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Short communication

Improvements in europium sensitized fluorimetric determination of demeclocycline and methacycline

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Abstract

Demeclocycline (DM) and methacycline (MT) have been determined by europium-sensitized fluorescence, using EDTA as co-ligand and cetyltrimethylammonium chloride as surfactant. The methods have been developed in slightly alkaline solutions, with the formation of a new chelate where the lanthanide ion is bound to the beta-diketone group. Calibration graphs between 0.01 and 0.1 $\mu g\,mL^{-1}$ have been obtained for DM and MT determination. Both methods have been applied to the determination of these tetracyclines in serum samples with satisfactory recovery results.

Keywords: Demeclocycline; Methacycline; Europium-sensitized fluorescence; Serum determination

1. Introduction

Tetracyclines (TCs) are an important antibiotic group used in medical and veterinary practice. The native fluorescence of TCs is well known and this property has been used for its determination [1–5].

Most of the proposed methods for DM and MT analysis are HPLC separation methods [6–14]. The intramolecular energy transfer from TCs to the Eu(III) ion has been described for TC detection by Hirschy et al. [15] and used as a detection system in HPLC and FIA methods [16].

The effect of several micellar systems on the enhancement of the emission fluorescence of various TC complexes with Eu(III) and Tb(III) has been previously described [17–22]. Thus, Georges and Ghazarian [17] proposed the use of Triton X-100. Enhancement of the fluorescence of the TCs–Eu(III) complex by DNA has been also used to determine DNA [18,19]. Thenoyltrifluoroacetone [20,21] and cetylpyridinium chloride [22] considerably enhanced the Eu(III)-sensitized luminescence of tetracyclines. Methods for the individual determination of tetracycline, oxytetracycline, chlortetracycline and doxycycline have been reported, based on the Eu(III)-sensitized fluorescence, using cetyltrimethylammonium chloride as surfactant and EDTA as co-ligand [23]. However, no study has been previously described to determine demeclocycline or methacycline.

While chromatographic methods allow the separation and quantification of several tetracyclines, other analytical methods reported for their determination cannot distinguish between the different tetracyclines. In spite of this limitation, the availability of fast screening methods which allow the individual control of these antibiotics in human biological fluids is desirable.

In this paper, as an extension of the previous reports on this subject, several micellar media, including cetyltrimethylammonium chloride, cetylpyridinium monohydrate and sodium dodecylsulfate, have been examined for the study of the complexes of demeclocycline and methacycline with Eu(III), in the presence of EDTA as co-ligand.

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2. Experimental

2.1. Instrumentation

Fluorescence spectral measurements were performed on a Fluorescence Spectrophotometer Varian Model Cary Eclipse. The instrument consists of two Czerny-Turner monochromators (excitation and emission), a xenon light source, a range of fixed width selectable slits, selectable filters, attenuators and two photomultiplier tubes as detectors. The fluorimeter is connected to a PC microcomputer via an IEE serial interface. The Cary Eclipse Version 1.0 software was used for data acquisition, data interpretation and graphical display.

2.2. Chemicals and reagents

For all experiments analytical grade chemicals and solvents were used. Stock solutions of demeclocycline (DM) (Sigma) and methacycline (MT) (Sigma) 2×10^{-4} M were prepared from their hydrochlorides in deionized water and stored at 4 °C. A 1.25×10^{-3} M europium(III) solution was prepared by dissolving the appropriate amount of europium nitrate (Sigma). Also, 0.1 M Tris/hydrochloride buffer solutions of different pH and 2.5×10^{-4} M EDTA (Panreac) were prepared. Aqueous solutions of cetyltrimethylammonium chloride (CTACl) (Sigma), cetylpyridinium monohydrate (CPC) (Sigma) and sodium dodecylsulfate solutions (SDS) (Sigma) (1%, v/v) were prepared.

2.3. Recommended procedure for DM and MT determination

2.3.1. DM and MT determination

An aliquot of DM or MT samples was placed in a 25 mL volumetric flask, 1 mL of 1% CTACl, 2 mL of pH 8.5 Tris–HCl 0.1 M buffer, 1 mL of 2.5×10^{-4} M EDTA and 0.2 mL of 1.25×10^{-3} M Eu(III) solution were added, followed by dilution with water to the mark.

Emission spectra of the samples were recorded, with an excitation wavelength of 396 nm, and the fluorescence signals were measured at an emission wavelength of 615 nm. Calibration graphs were obtained in the range of DM and MT concentrations between 0.01 and 0.1 $\mu g\,mL^{-1}$.

2.3.2. Analysis of serum samples

An aliquot of 0.5 mL of serum, previously fortified with DM or MT, was placed in a test-tube; each serum sample was treated with 1 mL of acetonitrile to remove proteins. The suspension was centrifuged at 2000 rpm for 10 min, and 1 mL of the supernatant was placed in a 25 mL volumetric flask. In this volumetric flask, 1 mL of 1% CTACl, 2 mL of pH 8.5 Tris–HCl 0.1 M buffer, 1 mL of 2.5×10^{-4} M EDTA and 0.2 mL of 1.25×10^{-3} M Eu(III) solution were added, followed by dilution with water.

In these conditions, emission spectra of the samples were recorded with an excitation wavelength of 396 nm, and the fluorescence signals at an emission wavelength of 615 nm were measured.

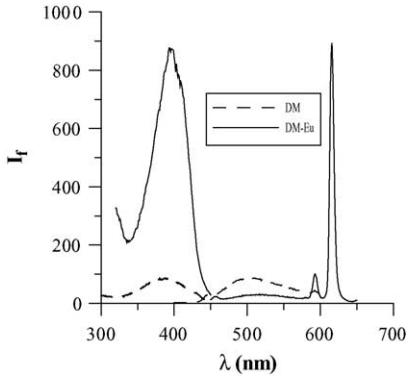

Fig. 1. Excitation and emission spectra of DM and DM–Eu(III) complex, pH 8.5, [Eu(III)] = 10^{-5} M, [EDTA] = 10^{-5} M, 0.04% CTACl.

3. Results and discussion

3.1. Optimization of experimental variables

The Eu(III) ion has the suitable energy level to allow intramolecular energy transfer from the lowest triplet level of the complex to a 4f level of the lanthanide ion, producing a fluorescence emission characteristic of the lanthanide ion.

The aqueous solutions of Eu(III) excited at 394 nm show a narrow emission band at 618 nm, and a less intense one at 594 nm. However, the fluorescence of the TCs–Eu(III) complexes is more intense than the Eu(III) fluorescence.

DM and MT show fluorescence emission in a basic pH and in presence of EDTA. Fig. 1 shows the excitation and emission spectra of the DM–Eu complex and DM alone. For both complexes; excitation at 396 nm and emission maxima at 615 nm were found.

The presence of EDTA allows the formation of a new complex where the Eu(III) ion is bound to the β-diketone group [15] giving an enhancement of the fluorescence. A 10^{-5} M EDTA concentration was fixed for the study of the complexes. In Fig. 2, the emission spectra of the MT complex in the absence and presence of EDTA are shown. For the DM complex a similar enhancement was found.

The effect of pH on the emission intensity is shown in Fig. 3. For the DM and MT complexes, the fluorescence emission increased with the pH value and the greatest fluorescence was found between pH 8 and 9. For higher pH values the fluorescence decreased. A pH value of 8.5 was chosen for the determination. This pH was fixed by addition of 2 mL of Tris/HCl 0.1 M buffer solution of pH 8.5, in a final volume of 25 mL.

For both complexes, the effect of the presence of several micellar media, CPC, CTACl and SDS was examined (Fig. 4). In all cases, the presence of a micellar medium enhances the

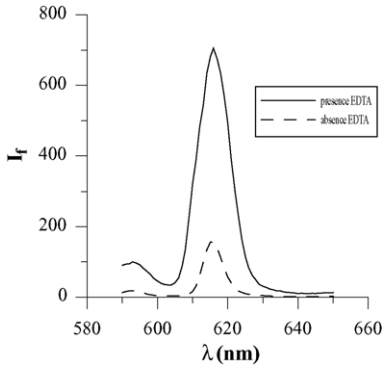

Fig. 2. Influence of the presence of EDTA in the sensitized fluorescence intensity of the MT–Eu(III) complex, pH 8.5, [Eu(III)] = 10^{-5} M, [EDTA] = 10^{-5} M, 0.04% CTACl.

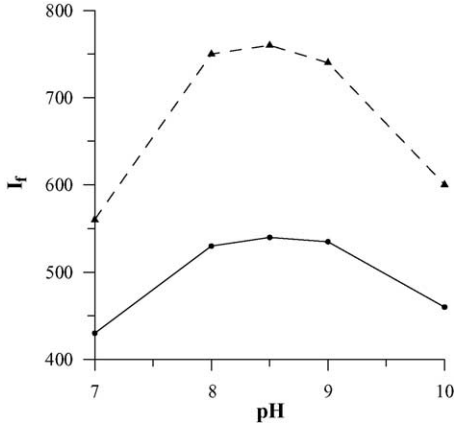

Fig. 3. Influence of pH on the sensitized fluorescence intensity of the DM–Eu(III) (- - -) and TM–Eu(III) (—) complexes, [Eu(III)] = 10^{-5} M, [EDTA] = 10^{-5} M, 0.04% CTACl.

fluorescence signal at 615 nm. The higher enhancement was found for CTACl. A concentration of 0.04% of CTACl was fixed for both complexes.

The influence of Eu(III) concentration shows that the fluorescence increased with the concentration of lanthanide ion. The intensity was maximum for an Eu/TC ratio comprised between 3 and 4, for the DM complex, and between 2 and 4 for the MT complex. In both cases, for higher Eu(III) concentration a decrease of the fluorescence was observed. A 10^{-5} M Eu(III) concentration was chosen and a 1:1 stoichiometry was found for both complexes.

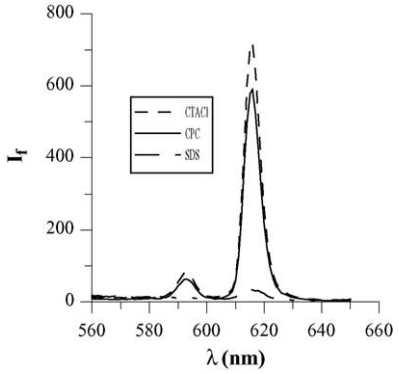

Fig. 4. Emission spectra of the DM–Eu(III) complexes in function of the nature of the surfactants employed, [Eu(III)] = 10^{-5} M, [EDTA] = 10^{-5} M, 0.04% surfactant.

Under these conditions linear calibration graphs were obtained for DM and MT determination, in the range between 0.01 and 0.1 μg mL^{-1}. The calibration graphs were constructed, with three replicates per point, by measuring the intensity of luminescence at 615 nm, exciting at 396 nm. In order to test the presence of outlier points, the least median of squares regression method was applied [24]. No outliers were detected.

The proposed methods were evaluated by statistical analysis of the experimental data by fitting the overall least squares line according to $y = a + bx$ ($y = 2.5 + 779x$, standard deviations, $s_a = 0.4$ and $s_b = 7$ for demeclocycline, and $y = 4.1 + 853x$, $s_a = 0.4$ and $s_b = 7$, for methacycline). According to the residual distribution obtained, it can be assumed that both calibration lines comply with homocedasticity criteria. In order to test the linearity of the overall least squares regression, the ANOVA test was performed by comparing the variation of set means about the line (lack of fit) with the variation within line (pure error). The precision of the method was established by testing the analytical signal corresponding to a tetracycline concentration of 0.06 μg mL^{-1}. For a series of 10 measurements, the relative error was 2.3% for the intra-days and 2.9% for the inter-days analysis (95% confidence level) for demeclocycline, and of 2.4 and 3.0% for methacycline, respectively.

The limit of detection (LOD) values found for both tetracyclines, were 0.002 μg mL^{-1}, according to Long and Winefordner [25], and 0.003 μg mL^{-1}, according to Clayton et al. [26], taking into account false positive and false negative error types ($\alpha = \beta = 0.05$), and the limit of quantification (LOQ) 0.01 μg mL^{-1}.

3.2. Applications

The proposed methods have been applied to determine the selected tetracyclines in serum samples. The therapeutic levels for DM and MT are between 1 and 5 μg mL^{-1} in serum. Urine samples were not tested with the proposed method, as the therapeutic levels of these drugs in urine are very high (300–500 μg mL^{-1}) and a 1:1000 dilution of the urine was necessary to perform the determination.

Samples belonged to different volunteer individuals, and collected in different days at the “Residencia Sanitaria Infanta Cristina” Hospital (Badajoz, Sapin), were used for the determination of DM and MT in serum. The serum samples were deproteinized with acetonitrile. The suspension was centrifuged and 1 mL of the supernatant was taken to be treated following the procedure above mentioned. The recoveries found for four different serum samples are summarized in Table 1.

Table 1
Recovery results for the sensitized-luminescence determination of demeclocycline and methacycline in fortified serum samples[a]

Sample	Added ($\mu g\,mL^{-1}$)	Demeclocycline		Methacycline	
		Found ($\mu g\,mL^{-1}$)	Recovery (%)	Found ($\mu g\,mL^{-1}$)	Recovery (%)
Serum 1	2.9	3.00 ± 0.06	103	3.09 ± 0.09	106
	3.8	3.87 ± 0.03	102	3.58 ± 0.02	94
	4.8	4.89 ± 0.07	102	4.70 ± 0.05	98
Serum 2	2.9	2.79 ± 0.09	96	2.8 ± 0.1	96
	3.8	3.99 ± 0.05	105	3.8 ± 0.1	100
	4.8	4.70 ± 0.07	98	4.98 ± 0.09	104
Serum 3	2.9	2.84 ± 0.04	98	3.04 ± 0.07	105
	3.8	3.68 ± 0.02	97	3.65 ± 0.07	96
	4.8	4.99 ± 0.07	104	4.70 ± 0.05	98
Serum 4	2.9	3.09 ± 0.08	106	2.80 ± 0.08	97
	3.8	3.70 ± 0.09	97	4.07 ± 0.07	107
	4.8	5.08 ± 0.03	106	4.60 ± 0.07	96

[a] Average of five determinations.

4. Conclusions

This paper presents a suitable method for routine analysis, in which the use of sensitized europium luminescence is extended to the determination of DM and MT in serum samples. In cases in which is necessary the individual determination of the tetracyclines studied in this work, it is of interest to dispose, as an alternative, a method of analysis simpler than a chromatographic method. EDTA was used simultaneously with europium, in order to prevent the formation of europium hydroxides in alkaline solutions, and acting as a co-ligand to exclude water molecules from the coordination sphere of the lanthanide. The use of micellar systems, particularly CTACl, providing an environment characterized by lower polarity and higher viscosity than the bulk aqueous phase, provides a simple means of enhancing the Eu(III)-sensitized luminescence from tetracyclines, and hence detection limits. In addition, the performed study may be interested as a preliminary study to develop a chromatographic method for tetracycline derivatives screening, using europium-sensitized luminescence as a detection system.

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References

[1] M.A. Elsayed, M.H. Barary, H. Mahgoub, Talanta 32 (1985) 1153–1155.
[2] W.B. Chang, Y.B. Zhao, Y.X. Ci, L.Y. Hu, Analyst 117 (1992) 1377–1378.
[3] S. Croubels, C. Van Peteghem, W. Baeyens, Analyst 119 (1994) 2713–2716.
[4] H.C. Goicoechea, A.C. Olivieri, Anal. Chem. 71 (1999) 4361–4368.
[5] R. Fernández-González, M.S. García-Falcón, J. Simal-Gandara, Anal. Chim. Acta 455 (2002) 143–148.
[6] N. Weng, E. Roets, J. Hoogmartens, J. Pharm. Biomed. Anal. 7 (1989) 1691–1703.
[7] G. Stubbings, J.A. Tarbin, G. Shearer, J. Chromatogr. B 679 (1996) 137–145.
[8] S. Tanase, H. Tsuchiya, J. Yao, S. Ohmoto, N. Takagi, S. Yoshida, J. Chromatogr. B 706 (1998) 279–285.
[9] A.G. Kazemifard, D.E. Moore, J. Pharm. Biomed. Anal. 16 (1997) 689–696.
[10] A.D. Cooper, G.W.F. Stubbings, M. Kelly, J.A. Tarbin, W.H.H. Farrington, G. Shearer, J. Chromatogr. A 812 (1998) 321–326.
[11] F. Kondo, S. Morikawa, S. Tateyama, J. Food. Prot. 52 (1989) 41–44.
[12] W. Naidong, K. Verresen, E. Roets, J. Hoogmartens, J. Chromatogr. 586 (1991) 61–66.
[13] A.G. Kazemifar, D.E. Moore, J. Pharm. Biomed. Anal. 16 (1997) 689–696.
[14] L. Monser, F. Darghouth, J. Pharm. Biomed. Anal. 23 (2000) 353–362.
[15] L.M. Hirschy, E.V. Dose, J.D. Winefordner, Anal. Chim. Acta 147 (1983) 311–316.
[16] T.J. Wenzel, L.M. Collete, D.T. Dahlen, S.M. Hendrickson, L.W. Yarmaloff, J. Chromatogr. Biomed. Appl. 77 (1998) 149–158.
[17] J. Georges, S. Ghazarian, Anal. Chim. Acta 276 (1993) 401–409.
[18] Y.X. Ci, Y.Z. Li, X.J. Liu, Anal. Chem. 67 (1995) 1785–1788.
[19] X.J. Liu, Y.Z. Li, Y.X. Ci, Anal. Chim. Acta 345 (1997) 213–217.
[20] P. Izquierdo, A. Gomez-Hens, D. Perez-Bendito, Anal. Chim. Acta 292 (1994) 133–139.
[21] B. Gala, A. Hens-Gomez, D. Perez-Bendito, Talanta 44 (1997) 1883–1889.
[22] R.D. Jee, Analyst 120 (1995) 2864–2872.
[23] N. Arnaud, J. Georges, Analyst 126 (2001) 694–697.
[24] P.J. Rousseeuw, A.M. Leroy, Robust Regression and Outlier Detection, Wiley, New York, 1987.
[25] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712A–724A.
[26] C.A. Clayton, J.W. Hines, P.D. Elkins, Anal. Chem. 59 (1987) 2506–2514.