Sensitive detection of tetracyclines using europium-sensitized fluorescence with EDTA as co-ligand and cetyltrimethylammonium chloride as surfactant

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The determination of tetracyclines (TC) in aqueous solutions, based on europium-sensitized fluorescence, has been improved using EDTA as co-ligand and cetyltrimethyl ammonium chloride as surfactant. The method involves working in slightly alkaline solutions with the formation of a new chelate where the lanthanide ion is bound to the β-diketone group. The method is about 6 times more sensitive than that with the Eu–TC–Triton system and LODs are 2.5 × 10^-10, 5 × 10^-10, 1.5 × 10^-9 and 2 × 10^-9 mol l^-1 for TC, oxytetracycline, chlortetracycline and doxycycline, respectively. The method has been applied to the determination of TC in calf serum without sample pretreatment. The mean recovery was close to 102% and the lowest concentration attainable in serum samples was better than 0.1 µg ml^-1.

Introduction

Tetracycline (TC) and its derivatives chlortetracycline (Chlor-TC), oxytetracycline (Oxy-TC) and doxycycline (Doxy-TC) are widely used antibiotics. An extensive literature is available on the native fluorescence of these compounds and numerous methodologies have been developed for analytical purposes.1–3 However, it is now well established that tetracyclines can be sensitively detected using europium-sensitized fluorescence in micellar solutions.4–8 Upon complexation with trivalent europium ions, tetracyclines form stable chelates which exhibit broad-banded absorption spectra and the narrow emission band centered at 615 nm and characteristic of the 5D0 → 7F2 transition within the lanthanide ion. Optimum conditions for enhanced fluorescence have been found to be pH 8 with Triton X-100 or cetylpyridinium chloride surfactant.6–8 Even so, thermal lens experiments have shown that only a very small part of the energy absorbed by the ligand is efficiently transferred to the central ion and converted into light.8 The overall fluorescence quantum yield of the Eu–tetracycline–Triton X-100 system is about 0.06 and further improvement in fluorescence efficiency is strongly desired.

Tetracyclines have several proton-donating groups which offer different possibilities of complexation with metal ions depending on the pH.9 The molecules contains three or even four distinct acid groups which are assigned to the A, B, C and D sites, respectively (Fig. 1). The corresponding pK values reported for tetracycline are approximately 3.3, 7, 9 and 13.9–11 The totally protonated form can be written as AB^+CD with one proton on the dimethylamino group (site B). pK1 corresponds to the deprotonation of OH in site A, pK2 is due to the deprotonation of the dimethylamino group, pK3 and pK4 are assigned to OH in the C and D sites, being not possible to distinguish these two sites. The conformation of the Eu–TC chelates can be compared with that of Gd–TC chelates as studied by NMR spectrometry.12 Below pH 8, the conformation of the complex is the same for both forms of tetracycline before and after the first dissociation constant. The metal is probably bound to oxygen atoms of site A. Above the third pK value, the metal lies close to the β-diketone group of site D. According to previous studies,4 the fluorescence intensity afforded by the Eu–TC chelate in the second conformation would be greater, but working at high pH is often difficult owing to the tendency of lanthanide ions to form insoluble hydroxides in alkaline solutions.

The aim of this work was to investigate the possibility to enhance the europium-sensitized fluorescence of tetracyclines using EDTA as co-ligand and cetyltrimethylammonium chloride (CTAC) as surfactant. The luminescence properties of the Eu–TC–EDTA–CTACl system are studied and compared to those of the Eu–TC–Triton system in terms of sensitivity and limits of detection. The new proposed method is then applied to the determination of tetracycline in spiked calf serum samples.

Experimental

Apparatus

Absorption spectra were recorded on a Shimadzu (Tokyo, Japan) UV-1605 spectrophotometer using a 1 cm quartz cell. 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. Fluorescence lifetimes were measured

Fig. 1  Chemical structure and possible acid groups of the tetracyclines.
using a Nd:YAG laser (Mimilite I, Continuum) operated at 355 nm with 2nd and 3rd harmonic generators. The laser beam was sent into a four-window fluorescence cell. Europium fluorescence was collected at right angles to the excitation beam, focused on the entrance of a monochromator, detected by a photomultiplier tube and processed with a boxcar integrator.

Reagents
The hydrochlorides of tetracycline, chlortetracycline, oxytetracycline and doxycycline, tris(hydroxymethyl)aminomethane (TRIS) and Triton X-100 were obtained from Sigma (St. Louis, MO, USA). Europium chloride, the disodium salt of EDTA and cetyltrimethylammonium chloride (CTACl) were obtained from Fluka (Buchs, Switzerland) and used without further purification. CTACl was preferred to CTABr because the Krafft temperature of the chloride is 11 °C instead of 20 °C for the bromide, which is a drawback to working at room temperature in saline solutions. The critical micelle concentration (c.m.c.) of CTACl was determined using the refractive index method. In pure water, one obtained the value of 0.05% which is close to 0.043% reported in the literature, but in the presence of TRIS buffer the c.m.c. is lowered to about 0.01%. The c.m.c. of nonionic Triton X-100 is 0.012%, as determined by the dye micellization method, and was expected not to depend on the salt composition. Samples of calf serum (Sigma) were used without a deproteinization step.

Methods
Stock standard solutions of tetracyclines (1 × 10⁻² mol l⁻¹) were prepared in slightly acidified (HCl) distilled water. Diluted standard solutions were then prepared daily by successive dilution of the stock standard solution. Working solutions were prepared in distilled water with 10⁻² or 10⁻¹ mol l⁻¹ TRIS buffer and the pH was continuously varied in the range 5–12 or adjusted to the required value by adding NaOH or HCl. When necessary, EDTA was used simultaneously with europium at the same concentration in order to prevent the formation of europium hydroxides in alkaline solutions. Depending on the pH, EDTA can also act as a co-ligand to exclude water from the coordination sphere of the lanthanide. Addition of EDTA at pH ≈ 8, i.e., when europium is expected to coordinate with site A of the ligand. On the contrary, when cationic CTACl is added simultaneously with EDTA, complexation is favoured at pH 8 and maximizes at pH 10.

Results and discussion
Absorption spectra
The absorption spectrum of free tetracycline exhibits a maximum ranging from 358 nm at pH 5 to 378 nm at pH 10 (Fig. 2). Complexation upon the addition of europium ions results in a red shift of the absorption spectrum, with peak absorption close to 378 nm at pH 5 and 388 nm at pH 8 and 10. In the absence of surfactant and co-ligand, complexation is observed at any pH but is easier at pH greater than 7 and both spectra are best resolved around pH 8. At this pH, complexation is complete when europium and tetracycline are present at equal concentration, giving evidence for a 1:1 complex as previously reported. On addition of Triton X-100, the absorption bands remained unchanged except for a slight increase in peak absorbance. In the presence of EDTA, complexation is nonexistent at pH 5, decreases strongly at pH 8 but is unchanged at pH 10. It is likely that EDTA prevents further complexation of europium with the tetracycline at pH ≈ 8, i.e., when europium is expected to coordinate with site A of the ligand. The c.m.c. of nonionic Triton X-100 is 0.012%, as determined by the dye micellization method, and was expected not to depend on the salt composition. Samples of calf serum (Sigma) were used without a deproteinization step.

Optimization of europium luminescence
All the tetracyclines studied showed europium-sensitized luminescence with the same wavelength for excitation (405 nm) and emission (615 nm). The pH dependence of europium fluorescence was first investigated in the presence and in the absence of surfactant and EDTA as co-ligand (Fig. 3). In the absence of surfactant, a weak fluorescence is observed in the 5–9 pH range. In the presence of Triton X-100, europium fluorescence increases slightly, maximizing around pH 8 and then decreasing in alkaline solutions due to the precipitation of europium hydroxides. Addition of EDTA at pH ≈ 8 has a
negative effect as expected from the absorption spectra. With CTACl as surfactant, the pH dependence is slightly different and luminescence is much greater. In the absence of EDTA, fluorescence maximizes around pH 9 and then decreases sharply due to precipitation of europium hydroxides. In the presence of EDTA, europium fluorescence is not improved but decreases slower in alkaline solution. Using CTACl instead of Triton X-100 probably facilitates the third deprotonation step leading to a new chelate with improved luminescent properties.

The effect of surfactant on the fluorescence intensity was determined for $1 \times 10^{-6}$ mol l$^{-1}$ tetracycline and $1 \times 10^{-5}$ mol l$^{-1}$ europium with Triton X-100 at pH 8 and CTACl at pH 9. In both cases, europium emission increased with surfactant concentration and became constant just above the critical micelle concentration, i.e., about 0.01% for both surfactants (Fig. 4), which means that the surfactant effect corresponds with the formation of micelles. However, subsequent experiments were carried out with a concentration of 0.1%.

Next, the influence of Eu$^{3+}$ and Eu$^{3+}$-EDTA concentrations was investigated with constant concentration of TC. At pH 8 in the absence of surfactant, fluorescence maximized when the concentration of Eu$^{3+}$ ions was equal that of TC, corroborating the formation of a 1:1 chelate. At higher europium concentration, fluorescence decreased due to a quenching effect of free europium ions. This is detrimental in the case of an analytical application because the concentration of Eu$^{3+}$ cannot be continuously adjusted to that of the tetracycline. On the contrary, in the presence of Triton X-100 at pH 8 or CTACl at pH 9, the europium fluorescence continues to increase after the stoichiometric ratio and then maximizes over a wide range of the molar ratio (Fig. 5).

The luminescence decay constant of europium ions in water has been reported to be 9.11 ms$^{-1}$ ($\tau = 110 \mu$s), corresponding to 9 coordinated water molecules. It is interesting to note that upon complexation with TC, the luminescence lifetime is smaller (Table 1), indicating an increase in the rate constant for non-radiative deactivation which does not involve OH oscillators. Moreover, the plot obtained for the chelate without surfactant exhibits two decay constants (Fig. 6), suggesting the presence of more than one species as previously reported. On the contrary, in the presence of Triton X-100 at pH 8 or with CTACl and EDTA at pH 9, fluorescence decay curves follow first-order kinetics and the luminescence lifetime increases as does the relative fluorescence intensity (Table 1).

### Calibration graphs, detection limits and recovery in serum samples

According to the above results, the best conditions for optimized europium fluorescence are with TRIS buffer at pH 9 in the presence of CTACl as surfactant and EDTA as co-ligand. The calibration graphs obtained in the test solution with standards prepared in water are linear over at least three orders of magnitude (Fig. 7). The response is slightly dependent on the tetracycline, decreasing in the order TC > Oxy-TC > Chlor-

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**Fig. 3** Influence of pH on europium fluorescence at 615 nm for $1 \times 10^{-6}$ mol l$^{-1}$ TC + $1 \times 10^{-5}$ mol l$^{-1}$ Eu$^{3+}$ in TRIS buffer with (x) 0.1% Triton X-100 and with 0.05% CTACl, (C) without EDTA, (O) with $1 \times 10^{-5}$ mol l$^{-1}$ EDTA and (C) with $1.2 \times 10^{-5}$ mol l$^{-1}$ EDTA.

**Fig. 4** Influence of surfactant concentration on europium fluorescence for $1 \times 10^{-6}$ mol l$^{-1}$ TC + $1 \times 10^{-5}$ mol l$^{-1}$ Eu$^{3+}$ in (O) Tris buffer at pH 8 and (O) TRIS buffer at pH 9 in the presence of $1 \times 10^{-5}$ mol l$^{-1}$ EDTA.

**Fig. 5** Influence of the [Eu]/[TC] ratio on europium fluorescence for $1 \times 10^{-6}$ mol l$^{-1}$ TC in TRIS buffer (x) at pH 8 with 0.1% Triton X-100 and (O) at pH 9 with 0.05% CTACl and EDTA ([EDTA] = [Eu$^{3+}$]).

**Fig. 6** Fluorescence decay curves for europium in TRIS buffer (O) at pH 8 without surfactant, (x) at pH 8 with Triton X-100 and (O) at pH 9 with CTACl and EDTA.

### Table 1 Lifetimes and relative intensities for luminescence of Eu–TC chelates in various pH and surfactant solutions. [Eu$^{3+}$] = $10^{-5}$ mol l$^{-1}$, [TC] = $10^{-6}$ mol l$^{-1}$, TRIS buffer

<table>
<thead>
<tr>
<th>Solution</th>
<th>Lifetime/µs</th>
<th>Relative intensity</th>
<th>LOD/mol l$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8, without surfactant</td>
<td>17, 27</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>pH 8, with 0.1% Triton</td>
<td>53</td>
<td>4.3</td>
<td>$2.0 \times 10^{-9}$</td>
</tr>
<tr>
<td>pH 9, with EDTA and 0.05% CTACl</td>
<td>72</td>
<td>25</td>
<td>$2.5 \times 10^{-10}$</td>
</tr>
</tbody>
</table>
TC > Doxy-TC. The LODs (three standard deviations of the blank) are $2.5 \times 10^{-3}$, $5 \times 10^{-3}$, $1.5 \times 10^{-2}$ and $2 \times 10^{-2}$ mol l$^{-1}$ for TC, Oxy-TC, Chlor-TC and Doxy-TC, respectively.

The addition of increasing amounts of calf serum in the test solution decreased the sensitivity up to about 17% when 0.5 ml of serum was added to 10 ml of the test solution. However, the calibration graphs remained perfectly linear and the best compromise between sensitivity and sample dilution was obtained with a dilution factor of 25. The results were also improved when the concentration of CTACl and Eu$^{3+}$–EDTA was increased to 0.5% and $1 \times 10^{-2}$ mol l$^{-1}$, respectively. Analysis of tetracycline in calf serum was made without sample pretreatment. The serum was spiked with appropriate amounts of tetracycline to obtain final concentrations ranging between $2 \times 10^{-7}$ and $5 \times 10^{-6}$ mol l$^{-1}$. Each measurement was carried out using 0.4 ml of spiked serum added to 10 ml of the test solution. The analytical signal was then measured and determination of tetracycline concentration was made by using the method of multiple standard additions. As reported in Table 2, the method is precise and sensitive. The mean recovery is close to 102% and the lowest concentration studied corresponds to about 0.1 mg ml$^{-1}$, which is far below the therapeutic levels in serum.

Table 2  Recovery of tetracycline (TC) added to calf serum samples. TRIS buffer, pH 9, 0.5% CTACl, [EDTA] = [Eu$^{3+}$] = $1 \times 10^{-4}$ mol l$^{-1}$; 0.4 ml of serum added to 10 ml of the test solution

<table>
<thead>
<tr>
<th>Added/mol l$^{-1}$</th>
<th>Found/mol l$^{-1}$</th>
<th>Recovery (%)</th>
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<tr>
<td>$2 \times 10^{-7}$</td>
<td>$2.07 \times 10^{-7}$</td>
<td>103.3</td>
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<tr>
<td>$8 \times 10^{-7}$</td>
<td>$8.27 \times 10^{-7}$</td>
<td>103</td>
</tr>
<tr>
<td>$2 \times 10^{-6}$</td>
<td>$2.01 \times 10^{-6}$</td>
<td>100.5</td>
</tr>
<tr>
<td>$5 \times 10^{-6}$</td>
<td>$5.01 \times 10^{-6}$</td>
<td>100.2</td>
</tr>
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* Average of three or five determinations.

References