Formation of a Chromophoric Complex Between Alendronate and Copper(II) Ions

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INTRODUCTION

Alendronate (4-amino-1-hydroxybutane-1,1-bisphosphonic acid; ABP) is an experimental drug with potential utility in treatment of diseases characterized by abnormal bone turnover, such as metastatic bone disease, hypercalcemia of malignancy, Paget's disease, periodontal disease, and osteoporosis. The lack of a detectable chromophore in alendronate, as well as in many other bisphosphonates, makes the analytical methods development for this class of compounds challenging. Most of the published analytical methods for determination of bisphosphonates require specialized equipment (1–5) or involve a derivatization step in order to introduce a chromophore into the molecule (6–9). For ABP alone there are three published reversed-phase HPLC methods involving precolumn (6,7) or postcolumn (8) derivatization. An ion-exchange HPLC method with conductivity detection for determination of ABP has also been developed (1).

Metal chelating properties of bisphosphonates are well documented (10–12). We discovered that alendronate and copper(II) ions form a complex with UV activity suitable for monitoring by conventional UV/Vis equipment. In this communication we describe a study of chromophoric complex formation between alendronate and copper(II) ions in acidic media and briefly discuss the applicability of our findings to analytical methods development.

MATERIALS AND METHODS

Materials. 4-Amino-1-hydroxybutane-1,1-bisphosphonic acid (alendronate; ABP), 4-amino-1-hydroxybutane-1,1-bisphosphonic acid sodium salt trihydrate (MK-0217), 3-amino-1-hydroxypropane-1,1-bisphosphonic acid (pamidronate; APD), and 1-hydroxyethane-1,1-bisphosphonic acid (etidronate; EDPH) were prepared by Merck Research Laboratories Process Research Department and used as received. All other materials and standard solutions were purchased from Aldrich Chemical Company or Fisher Scientific and used as received.

Methods. UV/Vis spectrophotometric measurements were conducted using a Perkin–Elmer Lambda 6 UV/Vis spectrophotometer. pH measurements were performed using an Orion Research microprocessor ionalyzer/901 equipped with a Fisher Scientific glass/Ag-AgCl combination electrode calibrated with Fisher Scientific standard buffer solutions.

All experiments were conducted at 25°C. Initial complexometric titrations were performed in 1.5×10^{-3} *M* aqueous HNO_3. The measurements of pH dependence of the equilibrium binding constants between ABP and Cu^{2+} in acidic media were performed in 1.5×10^{-3} *M* aqueous chloroacetate buffers. An example of a typical complexometric titration follows: A solution of ABP (3.0×10^{-4} *M*, 100 mL) in appropriate buffer thermostated at 25°C was titrated with a 0.5 *M* solution of $CuSO_4$ in the same buffer. UV/Vis spectra of the resulting solution were taken after each addition of $CuSO_4$ and absorbance was recorded as a function of total concentration of Cu^{2+}. The volume change during titration was less than 1% and it was neglected in equilibrium constant calculations. Molar absorptivities of Cu^{2+} (ϵ_{Cu}) as a function of pH in chloroacetate buffer solutions were determined independently. Molar absorptivities of the ABP–Cu complex ($\epsilon_{ABP\text{-}Cu}$) as a function of pH in chloroacetate buffer solutions were determined from absorbances of solutions of ABP and Cu^{2+} in which at least 90% of ABP was present as an ABP–Cu complex (*vide infra*).

RESULTS AND DISCUSSION

The initial titration of ABP with Cu^{2+} was performed in 1.5×10^{-3} *M* HNO_3 (pH 2.8). This solvent was chosen because it is currently used as a mobile phase in an ion-exchange HPLC method (1).

UV/Vis spectra of solutions of $CuSO_4$ in 1.5×10^{-3} *M* HNO_3, with and without the addition of ABP, and the relevant difference spectra are shown in Fig. 1. Also shown in Fig. 1 is the comparison of spectra of the ABP–Cu complex in 1.5×10^{-3} *M* HNO_3 and 1.5×10^{-3} *M*, pH 2.8, chloroacetate buffer. The titration curve of ABP with Cu^{2+} in 1.5×10^{-3} *M* HNO_3 determined spectrophotometrically at 240 nm is shown in Fig. 2. Initially, the concentration of ABP is higher than the concentration of Cu^{2+} and most of the absorbance increase corresponds to the increase in concentration of the ABP–Cu complex. The numerical value of the slope at higher Cu^{2+} concentrations is identical to the molar absorptivity of $CuSO_4$, which shows that at higher concentrations of Cu^{2+}, ABP is saturated with Cu^{2+} and that the absorbance increase is due only to the increase in Cu^{2+} concentration. The line representing the initial slope of the titration curve intersects the correlation line of the latter portion of the curve at a Cu^{2+} concentration of 3×10^{-4} *M*, which is the same as the concentration of ABP, showing that the ABP–Cu complex has a 1:1 stoichiometry [Eq. (1)].

$$\text{ABP} + \text{Cu}^{2+} \underset{}{\overset{K_{eq}}{\rightleftharpoons}} \text{ABP–Cu} \quad (1a)$$

$$K_{eq} = [\text{ABP–Cu}]/([\text{ABP}]_{free}[\text{Cu}^{2+}]_{free}) \quad (1b)$$

Equation (2) describes the total absorbance of a solution containing ABP and Cu^{2+}:

$$A_{tot} = \epsilon_{Cu} \times [\text{Cu}^{2+}]_{free} + \epsilon_{ABP\text{-}Cu} \times [\text{ABP–Cu}] \quad (2)$$

$$[\text{Cu}^{2+}]_{free} = [\text{Cu}^{2+}]_{tot} - [\text{ABP–Cu}] \quad (3)$$

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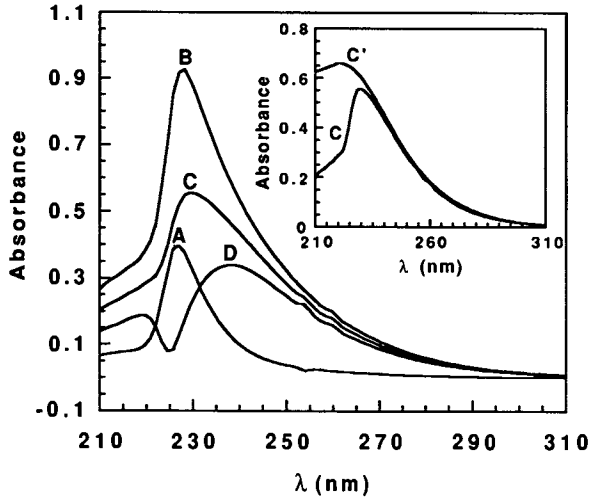


Fig. 1. UV/Vis spectra of (A) $CuSO_4$ (1.2×10^{-3} *M*) and (B) ABP (3×10^{-4} *M*) + $CuSO_4$ (1.5×10^{-3} *M*) in 1.5×10^{-3} *M* HNO_3, and difference spectra (C) = B − A = the spectrum of ABP–Cu complex and (D) = C − A, the difference spectrum between ABP–Cu complex and Cu^{2+} showing the maximum difference at ~240 nm, the wavelength used for analytical measurements. Insert: Comparison of the spectrum of ABP–Cu complex obtained in 1.5×10^{-3} *M* HNO_3 (C) and in 1.5×10^{-3} *M*, pH 2.8, chloroacetate buffer (C'). Spectral differences at lower wavelengths are due to differences in background absorbance.

Substitution of Eq. (3) into Eq. (2) gives Eq. (4):

$$A_{tot} = \epsilon_{Cu} \times [Cu^{2+}]_{tot} + (\epsilon_{ABP-Cu} - \epsilon_{Cu}) \times [ABP\text{–}Cu] \quad (4)$$

where [ABP–Cu] is derived from Eqs. (1b) and (3) and expressed in terms of equilibrium constant K_{eq} and known total concentrations of ABP and Cu^{2+} [Eq. (5)].

$$[ABP\text{–}Cu] = (K_{eq}[Cu^{2+}]_{tot} + K_{eq}[ABP]_{tot} + 1 - \text{sqrt}((K_{eq}[Cu^{2+}]_{tot} + K_{eq}[ABP]_{tot} + 1)^2 - 4K_{eq}^2[Cu^{2+}]_{tot}[ABP]_{tot}))/(2K_{eq}) \quad (5)$$

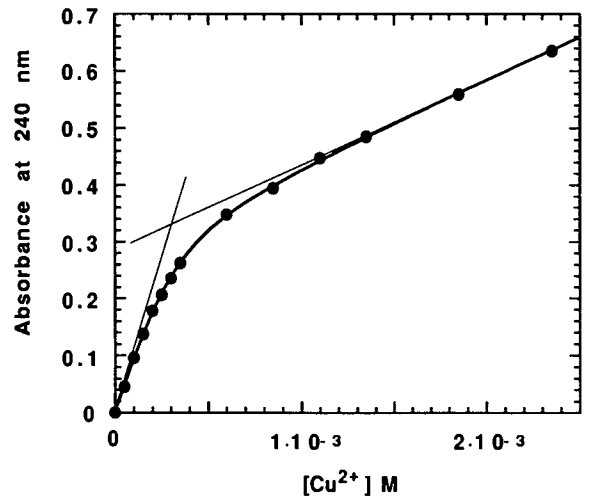
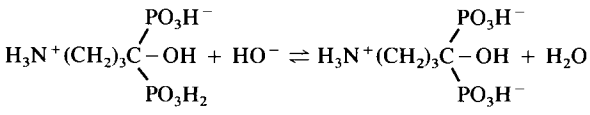


Fig. 2. Spectrophotometric titration curve of ABP with $CuSO_4$ in 1.5×10^{-3} *M* HNO_3. The points are experimental and the line is the nonlinear least-squares fit of the data to Eq. (4) [ϵ_{Cu} = 145.6 ± 1.2 M^{-1} cm^{-1}, ϵ_{ABP-Cu} = 1156 ± 12 M^{-1} cm^{-1}, K_{eq} = (1.67 ± 0.12) × 10^4 M^{-1}].

The equilibrium binding constant between ABP and Cu^{2+} in 1.5×10^{-3} *M* HNO_3 was obtained by nonlinear least-squares fitting of the data in Fig. 2 to Eq. (4); K_{eq} = (1.67 ± 0.12) × 10^4 M^{-1}. This value is similar to the reported $K_{eq} = 6.3 \times 10^4$ M^{-1} for complex formation between Cu^{2+} and EDPH determined under similar conditions (12).

The pH dependence of the equilibrium constant for Cu^{2+} binding by ABP was studied in 1.5×10^{-3} *M* chloroacetate buffers. Chloroacetate buffer was chosen because its pK_a is close to the pH range of interest for analytical work and because of its relatively small Cu^{2+} binding constant (13). Since chloroacetate can compete to a certain extent with ABP for Cu^{2+} binding, the measured equilibrium constants for Cu^{2+} binding by ABP in chloroacetate buffers are in fact the *apparent equilibrium constants* (K'_{eq}). The titration curves of ABP with Cu^{2+} are shown in Fig. 3, and the pH dependence of the log K'_{eq} is shown in Fig. 4. As the pH is raised, the phosphonate groups in ABP are deprotonated (Scheme I), and it is expected that the magnitude of K_{eq} for binding between ABP and Cu^{2+} will increase in analogy with the known pH dependence of calcium binding by ABP.



Scheme I

However, the apparent equilibrium constant (K'_{eq}) shows a parabolic dependence on pH, which suggests that, as the pH is raised, chloroacetate anion effectively competes with ABP for complexation with Cu^{2+}.

The magnitude of the apparent equilibrium constants in chloroacetate buffer in the 2.5–3.1 pH range (K'_{eq} = ~7000 M^{-1}) indicates that ABP is not completely saturated with Cu^{2+}. For example, at [Cu^{2+}] = 2×10^{-3} *M* and [ABP] = 1×10^{-4} *M*, only about 93% of ABP is present as ABP–Cu complex. Since the units for K_{eq} are M^{-1}, the saturation is

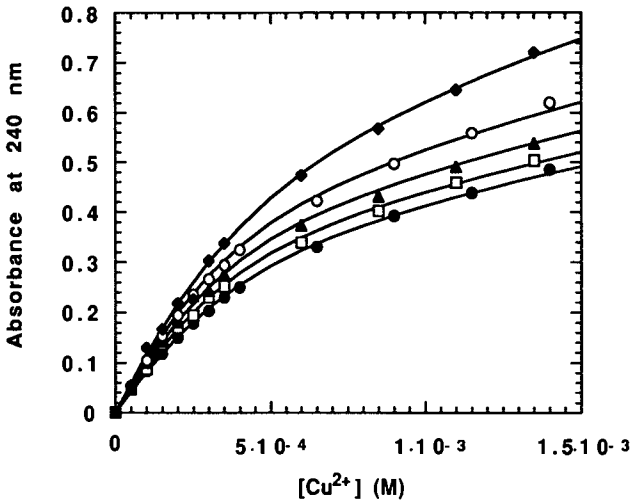


Fig. 3. Spectrophotometric titration curves of ABP with $CuSO_4$ in 1.5×10^{-3} *M* chloroacetate buffers. The points are experimental and the lines are the nonlinear least-squares fits of the data to Eq. (4).

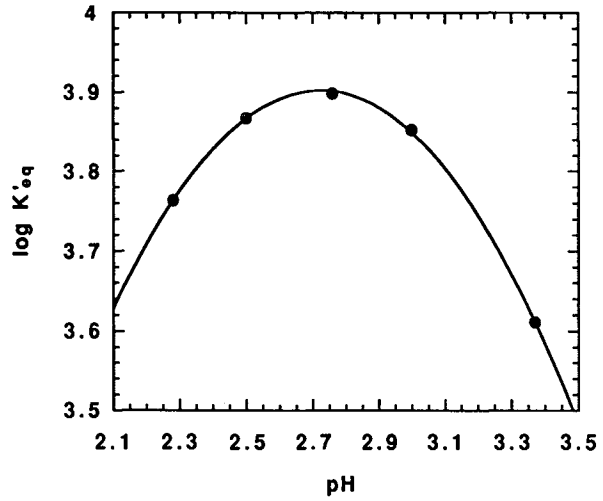


Fig. 4. pH dependence of the log of the apparent equilibrium constant (log K'_{eq}) for Cu^{2+} binding by ABP in 1.5×10^{-3} *M* chloroacetate buffers.

also a function of the total concentrations of ABP and Cu^{2+}. However, at constant Cu^{2+}, the predicted variation in saturation is negligible over the limited range of ABP concentrations suitable for analytical work and the absorbance should be a linear function of ABP concentration. In order to demonstrate this, a series of solutions was prepared with constant concentration of Cu^{2+} (2.5×10^{-3} *M*) and varying concentrations of bisphosphonates APD, EDPH, and ABP. A plot of absorbance of these solutions at 240 nm vs the concentration of bisphosphonates is shown in Fig. 5. There is no observable deviation from linearity, indicating that chromophoric complex formation between bisphosphonates and Cu^{2+} can be used as an analytical tool.

Possible limitations for quantitative utilization of complex formation between bisphosphonates and Cu^{2+} ions include (i) the presence of other ions which may compete for complexation with the bisphosphonate (12); (ii) the presence of competing ligands for Cu^{2+} ions such as citrate, which is

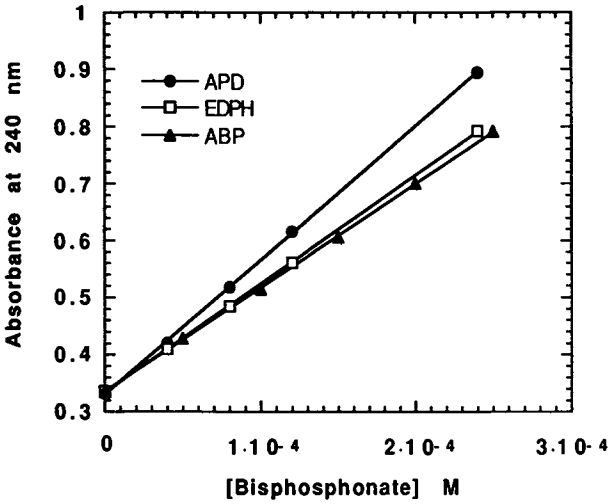


Fig. 5. Calibration curves for copper complexes of APD, EDPH, and ABP in 1.5×10^{-3} *M* chloroacetate buffer at pH 2.8 and $[Cu^{2+}] = 2.5 \times 10^{-3}$ *M*.

present in many pharmaceutical formulations (1); and (iii) the relatively low solubility of Cu–bisphosphonate salts, especially at higher pH's (10).

Our findings suggest that it may be possible to develop HPLC analytical methods for bisphosphonates, which would utilize direct UV detection for bisphosphonate–copper complexes. A capillary zone electrophoresis method for determination of ABP which utilizes the inclusion of Cu^{2+} in the electrolyte and UV detection of ABP–Cu complex has already been developed in our laboratories (14).

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