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Synthesis of a Terbium Fluorescent Chelate and Its Application to Time-Resolved Fluoroimmunoassay

Jingli Yuan,[†] Guilan Wang,[†] Keisuke Majima,[‡] and Kazuko Matsumoto*,[‡]

Department of Chemistry, Waseda University, Japan Science and Technology Corporation, Shinjuku-ku, Tokyo 169-8555, Japan, and Department of Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China

A new nonadentate ligand, *N*, *N*, *N*^{1}, *N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine]tetrakis(acetic acid) (BPTA) for a Tb^{3+} fluorescent complex was synthesized. The Tb^{3+} complex is strongly fluorescent, having a large fluorescence quantum yield of 1.00 and very long fluorescence lifetime of 2.681 ms in 0.05 M borate buffer of pH 9.1. Streptavidin (SA) was labeled with BPTA by using its succinimidyl monoester, and the BPTA−Tb^{3+}-labeled SA was used in sandwich-type time-resolved fluoroimmunoassay (TR-FIA) of α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) in human sera. The Tb^{3+}-labeled SA was also used in competitive-type TR-FIA of bensulfuron-methyl (BSM) in water. The detection limits of these assays are 42 pg/mL for AFP, 70 pg/mL for CEA, and 0.4 ng/mL for BSM. In addition, a new simultaneous measurement method for AFP and CEA in a human serum sample was developed by using 4,4′-bis(1″,1″,1″,2″,2″,3″,3″ -heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT)−Eu^{3+}-labeled anti-AFP antibody, biotinylated anti-CEA antibody, and BPTA−Tb^{3+}-labeled SA. The concentrations of AFP and CEA in 39 human serum samples were determined, and the results were compared with those of the independently determined AFP and CEA by TR-FIA with a single-label method. A good correlation was obtained with the correlation coefficients of 0.991 for AFP and 0.994 for CEA.

Lanthanide fluorescent chelates have been successfully developed as fluorescence probes for highly sensitive time-resolved fluoroimmunoassay (TR-FIA) and DNA hybridization assay with microsecond time-resolved fluorometric measurement.[1−8] The most important advantage of the method is that it allows easy distinction of the specific fluorescence signal of the long-lived fluorescent lanthanide probe from the short-lived background fluorescence present in most biological samples and also obviates the problems associated with the scattering light of the optical components. The representative lanthanide fluorescent chelates used in the current TR-FIA systems include europium, terbium, and samarium fluorescent chelates, such as the Eu^{3+}−β-diketonate (2-naphthoyltrifluoroacetonate)−trioctylphosphine oxide ternary fluorescent complex in the "DELFIA" system,[3] Eu^{3+} chelate with 4,7-bis(chlorosulfodiphenyl)-1,10-phenanthroline-2,9-dicarboxylic acid in the"FIAgen" system,[5] Eu^{3+}−tris(bipyridine) cryptate in the "time-resolved amplified cryptate emission (TRACE)" system,[9,10] and 5-fluorosalicylate−Tb^{3+}−EDTA in the "enzyme-amplified time-resolved fluoroimmunoassay" system.[11]

Recently, TR-FIA has been directed to multiple labeling for use in simultaneous detection of multiple biomolecules, and Eu^{3+}, Sm^{3+}, Tb^{3+}, and Dy^{3+} chelates were used as fluorescent labels having long-lived fluorescence at different wavelengths.[7] Dual-label TR-FIA using Eu^{3+} and Sm^{3+} chelates has been studied for many multiple-component simultaneous measurements.[12−17] Although the dual-label TR-FIA has been used successfully, the Sm^{3+} chelates need some improvement; i.e., their fluorescence is weaker and the fluorescence lifetime is shorter compared to those of Eu^{3+} chelates. Since the fluorescence quantum yields and lifetimes of Sm^{3+} chelates are smaller than those of Eu^{3+} chelates, the Eu^{3+}−Sm^{3+} pair is suitable only for the simultaneous measurement of a low-concentration component and a high-concentration component in a sample. Recently we developed a new TR-FIA method for

* To whom correspondence should be addressed. (tel) +81-3-5286-3108; (fax) +81-3-5273-3489; (e-mail) kmatsu@mn.waseda.ac.jp.

† Dalian Institute of Chemical Physics.

‡ Waseda University.

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the simultaneous measurement of two low-concentration components, α-fetoprotein (AFP) and carcinoembryonic antigen (CEA) in human serum, by using the Eu^{3+} and Sm^{3+} fluorescence chelates as labels,[18] in which the fluorescence of the Sm^{3+} label was amplified by multiple labeling using Sm-labeled streptavidin bound to biotinylated protein.

Compared with the Sm^{3+} fluorescent chelates, some Tb^{3+} fluorescent chelates are reported to have higher fluorescence quantum yields and very long lifetimes. Therefore, dual-label TR-FIA by using Eu^{3+} and Tb^{3+} chelates would be more preferable. Although $Eu^{3+}-Tb^{3+}$ dual-label TR-FIA is reported for the simultaneous measurement of free and total prostate-specific antigen (PSA),[19] the $Eu^{3+}-Tb^{3+}$ combination is not as widely used as the $Eu^{3+}-Sm^{3+}$ dual-label.

Recently, several Tb^{3+} fluorescent chelates having large fluorescence quantum yields and long fluorescence lifetimes have been synthesized.[20−22] The amino-active forms of these chelates have also been developed for protein labeling.[23] These ligands include polyacid derivatives of 2,6-bis(pyrazol-1-yl)pyridine, 2,6-bis(pyrazol-1-yl)pyrazine, and 1,3-bis(pyridin-2-yl)pyrazole, whereas polyacid derivatives of 2,2′:6′,2″-terpyridine are not effective for Tb^{3+} strong fluorescence. In the present work, a new ligand for Tb^{3+}, *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine]tetrakis(acetic acid) (BPTA) was synthesized, and the fluorescence properties of the Eu^{3+} and Tb^{3+} chelates were examined as well as the application of the BPTA−Tb^{3+} as a fluorescence label for TR-FIA. In the new ligand, a phenyl group is introduced to the pyridine ring of the formerly prepared *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)pyridine]tetrakis(acetic acid),[22] to increase the molar absorption coefficient. An additional usefulness of this phenyl group is that an amino-active group can be introduced to the phenyl group for protein labeling. The fluorescence property of the BPTA−Tb^{3+} complex shows that both the molar absorption coefficient and the fluorescence quantum yield are increased remarkably by introduction of the phenyl group.

To evaluate the usefulness of BPTA−Tb^{3+} as a fluorescence label for TR-FIA, human AFP in serum, CEA in serum, and bensulfuron-methyl (BSM) in water were measured by sandwich-type (AFP and CEA) and competitive-type (BSM) TR-FIA, by using BPTA−Tb^{3+}-labeled streptavidin (SA). The methods gave detection limits of 42 pg/mL for AFP, 70 pg/mL for CEA, and 0.4 ng/mL for BSM. In addition, a new TR-FIA method for simultaneous measurement of AFP and CEA in human serum was developed, in which 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT)−Eu^{3+}-labeled anti-AFP antibody, biotinylated anti-CEA antibody, and BPTA−Tb^{3+}-labeled SA were used. Compared with the previous method using the Eu^{3+} and Sm^{3+} chelates as labels,[18] the new method is simpler and more sensitive. The AFP and CEA concentrations in 39 human serum samples were determined, and the results were compared with those of the usual single-assay method. Good correlations were obtained between the simultaneous and single assays with the correlation coefficients of 0.991 for AFP and 0.994 for CEA.

EXPERIMENTAL SECTION

Synthesis of BPTA. The new ligand BPTA was synthesized following the six-step reaction shown in Figure 1. The details of the procedure are described in the following.

(i) Synthesis of 2,6-Dibromo-4-phenylpyridine (2). The starting compound 4-amino-2, 6-dibromopyridine (**1**) was synthesized from 2,6-dibromopyridine by using the literature methods.[24,25] Anal. Calcd for $C_5H_4Br_2N_2$: C, 23.84; H, 1.60; N, 11.16. Found: C, 24.00; H, 1.47; N, 11.02. ^{1}H NMR (acetone-d_6) δ 6.82 (s, 2H), 6.16 (b, 2H). To 180 mL of benzene containing 3.7 g (15 mmol) of **1** was added 30 mL of trifluoroacetic acid and 3.3 g (28 mmol) of isopentyl nitrite with stirring. After stirring for 20 min at room temperature, the solution was refluxed for 2.5 h. To the solution was added 50 mL of benzene, and the solution was washed with 3 × 100 mL of water and 100 mL of 5% Na_2CO_3. After the solution was dried with Na_2SO_4, the solvent was evaporated. The residue was purified by silica gel column chromatography using benzene as eluent and then was recrystallized from methanol. Pale yellow crystals of **2** were obtained (2.3 g, 49.0% yield). Anal. Calcd for $C_{11}H_7Br_2N$: C, 42.21; H, 2.25; N, 4.47. Found: C, 41.76; H, 2.11; *N*, 4.65. ^{1}H NMR (acetone-d_6) δ 7.95 (s, 2H), 7.88−7.84 (m, 2H), 7.58−7.53 (m, 3H).

(ii) Synthesis of 2,6-Bis(3′-methoxycarbonyl-1′-pyrazolyl)-4-phenylpyridine (3). Potassium (1.56 g, 40 mmol) was added in small portions to a solution of 5.04 g (40 mmol) of 3-methoxycarbonylpyrazole in 120 mL of dry THF at 60−70 °C with stirring. After the metal was dissolved, 3.13 g (10 mmol) of **2** was added, and the mixture was refluxed for 1 week. The solvent was evaporated, and the residue was extracted with 3 × 150 mL of CH_2Cl_2. After CH_2Cl_2 was evaporated, the residue was purified by silica gel column chromatography using $CH_2Cl_2-CH_3OH$ (98:2, w/w) as eluent and then was recrystallized from benzene. White crystals of **3** were obtained (0.55 g, 13.6% yield). Anal. Calcd for $C_{21}H_{17}N_5O_4$: C, 62.53; H, 4.25; N, 17.36. Found: C, 62.48; H, 4.01; N, 17.28. ^{1}H NMR ($CDCl_3$) δ 8.65 (d, *J*, 2.64 Hz, 2H), 8.31 (s, 2H), 7.86−7.82 (m, 2H), 7.57−7.50 (m, 3H), 7.05 (d, *J*, 2.64 Hz, 2H), 4.00 (s, 6H).

(iii) Synthesis of 2,6-Bis(3′-hydroxymethyl-1′-pyrazolyl)-4-phenylpyridine (4). To 250 mL of dry THF containing 500 mg $LiAlH_4$ was added 1.0 g (2.48 mmol) of **3**. After stirring for 3.5 h at room temperature, 0.45 mL of water, 0.45 mL of 15% NaOH, and 1.5 mL of water were added dropwise to the solution. The solution was filtered to remove precipitate, and the solvent was evaporated. The product was washed with CH_3CN and then dried. A white powder of **4** was obtained (0.66 g, 76.6% yield). ^{1}H NMR (DMSO-d_6) δ 8.91 (d, *J*, 2.31 Hz, 2H), 7.96 (s, 2H), 7.91−7.87 (m, 2H), 7.64−7.56 (m, 3H), 6.61 (d, *J*, 2.31 Hz, 2H), 5.31 (t, *J*, 5.94 Hz, 2H), 4.57 (d, *J*, 5.94 Hz, 4H).

(iv) Synthesis of 2,6-Bis(3′-bromomethyl-1′-pyrazolyl)-4-phenylpyridine (5). To 100 mL of dry THF containing 0.66 g of

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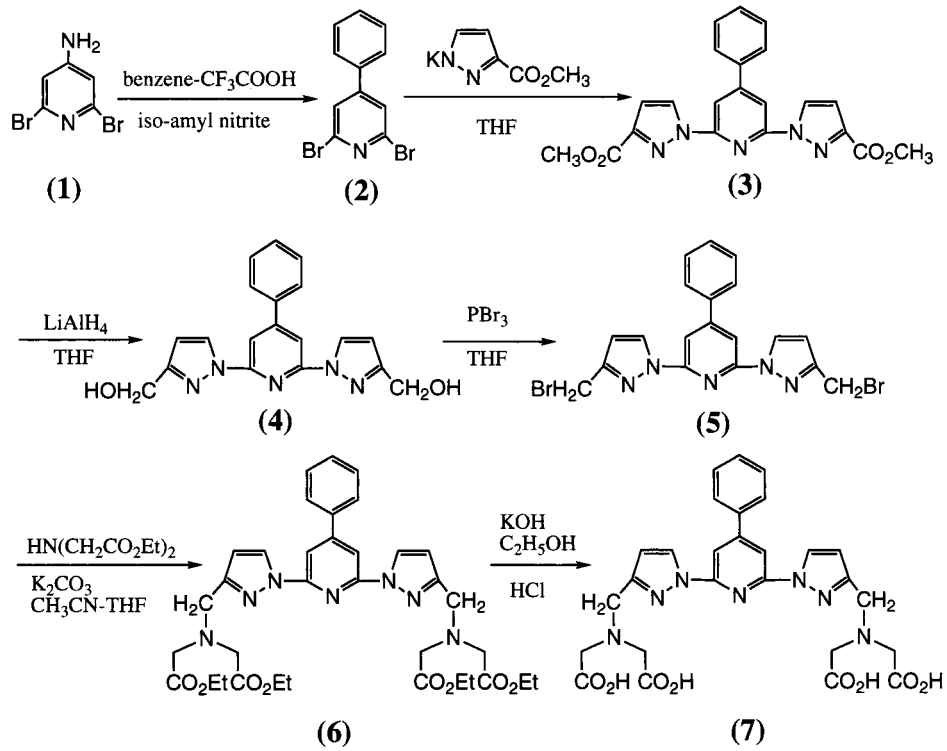

Figure 1. Synthesis of BPTA (**7**).

4 (1.9 mmol) was added 1.45 g of PBr_3 (5.36 mmol), and the solution was refluxed for 4 h with stirring. After the solvent was evaporated, the residue was washed with 10% Na_2CO_3 and then dissolved in 20 mL of benzene with slight warming. After filtering, the solvent was evaporated. The product was washed with hexane and then dried. A white powder of **5** was obtained (0.81 g, 90.0% yield). ^{1}H NMR ($CDCl_3$) δ 8.54 (d, *J*, 2.64 Hz, 2H), 8.08 (s, 2H), 7.84−7.80 (m, 2H), 7.60−7.50 (m, 3H), 6.58 (d, *J*, 2.64 Hz, 2H), 4.59 (s, 4H).

(v) Synthesis of Tetraethyl *N*,*N*,*N*^{1},*N*^{1}-[2,6-Bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine] Tetrakis(acetate) (6). To a solution of 100 mL of dry CH_3CN−20 mL of dry THF containing 0.75 g of **5** (1.58 mmol), 608 mg of diethyl iminodiacetate (3.2 mmol) dissolved in 20 mL of CH_3CN and 2.2 g of K_2CO_3 were added. The mixture was refluxed for 24 h with stirring. After filtering, the solvent was evaporated. The residue was dissolved in 200 mL of $CHCl_3$, and then the solution was washed with 2 × 100 mL of water. After the organic phase was dried with Na_2SO_4, the solvent was evaporated. The oily residue was purified by silica gel column chromatography using ethyl acetate as eluent to yield 0.57 g (52.3% yield) of **6**. ^{1}H NMR ($CDCl_3$) δ 8.54 (d, *J*, 2.31 Hz, 2H), 8.05 (s, 2H), 7.84−7.80 (m, 2H), 7.55−7.45 (m, 3H), 6.56 (d, *J*, 2.64 Hz, 2H), 4.18 (q, *J*, 7.26 Hz, 8H), 4.08 (s, 4H), 3.64 (s, 8H), 1.26 (t, *J*, 7.26 Hz, 12H).

(vi) Synthesis of *N*,*N*,*N*^{1},*N*^{1}-[2,6-Bis(3′-aminomethyl-1′-pyrazolyl)-4-phenylpyridine] Tetrakis(acetic acid). To a solution of 1.2 g of KOH dissolved in 40 mL of ethanol and 5 mL of water was added 550 mg of **6** (0.80 mmol), and then the resultant solution was refluxed for 3 h with stirring. After the solvent was evaporated, the residue was dissolved in 40 mL of water. To the solution was added dropwise 3 M HCl to adjust the pH to ∼1, and the solution was stirred for 3 h at room temperature. The precipitate of BPTA was collected by filtration and washed with 1% HCl. After drying, 429 mg of BPTA (90.0% yield) was obtained. Anal. Calcd for $C_{27}H_{29}N_7O_9$ (BPTA·H_2O): C, 54.45; H, 4.91; N, 16.45. Found: C, 54.12; H, 5.03; N, 16.18. ^{1}H NMR (DMSO-d_6) δ 8.92 (d, *J*, 2.64 Hz, 2H), 8.20 (s, 2H), 7.90−7.86 (m, 2H), 7.60−7.55 (m, 3H), 6.59 (d, *J*, 2.31 Hz, 2H), 4.00 (s, 4H), 3.53 (s, 8H).

Labeling of SA with BPTA. SA was labeled with BPTA by using succinimidyl monoester of BPTA (NHS−BPTA). The preparation of *N*-hrdroxysuccinimide (NHS)−BPTA and the labeling procedure are described in the following.

(i) Preparation of NHS−BPTA. Before preparation, BPTA and NHS were vacuum-dried for 24 h in a desiccator in the presence of P_2O_5. To a solution of 173 mg of BPTA (0.3 mmol) dissolved in 5 mL of dry DMF, 34.5 mg of NHS (0.3 mmol) and 61.9 mg of *N*,*N*′-dicyclohexylcarbodiimide (0.3 mmol) were added. The solution was stirred for 24 h at room temperature. After undissolved white powder was removed by filtration, the DMF solution was evaporated to dryness by vacuum evaporation, and the product was washed with 2-propanol and dried. The product was directly used for SA labeling without further purification. Since the esterification between BPTA and NHS is equimolar, and four carboxyl groups of BPTA are chemically equivalent, it can be considered that only one carboxyl group is esterified in NHS−BPTA.

(ii) Labeling of SA with NHS−BPTA. To a solution of 5 mg of SA dissolved in 10 mL of 0.1 M carbonate buffer of pH 9.1 was added 10 mg of NHS−BPTA. After stirring for 4 h at room temperature, the solution was dialyzed three times against 3 L of 0.1 M $NaHCO_3$ containing 0.25 g of NaN_3, at 4 °C each for 24 h. The concentration of BPTA in the labeled SA solution was measured by $TbCl_3$ fluorescence titration, and the labeling ratio of BPTA to SA was calculated to be ∼26 (26 BPTA per SA). The

solution of labeled SA was stored at −20 °C after 25 mg of NaN_3 was added. To the BPTA-labeled SA solution was added $TbCl_3$ (Tb^{3+}:BPTA = 1.5:1) and the resultant mixture was diluted 1000-fold with 0.05 M Tris-HCl buffer, pH 7.8, containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl, before use in immunoassay.

Physical Measurements. The ^1H NMR spectra were measured on a JEOL JNM-LA270 spectrometer. The fluorescence quantum yields (ϕ_1) of BPTA−Eu^{3+} and BPTA−Tb^{3+} were measured in 0.05 M borate buffer, pH 9.1, and calculated[22] by using the equation $\phi_1 = I_1\epsilon_2C_2\phi_2/I_2\epsilon_1C_1$ with a standard quantum yield of $\phi_2 = 0.110$ for [Eu(2,2′:6′,2″-terpyridine-6,6″-dicarboxylate)$_2$] (molar absorption coefficient $\epsilon_{327\ nm} = 1.75 \times 10^4\ cm^{-1}\ M^{-1}$) or $\phi_2 = 0.090$ for [Tb(2,2′:6′,2″-terpyridine-6,6″-dicarboxylate)$_2$] ($\epsilon_{327\ nm} = 1.75 \times 10^4\ cm^{-1}\ M^{-1}$).[22] In the equation, I_1 and I_2 are the fluorescence intensities for the measured chelate and the standard chelate, ϵ_1 and ϵ_2 are molar absorption coefficients of the measured chelate and the standard, and C_1 and C_2 are concentrations of the chelate and the standard, respectively. The fluorescence excitation and emission spectra were measured on a Hitachi F-4500 fluorospectrometer. The fluorescence lifetime was measured on a Perkin-Elmer LS 50B luminescence spectrometer. The time-resolved fluorometric immunoassay was performed on a DELFIA 1234 time-resolved fluorometer (Wallac). For the terbium fluorescence measurement, the measurement conditions were as follows: excitation wavelength, 340 nm; emission wavelength, 545 nm; delay time, 0.2 ms; window time, 0.4 ms; cycling time, 1.0 ms. The europium fluorescence measurement was carried out under the following conditions: excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 0.2 ms; window time, 0.4 ms; cycling time, 1.0 ms.

Application of BPTA−Tb^{3+}-Labeled SA to TR-FIA. The BPTA−Tb^{3+}-labeled SA was tested for both sandwich-type TR-FIA and competitive-type TR-FIA. By using BPTA−Tb^{3+}-labeled SA, human AFP and CEA in serum and BSM in water were measured by sandwich-type TR-FIA (AFP and CEA) and competitive-type TR-FIA (BSM). In addition, BHHCT−Eu^{3+}-labeled anti-AFP antibody, biotinylated anti-CEA antibody, and BPTA−Tb^{3+}-labeled SA were used for simultaneous determination of AFP and CEA in human serum. Labeling of the antibodies and each assay were carried out according to the following procedures.

Preparation of Biotinylated Anti-AFP Antibody. After two dialyses of 1.0 mL of goat anti-human AFP antibody solution (0.35 mg/mL, Nippon Bio-Test Laboratories, Inc.) for 24 h at 4 °C against 3 L of saline−water, 8.4 mg of $NaHCO_3$ and 5 mg of sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin, Pierce Chemical Co.) were added with stirring. After stirring for 1 h at room temperature, the solution was incubated for 24 h at 4 °C. The solution was twice dialyzed each for 24 h at 4 °C against 3 L of 0.1 M $NaHCO_3$ containing 0.25 g of NaN_3, and then 10 mg of NaN_3 was added. The solution was stored at −20 °C before use. When the biotinylated antibody solution was used for immunoassay, it was diluted 2000-fold with 0.05 M Tris-HCl buffer, pH 7.8, containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl.

Preparation of Biotinylated Anti-CEA Antibody and BHHCT-Labeled Anti-AFP Antibody. Biotinylated rabbit anti-human CEA antibody (Dako-immunoglobulins) and BHHCT-labeled goat anti-human AFP antibody (Nippon Bio-Test Laboratories, Inc.) were prepared using the methods described in a previous report.[18] When BHHCT-labeled anti-AFP antibody was used in a single-label assay of AFP, the stock solution was diluted 750-fold with 0.05 M Tris-HCl buffer, pH 7.8 containing 1.0×10^{-6} M $EuCl_3$, 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl. When biotinylated anti-CEA antibody was used in a single assay of CEA, the stock solution was diluted 1000-fold with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl. When BHHCT-labeled anti-AFP antibody and biotinylated anti-CEA antibody were used for the simultaneous measurement of AFP and CEA, a mixture of 1/750 BHHCT-labeled anti-AFP antibody solution and 1/1000 biotinylated anti-CEA antibody solution was prepared with 0.05 M Tris-HCl buffer, pH 7.8, containing 1.0×10^{-6} M $EuCl_3$, 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl.

Preparation of Biotinylated BSM−BSA Conjugate. The preparation was carried out by using the same method as described in the preparation of biotinylated anti-AFP antibody. In the reaction, 1.0 mL of the BSM−BSA conjugate solution (BSA concentration is ∼1 mg/ml, Iatron Laboratories, Inc.) and 5 mg of NHS-LC-biotin were used. After purification by dialysis, the biotinylated BSM−BSA was diluted to 2.0 mL with water, and 10 mg of BSA and 10 mg of NaN_3 were added. The solution was stored at 4 °C before use. When the biotinylated BSM−BSA solution was used for immunoassay, it was diluted 1000-fold with 0.05 M Tris-HCl buffer, pH 7.8, containing 0.4% BSA, 0.2% NaN_3, and 1.8% NaCl.

Immunoassay of Human AFP by Using BPTA−Tb^{3+}-Labeled SA. The standard solutions of human AFP were prepared by diluting human AFP (Nippon Bio-Test Laboratories, Inc.) with 0.05 M Tris-HCl buffer, pH 7.8, containing 5% BSA, 0.9% NaCl, and 0.1% NaN_3.

After anti-AFP monoclonal antibody (Oemconcepts Co., diluted to 5 μg/mL with 0.1 M carbonate buffer, pH 9.6) was coated on the wells (100 μL/well) of a 96-well microtiter plate (FluoroNunc plate) by physical adsorption,[18] 50 μL of human AFP standard solution was added to each well. After incubation at 37 °C for 1 h, the wells were washed twice with 0.05 M Tris-HCl buffer, pH 7.8, containing 0.05% Tween 20 (buffer 1) and once with 0.05 M Tris-HCl buffer, pH 7.8 (buffer 2). Then 50 μL of biotinylated goat anti-AFP antibody was added to each well, and the plate was incubated at 37 °C for 1 h. After the wells were washed with buffer 1 and buffer 2, 50 μL of BPTA−Tb^{3+}-labeled SA was added to each well and the plate was incubated at 37 °C for 1 h. After the wells were washed four times with buffer 1, the plate was subjected to solid-phase terbium time-resolved fluorometric measurement.

Immunoassay of Human CEA by Using BPTA−Tb^{3+}-Labeled SA. The standard solutions of human CEA were prepared by diluting human CEA (Seikagaku Co.) with 0.05 M Tris-HCl buffer, pH 7.8, containing 5% BSA, 0.9% NaCl and 0.1% NaN_3.

After anti-CEA monoclonal antibody (Nippon Bio-Test Laboratories, Inc., diluted to 5 μg/mL with 0.1 M carbonate buffer of pH 9.6) was coated on the wells (100 μL/well) of a 96-well microtiter plate by physical adsorption, 50 μL of human CEA standard solution was added to each well. After incubation at 37 °C for 1 h, the wells were washed twice with buffer 1 and buffer 2. Then 50 μL of biotinylated rabbit anti-CEA antibody was added to each well and the plate was incubated at 37 °C for 1 h. After the wells were washed with buffer 1 and buffer 2, 50 μL of BPTA-Tb^{3+} labeled SA was added to each well and the plate was

incubated at 37 °C for 1 h. After wells were washed four times with buffer 1, the plate was subjected to solid-phase terbium time-resolved fluorometric measurement.

Immunoassay of BSM by Using BPTA−Tb^{3+}-Labeled SA. The standard solutions of BSM were prepared by diluting 1 mg/mL BSM−acetonitrile solution with 0.05 M Tris-HCl buffer, pH 7.8.

After anti-BSM monoclonal antibody (Iatron Laboratories, Inc., diluted to 4 μg/mL with 0.1 M carbonate buffer, pH 9.6) was coated on the wells (100 μL/well) of a 96-well microtiter plate by physical adsorption, 50 μL of a 1:1 mixture of the BSM standard solution and the biotinylated BSM−BSA was added to each well. The plate was incubated at 37 °C for 2 h and washed with buffer 1 and buffer 2. Then 50 μL of the BPTA−Tb^{3+}-labeled SA was added to each well, and the plate was incubated at 37 °C for 1 h. After wells were washed four times with buffer 1, the plate was subjected to solid-phase terbium time-resolved fluorometric measurement.

Simultaneous Measurement of AFP and CEA Using BHHCT−Eu^{3+}-Labeled Anti-AFP Antibody and BPTA−Tb^{3+}-Labeled SA. The standard solutions containing AFP and CEA were prepared by diluting human AFP and CEA with 0.05 M Tris-HCl buffer, pH 7.8, containing 5% BSA, 0.1% NaN_3, and 0.9% NaCl.

After a solution containing anti-AFP monoclonal antibody and anti-CEA monoclonal antibody (5 μg/mL anti-AFP and 5 μg/mL anti-CEA in 0.1 M carbonate buffer, pH 9.6) was coated on the wells (100 μL/well) of a 96-well microtiter plate by physical adsorption, 50 μL of a human AFP−CEA standard solution (or human sera) was added to each well. After incubation at 37 °C for 1 h, the wells were washed with buffer 1 and buffer 2. Then a 50-μL mixture of BHHCT−Eu^{3+}-labeled anti-AFP and biotinylated anti-CEA was added to each well, and the plate was incubated at 37 °C for 1 h. After the wells were washed four times with 0.05 M Tris-HCl, pH 9.1, containing 0.05% Tween 20, the plate was subjected to solid-phase europium time-resolved fluorometric measurement. After measurement, 50 μL of BPTA−Tb^{3+}-labeled SA was added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed four times with buffer 1 and was subjected to solid-phase terbium time-resolved fluorometric measurement.

To compare the correlation between the simultaneous measurement and the independent single measurement of AFP and CEA in human serum, AFP and CEA concentrations in 39 human serum samples were determined by using both the dual-label method and single-label independent assay for AFP and CEA. In the single-label independent assay of AFP, the anti-AFP antibody-coated plate, AFP standard solutions (or serum samples) and BHHCT−Eu^{3+}-labeled anti-AFP antibody were used for measurement. After the anti-AFP antibody-coated wells were incubated with the AFP standard solutions or serum samples at 37 °C for 1 h, BHHCT−Eu^{3+}-labeled anti-AFP antibody was added, and the solution was incubated at 37 °C for 1 h. After the wells were washed four times with 0.05 M Tris-HCl, pH 9.1, containing 0.05% Tween 20, the plate was subjected to solid-phase europium time-resolved fluorometric measurement, and the AFP concentrations in the samples were calculated. The method of CEA independent assay by using biotinylated anti-CEA antibody and BPTA−Tb^{3+}-labeled SA has been described above.

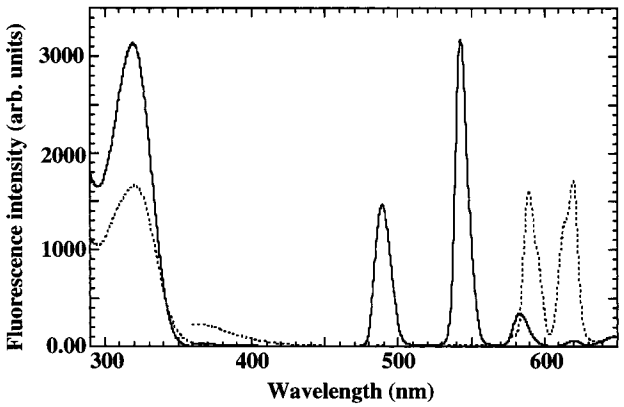

Figure 2. Excitation and emission spectra of BPTA−Tb^{3+} (1 μM, —) and BPTA−Eu^{3+} (20 μM, - - -) in 0.05 M borate buffer, pH 9.1.

Table 1. Fluorescence Properties of BPTA−Tb^{3+} and BPTA−Eu^{3+} in 0.05 M Borate Buffer, pH 9.1[a]

chelate	$\lambda_{ex,max}$ (nm)	ϵ (cm^{-1} M^{-1})	$\lambda_{em,max}$ (nm)	ϕ	$\epsilon\phi$ (cm^{-1} M^{-1})	τ_{H_2O}[b] (ms)	τ_{D_2O}[c] (ms)
BPTA−Tb^{3+}	325	9290	543	1.00	9290	2.681	3.031
BPTA−Eu^{3+}	325	9290	620	0.134	1245	1.353	2.427
BPTA−Tb^{3+} (bound to SA)	322		543		6460	2.410	2.920
BPTA−Eu^{3+} (bound to SA)	322		619		731	1.213	2.285

[a] The experimental uncertainty for quantum yield is <15%. [b] Measured in 0.05 M $NaHCO_3$−H_2O buffer. [c] Measured in 0.05 M $NaHCO_3$−D_2O buffer.

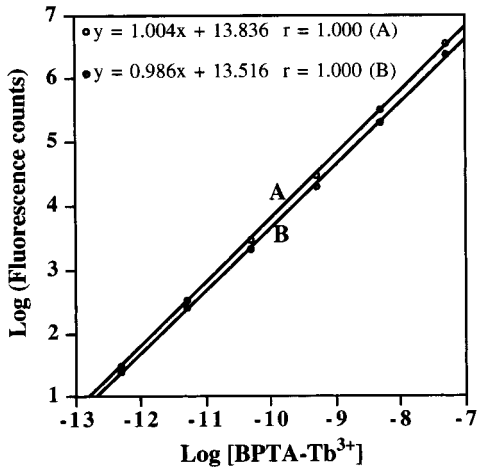

Figure 3. Log(fluorescence count) vs log[BPTA−Tb^{3+}] in 0.05 M Tris-HCl buffer, pH 8.0: (A) free BPTA−Tb^{3+}. (B) BPTA−Tb^{3+} bound to SA. The concentration of BPTA−Tb^{3+} is in molar and the fluorescence count is in arbitrary units.

RESULTS AND DISCUSSION

Fluorescence Properties of BPTA Chelates with Eu^{3+} and Tb^{3+}. The excitation and emission spectra of BPTA−Tb^{3+} and BPTA−Eu^{3+} are shown in Figure 2, while the fluorescence properties are shown in Table 1. Both BPTA−Tb^{3+} and BPTA−Eu^{3+} show the excitation maximum wavelength at 325 nm (ϵ = 9.29×10^3 cm^{-1} M^{-1} for both). The BPTA−Tb^{3+} complex shows four sharp emissions at 489, 543, 582, and 620 nm with the intensities in the order of 543 > 489 > 582 > 620 nm. The BPTA−Eu^{3+} complex shows two sharp emissions at 589 and 620 nm with

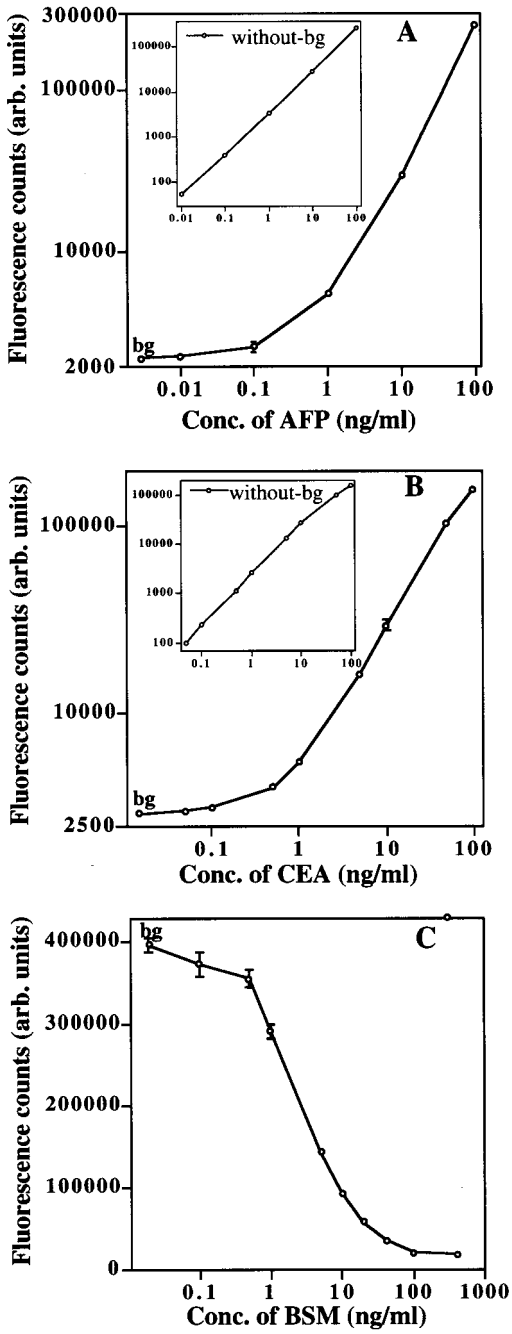

Figure 4. Calibration curves of TR-FIA by using BPTA−Tb^{3+}-labeled SA for AFP (A), CEA (B), and BSM (C). bg, background. The inset curves in (A) and (B) are the plot of the background-subtracted signals vs the concentrations of AFP and CEA.

the intensity order of 620 > 589 nm. Compared with the Tb^{3+} complex, the Eu^{3+} complex is weakly fluorescent. In 0.05 M borate buffer, pH 9.1, the fluorescence quantum yield and lifetime of free BPTA−Tb^{3+} are 1.00 and 2.681 ms, and those of BPTA−Eu^{3+} are 0.134 and 1.353 ms, respectively. These fluorescence quantum yield and molar absorption coefficient of BPTA−Tb^{3+} are clearly larger than those of *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)pyridine]tetrakis(acetic acid) chelate with Tb^{3+} (the fluorescence quantum yield and the molar absorption coefficient of the Tb^{3+} chelate are 0.580 and 8100 cm^{-1} M^{-1}, respectively).[22] After the two BPTA chelates were bound to SA, their fluorescence intensities ($\epsilon\phi$) and lifetimes decreased to about 70 (Tb chelate) and 58% (Eu chelate) and 2.410 and 1.213 ms, respectively. Using the fluorescence lifetimes of BPTA−Eu^{3+} (free and bound to SA) in D_2O and in H_2O, the average number (q) of coordinated water molecules in the first coordination sphere of Eu^{3+} was calculated from the equation[22] of $q = 1.05(1/\tau_{H_2O} - 1/\tau_{D_2O})$ to be 0.34 (free chelate) and 0.41 (bound to SA).

Table 2. Measurement Precision and Analytical Recovery of AFP Added to Serum Samples

added (ng/mL)	found (ng/mL)	CV (%) ($n = 4$)[a]	recovery (%)
	1.81	9.19	
25.0	28.6	6.92	107.2
10.0	10.9	5.82	91.9
5.00	7.06	7.80	105.0
2.50	4.02	6.98	88.4
1.00	2.72	9.85	91.0
	3.28	4.15	
25.0	26.2	7.81	91.7
10.0	14.2	5.82	109.2
5.00	8.22	4.15	98.8
2.50	5.57	7.21	91.6
1.00	4.19	7.12	91.0
	5.12	5.55	
25.0	26.4	5.18	85.1
10.0	16.2	4.19	110.8
5.00	10.1	4.15	99.6
2.50	7.41	7.20	91.6
1.00	6.05	3.15	93.0

[a] Intraassay using four different wells in a plate.

The effect of pH on the fluorescence intensity and the lifetime was measured by using a solution of 1.0 μM BPTA−Tb^{3+} (free and bound to SA) in 0.05 M Tris-HCl buffer with several pHs (from 5.5 to 9.5). The results show that both fluorescence intensity and lifetime are not affected (the change of fluorescence intensity <5%) by the pH in the range of pH 6.0−8.5. In addition, Tris-HCl, carbonate, and borate buffers did not change the fluorescence intensity and lifetime of BPTA−Tb^{3+}. These experiments also proved that BPTA chelates of Tb^{3+} and Eu^{3+} are highly stable in these buffers.

The calibration curve of BPTA−Tb^{3+} (free and bound to SA) in 0.05 M Tris-HCl buffer, pH 8.0, was measured and is shown in Figure 3. The detection limits of free BPTA−Tb^{3+} and BPTA−Tb^{3+} bound to SA were calculated as the concentration corresponding to 2 standard deviations (SD) of the background intensity and are 5.8×10^{-13} (free BPTA−Tb^{3+}) and 1.6×10^{-12} M (BPTA−Tb^{3+} bound to SA) (which are 8.7×10^{-13} and 2.4×10^{-12} M when 3 SD of the background intensity was used).

TR-FIA of AFP, CEA, and BSM by Using BPTA−Tb^{3+}-Labeled SA. The calibration curves of AFP, CEA, and BSM are shown in Figure 4. The detection limits defined as the concentration corresponding to 2 SD of the background intensity were 42 pg/mL for AFP, 70 pg/mL for CEA, and 0.4 ng/mL for BSM (which are 65 pg/mL for AFP, 106 pg/mL for CEA, and 0.68 ng/mL for BSM when 3 SD of the background intensity is used). These detection limits are low enough to measure AFP and CEA in human sera and BSM in environmental samples. The standard deviations of the background signals were calculated by using eight date points measured in eight different wells in a 96-well microtiter plate. As panels A and B of Figure 4 show, the plot of the background-subtracted signals versus antigen concentrations

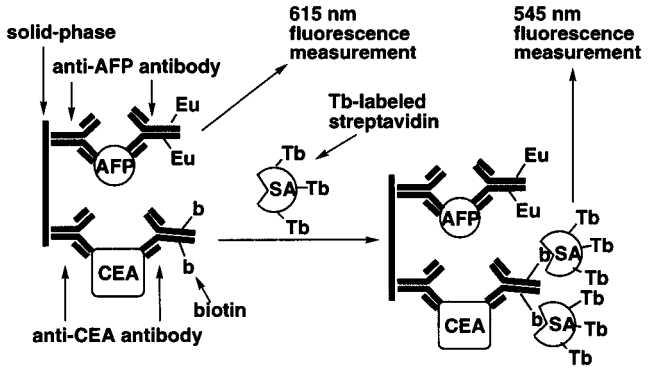

Figure 5. Schematic representation of the simultaneous measurement system for AFP and CEA. Eu, BHHCT−Eu^{3+}; Tb, BPTA−Tb^{3+}. For the details of the procedure, see the Experimental Section.

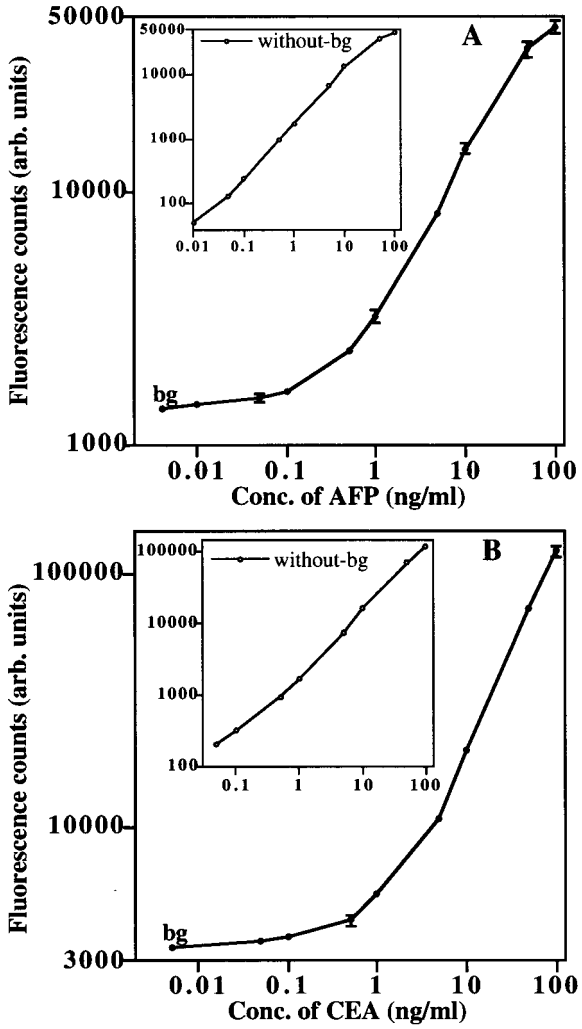

Figure 6. Calibration curves of the simultaneous measurement of AFP (A) and CEA (B) using BHHCT−Eu^{3+}-labeled anti-AFP antibody, biotinylated anti-CEA antibody, and BPTA−Tb^{3+}-labeled SA; bg, background. The inset curves are the plot of the background-subtracted signals vs the concentrations of AFP and CEA.

shows straight lines expressed as log(signal) = 0.919 log[AFP] + 3.535 (r = 1.000) for AFP and log(signal) = 0.979 log[CEA] + 3.353 (r = 0.998) for CEA. The upper limits of the three calibration curves are ~100 ng/mL for AFP, CEA, and BSM. The analytical precision and recovery of TR-FIA using BPTA−Tb^{3+}-labeled SA

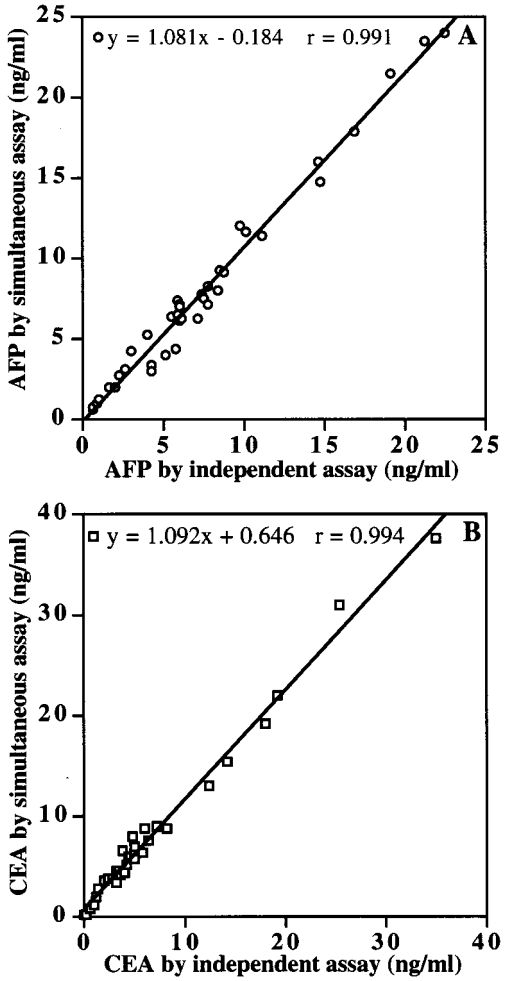

Figure 7. Correlation between the independent measurement and the simultaneous measurement of AFP (A) and CEA (B) in 39 human serum samples.

measured for AFP are summarized in Table 2. The CVs of all the assays are below 10%, and the analytical recoveries are in the range of 85−110%. These results indicate that TR-FIA by using BPTA−Tb^{3+}-labeled SA is highly sensitive with good accuracy and precision.

Simultaneous Measurement of AFP and CEA by Using BHHCT−Eu^{3+}-Labeled Anti-AFP Antibody and BPTA−Tb^{3+}-Labeled SA. The assay format for the simultaneous measurement of AFP and CEA in sera is shown in Figure 5, in which BHHCT−Eu^{3+}-labeled anti-AFP antibody, biotinylated anti-CEA antibody, and BPTA−Tb^{3+}-labeled SA are employed. As shown in Figure 2, the emission of BPTA−Tb^{3+} at 620 nm overlaps the emission of the Eu^{3+} chelate (the fluorescence intensity of BPTA−Tb^{3+} measured with the Eu^{3+} fluorescence measurement mode (615 nm) of DELFIA 1234 is ~1/30 of the intensity obtained with the Tb^{3+} fluorescence measurement mode (545 nm)), and therefore, the Eu^{3+} fluorescence intensity at 615 nm is affected by the presence of the Tb^{3+} chelate. To overcome this problem, a separated assay format as shown in Figure 5 was used for the simultaneous measurement of AFP and CEA by sequential addition of the tracers and by Tb^{3+} fluorescence measurement at 545 nm since Tb^{3+} fluorescence measurement at this wavelength is not affected by the presence of the Eu^{3+} fluorescence label.

Using this assay format, the sensitivity of CEA measurement is also increased by the multiple labeling via the biotin−streptavidin binding.

The calibration curves for the simultaneous measurement of AFP and CEA are shown in Figure 6. The plot of the background-subtracted signals versus antigen concentrations shows almost linear calibration curves for both AFP and CEA. The detection limits of the present method are calculated[26] to be 44 pg/mL for AFP and 76 pg/mL for CEA. These detection limits are low enough to measure AFP and CEA in human sera. Compared with our previous method for simultaneous determination of AFP and CEA by using Eu^{3+} and Sm^{3+} chelates as the labels,[18] the present method is simpler and more sensitive, especially for CEA, for which the previous Sm^{3+} label has been replaced by the Tb^{3+} label. The AFP and CEA concentrations in 39 human serum samples were determined with both the simultaneous measurement method and the single-label assay method. Correlation of the two methods is shown in Figure 7. The correlation coefficients of AFP and CEA are 0.991 and 0.994 with the correlation equations of $y = 1.081x - 0.184$ and $y = 1.092x + 0.646$ (y, the results of simultaneous measurement; x, the results of single-label assay), respectively. Although the slopes of the two statistical straight lines are somewhat higher than 1.00, and their intercepts are not zero, these might be caused by some unexpected errors of the two measurement methods, and the results are in the range of normal measurement errors. These results indicate that the two antigens can be simultaneously determined in the present method with enough accuracy and precision. Based on the above results, it is concluded that the TR-FIA by using BPTA−Tb^{3+} is highly sensitive and can be practically applied to the general clinical analysis. Although the sensitivity of the measurement is lower than that of our previous label, BHHCT−Eu^{3+},[27,28] the present Tb^{3+} label is more sensitive than BHHCT−Sm^{3+} chelate.[18]

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