Ultrasensitive Determination of Europium Using Microsecond Time-resolved Spectrofluorimetry

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4,7-Bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) is proposed as a simple, highly sensitive and specific reagent for the ultrasensitive determination of Eu^{3+} with microsecond time-resolved spectrofluorimetry. The optimum reaction pH was 9.0 in a glycine buffer. Detection limit for Eu^{3+} was 3×10^{-13} mol l^{-1}. No major interferences from other metals were found when the interferent was present in a 500-fold molar excess. Negative interference was observed at high ($>1 \times 10^{-3}$ mol l^{-1}) concentrations of Fe^{3+} and Ca^{2+} presumably through the formation of non-fluorescent complexes with BCPDA.

Keywords: *Lanthanide-ion chelates; time-resolved spectrofluorimetry; europium; ultrasensitive assay; trace metal determination*

Lanthanide-ion chelates have already been widely used as fluorescent probes in studies of alkaline earth metal interactions with proteins and enzymes,[1] in monitoring nucleic acid conformations[2] and in the characterization of metal-ion binding sites in fulvic acids.[3] The Eu^{3+} ion has been the lanthanide of choice in most of these studies and its spectral features are well established.[4,5] In addition, valuable information is available about the solution properties of aqueous nuclear magnetic resonance (NMR) shift reagents[6] when combining lifetime and high-resolution luminescence spectral measurements.

Another area of wide application of Eu^{3+}, of greater analytical interest, is its use for the labelling of antibodies in immunoassays and nucleic acid probes in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) hybridization techniques.[7] In these methods, the Eu^{3+}-labelled biospecific reagent is usually produced by the covalent bonding of the macromolecule with an ethylenediaminetetraacetic acid (EDTA) derivative and then incubation of the product with Eu^{3+}. After the immunoreaction or following nucleic acid hybridization to a specific target, bound Eu^{3+} is released by lowering the pH. It can then be complexed to a suitable organic ligand (*e.g.*, β-naphthoyltrifluoroacetone) that is capable of energy coupling with Eu^{3+}, to produce a highly fluorescent chelate, which is subsequently measured in solution by using microsecond time-resolved spectrofluorimetry. This measuring technique is applicable because of the very long decay time of the fluorescent Eu^{3+} chelates (0.4–1 ms).

The determination of Eu^{3+} in biological fluids is also of interest, as recently the lanthanides have been related to some pathological conditions.[8]

In the present work, a simple, highly sensitive and specific time-resolved spectrofluorimetric determination of Eu^{3+} is proposed, by using a recently synthesized[9] compound, 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2.9-dicarboxylic acid (BCPDA) (Fig. 1). In the Eu^{3+}–BCPDA complexes, the excitation radiation is absorbed by BCPDA and is then transferred to the Eu^{3+} ion by an internal energy transfer process resulting in europium-ion emission. The fluorescence lifetime of the complex has been found to be in the 0.44–0.76 ms range,[9] which makes it suitable for microsecond time-resolved spectrofluorimetric measurements. Furthermore, BCPDA carries two sulphonyl chloride groups capable of reaction with the amino groups of protein molecules and thus it has been successfully applied to the labelling of antibodies or streptavidin for immunoassays.[10–12] In all these applications, an excess of Eu^{3+} is added after the completion of the immunoreaction and BCPDA is quantified by the fluorescence emitted from the complexed Eu^{3+}. In this work the prospect of BCPDA as a highly sensitive and specific reagent for the determination of Eu^{3+} in aqueous solutions is examined.

Experimental

Reagents

Europium chloride, 1 mmol l^{-1} stock solution, was prepared by dissolving 99.99% pure Eu_2O_3 (Sigma, St. Louis, MO, USA) in 10 mmol l^{-1} HCl. Working solutions of Eu^{3+} in the range from 1×10^{-12} to 1×10^{-7} mol l^{-1} were prepared by successive dilutions of the stock solution in 1×10^{-4} mol l^{-1} HCl.

Ethanolic solution of BCPDA (5×10^{-3} mol l^{-1}). A 0.077 g portion of BCPDA (CyberFluor, Toronto, Canada) was dissolved in 25 ml of ethanol. Solutions of BCPDA of various concentrations and pH were prepared by dilution of this stock solution in the appropriate buffer.

Buffers. Citrate, pH 2.5 and 3.0; acetate, pH 4.5; 2-morpholinoethanesulphonic acid (MES), pH 6.0; 3-morpholinopropanesulphonic acid (MOPS), pH 7.0; *N*-(2-acetamido)-2-iminodiacetic acid [ADA, *N*-(carbamoylmethyl)imino-

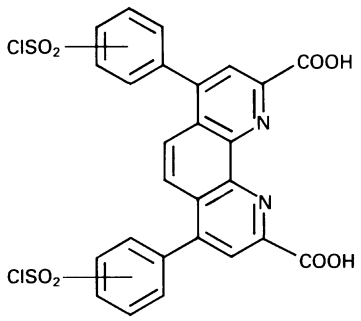

Fig. 1 Structure of the Eu^{3+} chelator, 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA)

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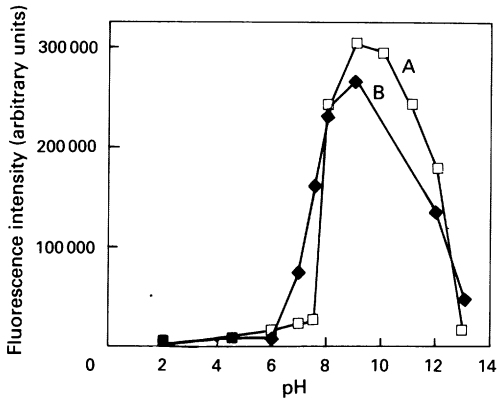

Fig. 2 Effect of pH on the fluorescence intensity of the Eu^{3+}–BCPDA complex. Concentration of Eu^{3+}, 1×10^{-9} mol l^{-1}. Concentration of BCPDA: A, 1×10^{-6}; and B, 1×10^{-4} mol l^{-1}

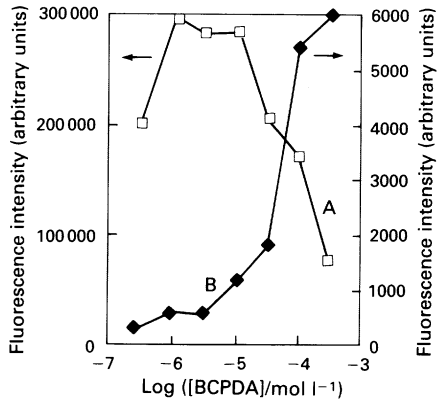

Fig. 3 Effect of BCPDA concentration on the fluorescence intensity of the Eu^{3+}–BCPDA complex at a constant Eu^{3+} concentration of 1×10^{-9} mol l^{-1} (A). Background signal (B) was obtained in the absence of Eu^{3+}

diacetic acid], pH 7.0; 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES), pH 7.5; tris(hydroxymethyl)-aminomethane (Tris), pH 8.0; glycine, pH 9.0; and carbonate, pH 10.0 and 11.0, were prepared at a final concentration of 1×10^{-2} mol l^{-1}, by weighing the acid or the corresponding salt and adjusting the pH with NaOH or HCl, respectively. For pH 12.0 and 13.0, 0.010 and 0.10 mol l^{-1} NaOH solutions were used, respectively.

Apparatus

Time-resolved spectrofluorimetic measurements, of the solutions, were performed on an Arcus Model 1230 fluorimeter (LKB Wallac, Turku, Finland).

Procedures

Europium determination, under various conditions, was carried out by mixing 200 μl of the reagent (BCPDA in a suitable buffer) with 20 μl of aqueous Eu^{3+} solution, followed by vortexing and pipetting 200 μl into clear plastic microtitre wells (Dynatech Laboratories, Chantilly, VA, USA). All results refer to the final Eu^{3+} concentration in the measured solutions.

Results and Discussion

The Eu^{3+} binding site of the BCPDA molecule (Fig. 1) consists of four ionizable groups, *i.e.* two carboxyl and two heteroaromatic nitrogens.

Therefore, at various pH values, different ionic species of BCPDA are present and this might influence the affinity of the molecule for Eu^{3+} and/or the fluorescence of the complex. The effect of pH on the fluorescence of the BCPDA–Eu^{3+} chelate was studied for the pH range from 2.0 to 13.0. The results are presented in Fig. 2. There is a dramatic increase in the signal at pH >7.0 indicating that the dissociation of the carboxylic groups plays a key role in the affinity of BCPDA for Eu^{3+} and/or the energy coupling process. The optimum pH for the fluorescence signal maximum is 9.0. In more alkaline solutions and especially at pH >11, the signal decreases dramatically. This can be attributed to the formation of complexes between Eu^{3+} and the hydroxyl ions at high pH values.

The effect of the concentration of the buffer species, was tested by measuring the fluorescence of a Eu^{3+} solution in 1×10^{-3}, 1×10^{-2} and 1×10^{-1} mol l^{-1} glycine, pH 9.0. No

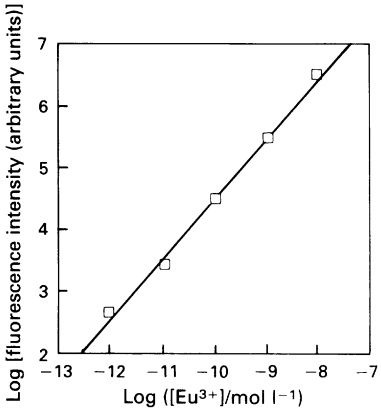

Fig. 4 Calibration graph for the determination of Eu^{3+} at pH 9.0 (1×10^{-2} mol l^{-1} glycine). Concentration of BCPDA, 1×10^{-6} mol l^{-1}

significant change in the signal was observed at the various glycine concentrations. Therefore, a 1×10^{-2} mol l^{-1} glycine buffer of pH 9.0 was selected for further studies.

The optimum concentration of BCPDA in the Eu^{3+} assay was established by varying the concentration in the reaction mixture, in the range from 5×10^{-7} to 5×10^{-4} mol l^{-1} and by measuring the fluorescence of a fixed concentration of Eu^{3+} solution (1×10^{-9} mol l^{-1}). Fig. 3 reveals that at BCPDA concentrations of lower than 1×10^{-6} mol l^{-1}, the complexation is incomplete and the fluorescence relatively low. At BCPDA levels of higher than 2×10^{-5} mol l^{-1}, a decrease in the fluorescence is observed. At such high BCPDA concentrations, the large excess of free BCPDA molecules absorbs most of the available excitation energy so that the remaining energy is not sufficient for the excitation of the relatively small number of Eu^{3+}–BCPDA complexes. The number of the europium complexes that can be excited decreases as the level of free BCPDA becomes progressively higher. Maximum fluorescence readings were taken with BCPDA in the range 1×10^{-6}–1×10^{-5} mol l^{-1} (Fig. 3). Fig. 3 also shows that by increasing the BCPDA concentration above 1×10^{-5} mol l^{-1}, the signal originating from the reagent only (background), increases progressively so that the signal-to-noise ratio deteriorates.

A calibration graph at the optimum pH and BCPDA concentration is shown in Fig. 4. The detection limit for Eu^{3+}, defined as the concentration of Eu^{3+}, which can be distinguished from zero with 95% confidence by using a two-tailed

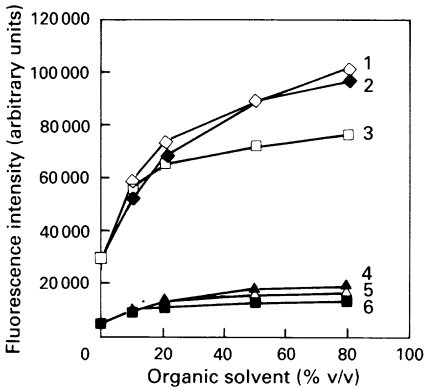

Fig. 5 Effect of organic solvents on the fluorescence intensity of the Eu^{3+}–BCPDA complex at a BCPDA level of 1×10^{-4} mol l^{-1}. 1–3, in the presence of Eu^{3+} (1×10^{-10} mol l^{-1}); and 4–6, in the absence of Eu^{3+}. 1 and 4, Dimethylformamide; 2 and 5, dimethyl sulphoxide; and 3 and 6, acetonitrile

t-test, is as low as 3×10^{-13} mol l^{-1} and the linearity extends to 1×10^{-8} mol l^{-1}. Above this concentration, saturation of the detector occurs.

The possible effect of the presence of organic solvents was studied by measuring a solution of constant Eu^{3+} concentration using reagents containing BCPDA (1×10^{-4} mol l^{-1}), glycine (1×10^{-2} mol l^{-1}, pH 9.0) and dimethyl sulphoxide, dimethylformamide or acetonitrile at various concentrations from 10 to 80% v/v. The results are shown in Fig. 5. There is always an increase in the fluorescence signal with an increase in the organic solvent content. However, a parallel increase in the background signal also occurs and therefore, there is no improvement in the signal-to-noise ratio.

Previous studies[7,13] of the Eu^{3+} complexes with β-diketones (*e.g.*, β-naphthoyltrifluoroacetone) have shown that molecules that have an oxygen atom available (*e.g.*, trioctylphosphine oxide, TOPO) can exclude water from the coordination sphere of Eu^{3+}, resulting in an enhancement of the Eu^{3+} fluorescence. Solubilization of these complexes in surfactant micelles (*e.g.*, 0.1% Triton X-100) causes a further increase in fluorescence. Therefore, the effect of various surfactants on the fluorescence of the Eu^{3+}–BCPDA complexes was tested. Tween-20, -60, -80 and -85, Triton X-15, X-45, X-100, X-305, X-405 and CF-32, Tergitol-4, -8 and NP-14, Lubrol-PX, Brij-35 and Nonidet P-40 (all obtained from Sigma, except Lubrol-PX which was obtained from Pierce) were added to a final concentration of 0.2% in the optimized BCPDA reagent. No change in the signal measured was observed. Addition of 50 μmol l^{-1} of TOPO did not cause any change. This suggests that with BCPDA as a ligand, the coordination sphere of the metal is presumably saturated because there are four atoms (two nitrogens and two oxygens) per BCPDA molecule available for coordination. This gives a total of 8 atoms around Eu^{3+} in the $Eu(BCPDA)_2$ complexes.[14] When β-diketones are used as Eu^{3+} chelators, there are only two oxygen atoms per diketone available for coordination and a maximum of three ligands per metal ion can be complexed which are not enough to complete the coordination sphere of Eu^{3+}.

The interference from other metal ions in the determination of Eu^{3+} was studied by preparing mixed solutions containing 1.7×10^{-9} mol l^{-1} of europium and 8.7×10^{-7} mol l^{-1} of interferent (a 500-fold excess of interferent). It was found that Er^{3+}, Dy^{3+}, Mn^{2+}, Tb^{3+}, Tm^{3+}, Li^{+}, Cs^{+}, Pr^{3+}, Cu^{2+}, Mg^{2+}, In^{3+}, Rh^{3+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, Gd^{3+}, Nd^{3+}, Co^{2+}, Ni^{2+}, Lu^{3+}, Cd^{3+}, Y^{3+}, Ba^{2+}, La^{3+}, Sr^{2+}, Al^{3+}, Ho^{3+}, Rb^{+}, Ce^{4+}, Zr^{3+} and Mg^{2+} caused less than a 5% change in the signal measured. The Ru^{3+} and Sm^{3+} ions caused a −24 and +7% change, respectively. Interference from Ca^{2+} and Fe^{3+} was tested in the concentration range from 1×10^{-6} to 1×10^{-2} mol l^{-1}. The Fe^{3+} ion at 1×10^{-4} mol l^{-1} causes a 20% reduction of the fluorescence which becomes 98% when the concentration is elevated to 1×10^{-2} mol l^{-1}. The Ca^{2+} ion interferes at concentrations of higher than 1×10^{-3} mol l^{-1} causing complete quenching. The reduction in fluorescence caused by Fe^{3+} and Ca^{2+} presumably occurs because these two metals form nonfluorescent complexes with BCPDA thus excluding Eu^{3+} from the complexation site.

The effect of various amino acids on the fluorescence of the Eu^{3+}–BCPDA complexes was also studied, at a Eu^{3+} concentration of 1×10^{-9} mol l^{-1}. Histidine, proline, phenylalanine, hydroxyproline, threonine, glutamine, arginine, aspartic acid, leucine, alanine, methionine, valine and isoleucine at a concentration of 1×10^{-2} mol l^{-1} do not interfere. Tyrosine, at the same concentration, causes a 50% reduction of the fluorescence; tryptophan causes complete quenching at concentrations of higher than 1×10^{-4} mol l^{-1}. These two amino acids absorb strongly at the absorption maximum of BCPDA and presumably interfere by absorbing part of the excitation energy.

Europium is one of the four (Eu^{3+}, Tb^{3+}, Sm^{3+} and Dy^{3+}) rare earth metal ions that are fluorescent when excited by ultraviolet radiation at 615 nm (metal-ion fluorescence). However, the fluorescence of inorganic salts of Eu^{3+} is relatively weak. The fluorescence is dramatically enhanced when the metal ion forms a chelate with appropriate organic ligands. An important property of these chelates is that the radiation is absorbed at a wavelength characteristic of the ligand and it is emitted as the line spectrum of Eu^{3+}. This is owing to an intramolecular energy transfer from the ligand to Eu^{3+} which results in the excitation of the ion. Furthermore, Eu^{3+} chelates emit long-lived fluorescence which allows for microsecond time-resolved spectrofluorimetric measurements.

β-Naphthoyltrifluoroacetone (β-NTA), thenoyltrifluoroacetone[15,16] and dipicolinc acid[17] have been used successfully for the conventional (non-time-resolved) spectrofluorimetric determination of Eu^{3+}. The detection limit of these methods is in the range 6×10^{-11}–6×10^{-10} mol l^{-1}. Time-resolved spectrofluorimetry removes the short-lived undesirable background signals, this, therefore, gives improved sensitivity over conventional spectrofluorimetry. By using the β-NTA–TOPO–Triton X-100 system and time-resolved spectrofluorimetry, detection limits of 5×10^{-14} mol l^{-1} of Eu^{3+} have been achieved.[13] The proposed method is a single reagent assay for Eu^{3+} in aqueous solutions, based on the formation of a highly fluorescent complex between Eu^{3+} and the Eu^{3+} chelator, BCPDA. The signal is measured by using microsecond time-resolved spectrofluorimetry because of the long fluorescence lifetime of the complex. The method is highly sensitive (detection limits of 3×10^{-13} mol l^{-1} for Eu^{3+}) and specific.

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Paper 0/03736B
Received August 14th, 1990
Accepted February 4th, 1991