Complex Formation between Gallium(III) and Adriamycin in Aqueous Solutions

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The complex formation between gallium(III) and adriamycin (ADR) was investigated in 0.1 M KNO_3 at 25°C. With excess concentration of Ga(III) compared to ADR, Ga(III) was found to be coordinated with ADR in the low pH region below about 4.0. The spectrophotometric titration data supported the hypothesis that one proton is released as a result of complexation between Ga(III) and ADR via a 1:1 stoichiometry, and a phenolic oxygen on the ADR is involved. The stability constant of the complex was obtained by two different spectrophotometric methods. First, the absorbance development and pH changes reflecting the release of a proton due to complexation were monitored and the stability constant was estimated using the method of Hildebrand-Reilley. Secondly, ADR was titrated with increasing Ga(III) at constant pH 3.0 and analyzed by the Benesi-Hildebrand method. We took into consideration gallium hydrolysis in the calculation. The stability constants, 1.24×10^8 and $3.06 \times 10^8 \text{ M}^{-1}$ obtained by the two different methods, showed good agreement within an order-of-magnitude range.

Keywords adriamycin; deprotonation constant; gallium; chelate; metal complex; complex formation

Introduction

Adriamycin (ADR) is an antitumor antibiotic exhibiting activity against a variety of human cancers.¹⁾ However, the presence of dose-limiting toxic side effects has stimulated the development of analogs with the hope of improved efficacy and tolerability without cardiotoxicity.

Complexation of ADR with metal ions has been examined for this purpose. Quelamycin, consisting of three ADR molecules chelated to one Fe(III) ion, was reported to produce less cardiotoxicity as compared to ADR alone.²⁾ Physicochemical characteristics of Fe(III)–ADR and Fe(III)–daunorubicin complexes have been extensively investigated.³⁾ Complexation with Cu(II) and Pd(III) has been also studied.^{4,5)}

The radionuclide gallium-67 has been used extensively as a tumor-imaging reagent.⁶⁾ Injection of the citrate complex $\text{Ga(III)(citrate)}_2$ results in enhanced uptake of the metal into a variety of malignant tumors.⁷⁾ Similar uptake of gallium complexes with other organic ligands may be expected, as a possible antitumor activity of the Fe(III)–ADR complex is suggested.³⁾ In this report we examined the complex formation between Ga(III) and ADR, and the stability constant was determined by spectrophotometric titration methods.

Experimental

Chemicals Purified ADR was kindly provided by Kyowa Hakko Kogyo Co., Tokyo. $\text{Ga(NO}_3)_3 \cdot x\text{H}_2\text{O}$, ethylenediaminetetraacetic acid (EDTA), and 1-(2-pyridylazo)-2-naphthol (PAN) were purchased from Wako Pure Chemical Industries, Ltd., Osaka. All other chemicals were of reagent grade.

Apparatuses Absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer (Kyoto). Circular dichroism (CD) spectra were obtained on a Jasco J-600 spectrometer (Tokyo). Spectral changes were expressed in terms of the molar absorptivity for absorption spectra, and as $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficient) where ϵ_L and ϵ_R are the molar absorptivities for left and right circularly polarized lights. Fluorescence spectra were recorded on a spectrofluorophotometer (RF-520, Shimadzu, Kyoto) at an excitation wavelength of 460 nm. pH measurements were made with a Horiba F-7_{LC} digital pH meter having a precision of ± 0.005 pH unit (Tokyo).

Determinations of Ga(III) and ADR The content of Ga(III) was determined by means of EDTA titrations with PAN as an indicator.⁸⁾ ADR concentration was determined based on the molar absorptivity at 480 nm (ϵ , 11500).⁹⁾ As anthracycline solutions are generally sensitive to light and oxygen, the stock solution was prepared with water bubbled by

nitrogen gas for 10 min and stored at -20°C .

Spectrophotometric Titration of ADR Solution All titrations were carried out in KNO_3 (ionic strength 0.1) at $25 \pm 0.1^\circ\text{C}$. ADR ($9.9 \mu\text{M}$) was titrated with a KOH solution. Carbonate-free $0.0748 \pm 0.0002 \text{ M}$ KOH was prepared adding barium hydroxide to remove the CO_2 from the KOH solution and exchanging barium for potassium by passing a column of Amberlite IR 120B in the potassium form.¹⁰⁾ The KOH solution was successively increased to $1400 \mu\text{l}$ by a microsyringe. The mixing of the solution was carried out by gently bubbling nitrogen gas. Once the volume of KOH solution was added, the pH, absorption spectra and absorptivity at 590 nm of ADR solution were measured. The decreased concentration of ADR by the addition of a KOH solution was counterbalanced by changing the full scale of a spectrophotometric recorder. Since ADR decomposes at alkaline pHs,¹¹⁾ the spectrum was recorded as quickly as possible after the pH was raised to the desired value. Titrations were performed in triplicate. Protolytic dissociation constants were obtained for each run and the average values were determined by nonlinear least-squares computer analysis.^{12,13)}

Spectral Changes of ADR with $\text{Ga(NO}_3)_3$ ADR ($40 \mu\text{M}$ in 0.1 M KNO_3) was titrated by successively adding 10 mM $\text{Ga(NO}_3)_3$ at the molar ratio up to 200. The absorption and fluorescence spectra were recorded after about 10 min standing, at which time spectral changes were generally settled. The pH changes were also measured.

Spectrophotometric Titration of ADR–Ga(III) Complex An ADR ($40 \mu\text{M}$) solution containing 4 mM $\text{Ga(NO}_3)_3$ was titrated with a small amount of 0.02 M HCl or 0.02 M KOH. The absorbance changes at 567 nm in the pH region from 2.3 to 3.9 (final pHs after titration) were monitored and a stability constant was derived according to the Hildebrand-Reilley method.¹⁴⁾

In the meantime, ADR ($40 \mu\text{M}$) was titrated by Ga(III) at molar ratios up to 175 where the pH was always maintained at 3.0 by adding 0.02 M HCl or 0.02 M KOH. The absorptivity at 567 nm was monitored and the stability constant estimated according to the Benesi-Hildebrand method.¹⁵⁾

Results and Discussion

Deprotonation Constants of ADR The interactions of the ADR with metal ions and other biologically important substances depend on its various prototropic forms. The protolytic equilibria of the drug in citrate and phosphate buffers and sulfuric acid solution were intensively investigated in this respect.¹²⁾ Before studying a Ga(III)–ADR system where gallium was used as $\text{Ga(NO}_3)_3$, deprotonation constants of ADR were determined by spectrophotometric titration in 0.1 M KNO_3 .

Figure 1 shows the individual stage of protolytic equilibria of ADR with the microscopic dissociation constants, K_{CN} , K_{CZ} , K_{NA} and K_{ZA} . Defined similarly to previous literature,¹²⁾ the K_{CN} and K_{NA} correspond to the equilibria

Fig. 1. Structure of Adriamycin and a Chart of Its Protolytic Equilibria

Fig. 2. Absorption Spectra of Adriamycin without Ga(III) as a Function of pH in 0.1 M KNO₃

pH: (1), 6.39; (2), 8.33; (3), 8.79; (4), 9.26; (5), 9.57; (6), 9.82; (7), 10.06; (8), 10.34.

between the singly charged cation and the neutral species, and between neutral species and the singly charged anion, respectively. The K_{CZ} and K_{ZA} correspond to the equilibria between the singly charged cation and zwitterionic species, and between zwitterionic species and singly charged anion, respectively.

Figure 2 shows the visible absorption spectra of ADR in the pH range 6.39–10.34. As the pH was raised, the absorbance at 480 nm decreased and the band shifted to a higher wavelength at maxima 555 and 590 nm with the isosbestic points at 398 and 517 nm (Fig. 2). The color of ADR solution changed from red to purple at basic pH. This large shift can be attributed to the deprotonation of one phenolic group.^{4b,d} Since the amino group of the amino sugar moiety is located apart from the aromatic chromophore of the aglycone, the deprotonation of the amino group is unlikely to affect the spectrum of ADR.

The microscopic constants represented in Fig. 1 have the following relation to the macroscopic constants, K_1 and K_2 , which represent first and second deprotonation, respectively.¹²⁾

$$K_1 = K_{CN} + K_{CZ} \quad (1)$$

$$\frac{1}{K_2} = \frac{1}{K_{NA}} + \frac{1}{K_{ZA}} \quad (2)$$

Fig. 3. Spectrophotometric Titration of Adriamycin at 590 nm in 0.1 M KNO₃

ADR concentration was 9.9×10^{-6} M. Titration was carried out using 74.8 mM KOH. A solid line represents the calculated absorbance changes using the parameters obtained.

TABLE I. Negative Logarithms of Dissociation Constants of Adriamycin

Constant	Value
pK_1	9.10
pK_2	9.97
pK_{CN}	9.25
pK_{CZ}	9.64
pK_{ZA}	9.43
pK_{NA}	9.82

The reproducibilities of the K_1 and K_2 values obtained from Eq. 3 by nonlinear least-squares analysis were within 9% and 7%, respectively.

Based on these equilibria, the observed molar absorptivity of ADR, ϵ , corresponding to total absorbance at an analytical wavelength may be expressed by

$$\epsilon = \frac{\epsilon_C [H^+]^2 + K_1 [H^+] \epsilon_{N'} + K_1 K_2 \epsilon_A}{[H^+]^2 + K_1 [H^+] + K_1 K_2} \quad (3)$$

where $\epsilon_C [N] + \epsilon_A [Z] = \epsilon_{N'} ([N] + [Z])$, and ϵ_C , $\epsilon_{N'}$ and ϵ_A refer to the species $ADRH_3^+$, $ADRH_2$ and $ADRH^-$, respectively. As the fully protonated species does not absorb at 590 nm ($\epsilon_C = 0$), the absorptivity at 590 nm was used to determine deprotonation constants, K_1 and K_2 . Figure 3 shows the plot of ϵ_{590} as a function of pH. When the value of ϵ_A , 10800 was held constant, the parameters $\epsilon_{N'}$, K_1 and K_2 were estimated using Eq. 3. The ratio of $[N]$ to $[Z]$, represented as $(\epsilon_A - \epsilon_{N'}) / (\epsilon_{N'} - \epsilon_C)$, was calculated to be 2.45. Thus, according to Eqs. 1 and 2, the microscopic constants were obtained as summarized in Table I. A solid line in Fig. 3 represents the calculated curve based on these values.

Spectral Changes of Ga(III)–ADR Complex Formation

Fig. 4. Absorption Spectra of Adriamycin Titrated by Gallium Nitrate

ADR concentration was 4.0×10^{-5} M. $[\text{Ga}(\text{NO}_3)_3]/[\text{ADR}]$ and pH: (1), 0, 6.46; (2), 10, 3.43; (3), 20, 3.28; (4), 50, 3.11; (5), 100, 3.00; (6), 150, 2.95; (7), 200, 2.91 in 0.1 M KNO_3 .

Fig. 5. Fluorescence Spectra of Adriamycin in the Presence of $\text{Ga}(\text{NO}_3)_3$

ADR concentration was 4.0×10^{-5} M. $[\text{Ga}(\text{NO}_3)_3]/[\text{ADR}]$ and pH: (1), 0, 6.46; (2), 50, 3.11; (3), 100, 3.00 in 0.1 M KNO_3 , $\lambda_{\text{ex}} = 460$ nm.

Figure 4 shows the spectral changes of ADR in the presence of various amounts of $\text{Ga}(\text{NO}_3)_3$ in which the molar ratio of the metal ion to the drug varied from 0 to 200. The scanning spectra exhibited new absorption maxima at 496, 526 and 567 nm, accompanied by color changes from red to red-purple. The spectral shift was more appreciable, especially when $\text{Ga}(\text{III})$ was more than 10-fold the amount of ADR. As no spectral changes of the drug were observed in the acidic region, these shifts are the result of induction by $\text{Ga}(\text{III})$ and suggest the formation of a complex between $\text{Ga}(\text{III})$ and ADR. The spectra also exhibited isosbestic points at 387 and 487 nm, showing that a single gallium-ADR complex is formed. We also performed a spectroscopic study at physiological pH, but a shift of spectra was not detected. This may have been due to hydrolysis of gallium ion, which will be discussed later.

Concerning the complex formation, there are three possibilities: $\text{Ga}(\text{III})$ ion is bound to ADR through (i) the

Fig. 6. CD Spectra of Adriamycin in the Absence and Presence of $\text{Ga}(\text{NO}_3)_3$ at pH 3.0

ADR concentration was 4.0×10^{-5} M. $[\text{Ga}(\text{NO}_3)_3]$: ---, 0 M; —, 6.0×10^{-3} M in 0.1 M KNO_3 .

Fig. 7. Spectrophotometric Titration of $\text{Ga}(\text{III})$ -Adriamycin Complex

$[\text{ADR}]$, 4.0×10^{-5} M; $[\text{Ga}(\text{NO}_3)_3]$, 4.0×10^{-3} M. Titration was carried out using 0.02 M KOH or HCl in 0.1 M KNO_3 .

hydroxyl group on C_{11} , (ii) the hydroxyl group on C_6 , or (iii) the amino group of the sugar. The shift of the band to higher wavelengths could be assigned to deprotonation of the phenolic groups.^{4b,d} Thus, the observation suggests that the first or the second is feasible.

Figure 5 shows representative fluorescence spectra of ADR in the presence of $\text{Ga}(\text{NO}_3)_3$ under similar conditions to Fig. 4. It exhibits an isoemissive point at 572 nm. This also strongly suggests the involvement of phenolic chromophores in reference to the fluorescence spectrum of ADR at alkaline pH.¹² Considering the similarity to the complexes formed between ADR and $\text{Fe}(\text{III})$, $\text{Cu}(\text{II})$, or $\text{Pd}(\text{II})$,^{3,4b,5} $\text{Ga}(\text{III})$ is most probably bound to the C_{11} -phenolate oxygen and the C_{12} -carbonyl oxygen of ADR.

It has been shown that the dimeric form of ADR could accommodate two $\text{Pd}(\text{II})$ ions.⁵ In the case of the $\text{Ga}(\text{III})$ -ADR system, the existence of the dimer of ADR was examined by CD spectrum. Figure 6 shows the CD spectra at the molar ratios of $\text{Ga}(\text{III})$ to ADR 0:1 and 150:1 when the pH was maintained at 3.0. If ADR were in the dimeric form, the CD signal should be of the couplet type with a positive band at 445 nm and a negative one at 530 nm, while if it were in the monomeric form, only one positive band at 480 nm should appear.¹⁶ As the CD spectrum exhibited

no negative band at 530 nm in the Ga(III)–ADR system, it can be ruled out that Ga(III) ions are bound to the dimeric form of ADR.

Stability Constants for Ga(III)–ADR Complex Formation Figure 7 shows spectrophotometric titration by the addition of 0.02 M KOH or 0.02 M HCl when the molar ratio of Ga(III) to ADR was held constant at 100. The absorbance at 567 nm reflecting the complex formation was monitored in the pH region 2.3–3.8. At pHs ranging from 2.3 to 3.0 the absorbance increased with increasing pH value as the result of complexation. The maximum absorbance was shown at pH 3.0, followed by diminished absorbance with further increase in pH. The decreased absorbance observed in the pH region greater than 3.0 could be explained by hydrolysis of the Ga(III) ion. The gallium ion is hydrolyzed to form $\text{Ga}(\text{OH})_2^+$, $\text{Ga}(\text{OH})_2^+$ and $\text{Ga}(\text{OH})_3$.^{17,18} The values of the hydrolysis constants of gallium have been reported previously,¹⁸ with $\log K_{\text{OH}}^1 = 2.91$, $\log K_{\text{OH}}^2 = 3.70$ and $\log K_{\text{OH}}^3 = 4.40$ corresponding to the formation of the species described above, respectively. The prevailing forms of the metal in the pH region examined (2.3–3.8, Fig. 7) are Ga^{3+} and $\text{Ga}(\text{OH})_2^+$. Therefore, the concentration of the unhydrolyzed metal ion decreases with increasing pH.

To estimate the stability constant of the complex, the following two methods were used. First, the pH-absorption profile reflecting the complex formation shown in Fig. 7 was analyzed according to the method of Hildebrand and Reilley.¹⁴ Assuming that the Ga^{3+} species is responsible for the complex formation and the complex is formed at a 1:1 stoichiometry, the stability constant, β , is defined as follows:

$$\beta = \frac{[\text{Ga}(\text{ADRH}_2)^{3+}]}{[\text{Ga}^{3+}][\text{ADRH}_2]} \quad (4)$$

$$\beta^* = \frac{[\text{Ga}(\text{ADRH}_2)^{3+}][\text{H}^+]}{[\text{Ga}^{3+}][\text{ADRH}_3]} \quad (5)$$

where β^* is the equilibrium constant. The logarithmic form of Eq. 5 is given by

$$\log \frac{[\text{Ga}(\text{ADRH}_2)^{3+}]}{[\text{ADRH}_3]} - \log [\text{Ga}^{3+}] = \text{pH} - \text{p}\beta^* \quad (6)$$

in which $\text{p}\beta^*$ means $(-\log \beta^*)$. The first term of the left member can be represented by

$$\log \frac{[\text{Ga}(\text{ADRH}_2)^{3+}]}{[\text{ADRH}_3]} = \log \frac{A - A_f}{A_\infty - A} \quad (7)$$

where A is absorbance for ADR in the presence of excess Ga(III) concentrations, A_f the absorbance for free ADR which is constant in the acidic region, and A the absorbance when ADR is fully complexed with Ga(III). On plotting the absorbance changes according to Eqs. 6 and 7, it should be born in mind that the concentration of unhydrolyzed metal species, $[\text{Ga}^{3+}]$, in Eq. 6 could not always be approximated to the initial concentration of the metal ion if the metal ion were in excess of the drug. As mentioned earlier, this is because the concentration of the Ga^{3+} species is determined by the hydrolysis constants and pH examined. A complex formation may also diminish the unhydrolyzed

metal ion. However, the influence of the pH changes is likely to be much larger than that of the complex formation because of an excess metal ion compared to the drug. Therefore, the analysis of the spectral changes was made in the lower pH region (2.28–2.62) to avoid such complexity where the effective concentration of the Ga^{3+} species was adjusted by only the hydrolysis constant at each pH examined.

Figure 8 shows the relationship between the absorbance changes reflecting the complex formation monitored at 567 nm and the pH according to Eqs. 6 and 7. The plot showed a linear dependency on the pH, producing a slope of 1.21. This result supports the idea that one proton is most likely to be released from the ADR as a result of the 1:1 complex formation. Subsequently, the equilibrium constant β^* could be estimated from the intercept on the ordinate according to Eq. 6. Taking in the first deprotonation constant, K_1 and the β^* value obtained, 9.82×10^{-2} , the stability constant ($\beta = \beta^*/K_1$) thus obtained was given as $1.24 \times 10^8 \text{ M}^{-1}$.

Secondly, the stability constant was determined by the method of Benesi-Hildebrand.¹⁵ In a set of experiments in which ADR (40 μM) was titrated by $\text{Ga}(\text{NO}_3)_3$ at molar ratios up to 175, the pH was held constant at 3.0 by adding

Fig. 8. Plots for Determining the Equilibrium Constant (β^*) of Ga(III)–Adriamycin Complex According to Eqs. 7 and 8

The effective concentration of Ga(III) was adjusted by the hydrolysis constant of the metal ion with changes of pH. Temperature: 25 °C.

Fig. 9. Absorption Spectra of Ga(III)–Adriamycin Solution at pH 3.0

ADR concentration was $4.0 \times 10^{-5} \text{ M}$. $[\text{Ga}(\text{NO}_3)_3]/[\text{ADR}]$: (1), 50; (2), 75; (3), 100; (4), 125; (5), 150; (6), 175 in 0.1 M KNO_3 .

Fig. 10. Double-Reciprocal Plots for Determining the Stability Constant (β) of Ga(III)-Adriamycin Complex According to Eq. 9

Temperature: 25°C.

0.02 M HCl or 0.02 M KOH. Figure 9 shows spectral changes as a function of the metal concentration. Assuming that the 1 : 1 complex formation is responsible for these changes, the following equilibrium equations can be derived:

$$K = \beta^* / [\text{H}^+] = \frac{[\text{Ga}(\text{ADRH}_2)]^{3+}}{[\text{Ga}^{3+}][\text{ADRH}_3^+]} \quad (8)$$

$$\frac{[\text{ADR}]_0}{A - \epsilon_f[\text{ADR}]_0} = \frac{1}{K(\epsilon_b - \epsilon_f)} \cdot \frac{1}{[\text{Ga}^{3+}]} + \frac{1}{\epsilon_b - \epsilon_f} \quad (9)$$

where K is an arbitrary constant. A is the absorbance at 567 nm. ϵ_f and ϵ_b represent molar absorptivities for free ADR and complexed ADR, respectively. $[\text{ADR}]_0$ is the initial concentration of ADR. $[\text{Ga}^{3+}]$ is the concentration of the unhydrolyzed metal ion calculated from the initial concentration and the hydrolysis constant at pH 3.0.

Figure 10 shows a plot according to Eq. 9, which allowed calculation of the K value, $2.43 \times 10^2 \text{ M}^{-1}$, from the slope and subsequently the stability constant. The stability constant ($\beta = K[\text{H}^+]/K_1$) $3.06 \times 10^8 \text{ M}^{-1}$, finally obtained was in good agreement with that obtained by the previous method.

The complex formation between ADR and Ga(III) was only observed in the low pH region. It is therefore most probable that ADR is unable to form complexes with Ga(III) as the pH increases because of the hydrolysis of the metal ion. In contrast with the above, such a phenomenon was not found in the complexation of ADR-Fe(III), in which the spectroscopic titration data showed an absorbance plateau with increasing pH up to 7, although

Fe(III) also formed ferric hydroxide.³⁾ Precise kinetic data including the hydration rate of the metal ions may be needed to explain this difference. The intrinsic difference of these metal ions is that Ga(III) is a trivalent d^{10} -system metal ion while Fe(III) belongs to transition elements, possibly making use of its $3d$, $4s$ and $4p$ orbitals in forming hybrids. Finally, the lack of complex formation of ADR and Ga(III) in the neutral pH region seems to be at a disadvantage for therapeutic use because the complex would not be able to remain unmodified in biological fluids.

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References and Notes

- 1) F. Arcamone, "Metabolism of Xenobiotics," ed. by J. W. Gorrod, H. Oelschläger, and J. Caldwell, Taylor & Francis, London, 1988, pp. 115-119.
- 2) M. Gosalvez, M. F. Blanco, C. Vivero, and F. Valles, *Eur. J. Cancer*, **14**, 1185 (1978); C. E. Myers, L. Gianni, C. B. Simone, R. Klecker and R. Greene, *Biochemistry*, **21**, 1707 (1982).
- 3) H. Beraldo, A. Garnier-Suillerot, L. Tosi, and F. Lavelle, *Biochemistry*, **24**, 284 (1985).
- 4) a) P. M. May, G. K. Williams, and D. R. Williams, *Inorg. Chim. Acta*, **46**, 221 (1980); b) F. T. Greenaway and J. V. Dabrowiak, *J. Inorg. Biochem.*, **16**, 91 (1982); c) R. Kiraly and R. B. Martin, *Inorg. Chim. Acta*, **67**, 13 (1982); d) H. Beraldo, A. Garnier-Suillerot, and L. Tosi, *Inorg. Chem.*, **22**, 4117 (1983).
- 5) M. M. L. Fiallo and A. Garnier-Suillerot, *Biochemistry*, **25**, 924 (1986).
- 6) C. L. Edwards and R. L. Hayes, *J. Nucl. Med.*, **10**, 103 (1969).
- 7) E. Aulbert, A. Gibhardt, D. Shulz, and U. Hanbold, *Nucl. Med.*, (Amsterdam) **15**, 183 (1976); P. Bickel and H. A. Hansen, *Br. J. Radiol.*, **45**, 182 (1972).
- 8) H. Onishi, "Treatise on Analytical Chemistry," Part II, Vol. 2, ed. by I. M. Kolthoff and P. J. Elving, Interscience Publishers, Inc., New York, 1962, pp. 1-105.
- 9) J. B. Chaires, N. Dattagupta, and D. M. Crothers, *Biochemistry*, **21**, 3927 (1982).
- 10) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co., Ltd., Great Britain, 1962.
- 11) J. H. Beijnen, O. A. G. J. van der Houwen, and W. J. M. Underberg, *Int. J. Pharmaceut.*, **32**, 123 (1986).
- 12) R. J. Sturgeon and S. G. Schulman, *J. Pharm. Sci.*, **66**, 958 (1977).
- 13) K. Yamaoka, Y. Tanigawara, T. Nakagawa, and T. Uno, *J. Pharmacobio-Dyn.*, **4**, 879 (1981).
- 14) G. P. Hildebrand and C. N. Reilly, *Anal. Chem.*, **29**, 258 (1957).
- 15) H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1949).
- 16) S. R. Martin, *Biopolymers*, **19**, 713 (1980).
- 17) W. R. Harris and A. E. Martell, *Inorg. Chem.*, **15**, 713 (1976).
- 18) V. A. Nazarenko, V. P. Antonovich, and E. M. Nevskaya, *Russ. J. Inorg. Chem. (Engl. Transl.)*, **13**, 825 (1968).