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Short Communication

Evaluation of lanthanide-sensitized luminescence spectrometry for the measurement of tetracycline in serum

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Introduction

Tetracycline is a drug that is widely used in the treatment of many different infections. However, patients to whom tetracycline has been administered over a long period of time have often exhibited undesirable side-effects, including hepatic and renal toxicity [1]. A correlation has been found [1] between the level of tetracycline in the serum of these patients and their clinical condition. These findings indicate the need for an accurate, reliable method for the determination of tetracycline in serum. Such a method would also make studies on the pharmacokinetics of tetracycline more feasible.

Many different methods for the determination of tetracycline in urine, serum, and even tissue samples have been developed; references are cited by Faraj and Ali [1] and by Charles *et al.* [2]. These methods include different types of chromatographic, radioimmunoassay, UV-absorbance and fluorescence techniques. Most of these techniques involve extraction steps or the production of an antibody, which makes them complicated and time-consuming. The method described in the present paper is comparable in sensitivity to these techniques, but is much simpler and faster.

The present method is based on the formation of an europium(III)-tetracycline complex, in which the excited ligand (tetracycline) undergoes intersystem crossing to the triplet state; energy transfer then occurs to the 4f-level of the europium(III) ion. This ion exhibits a narrow emission profile which is much more intense than the broad-band emission of the uncomplexed ligand. The formation and properties of this complex are discussed by Hirschy *et al.* [3].

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Experimental

Materials

The hydrochloride salts of tetracycline, doxycycline and chlortetracycline were obtained from Sigma Chemical Company (St. Louis, MO, USA). Europium-(III) nitrate hexahydrate was obtained from Alfa Products, Ventron Division (Danvers, MA, USA).

'Validate and Calibrate Automated Lock In(II) Sera' were obtained from General Diagnostics, Division of Warner-Lambert (Morris Plains, NJ, USA). The 'Validate' serum was reconstituted with distilled water according to the manufacturer's directions; a reconstitution fluid was provided with the 'Calibrate' serum.

Apparatus

Fluorescence measurements were made using a laboratory-constructed fluorimeter, the components of which have been described earlier [3]. In addition to this equipment, a digital voltmeter was used when necessary so that the standard deviation of signals could be determined.

Analytical figures of merit

Stock solutions of tetracycline, doxycycline and chlorotetracycline were made by dissolving appropriate amounts of their hydrochloride salts in distilled water to give concentrations of 0.001 M. Various volumes of these solutions (1 μl, 2 μl, 3 μl, and so on) were added to 3-ml samples of serum, and the signals recorded at the appropriate excitation wavelength, using 617 nm as the emission wavelength. These signals represented blanks. To each mixture 100 μl of 0.01 M europium(III) nitrate hexahydrate was added, and the signals were again recorded. Calibration graphs (see Fig. 1) were constructed by plotting the difference between these two signals against the concentration of antibiotic in serum.

Limits of detection (L.O.D.) were established according to the IUPAC definition [4]:

$$\text{L.O.D.} = \frac{k(s_b)}{m}$$

where s_b = standard deviation of 16 blank readings, m = slope of calibration curve, and k = a constant, chosen to be 3 in this analysis.

Results and Discussion

Figure 2 shows the structural differences between the three antibiotics, and Table 1 gives the limits of detection and excitation wavelengths used for each antibiotic in aqueous solution. The linear dynamic range spanned approximately two decades for each antibiotic.

If europium is added to serum which does not contain any antibiotic, a scan of the emission spectrum reveals a similar scan to that of serum alone. If tetracycline is added to a sample which already contains europium, the signal observed is not as large as that obtained when the europium is added to the tetracycline. In a normal analysis, this effect does not cause a problem because the antibiotic will already be present in the serum; but the effect does eliminate the possibility of making standard additions of the antibiotic to a sample. It appears that some of the europium binds to other serum components and is therefore unavailable to complex with the additional antibiotic.

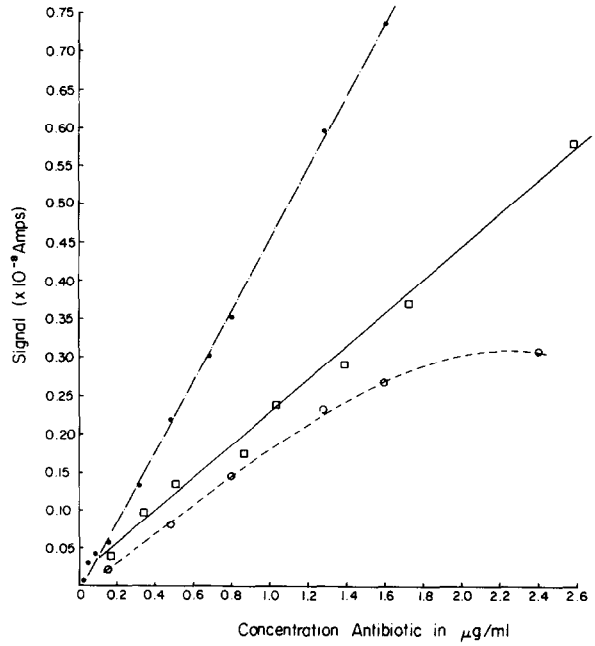
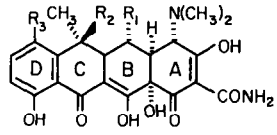


Figure 1
Calibration graphs for: (a) tetracycline; (b) chlortetracycline; and (c) doxycycline. The Eu^{3+} concentration was 149 μg/ml for each standard. The linear regression equations were, respectively; $y = 4.59 \times 10^{-9}x - 2.65 \times 10^{-11}$; $y = 2.16 \times 10^{-9}x + 1.04 \times 10^{-10}$; and $y = 1.89 \times 10^{-9}x + 8.61 \times 10^{-11}$; y = signal level, in units of A and x = antibody concentration in μg/ml.



R_1	R_2	R_3	
H	OH	H	Tetracycline
H	OH	Cl	Chlortetracycline
OH	H	H	Doxycycline

Figure 2
The structure of tetracycline (TC) and its analogues chlortetracycline (CTC) and doxycycline (DC).

These conclusions were verified by examination of standard addition graphs (Fig. 3). The signals increased upon addition of tetracycline, but the slopes of these graphs were much smaller than those obtained from calibration graphs constructed using separate samples for each concentration of tetracycline.

One alternative to the use of standard additions is to use standard dilutions. In this method, 1.00 ml of sample containing the antibiotic and europium was removed and replaced by 1.00 ml of control serum containing no tetracycline or europium. This method can be compared to an analytical calibration graph derived from standards and eliminates problems with samples that might contain an amount of antibiotic which is not within the linear dynamic range. Figure 3 (curve (b)) illustrates the decrease in slope of the calibration curve when standard additions of tetracycline are made to a sample which already contains europium and tetracycline. Figure 3 (curve (a)) illustrates the linearity

Table 1
Detection limits for antibiotics studied by lanthanide-sensitized luminescence spectrometry

Antibiotic	Excitation wavelength (nm)*	Detection limit (ng/ml)	Detection limit Absolute (ng)†
Tetracycline	398	10	0.6
Chlortetracycline	395	40	2
Doxycycline	385	70	3

* Emission wavelength, 617 nm; excitation and emission bandwidths, 2 mm (equivalent to 4 nm).
† Absolute limits of detection are based on the illuminated volume, i.e. for 0.2-cm slit widths and 1.2-cm slit heights.

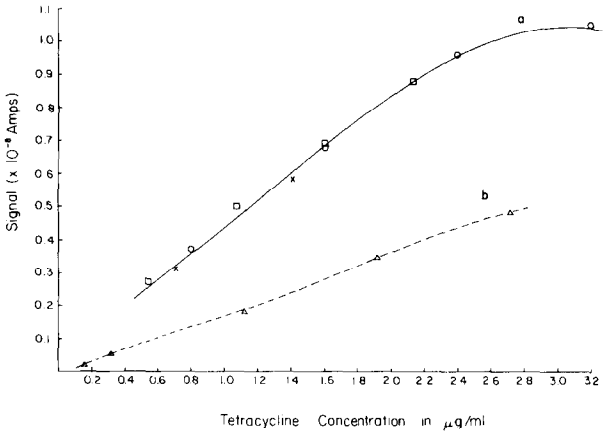


Figure 3
Calibration graphs: (a) Standard dilutions of four tetracycline solutions having initial concentrations (○) of 3.20, 2.40, 1.60 and 0.80 μg/ml, respectively; after one dilution (□), the concentrations were 2.13, 1.60, 1.07 and 0.54 μg/ml; after the second dilution (×), the concentrations were 1.41 and 0.71 μg/ml. The initial Eu^{3+} concentration was 149 μg/ml. (b) Standard additions, where tetracycline is added progressively to a serum sample containing Eu^{3+}.

of the graph obtained by standard dilutions and the advantage of diluting a concentrated sample, which is initially outside the linear dynamic range, so that signals can be obtained that are proportional to concentrations in the linear dynamic range.

It is well known that tetracycline also chelates calcium, a fact that has been used for the determination of tetracyclines [5], although the reported limit of detection (150 ng) is not as good as that reported for the present method. Although tetracycline will bind to the serum calcium to some extent, the stability constant for the europium–tetracycline complex is much larger [6] than that of the calcium–tetracycline complex. Thus the europium should displace the calcium to give the desired effect. This proposal was verified by adding first the tetracycline and then, up to 90 min later, the europium. The fluorescence signal continued to rise for approximately 3 min until binding (or substitution) was essentially complete; at this point the signal stabilized and remained constant.

In all experiments the europium was employed in very large excess with respect to the tetracycline concentration (approximately 100 to 1000-fold). However, by adding 100-μl volumes of the stock europium solution to a sample which already contained tetracycline and europium, the free europium ion began to quench the fluorescence signal of the complex after several 100-μl additions. Therefore, for all other studies, only one 100-μl portion of the stock europium solution was added to each sample. This method gave linear, reproducible calibration graphs, and was the only way found to analyse real samples, where the concentration of tetracycline was unknown.

It was hoped that some specificity could be obtained by the use of different excitation wavelengths for each of the antibiotics. However, it was found by scanning samples containing each of the different antibiotics with added europium, that although each antibiotic had an optimum excitation wavelength, there was an excitation band (about 370–400 nm) where all were excited. Monochromator slit widths of 2 mm were used in the calibration analysis which yielded bandwidths of 4 nm. Fortunately, specificity is not usually a problem because a mixture of tetracyclines is seldom used medically [7].

Figure 4 shows a scan of the typical emission spectra obtained. Sample (a) comprised serum with tetracycline. Sample (b) comprised serum with tetracycline and europium; the emission peak at 617 nm was used in this analysis, the peak at about 595 nm being also attributable to the tetracycline europium complex. Sample (c) was serum alone; because the tetracycline transfers its energy to the europium, the luminescence signal is observed at a wavelength removed from that of the background signal arising from proteins and other serum components.

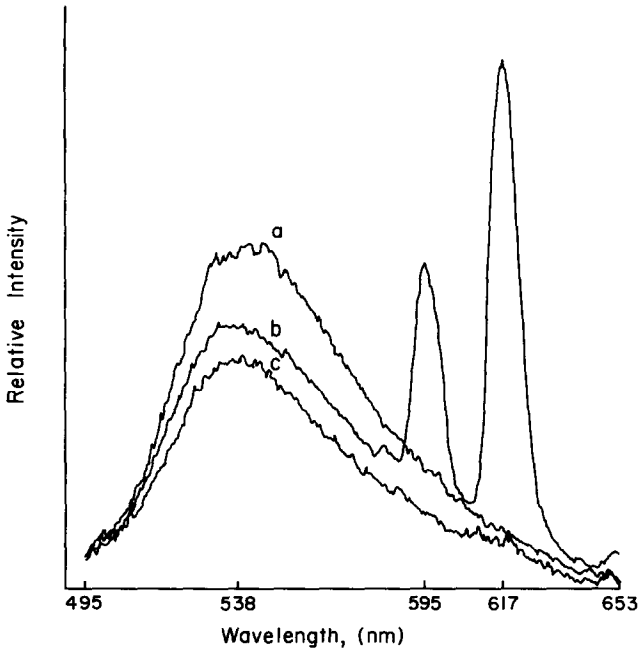


Figure 4
Fluorescence spectra: (a) serum + 1.60 μg/ml tetracycline; (b) serum + 1.60 μg/ml tetracycline + 149 μg/ml europium, and (c) serum alone. Excitation wavelength, 398 nm; spectral bandwidth, 4 nm.

The low limits of detection allow the use of small sample sizes. For example 0.5 ml of serum obtained from a patient can be diluted to 3 ml with control serum prior to analysis. This consideration is important where it is difficult to obtain large blood samples.

It is suggested that this technique should potentially offer a simple, rapid and specific assay for the determination of tetracyclines in serum.

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