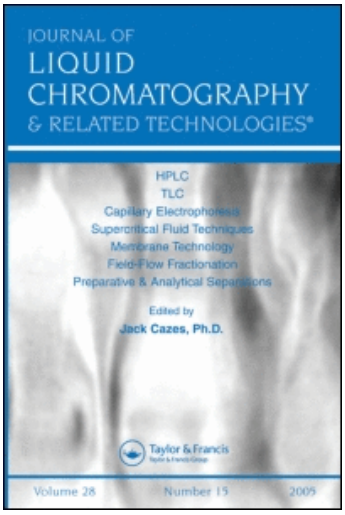

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SYNTHESIS, PURIFICATION, AND STABILITY OF β-DIKETONATE EUROPIUM CHELATE REAGENT, DETERMINED BY RP-HPLC

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ABSTRACT

Various estimation conditions concerned with a europium chelate labeling agent, 4-(1,1,1,2,2-pentafluoro-3,5-dioxo-pentyl)-[1,11′-biphenyl]-sulfonyl chloride (PDB-SCl), was studied, by using reversed-phase HPLC-time-resolved fluorescence detector system. Samples dissolved in acetonitrile were introduced to a TMS column; the eluent was monitored with absorbance at 280 nm, and then collected. To the fractions was added europium (Eu) solution containing tri-*n*-octylphosphine oxide (TOPO) and Triton X-100, and the time-resolved fluorescence intensity of the PDB-Eu chelate was measured with an off-line time-resolved fluorometer. In time-resolved fluorescence measurement, one major peak of PDB-SCl was observed, but in absorbance measurement, many peaks were observed. The fractions having time-resolved fluorescence were hydrolyzed, and the reaction solutions were analyzed by an HPLC system connected to an on-line time-

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resolved fluorescence detector (TRFD). There were little byproduct peaks, which were produced by reacting PDB-SCl with contaminants in the synthesized PDB-SCl powder. The stability of chlorosulfonyl group of PDB-SCl in aqueous acetonitrile solutions was also studied, and 80% of chlorosulfonyl group of PDB-SCl dissolved in 90% aqueous acetonitrile solution was hydrolyzed to sulfonic acid. However, PDB-SCl dissolved in 100% acetonitrile was stable at ambient temperature for at least 96 hours in the dark.

Key Words: Eu chelate; Time-resolved fluorescence; RP-HPLC; Purification

INTRODUCTION

Fluorescent lanthanide chelates have been used as a powerful labeling reagent for fluoroimmunoassay (FIA) or DNA hybridization assay,[1–8] since the sensitivity is much higher, and has longer fluorescent lifetime than traditional fluorescent reagents, and can be discriminated from other naturally existing fluorescent contaminants.

This kind of reagent was applied as a pre-labeling reagent for highly sensitive detection, and for this purpose it should have the higher purity, since even a minute amount of impurities or byproducts may interfere with the sample peaks. The synthesis of a new europium (Eu) fluorescent label 4-(1,1,1,2,2-pentafluoro-3,5-dioxo-pentyl)-[1,11′-biphenyl]-sulfonyl chloride (PDB-SCl*, Compound II in Fig. 1) and its reaction with analyte were indicated in Fig. 1. Note: PDB-SCl (CDPP): the same compound as used previously,[9] but the nomenclature is changed to indicate the reactive group, sulfonyl chloride group.

An optimization of the synthesis, the purity control, and the stability of the PDB-SCl, using a reversed-phase HPLC-time-resolved fluorescence detector system, is described in this paper.

EXPERIMENTAL

Instruments

The main part of the chromatograph was a Shimadzu LC-10AT (Kyoto, Japan), which consisted of an LC-10AT$_{VP}$ HPLC pump (Simadzu), a Rheodyne (Cotati, CA, USA) Model 7725I syringe-loading sample injector valve (10-μL loop), a time-resolved fluorescence detector (TRFD) equipped with a 16 μL flow cell (Tosoh, Tokyo, Japan). The operation and the data recording were performed

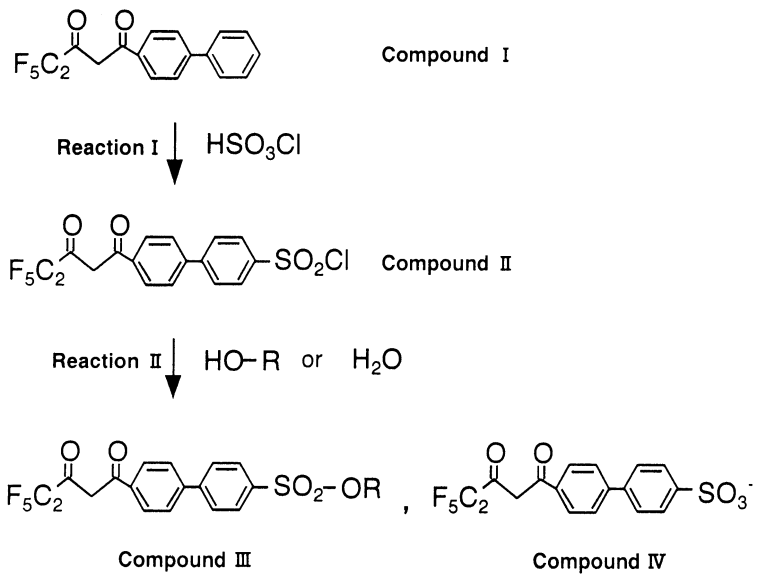

Figure 1. Reagent synthesis and hydrolysis.

by PC computer, which was connected to TRFD via RS-232C cable line. Time-resolved fluorescence detector and computer software for time-resolved fluorescence measurement, were Hamamatsu Photonics K.K. (Hamamatsu, Japan) made and designed only for analysis. Teflon tubing of 0.25 mm I.D. was generally used for all flow circuit connection. The measurement conditions were as follows: excitation at 340 nm, emission at 615 nm, excitation gate time of 0.01 ms, delay time of 0.1 ms, and signal gate time of 1.8 ms, and excitation interval of 2 ms. Absorbance spectrum was measured with a 112 UV/VIS detector (Gilson, WI, USA), and data were recorded with a chart recorder (M&S Instruments Inc., Osaka, Japan).

For off-line analysis, a Arcus 1234 time-resolved fluorometer (Wallac Oy., Turku, Finland) was used for measuring the fluorescence of Eu chelates in 96-well microtitration wells. The measurement conditions were delay time 0.20 ms, window time 0.40 ms, and flash rate 1.00 ms.

Reagents and Chemicals

A 96-well microtitration plate was purchased from Nalge Nunc International (Rochester, NY, USA). Organic solvents and distilled water of HPLC

grade (Kanto Chemicals Co., Tokyo, Japan) were used without further purification. Dry diethyether, sodium methoxide, and ethyl pentafluoropropionate were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Reagent 4′-phenylacetophenone was purchased from Merk (Darmstadt, Germany). Chlorosulfonic acid was from Wako Pure Chemicals Industries (Osaka, Japan). All other chemicals were of analytical reagent grade. PDB-SCl was synthesized as described previously.[9]

Reversed-Phase Chromatography of β-Diketone Compounds

β-Diketone structure was estimated by Eu chelation and time-resolved fluorescence measurement. Phenyl structure was estimated by UV analysis, and sulfonyl chloride structure was analyzed through the reaction with phenol compound and further HPLC.

Sample solution (10 μL) was applied on a reversed-phase column, 4.6 mm I.D. × 150 mm YMC-Pak TMS (YMC, Kyoto, Japan) by isocratic elution with acetonitrile–water (65 : 35, v/v) containing 0.05% TFA as the mobile phase. The flow rate of mobile phase was set at 0.6 mL/min and the column temperature was ambient (24 ± 3°).

Eluent from the LC column was monitored by absorbance at 280 nm for analysis of phenyl structure (Compound I, II, III, and IV), and then for analysis of the *β*-diketone structure (Compound I, II, III, and IV), the eluent was collected. The monitoring of time-resolved fluorescence intensity in the purification of PDB-SCl was performed by an Arcus 1234 time-resolved fluorometer, since the fractions were further analyzed for the purity of PDB-SCl. A Gilson Model 201 fraction collector was used for collection of the eluted solution. Fractions of 0.15 mL were collected, and were diluted 20,000-fold with distilled water containing 1% Triton X-100, 0.2 mM $EuCl_3$, and 0.2 mM tri-*n*-octylphosphine oxide (TOPO). A hundred microliter of the solution was placed into the 96-wells microtiter plate well, and the time-resolved fluorescent intensity of PDB-SCl-Eu chelate was measured with the Arcus 1234 fluorometer.

The purity of the PDB-SCl was studied by using a UV detector for contaminating aromatic compounds and an off-line TRFD for *β*-diketone compounds. The sample solution containing 50 μg of PDB-SCl (5 μL) was applied on reversed-phase columns, 4.6 mm I.D. × 250 mm TSK-gel ODS 120-A (Tosoh, Tokyo, Japan) and 4.6 mm I.D. × 150 mm TSK-gel ODS-80Ts (Tosoh), and was eluted isocratically with 100% acetonitrile containing 0.05% TFA as a mobile phase. The flow rate of mobile phase was set at 0.6 mL/min and the column temperature was ambient (24 ± 3°).

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On-Line Analysis of Time-Resolved Fluorescence

Eluent from the LC column was mixed with a solution containing 0.2 mM $EuCl_3$, 0.2 mM TOPO, and Triton X-100 in distilled water by a T-type mixing device, delivered by a Shimadzu LC-10AT$_{VP}$ HPLC pump at a flow rate of 0.8 mL/min. The generated fluorescence was measured with TRFD. Teflon tubing coil (0.25 mm I.D. × 5 m) was used between the pump for the Eu reagent and TRFD. The sulfonylchloride structure (Compound II) was analyzed throughout the reaction with phenol compound (Compound III) and further HPLC. The HPLC system is illustrated in Fig. 2.

RESULTS AND DISCUSSION

Minimum Reaction Time of HSO_3Cl. Time Course Analysis of Chlorosulfonation of PDB

4-(1,1,1,2,2,-pentafluoro-3,5-dioxo-pentyl)-[1,11′-biphenyl]-sulfonyl chloride and PDB (PDB, Compound I in Fig. 1) were synthesized as described

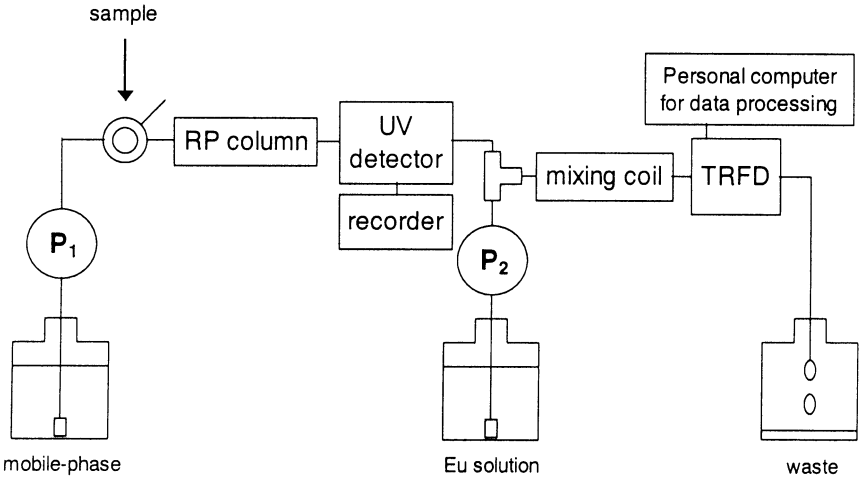

Figure 2. Post-column Eu derivatization HPLC system. P1, Shimadzu HPLC Pump (LC-10ATvp); P2, Shimadzu HPLC Pump (LC-10ATvp); RP column, YMC TMS (4.6 mm I.D. × 150 mm); mixing coil, teflon tubing coil (0.25 mm I.D. × 5 m); TRFD, time-resolved fluorescence detector.

previously.[9] To 10 mL of HSO_3Cl was gradually added 2.50 g (7.3 mmol) of PDB, with stirring. At the various intervals of reaction at ambient temperature, each solution was added, dropwise with stirring, to 200 mL of iced-water. The residue was collected by centrifugation, rinsed with cold water, and twice centrifuged. The precipitate was dissolved in acetonitrile, and the solutions were analyzed by the on-line time-resolved fluorometric HPLC method.

As shown in Fig. 3(A), a main PDB-SCl peak (Compound II in Fig. 1) was eluted at 9 min from a reversed-phase HPLC column. The relationship between the reaction time of HSO_3Cl to PDB and the amounts of PDB-SCl was examined. The PDB was added to HSO_3Cl solution with stirring, and the reaction solutions were added to iced-water at the different reaction periods. Thereby, the chlorosulfonation of PDB was stopped. An orange product of PDB-SCl was dissolved in acetonitrile. Then, the resulting PDB-SCl was hydrolyzed to PDB-SO_3^- (Compound IV in Fig. 1) in an aqueous acetonitrile solution, containing 23% of 20 mM carbonate buffer of pH 10.0, at 70°C for 30 min. After that, the solutions (1.6 μg/mL) of PDB-SCl and PDB-SO_3^- were introduced into the reversed-phase HPLC system as described in Experimental. Two hours of reaction were enough for the chlorosulfonation of PDB (Compound I in Fig. 1).

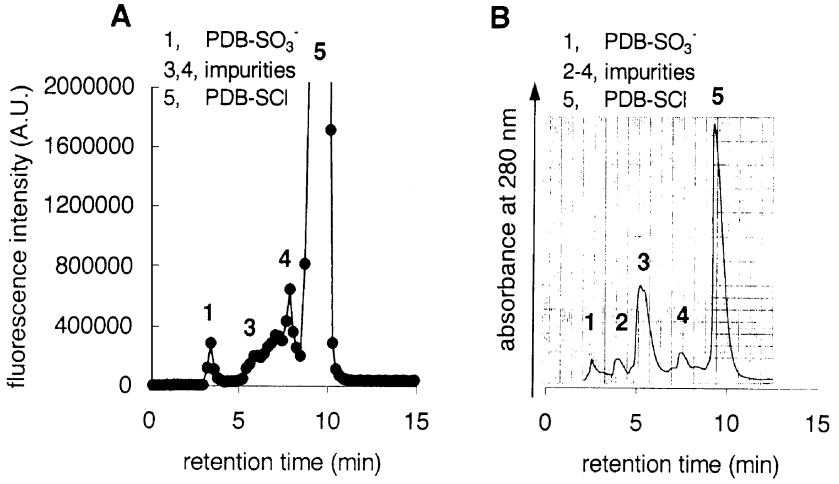

Figure 3. Chromatograms of synthesized PDB-SCl. Column, YMC TMS (4.6 mm I.D. × 150 mm); mobile-phase, 65(v/v)% acetonitrile containing 0.05% TFA; flow rate, 0.6 mL/min; (A) off-line time-resolved fluorescence analysis; (B) UV analysis.

Purity of the Synthesized PDB-SCl

The purity of the PDB-SCl was analyzed by a reversed-phase HPLC using a TMS column as described in Experimental. Figure 3 shows the HPLC chromatograms of PDB-SCl dissolved in acetonitrile. The eluent was monitored at absorbance at 280 nm [Fig. 3(B)], since the PDB compounds (Compound I, II and III in Fig. 1) have absorption around the UV region (260 nm–400 nm), and five peaks were observed. Then, the eluent was collected every 15 sec, and the time-resolved fluorescence intensity of the fractions was measured with Arcus 1234 fluorometer after addition of Eu solution containing TOPO and Triton X-100 [Fig. 3(A)]. In time-resolved fluorescence measurement, a main peak (PDB-SCl) was eluted at 9.2 min and minor peaks at 3 to 9 min were observed as shown in [Fig. 3(A)]. On the other hand, a number of peaks were observed in the absorbance measurement [Fig. 3(B)]. More than 90% of fluorescence intensity in the time-resolved fluorescence measurement was from the peak of PDB-SCl, but the signal of PDB-SCl was approximately 70% in the absorbance measurement. The time-resolved fluorescence intensity to the absorbance signal ratio in peak 2 to 4, was remarkably low compared to that in the PDB-SCl peak. It was suggested that at least three contaminants (peak 2 to 4) were contained in the synthesized PDB-SCl powder. And these contaminants could not be removed by recrystallization.

The synthesized PDB-SCl was hydrolyzed, and the purity of PDB-SCl was studied by a reversed-phase HPLC system connected to TRFD (Fig. 2). The hydrolysis of PDB-SCl was performed as described previously.[9,10] The PDB-SCl was hydrolyzed in diethylamine/triethylamine solution, aqueous acetone solution, aqueous acetonitrile solution, and aqueous tetrahydrofurane (THF) solution, and the reaction mixtures were analyzed by the HPLC system. The eluent was reacted with Eu by introducing the postcolumn reagent containing Eu, TOPO, and Triton X-100. The results were shown in Fig. 4. The hydrolyzed form of PDB-SCl, PDB-SO_3^- (Compound IV in Fig. 1), was eluted at 3.5 min. When PDB-SCl was hydrolyzed to PDB-SO_3^- in the above four solutions, many byproduct peaks were observed in the HPLC profiles. The many byproduct peaks were observed when the reagent was reacted with diethylacetoamine/triethylamine solution compared with aqueous acetone, aqueous acetonitrile, and aqueous THF solution. It was suggested, that the peaks eluted at 5 min to 9 min in Fig. 4(A), were produced by reacting PDB-S with the contaminants in diethylacetamide and/or triethylamine, and that the peaks eluted at 5 min to 8 min, and at 13 min, were produced by reacting PDB-SCl with the contaminants in an orange product of PDB-SCl [Fig. 4(B), (C), and (D)], since the peaks having little time-resolved fluorescence intensity were observed in absorbance measurement as shown in Fig. 3. It was suggested,

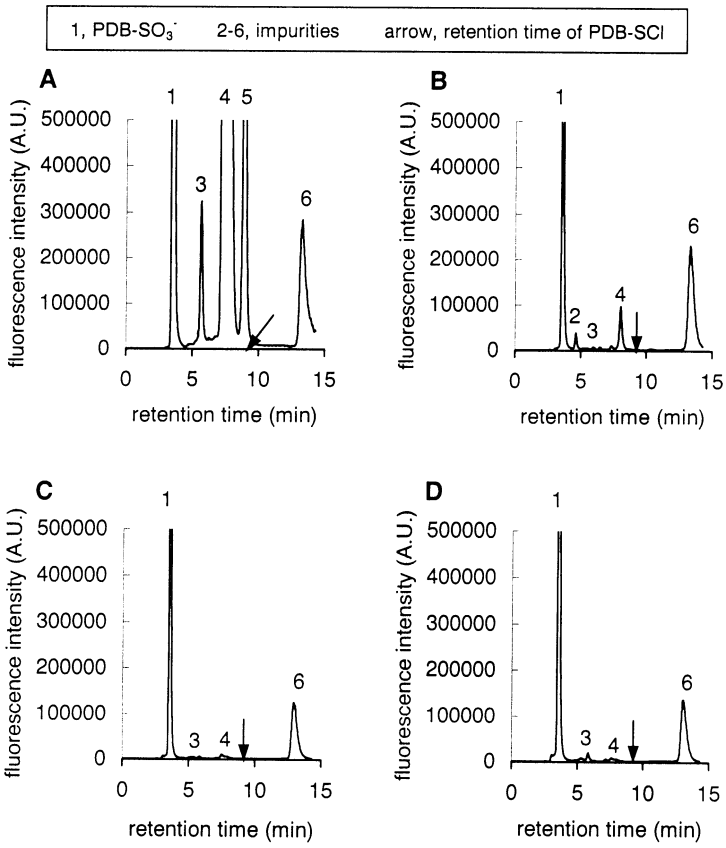

Figure 4. HPLC chromatograms of the hydrolyzed PDB-SO_3^-. Column, YMC TMS (4.6 mm I.D. × 150 mm); mobile-phase, 65(v/v)% acetonitrile containing 0.05% TFA; flow rate, 0.6 mL/min; (A) DMA/TEA; (B) acetone; (C) acetonitrile; (D) THF. The arrows indicate the retention time for PDB-SCl.

that the minor peaks observed in [Fig. 4(C) and (D)] (peak 3, 4) may come from the impurities in Fig. 3 (peak 3, 4).

Purification of PDB-SCl

After recrystallization, PDB-SCl was further purified using a reversed-phase column. The acetonitrile solution containing PDB-SCl was loaded on

TSK-gel ODS 120-A column, and eluted isocratically with 100% acetonitrile containing 0.05% TFA. In this case, it was expected that addition of water to the eluent induced the hydrolysis of sulfonyl chloride group of PDB-SCl. The eluted solution was collected, and the time-resolved fluorescence intensity was measured with Arcus 1234 fluorometer after addition of europium chloride solution containing TOPO and Triton X-100. The chromatograms were shown in Fig. 5, and the recovery of PDB-SCl was listed in Table 1. The recovered PDB-SCl amounts were calculated as follows; (time-resolved fluorescence intensity of recovered PDB-SCl/time-resolved fluorescence intensity of applied PDB-SCl) × applied PDB-SCl amounts (50 μg).

In order to examine the purity of PDB-SCl in every fraction, fractions No. 27 to 30 obtained from ODS 120-A column (4.6 mm I.D. × 250 mm), were hydrolyzed in aqueous acetonitrile solution, and the reacted solutions were introduced to the reversed-phase HPLC system connected TRFD (on-lined time-resolved fluorescence measurement). The results were shown in Fig. 6. As shown in [Fig. 6(B)], most byproduct peaks in fraction No. 28 were removed, and it was expected that the minor peaks at 5.5 to 7 min could be completely removed by using a larger theoretical plate column. On the other hand, it could not remove the byproduct peaks in any fractions by using a 4.6 mm I.D. × 150 mm ODS 80Ts column (data not shown). The capacity ratio of the PDB-SCl peak in the two ODS columns was equal ($k' = 0.5$). Then, it was suggested that the byproduct peaks could be removed when a 250 mm length column of ODS-80Ts was used.

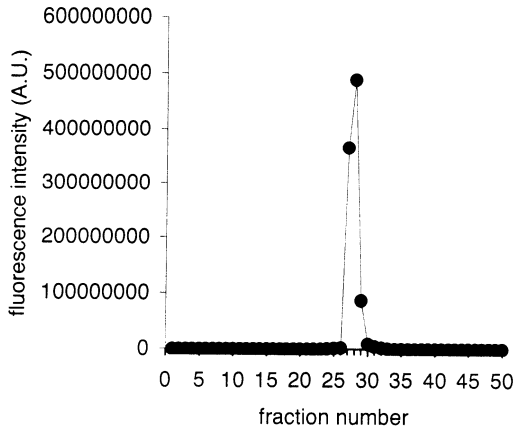

Figure 5. HPLC profile for the synthesized PDB-SCl by off-line time-resolved fluorescence analysis. Column, TSK-gel ODS 120-A (4.6 mm I.D. × 250 mm); mobile-phase, acetonitrile; flow rate, 0.6 mL/min.

Table 1. Recoveries of PDB-SCl from the Reversed-Phase Columns

Column	Fraction No.	Time (min)	PDB-SCl (mg)	Recovery (%)
ODS-120A	27	6.75	18.8	37.6
	28	7.00	25.2	50.4
	29	7.25	4.5	9.0
	30	7.50	0.4	0.01
ODS-80Ts	15	3.75	7.6	1.5
	16	4.00	21.3	42.6
	17	4.25	11.5	23.0
	18	4.50	4.3	8.6

The recovery of PDB-SCl and the recovered PDB-SCl amounts were calculated as follows; recovery (%) = [(time-resolved fluorescence intensity of every fraction) × fraction volume (mL)/(time-resolved fluorescence intensity of the applied PDB-SCl solution) × applied volume (mL)] × 100, recovered PDB-SCl amounts = [recovery (%)/100] × applied PDB-SCl amounts (50 μg). The recovery of PDB-SCl in fraction No.28 was 50.4% (Table 1).

Figure 7 shows the HPLC chromatograms of purified PDB-SCl, PDB-SO_3^-, and mixture of PDB-SCl and PDB-SO_3^-. The hydrolyzed form of PDB-SCl, PDB-SO_3^-, was prepared as follows; PDB-SCl was dissolved in 35 μL of acetonitrile solution containing 23% of 20 mM carbonate buffer of pH 10.0, and the PDB-SCl solution was incubated at 70°C for 1 hour in the dark. To the reaction mixture was added 5 μL of 10% HCl, prior to the HPLC analysis. The peaks of PDB-SCl and PDB-SO_3^- were eluted at 4 min and 9 min, respectively. The change of PDB-SCl to PDB-SO_3^- by the hydrolysis could be analyzed in the reversed-phase HPLC system connected TRFD. As shown in Fig. 7, no byproduct peaks in the purified reagent was observed.

Stability of SO_2Cl Group in Aqueous Acetonitrile Solution

If samples are reacted with PDB-SCl by on-line prederivatization method in the time-resolved fluorometric HPLC system, the PDB-SCl reagent has to be stable in solution-phase. In Ref. 11, the prederivatization of PDB-SCl to phenolic compounds was performed in 20% aqueous acetonitrile solution. Therefore, the stability of sulfonylchloride group of PDB in 100% acetonitrile and aqueous acetonitrile solutions was investigated.

Five milligrams of PDB-SCl was dissolved in 1 mL of acetonitrile. The PDB-SCl solution was diluted 250-fold by different concentrations (100 v/v%,

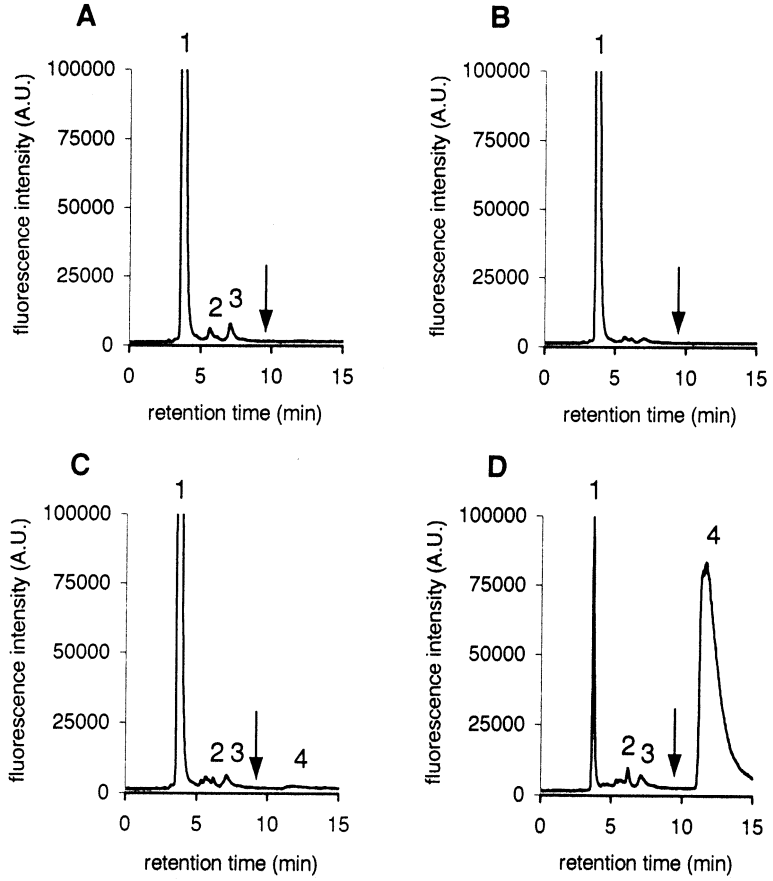

Figure 6. HPLC profiles of the PDB-SCl fractions chromatographed by ODS-120A column. Column, YMC TMS (4.6 mm I.D. × 150 mm); mobile-phase, 65(v/v)% acetonitrile containing 0.05% TFA; flow rate, 0.6 mL/min; (A) fraction 27; (B) fraction 28; (C) fraction 29; (D) fraction 30. The arrows indicate the retention time for PDB-SCl.

90 v/v%, 80 v/v%, and 70 v/v%) of acetonitrile solutions. The prepared PDB-SCl solutions stood at room temperature for 24 hours in the dark. Then, the solutions were diluted 3-fold by 100% acetonitrile, and the PDB-SCl solutions were applied on the reversed-phase TMS column, and the time-resolved fluorescence intensity of PDB-SCl and PDB-SO_3^- was measured by on-line analysis (Fig. 8). Approximately 80% of PDB-SCl dissolved in aqueous 90% acetonitrile solution was hydrolyzed to PDB-SO_3^- after incubation for 24 hours,

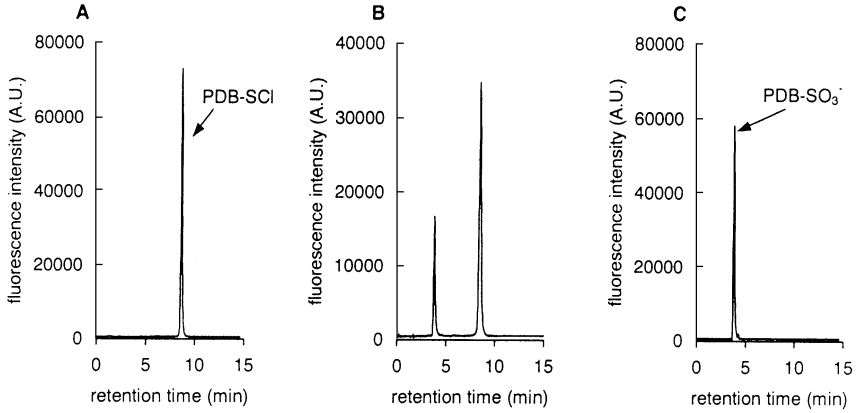

Figure 7. HPLC chromatograms of PDB-SCl and PDB-SO_3^-. Column, YMC TMS (4.6 mm I.D. × 150 mm); mobile-phase, 65(v/v)% acetonitrile containing 0.05% TFA; flow rate, 0.6 mL/min; (A) PDB-SCl; (B) PDB-SCl and PDB-SO_3^-; (C) PDB-SO_3^-.

at room temperature, in the dark. The hydrolysis of PDB-SCl to PDB-SO_3^- was accelerated with increasing the concentration of water in the aqueous acetonitrile solutions. The PDB-SCl solution, dissolved in 100% acetonitrile, was further incubated at room temperature for 72 hours in the dark. The peak height of PDB-SCl did not change at least for 96 hours at room temperature, in the dark. When PDB-SCl dissolved in 100% acetonitrile, was standing at room

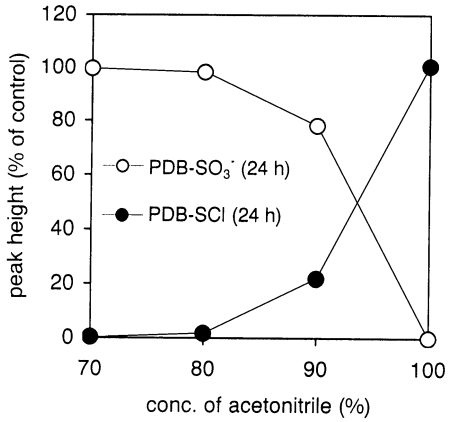

Figure 8. Time course analysis of chloro-sulfonation of PDB.

temperature for 24 hours in daylight, the time-resolved fluorescence intensity of PDB-Eu chelate was remarkably decreased (data not shown).

In order to study the activity of sulfonylchroride group, 17β-estradiol was labeled with the PDB-SCl solutions, and the fluorescence intensity of the PDB-S-17β-estradiol was measured with the time-resolved fluorometric HPLC system. The PDB-SCl solutions were prepared by being dissolved in different concentrations of aqueous acetonitrile solution, and were incubated for 24 hours at room temperature in the dark. The prederivatization of PDB-SCl to 17β-estradiol was performed as follows; to 22 μL of 17β-estradiol solution dissolved in acetonitrile was added 8 μL of 20 mM carbonate buffer of pH 10.0 and 5 μL of PDB-SCl solution (12.5 μg/mL), and the solution was incubated for 30 min at 70°C. To the solution, was added 5 μL of 10% HCl, and this was introduced into the TMS column. The LC separation and the detection conditions were described in Experimental. The peak height for the 17β-estradiol derivative was decreased in dependence on increasing the concentration of water in the aqueous acetonitrile solutions, and the 17β-estradiol derivative using PDB-SCl dissolved in 100% acetonitrile, was determined by the time-resolved fluorometric HPLC system, as well as the freshly prepared PDB-SCl solution (data not shown). The PDB-S derivatives of phenolic compounds in the final mixture were stable, and still gave constant time-resolved fluorescence intensities after standing for at least 24 hours in the dark at room temperature.

CONCLUSIONS

The optimal conditions for the chlorosulfonylation of the PDB-SCl were obtained and, also, the extreme purification was performed by reversed-phase HPLC. The purified PDB-SCl was pure enough for highly sensitive chromatographed analysis of estrogens.[11]

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