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Comparative study of fluorescent ternary terbium complexes. Application in enzyme amplified fluorimetric immunoassay for α-fetoprotein

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Abstract

A systematic spectrofluorimetric study of the formation of ternary complexes of terbium ions (Tb^{3+}) with ethylenediamine tetraacetic acid (EDTA) and a variety of bidentate organic ligands has been performed. Fourteen ligands (salicylic acid, quinolones and hydroxybenzene sulfonic acid derivatives) were examined as efficient energy donating chelators for Tb^{3+}. The fluorescence properties of the ligands, alone as well as of the ternary complexes with Tb^{3+} and EDTA have been examined. Optimization studies, including the effect of pH, buffer systems and the use of EDTA analogs, were performed. A possible relationship between the structure and the ability of the ligand to act as an efficient light absorbing and energy donating chelator for Tb^{3+} is discussed. The phosphate ester of diflunisal, one of the most sensitive ligands for Tb^{3+}, was used as an alkaline-phosphatase substrate for the determination of α-fetoprotein (AFP) in serum by a highly sensitive enzyme amplified lanthanide luminescence immunoassay by means of second derivative synchronous fluorescence spectroscopy as a detection technique. By using this substrate, AFP was determined in serum with a limit of detection (3×s.d. blank) of 5 pg ml^{-1}, coefficients of variation in the 2.4–5.3% range and mean recovery from four pooled serum samples at four concentration levels (5, 40, 100 and 250 ng ml^{-1}) equal to (99±3)%.

Keywords: Terbium ternary complexes; α-Fetoprotein (AFP); Enzyme amplified lanthanide luminescence immunoassay

1. Introduction

The unique fluorescent properties of some lanthanide chelates and especially those of Eu^{3+} and Tb^{3+} are well documented [1]. The strong lanthanide-ion emission of these complexes originates from intra-chelate energy transfer, where an organic ligand is first excited by absorption of light, followed by energy transfer to the excited energy levels of the emitting ion [2]. Most of the ligands proposed for lanthanide sensitization contain aromatic moieties which strongly absorb in the UV-region [2,3]. To prevent the formation of the relatively strong metal-water bond, which acts as an efficient energy sink, co-chelators or synergistic agents such as aminopo-

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lycarboxylates or tri-*n*-octylphosphine oxide (TOPO), are also included in the complexes [4]. These molecules have strong coordinating properties and can fill the unoccupied slots in the metal-ion coordination sphere.

Lanthanide-sensitized luminescence has been successfully applied in analytical practice for the determination of trace amounts of lanthanide ions [5,6] and of several organic compounds [7], as well as for the development of highly sensitive detection systems for time resolved fluorescence immunoassays [8]. The introduction by Evangelista and co-workers of the enzyme amplified lanthanide luminescence (EALL) method [9,10] has opened new, quite advantageous perspectives in the application of lanthanide complexes and especially those of terbium ions.

The basic principle of the EALL method is that under the action of an enzyme used as a label in immunoassay, a substrate which under the conditions of measurement does not form a highly luminescent chelate with a lanthanide ion such as Tb^{3+} or Eu^{3+}, is converted to a product which does form such a chelate. The amplification provided by using an enzyme as label results in exceptionally low detection limits for the analyte [11]. The applicability of the method has been demonstrated by means of a variety of hydrolytic and oxidative enzymes combined with fluorosalicylic acid as ligand for Tb^{3+} in the form of an appropriate substrate [9]. In an extensive study reported by Diamandis [12] several organic ligands for Eu^{3+} and Tb^{3+} were tested as candidate substrates for EALL-type immunoassay methods.

It is of interest to note that since the introduction of EALL methods, only three alkaline phosphatase (ALP) substrates, i.e. the phosphate ester of fluorosalicylic acid (FSAP) [9,11], diflunisal (DIFP) [13], and salicylic acid (SAP) [14], have been used for EALL-type immunoassays. This fact could be explained by (i) the limited number of enzymes commonly used for labeling and (ii) the limited number of lanthanide ligands with chelating groups capable of being easily converted to appropriate substrates. From this point of view, terbium ligands in which the hydroxyl group is involved in complexation with the lanthanide ion, such as salicylate derivatives or hydroxybenzene sulfonic acids derivatives, seem to be the most promising for EALL methods. The formation of highly sensitive terbium complexes with such ligands occurs in alkaline solutions in the presence of EDTA acting as co-chelator [15,16]. Another group of highly sensitive ligands for Tb^{3+} are those containing at least one carboxyl group or a combination of carboxyl and carbonyl groups. These ligands usually form complexes with Tb^{3+} in weakly acidic or weakly alkaline solutions either in the presence of EDTA or TOPO or their analogs [17,18]. It is of interest to examine such ligands as candidate substrates in EALL-type immunoassay methods.

Here, we report the first part of a comparative study of ligands for Tb^{3+} with different chelating groups, including salicylic acid derivatives, hydroxybenzene sulfonic acid derivatives and quinolones, either for their use as substrates for EALL methods or for the development of highly sensitive methods for the determination of the ligands themselves, since some of them are compounds of pharmaceutical interest. One of the most sensitive ligands for terbium ions, diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) (DIF), was converted to its phosphate ester and a detailed study concerning its kinetic parameters as an alkaline phosphatase substrate, as well as of its self-hydrolysis, was performed. The DIF–Tb^{3+}–EDTA detection system is found useful in an EALL-type immunoassay for the determination of α-fetoprotein (AFP) by means of second derivative synchronous fluorescence spectrometry (SDSFS) as the detection technique [14].

2. Experimental

2.1. Apparatus

A model 512-A double-beam fluorescence spectrophotometer (Perkin–Elmer, Norwalk, CT) equipped with a 150 W xenon arc lamp and a magnetic stirrer under the cell holder was used. The instrument was interfaced to an IBM-PC 386DX microcomputer for data acquisition, estimation of the spectrum derivatives, and automatic evaluation and presentation of the analytical signals. Excitation and emission monochromators were brought under computer control by means of 5-phase 1000-step per resolution stepper-motors, type 564/50 LH (Berger-Lahr GmbH) and a stepping-motor control card, type PCL-838

[19]. For SDSFS measurements, excitation and emission monochromators were locked together and scanned simultaneously with a constant wavelength difference, $\Delta\lambda$=262 nm.

2.2. Reagents

The organic ligands tested are shown in Scheme 1. The ligands were obtained from Aldrich (Steinheim), Sigma (St. Louis, MO), Merck (Darmstadt), Serva (Heidelberg) and Fluka (Buchs). Quinolone antibiotics were donated by pharmaceutical companies. The monoclonal antibodies used for the immunoassay were kindly donated by Dr. E. Diamandis, Clinical Biochemistry Deptartment, University Of Toronto, Canada. The antibiotin-alkaline phosphatase conjugate was obtained from Abbott Laboratories (IMx/HBsAg assay). DIFP was synthesized and purified by continuous recrystallization as previously described [10].

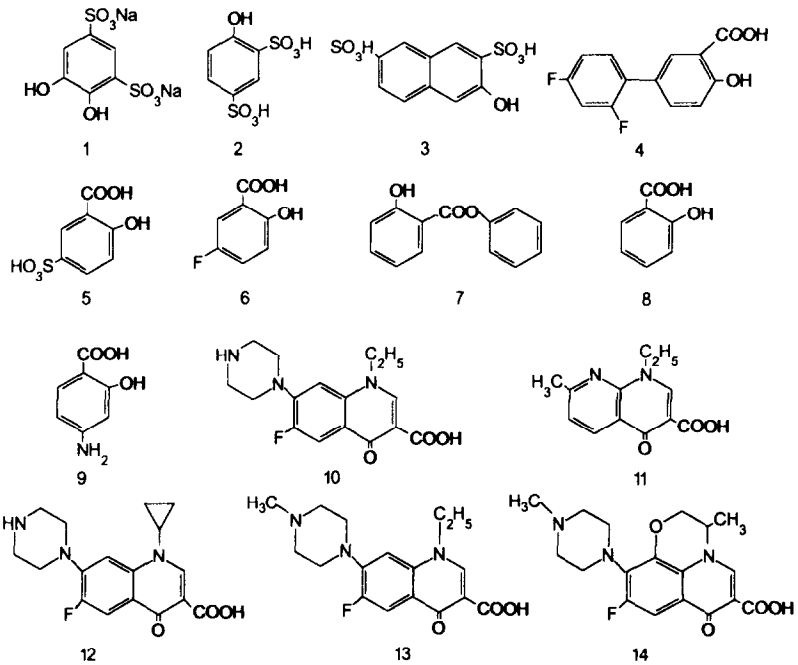
2.3. Solutions

All solutions were prepared in deionized distilled water (DDW) from reagent-grade materials, unless otherwise stated.

The appropriate amounts of the ligands were dissolved in DDW and NaOH (pH~12) to prepare stock solutions of 10 mmol l^{-1}. Ligand working solutions of 0.10 mmol l^{-1} were prepared daily by dilution with DDW. A terbium stock solution of 30 mmol l^{-1} was prepared from terbium oxide [19]. An EDTA stock solution of 30 mmol l^{-1}, a CAPS (3-(cyclohexyl-amino)-1-propanesulfonic acid) buffer solution 125 mmol l^{-1}, of pH 12.6, a tris (tris(hydroxymethyl)-aminomethane) buffer solution 125 mmol l^{-1}, of pH 7.8 and an acetate buffer solution 125 mmol l^{-1}, of pH 5.5 were also prepared.

Mixed working solutions of Tb^{3+} (3 mmol l^{-1}) – EDTA (3 mmol l^{-1}) – CAPS (100 mmol l^{-1}), pH 12.6, Tb^{3+} (3 mmol l^{-1}) – EDTA (3 mmol l^{-1}) – tris (100 mmol l^{-1}), pH 7.8 and Tb^{3+} (3 mmol l^{-1}) – EDTA (3 mmol l^{-1}) – acetate (100 mmol l^{-1}), pH 5.5, were prepared and the final pH was readjusted as needed. Each of the mixed working solutions was used as a single reagent for the measurements. These solutions were stable at 4°C for at least 1 month.

The solutions used for the AFP immunoassay, i.e. coating solution, samples diluent, buffer for ALP substrate and wash solution, were prepared in the manner already described [14]. A DIFP stock



Scheme 1. Chemical structures of the organic ligands tested (the names of the compounds are given in Table 1).

solution, 10 mmol l^{-1}, was prepared by dissolving 13.2 mg of DIFP in 4.00 ml of 0.10 mol l^{-1} NaOH and was kept at 4°C. Working solutions of DIFP were prepared just before use by tenfold dilution of the stock solution in the buffer solution used for the ALP substrate.

The mixed working solution used for the determination of AFP contained 3 mmol l^{-1} Tb^{3+}– EDTA complex and 100 mmol l^{-1} CAPS buffer, pH 12.60.

2.4. Procedures

2.4.1. Study of the ligands

All ligands were studied by mixing 20 µl of the working solution of the ligand under examination with 2.0 ml of the appropriate terbium working solution (final concentration of the ligand: 1 µmol l^{-1}) of various pH values and measuring the fluorescence intensity vs. blank at λ_{em}=546 nm and at the optimum excitation wavelength for each ligand.

2.4.2. Immunoassay procedure

The procedure followed for the AFP immunoassay was identical to the one already described [14], except that DIFP was used as an ALP substrate. Aliquots of 180 µl of the DIF–Tb^{3+}–EDTA complex solution, developed finally in each well, and 700 µl of the working buffer solution were brought into a 1.00 ml cuvette and mixed thoroughly. The synchronous fluorescence spectra were obtained by simultaneously scanning both monochromators at a constant wavelength difference $\Delta\lambda$=262 nm (λ_{exc}=260–320 nm), and the second derivative signals within 280–306 nm range were evaluated.

3. Results and discussion

3.1. Luminescence spectra

The uncorrected luminescence spectra of terbium complexes with three of the most sensitive ligands (Scheme 1, ligands 1, 4, 10) are shown in Fig. 1. Emission spectra in the 480–600 nm range (ascribed to the *f–f* transition) seem to be identical with those so far reported for various terbium chelates (or ions) [1], whereas the excitation spectra are characteristic

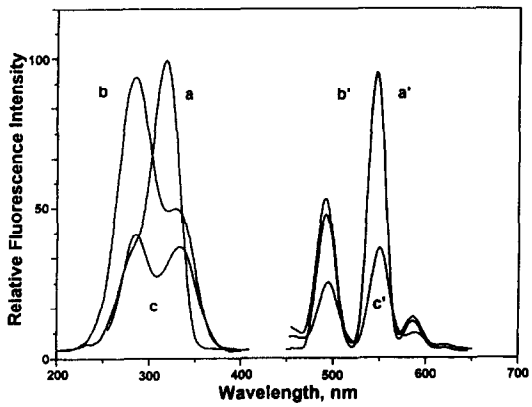


Fig. 1. Fluorescence excitation (a,b,c) and emission (a′,b′,c′) spectra of Tb^{3+}-EDTA-L complexes: a,a′:L=Tiron, λ_{ex}=324 nm, b,b′:L=DIF, λ_{ex}=284 nm c,c′: L=NOR, λ_{ex}=281 nm. C_{Tiron}= 1 µmol l^{-1}, C_{DIF}=1 µmol l^{-1},C_{NOR}=1 µmol l^{-1}, $C_{Tb^{3+}-EDTA}$= 3 mmol l^{-1}, λ_{em}=546 nm.

for each ligand. The maximum excitation wavelengths and the relative fluorescence intensities of terbium complexes measured at λ_{em}=546 nm with all the ligands under investigation are summarized in Table 1. Most of the ligands show an intrinsic fluorescence in the 420–500 nm range, which effectively decreases in the presence of Tb^{3+} due to energy transfer from the organic molecule acting as an *antenna* to the emitting terbium ion.

The luminescence of terbium ions originating within $4f^n$ configurations, when optically excited, is extremely weak due to the low oscillatory strength of their absorption bands [6]. However, an increase in terbium-ion fluorescence could be achieved by its complexation with appropriate ligands, as a result of the ligand field effect or by energy transfer from the triplet state of an organic ligand to the lanthanide ion. Tb^{3+}, as a typical *hard* acid, interacts in aqueous solutions preferentially with hard bases such as oxygen. An interaction with softer bases like nitrogen also occurs in aqueous solutions with ligands such as aminopolycarboxylates since carboxylate interactions produce sufficient dehydration of the lanthanide cation [1]. When mixed ligand complexes of Tb^{3+} with aromatic donors and aminopolycarboxylates are formed, the aromatic ligand, which is usually an oxygen donor, replaces the remaining hydration water molecules from the primary coordination sphere of the terbium-aminopolycarboxylate com-

Table 1
Relative fluorescence intensity and optimum assay conditions of the complexes of various organic ligands with Tb^{3+} and EDTA, (λ_{em}=546 nm)

Number[a]	Organic ligand	Relative fluorescence intensity	Optimum pH	λ_{ex} (nm)
1	**Tiron**	**100**	**12.0**	**324**
2	3-Phenol disulfonic acid	23	12.5	314
3	2-Naphthol disulfonic acid	13	8–12	320
4	**Diflunisal (DIF)**	**92**	**12.6**	**284**
5	5-Sulfosalicylic acid (SSA)	53	12.6	329
6	5-Fluorosalicylic acid	49	12.6	338
7	Salol	32	12.6	328
8	Salicylic acid (SA)	31	12.6	329
9	4-Aminosalicylic acid (ASA)	28	12.6	324
10	**Norfloxacin (NOR)**	**56**	**7.8**	**281**
11	Nalidixic acid	52	7.2	328
12	Ciprofloxacin	40	7.8	283
13	Pefloxacin	19	7.8	283
14	Ofloxacin	11	7.8	300

[a] The numbers correspond to the chemical structures shown in Fig. 1" type="SCHEME.

plexes. The effectiveness of this replacement, which results in a strong enhancement of terbium-ion fluorescence, is mainly brought about by the basicity as well as by the number of the donor groups involved in the complexation process.

Most of the ligands under investigation form fluorescent complexes with terbium in the presence of EDTA, which acts as a co-chelator both by excluding water molecules from the coordination sphere of Tb^{3+} and by preventing the precipitation of terbium hydroxide in alkaline solutions.

It is found that *o*-hydroxycarboxylate ligands form chelates involving the phenolic and carboxylic groups, thus resulting in more stable complexes than those expected from the pK_a values of the aromatic ligand. The magnitude of signals obtained in our study for salicylate derivatives under the optimal conditions for each ligand are in the following order: DIF>SSA>SA≈ASA, while the pK_a values for the corresponding carboxylic groups – ASA>DIF>SA>SSA. The strongest signal obtained with DIF could be explained by both the pK_a value and the presence of fluoro substituents which decrease the effectiveness of the non-radiative processes [10]. SSA, despite the lower basicity of its donor groups compared to SA and ASA, shows greater signals when complexed with terbium. A possible explanation of this fact may be the electron-acceptor properties of sulfonate group which promote the exclusion of water molecules from the coordination sphere of the mixed ligand terbium complex.

o-Dihydroxyphenyl compounds, in which the coordination of the lanthanide ion is proposed to be through the hydroxyl groups [21], represent another group of highly sensitive reagents for the determination of trace amounts of Tb^{3+}. When sulfonate groups are present in such molecules, a much greater increase in fluorescence signal is observed due to the effect of the sulfonate group on the electron-donor capacity of the *o*-dihydroxyphenyl group. For the purposes of our study we have chosen three hydroxysulfonated compounds containing one or two hydroxyl groups on benzene or naphthalene rings. Of these compounds only *Tiron* forms a strongly fluorescent complex with Tb^{3+} and EDTA. In the absence of the second hydroxyl group no complexation with Tb^{3+} was observed, confirming the aforementioned coordination of terbium ion through the *o*-dihydroxy group.

Quinolone derivatives are involved in complexation with Tb^{3+} through the carboxylic and the keto oxygens instead of the phenolic hydroxyl. The optimum pH for complex formation is thus related only to the pK_a value of the carboxylic group. By comparing the magnitude of the fluorescence signals obtained with ligands 10–14, it is obvious that this property is mainly dependent on the substituent attached to the nitrogen atom of the pyridone ring.

The most promising ligands of this group are norfloxacin, nalidixic acid and ciprofloxacin.

3.2. Optimization studies

3.2.1. Effect of pH

The fluorescence intensity of the complexes is pH dependent; the optimum pH for each ligand is given in Table 1. Terbium complexes with salicylate derivatives (ligands 4–9), as well as with *Tiron* (ligand 1) exhibit maximum fluorescence in alkaline solution (pH~12). Optimum pH for the complexes with quinolone derivatives (ligands 10–14) was within the range 7–8.

3.2.2. Effect of terbium and EDTA concentration

The effect of terbium concentration at various EDTA concentrations on the fluorescence intensity of the DIF–Tb^{3+}–EDTA complex was studied. The fluorescence intensity of the complexes at the optimum pH increases with increasing terbium concentration, given a Tb^{3+}-EDTA ratio of at least 1 : 1, as can be seen in Fig. 2. The maximum fluorescence signal is observed at a concentration of Tb^{3+} and EDTA of 3 mmol l^{-1} each.

3.2.3. Effect of EDTA analogs

The effect of other aminopolycarboxylate ligands, when used instead of EDTA, on the fluorescence intensity of terbium complexes with *Tiron* and DIF was examined. The ligands tested were DCTA (1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid), EGTA (ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), IDA (iminodiacetic acid), NTA (nitrilotriacetic acid) and DTPA (diethylenetriaminepentaacetic acid). The fluorescence intensity increased in the following order: DTPA<NTA≈IDA<EGTA<DCTA≈EDTA.

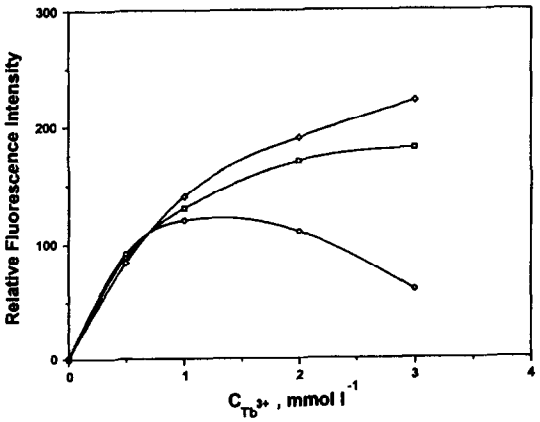


Fig. 2. Effect of terbium concentration on the analytical signal of the DIF–Tb^{3+}–EDTA ternary complex (λ_{ex}=284 nm, λ_{em}=546 nm) at the following EDTA concentrations: 1 mmol l^{-1} (○); 2 mmol l^{-1} (□) and 3 mmol l^{-1} (◇).

The effect of the structure of the aminopolycarboxylic acids, acting as co-chelators on the fluorescence signal of the terbium complexes, on the measured signal is correlated with both the formation constants of their chelates with Tb^{3+} [22], and the effectiveness of the energy transfer from the ligand to the ion. In general, the greater the formation constants, the stronger the signal observed. However, in the case of DTPA, the signals are lower than those expected, taking into account the greater formation constant of its complex with Tb^{3+} in comparison to the other EDTA analogs. This could be explained by the low effectiveness of the energy transfer from DTPA to Tb^{3+} [23].

3.3. Analytical features

Linear calibration ranges for three of the most sensitive ligands for Tb^{3+}, i.e. Tiron, diflunisal and norfloxacin, were studied and limits of detection, defined as the concentration corresponding to a signal equal to three times the standard deviation of the blank, calculated. The linear range for the ligands extended up to 10 μmol l^{-1} and the detection limits were in the $1–3\times10^{-9}$ mol l^{-1} range.

The phosphate ester of diflunisal was recently introduced in EALL methods as a substrate for ALP for the development of an ultrasensitive time resolved immunofluorimetric assay of prostate specific antigen (PSA) [13]. Despite the better sensitivity obtained with this substrate when compared to FSAP, no detailed study concerning the kinetic parameters for DIFP as an ALP substrate, nor its self-hydrolysis has been so far carried out.

3.4. DIFP kinetic study

The kinetic parameters for DIFP as an ALP substrate were determined. Kinetic measurements were obtained at (30±1)°C for 1 U ml^{-1} ALP in the ALP

substrate buffer. K_m and V_{max} values for DIFP were determined as 60 μmol l^{-1} and 0.26 μmol min^{-1} U^{-1}, respectively. As is well known, the substrate concentration must be significantly higher than its K_m value ($S \gg K_m$), so that the signal taken would be proportional to the catalytic activity of the enzyme to be determined [20]. For this reason, four different DIFP concentrations of 0.25, 0.50, 1.0 and 3.0 mmol l^{-1} were studied, corresponding to 4, 8, 17 and 50 K_m, respectively. The entire procedure for the AFP–EALL immunoassay was followed [11,14] and the optimum concentration for DIFP was found to be 1.0 mmol l^{-1}, identical to that proposed in the literature [13].

3.5. DIFP and SAP self-hydrolysis study

Self-hydrolysis of DIFP is very important since it may have a significant contribution to the background signal, thus reducing the sensitivity of the method. A comparative study, concerning the self-hydrolysis of SAP and DIFP in ALP substrate buffer, was per formed. Working substrate solutions were incubated at room temperature for various time periods, followed by addition of Tb^{3+}–EDTA–CAPS working solution and measurement of the fluorescence intensity. As shown in Fig. 3, DIFP is practically stable, unlike SAP.

3.6. Sensitivity

Typical calibration graphs obtained for the determination of AFP by the one step EALL immunoassay, as previously described by means of SDSFS technique [14] using SAP and DIFP as ALP substrates are shown in Fig. 4. The superiority of DIFP as an ALP substrate is clear from Fig.4. The detection limit, defined earlier [14], was found to be 3 pg ml^{-1} and 7 pg ml^{-1} for AFP when using DIFP and SAP as ALP substrates, respectively.

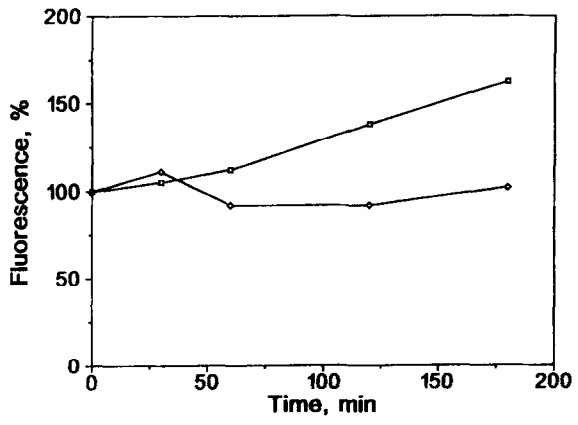


Fig. 3. Self-hydrolysis study of: DIFP (◇) and SAP (□) in ALP substrate buffer (C_{DIFP}=1 mmol l^{-1}, C_{SAP}=1 mmol l^{-1}).

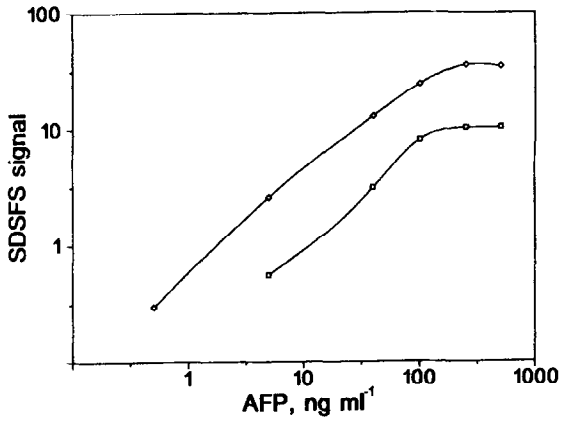


Fig. 4. Typical calibration graphs for AFP with: DIFP (1 mmol l^{-1}) (◇) and SAP (1 mmol l^{-1}) (□) as ALP substrates, with SDSFS detection.

From a comparative study of DIFP and SAP as ALP substrates for the EALL-type immunoassays, it is obvious that the main criteria for choosing new substrates must be not only the low detection limits, but also their kinetic behavior as enzyme substrates as well as their stability under non-enzymatic conditions. DIFP fulfills the aforementioned criteria in terms of sensitivity, K_m value and stability to self-hydrolysis when compared to SAP (Figs. 3 and 4), and thus is the preferred substrate for ALP.

3.7. Recovery and precision

AFP-supplemented serum samples were prepared by adding known concentrations of exogenous AFP to four pooled serum samples at four concentration levels (5, 40, 100 and 250 ng ml^{-1}). Analytical recovery was assessed by analyzing the serum samples in triplicate before and after the additions. The mean (±SD) recovery was (99±3)% (n=12).

Precision was assessed by analyzing six times pooled serum samples spiked with AFP at three concentration levels (5, 40, and 100 ng ml^{-1}). The within-run coefficients of variation were in the 2.4–5.3% range.

4. Conclusion

In this comparative study, the terbium luminescence sensitized by a variety of bidentate organic ligands in the presence of aminopolycarboxylates was examined. The effect of the structure of the ligands involved in the formation of terbium ternary chelates has been discussed and optimization of the experimental variables performed. One of the most sensitive ligands for terbium, DIF, in the form of its phosphate ester, DIFP, has been used as an alkaline phosphatase substrate in EALL-type fluorescence immunoassay for determining of AFP by means of SDSFS as a detection technique. A comparative study between two ALP substrates, viz. DIFP and SAP, has also been performed.

Work is now in progress to exploit other groups of ligands for terbium ions capable of being used as substrates for the development of highly sensitive EALL methods as well as for developing simple and sensitive methods for the determination of fluoroquinolone antibiotics in biological fluids.

Acknowledgements

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