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Fluorometric Investigation of the Acid-Base and Complexation Behaviour of Tetracycline and Oxytetracycline

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The widely used antibiotics tetracyclines have been effectively used for ailing heart attack, ulcer cure and gene therapy. The actual mechanism of their activity has been proposed to link with the complexes with many metal ions. However, the sites at which complex formation takes place are not well established. In the present work, the deprotonation sequence of tetracycline (TC) and oxytetracycline (OTC), and their specific group used to bind europium ion were investigated by examining the character of fluorescence of TC and OTC as well as that of their complexes. It was concluded that the site of complexation is coordinated with the deprotonation sequence changing with the acidity/basicity of the solution. And it was inferred that five hydrogens in TC and OTC could be dissociated. The deprotonation sequence is as follows: C(3) hydroxy, C(10) phenol, C(4) dimethylamine, C(12) hydroxy and C(12a) hydroxy. The corresponding complexation site changed with pH increase in solution as follows: C(2) acylamino and C(3) hydroxy moiety, C(10)—C(11) ketophenol moiety, C(4) dimethylamine and C(3) hydroxy moiety, C(11)—C(12) β-diketone moiety, C(12) hydroxy and C(12a) hydroxy moiety, and C(12) hydroxy and C(1) ketone moiety respectively.

Keywords tetracycline, oxytetracycline, fluorescence, europium

Introduction

Tetracycline (TC) and oxytetracycline (OTC) are widely used broad-spectrum antibiotics for the treatment of infections. Recently it was found that tetracycline antibiotics have some other important functions. The medicines give new hopes for ailing heart attack[1] and ulcer[2,3] patients. A novel single tetracycline-regulative adenoviral vector was investigated by Fang group[3] for tumor-specific Bax gene expression and cell killing *in vitro* and *viro*, which may become a potential therapeutic agent for the treatment of cancers. The tetracycline-inducible gene expression system has become a commonly used approach to experimenter-controlled expression of genes for functional evaluation in mammalian cells.[4,5] TC is used to switch gene activity on and off. Through such precise regulation, researchers can learn more about what specific substances do during different stages of an animal's life.[6] Knott group[7] investigated tetracycline-dependent gene regulation, and reported that combinations of *trans*-regulators yield a variety of expression windows. The structural formulas of tetracycline and oxytetracycline are shown in Figure 1.

The protonation scheme of TC has been the subject of controversy and intense study.[8-13] Stephens *et al.*[8]

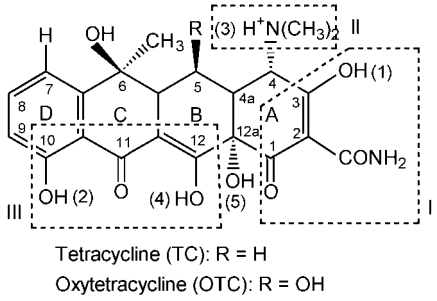

Figure 1 Structural formulas of tetracycline and oxytetracycline.

proposed that pK_1 is due to the protonation of –OH group at C(3), pK_2 is due to the protonation of –NH-$(CH_3)_2$ group at C(4), and pK_3 is due to the protonation of *β*-diketone moiety at C(10)—C(12). Rigler *et al.*[9] used ^{1}H NMR chemical shift information to suggest that pK_2 and pK_3 were contributed from the dimethylamino group at C(4) and phenolic group at C(10), respectively. By using ^{13}C NMR, as for the protonation sites of pK_2 and pK_3, Asleson, Frank[10] are in agreement with Rigler *et al.*,[9] and further proposed that the protonation of the-

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oxygen in C(12) is preferential with respect to the phenolic group C(10). And they used the chemical shift of C(8) as a measure of protonation of the phenolic group C(10) and assumed that pK_1 is due to the tricarbonyl system of ring A. However, Lesson *et al*.[11] proposed a reversal in assignments for pK_2 and pK_3 based upon a comparison of the pK_a values of different TC derivatives. These conclusions were supported by Garrett,[12] who investigated the effect of dielectric constant on the pK_a values of TC in dimethylformamide-water mixtures. The pK_a values of uncharged acids decreased with increasing dielectric constants. On the other hand, Smyth *et al*.[13] provided evidence for a hydrogen-bonded species and determined a fourth pK_a value that was assigned to the $-$OH group at C(12). Suyan group[14] proposed TC underwent quaternary deprotonation by means of potentiometric titration.

Buckley and Smyth[15] studied an ultraviolet spectrum of the acid-base of oxytetracycline hydrochloride yielding pK_a values for these compounds of pK_1=3.3, pK_2=7.5, pK_3=9.7 and pK_4=10.4. The pK_1 value was assigned to deprotonation of the $-$OH group at C(3), the pK_2 value to deprotonation of the $HN(CH_3)_2$ group at C(4), the pK_3 value to deprotonation of the $-$OH group at C(9) and the pK_4 value to deprotonation of the $-$OH group at C(11).

The actual pharmaceutical mechanism of TC and its derivatives has not been definitely established. However, it appears to be linked to the ability of the molecule to form complexes with a large variety of metal ions.[16-21] The question of which specific group of TC uses to bind the metal has not been completely affirmed, and therefore the behavior of metal ion coordination by TC and its analogs has received appreciable attention.[17]

Many possibilities of the sites of complexation have been proposed. Based on a comparison of the effects of metal ions on the absorption spectra, specifically the band at 370 nm of OTC with the corresponding effect of the ions on model compounds, Connover[22] concluded that the binding group is the enolized β-diketone group at C(11) and C(12). This result was supported by Colaizzi group.[23] Smyth group[12] pointed out that the complexation takes place at the C(10)—C(11) ketophenol moiety. Based on the assignment of the acidity constants made by Stephens, *et al*.,[8] Dolusio and Martin[24] concluded on the basis of potentiometric titrations of tetracycline and some of its analogs in the presence and absence of certain metal ions that the binding group, at least for Cu(II), Ni(II), and Zn(II), was the dimethylamino at C(4) and the hydroxyl at either C(3) or C(12a). Baker[25] attacked the problem of the coordination site through the study of the d-d spectra of transition metal complexes of TC and its certain analogs. The electronic spectra of the Co(II) and Ni(II) complexes of three tetracyclines indicated that the molecules coordinate through oxygen, probably in the C(1)—C(3) tricarbonyl methane moiety. Caswell and Hutchinson[26] indicated that tetracyclines form complexes with calcium and magnesium in blood plasma through multidentate combination of the C(11)—C(12) β-diketone and C(1)—C(3) tricarbonyl methane moiety achieved through folding of the molecule along the C(4a)—C(12a) axis.

In our previous work,[27,28] tetracycline antibiotics were used as ligands to form complexes with europium. The complex of Eu^{3+}-TC (OTC) gave out very strong characteristic fluorescence of europium by which the quantity of europium in material was detected. In present work, we use europium as a fluorescence probe of tetracycline antibiotics to investigate the acid-base and complexation behaviour of tetracycline and oxytetracycline with europium. The deprotonation sequence of TC and OTC, and their specific group used to bind europium ion were investigated by examining the character of fluorescence of TC and OTC as well as that of their complexes.

Experimental

Preparation of tetracycline and oxytetracycline solutions

Tetracycline hydrochloride and oxytetracycline (chromatographic grade) were purchased from Beijing Institute for the Control of Biological Products, China. Separate standard solutions were prepared by accurately weighing tetracycline hydrochloride and oxytetracycline and dissolving them in distilled water to give solutions of 1.0×10^{-4} mol/L. These solutions were kept in refrigerator for a three-day use.

Preparation of europium standard solution

Europium oxide (Eu_2O_3, 0.0579 g) (primary standard, 99.9%) was dissolved in 5 mL of 2 mol/L hot hydrochloric acid, then diluted with 0.1 mol/L hydrochloric acid to 50 mL to obtain a stock solution of 1 mg/mL. This solution was then diluted using 0.1 mol/L hydrochloric acid to give solution of 10 mg/L.

Acetic acid and sodium acetate buffer (pH=5.0) and ammonia and ammonia chloride buffer (pH=10)

General procedures Europium solution (1 mL, 10 mg/L), tetracycline hydrochloride or oxytetracycline solution (1 mL, 1.0×10^{-4} mol/L), and 2.5 mL of buffer solution were added successively in 25-mL beaker. Water was added to a volume of about 20 mL. Adjusting solution on a pH meter by dropwise addition of either HCl (0.01—1 mol/L) or NaOH (0.01—1 mol/L) to the needed pH. The pH meter used was a PHS-3D digital acidity/ion meter (Shanghai Leici Instrument Factory) with an saturated calomel electrode (SCE) as a reference electrode, a glass electrode as an indicating electrode, and a temperature compensating device, which was calibrated before use with standard buffer solutions of pH=4.30, 6.18 and 9.18, respectively. After that, transfer these solutions into 25-mL volumetric flasks, and dilute to volume mark with distilled water. The solutions were shaken and allowed to stand for 15 min at room temperature. After determination of fluorescence, the pH values of these solutions are detected at pH me-

ter again. Prepare and measure a blank solution in the same way. Hydrochloric acid (1 mL, 0.1 mol/L) was used to instead of europium solution (1 mL, 10 mg/L).

For the solution of pH=3—7, acetic acid and sodium acetate buffer (pH=5) was used. At a range of pH =7—11.5, ammonia and ammonia chloride buffer (pH =10) was utilized. At pH=11.5—12.5, the solution was adjusted with 0.1 mol/L NaOH. When the solution was in strong basic condition, all the reagents were added directly in 25-mL volumetric flask. A portion of volume of 5 mol/L NaOH solution was used to instead of 2.5 mL of buffer, and filled with distilled water to the mark.

Determination of characteristic fluorescence

Hitachi 850 fluorospectrophotometer (made in Japan) was used for measurements of fluorescence. The instrumental condition was as follows. The spectral bandwidths were 5 nm for both excitation and emission. PM gain was high. And EM filter was 430 nm.

An aliquot of solutions was transferred to 1 cm×1 cm quartz cell and the excitation spectrum and fluorescence spectrum were recorded. The excitation spectra of each solution were scanned with fluorescence at λ_{em}= 617 nm, and the fluorescence spectra were scanned with excitation at the maximum wavelength of long range of wavelength of each excitation spectrum.

Results and discussion

Excitation and fluorescence spectra of TC (OTC) and Eu^{3+}-TC (OTC)

The excitation and fluorescence spectra of the complex of Eu^{3+}-TC differ in different condition of solution, which were shown in Figures 2—4. The excitation and fluorescence spectra of Eu^{3+}-OTC complex were the same as those of Eu^{3+}-TC.

For Eu^{3+}-TC (OTC) system, there was a strong peak at 270 nm in the range of short wavelength of the excitation spectra under the acidic condition. When pH increased up to about 5, a shoulder peak at 240—245 nm appeared. The intensity of this peak increased as pH increased, whereas the intensity of the peak at 270 nm decreased. When pH of the solution increased up to about 12.5, the peak at 270 nm disappeared. In the range of long wavelength of the excitation spectrum, there was a peak at 350 nm when pH=2—7. When pH>7, this peak shifted to about 380—390 nm. When the pH increased to pH>8, there appeared a weak peak at 310 nm. The excitation spectra of TC and OTC were the same as those of Eu^{3+}-TC (OTC).

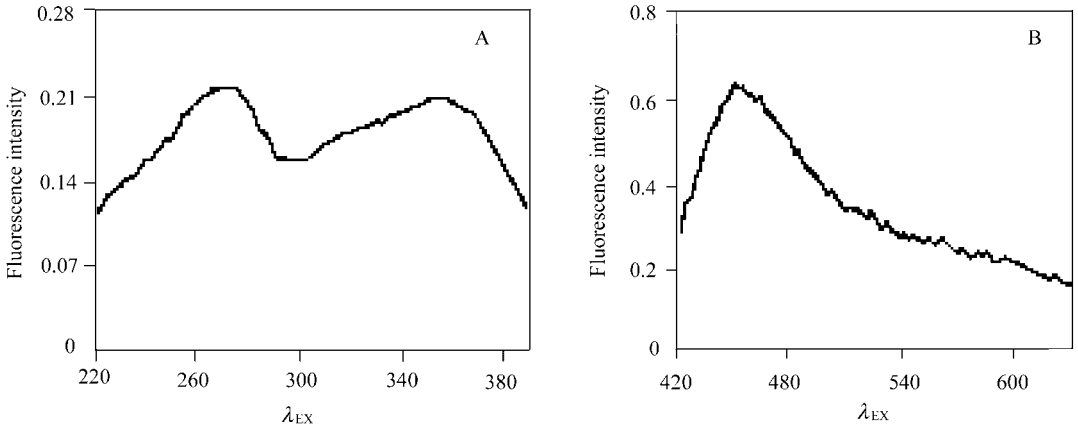


Figure 2 Excitation and fluorescence spectra of the complex of Eu^{3+}-TC (pH=4.0). A: excitation spectra at λ_{EM}=617 nm; B: fuorescence spectra at λ_{EX}=353 nm.

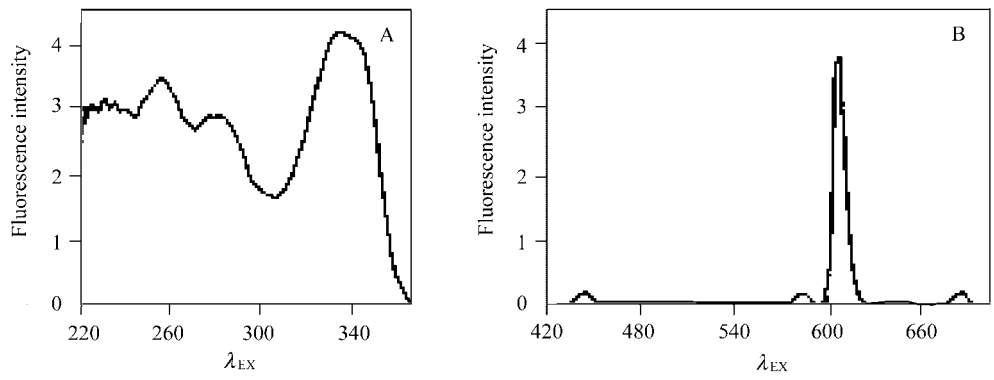


Figure 3 Excitation and fluorescence spectra of the complex of Eu^{3+}-TC (pH=8.65). A: excitation spectra at λ_{EM}=617 nm; B: fluorescence spectra at λ_{EX}=390 nm.

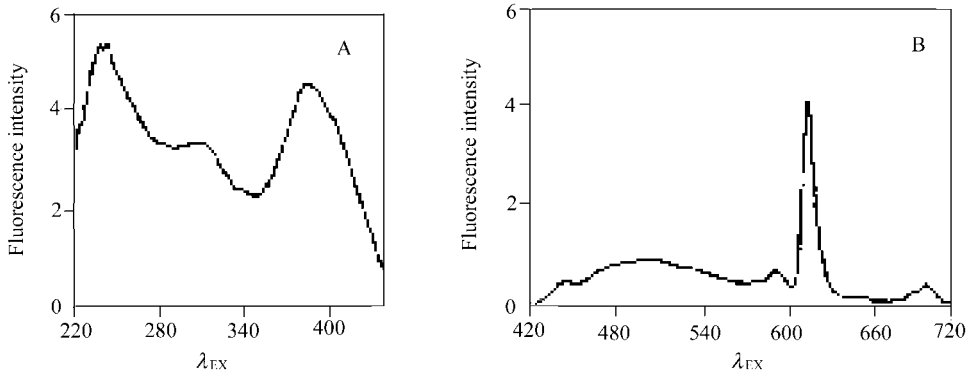


Figure 4 Excitation and fluorescence spectra of the complex of Eu^{3+}-TC (0.2 mol/L NaOH). A: excitation spectra at λ_{EM}=617 nm; B: fluorescence spectra at λ_{EX}=383 nm.

The peaks at 350—380, 245 or 270, and 310 nm on the excitation spectra are π-π electron transition at part III of molecular TC (OTC), π-π electron transition at part I, and the *n*-π electron transition of nitrogen and oxygen at part II and part III, respectively (as shown in Figure 1).

For fluorescence spectra of TC (OTC) and the complex of Eu^{3+}-TC (Eu^{3+}-OTC), when pH<8, the maximum wavelength of fluorescence of TC (OTC) was at 450 nm. When 8<pH<12, it changed to 510—520 nm. When pH>12, this peak took a blue shift to 500—505 nm. For the fluorescence spectra of Eu^{3+}-TC (Eu^{3+}-OTC) systems, there are another four characteristic fluorescence peaks of europium, located at 592, 617, 652 and 697 nm respectively. The peak at 617 nm was the maximum, which was chosen as the emission wavelength.

The peaks on the excitation and fluorescence spectra changed with the change of pH of the solution because of the various microscopic forms of TC (OTC) at different acidity/basicity situations.

Influence of acidity and basicity of the solution on the fluorescence of Eu^{3+}-TC (OTC) complex

The influence of the acid-base of the solution on the characteristic fluorescence intensity of europium in the complex of Eu^{3+}-TC (OTC) was shown in Figures 5, 6. There were two maximum emissions at pH=8—9 and strong basicity, respectively.

Site of complex of Eu^{3+}-TC (OTC) and the sequence of ionization of TC (OTC)

We inferred the deprotonation sequence of tetracycline and oxytetracycline, and the sites of complex of TC (OTC) with europium by means of studying the characteristic fluorescence of europium of Eu^{3+}-TC (OTC). As shown in Figures 2—6, the excitation and fluorescence spectra of the complex of Eu^{3+}-TC (OTC) show that the characteristic fluorescence intensity of europium of the complex differs with different acid-base in the solution. That was because the characteristic fluorescence of europium of complex Eu^{3+}-TC (OTC) much influenced by the site of complexation. Therefore, it is concluded that the complex site of TC and OTC with europium changed with the difference of the acidity/basicity of the solution and was related to the deprotonation sequence of TC and OTC.

The mechanism of the fluorescence emission of TC and OTC belongs to $l^* \rightarrow l$ model (shown in Figure 7A). Following light absorption, the electrons in TC (OTC) molecules are excited to the excited states from their ground state. The electrons at higher level of the excited

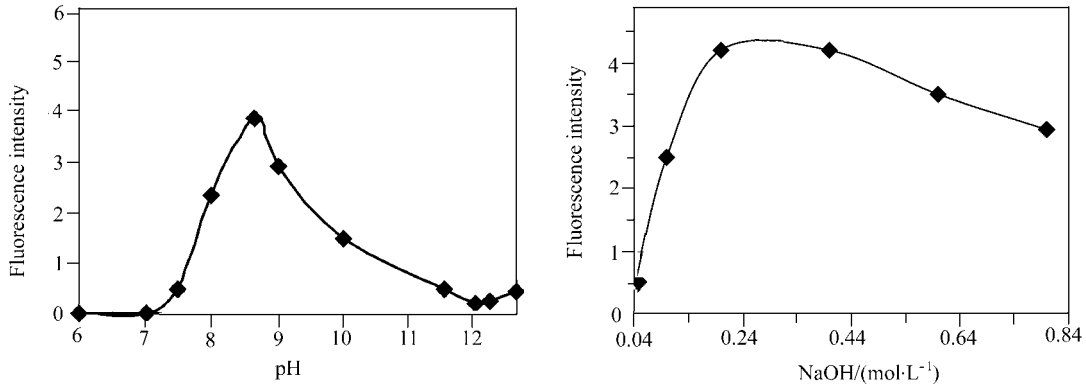


Figure 5 Influence of the acid-base of the solution on the characteristic fluorescence intensity of europium of Eu^{3+}-TC.

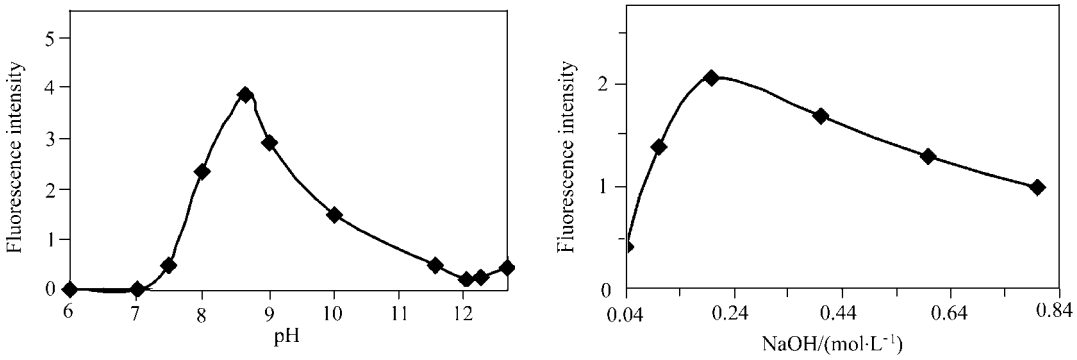


Figure 6 Influence of the acid-base of the solution on the characteristic fluorescence intensity of europium of Eu^{3+}-OTC.

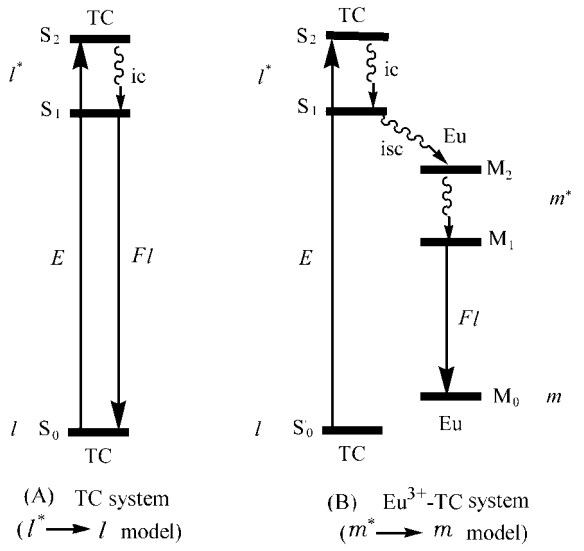


Figure 7 Mechanism of the fluorescence emission of TC (OTC) and Eu^{3+}-TC (OTC).

state return to the first excited state (S_1) through the internal conversion, then return to the ground state by emitting fluorescence. However, the mechanism of the fluorescence emission for the complex of TC (OTC) with europium belongs to $m^* \rightarrow m$ model (shown in Figure 7B). TC (OTC) molecules in the ground state are excited to the excited states by absorbing light. The electrons at higher level of the excited state also return to the first excited state (S_1) through the internal conversion. Because the excited state (S_1) of TC（OTC）is higher than that of europium, the excited electrons of TC (OTC) molecule transfer to the higher level excited states of europium by intersystem crossing, then return to the first excited state (S_1) of europium through the internal conversion. The electrons of europium ion in the first excited state returned to the ground state by emitting the characteristic fluorescence of europium.

The possible sites of complexation of TC and OTC with europium are supposed to be: (a) the C(2) acylamino and C(3) hydroxy moiety, (b) the C(10)—C(11) ketophenol moiety, (c) the C(4) dimethylamine and the C(3) hydroxy moiety, (d) the C(10)—C(12) β-diketone moiety, (e) the C(12) hydroxy and C(12a) hydroxy moiety, and (f) the C(12) hydroxy and C(1) ketone moiety.

According to the above analysis, the characteristic fluorescence of europium emission takes place only when the complexation of TC and OTC with europium ion forms in the forms of (b), or (f). Because the rigid conjugate planes of TC (OTC) and europium could be formed when complexation is in the forms of (b), or (f), which is benefit to the electrons in excited state of TC (OTC) to transfer to the excited state of europium by intersystem crossing and the emission of characteristic fluorescence of europium. The conjugate planes between europium and TC (OTC) molecule could also be formed when complexation is in the form of (d), and the rigidity of conjugate planes was poor. And therefore, there was no or little emission of characteristic fluorescence of europium. Whereas, the conjugate planes between TC (OTC) and europium could not be formed when complexation is in the forms of (a), (c) or (e). Therefore, the electrons in the excited state of TC (OTC) could not transfer to the excited state of europium and the emission of characteristic fluorescence of europium could not take place.

Based on the pK_a values of OTC,[15] the plot showing percent of their various microscopic forms is shown in Figure 8.

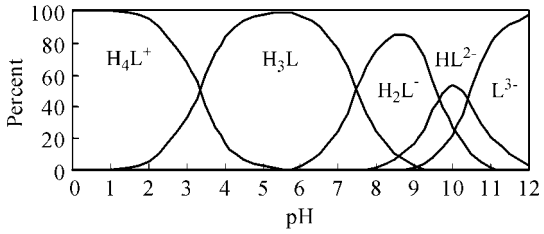


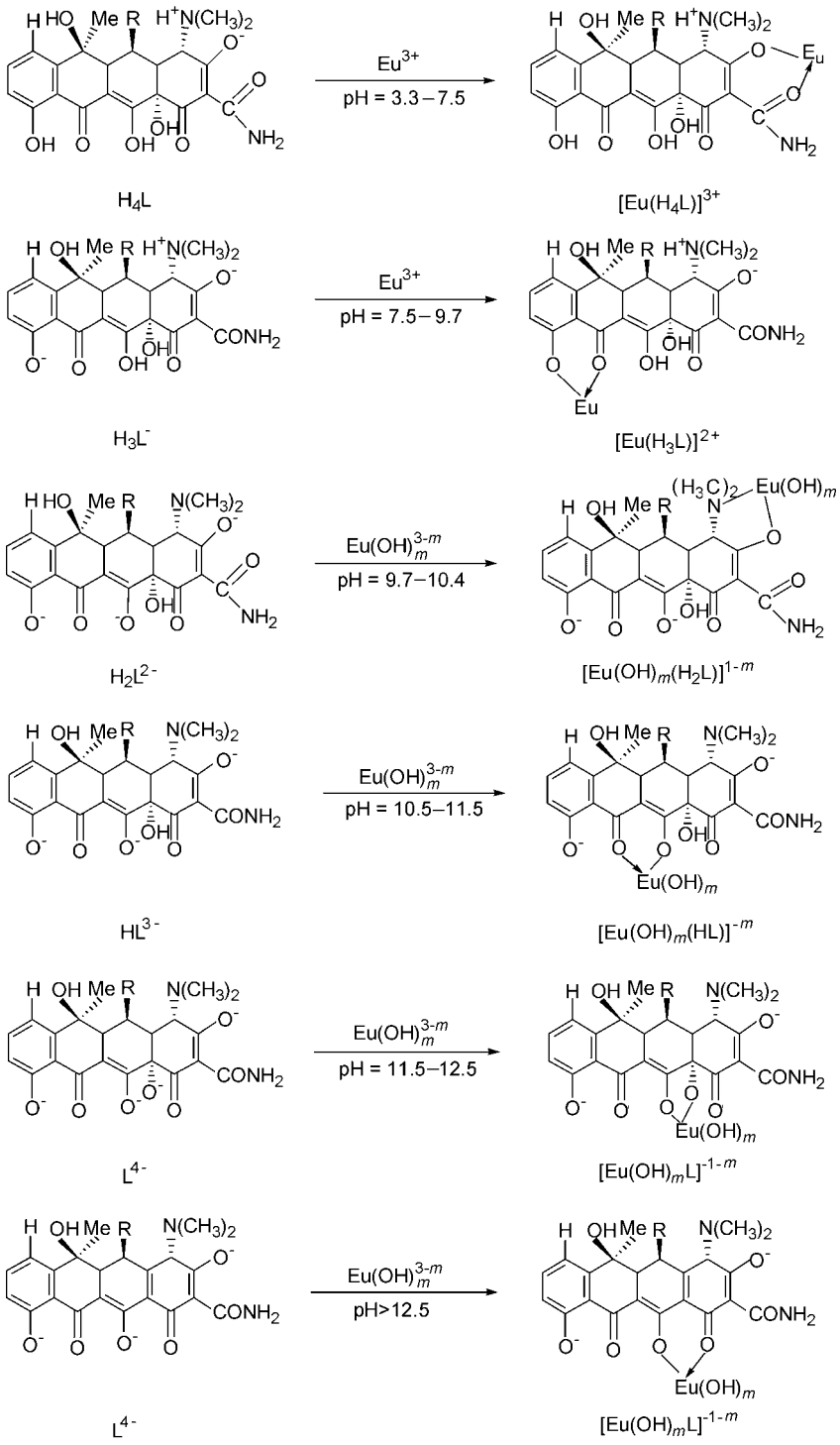
Figure 8 Plot showing percent of their various microscopic forms of oxytetracycline.

The deprotonation sequence of tetracycline and oxytetracycline, and the sites of complexation of TC (OTC) with europium were inferred by comprehensive

analysis of Figures 2—8. It is concluded that five hydrogens in TC and OTC could be dissociated. The course of deprotonation of TC and OTC, and the course of complexation of TC and OTC with europium are summary in Scheme 1.

When pH<3.3, H_5^+L is the mainly existing form of OTC and TC, which could not form complex with europium. When 3.3<pH<12.5, the sequences of five deprotonations of OTC and TC were in according to Eqs. (1—5), and the forms of complexation of TC and OTC with europium were in the form (a), (b), (c), (d) and (f), respectively. When the basicity of solution continued to increase, a majority of OH^- affected the hydrogen in C(4a). This hydrogen is in tertiary carbon, which is more active. On the other hand, the bond of C(12a)—O is weakened because of the formed bond of europium and

Scheme 1



C(12a) hydroxy moiety. Therefore, these result in the break of the bonds of C(4a)—H and C(12a)—O, and formation of a double bond between C(4a) and C(12a). So, the complex formed in the form (f) (shown in Scheme 1).

Conclusion

The traditional medicine tetracycline and its derivatives attract interesting to scientists, because they play the important role in cure cancer and gene expression. The mechanisms are seemed to be linking with their property and ability to form complex with metals. The deprotonation of the second and the third hydrogens and the specific group of tetracycline uses to bind the metal were controversy and intensely studied. By examining the character of the fluorescence of TC or OTC as well as that of their complexes with europium, the site of complexation can be linked with the deprotonation sequence, which changed with the acidity/ basicity of the solution. From our results, it is concluded that inferred five hydrogens in TC and OTC could be dissociated. The deprotonation sequence is as follows: (1) the C(3) hydroxy moiety, (2) the C(10) phenol moiety, (3) the C(4) dimethylamine, (4) the C(12) hydroxy moiety, and (5) the C(12a) hydroxy moiety (shown in Figure 1). The corresponding complexation forms changed with increased pH of the solution as follows: (a) the C(2) acylamino and C(3) hydroxy moiety (pH＝3.3—7.5); (b) the C(10)—C(11) ketophenol moiety (pH＝7.5—9.7), (c) the C(4) dimethylamine and the C(3) hydroxy moiety (pH＝9.7—10.4), (d) the C(11)—C(12) β-diketone moiety (pH＝10.4—11.5), (e) the C(12) hydroxy and C(12a) hydroxy moiety (pH＝11.5—12.5), and (f) the C(12) hydroxy and C(1) ketone moiety (strong basicity), respectively (shown in Scheme 1).

The emission of the characteristic fluorescence of europium of complexes Eu^{3+}-TC (OTC) is much related to the complex structure. So, the results received by comprehensive analyzing of the character of the fluorescence spectra of the complex of Eu^{3+}-TC (OTC) and characteristic fluorescence intensity of europium in different acid –base solutions are more convincing.

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