without an enhancer solution should be developed;
2. the sensitivity of detection might be improved by using another kind of eluent system or reagents such as TTA or other β-diketones;
3. a separation mode without using an organic solvent must be investigated to obtain better separation and sensitivity;
4. the comparison of the competitive binding, and the binding ability of analyte and ligands to Eu^{3+} in this system should be investigated, because the results may give the potential for qualitative and quantitative analysis.

At present, we are attempting to solve these problems.

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References

[1] Y. Okabayashi, T. Kitagawa, Anal. Chem. 66 (1994) 1448–1453.
[2] M. Schreurs, G.W. Somsen, C. Gooijer, R.W. Velthorst, R.W. Frei, J. Chromatogr. 482 (1989) 351–359.
[3] T.J. Wenzel, L.M. Collette, D.T. Dahlen, S.M. Hendrickson, L.W. Yarmaloff, J. Chromatogr. 433 (1988) 149–158.
[4] Y-X. Ci, Y-Z. Li, X-J. Liu, Anal. Chem. 67 (1995) 1785–1788.
[5] T. Taketatsu, Talanta 29 (1982) 397–400.
[6] M. Aihara, M. Arai, T. Taketatsu, Analyst 111 (1986) 641–643.
[7] M. Schreurs, L. Hellendoorn, C. Gooijer, N.H. Velthorst, J. Chromatogr. 552 (1991) 625–634.
[8] T. Iwata, M. Senda, Y. Kurosu, A. Tsuji, M. Maeda, Anal. Chem. 69 (1997) 1861–1865.
[9] S. Mho, E.S. Yeung, Anal. Chem. 57 (1985) 2253–2256.
[10] S.Y. Su, A. Jurgensen, D. Bobbitt, J.D. Winefordner, Anal. Lett. 14(A1) (1981) 1–6.
[11] W.G. Kuhr, E.S. Yeung, Anal. Chem. 60 (1988) 2642–2646.
[12] W.R. Gray, 1972. in: C.H.W. Hirs, S.N. Timasheff (Eds.), Methods in Enzymology, vol. 25, Academic Press, New York, p. 121.
[13] Y. Kurosu, K. Murayama, N. Shindo, Y. Shisa, N. Ishioka, J. Chromatogr. A. 752 (1996) 279–286.