Improved detection of salicylic acids using terbium-sensitized luminescence in aqueous micellar solutions of cetyltrimethylammonium chloride

Nadine Arnaud and Joseph Georges*

Laboratoire des Sciences Analytiques (UMR 5619), Université Claude Bernard–Lyon 1, 69622 Villeurbanne Cedex, France. E-mail: j.georges@cpe.fr

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The determination of salicylic, *p*-aminosalicylic and 5-fluorosalicylic acids was investigated using terbium-sensitized luminescence in aqueous solutions. Formation of a ternary chelate between terbium, EDTA and the salicylic acid requires dissociation of the phenol group which is adjacent to the dissociated carboxylic group. The reaction is obtained in alkaline solutions and is enhanced in the presence of cetyltrimethylammonium chloride. As evidenced by absorbance and fluorescence measurements, the cationic surfactant plays an important role in the formation of the ternary chelate and then terbium luminescence depends mainly on the extent of chelate formation. Linearity is found over more than four orders of magnitude and detection limits are in the range (2–4) $\times$ 10^{-10} mol l^{-1} for the three acids.

Introduction

Sensitization of europium or terbium luminescence by organic donor chromophores has been extensively studied with the aim of developing spectrofluorimetric methods for the detection of organic analytes in batch solutions or coupled with separation techniques[1–3] and for fluorescence labelling in clinical chemistry and molecular biology.[4–8] Europium and, especially, terbium ions can form stable complexes or chelates with various organic ligands and undergo intramolecular energy transfer through the triplet state of the ligand to the emitting level of the lanthanide ion. The main features of the absorption–energy transfer–emission process are a large Stokes shift, a narrow-band emission which is specific of the lanthanide and a long fluorescence lifetime.

Salicylic acid and its substituted derivatives can form luminescent terbium chelates in the presence of EDTA at high pH. Terbium-sensitized luminescence has been used for monitoring salicylic acid (SA) in human blood in cases of long-term therapy or accidental poisoning with aspirin.[9] The same method has been proposed to determine salicylic acid and its derivatives either without a separation step[10,11] or after separation using capillary electrophoresis.[12,13] *p*-Aminosalicylic acid (*p*-ASA) has been used as a sensitizing reagent in the determination of bovine serum albumin.[14,15] 5-Fluorosalicylic acid (FSA) has been found to form highly fluorescent chelates with terbium for application as labels in time-resolved fluorimetric immunoassays.[6] The phosphate derivatives of salicylic acids have been investigated for the detection of alkaline phosphatase and other enzymes using the enzyme amplified lanthanide luminescence (EALL) method.[16–18] In this method, the phosphate derivative (substrate) of a salicylic acid is converted by an alkaline phosphatase to the salicylic acid (product), which then can form a fluorescent ternary complex with terbium and EDTA. The use of salicylic acid and of its derivatives is desirable to extend the variety of enzymes which can be detected. The nature and position of the substituent on the benzene ring can change the performance of the substrate–product system with respect to (i) their ability to chelate the lanthanide ion, (ii) the shift between their absorption maxima and (iii) the position of the excited state energy levels.

In order to improve the sensitivity of bioanalytical assays, it is therefore necessary to improve the detectability of salicylic acid derivatives. It is known that these chelates form only at pH $>$ 12, and that terbium emission is unstable because of the tendency for terbium ions to form hydroxides in alkaline solutions. The purpose of this work was to compare the fluorimetric determination of SA, *p*-ASA and FSA with the aim of improving the performance of the method. pH requirements were investigated in pure water and in the presence of cetyltrimethylammonium chloride (CTACl) as cationic surfactant. The results are discussed with respect to the optimum conditions necessary not only to form the ternary chelate but also to achieve the best signal-to-blank ratio.

Experimental

Apparatus

Absorption spectra were recorded on a Beckman (Fullerton, CA, USA) M25 UV/VIS spectrophotometer. Steady-state fluorescence measurements were carried out on a Jobin-Yvon (Longjumeau, France) JY3 spectrofluorimeter with a 1 cm quartz cell and an R928 (Hamamatsu, Tokyo, Japan) photomultiplier tube. All pH measurements were performed using a digital readout meter and a TC 200 glass and reference unitubular electrode.

Reagents

SA, *p*-ASA and tris(hydroxymethyl)aminomethane (TRIS) (Sigma, St. Louis, MO, USA), FSA, terbium chloride and EDTA, disodium salt (Fluka, Buchs, Switzerland) were used without further purification. The p*K* values of SA are 2.97 and 13.40 for the carboxylic and phenol groups, respectively.[19] The dissociation constants of *p*-ASA and FSA are not known and are expected to be of the same order.

The cationic surfactant was a solution of CTACl 25% in water (Fluka). The chloride was preferred to the bromide because the Krafft temperature of the chloride is 11 °C instead

of 20 °C for the bromide,[20] which was a drawback to working at room temperature in saline solutions. The required amount of surfactant in the test solution was obtained by dilution of the commercial solution. Stock standard solutions of terbium(III) were prepared by dissolving the chloride salt in de-ionized water which was slightly acidified with HCl.

Methods

In order to form luminescent chelates between salicylic acids and Tb^{3+}, it is necessary to work in highly alkaline media, probably because of the high p*K* value of the phenol group which is adjacent to the carboxylic group. However, in the absence of EDTA, terbium hydroxides begin to form at pH higher than 7–8.[21] EDTA not only prevents the formation of terbium hydroxides, but also acts as a coligand to exclude water molecules from the coordination sphere of the lanthanide. In the resulting Tb–EDTA–SA complex, the terbium ion should be coordinated in eight positions.[17] Diethylenetriaminepentaacetic acid (DTPA) could be used instead of EDTA in order to protect more efficiently terbium ions from non-radiative decay processes.[22] However, these attempts failed because Tb^{3+} has a very high affinity for DTPA with a K_a value of about 10^{23}, which then would prevent further association with the salicylic acids. Indeed, addition of DTPA in a Tb–EDTA–FSA–CTACl solution was followed by a decrease and even complete disappearance of the absorption band of the ternary chelate. Working solutions were prepared in de-ionized water with 10^{-2} mol l^{-1} TRIS buffer adjusted to the required pH by adding NaOH or HCl. Because the pH of the buffer solution could be slighly changed upon addition of the reagents, the pH values reported in this work are those of the final solution. Separate stock standard solutions of terbium chloride and EDTA were first prepared in slightly acidic and basic water, respectively, then the required amounts of EDTA and terbium were added to the buffer solution while keeping always a small excess of EDTA with respect to terbium (1.2 :1 concentration ratio). The fluorescence intensity of the ternary chelate at a fixed concentration of the salicylic acid and at the optimum pH was shown to increase as the Tb–EDTA concentration was increased. However, the blank signal due to weak absorption of EDTA at the excitation wavelength increased in a similar manner and the best compromise in terms of blank and analyte signal separation was obtained for a terbium concentration close to 10^{-4} mol l^{-1}.

The analytical procedure used to construct the calibration graphs was as follows. A 10 ml volume of the blank solution (pH adjusted at 12) containing the TRIS buffer, the required excess of EDTA and Tb^{3+} and 0.1% m/m CTACl was pipetted in a polystyrene flask. Incremental additions of salicylic acids were made using appropriate stock standard solutions in order to avoid dilution effects during the course of an experiment. The solutions were stirred for 1 min, after which the terbium emission was recorded. The fluorescence was measured using the peak height at 545 nm. All measurements were corrected for the background fluorescence of the blank. The excitation wavelengths were 330, 310 and 335 nm for SA, *p*-ASA and FSA, respectively. Experiments were carried out in plastic rather than glass containers in order to avoid memory effects of terbium adsorbed on glass vessels. Fluorescence cuvettes were cleaned with dilute nitric acid.

Results and discussion

Formation of the ternary chelate

The complexation of salicylic acids by terbium(III) results in a red shift of the absorption spectrum of the free acid (Fig. 1). As expected, complexation depends greatly on the pH and is favoured when CTACl is present in the solution at a concentration greater than the critical micelle concentration. In the absence of surfactant, complexation is very weak even in alkaline solutions. Owing to the p*K* value of the phenol group (12–13), the optimum pH for complex formation seems to be related to the dissociation of the phenol group which is adjacent to the carboxylic group. This result corroborates that complexation involves two negatively charged oxygens available in a single ligand to form a chelate. In contrast, when CTACl is present in the solution, complexation begins to occur at pH 9 and is complete at pH 10.5. The red shifts between the free ligand and chelate spectra are 25, 15 and 7 nm for FSA, SA and *p*-ASA, respectively. Other surfactants such as anionic SDS or non-ionic Brij 35 had no effect on the formation of the ternary chelate. It is likely that cationic micelles of CTACl produce strong electrostatic interactions with dissociated salicylic acids. Micelles would then make complex formation easier either because of an increase in the pH locally at the micellar interface or by decreasing the p*K* value of the phenol group of the free acid.[23,24]

Optimization of terbium luminescence

The fluorescence of terbium exhibits three sharp bands around 490, 545 and 590 nm corresponding to the $^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$ and $^5D_4 \rightarrow {}^7F_4$ transitions, respectively. Under optimum conditions, the relative peak intensities are 1, 3 and 0.4, respectively, and further measurements were made at 545 nm. The pH dependence of terbium fluorescence was investigated in the absence and the presence of CTACl (Fig. 2). In the absence of surfactant, fluorescence is weak all over the pH range and maximizes at pH 12–12.5, which corroborates previous results obtained with FSA.[16] As expected from the absorption spectra, the cationic surfactant has a significant effect on the dependence of fluorescence upon pH. Fluorescence increases slowly at pH > 8.5, maximizes at pH 11.5–12.5 and then decreases sharply in more basic solutions. One can conclude that the increase in fluorescence is directly related to the extent of complex formation. Similarly to that observed for the absorbance at 340 nm (Fig. 1), the fluorescence intensity at pH 9 in the presence of CTACl equals the fluorescence obtained at pH 12 without surfactant. At the optimum pH, the addition of CTACl produces

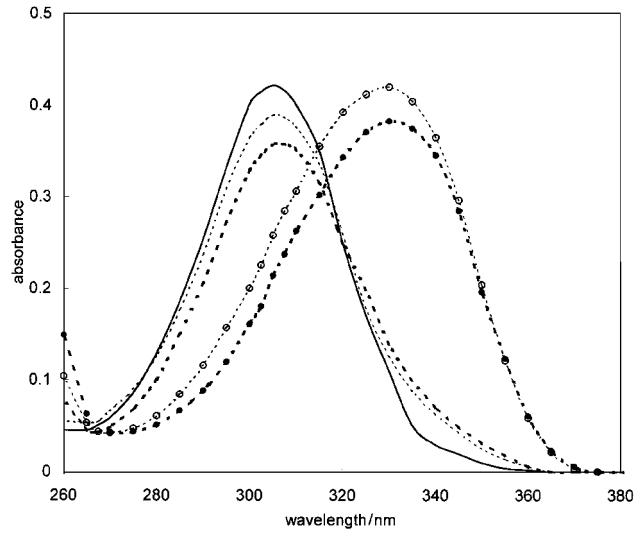

Fig. 1 Absorption spectra of (——) 1×10^{-4} mol l^{-1} FSA and 1×10^{-4} mol l^{-1} FSA + 1.2×10^{-3} mol l^{-1} EDTA + 1×10^{-3} mol l^{-1} Tb^{3+} in (– – – –) water + TRIS buffer at pH 12.5, (- - - -) water + CTACl + Tris buffer at pH 9, (○··○) water + CTACl + TRIS buffer at pH 10.5 and (●--●) water + CTACl + TRIS buffer at pH 12.5.

a 10-fold increase in the peak fluorescence intensity. The influence of CTACl is similar for the three salicylic acids, and the optimum surfactant concentration is about 0.05% (Fig. 3), which is close to the critical micelle concentration (~0.04%).[20]

Calibration graphs and limits of detection

In water, the sensitivity for salicylic acids under the optimum conditions decreases in the order FSA > SA ≈ *p*-ASA (Fig. 4). For SA and ASA, linearities extend over about three orders of magnitude and the limits of detection (LODs) are close to or even less than 10^{-8} mol l^{-1}. For FSA, the sensitivity is significantly better and the LOD is about 5×10^{-9} mol l^{-1}, which is close to the best value reported elsewhere (Table 1). In the presence of CTACl, the sensitivity is greater and is approximately the same for the three acids with linearity ranges extending over at least four orders of magnitude (Fig. 5). It is likely that, in the absence of surfactant, the fluoro substituent decreases the effectiveness of non-radiative deactivation. This effect would be cancelled out in the micellar solution because micelles produce their own protecting effect against non-radiative processes. The slopes of the log–log calibration plots were significantly lower than unity (~0.91) and the regression coefficients were always >0.999. It is known that terbium emission in alkaline solutions is not very stable because of hydroxide precipitation even in the presence of EDTA. The presence of CTACl did not improve the stability and terbium emission was expected to decrease slowly during the course of an experiment. However, the fluorescence intensity–concentration relationships were linear over almost two orders of magnitude except in the highest concentration range where primary absorption effects due to significant absorbance of the solutions may contribute to the downward curvature of the calibration graph. Taking a reaction time of 1 min, the relative standard deviations were between 1.5 and 2.5% depending on the concentration. The LODs defined as the concentration producing a signal which is equal to the background signal plus 3σ of the background signal were in the range (2–4) $\times 10^{-10}$ mol l^{-1} for the three acids, which is far better than the results obtained previously using terbium-sensitized luminescence.

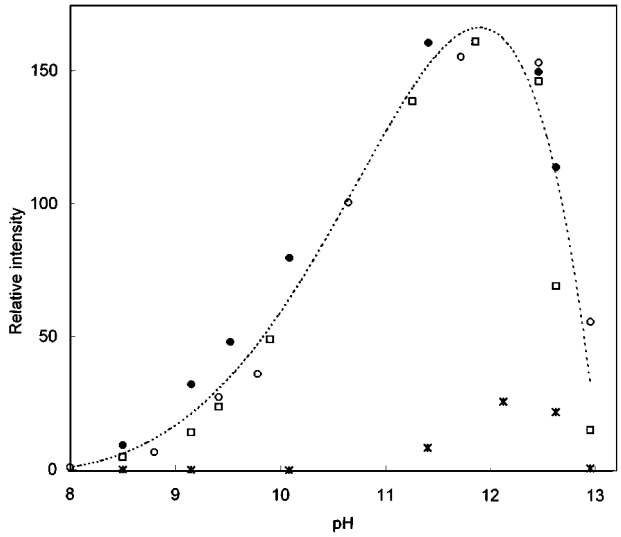

Fig. 2 Influence of pH on terbium fluorescence at 545 nm for 1×10^{-7} mol l^{-1} (○) SA, (●) FSA and (□) *p*-ASA in water + 1×10^{-4} mol l^{-1} Tb^{3+} + 1.2×10^{-4} mol l^{-1} EDTA + 0.1% CTACl + TRIS buffer at pH 12. The asterisks are the values obtained for FSA in the same medium in the absence of surfactant.

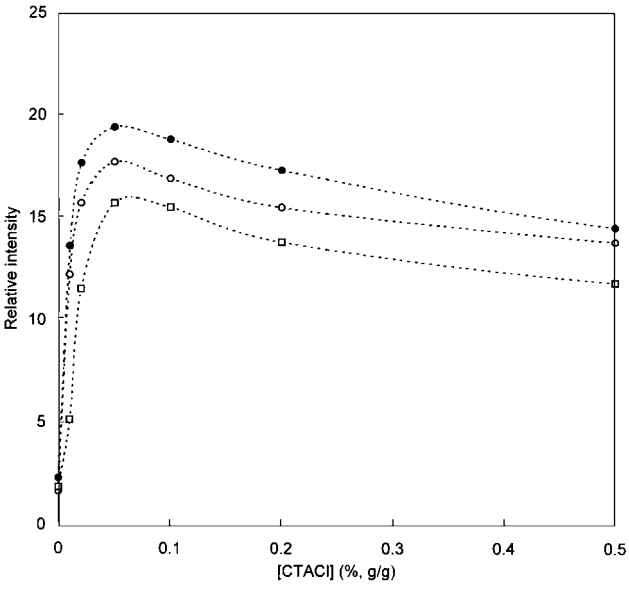

Fig. 3 Influence of CTACl concentration on terbium fluorescence for 1×10^{-7} mol l^{-1} (○) SA, (●) FSA and (□) *p*-ASA in water + 1×10^{-4} mol l^{-1} Tb^{3+} + 1.2×10^{-4} mol l^{-1} EDTA + TRIS buffer at pH 12.

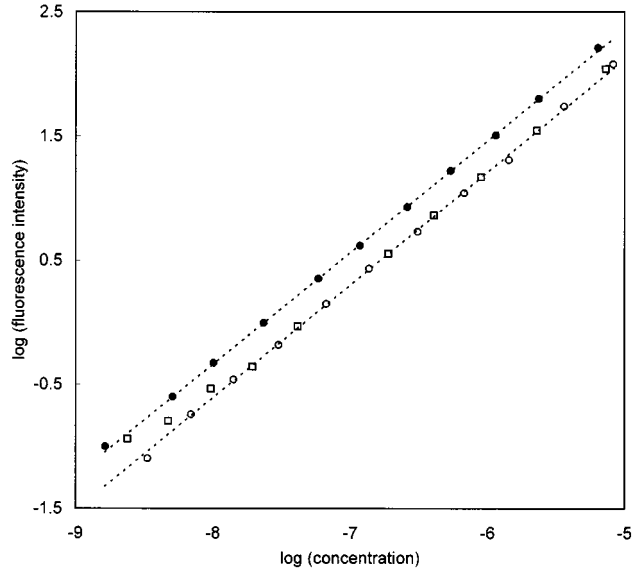

Fig. 4 Calibration graphs for (○) SA, (●) FSA and (□) *p*-ASA in water + 1×10^{-4} mol l^{-1} Tb^{3+} + 1.2×10^{-4} mol l^{-1} EDTA + TRIS buffer at pH 12. The straight lines are linear regression results with log–log slopes of 0.91 and $r > 0.999$.

Table 1 Limits of detection obtained for salicylic acids and comparison with previously published values.

Acid	[Tb–EDTA]/ mol l^{-1}	LOD/ mol l^{-1}	Ref.
Salicylic	10^{-4}	4×10^{-10}	This work
	10^{-2}	2.9×10^{-6}	9[a]
	10^{-3}	8.7×10^{-6}	10[a]
	10^{-3}	2.0×10^{-6}	13[b]
	2×10^{-3}	1.0×10^{-7}	12[c]
	10^{-3}	1.5×10^{-8}	11
Fluorosalicylic	10^{-4}	1.8×10^{-10}	This work
	5×10^{-4}	5.0×10^{-9}	6
	10^{-3}	5.0×10^{-9}	16
Aminosalicylic	10^{-4}	3×10^{-10}	This work
	2×10^{-3}	1.0×10^{-7}	12[c]

[a] Serum samples. [b] Using capillary electrophoresis with post-column addition of Tb and CTABr. [c] Using capillary electrophoresis with reagents in the running medium.

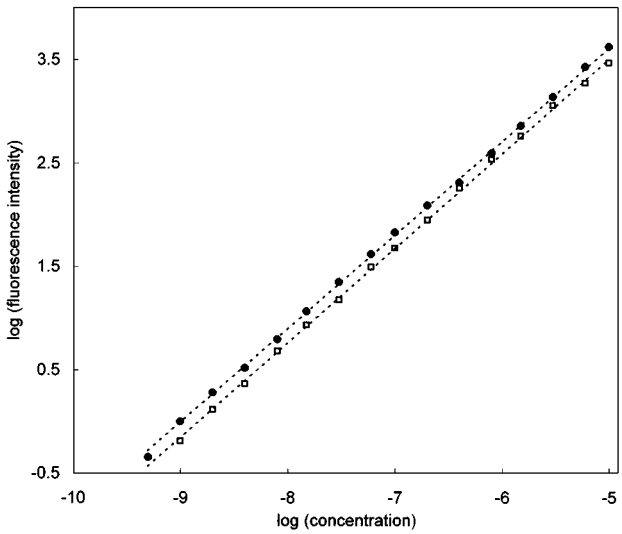

Fig. 5 Calibration graphs for (●) FSA and (□) *p*-ASA in water + 1 × 10^{-4} mol l^{-1} Tb^{3+} + 1.2 × 10^{-4} mol l^{-1} EDTA + 0.1% CTACl + TRIS buffer at pH 12. The straight lines are linear regression results with log–log slopes of 0.91 and $r > 0.999$.

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