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Synthesis of Zn(II)–Cloxacillin Sodium Complex and Study of Its Interaction with Calf Thymus DNA

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Abstract—In this paper, a solid complex of cloxacillin sodium (CS) with Zn(II) was prepared by coprecipitation and characterized by UV, fluorescence, IR, and thermal spectra. Furthermore, the nature of the complex has been studied by ^{1}H-NMR and ^{13}C-NMR spectroscopy. The influence of Zn(II) on the combination of CS and calf thymus DNA (CT DNA) was studied using fluorescence spectrophotometry, and the formation of binary CS–Zn(II) and CS–CT DNA complexes and ternary CS–Zn(II)–CT DNA complex was studied. The results show that the fluorescence intensity of CS can be quenched in the presence of Zn(II) or DNA. In the presence of Zn(II), the fluorescence quenching action of DNA on CS was strongly enhanced. Based on the fluorescence intensity, the formation constants of CS–Zn(II) and CS–CT DNA complexes were calculated, and the mechanism of interaction between CS, Zn(II), and DNA is discussed.

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Key words: cloxacillin sodium, Zn(II), DNA, complexes, NMR, fluorescence

In the 1960s American scientists Rosenberg and colleagues discovered that paramagnetic platinum was active against testicular and ovarian carcinoma, urinary bladder carcinoma, breast cancer, malignant lymphoma, and leucosis, and that it presents a broad spectrum of anti-cancer activity. This research was the first to reveal that inorganic metal complexes could also be anti-carcinogenic, which laid the foundation of bioinorganic pharmacochemistry [1]. Afterwards, people came to realize that metal complexes, especially multiple metal complexes, play a very important role in vital activity. For example, enzyme and substrate can form supramolecular complexes. Many researchers studying pharmaceuticals and analytical chemistry have shown great interest in pharmacology as well as in the physicochemical properties of these metal complexes [2]. When organic pharmaceutical molecules are absorbed by the human body, they interact with microelements, bacteria, and viruses in the human body or metalloprotein and metal nucleic acidase in cancer cells and, therefore, accelerate the recovery of the body's normal metabolism or destroy the normal metabolism of pathogens, which is how the metal complexes take effect on the human body. That is why it has been said: "Metal complexes are the basis of life phenomenon" [3]. As we all know, metal complexes have been widely employed in medical and pharmaceutical research. For example, ligand chemotherapy uses the complexing action between ligand and metal ions to eliminate poisonous elements, such as Hg, Pb, etc. in the human body [4]. Some metal complexes have already been used in clinical medicine or diagnostic reagents, and among them early tumor diagnostic reagent is a good case in point.

Cloxacillin sodium (CS) is a semisynthetic antibiotic. It is an enzymatic depressant of gram-positive bacteria that can produce β-lactamase [5]. So it can be used to protect ampicillin from β-lactamase and remedy some clinical disorders like septicemia, pneogaster infection, meningitis and soft tissue infection, etc. [6]. Zinc is one of the microelements that are indispensable to the human body, and it plays an important role in the regulation of metabolism. In this paper a solid complex of CS with Zn(II) was prepared by coprecipitation [7-9], and it was characterized by UV, fluorescence, IR, and thermal spectra. Furthermore, the complex site and the complex mechanism were also studied by means of ^{1}H-NMR and ^{13}C-NMR. All the studies we have done can provide

Abbreviations: CS) cloxacillin sodium; CT DNA) calf thymus DNA; DSC) differential scanning calorimetry.

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information on the interaction between CS and the indispensable microelements required by the human body like Zn(II), etc., and the interaction mechanism and possible effect of microelements like zinc on the drug action of CS. It is known that CS is a therapeutic pharmaceutical against drug-resistant *Staphylococcus aureus* infection. Research of its effect is deepening, since its clinical function is constantly extended. The latest investigation indicates that ionic strength or other factors may affect the combination of antibacterial pharmaceutical and bacterial DNA [10, 11]. Though the role of metal on the combination of pharmaceutical and DNA has been studied by others, most research concerns pharmacology [10, 12]. In our work we studied the complexing reaction of CS, calf thymus DNA (CT DNA), and Zn(II) using a fluorescence method.

MATERIALS AND METHODS

Materials. Cloxacillin sodium was purchased from Bokang Pharmaceutical Industry (China). Zn(II) chloride and methanol were used as received without further purification. CT DNA and Tris were purchased from Sino-American Biotechnology Company. The purity of CT DNA was A_{260}/A_{280} = 1.8-2.0. All other reagents were analytical reagent grade without further purification.

Fluorescence spectra were measured with a Platinum-Amer LS-50B spectrometer using 10 mm quartz cells. The UV spectra were recorded in 10 mm quartz cells on a Shimadzu UV-265 double-beam spectrophotometer. The IR spectra were recorded as KBr pellets on an FTIR-8400S infrared spectrometer. The thermal spectra were measured using a DT-40 thermal analysis system (Japan). The ^{1}H-NMR and ^{13}C-NMR spectra were taken at 300 MHz with a Bruker DRX-300 (Switzerland).

Preparation of solutions. Stock solutions of CS and $ZnCl_2$ with final concentration 10^{-3} M were prepared on doubly-distilled water. The DNA from calf thymus was dissolved in 0.1 M NaCl. Before use, the solution was diluted to 0.02 M final NaCl concentration. DNA concentration was measured by absorption at 260 nm using extinction coefficient of 6600 $M^{-1}\cdot cm^{-1}$ [13, 14]. All solutions were stored at 4°C.

Synthesis of solid complex. The appropriate weight of CS dissolved in CH_3OH was put in a beaker, then $ZnCl_2$ dissolved in CH_3OH was added into the beaker to make the molar ratio of ligand/Zn^{2+} 1 : 1. The reaction mixture was stirred at room temperature for 6 h and then cooled in an ice bath, allowed to stand overnight at room temperature until a precipitate was formed, and filtered. The precipitate was washed with CH_3OH repeatedly. After the wet precipitate was dried, the complex was finally obtained. The solid metal complex stored in a desiccator was used for measuring IR spectra, differential scanning calorimetry (DSC) analysis, and NMR spectra.

RESULTS AND DISCUSSION

Absorption spectra. In aqueous solutions at room temperature, the absorption spectra of the ligand and the complex were scanned from 190 to 350 nm. As shown in Fig. 1, the ligand and the complexes have similar absorption features, but with increasing concentration of Zn(II) the absorption at 220 nm increases gradually and a small red shift can be observed. In addition, a new absorption peak is observed at 275 nm when Zn(II) is added to the ligand solution. This indicates that a complex is formed after Zn(II) is added into the CS solution.

Fluorescence spectra. One milliliter of 10^{-3} M CS solution was added to a series of test tubes and different volumes of 10^{-3} M Zn(II) were added to the test tubes. Then, double-distilled water was added until the volume of solution in each test tube was 5 ml. The fluorescence

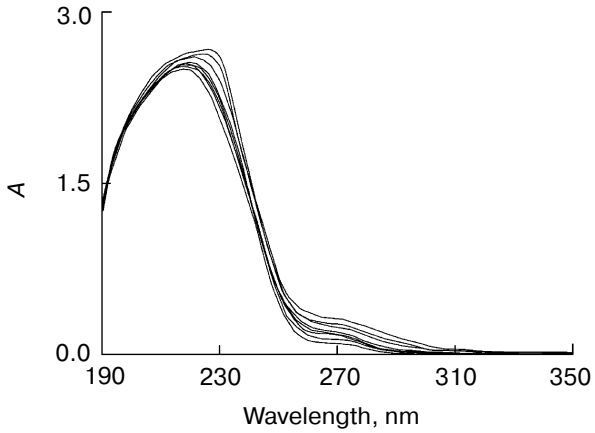


Fig. 1. UV spectra of CS and its complex with Zn(II). Concentrations: CS, $1\cdot10^{-4}$ M; Zn(II), $1\cdot10^{-5}$, $3\cdot10^{-5}$, $5\cdot10^{-5}$, $7\cdot10^{-5}$, and $9\cdot10^{-5}$ M.

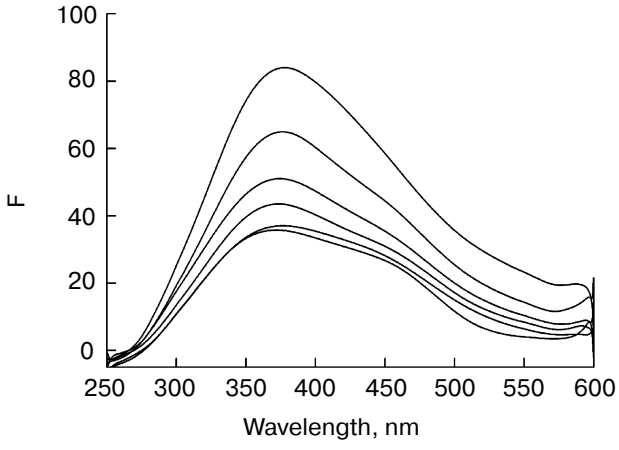


Fig. 2. Fluorescence spectra of CS and its complex with Zn(II). Concentrations: CS, $1\cdot10^{-4}$ M; Zn(II), $1\cdot10^{-5}$, $3\cdot10^{-5}$, $5\cdot10^{-5}$, $7\cdot10^{-5}$, and $9\cdot10^{-5}$ M.

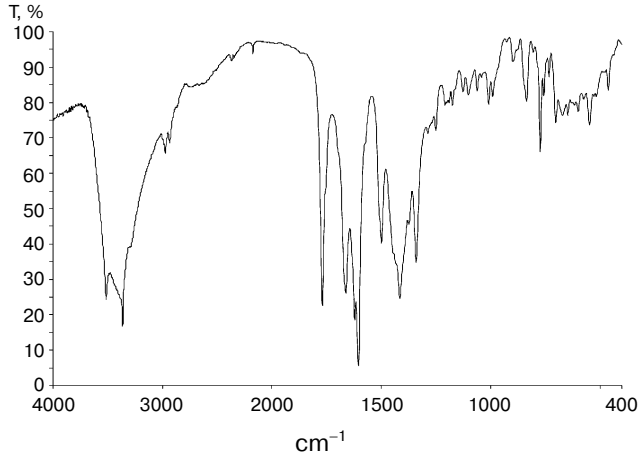


Fig. 3. IR spectrum of cloxacillin sodium (CS).

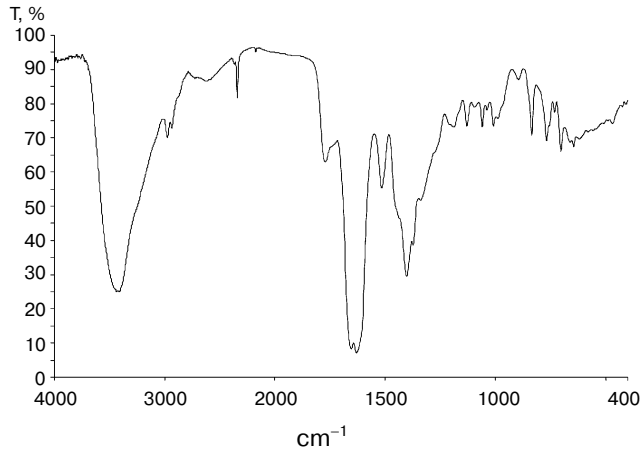


Fig. 4. IR spectrum of the CS–Zn(II) complex.

spectra were measured after the solutions were stirred adequately and left to stand for half an hour.

At room temperature, when CS was excited at its maximum excitation wavelength of 220 nm, a corresponding strong maximum emission was registered at 370 nm. As shown in Fig. 2, the fluorescence intensities of CS–Zn^{2+} decrease with the increase in Zn(II) and tend to remain constant at high concentration of Zn(II). Moreover, the maximum emission wavelength of the complex blue shifts gradually by 5-6 nm. This illustrates that the fluorescence is gradually quenched when more Zn(II) is added into the CS solution, which indicates that a complex is formed.

IR spectra. IR spectra were measured in KBr pellets on an FTIR-8400S infrared spectrometer. Compared with the IR spectrum of the ligand (Fig. 3), the IR spectrum of the complex (Fig. 4) has several significant changes. Two sharp absorption peaks at 3514 and 3363 cm^{-1} that are related to the stretching vibration of N–H of secondary amide (-CONH-) in the ligand are integrated into a relatively wider absorption peak in the complex, and three absorption peaks ($\nu_{C=O}$ of secondary amide, $\nu_{C=O}$ of carboxylic acid (-COO^-), and $\nu_{C=C}$ of benzene ring) in the ligand form a strong absorption peak near 1630 cm^{-1}. Two other absorption peaks (1414 and 1338 cm^{-1}) of ν_{C-O} of carboxylic acid in the ligand are also integrated into a medium strong peak (1401 cm^{-1}) in the complex. Also, the absorption intensity of $\nu_{C=O}$ (1768 cm^{-1}) of tertiary amide (-CON<) in the ligand is much stronger than that in the complex. However, although other absorption peaks reveal some changes, the changes are rather insignificant.

Through the comparison of the IR spectra in Figs. 3 and 4, a preliminarily hypothesis can be drawn: there is a possibility that it is the carbonyl of the amide, the carboxylic acid functional group, and the nitrogen (N) that complex with Zn(II).

Thermal analysis. Samples containing 4.1 mg CS plus 8.12 mg α-Al_2O_3 and 4.2 mg the complex plus 8.68 mg α-Al_2O_3 were subjected to thermal analysis. The DSC spectra of the ligand and of the complex are shown in Figs. 5 and 6, respectively. Comparing Figs. 5 and 6 (each figure contains a trace of the temperature and a corresponding trace showing sample enthalpy), we see that CS has two endothermic peaks, at 180 and 235°C, but the complex only has one small exothermic peak at 240°C. These results indicate that a complex is formed, that it is a new substance different from CS.

NMR spectra. The UV and fluorescence spectra and the thermal analysis clearly indicate that a complex of CS with Zn(II) is formed, and the nature of the complex is tentatively identified by the IR spectra. To clearly eluci-

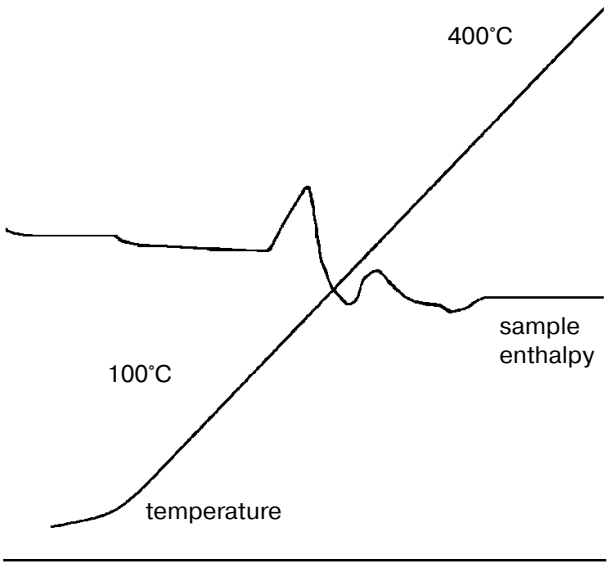


Fig. 5. DSC spectrum of cloxacillin sodium (CS).

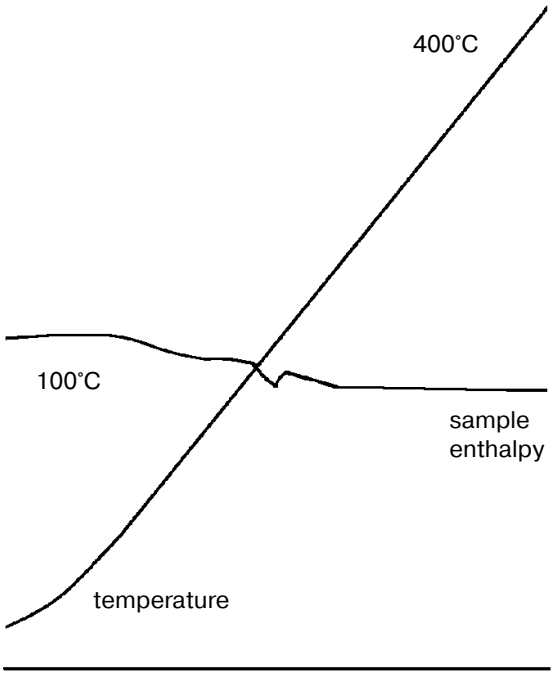


Fig. 6. DSC spectrum of CS–Zn(II) complex.

date the chemical nature of the complex, we measured the ^{1}H-NMR and ^{13}C-NMR spectra of CS both in the absence and in the presence of Zn(II); changes in chemical shifts were observed.

First, the ^{1}H-NMR and ^{13}C-NMR spectra of CS alone were measured. The numeration of the hydrogen atoms is provided in Scheme 1, and the numeration of the carbon atoms is provided in Scheme 2. Chemical shifts for the ^{1}H-NMR and ^{13}C-NMR spectra are given in Tables 1 and 2, respectively.

After measuring the wide-band decoupling spectra, the position of each carbon atom was determined using some additional methods.

Samples of the complex were prepared by adding 5 or 10 μl of $1\cdot10^{-3}$ M Zn(II) to 0.5 ml of $1\cdot10^{-3}$ M CS solution, and the spectrum was measured to compare it with that of the ligand alone. Figure 7 shows that hydrogen atoms 7 and 8 are the most affected, moving toward lower field; the other hydrogen atoms, except for the four hydrogen atoms in the aromatic ring, are also shifted, but these shifts are clearly less than those of atoms 7 and 8. The reason for this is probably that the lone pair of electrons of the two oxygen atoms in the secondary amide and the tertiary amide fill the empty orbit of Zn(II), which results in the redistribution of electrons of the entire molecular framework. Therefore, some hydrogen atoms are shielded, others deshielded, and the hydrogen atoms 7 and 8, which are close to the complex site, experience greater shielding effect.

To further define the complexing site and mechanism, we measured the ^{13}C-NMR spectra of similarly prepared samples (Fig. 8). Figure 8 shows that only carbon atoms 7, 8, 10, 11, and 13 undergo significant chemical shifts, while nearly no discernable shift occur for the other carbons (e.g., carbon atom 19). Among those carbon atoms that have noticeable chemical shifts, carbon atoms 7, 9, and 11 shift toward higher field, while carbon atoms 10 and 13 shift to lower field. This experimental result completely confirms what we inferred from the ^{1}H-NMR spectrum in Fig. 7. Namely, it is the oxygen of the secondary amide (-CONH-) and the oxygen of the tertiary amide (-CON<) in the ligand that complex with Zn(II); the complex site is shown in Scheme 3.

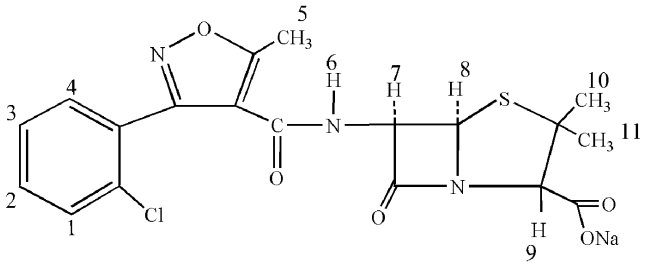
We can explain these results as follows. When Zn(II) is complexed with CS, the chemical shifts of carbon atoms 7, 9, and 11 move to higher field because their conjugate structures are to some extent destroyed and their shielding effect increases, while carbon atoms 10 and 13 shift to lower field because of the decreasing electron cloud density and hence deshielding effect. Also, the fact

Table 1. ^{1}H chemical shifts of cloxacillin sodium

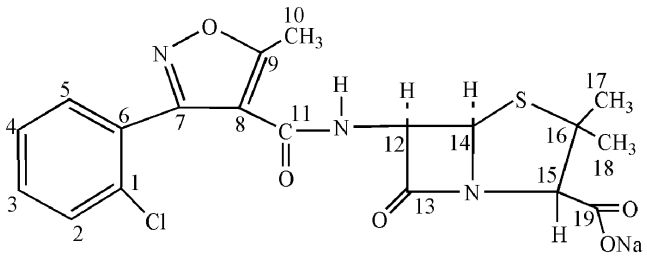
Proton number	Chemical shift	Splitting	Number of atoms
H (1-4)	7.31-7.41	m	4
H (10, 11)	1.27	s	6
H5	2.47	s	3
H9	3.94	s	1
H7	5.30	s	1
H8	5.44	s	1

Table 2. ^{13}C chemical shifts of cloxacillin sodium

Carbon number	Chemical shift (from low field to high field)	Carbon number	Chemical shift
C19	174.5	C6	125.70
C11	174.32	C8	111.77
C13	173.53	C15	73.00
C9	163.38	C12	65.79
C7	159.52	C16	64.44
C1	132.91	C14	57.52
C5	132.38	C17	30.41
C3	131.42	C18	26.30
C4	130.36	C10	11.89
C2	127.75		



Numbering of hydrogen atoms in CS
Scheme 1



Numbering of carbon atoms in CS
Scheme 2

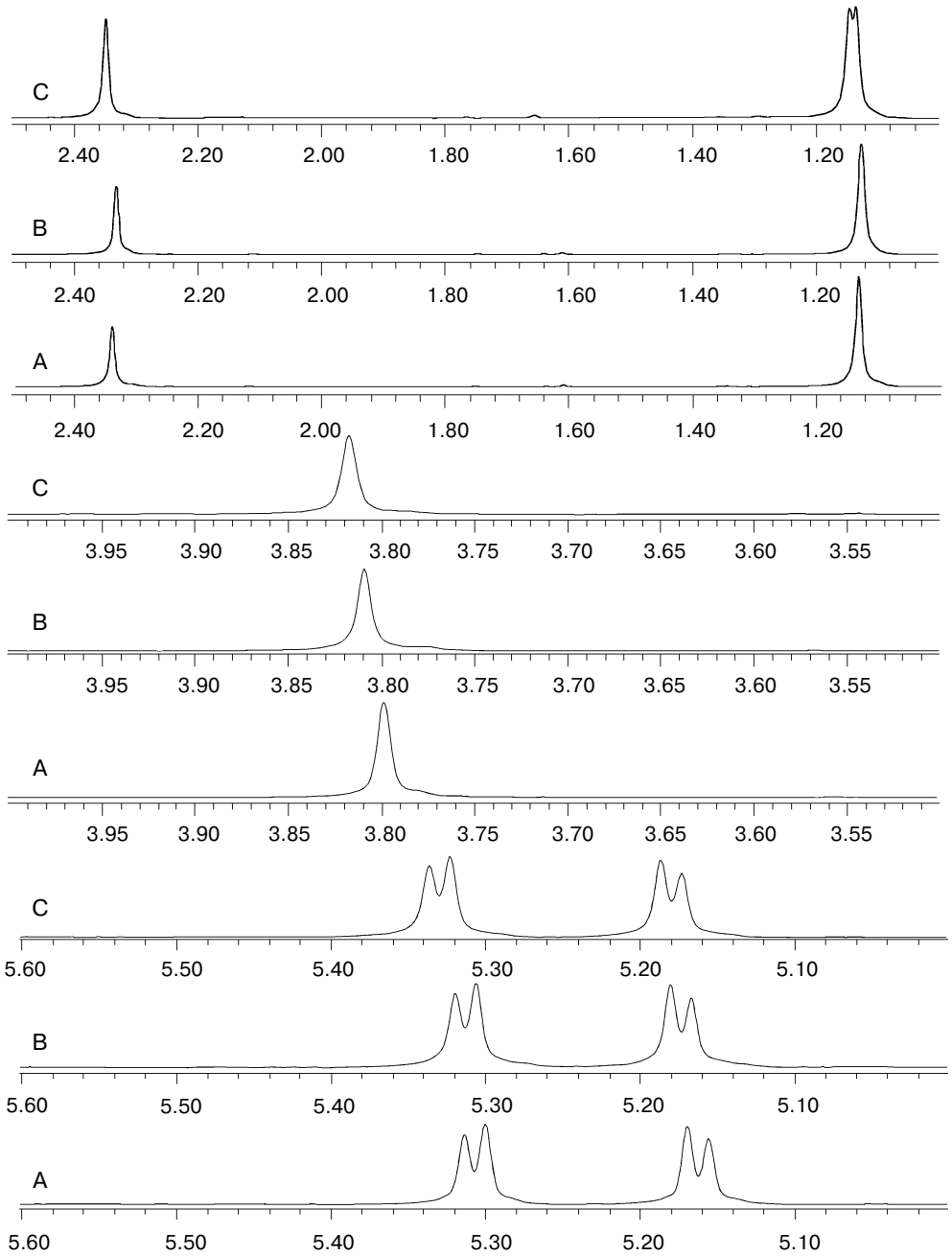


Fig. 7. ^{1}H-NMR spectra of CS and its complex: A) $1 \cdot 10^{-3}$ M CS; B) $1 \cdot 10^{-3}$ M CS + $1 \cdot 10^{-5}$ M Zn^{2+}; C) $1 \cdot 10^{-3}$ M CS + $2 \cdot 10^{-5}$ M Zn^{2+}.

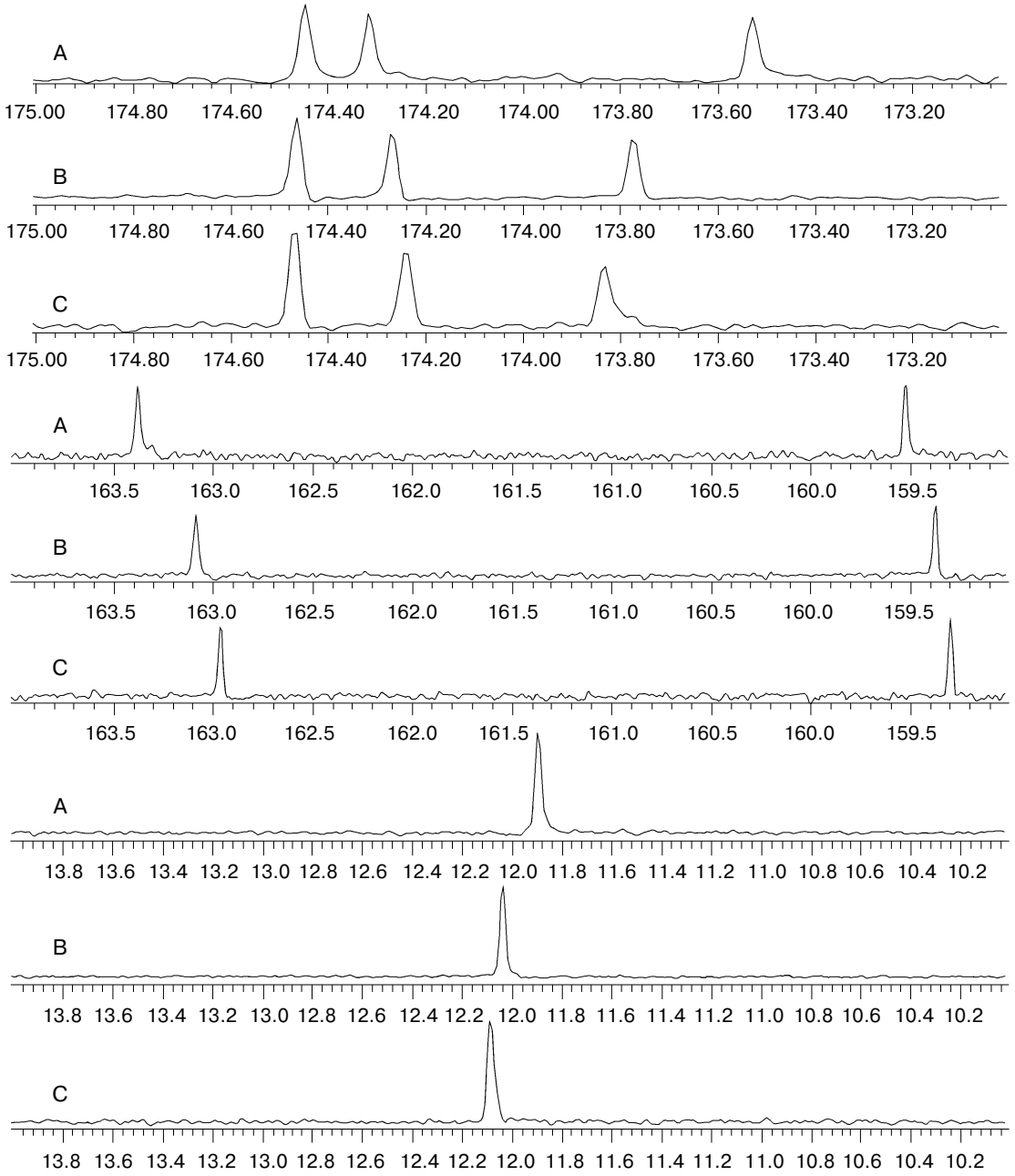
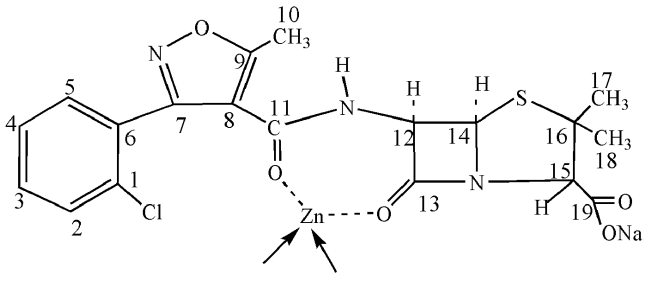


Fig. 8. ^{13}C-NMR spectra of CS and its complex: A) $1\cdot10^{-3}$ M CS; B) $1\cdot10^{-3}$ M CS + $1\cdot10^{-5}$ M Zn^{2+}; C) $1\cdot10^{-3}$ M CS + $2\cdot10^{-5}$ M Zn^{2+}.



Structure showing the site of complexing of Zn(II) with CS
Scheme 3

that the chemical shift of carbon atom 19 was negligible indicates that our hypothesis drawn from the IR spectra is not reliable.

Interaction between CS, Zn(II), and CT DNA. A solution was prepared containing $1\cdot10^{-4}$ M $ZnCl_2$, $1\cdot10^{-4}$ M cloxacillin sodium, $4\cdot10^{-4}$ M DNA, and 0.1 M NaCl in 0.1 M Tris-HCl, pH 7.4. UV and fluorescence spectra were measured. For the fluorescence spectrum the excitation wavelength was 205 nm, emission wavelength 380 nm, and excitation and emission slits 10 nm; the measurements were performed at room temperature.

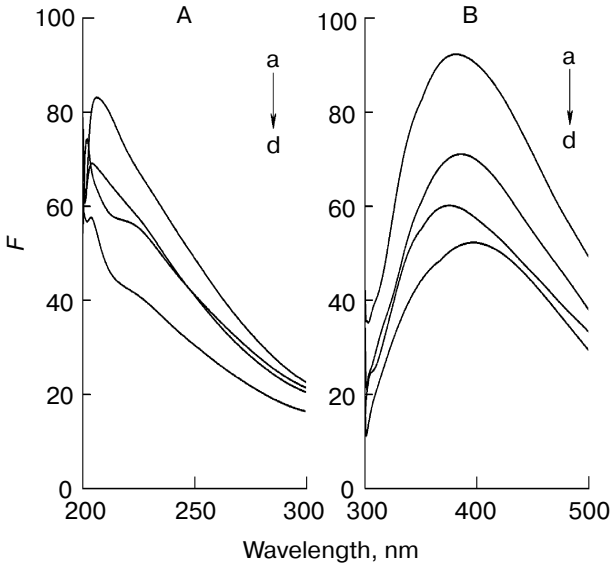


Fig. 9. Fluorescence excitation (A) and emission (B) spectra of CS in the presence of Zn(II) and DNA. a) CS; b) CS + Zn(II); c) CS + DNA; d) CS + Zn(II) + DNA. Concentrations: CS, $5 \cdot 10^{-6}$ M; DNA, $4 \cdot 10^{-5}$ M.

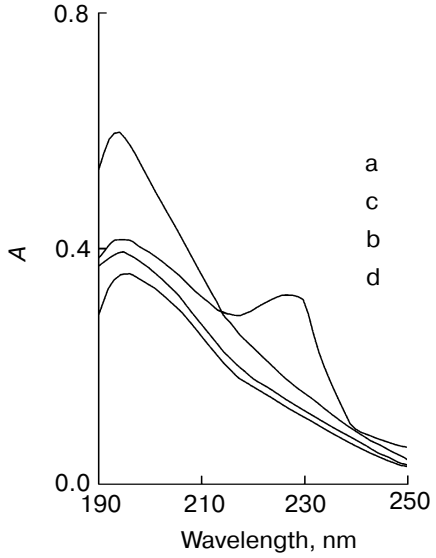


Fig. 10. UV absorption spectra of CS in the presence of Zn(II) and DNA. Zn^{2+} concentration is 10^{-5} M. a) CS; b) CS + Zn(II); c) CS + DNA; d) CS + Zn(II) + DNA.

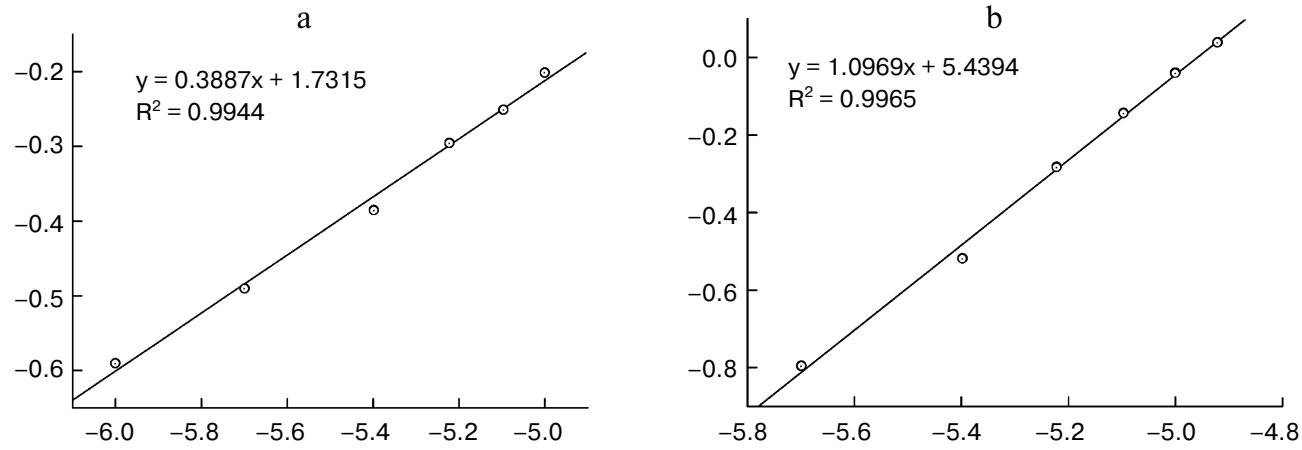


Fig. 11. Log-log curve of fluorescence quenching action of Zn(II) (a) and DNA (b) on CS.

Adding Zn(II) into the buffer solution containing CS and DNA formed a ternary CS–Zn(II)–CT DNA complex.

Zn^{2+}–CS complex. Zinc ions quenched the fluorescence intensity of CS quench (Fig. 9, A and B). Moreover, the UV spectrum is altered (Fig. 10). These results indicate that there is a ground state complex between CS and Zn^{2+} [15, 16]. We can deduce the relation of fluorescence intensity and quenching reagent from the association constant equation of CS and Zn^{2+}:

$$\log[(F_0 - F)/F] = \log K_A + n \cdot \log[M], \qquad (1)$$

where F_0 and F are the fluorescence intensities of free CS and complexed CS, K_A is the association constant of CS and Zn^{2+}, n is the number of complexing centers, and $[M]$ is equilibrium concentration of Zn(II). Using Eq. (1) we can determine the association constant of CS–Zn^{2+} by the slope and intercept: $K_A = 2.75 \cdot 10^5$ liter·mol^{-1} and $n \approx 1$ (Table 3 and Fig. 11).

Interaction of CS and CT DNA. DNA can quench the fluorescence of CS in the binary CS–Zn^{2+} complex (Figs. 9a and 9c). Furthermore, the UV absorption intensity is decreased at λ_{195}, but a new peak appears in the UV spectra (228 nm) (Figs. 10a and 10c). This indicates that a ground state complex forms between CS and DNA. We also get the association constant $K_B = 53.89$ liter·mol^{-1} and $n \sim 0.4$ of fluorescence and quenching reagent concentration from Eq. (1) (Table 4 and Fig. 11). The lower association con-

Table 3. Fluorescence spectral parameters of cloxacillin sodium in the presence of Zn(II)

$V_{Zn^{2+}}$, ml	$C_{Zn^{2+}}$, M	Molar ratio of Zn^{2+} and cloxacillin sodium	Fluorescence intensity (F)	log([Zn^{2+}], M)	log[($F_0 - F$)/F]
0	–	control	82.5	–	–
0.02	$2 \cdot 10^{-6}$	0.2	72.1	–5.699	–0.795
0.04	$4 \cdot 10^{-6}$	0.4	63.3	–5.398	–0.518
0.06	$6 \cdot 10^{-6}$	0.6	54.2	–5.222	–0.282
0.08	$8 \cdot 10^{-6}$	0.8	48.0	–5.097	–0.143
0.10	$1 \cdot 10^{-5}$	1.0	43.1	–5.000	–0.039
0.12	$1.2 \cdot 10^{-5}$	1.2	39.4	–4.921	+0.039

Table 4. Fluorescence spectral parameters of cloxacillin sodium in the presence of DNA

No.	$V_{Zn^{2+}}$, ml	C_{DNA}, M	Molar ratio of Zn^{2+} and cloxacillin sodium	Fluorescence intensity (F)	log([Zn^{2+}], M)	log[($F_0 - F$)/F]
1	0	–	control	82.6	–	–
2	0.25	$1 \cdot 10^{-6}$	0.1	65.7	–6.000	–0.590
3	0.50	$2 \cdot 10^{-6}$	0.2	62.4	–5.699	–0.490
4	1.00	$4 \cdot 10^{-6}$	0.4	58.5	–5.398	–0.385
5	1.50	$6 \cdot 10^{-6}$	0.6	54.8	–5.222	–0.295
6	2.00	$8 \cdot 10^{-6}$	0.8	52.9	–5.097	–0.251
7	2.50	$1 \cdot 10^{-5}$	1.0	50.7	–5.000	–0.201

stant and n value indicate that there is weaker interaction between CS and the DNA than between CS and Zn(II).

Effect of Zn(II) on the interaction between CS and DNA. Our results indicate obvious changes in excitation and emission spectra among ternary Zn(II)–CS–DNA complex (Fig. 9d) and binary Zn(II)–CS and CS–DNA complexes. The UV spectrum of Zn(II)–CS–DNA is also clearly changed (Fig. 10d).

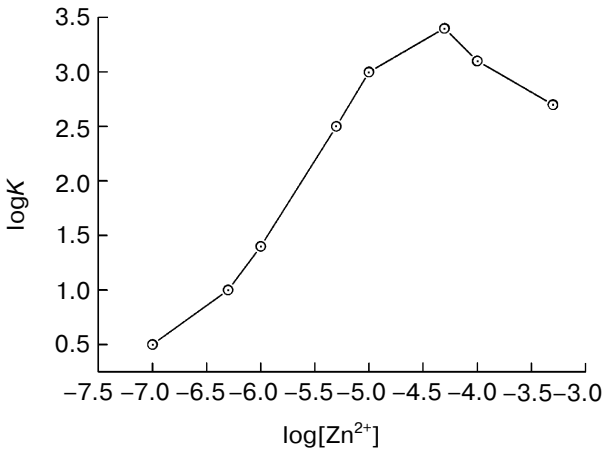


Fig. 12. Binding constants of CS and DNA versus Zn(II) concentration.

These results prove that Zn(II), CS, and DNA form a ternary complex and quench the fluorescence of CS.

To discuss and confirm the effect of Zn(II) on the interaction of CS and DNA, we calculated the changes in association constants between CS and DNA in the presence of Zn(II) at different concentrations according to theory and results deduced from Eq. (1) (Fig. 12). From Fig. 12 we can see that the association interaction of CS and DNA is first enhanced and then weakened as the concentration of Zn(II) increases. There is maximum interaction when log([Zn(II)], M) ≈ –4.25. So, Zn(II) becomes important in a certain concentration range.

Based on all the experimental results listed above, a complex of CS with Zn(II) is formed, and the molecular site of the complex is shown in Scheme 3. Moreover, the seven-element chelate structure contributes to the stability of the complex. This metal complex can be formed because there are lone pairs of electrons on the two oxygen atoms of the secondary amide (-CONH-) and the

tertiary amide (-CON<), while Zn(II) has an empty orbit which is open to the lone pair of electrons. Therefore, the complex can be formed as we already confirmed by our measurements of fluorescence and UV spectrum. The Zn(II) ion is important within a certain concentration range.

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