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Highly sensitive time-resolved fluorometric determination of estrogens by high-performance liquid chromatography using a β-diketonate europium chelate

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Abstract

A new fluorescent europium chelate labeling reagent, 5-(4″-chlorosulfo-1′,1″-diphenyl-4′-*yl*)-1,1,1,2,2-pentafluoro-3,5-pentanedione (CDPP), was synthesized for the time-resolved fluorometric detection of HPLC. The label can be directly bound to amino or phenolic hydroxyl groups of analytes with its chlorosulfonyl group, and the labeled analytes are separated on a HPLC column. After separation, $EuCl_3$, TOPO (tri-n-octylphosphine oxide), and Triton X-100 were added by post-column introduction to the eluent, and the fluorescence of the europium chelate was measured with the time-resolved fluorometric detector. Estrone (E1), 17β-estradiol (E2), ethynylestradiol (EE2) and estriol (E3) were measured with the detection limits of 0.65, 0.65, 0.65 and 0.60 ng/ml, respectively. The recovery for river water samples was in the range of 86.0–105.1% with the RSD of 1.9–5.8%. The method was applied to the analysis of a river water sample and estrone (E1) was determined to be 2.1 ng/l. The results and processing have been compared with those of a GC–MS method and a high degree of correlation ($r \geqq 0.98$) was observed.

Keywords: Derivatization, LC; Estrogens; β-Diketonate europium

1. Introduction

Lanthanide fluorescent chelates have been developed as fluorescence labels for time-resolved fluoroimmunoassay (TR-FIA) and DNA hybridization assay [1–8]. Owing to the long lifetime of the lanthanide fluorescent chelates (100 μs–2 ms), background fluorescence due to the cuvette wall or coexisting components in the sample solution can be effectively removed by employing time-resolved fluorometric detection. The combination of our strongly fluorescent Eu chelate, which can be direct-

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ly bound to analyte and time-resolved detection, has improved the detection limits of TR-FIA and DNA hybridization assay by 2 to 4 orders of magnitude [9–11] and the method is expected to be applicable to other chemical and biomedical fluorometric assays. As one such new application, a HPLC system having a time-resolved fluorometric detector has been constructed in the present study. A new Eu fluorescent label 5-(4″-chlorosulfo-1′,1″-diphenyl-4′-*yl*)-1,1,1,2,2-pentafluoro-3,5-pentanedione (CDPP) having a covalently binding group to amino and phenolic hydroxyl groups of the analyte, has been synthesized and used as a label for HPLC.

Several HPLC systems using lanthanide fluorescence were reported. In some systems, terbium (Tb) fluorescence sensitized by the analyte in the SDS or TOPO (tri-n-octylphosphine oxide) micelle is used. The system is specific to the analytes having the α–β unsaturated carbonyl structure, since only such a structure can efficiently transfer the absorbed UV light energy to the terbium [12,13]. In other HPLC systems, a non-fluorescent europium chelate is covalently bound to the analytes, which are then separated on the column. After separation, the non-fluorescent europium chelate is converted to fluorescent chelate by post-column introduction of a large excess of another ligand and the fluorescence produced is measured [14–16]. In the most recent variation, non-fluorescent Tb chelate is bound to silica gel surface, and the analytes, which can transfer the absorbed UV light to the Tb ion to emit the Tb fluorescence, can be detected as they move on the surface [17]. In contrast to these systems, the present study we have developed in a new ligand label CDPP, whose europium chelate complex is fluorescent as it is bound to the analyte. This situation is superior to the previous non-fluorescent europium labeling combined with post-column conversion to the fluorescent chelate, since in the latter system, the large excess of the free ligand in the measured solution raises the background level, even if time-resolved measurement is employed. In our direct fluorescent labeling HPLC system, the background is low, and the detection limits have been remarkably improved.

The new HPLC system was applied to the determination of estrogens, i.e. estrone (E1), 17β-estradiol (E2), ethynylestradiol (EE2) and estriol (E3). The phenolic hydroxyl groups of the estrogens can be covalently bound to the chlorosulfonyl group of CDPP. The results and processing have been compared with those of a GC–MS method [18].

2. Experimental

2.1. Instruments

The time-resolved phosphorescence detector was purchased from Hamamatsu Photonics K.K. (Shizuoka, Japan). The excitation light source, a Xe flash lamp, was used to obtain the excitation light of 340 nm with the frequency of up to 500 Hz.

The flow-cell is made of non-fluorescent quartz, and the fluorescence of the CDPP-Eu chelate at 615 nm is detected with a photomultiplier. The lifetime of the CDPP-Eu is ca. 500 ms, whereas the background fluorescence of the coexisting materials is several ns, to remove the background signal and obtain the high S/N ratio. The signal from the detector is sent to the computer every 10^{-1} s after amplification and digitization. The system processes the data automatically for 10 h.

In the present experiment, the measurement conditions were as follows: excitation at 340 nm, emission at 615 nm, the excitation gate time of 0.01 ms, the delay time of 0.1 ms, the signal gate time of 1.8 ms, and the excitation interval of 2 ms.

The HPLC system used was a Jasco liquid chromatograph (Jasco Intelligent HPLC System GULLIVER 900 series, Tokyo, Japan) with a Jasco DG-980-50 degasser, a Jasco HG-980-31 solvent mixing module, Jasco PU-986 preparation pumps, a Jasco PU-980 HPLC pump for post-column reagent, a Jasco CO-966 column thermostat, a Rheodyne (Cotati, CA, USA) Model 7725I injector fitted with a 10-μl sample loop, and a time-resolved phosphorescence detector described above equipped with a 16-μl flow-cell.

The Arcus 1234 time-resolved fluorometer from Wallac Oy. (Turku, Finland) was used for measuring the fluorescence of the Eu chelate in 96-well microtiter wells. The measurement conditions were delay

time 0.20 ms, window time 0.40 ms and flash rate 1.00 ms.

2.2. Reagents and chemicals

Organic solvents and distilled water of HPLC grade (Kanto Chemicals Co., Tokyo, Japan) were used without further purification. Estrogens E1, E2, EE2, E3 and 1,4-dithioerythritol (DTE) were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Stock solutions of each estrogen (1000 μg/ml) were prepared in acetone. Each estrogen or a mixture of the estrogens working solutions (150, 50, 15 and 5 ng/ml) were prepared in acetonitrile. The stock solutions were stored in screw-capped centrifuge tubes at −20 °C in the dark. Reagents *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and tri-methylchlorosilane (TMCS) were from Fluka (Neu-Ulm, Germany). Sep-Pak cartridges were purchased from Waters (MA, USA). All other chemicals were of analytical reagent grade.

The derivatization mixture for GC–MS was prepared as follows: approximately 2 mg DTE was dissolved in 1 ml MSTFA. A 2-μl volume of TMCS were added under the liquid surface.

2.3. Synthesis of CDPP

The labeling reagent CDPP was prepared according to the two-step reaction in Fig. 1.

2.3.1. Preparation of 5-(1′,1″-diphenyl-4′yl)-1,1,1,2,2-pentafluoro-3,5-pentanedione (DPP)

A solution (70 ml) of dry diethyl ether containing 3.0 g (55.5 mmol) of $NaOCH_3$, 3.8 g (20 mmol) of $C_2F_5COOC_2F_5$ and 3.88 g (20 mmol) of 4′-phenylacetophenone was stirred for 24 h at ambient temperature in a sealed flask. After ether was evaporated, 100 ml of 15% H_2SO_4 was added to the residue and the solution was stirred at ambient temperature. The solid product was collected by filtration, washed with water and recrystallized from 100 ml of ethanol. The yellow product was obtained (2.88 g, 42.4% yield). Anal. Calcd. for $C_{17}H_{11}F_5O_2$: C, 59.65; H 3.24. Found: C, 60.82; H, 3.29. ^{1}H NMR (270 MHz, acetone-d_6) δ 8.27 (*d*, 2H, *J*=8.4 Hz); 7.92 (*d*, 2H, *J*=8.4 Hz); 7.82–7.70 (*m*, 2H); 7.58–7.43 (*m*, 3H); 7.07 (*s*, 1H). FAB MS: *m*/*z* 343 ($M+H^+$).

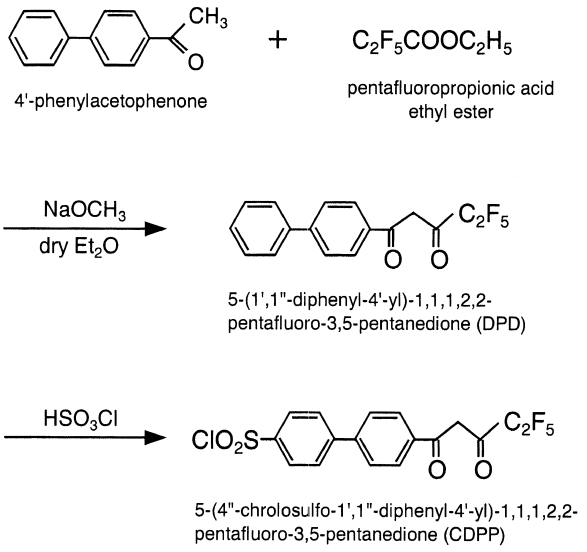

Fig. 1. Synthesis of CDPP.

2.3.2. Preparation of 5-(4″-chlorosulfo-1′,1″-diphenyl-4′yl)-1,1,1,2,2-pentafluoro-3,5-pentanedione (CDPP)

To 10 ml of HSO_3Cl was gradually added 2.50 g (7.3 mmol) of DPD with stirring. After the solution was stirred for 7 h at ambient temperature, it was added dropwise with stirring to 200 ml of ice-water. The residue was collected by centrifugation, rinsed with cold water and twice centrifuged. The precipitate was filtered and vacuum-dried for more than 48 h. An orange product of CDPP was obtained with a yield of 73.2% (2.46 g). Anal. Calcd. for $C_{17}H_{11}F_5O_{4.5}SCl$ (as CDPP-1/2 H_2O): C, 45.40; H, 2.46. Found: C, 45.33; H, 2.01. ^{1}H NMR (270 MHz, acetone-d_6 r.t.) δ 8.35 (*d*, 2H, *J*=8.8 Hz); 8.28 (*d*, 2H, *J*=8.9 Hz); 8.20 (*d*, 2H, *J*=8.9 Hz); 8.06 (*d*, 2H, *J*=8.9 Hz); 7.12 (*s*, 1H). FAB MS: *m*/*z* 441 ($M+H^+$).

2.3.3. Purification of CDPP by reversed-phase chromatography

The CDPP prepared as above was purified by reversed-phase chromatography. A preparation pump

Jasco PU-986, a column thermostat Jasco CO-966, a Rheodyne Model 7725I injector fitted with a 10-μl sample loop, and a Gilson (WI, USA) Model 201 fraction collector were used. A total of 5 μl of an acetonitrile solution containing 50 μg of CDPP was chromatographed by injecting 10 μl through a 4.6-mm I.D.×150 mm TSK-gel ODS 120-A column (Tosoh, Tokyo, Japan) at ambient temperature with isocratic elution. The mobile-phase was acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 0.6 ml/min. Fractions of 0.15 ml were collected, and were diluted 20 000-fold with distilled water containing 1% Triton X-100, 0.2 m*M* $EuCl_3$ and 0.2 m*M* TOPO. A 100-μl of the solution was placed into the 96-wells microtiter plate well, and the time-resolved fluorescent counts of CDPP-Eu chelate were measured with an Arcus 1234 fluorometer. The fraction No. 28 gave the strongest fluorescence and was used for the derivatization of estrogens.

2.4. Labeling of estrogens

The CDPP labeling procedure for estrogens was based on the slightly modified protocol for dancylation of catecholamines [19,20]. To 10 μl of estrogen solution was added 8 μl of 20 m*M* carbonate buffer (pH 10.0) and 22 μl of acetonitrile. To the mixture was added 5 μl of a CDPP solution (12.5 μg/ml), and the mixture was incubated for 30 min at 70 °C. After cooling, 5 μl of 10% HCl was added and 10 μl of the reaction mixture was directly applied onto the column.

2.5. Analysis of the labeled estrogens by isocratic HPLC

The 10 μl of the sample solution was chromatographed on a 4.6-mm I.D.×150 mm YMC-Pak TMS column (YMC Co. Ltd., Tokyo, Japan) at 30 °C with isocratic elution. The mobile-phase was 70% acetonitrile in distilled water containing 0.05% trifluoroacetic acid at a flow rate of 0.6 ml/min. The eluent was measured with post-column introduction of a distilled water solution containing 0.2 m*M* $EuCl_3$, 0.2 m*M* TOPO and 1% Triton X-100. The post-column reagent was introduced at a flow rate of 0.8 ml/min. The time-resolved fluorescent counts of the Eu chelate-labeled estrogens were measured with the time-resolved phosphorescence detector.

2.6. Sample preparation

2.6.1. Sampling of the river water samples

The river water was sampled near the Johsoh bridge of Tone River in Ibaraki prefecture on April 25th, 2001. The sample was stored in a clean bottle previously used for commercial HPLC-grade distilled water, and was stored at 4 °C. The analysis was carried out within 48 h after sampling, and no preservative was added.

2.6.2. Extraction of the river samples

The solid-phase extraction was based on the slightly modified literature method [21,22] and is as follows. The sample water was first filtered through a 48-mm Whatmann (Maidstone, UK) GF/C filter with a pore size of 1.2 μm, and to 500 ml of the filtrate was added 0.5 ml of HCl and 0.5 g of ascorbic acid. The solution was passed at 20 ml/min flow-rate through a Sep-Pak C_{18} cartridge (Waters, MA, USA), which had been pre-conditioned with 20 ml of acetonitrile, 5 ml of distilled water, 5 ml of ethylacetate and 5 ml of distilled water. After the extraction, the remaining water in the column was removed by pumping in the air with a 10-ml syringe. The material on the column was extracted with 4 ml of ethylacetate into a 10-ml test tube. The extract solution was vortexed for 30 s, and was centrifuged at 1000 *g* at 10 °C. The upper ethylacetate layer was transferred to a 10-ml test tube, and the remaining aqueous phase was again extracted with 3 ml of ethylacetate, vortexed, centrifuged, and the ethylacetate layer was combined into the previous ethylacetate solution. The solution was condensed to 1 ml under N_2 flow, and was applied to a Sep-Pak NH_2 cartridge (Waters), which had been conditioned with 10 ml of methanol, 5 ml of ethylacetate, 10 ml of methanol and 5 ml of ethylacetate. The analyte on the cartridge was extracted with 6 ml of methanol into a 10-ml test tube, and the solution was dried under N_2 flow. The solid was dissolved into 0.2 ml of acetonitrile and was processed for CDPP labeling.

2.7. Analysis by GC–MS

For comparison, the water sample was analyzed by derivatization and GC–MS detection, as described by Hartmann et al. [18]. The prepared river water sample solutions (50 μl) with or without standard estrogens and acetonitrile solutions (50 μl) containing standard estrogens were added to the eppendorf sampling tubes, and were evaporated under a stream of nitrogen gas. Silylation of estogens was carried out directly in the eppendorf tubes. After adding a 50 ml derivatization mixture containing MSTFA–TMCS–DTE (1000:2:2, v/v/w), the tubes were sealed tightly and heated at 60 °C for 30 min, and were further incubated at 4 °C overnight to complete the reaction at the C_{17} position of estrogens. The solution was injected directly into the gas chromatograph (injection volume: 1 μl).

A Hitachi G-3000 gas chromatograph (Hitachi Ltd. Instruments, Tokyo, Japan) equipped with a Hitachi M-2000 mass spectrometer (Hitachi Ltd. Instruments), and a 25-m×0.2 mm I.D.×0.33 mm film thickness Ultra-2 column (Hewlett-Packard), was used for quantitation of the estrogens. GC conditions were as follows: carrier gas, helium; oven temperature from 130 °C (1 min) to 290 °C at 12 °C/min, 15 min isotherm; injector temperature, 260 °C (splitless injection); transfer line, 290 °C. The MS conditions were as follows: ionization potential, 70 eV; ion source temperature, 170 °C; acceleration electrical potential, 4 kV; ion source slit width, 500 μm; collector slit width, 500 μm; secondary electronic step-up tube electrical potential supply, 2.0 kV.

Masses for selected ion monitoring: E1, *m*/*z* 342; E2, *m*/*z* 416; EE2, *m*/*z* 425; E3, *m*/*z* 504.

3. Results and discussion

3.1. The HPLC profiles of the CDPP-labeled estrogens

Fig. 2 shows the HPLC profile of the four CDPP-labeled estrogens (0.7 ng each). The peak at 4 min is the free hydrolized CDPP (CDPP-SO_3^-). The CDPP-labeled E1, E2, EE2 and E3 are eluted at 12.5, 11, 11.5 and 7 min, respectively.

The CDPP derivatives of estrogens in the final reaction mixture were stable, and still gave constant time-resolved fluorescence intensities after standing for at least 24 h in the dark at room temperature.

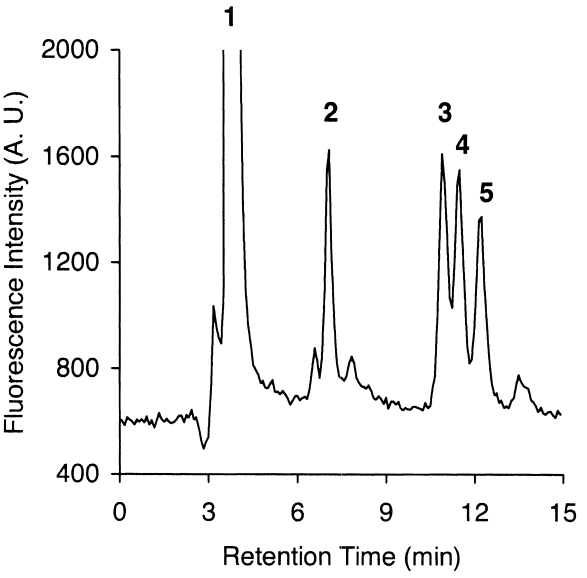

Fig. 2. The HPLC profile of the CDPP-labeled estrogens. A total of 10 μl of the standard solution containing the four estrogens (each 0.7 ng) labeled with CDPP was directly applied on the YMC-Pak TMS column (4.6 mm I.D.×150 mm). (1) CDPP-SO_3^--Eu; (2) estriol-CDPP-Eu; (3) 17β-estradiol-CDPP-Eu; (4) ethynylestradiol-CDPP-Eu; (5) estrone-CDPP-Eu.

3.2. The calibration curves and the detection limits of estrogens in pure water

Good linearity of the calibration curves were obtained in the concentration range from 5 ng/ml to 500 ng/ml (E1, 13.5 x+695, r^2=0.999; E2, y=13.8 x+664, r^2=0.999; EE2, y=13.8 x+668, r^2=0.998; E3, y=15.8 x+676, r^2=0.999). The standard deviations in slope and intercept for the calibration equations were less than 0.729 for slope and 29 for intercept.The detection limits for the estrogens, which were defined as the concentration corresponding to the three SD of the baseline signal, were 0.65, 0.65, 0.65 and 0.60 ng/ml for E1, E2, EE2 and E3, respectively. The relative standard deviations of the peak intensities of E1, E2, EE2 and E3 at 75 ng/ml for 5-times measurement are 1.3, 1.6, 2.5 and 1.4, respectively.

3.3. The recovery test and the detection limits of estrogens in a river water sample

The standard addition and recovery measurement were carried out by using 500 ml of the river water sample according to the procedure described in Section 2.6. After solid-phase extraction and labeling reaction, the solution was analyzed. The recovery was calculated from the difference of the intensities of added and non-added samples. The chromatograms after subtraction of that of the non-added sample are shown in Fig. 3. The signal for E3 could not be detected due to the matrix effect of the river water. The result of the recovery test is summarized in Table 1.

The matrix of the river water influenced the detection limits for E1, E2 and EE2. The detection limits (the concentration corresponding to the three SD of the baseline signal) in the river water sample were 1.6 ng/l for the three estrogens.

3.4. Comparison of the time-resolved fluorometic HPLC and GC–MS

The method presented here was compared with the established GC–MS method [21]. The detection limits of the gas chromatographic method for river water sample coupled with the extraction method using two solid-phase extraction cartridges were 0.3 ng/l for E1, 3.2 ng/l for E2 and 13.4 ng/l for EE2. The river water sample spiked with several concentrations of estrogens (E1, E2 and EE2) [23], was used in this work for comparison of the results of the present method with those of GC–MS. The amounts of estrogens, which were spiked into the river water sample and were recovered with the two methods, are listed in Table 2, and the resulting correlation plots are shown in Fig. 4. In the analysis of EE2, the peak could not be detected at all by the GC–MS method, since the spiked amounts were less than the detection limit of the GC–MS method studied. On the other hand, the peak of EE2 could not be detected quantitatively in the present method due to the interference of the peak originating from the river water sample. For E1, the analysis of five samples with the two methods gave a correlation plot with a slope of 0.57, an intercept of 8.06, and a correlation coefficient of 0.98 over the concentration range of 6–60 ng/l (Fig. 4A). For E2, the analysis of five samples with both methods gave a correlation plot with a slope of 0.45, an intercept of 2.54, and a correlation coefficient of 0.99 over concentration range of 3–30 ng/l (Fig. 4B). The correlation coefficients were high, but the slopes of the correlation plots were approximately 0.5. The difference between the spiked amounts and the recovered amounts is little as shown in Table 2. Mol et al. [24] reported that lower recoveries (approximately 60%) were obtained for the phenolic compounds including estrogens when the extract from a surface water sample was evaporated to dryness in the GC–MS analysis by using *N*-methyl-*N*-(*tert*.-butyl-

Table 1
Recovery and reproducibility of the estrogens added to the river water ($n=3$)

Compounds	Added (ng/l)	Recovery (%)	RSD (%)
Estrone	30	86.0	4.0
	100	95.5	1.9
17β-Estradiol	30	105.1	4.0
	100	90.8	5.4
Ethynylestradiol	30	86.0	5.8
	100	86.3	2.3

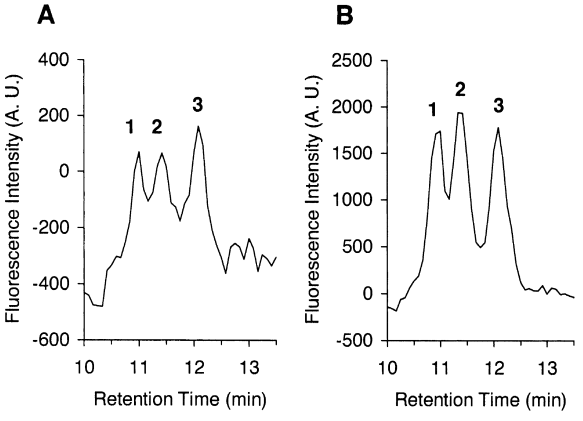

Fig. 3. The HPLC profiles of the estrogen-added water samples. The profiles show the difference of the added and non-added samples. (A) each estrogen (15 ng each) added to 500 ml of the water sample; (B) each estrogen (50 ng each) added to 500 ml of the sample. (1) 17β-estradiol-CDPP-Eu; (2) ethynylestradiol-CDPP-Eu; (3) estrone-CDPP-Eu.

Table 2
Comparison of estrogens values in the river water samples spiked with estrogens

Sample	Added (ng/l)			Present method (ng/l)			GC–MS (ng/l)		
	E1	E2	EE2	E1	E2	EE2	E1	E2	EE2
A	6.0	3.0	1.2	5.0	3.9	n.d.	7.5	3.2	n.d.
B	15.0	6.0	3.0	14.4	6.5	2.9	18.2	6.2	n.d.
C	30.0	12.0	6.0	31.0	13.7	3.3	28.2	9.5	n.d.
D	45.0	18.0	9.0	42.7	18.5	3.6	33.6	10.9	n.d.
E	60.0	30.0	12.0	62.5	29.6	4.0	41.4	15.6	n.d.

The river water samples were spiked with an appropriate amount of standard solutions of four estrogens, and concentrated to 200 μl by the clean-up procedure. The samples were then derivatized with CDPP for the present method or MSTFA for GC–MS method. The values of three estrogens were determined by comparing the peak height in the present method or the peak area in GC–MS method with the calibration curves of each estrogen. n.d., not detected.

dimethyltrifluoro-acetamide (MTBSTFA) as the derivatization reagent, and that the recoveries were quantitative when the extract volume was 100–200 μl, irrespective of the amount of reagent added. In this study, the analytes extracted from the river water were evaporated to dryness prior to the derivatization with MSTFA. Therefore, the slopes of the correlation plots between the present method and the GC–MS method would be nearly 1, if the extract volume were 100–200 μl.

3.5. Analysis of estrogens in the river water sample

In the present method, the amounts of estrogens in the river water sample were determined by means of standard addition. The water sample was concentrated according to the procedure described in Section 2.6 and the prepared sample was introduced into the HPLC system after the addition of different concentrations of standard estrogens. The linear correlation coefficients were more than 0.998 for the three estrogens in the analysis of the river water sample. The each line was extrapolated to the *x*-axis, and the concentration of the intersecting point with the axis was read, 2.1 ng/l of E1 was detected in the original river water.

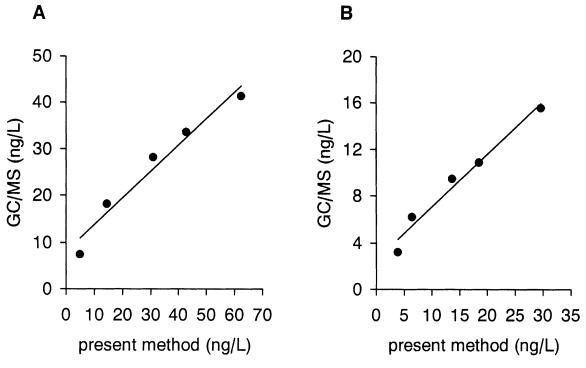

Fig. 4. Correlation plots for river water samples containing (A) estrone and (B) 17β-estradiol. The best-fit parameters for the each plot are given in the text.

4. Conclusions

The use of fluorescent lanthanide chelate together with time-resolved detection has been proved to provide a highly sensitive detection method for HPLC. The processing method and the results were compared with those of the previously described GC–MS method [18], and the correlation coefficients are more than 0.98 between the two methods. Advantages of the present method in comparison with the GC–MS methods are the inexpensive detector and the cost of the maintenance. The concentration of E1 in a river water sample was determined to be 2.1 ng/l with the present method.

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