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Bisphosphonate binding affinity as assessed by inhibition of carbonated apatite dissolution *in vitro*

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Abstract

Bisphosphonates (BPs), which display a high affinity for calcium phosphate surfaces, are able to selectively target bone mineral, where they are potent inhibitors of osteoclast-mediated bone resorption. The dissolution of synthetic hydroxyapatite (HAP) has been used previously as a model for BP effects on natural bone mineral. The present work examines the influence of BPs on carbonated apatite (CAP), which mimics natural bone more closely than does HAP. Constant composition dissolution experiments were performed at pH 5.50, physiological ionic strength (0.15M) and temperature (37°C). Selected BPs were added at (0.5×10^{-6}) to $(50.0 \times 10^{-6})M$, and adsorption affinity constants, K_L , were calculated from the kinetics data. The BPs showed concentration-dependent inhibition of CAP dissolution, with significant differences in rank order zoledronate > alendronate > risedronate. In contrast, for HAP dissolution at pH 5.50, the differences between the individual BPs were considerably smaller. The extent of CAP dissolution was also dependent on the relative undersaturation, σ , and CAP dissolution rates increased with increasing carbonate content. These results demonstrate the importance of the presence of carbonate in mediating the dissolution of CAP, and the possible involvement of bone mineral carbonate in observed differences in bone affinities of BPs in clinical use.

Keywords

constant composition; bisphosphonates; dissolution kinetics; adsorption affinity; carbonated apatite; hydroxyapatite; bone mineral

INTRODUCTION

Based on X-ray diffraction, infrared spectroscopy, and chemical analysis, calcium hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$, is often regarded as the principal inorganic constituent of bone mineral.^{1–3} Thus HAP and its derivatives have been used extensively in *in vitro* studies to investigate the complex nature of skeletal mineral scaffolding. However, this ignores the role of minor components (e.g. Na^+ , K^+ , Mg^{2+} , HPO_4^{2-} , CO_3^{2-} , Cl^- , F^- , H_2O) and

trace impurities (e.g. Sr^{2+} , Ba^{2+} , Pb^{2+} , Mn^{2+} , Cd^{2+} , Li^+ , $\text{P}_2\text{O}_7^{4-}$) in bone mineral,⁴ and the fact that the inorganic portion of bone does not have pure HAP stoichiometry. Human skeletal mineral contains ~4–7% (see Ref. 5, p 110, Table 6.1)^{6,7} carbonate by mass. This has prompted the recent use of carbonated apatite (CAP), specifically B-type CAP containing 3.00% and 7.96% carbonate, where carbonate has been substituted for phosphate; this appears to be more biologically relevant based on numerous natural bone studies.^{6,8} In our work, FTIR spectroscopy has shown that the characteristic vibrational frequencies of natural bone powder are closer to CAP than HAP (Fig. 3), as suggested by the results of other studies.^{3,6,7} Although the biological importance of carbonate in bone is not fully understood, it has been shown to act as a hydrogen ion buffer,⁹ to increase crystal lattice strain, and to reduce the overall crystallite size.^{10,11}

Bisphosphonates (BPs) are nonhydrolyzable analogues of naturally occurring inorganic pyrophosphates; they display strong affinity for bone mineral, and are potent inhibitors of bone mineral resorption. BPs are used clinically to treat disorders of excessive bone resorption, including Paget's disease, bone metastases, and osteoporosis.^{4,12} It was first thought that the antiresorptive action resulted from a direct inhibition of mineral dissolution, but more recent studies have shown an additional direct intracellular inhibition of osteoclasts.^{4,13–15} The chemical composition of the R_1 and R_2 side chains (Fig. 1) plays an important role in both the mineral binding and osteoclast inhibition.⁴ At the molecular level, N-containing BPs inhibit farnesyl diphosphate synthase in the mevalonic acid pathway, thereby inhibiting prenylation of selected G-proteins.¹³ BPs also exhibit highly selective localization and prolonged storage within bone.¹⁵

The purpose of this study was to determine the effects of relative undersaturation, carbonate content, and BP concentration on the dissolution of CAP, and to complement recent studies on HAP.¹⁶

EXPERIMENTAL METHODS

CAP, $(\text{Ca}_{10-x}\text{Na}_x)(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{OH})_2$, and HAP, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, were prepared by direct precipitation from solution using methods described by LeGeros (see Ref. 5, pp 22–24). Physical characteristics and elemental compositions are shown in Table I. Specific surface areas (SSAs) of the seed crystals were determined by a single point BET (Brunauer, Emmett, and Teller) adsorption method with a 30/70 N_2/He gas mixture (Monosorb; Quantachrome Corporation).¹⁷ Crystals were also examined by scanning electron microscopy (Hitachi S-4000 Field Emission SEM) [Fig. 2(a,b)], and FTIR spectroscopy (Perkin–Elmer 1760×) (Fig. 3).^{18,19} Phosphate and calcium analyses were made using UV–vis and atomic absorption spectroscopy, respectively.²⁰ Carbonate analysis was performed using carbon coulometry.²¹ Stock solutions, prepared in Grade A glassware using reagent grade chemicals, were vacuum-filtered twice through 0.22- μm Millipore® filter papers.

Constant composition (CC) dissolution experiments were conducted in Teflon® covered, double-walled, Pyrex® jacketed cells maintained at $(37.0 \pm 0.05)^\circ\text{C}$.²² Under-saturated solutions were prepared by introducing triple distilled water to the cell, followed in succession by NaCl , KH_2PO_4 , and CaCl_2 solutions to yield a calcium/phosphate molar ratio of 1.67 and an ionic strength of 0.15M. Solutions were bubbled with water-saturated N_2 gas, and the pH was adjusted to 5.50 ± 0.01 by the dropwise addition of 0.100M KOH. Experimental conditions and results are summarized in Table II and Tables III and IV, respectively.

Hydrogen-ion activity was monitored using a pH electrode (Orion model 91) with a Ag/AgCl single junction reference electrode (Orion model 90-01) and an Orion model 720A+ pH meter. The electrodes were calibrated using pH 6.84 (0.025M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$), and pH 4.03

(0.050M KHP, potassium hydrogen phthalate) buffers.²³ When the reaction solutions had equilibrated, the potentiometer voltage bias was set, and following the subsequent addition of seed, a change of -0.05 mV from the set value triggered the addition of HCl/NaCl titrant. Periodically, an aliquot of solution was removed, rapidly filtered, and analyzed for calcium and phosphate to verify constancy of the composition.

The reaction solution concentration, W_i , of the free ions “i,” after titrant addition is given by Eq. (1)

$$W_i = \frac{W_i V_T + T_i dV_t + x_i dn_i}{V_T + n_b dV_t} \quad (1)$$

where V_T is the volume of reaction solution, T_i the titrant concentration, dV_t the volume of titrant added, x_i the stoichiometric coefficient, dn_i the number of moles dissolved, and n_b the number of titrant burets. T_i is given by Eq. (2)

$$T_i = W_i N_t + C_{\text{eff}} \quad (2)$$

where C_{eff} is the number of moles of equivalent HAP dissolved per liter of added titrant

$$C_{\text{eff}} = \frac{dn}{dV_t} \quad (3)$$

Titrant concentrations for sodium chloride (T_{NaCl}) and hydrochloric acid (T_{HCl}) were calculated from Eqs. (4) and (5)

$$T_{\text{NaCl}} = n_b W_{\text{NaCl}} + 6 C_{\text{eff}} \quad (4)$$

$$T_{\text{HCl}} = n_b W_{\text{HCl}} + 14 C_{\text{eff}} \quad (5)$$

The uncorrected rate of dissolution, R_D ($\text{mol m}^{-2} \text{min}^{-1}$), was calculated from the titrant volume, V_t , using Eq. (6)

$$R_D = \frac{\left(\frac{dV_t}{dt}\right) (C_{\text{eff}})}{(m_o)(SSA_o)} \quad (6)$$

where dV_t/dt is the rate of titrant addition, and m_o the mass of seed added, normalized for the SSA_o of the seed crystals. As dissolution proceeded, the decrease in crystal size resulted in rate reduction and possible changes in shape. Assuming isotropic, uniform dissolution of simple cubes or spheres, and HAP stoichiometry as in Eq. (7)

the corrected rate of dissolution, R_C ($\text{mol m}^{-2} \text{min}^{-1}$), was calculated using Eq. (8)

$$R_C = \frac{(V_t)(T_{\text{HCL}})}{14(m_o)(\text{SSA}_o) \left[1 - \frac{(V_t)(T_{\text{HCL}})(\text{MW})}{14(m_o)} \right]^{2/3}} \quad (8)$$

where MW is the molecular weight of HAP. The titrant solutions maintained constant ionic activities for all species.

Inhibition data were interpreted in terms of surface adsorption following a Langmuir model to yield adsorption affinity constants, K_L , in Eq. (9)

$$\frac{R_o}{R_o - R_i} = 1 + \frac{1}{K_L C_i} \quad (9)$$

in which R_o and R_i are the dissolution rates in the absence and presence of additives, respectively, and C_i is the concentration of BP.16

RESULTS AND DISCUSSION

Dissolution experimental conditions at pH 5.50 and 37.0°C are summarized in Table II and typical plots of moles of seed dissolved (per unit area) as a function of the time are shown in Figures 4, 6, and 7. The relative undersaturation, σ , is given by Eq. (10)

$$\sigma = 1 - S = 1 - \left[\frac{\text{IAP}}{K_a} \right]^{1/\nu} \quad (10)$$

where S is the degree of saturation, IAP the ionic activity product of the free lattice ions in solution, K_a the activity solubility product, and ν the number of ions in a formula unit,24 in terms of HAP stoichiometry.

As the HAP relative undersaturation, σ_{HAP} , was decreased from -0.12 to $+0.52$, the overall rate of dissolution of 3.00% CAP decreased from 1.24×10^{-8} to $0.10 \times 10^{-8} \text{ mol m}^{-2} \text{min}^{-1}$ [Table III, Fig. 4(a)]. Interestingly, the resulting plot of the dissolution rate against undersaturation [Fig. 4(b)] suggested that at higher driving forces ($\sigma_{\text{HAP}} \leq 0.16$), the mechanism of dissolution was best explained by the Burton, Cabrera, and Frank (BCF) model by which dissolution is controlled by the surface diffusion of dissolved lattice ions.^{25,26} Here the hydration of Ca^{2+} , PO_4^{3-} , CO_3^{2-} , and OH^{-} ions is followed by detachment, diffusion across the surface, and subsequent release into the bulk solution. This was previously reported by Tang et al.,²⁶ who considered dissolution to be associated with the hydration of carbonate and subsequent release from aqueous solution, and suggested that this equilibrium may be a rate-controlling step. At lower driving forces ($\sigma_{\text{HAP}} \geq 0.30$), the rate of dissolution appeared to be driven by a polynucleation dissolution mechanism. These observations suggest that carbonate

plays an important role in the dissolution of CAP, in contrast to the dissolution of stoichiometric HAP which follows a polynucleation dissolution mechanism over the range of conditions used in the present work.²⁷

In CAP, substitution of carbonate for phosphate causes a contraction of the *a*-axis and expansion of the *c*-axis dimensions of the HAP crystal lattice, decreasing crystallite size and increasing crystallite strain.¹⁸ This results in an increased solubility, which influences the overall dissolution kinetics of the mineral²⁸; all CAP samples used in these experiments exhibited this tendency (Table III, exp. groups H–L). Rate data were normalized with respect to the sample containing the lowest weight percent carbonate (0.52%), allowing for direct comparison of the increase in dissolution rates. At $\sigma_{\text{HAP}} = 0.02$, pH = 5.50, and 37.0°C, the rate of dissolution of 7.96% CAP ($R_C = 1.19 \times 10^{-7} \text{ mol m}^{-2} \text{ min}^{-1}$) was ~15.6 times greater than that of the 0.52% CAP ($R_C = 7.60 \times 10^{-9} \text{ mol m}^{-2} \text{ min}^{-1}$). A linear relationship between the dissolution rate and carbonate substitution, illustrated in Figure 5, suggested that the solubility was directly related to the amount of carbonate in the lattice. These observations confirm the importance of carbonate in bone, and point to the relevance of using CAP as an *in vitro* model for bone mineral.

The addition of BPs to the reaction solutions reduced the dissolution rates of both HAP and CAP, the extent dependent on the nature of the R_1 and R_2 side chains. Although all BPs had the same R_1 hydroxyl group, the substitution of different R_2 side chains displayed different degrees of inhibition. At a BP concentration of $0.5 \mu\text{mol L}^{-1}$ BP and $\sigma_{\text{HAP}} = -0.42$ (pH = 5.50, 37.0°C), all three BPs decreased the overall dissolution rate of HAP to a similar degree, zoledronate by 60.8% [$K_L = (3.10 \times 10^6)M^{-1}$], risedronate by 57.7% [$K_L = (2.73 \times 10^6)M^{-1}$], and alendronate by 57.0% [$K_L = (2.65 \times 10^6)M^{-1}$] [Fig. 6(a)]. It is not yet known how carbonate influences the binding of BPs to CAP surfaces; however, the effects on dissolution were similar to those for HAP. Thus, at a concentration of $5.0 \mu\text{mol L}^{-1}$, the three BPs behave differently; zoledronate was the most potent with ~86.0% [$K_L = (1.23 \times 10^6)M^{-1}$] inhibition of 7.96% CAP dissolution, followed by alendronate with 52.1% [$K_L = (0.22 \times 10^6)M^{-1}$] and risedronate with 17.8% [$K_L = (0.043 \times 10^6)M^{-1}$] [Fig. 6(b)]. It should be noted that the condition of $\sigma_{\text{HAP}} = 0.02$ for CAP dissolution was equivalent to approximately $\sigma_{\text{CAP}} = -0.44$ (based on unpublished solubility estimations by Tang et al.²⁶).

It is interesting to note that it required a 10-fold increase in BP concentration for the same degree of inhibition of 7.96% CAP dissolution when compared with HAP (alendronate inhibition: $5.0 \mu\text{mol L}^{-1}$ BP required to achieve 52.1% inhibition of CAP vs. $0.5 \mu\text{mol L}^{-1}$ BP for 57.0% inhibition of HAP). Binding affinity data also suggest that BPs have much lower affinities for CAP under these conditions, when compared with HAP [zoledronate: $K_{L,\text{CAP}} = (1.23 \times 10^6)M^{-1}$ vs. $K_{L,\text{HAP}} = (3.10 \times 10^6)M^{-1}$, alendronate: $K_{L,\text{CAP}} = (0.22 \times 10^6)M^{-1}$ vs. $K_{L,\text{HAP}} = (2.65 \times 10^6)M^{-1}$, and risedronate: $K_{L,\text{CAP}} = (0.043 \times 10^6)M^{-1}$ vs. $K_{L,\text{HAP}} = (2.73 \times 10^6)M^{-1}$]. Although a clear binding mechanism has not yet been established, molecular geometry and stereochemical factors probably contribute; the presence of carbonate is clearly a contributing factor.

Recent work by Fernandez et al. has shown that the molecular conformation of alendronate plays an important role in its HAP binding ability.²⁹ The zwitterionic character of alendronate allows the negatively charged terminus to approach the calcium ions in the lattice, while the position of the positively charged nitrogen enables phosphate to be involved in multidentate binding.³⁰ Mathew et al. investigated possible interaction models between bis (acylphosphonate) ions and HAP. The data showed a nearly perfect lattice match between the calcium and the phosphate/phosphonate ions on the most prominent faces for HAP, (001) and (100), allowing optimal binding of incoming acylphosphonate inhibitor molecules.³¹ Further, they established that the (100) face of HAP had a plane of calcium ions with similar Ca–Ca

distances as in the (010) face of Ca-bis(acylphosphonate) salt, indicating that this may be the most probable binding site. A simple growth inhibition mechanism was proposed in which the BPs selectively block active growth sites on the HAP surface.

Recent modeling studies of BP binding to HAP surfaces have implicated hydrogen bonding between the nitrogen of the R_2 -side chain and labile OH on the HAP surface; these interactions probably play a key role in the differential binding of BPs to HAP. Conceptually, this trend found for HAP is likely to occur with CAP due to similarities in lattice structure^{32,33}; however, lattice dimensions for CAP may be significantly different allowing some BPs to bind more strongly than others. The differences in calculated inhibitory capacity and adsorption affinity for this CAP model, compared with HAP, might be explained using a similar approach. Changes in the crystalline lattice, resulting from carbonate substitution, may affect the ability of BPs to bind to the CAP surface, that is, stereochemical mismatch between the BP and substrate could affect the number of BP molecules able to form bonds. This might explain why some BPs have comparatively higher binding affinities for CAP (zoledronate > alendronate > risedronate).

Interestingly, solid-state ^{31}P NMR experimental results have shed light on the binding of BPs to crystal surfaces.³⁴ Using magic angle spinning, Grossman et al. were able to distinguish between pure BPs and those specifically adsorbed to HAP. This capability allowed them to estimate the molar ratio of surface-adsorbed to nonadsorbed phosphonates. Their research also showed that the atomic arrangement of the BPs appeared to change to better match the crystal structure of the HAP, and that charge changes were overcompensated by conformational changes. The results observed in our experiments, namely that certain BPs bind more strongly than others, may be due to similar trends; geometrical changes to the apatite lattice following carbonate incorporation may influence which BP molecules are able to adsorb more readily.

The dependence of dissolution inhibition on BP concentration was also investigated. Concentrations ranging from 0.1 to 50.0 $\mu\text{mol L}^{-1}$ of zoledronate were examined with 3.00% CAP [CAP no. 1; Table IV; Fig. 7(a)]. A plot of % inhibition as a function of zoledronate concentration indicates that zoledronate inhibitory capacity follows a logarithmic trend with increasing concentration [Fig. 7(b)]. Table IV shows that a 50.0 $\mu\text{mol L}^{-1}$ concentration of zoledronate inhibits the rate of 3.00% CAP dissolution by ~92.4%. Interestingly, a concentration as low as 0.60 $\mu\text{mol L}^{-1}$ shows a 50% inhibition of dissolution, whereas a limiting concentration appears to be reached at concentrations > 10.0 $\mu\text{mol L}^{-1}$ [Fig. 7(b)]. Explanation for such a tendency invokes surface loading effects and nearest neighbor interactions between BP molecules. At zoledronate concentrations > 50.0 $\mu\text{mol L}^{-1}$, dissolution appears to be terminated, but thermodynamic considerations require it to continue at a rate probably undetectable using current methods.

CONCLUSIONS

The rate of dissolution of CAP was found to be dependent on relative undersaturation, carbonate content, and the presence of BPs. A rate-limiting mechanism involving ionic hydration and subsequent diffusion of lattice ions into the bulk solution is proposed for the dissolution of CAP at $\sigma_{\text{HAP}} \leq 0.16$. This hypothesis, which is supported by the work of Tang et al.,²⁶ can best be interpreted in terms of a BCF model. Data also indicated that 7.96% CAP dissolved much faster than 0.52% CAP under similar conditions. The solubility increased with increasing carbonate content and may be attributed to lattice strain, a result of carbonate substitution for phosphate. The degree of inhibition of CAP dissolution was also shown to be dependent on BP concentration and the nature of the R_2 side chain. The order of K_L values was zoledronate > alendronate > risedronate. This trend was also found for HAP, but the relative rate differences between the BP additives were significantly larger for CAP under similar

conditions. The data suggest that the high affinity of BPs for calcium phosphate minerals prevents dissolution by blocking active dissolution sites on the CAP surfaces. However, the role of this inhibitory action on the *in vivo* effects of the BPs remains to be determined. Since CAP mimics natural bone more closely than does HAP, these results provide strong support for the continued use of CAP in *in vitro* studies.

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Bisphosphonate	R ₁	R ₂
Alendronate	-OH	-CH ₂ CH ₂ CH ₂ NH ₂
Risedronate	-OH	-CH ₂ -
Zoledronate	-OH	-CH ₂ -

Figure 1.
Bisphosphonates and their side chains.

Figure 2.
SEM micrographs of seed material (a) CAP and (b) HAP.

Figure 3.
FTIR spectrum of HAP, B-type CAP, and bone (2 mg sample in 200 mg KBr).

Figure 4.
 (a) CC dissolution of 3.00% CAP at various relative undersaturations, and (b) $\ln R_C$ plotted against $\ln$ saturation.

Figure 5.

Rate of CAP dissolution plotted against % carbonate ($\sigma_{\text{HAP}} = 0.02$, pH = 5.50, 37°C, error bars $\leq 5.0\%$).

Figure 6. CC dissolution of (a) HAP at $\sigma_{\text{HAP}} = -0.42$ and (b) 7.96% CAP at $\sigma_{\text{HAP}} = 0.02$ ($\sigma_{\text{CAP}} = -0.44$).

Figure 7.

(a) CC dissolution of 3.00% CAP in the presence of zoledronate ($\sigma_{\text{HAP}} = 0.02$) and (b) plot of percent inhibition against concentration of zoledronate.

TABLE I

Physical and Chemical Analysis of Seed Materials (% by Mass)

Mineral	Calcium (%)	Phosphate (%)	Carbonate (%)	SSA (m ² /g)
HAP	39.90	56.70	0.00	27.206
CAP no. 1	34.80	46.00	3.00	74.192
CAP no. 2	34.40	42.90	7.96	60.870
Bone ^a	34.80	46.60	7.40	n/a

^aRef. 5.

TABLE II

Dissolution Experimental Conditions at pH 5.50 and 37.0°C

σ_{HAP}	$T_{\text{Ca}} (10^{-3} \text{ mol L}^{-1})$	$T_{\text{P}} (10^{-3} \text{ mol L}^{-1})$	$T_{\text{NaCl}} (\text{mol L}^{-1})$	$T_{\text{HCl}} (10^{-3} \text{ mol L}^{-1})$
-0.42	1.00	0.60	0.146	1.40
-0.17	1.50	0.90	0.145	2.10
-0.03	1.80	1.08	0.143	2.52
0.02	1.90	1.14	0.143	2.66
0.16	2.20	1.32	0.142	3.08
0.30	2.50	1.50	0.141	3.50
0.39	2.70	1.62	0.140	3.78
0.52	3.00	1.80	0.139	4.20

TABLE IIIExperimental Data for the Dissolution of CAP Samples ($SD \leq 4\%$)

Exp. Group	σ_{HAP}	CAP (% CO_3)	R_C ($10^{-8} \text{ mol m}^{-2} \text{ min}^{-1}$)
A	-0.12	3.00	1.24
B	-0.03	3.00	1.19
C	0.02	3.00	1.06
D	0.16	3.00	0.79
E	0.30	3.00	0.60
F	0.39	3.00	0.27
G	0.52	3.00	0.10
H	0.02	0.52	0.76
I	0.02	1.43	1.52
J	0.02	2.32	3.94
K	0.02	3.58	5.92
L	0.02	7.96	11.85

TABLE IV
Experimental Data for the Dissolution of Apatites with Bisphosphonates

Exp. Group	Phase	σ_{HAP}	BP	Conc. (μM)	$R_C (10^{-7} \text{ mol m}^{-2} \text{ min}^{-1})^a$	% Inhibition ^b	$K_L (10^6 M^{-1})$
M	HAP	-0.42	Control	–	2.93	0.0	–
N	HAP	-0.42	Alend	0.5	1.26	57.0	2.65
O	HAP	-0.42	Rised	0.5	1.24	57.7	2.73
P	HAP	-0.42	Zoled	0.5	1.15	60.8	3.10

Exp. Group	Phase CAP (%)	σ_{HAP}	BP	Conc. (μM)	$R_C (10^{-8} \text{ mol m}^{-2} \text{ min}^{-1})^a$	% Inhibition ^b	$K_L (10^6 M^{-1})$
Q	7.96	0.02	Control	–	5.35	0.0	–
R	7.96	0.02	Rised	5.0	4.40	17.8	0.043
S	7.96	0.02	Alend	5.0	2.56	52.1	0.22
T	7.96	0.02	Zoled	5.0	0.75	86.0	1.23
U	3.00	0.02	Control	–	1.98	0.0	–
V	3.00	0.02	Zoled	0.1	1.37	30.8	–
W	3.00	0.02	Zoled	1.0	0.88	55.6	–
X	3.00	0.02	Zoled	5.0	0.61	69.2	–
Y	3.00	0.02	Zoled	10.0	0.34	82.8	–
Z	3.00	0.02	Zoled	50.0	0.15	92.4	–

^aSD $\leq 4\%$.

^b% Inhibition calculated from integrated area under dissolution curve versus control.