

A dissociative fluorescence enhancement technique for one-step time-resolved immunoassays

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Received: 6 October 2010 / Revised: 19 November 2010 / Accepted: 26 November 2010 / Published online: 15 December 2010
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Abstract The limitation of current dissociative fluorescence enhancement techniques is that the lanthanide chelate structures used as molecular probes are not stable enough in one-step assays with high concentrations of complexones or metal ions in the reaction mixture since these substances interfere with lanthanide chelate conjugated to the detector molecule. Lanthanide chelates of diethylenetriaminepentaacetic acid (DTPA) are extremely stable, and we used EuDTPA derivatives conjugated to antibodies as tracers in one-step immunoassays containing high concentrations of complexones or metal ions. Enhancement solutions based on different β -diketones were developed and tested for their fluorescence-enhancing capability in immunoassays with EuDTPA-labelled antibodies. Characteristics tested were fluorescence intensity, analytical sensitivity, kinetics of complex formation and signal stability. Formation of fluorescent complexes is fast (5 min) in the presented enhancement solution with EuDTPA probes withstanding strong complexones (ethylenediaminetetra acetate (EDTA) up to 100 mM) or metal ions (up to 200 μ M) in the reaction mixture, the signal is intensive, stable for 4 h and the analytical sensitivity with Eu is 40 fmol/L, Tb 130 fmol/L, Sm 2.1 pmol/L and Dy 8.5 pmol/L. With the improved fluorescence enhancement technique, EDTA and citrate plasma samples as well as samples containing relatively high concentrations of metal ions can be analysed using a one-step immunoassay format also at elevated temperatures. It facilitates four-plexing, is based on one chelate structure for detector molecule labelling and is suitable for

immunoassays due to the wide dynamic range and the analytical sensitivity.

Keywords Time-resolved fluorometry · Dissociative fluorescence enhancement · Immunoassay · Multiplexing · Eu · Sm · Tb · Dy · Antibody

Introduction

Ions of the lanthanides Eu, Sm, Tb and to a lesser degree Dy have successfully been exploited as labels in bioanalytical assay for the last two decades [1–3]. Of all the different ways to apply lanthanide labels to bioanalytical assays, the most common technique is the one which is based on non-fluorescent lanthanide chelates [4, 5]. In this technique, the ion is chelated to polyaminopolyacetic acids which in turn are conjugated to the detector molecule. When the bioaffinity reaction is completed, the lanthanide ions are dissociated from the chelates on the detector molecule, into a fluorescence-enhancing solution containing ligands that form a fluorescent metal complex with the ions. Due to a long fluorescence decay time and intensive fluorescence, time-resolved fluorometry is the detection method of choice, i.e. the fluorescence is measured after the background fluorescence has decayed. In this way, the autofluorescence can be omitted resulting in ultimate analytical sensitivity [4, 6]. This technique has been widely employed in immunoassays, nucleic acid hybridisation assays, receptor-ligand assays, enzyme activity measurements and in various other applications [1, 6–8]. The physicochemical properties of different enhancement solutions and chelates have been discussed in several papers [5, 9, 10–12].

The lanthanide chelates attached to the detector molecule must be stable enough to withstand the

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conditions in the reaction mixture without dissociation of the lanthanide ion from the chelate. On the other hand, the ion should be rapidly dissociated into the enhancement solution before the fluorescence measurement; otherwise, the technique is not fast enough for routine laboratory applications and automation. Commercially available dissociated-enhanced lanthanide fluorescent immunoassay (DELFLIA®) chelates and enhancement solutions meet these criteria in that serum and plasma samples not containing strong complexones can be used whereas samples containing high concentrations of complexones or metal ions should be avoided in one-step assays. This is sometimes a limitation in the clinical laboratory as blood is taken into both serum and plasma tubes and thus there is a need for a technology that works with all types of samples [13].

The aim of this study was to develop an enhancement solution that quickly and efficiently dissociates lanthanide ions from chelates that are stable at harsh assay condition involving strong complexones and heavy metals. One-step assays with high concentrations of citrate, ethylenediaminetetra acetate (EDTA) or metal ions, require that the detector molecule is labelled with an extremely stable lanthanide chelate. Such assays are immunoassays based on citrate and EDTA plasma samples, enzyme activity assays relying on high concentrations of metal ions in order for the enzyme to be active, soil analysis with immunoassays, cytotoxicity assays and nucleic acid assays carried out partly at relatively high temperatures [7–10, 14].

Materials and methods

Reagents and buffers

Ten β -diketone molecules of the formula $R_1-C(=O)-CH_2-C(=O)-R_2$ where R_1 was a naphthyl, benzofuryl, furyl or thienyl group and R_2 was an alkyl chain with two or three carbon atoms substituted with five to seven fluorine atoms,

tri-*n*-octylphosphineoxide (TOPO), Eu (100 nmol/L), Sm (10 μ mol/L), Tb (100 nmol/L) and Dy (10 μ mol/L) standard solutions, isothiocyanate-activated diethylenetriaminepentaacetic acid (DTPA) lanthanide chelates, DELFLIA® clear microtitration strip plates and DELFLIA enhancement solution were obtained from PerkinElmer Inc. Anti-human chorionic gonadotropin beta unit (hCG β) antibody 2,764, alpha-fetoprotein (AFP) antibody IF5 and assay buffer, washing solution and hCG β and AFP-coated cups and calibrators were from the DELFLIA Xpress Free hCG β and AFP Immunoassay Kits (PerkinElmer). EDTA, CuSO₄ and Triton X-100 were purchased from Aldrich.

Characterisation of the enhancement solutions

Fluorescence characteristics of the synthesised β -diketones with Eu were tested in a glycine buffer at pH 2.3 containing various concentrations of the water protecting agent TOPO (25–120 μ M), detergent Triton X-100 (0.05–0.2% v/v) and the β -diketone (1–100 μ M) to be tested. The concentrations of the components of the enhancement solutions were primarily optimised with respect to fluorescence intensity of Eu, fluorescence signal stability, dissociation kinetics of EuDTPA chelates and Eu(β -diketone)₃ complex formation velocity. Fluorescence intensity and analytical sensitivity were determined by first diluting one volume of Eu standard with nine volumes of the enhancement solution to be tested, and then the obtained solution were serially diluted with the enhancement solution. The fluorescence from 200 μ l of the serial dilutions of Eu standards was measured in 96-well microtitration strip plates with a time-resolved fluorometer (Victor D, PerkinElmer). Standard dilutions of Sm, Tb and Dy were prepared and tested as described for Eu in the enhancement solution with the most intensive Eu fluorescence. The fluorescence from Tb and Dy were measured after addition of 50 μ l of dipicolinic acid derivative (DELFLIA Enhancer, PerkinElmer) to the serially diluted standards. Instrument settings used are shown in Table 1. Excitation and emission spectra for the

Table 1 Fluorescence of Eu and Sm in the BFPP-based enhancement solution

Fluorometer settings						Fluorescence ($n=4$)		Limit of blank	
Lanthanide	Excitation filter (nm)	Emission filter (nm)	Delay time (μ s)	Window time (μ s)	Cycle time (μ s)	Counts at 1 nmol/L	Background Signal	Concentration (fmol/L)	95% CI
Eu	340	615	400	400	1,000	1,112,000	113	40	30–64
Sm	340	642	50	100	1,000	6,092	62	2,100	1,610–3,370
Tb	340	545	500	1,400	2,000	571,300	825	130	86–181
Dy	340	572	30	30	1,000	8,824	350	8,500	6,310–13,200

Tb and Dy were measured after addition of dipicolinic acid

lanthanides in the developed enhancement solutions were measured with a PerkinElmer LS5 fluorescence spectrometer. The velocity of fluorescent complex formation and the stability of the fluorescence signal in the enhancement solutions were determined by adding 5 μL of EuDTPA-labelled AFP antibody and 195- μL enhancement solution into microtitration plate wells that were shaken for 5 min at room temperature and measuring the fluorescence as a function of time. Enhancement solutions were stored in dark at 4 °C and the fluorescence from 1 nmol/L of Eu in the enhancement solution was measured at various time points as described above.

Antibody labelling

Detector antibodies in these two assays were labelled with europium chelate of 1-(4-isothiocyanatobenzyl) diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid. The antibody at a concentration of 5 mg/mL was incubated with a 55-fold molar excess of the chelate in 50 mM carbonate buffer pH 9.5 over night at 4 °C. Labelled antibody was separated from unreacted chelates by gel filtration (Superdex 200, 1.6 \times 60 cm, Pharmacia) with a buffer containing 50 mmol/L Tris-HCl, 9 g/L NaCl, pH 7.8, as elution buffer. When the ratio Eu/IgG was determined by measuring the Eu concentration of conjugated antibody against an Eu standard and the protein concentration with absorbance, a conjugation level of ten was achieved. The principle of protein labelling with bifunctional chelating agents and coating of microtitration plates have in great detail been described elsewhere [7].

Immunoassays

Blood samples were taken from volunteers into plain tubes and EDTA-anticoagulant tubes. Informed consent was obtained from all volunteers and the samples were deidentified to ensure privacy. The study site has obtained approval to conduct studies for research purpose from the local ethical committee. The samples were measured in duplicate on a DELFIAXpress[®] immunoanalyzer (PerkinElmer). In the DELFIA, Xpress analyzer sample (10 μL) and assay buffer (25 μL) are pipetted simultaneously into anti-analyte antibody coated cups. In 5 μL , 50 ng of Eu-labelled detector antibody is added and the cups are incubated with shaking at +35 °C for 20 min after which they are washed four times. The fluorescence from the cups is measured after 100 μL of the developed enhancement solution was dispensed and the cups incubated for 5 min at +35 °C. Antibody bound Eu is dissociated from DTPA into the enhancement solution in which it forms intensively fluorescent complexes with the β -diketone. The effect of the complexone

EDTA and heavy metal Cu^{2+} on the stability of the detector molecule label EuDTPA was also tested by adding EDTA or Cu at various concentrations to the assay buffer in order to get total concentrations in the assay mixture ranging from 1–200 mmol/L of EDTA or 0–1,000 $\mu\text{mol/L}$ of Cu. The effect was tested with the analyzer at three different hCG β concentrations in duplicate, 236, 112 and 22 ng/mL.

Results

Fluorescence intensity and signal stability

Of all the β -diketones tested, the structure 1-(2-benzofuryl)-4,4,5,5,5-pentafluoro-1,3-pentanedione (BFPP) had the most favourable properties with respect to fluorescence intensity, analytical sensitivity, complex formation kinetics and signal stability in a glycine buffer at pH 2.3 with 50 μM TOPO and 0.2% (v/v) Triton X-100. This enhancement solution dissociates the lanthanide ions from LnDTPA and forms in 5 min an intensively fluorescing complex between BFPP and lanthanide. Once the Ln(BFPP)₃ complex is formed, the signal is stable for several hours in the solution (Table 2.) and the enhancement solution itself is stable upon storage at +4 °C for at least 18 months (Table 3).

Spectra

Excitation and emission spectra for the lanthanides are depicted in Fig. 1. All four lanthanides emit at one principal wavelength and the emission peaks are narrow and well separated from each other.

Limit of blank and dynamic range

The limit of blank (mean+3SD with 95% CI) for Eu in developed enhancement solution, 40 (30–64) fmol/L, is of the same order of magnitude than that of DELFIA enhancement solution, 80 (115–200) fmol/L. The lowest

Table 2 Kinetics of fluorescent complex formation in the BFPP-based fluorescence enhancement solution at RT

Incubation time (min)	Fluorescence (counts, 10 ³)	CV% (n=3)
5	1,800	4.5
15	1,850	4.2
30	1,853	4.0
60	1,858	4.4
120	1,869	4.7
240	1,849	4.7

Table 3 Storage stability of the BFPP-based enhancement solution at +4 °C

Storage time (days)	Fluorescence (counts, 10 ³)	CV% (n=6)
0	1,252	0.9
6	1,316	1.9
13	1,256	1.1
27	1,237	0.9
41	1,263	1.1
83	1,263	0.3
178	1,259	0.2
345	1,158	0.3
539	1,267	0.6

limit of blank is achieved with Eu followed by Tb, Sm and Dy (Table 1). In the developed enhancement solution the relationship between the Ln concentration and the fluorescent signal is linear up to 10 nmol/L of La and the fluorometer is linear up to 5×10^6 fluorescence counts with the instrument settings shown in Table 1. These characteristics result in a dynamic range that is about 5 logs for Eu and Tb and 4 logs for Sm and Dy, respectively.

Effect of edta plasma and metal ions

In immunoassays of hCG β and AFP the regression equations show no difference in the measured concentration levels between serum and EDTA plasma samples, indicating that the EuDTPA chelate conjugated to the detector

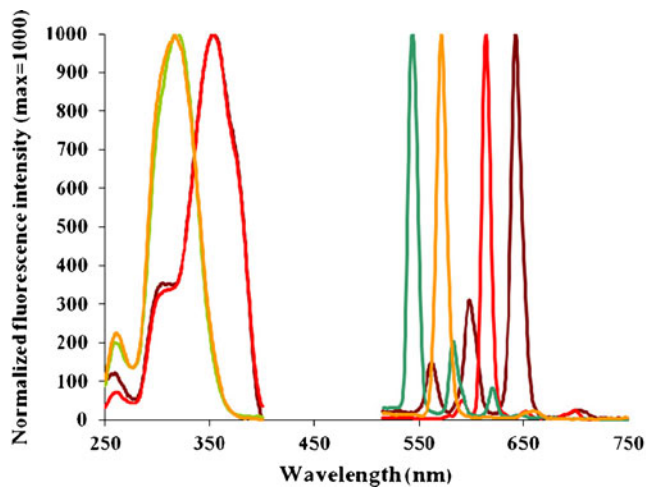


Fig. 1 Excitation and emission spectra for Eu, Sm, Tb and Dy. The spectra for Eu (red) and Sm (dark red) were measured in the BFPP-based enhancement solution. Excitation maxima, emission maxima and decay times obtained were 354 nm, 614 nm and 570 μ s for Eu and 353 nm, 643 nm and 43 μ s for Sm, respectively. The spectra for Tb (green) and Dy (orange) were determined after addition of dipicolinic acid to the BFPP enhancement solution. Excitation maxima, emission maxima and decay times for Tb were 319 nm, 544 nm and 1,190 μ s and for Dy 317 nm, 572 nm and 20 μ s, respectively

antibody is stable and withstands the chelating effect of EDTA in plasma samples (Fig. 2). When EDTA was added to the reaction mixture in an assay of serum hCG β the signal is stable up to 100 mmol/L of EDTA for all three hCG β concentration levels measured. In an assay of hCG β in serum samples, the measured concentrations are unchanged with up to 200 μ mol/L Cu²⁺ present at three different hCG β concentrations (Fig. 3). The criterion for stability was the *P* value for the slopes from the regression analysis of EDTA or Cu²⁺ concentrations present vs. the response in the hCG β assay for all three hCG β concentrations tested. We concluded that if *P*>0.05, the slopes were not significantly different from zero, and hence there were no evidence for dissociation of Eu from the labelled antibody.

Discussion

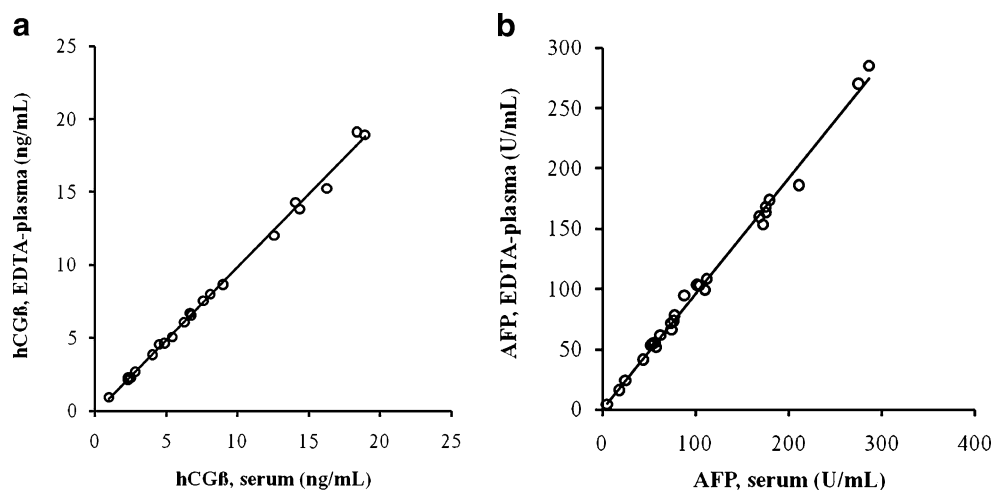
In dissociative fluorescence enhancement techniques relying on lanthanide probes, the fluorescence is measured in an enhancement solution after completion of the biospecific reaction. Immunoassays using this technique are usually performed on microtitration plates in which bound and free tracer is separated by washing and the fluorescence of the bound fraction (solid phase) is measured after addition of enhancement solution to the wells of the plates. An enhancement solution useful for routine laboratory applications is characterized by an intensive fluorescence (absorptivity \times quantum yield), wide dynamic range, rapid dissociation in the enhancement solution of the lanthanide ions from the chelates that are conjugated to detector molecules, fast formation of fluorescent Ln complexes with the components of the enhancement solution, a stable fluorescence signal and long storage stability.

The enhancement solution and diethylenetriaminetetraacetate (DTTA) chelates used in the DELFIA technology meet these criteria to a high degree. In the enhancement solution of this technology, lanthanide ions rapidly dissociate from the DTTA chelates due low pH (3.2). Eu and Sm form a fluorescent complex with the light absorbing trifluorinated β -diketone 2-naphtoyltrifluoroacetone (2-NTA). Lanthanide chelates of DTTA are stable in a reaction mixture not containing strong complexones such as EDTA and dissociate and form highly fluorescent 2-NTA complexes in less than 5 min in the enhancement solution.

In immunoassays intended for the clinical laboratory the main limitation of the present DELFIA technique is that citrate and EDTA plasma samples cannot be used in one-step assay formats since Ln ions may dissociate from DTTA at these conditions.

In this study the goal was to develop a variant of the dissociative enhancement technique that is robust and fast

Fig. 2 Linear regression analysis of serum and EDTA plasma samples analysed for hCG β and AFP using a one-step assay format. Concentration level difference between the two sample types was not observed. **a** hCG β assay, $y=1.001x-0.16$; $y/x=0.329$; $n=22$; $r=0.998$. **b** AFP assay, $y=0.958x+0.611$; $y/x=5.990$; $n=26$; $r=0.996$



enough with samples containing complexones and metal ions at concentrations interfering with LnDTTA chelates. The interference from complexones and metal ions in the biospecific reaction mixture can be avoided by replacing

LnDTTA with chelates possessing a higher stability. DTPA chelates of Ln withstand the citrate and EDTA in plasma samples due to a high stability constant ($K=10^{23}$) [15], but the drawback is the slow dissociation at $\text{pH}>3.0$ (120 min) [16], the lowest pH at which for instance 2-NTA-based enhancements work. The complex formation between Ln ions and 2-NTA is incomplete at lower pH. A long dissociation and complex formation time is unpractical in the laboratory and makes automation difficult. Therefore, a 2-NTA-based enhancement solution together with LnDTPA conjugates is not the best of choices.

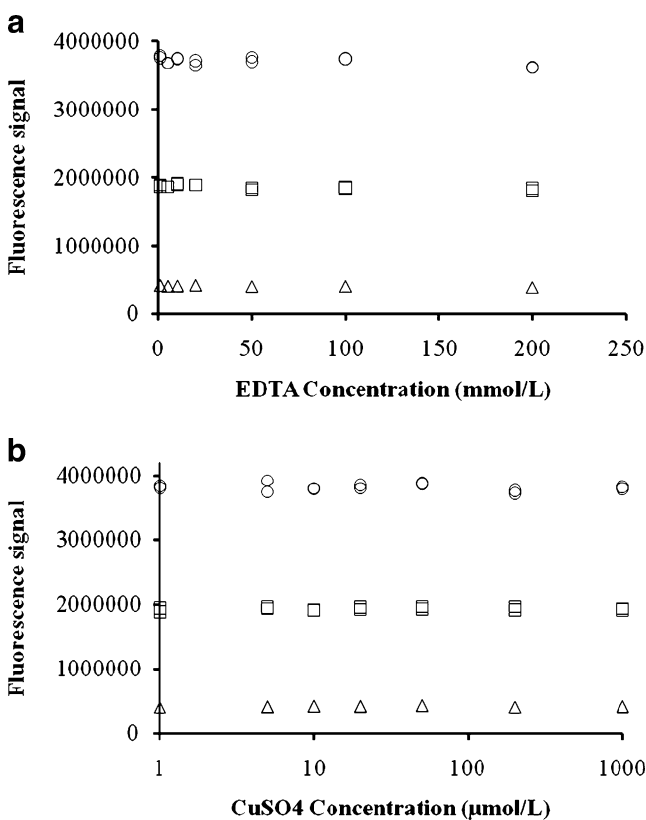


Fig. 3 Effect of EDTA and Cu^{2+} on the stability of the EuDTPA derivative used for detector antibody labelling in the hCG β assay. **a** The P value was >0.05 for up to 100 mmol/L EDTA for all three hCG β concentrations tested (from the highest to the lowest signal level, $P=0.68$ (circles); $P=0.07$ (squares); $P=0.67$ (triangles)). **b** The P value was >0.05 for up to 200 $\mu\text{mol/L}$ Cu^{2+} for all three hCG β concentrations tested (from the highest to the lowest signal level, $P=0.11$ (circles); $P=0.27$ (squares); $P=0.42$ (triangles))

A number of potential β -diketones were screened for their complex formation ability with Ln at pH values below 3. Acidic conditions increase the dissociation of LnDTPA chelates as the equilibrium is towards protonated DTPA and an excess of β -diketone further shifts the equilibrium towards Ln- β -diketone complexes. The β -diketones investigated have three essential features: a light absorbing aryl group, two ketone groups that binds to the coordination sites of the Ln ion and a fluorinated alkyl group that shifts the ketone-enole equilibrium towards the enole form. Five or seven strongly electronegative fluorine atoms make these β -diketones more acidic than 2-NTA with three fluorine atoms. These properties ensure efficient absorption of light upon excitation and complex formation at low pH. Of all the candidate β -diketones tested, the characteristics of BFPP met the requirements best. The benzofuryl group absorbs excitation light efficiently and the pentafluorinated alkyl group shifts the equilibrium towards the enole form also at low pH, which facilitates complex formation with Eu. The developed enhancement solution was primarily optimised for Eu detection and contains BFPP, TOPO and Triton X-100 in a glycine buffer at pH 2.3. Also, Sm fluoresces strongly in this solution. The synergistic agent TOPO and the micelle-forming detergent Triton X-100 further protects the Ln (BFPP) $_3$ complex from fluorescence quenching water molecules by replacing them at remaining coordination sites in

the complex and by solubilizing the complexes in micelles. LnDTPA chelates dissociate rapidly in this solution. Dipicolinic acid derivative at neutral pH is a better fluorescence-enhancing ligand than BFPP for Tb and Dy because of its higher excited triplet state energy level. In quadruple label assays Eu and Sm are measured in the BFPP-based enhancement solution subsequent to the completion of the biospecific reaction and the fluorescence of the dissociated Tb and Dy are detected after addition of a dipicolinic acid derivative containing solution that raises the pH to neutral.

The fluorescence intensities of Eu and Sm in the developed enhancement solution as well as the achieved analytical sensitivities compare well with the established 2-NTA-based enhancement solutions. Tb and Dy can be detected after addition of dipicolinic acid. The dynamic range for Eu and Tb is about 5 logs and for Sm and Dy 4 logs, which is identical to the dynamic range for 2-NTA-based DELFIA enhancement solution. Most of the fluorescence is emitted at one wavelength as a narrow peak and all four lanthanides emit at different wavelengths. They can be distinguished from each other, due to the favourable spectra (spectral resolution) and by utilising their different fluorescence decay times for temporal resolution i.e. employing different delay times when measuring the lanthanides. This permits quadruple-plex assays to be designed, although overlapping spectra must be accounted for and corrected for when detecting Sm in wells with high Eu concentrations and Dy in presence of high Tb concentrations. The assay requiring the lowest limit of detection should be labelled with Eu, followed by Tb, Sm and Dy. Usually up to 20 DTPA chelates can be conjugated to an antibody without any severe effects on the affinity which makes it easy to set up sandwich immunoassays that work in the picomolar range. In the assays for hCG β and AFP the detector antibodies were labelled with an isothiocyanate derivative of DTPA which reacts with amino groups. SH and COO groups can be labelled with iodoacetamido and amino derivatives of DTPA, respectively. There is no dissociation of Eu from DTPA at 35 °C in an assay mixture with human serum containing EDTA, nor is any dissociation observed with 200 μ M of Cu²⁺ present, showing that this enhancement technique can be used in one-step assays containing complexes or metal ions, and at physiological temperatures.

The presented fluorescence enhancement technique for bioanalytical assays offers advantages over current techniques, such as, one-step assays with EDTA and other plasma samples containing complexones as anti-coagulants, lanthanide labelling chelate stability at high concentrations of metal ions and at elevated temperatures, important in some DNA hybridisation assays as well as some enzymatic assays and rapid immunoassays. Four response parameters can be measured from the same assay mixture with the presented technique. The lanthanides are chelated to DTPA

which is stable at a vast range of assay condition resulting in that one chelate structure works for almost all assay designs and applications. Automation is easier with the improved fluorescence enhancement technique due to fewer steps and hence shorter assay protocols.

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