

Optimisation of Europium Sensitized Fluorescence Assay for Detection of Tetracycline Antibiotics

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Abstract

Objective: Europium sensitized fluorescence method for tetracycline antibiotics determination in various samples was optimized.

Method: The factors that influence intensity of the europium–tetracycline fluorescence have been studied in detail. Fluorescence intensity measurement was carried out using Shimadzu RF-5301-PC spectrofluorometer (Kyoto, Japan) with Panorama fluorescence 1.1 software.

Results: The parameters that can affect the fluorescence of europium-tetracycline complex are optimized. Best results were obtained using tris buffer and 1 mm of citric acid as co ligands. The method is linear in the range of 5-2500 ng/ml for tetracycline and oxytetracycline and 5-1000 ng/ml for chlortetracycline and can be used for their determination in various samples.

Conclusion: Europium–tetracycline sensitized fluorescence is a very sensitive that can be used for determination very low tetracycline concentrations. It is necessary to optimize all parameters that can affect the fluorescence, in order to achieve low detection limits.

Keywords: Tetracycline, Resistance, Europium sensitized fluorescence, Method optimisation

Introduction

Today, human and veterinary medicine uses more than 250 different antibiotics and antimycotic agents in the treatment of a large number of infectious diseases.¹ It is known that about 50% of the total amount produced antibiotics is used in veterinary medicine and for the growth stimulation.² Many antibiotics are excreted unchanged or as metabolites in the faeces and urine during the grazing animals and are due directly to agricultural land. Depending on the chemical properties of individual groups of antibiotics excretion of unchanged active substance is 10-90%.^{3,4} The widespread use of antibiotics in medicine and animal husbandry is the most important factor for the emergence, selection and dissemination of antibiotic resistant bacteria.⁵⁻⁷

Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) which is a kind of tetracycline antibiotics group were long time, the most used throughout the world.⁸⁻¹⁰ They have been widely used for the past sixty years as therapeutic agent in human and veterinary medicine and also as growth promotor in animal husbandry. Their use for humans was nowadays limited due the emergence of bacterial resistances. In human

medicine, tetracyclines have been prescribed for prophylaxis and treatment of respiratory infection. They have been the drugs of choice for treatment of pneumonia due to *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Chlamydia psittaci*.¹¹ Antibacterial activity of typical tetracyclines is associated with the reversible inhibition of the protein synthesis, but bacterial resistance to tetracycline was identified shortly after the introduction of therapy.¹² The first tetracycline resistant bacterium was *Shigella dysenteriae* isolated in 1953.¹³ Three different specific mechanisms of tetracycline resistance have been described: tetracycline efflux, ribosomal protection as the most common resistance mechanisms and tetracycline modification-enzymatic inactivation.¹⁴

Presence of antibiotic resistant genes, such as *TET* or *OTET* genes, have been reported in wastewater, surface water and sediments.¹⁵⁻¹⁷ Tetracyclines are strong complexing agents that easily bind with metal ions as calcium or magnesium from water or soil, which can reduce their mobility in the environment, and therefore the detection.¹⁸

There are numerous literature data that describe methods for the identification and quantification of tetracyclines antibiotics in various samples.¹⁹⁻²² They form fluorescent complex with the europium ion via intramolecular energy transfer from the ligand to europium, which yields a characteristic emission at 619 nm.²³ Europium-tetracycline fluorescence intensity depends on the concentration of metals and ligands, use of the co-ligands or surfactants and pH and is therefore necessary to optimize the conditions to maximize the sensitivity of the method.²⁴

Materials and Methods

All reagents used were analytical grade. Tetracycline, Oxytetracycline and Chlortetracycline (Sigma) was min. 98% pure. Ultra-pure water was used throughout. Stock solutions were prepared by dissolving accurate quantities of the powdered standards in 1 ml ultra-pure water and then diluted with acetonitrile. Mass concentration of standard solutions was 500 µg/ml. Stock solutions stored were protected from light at 4°C. Working standard solutions were made by diluting the stock standard solutions with acetonitrile in concentration range 5–2500 ng/ml. Stock solution of europium (1,6 mm) was prepared from $\text{EuCl}_3 \times 6\text{H}_2\text{O}$ (Aldrich). Citric acid, oxalic acid and tartaric acid stock solutions (1 mm) were prepared by dissolving appropriate amount of powdered substances in ultra-pure water. Tris and borate buffer solutions pH 6-9 were prepared in ultra-pure water according to the Ph. Eur. procedure.

Fluorescence intensity measurement was carried out using Shimadzu RF-5301-PC spectrofluorometer (Kyoto, Japan) with Panorama fluorescence 1.1 software.

Results

Effect of europium (III) concentration

The influence of europium (III) concentration was determined by measuring the fluorescence intensity at a concentration of europium (III) 1×10^{-6} M, 2.5×10^{-6} M, 5×10^{-5} M and 1.6×10^{-3} M, wherein the concentration of the antibiotic and buffer pH was kept constant.

Figure 1 shows the fluorescence spectra of europium complexes with different europium (III) concentrations. As can be seen, the lowest fluorescence intensity was with highest europium concentration. By reducing the concentration, fluorescence intensity increases in the following order 5×10^{-5} M > 1×10^{-5} M > 2.5×10^{-6} M. In accordance with obtained results europium concentration 5×10^{-5} M was chosen for further work.

Effect of pH and buffer solution

Figure 2 show the results of buffer selection. Tris buffer and borate buffer with different pH values (6.0, 7.0, 7.5, 8.0, 8.5, 9.0), were tested.

Emission intensity of europium-tetracycline complex with both tested buffer was very similar, increases to pH 8.5, than for the both buffer begins to drop. Fluorescence intensity with tris buffer, increases by about 30% at the same pH values (Figure 3). For further measurement tris buffer pH 8.5 was used.

Effect of coligands

The influence of bidentate ligands oxalic and tartaric acid and polydentate ligand citric acid on the fluorescence intensity of europium-tetracycline complex, were also investigated. An increase in fluorescence intensity occurs only in the presence of citric acid.

As can be seen from Figure 4 fluorescence intensity increased about 3.5 times if added citric acid to mixture of europium (III) and tetracycline antibiotics.

Effect of antibiotic concentrations

The optimum antibiotic concentration was investigated in the range of 5-2500 ng/mL of each antibiotic at a constant concentration of europium (III), 1 mM citric acid and tris buffer pH 8.5. The linear relationship of the fluorescence intensity and the antibiotic concentration usefull for analytical purposes was 5-2500 ng/ml for TC and OTC and 5-1000 ng/ml for CTC, respectively. Corellation coefficients were 0.999 for TC and 0.998 for OTC and CTC (Figure 5).

Discussion

The emission spectrum of europium-tetracycline complex show a maximum emission characteristic for $5d_0 \rightarrow 1F_7$ at 590 nm, and $5d_0 \rightarrow 2F_7$ at 619 nm. The $5d_0 \rightarrow 2F_7$ transition is strongest (red-orange emission) and the emission maximum at 619 nm is used fluorescence measurement.²⁵ The fluorescence of the europium complex is very sensitive.

Low reduction potential of europium cause non-radiative energy transfer from the ligand to the metal with a significant decrease in fluorescence intensity.²⁵ It is therefore necessary to optimize the parameters for fluorescence useful in analytical measurement.

Earlier investigations showed that fluorescence of europium complex dependence of the europium concentration, concentration of ligands, coligands and pH.²⁶

Tetracyclines show fluorescence in a neutral media. In basic media fluorescence considerably increased due to the deprotonation electron donor group.²⁴ Considering this fact, the influence of tris and borate buffer at pH 7.0, 7.5, 8.0, 8.5 and 9.0 were studied.

It was found that tris buffer increases fluorescence, probably because it penetrates the coordination sphere of europium, acting synergistically and in the same time reducing the impact of water on the complex.²⁷ In this case, its function is to achieve the optimal pH for the formation of the complex and the fluorescence.

As can be seen from Figure 3 the increase of pH of tris and borate buffer to 8.5 increase the fluorescence intensity of the europium-tetracycline complex. It can be explained by deprotonating of tetracycline molecule or groups involved in the formation of complex. Tetracycline forms complex with multivalent cations over tricarbonyl or β -diketone ring, which become nucleophilic after deprotonation. At pH 9.0 the fluorescence intensity

begins to drop, so that the pH 8.5 can be considered as optimal for use in analytical purposes. To increase the fluorescence intensity of europium tetracycline complex, is possible to use co-ligand such neutral, anionic or cationic surfactants.²⁵ In this study were investigated influence of citric, oxalic and tartaric acid as co-ligands. The obtained results showed that an increase in fluorescence intensity occurs only in the presence of citrate.

Oxalate has no effect on the fluorescence intensity of the europium-tetracycline complex at 619 nm and the fluorescence originating from $5d_0 \rightarrow 7F_2$ transitions, however, the intensity of fluorescence derived from $5d_0 \rightarrow 7F_1$, is about 25% higher. The tartrate increases the fluorescence intensity at 619 for about 20% and at 594 nm fluorescence is 20% lower. In contrast, with citric acid the fluorescence intensity was 3.5 times higher for both emission maximum, as europium – tetracycline fluorescence. With three oxygen atoms of the carboxyl groups and the oxygen of the hydroxyl group citrate is coupled with europium and removes the water molecules that occupy the coordination sphere of the europium ion, thereby forming a new complex of europium- tetracycline-citrate. In the europium-tetracycline-citrate complex, both, the tetracycline and citrate are protonated and sensitive to pH. Tetracycline has three pKa values, about 3.3, 7.7 and 9.5 and citric acid 3.1, 4.7 and 6.4. However, the formed complex is stable in a broad pH range from 7.4 to 9.225. As the pKa of oxalic (1.23 and 4.19) and the tartaric acid (3.03 and 4.37) are lower than pKa values of citric acid it is possible that protonation at pH 8.5 is only partially, which results in limited binding to europium ion and weak increase in emission intensity. This data is very useful for further use of europium–tetracycline–complex especially for the possible development and optimization of the HPLC method. It is known that mobile phase for tetracycline chromatography, very often used, some of the previously investigated acid.

For the practical analytical use the linear relationship of the fluorescence intensity and the tetracyclines (TC; OTC; CTC) concentration were established in the range of 5-2500 ng/ml for TC and OTC, and 5-1000 ng/ml for CTC.

Conclusion

Europium–tetracycline sensitized fluorescence is a very sensitive and therefore very applicable for the tetracycline antibiotics determination in very low concentrations. It is necessary to optimize all parameters that can affect the fluorescence, in order to achieve low detection limits.

Linearity in a wide range of concentrations and good correlation coefficient can be the basis for the development and validation of analytical methods for the determination of tetracycline antibiotics in different samples.

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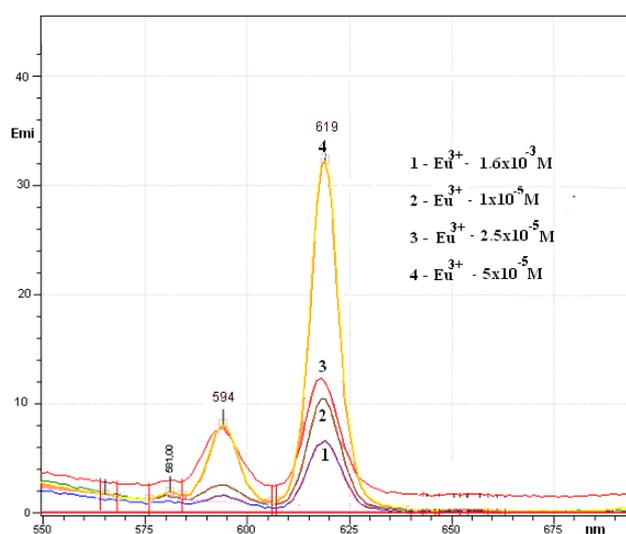


Figure 1: Effect of europium concentration.

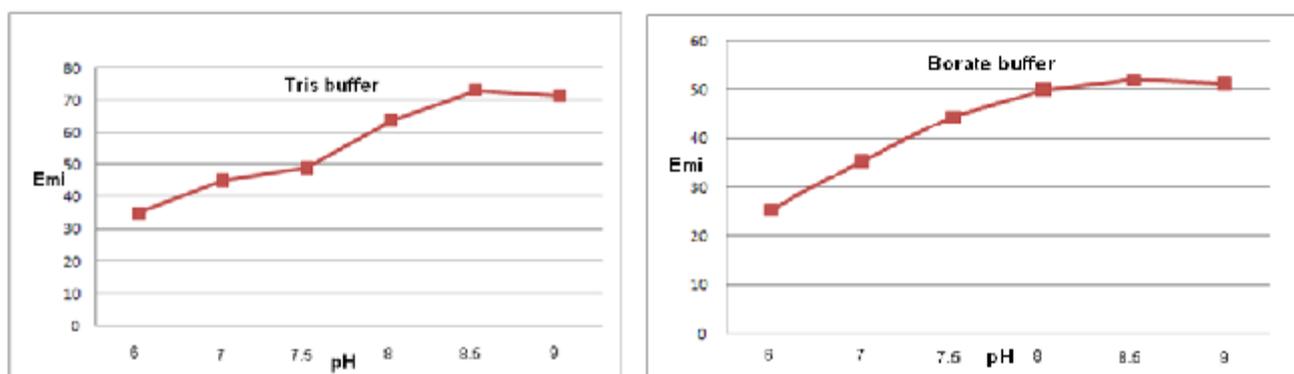


Figure 2: Fluorescence intensity with tris and borate buffer at pH 6.0-9.0.

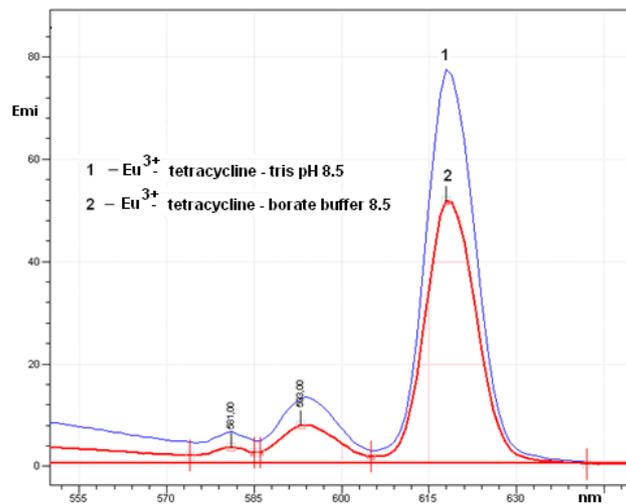


Figure 3: Fluorescence intensity at pH 8.5.

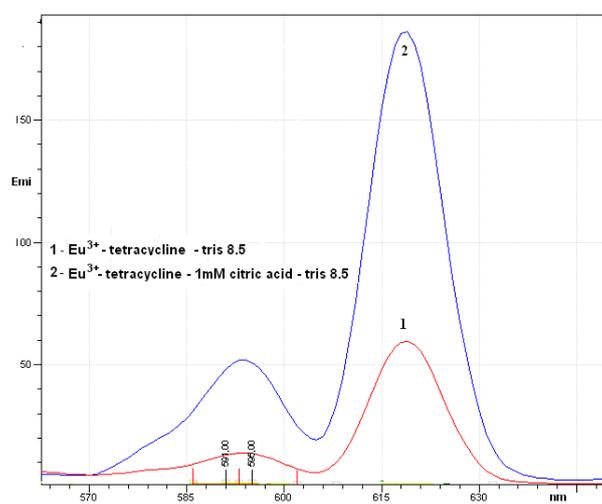


Figure 4: Effect of citric acid on the Europium-tetracycline fluorescence.

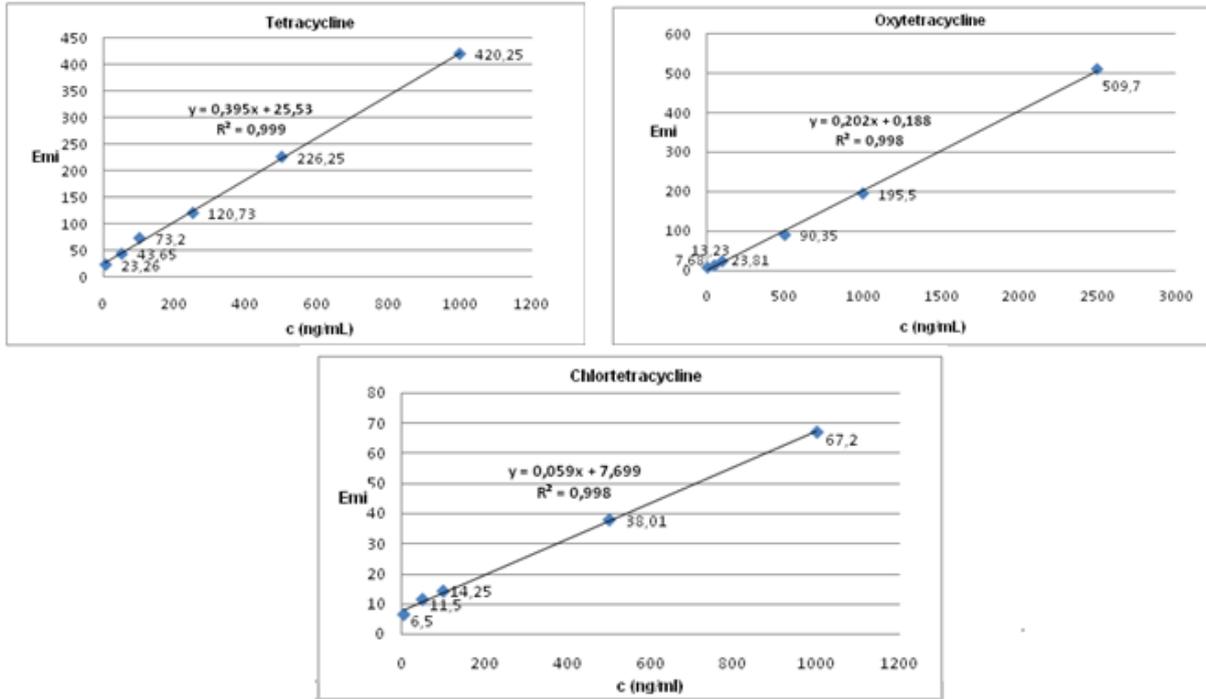


Figure 5: Tetracyclines calibration curve (TC, OTC 5-2500 ng/ml, CTC 5-1000 ng/ml).