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# Degradation kinetics of 4-dedimethylamino sancycline, a new anti-tumor agent, in aqueous solutions

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**Abstract**

The kinetics of degradation of the new anti-tumor drug, 4-dedimethylamino sancycline (col-3) in aqueous solution at 25°C were investigated by high-pressure liquid chromatography (HPLC) over the pH-range of 2–10. The influences of pH, buffer concentration, light, temperature, and some additives on the degradation rate were studied. The degradation of col-3 was found to follow first order kinetics. A rate expression covering the degradation of the various ionic forms of the drug was derived and shown to account for the shape of the experimental pH-rate profile. Under basic conditions, the degradation of col-3 involves oxidation, which is catalyzed by metal ions and inhibited by EDTA and Sodium bisulfite. 

*Keywords:* 4-Dedimethylamino sancycline; Anti-tumor agent; Aqueous solutions

## 1. Introduction

4-Dedimethylamino sancycline (col-3) exhibits in vitro and in vivo activity as an inhibitor of matrix metalloproteinases, tumor invasion, and metastasis of a variety of tumor types. Col-3 is of interest to the National Cancer Institute (NCI) and is in the Phase I clinical trials.

Col-3 is a new synthetic compound obtained by chemical synthesis from sancycline methiodide. The chemical structures of col-3, sancycline, and tetracycline are shown in Fig. 1.

Col-3, the simplest tetracycline, differs from tetracycline by the absence of the 4-dimethylamino, 6-hydroxyl, and 6-methyl groups. Col-3 is a yellow, odorless crystalline compound with a

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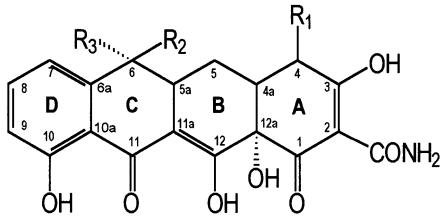
molecular weight of 371.35. It is slightly soluble in water (approx. 0.01 mg/ml) and readily soluble in organic solvents such as methanol, polyethylene glycol, and benzyl alcohol. Although the solubility of col-3 increases with increasing pH, its stability decreases with increasing pH. The exposure of col-3 to alkaline conditions results in the discoloration of the solution to reddish brown. In general, the tetracycline group of antibiotics is known to possess limited stability in aqueous solutions (Mitscher, 1978; Vej-Hansen and Bundgaard, 1978, 1979; Ali, 1984; Vej-Hansen et al., 1987). However, due to the lack of the 4-dimethylamino group and the 6-hydroxyl group, col-3 cannot undergo the normal degradation pathways of the tetracyclines such as epimerization at the C4 position (Doerschuk et al., 1955; McCormick et al., 1956; Sterphens et al., 1956; Kaplan et al., 1957; McCormick et al., 1957; Remers et al., 1963; Hussar, 1968), dehydration at the C5a–6 position in acidic media (Waller et al., 1952; Boothe et al., 1953; Conover et al., 1953; Green et al., 1960; Miller and Hochstein, 1962; Hlavka and Krazinski, 1963; Blackwood and Stephens, 1965), and lactonization of the 6-hydroxy and 11-keto group under basic conditions (Peradejordi et al., 1971; Mitscher, 1978). To date, no details of the degradation process of col-3 are available in the literature.

In this study, the degradation kinetics of col-3 was examined as a function of pH, buffer concentrations, light, temperature, and the presence of some additives.




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Tetracycline | $N(CH_3)_2$ | OH | $CH_3$ |
| Sancycline | $N(CH_3)_2$ | H | H |
| Col-3 | H | H | H |

Fig. 1. Chemical structure of col-3, sancycline and tetracycline.

## 2. Materials and methods

### 2.1. Materials

Col-3 was provided by the National Cancer Institute (Bethesda, MD). Methanol (high-pressure liquid chromatography (HPLC) grade) and glacial acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Acetonitrile was purchased from Baxter (Muskegon, MI). The 0.1 N hydrochloric acid (HCl) and sodium hydroxide (NaOH) solutions (certified grade) were purchased from Fisher Scientific (Fairlawn, NJ). Phosphoric acid, potassium chloride, potassium phosphate (monobasic), potassium phosphate (dibasic), and Edetic acid (EDTA, disodium salt) were purchased from Sigma (St. Louis, MO). All reagents were used as received without further purification.

### 2.2. Stability indicating assay

The degradation rate of col-3 was followed by measuring the remaining drug concentration by a reversed-phase HPLC. The chromatographic system consists of a Beckman 126 solvent delivery system equipped with a Beckman 168 diode array detector (Beckman Instruments, Fullerton, CA) and Rheodyne 710 manual injector (Rheodyne, Cotati, CA). The column was a $4.6 \times 150$-mm, Mixmode column packed with 5 μm, C-8 (cation), bonded silica (Alltech Associates, Deerfield, IL). The mobile phase was 35% acetonitrile and 65% 0.1 M acetic acid (containing 0.005% disodium EDTA). The analysis was performed at ambient temperature, at the flow rate of 1 ml/min. The wavelength of detection was 268 nm, with an injection volume of 100 μl.

### 2.3. Aqueous solution kinetic studies

For the kinetic studies phosphate buffer solutions ranging in pH from 2.0 to 10.0 were used. The buffer concentration was 0.01 M except for

the experiments where the influence of the buffer concentration was tested. The pH of each buffer solution was measured using a Corning pH meter (model # 140) which was standardized with NBS buffer solutions. A constant ionic strength of 0.2 was maintained by adding an appropriate amount of potassium chloride.

All kinetic studies were conducted at 25 ± 0.2°C. The reaction was initiated by adding 150 μl of a stock solution of col-3 (1.0 mg/ml in methanol) to 25 ml buffer solutions. All sample solutions were sealed in clear glass ampoules. Degradation was carried out in a thermostatically controlled water-bath, protected from light. The reaction samples were withdrawn at suitable time intervals. The concentration of residual compound was then determined by HPLC as described above.

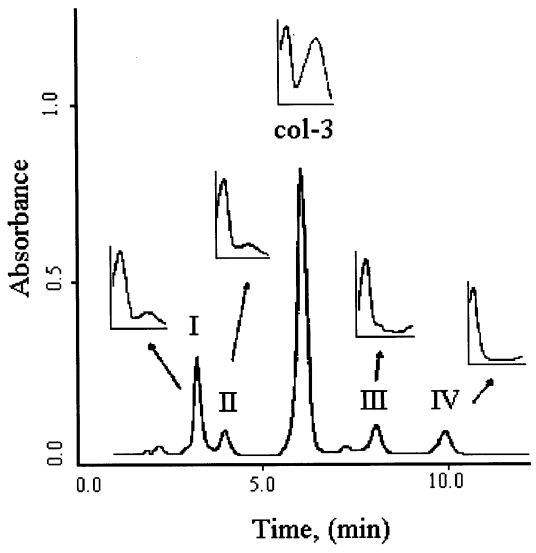



Fig. 2. Chromatogram of col-3 under basic condition after 48 h; I, II, III and IV are major degradates.

## 3. Results and discussion

### 3.1. Stability-indicating assay

Under basic conditions, four major degradation products were detected by HPLC. However, since they were formed in very small quantities, the quantitative detection of these compounds was carried out in concentrated solutions ( > 40 μg/ml). A typical chromatogram of partially degraded col-3 under basic conditions is shown in Fig. 2. The retention time of the drug is about 6.3 min. The major degradation peaks appear at 3.0 (I), 4.0 (II), 8.0 (III) and 10.0 (IV) min. No detectable amounts of the degradation products were produced in acidic media, over the time studied.

EDTA was found to be necessary for the elution of col-3. Without EDTA, the peaks tailed badly (peak tailing factor > 2.5) and very high concentration of organic solvents (up to 95% of acetronitrile) were needed to completely elute the required compounds. Similar phenomena have been reported in the assay of most tetracycline analogues (Butterfield et al., 1973; Tsuji et al., 1974; Tsuji and Robertson, 1976; Ali and Strittmatter, 1978; Hermansson and Andersson, 1982; Yasin and Jefferies, 1988; Ray and Harris, 1989). The tailing is believed to be the result of the complex formation with divalent cations, such as calcium, which are present in the column (Leenheer and Nelis, 1977).

The calibration curve of col-3 was linear over the concentration range of 0.5–10.0 μg/ml. The correlation coefficient ($r^2$) values were in the range from 0.9992 to 0.9999. The mean slope was 3.534 ml/μg (S.D. = 0.087, $n = 18$), and the mean intercept was 0.084 (S.D. = 0.017, $n = 18$). The resolution was greater than 1.7 for the poorest separation of the compounds I and II.

### 3.2. Degradation products

The degradation of col-3 in basic medium (pH > 8) is indicated by color change of the solution from yellow to pink, red and brown depending on the extent of degradation. Four major degradation products are detected by HPLC in partially degraded solution where the red color observed. Further degradation shows extensive formation of minor products.

Rapid scanning with the photo diode array detector on line with the HPLC system enabled the determination the UV absorption spectra of the four major degradation products of col-3. As

shown in Fig. 2, the first absorption band at about 260 nm is seen in the parent drug and in all four degradation products. However, the second absorption band at about 360 nm is decreased in degradates I and II and is totally absent in degradates III and IV. In addition, a new absorption band in the 400–500 nm region is observed in compounds III and IV.

The absence of the second absorption peak in the degradates III and IV indicates that the responsible chromophore for this peak was altered in the degradation process. For tetracycline-type compounds, the absorption at 360 nm is produced by the conjugated system composed of the aromatic ring D and the diketone moiety (Conover et al., 1953; Ali, 1984).

### 3.3. Observed kinetic order

At constant pH and temperature the overall loss of col-3 follows apparent first-order kinetics. The observed first order rate constants ($k_{obs}$) for the overall degradation of col-3 were calculated from the linear decrease of the logarithm of col-3 concentration with time. Representative concentration versus time plots at various pH-values for col-3 are shown in Fig. 3.

### 3.4. General acid/base catalysis

The effect of phosphate buffer concentration at several pH values on the rate of degradation of col-3 is given in Table 1, with some sample plots shown in Fig. 4.

Significant buffer catalysis was observed in the pH range of 4–10 ($p < 0.05$), whereas no significant buffer effect was observed at pH 2 and 3. This indicates that the degradation of col-3 is catalyzed not only by specific hydroxide ions but also by general bases, including the ionized buffer species, $H_2PO_4^-$, $HPO_4^{2-}$ and $PO_4^{3-}$.

In order to remove the contribution of each buffer species, the observed catalytic rate constants ($k_{cat}$) were calculated from the slope of linear plots of the $k_{obs}$ versus buffer concentration (Fig. 4). These values were related to the observed rate constants and the fraction of the individual buffer species ($f_i$) present at each pH value. For phosphate buffer, $f_i$ can be calculated as follows:

$$f_{H_3PO_4} = \frac{[H^+]^3}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1 K_2 K_3} \quad (1)$$

$$f_{H_2PO_4^-} = \frac{[H^+]^2 K_1}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1 K_2 + K_1 K_2 K_3} \quad (2)$$

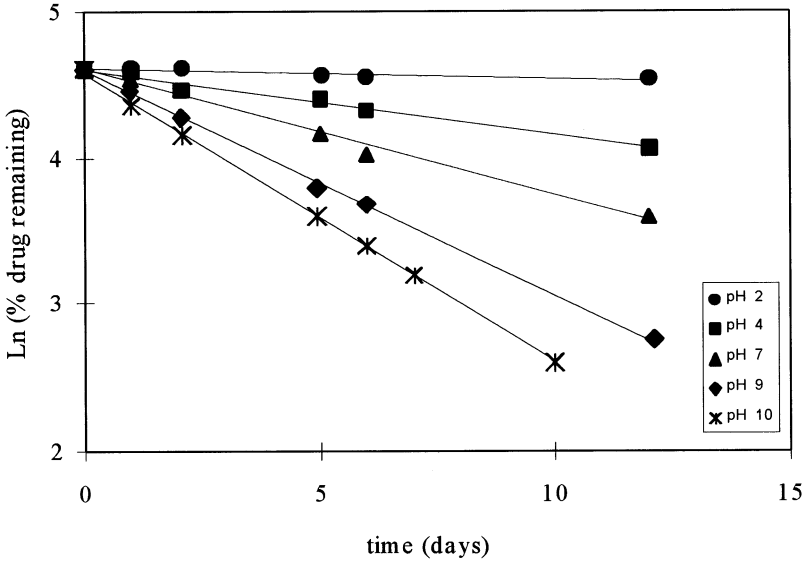



Fig. 3. Apparent first-order reaction of col-3 in aqueous solutions at various pH values.

Table 1
Observed first-order rate constant ($k_{obs}$) for the degradation of col-3 in phosphate buffer of various pH-values and buffer concentrations (25°C, $\mu = 0.2$).

| Buffer conc. (M) | pH[a] | $K_{obs}$[b] ($day^{-1}$) | S.D. | $r^{2}$[b] |
|---|---|---|---|---|
| 0.01 | 2.0 | 0.006 | 0.001 | 0.899 |
| | 3.0 | 0.006 | 0.002 | 0.991 |
| | 4.0 | 0.046 | 0.006 | 0.952 |
| | 5.0 | 0.055 | 0.008 | 0.995 |
| | 5.8 | 0.068 | 0.006 | 0.988 |
| | 6.5 | 0.074 | 0.013 | 0.968 |
| | 7.2 | 0.087 | 0.007 | 0.972 |
| | 8.2 | 0.101 | 0.010 | 0.984 |
| | 9.0 | 0.155 | 0.005 | 0.993 |
| | 10.0 | 0.198 | 0.011 | 0.997 |
| 0.03 | 2.0 | 0.015 | 0.003 | 0.910 |
| | 3.0 | 0.021 | 0.004 | 0.978 |
| | 4.0 | 0.128 | 0.007 | 0.982 |
| | 5.0 | 0.141 | 0.008 | 0.991 |
| | 5.8 | 0.161 | 0.018 | 0.974 |
| | 6.5 | 0.156 | 0.006 | 0.998 |
| | 7.2 | 0.129 | 0.009 | 0.976 |
| | 8.2 | 0.172 | 0.004 | 0.994 |
| | 9.0 | 0.236 | 0.008 | 0.992 |
| | 10.0 | 0.262 | 0.009 | 0.995 |
| 0.05 | 2.0 | 0.021 | 0.003 | 0.937 |
| | 3.0 | 0.031 | 0.002 | 0.969 |
| | 4.0 | 0.197 | 0.007 | 0.994 |
| | 5.0 | 0.205 | 0.009 | 0.995 |
| | 5.8 | 0.221 | 0.019 | 0.938 |
| | 6.5 | 0.209 | 0.012 | 0.988 |
| | 7.2 | 0.199 | 0.006 | 0.979 |
| | 8.2 | 0.209 | 0.007 | 0.988 |
| | 9.0 | 0.265 | 0.010 | 0.996 |
| | 10.0 | 0.289 | 0.004 | 0.995 |

[a] pH within 0.1 units of target values
[b] Average value ($n = 3$).

$$f_{HPO_4^{2-}} = \frac{[H^+]^2 K_1 K_2}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1 K_2 + K_1 K_2 K_3} \quad (3)$$

$$f_{PO_4^{3-}} = \frac{K_1 K_2 K_3}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1 K_2 + K_1 K_2 K_3} \quad (4)$$

where $K_1$, $K_2$ and $K_3$ are the first-, second-, and third-ionization constants of phosphate, respectively.

The catalytic components of phosphate buffer can be described as:

$$k_{obs} = k_o + \sum k_{phosphate}[\text{Phosphate}] \quad (5)$$

or

$$k_{obs} = k_o + k_{H_3PO_4}[H_3PO_4] + k_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^{2-}}[HPO_4^{2-}] + k_{PO_4^{3-}}[PO_4^{3-}] \quad (6)$$

$$k_{obs} = k_o + k_{H_3PO_4} f_{H_3PO_4}[Buf] + k_{H_2PO_4^-} f_{H_2PO_4^-} f_{H_2PO_4^-}[Buf] + k_{HPO_4^{2-}} f_{HPO_4^{2-}}[Buf] + k_{PO_4^{3-}} f_{PO_4^{3-}}[Buf] \quad (7)$$

Values for the observed buffer catalytic rate constants of col-3 and the fraction of buffer species at different pH values are listed in Table 2. The highest values of the observed catalytic rate constants are observed between approximately pH 4 and 6. This suggests that $H_2PO_4^-$ is the most catalytic of the phosphate species.

### 3.5. pH-rate profile

The pH-rate profile of col-3 at 25°C is shown in Fig. 5. The data points of this plot represent the intrinsic rate constants ($k_o$) obtained by extrapolating the apparent first-order rate constant ($k_{obs}$) to zero buffer concentration (Fig. 4), while the curve drawn through the data points represents the mathematical model developed in the following discussion.

As seen in Fig. 5, the degradation rate constant of col-3 increases in a non-linear manner as pH increases. The pH of maximum stability is below pH 4. The degradation rate increases with pH between 3 and 6, reaching a plateau between about pH 6 and 7, after which the rate increases again until minimum stability is observed above pH 10. This shape of the profile suggests that the dissociation equilibrium of the drug and the hydroxide ion concentration are the major factors, which influence the degradation rate. The mathematical model was, therefore, developed by considering the degradation rates of various ionic species of the drug whose concentrations change with pH.

In aqueous solution, col-3 exists as the unionized, the monovalent anion, and the divalent anion species depending on the pH of the solution.

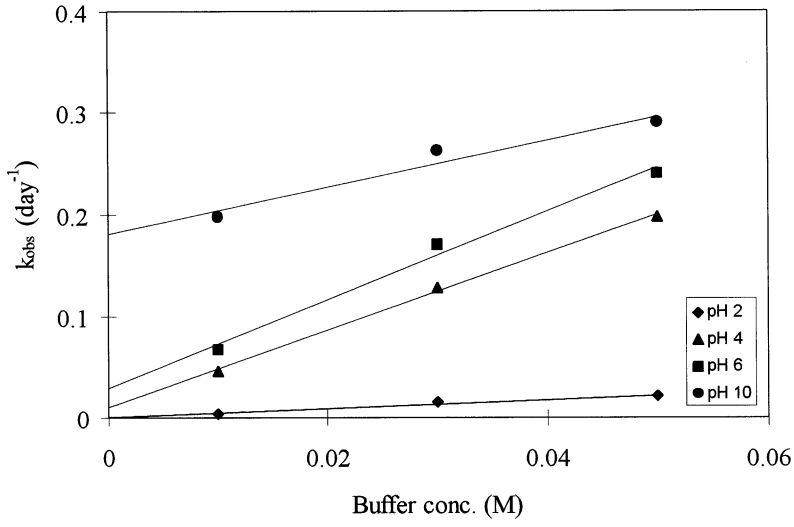



Fig. 4. Plots of observed rate constant versus phosphate buffer concentration at 25°C and $\mu = 0.2$.

The total rate equation for col-3 can be written as:

$$k_o = k_A f_A + k_B f_B + K_C f_C \quad (8)$$

where the subscripts A, B and C denote the unionized, the monovalent and divalent anions species, respectively; $k$ and $f$ are the macroscopic rate constant and the fraction of the species in the solution, respectively.

The fraction of each individual species can be calculated as a function of pH and the ionization constants ($K_1$ and $K_2$) of the drug:

$$f_A = \frac{[H^+]^2}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad (9)$$

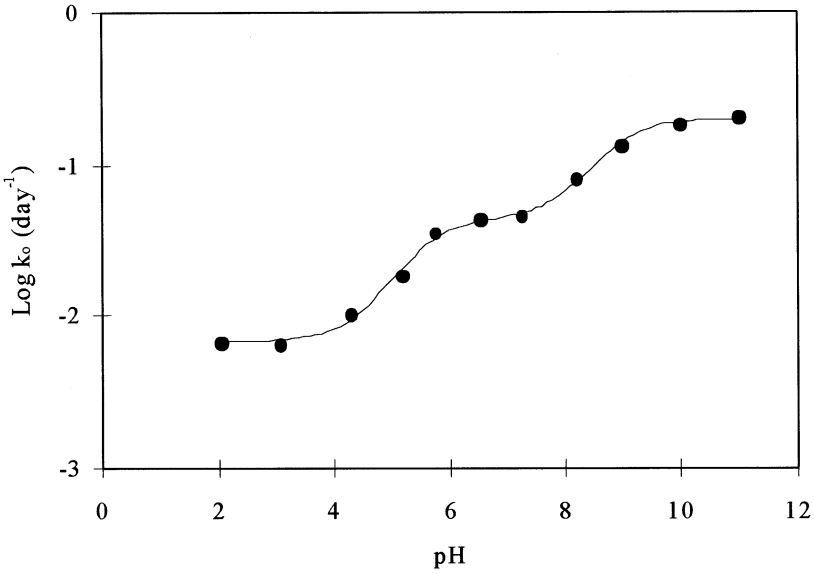



Fig. 5. pH-rate profile of col-3 at zero buffer concentration ($\mu = 0.2$, 25°C).

Table 2
Observed buffer catalytic rate constant ($k_{buf}$) for the decomposition of col-3 in phosphate buffer solutions ($\mu = 0.2$, 25°C) as a function of pH and fraction ($f_i$) of the individual buffer species

| pH[a] | $K_{buf}$[b] ($day^{-1}$ $M^{-1}$) | $f_i$ (%) | | | |
|---|---|---|---|---|---|
| | | $H_3PO_4$ | $H_2PO_4^{2-}$ | $HPO_4^{-}$ | $PO_4^{3-}$ |
| 2.0 | 0.425 | 58.79 | 41.21 | – | – |
| 3.0 | 0.617 | 12.48 | 87.51 | 0.01 | – |
| 4.0 | 3.775 | 1.40 | 98.51 | 0.08 | – |
| 5.0 | 3.824 | 0.14 | 99.07 | 0.79 | – |
| 5.8 | 3.826 | 0.01 | 92.59 | 7.39 | – |
| 6.5 | 3.401 | – | 79.83 | 20.17 | – |
| 7.2 | 2.973 | – | 55.58 | 44.41 | – |
| 8.2 | 2.725 | – | 11.13 | 88.87 | – |
| 9.0 | 2.732 | – | 1.23 | 98.72 | 0.05 |
| 10.0 | 2.305 | – | 0.12 | 99.40 | 0.47 |

[a] pH within 0.1 unit of target values.
[b] Obtained from the slope of the plot of $k_{obs}$ versus buffer concentration.

$$f_B = \frac{[H^+]K_1}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad (10)$$

$$f_C = \frac{K_1K_2}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad (11)$$

Substituting the appropriate fractional compositions into Eq. (8) yields:

$$k_{obs} = \frac{k_A[H^+]^2 + k_BK_1[H^+] + k_CK_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (12)$$

The ionization constants and the macroscopic rate constants can be obtained from the fit of this model to the experimental data. The calculated values of these parameters are shown in Table 3. The $pK_a$ values determined from this kinetic model are 5.41 for $pK_{a1}$ and 8.7 for $pK_{a2}$. These values are comparable to the values previously determined by the spectrophotometric method, 5.89 for $pK_{a1}$ and 8.01 for $pK_{a2}$ (Pinsuwan et al., 1998). The relative agreement of the $pK_a$ values supports the validity of the proposed model.

### 3.6. Effect of light

The effect of light on the degradation of col-3 in aqueous solutions was also investigated at several pH values. The results shown in Table 4 indicate that light has a significant effect on the degradation of col-3 only in basic conditions (pH > 8.0).

The degradation of col-3 in basic solutions is evidenced by the change of color from yellow to red, which is more rapid in the presence of light. With addition of acid, the solution reverts back to

Table 3
Macroscopic rate constants for the decomposition of each ionic species of col-3 and the ionization constants obtained from non-linear fit ($r^2 = 0.99$) of the experimental data to the kinetic model (Eq. (6))

| | Macroscopic rate constants ($day^{-1}$) | | | Ionization constants | |
|---|---|---|---|---|---|
| | $k_A$ | $k_B$ | $k_C$ | $K_1$ | $K_2$ |
| Values | $6.07 \times 10^{-3}$ | $4.33 \times 10^{-2}$ | $1.99 \times 10^{-1}$ | $3.94 \times 10^{-6}$ | $1.69 \times 10^{-9}$ |
| S.E.[a] | $1.30 \times 10^{-3}$ | $20.8 \times 10^{-3}$ | $1.49 \times 10^{-3}$ | $1.01 \times 10^{-6}$ | $3.86 \times 10^{-10}$ |
| %CV[b] | 2.14 | 4.86 | 0.75 | 25.5 | 2.28 |

[a] Standard error.
[b] Coefficient of variation (%).

Table 4
Effect of light on the degradation rate constants of col-3 in phosphate buffer solutions (0.01 M, $\mu = 0.2$, 25°C) of various pH-values

| pH[a] | $k_{obs}$[b], day$^{-1}$ (S.D.) | |
|---|---|---|
| | Dark | Light |
| 2.0 | 0.006 (0.001) | 0.005 (0.003) |
| 3.0 | 0.006 (0.002) | 0.008 (0.001) |
| 4.0 | 0.046 (0.006) | 0.040 (0.009) |
| 5.0 | 0.055 (0.008) | 0.054 (0.008) |
| 5.8 | 0.068 (0.006) | 0.061 (0.010) |
| 6.5 | 0.074 (0.013) | 0.074 (0.009) |
| 7.2 | 0.087 (0.007) | 0.083 (0.012) |
| 8.2[c] | 0.101 (0.010) | 0.189 (0.015) |
| 9.0[c] | 0.154 (0.005) | 0.245 0.009) |
| 10.0[c] | 0.198 (0.011) | 0.498 (0.005) |

[a] pH within 0.1 unit of target values.
[b] Average value ($n = 3$).
[c] Significant difference between light and dark ($p < 0.05$).

the original yellow color, even though all degradation products are still detected by HPLC analysis. These results suggest the formation of a metastable red product in the degradation of col-3 under alkaline conditions. This is consistent with the fact that a metastable red product has been reported for the oxidative degradation of tetracyclines, which occurs in alkaline media (Wiebe and Moore, 1977).

### 3.7. Effect of additives

To confirm whether or not oxidation is involved in the degradation of col-3, the effect of antioxidants (e.g. ascorbic acid and sodium bisulfite) was investigated. Additionally, the effect of a chelating agent (disodium EDTA) was studied because it complexes with heavy metal ions that may catalyze the oxidative degradation of col-3.

The studies were performed by determining the stability of col-3 in 0.05 M phosphate buffer pH 10.3 with and without 0.005% of either ascorbic acid, sodium bisulfite, or disodium EDTA. The experiments were conducted in the presence of light at 25°C. The results are graphically presented in Fig. 6.

In all cases, col-3 was more stable in the solution with the additive. The greatest stability was observed with EDTA, suggesting that the degradation of col-3 in basic solutions is due to oxidation, which is catalyzed by trace heavy metal ions. Ascorbic acid showed the smallest effect on increasing the stability of col-3 because it itself is

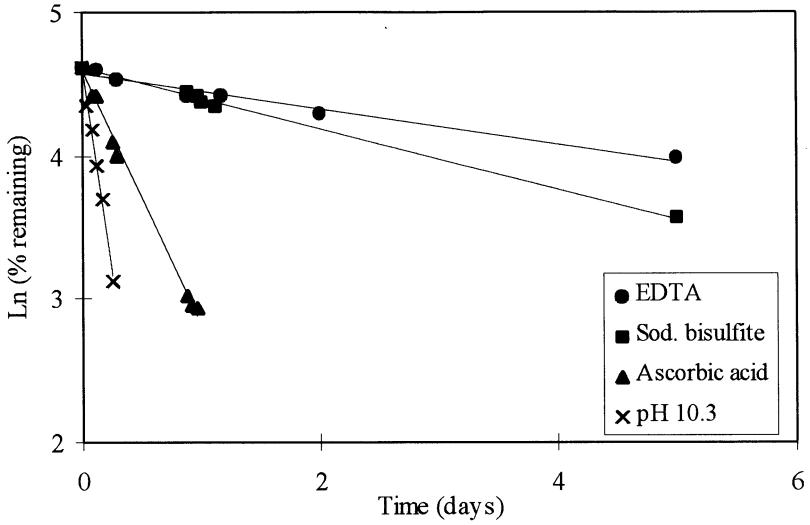



Fig. 6. Stability profiles of col-3 in basic solutions with and without additives.

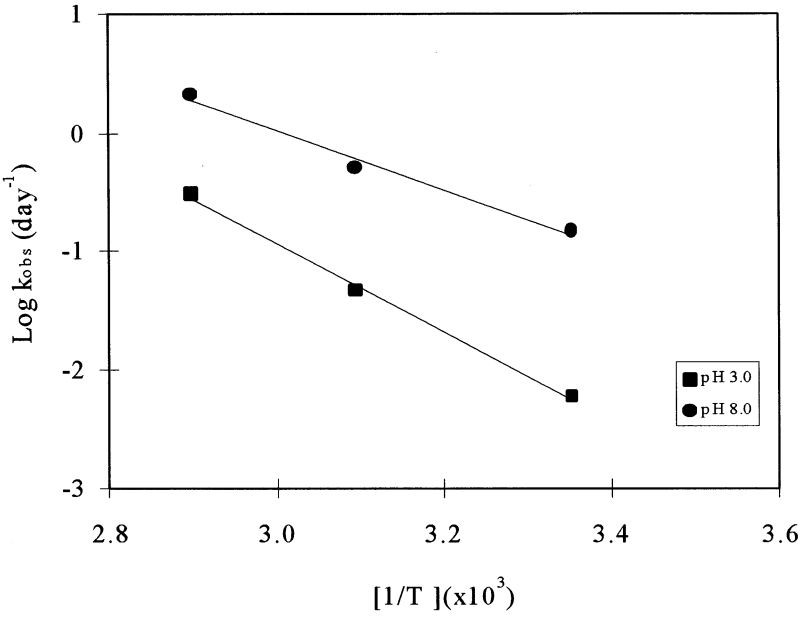



Fig. 7. Arrhenius plots for the apparent first order rate constant of col-3 in aqueous solution (0.05 M phosphate buffer, $\mu = 0.2$, dark).

not stable in aqueous solutions at high pH values (Carstensen, 1995).

The postulated mechanism for the photo-oxidation of tetracycline derivatives is primarily due to the photolytic reduction of the 4-dimethylamino group by photo-reaction, resulting in a formation of free radicals which consequently undergo oxidation and hydroxyl-catalyzed dehydration (Wiebe and Moore, 1977).

### *3.8. Effect of temperature*

The temperature dependence of the degradation of col-3 was examined in phosphate buffer pH 3 and 8 over the temperature range of 25–72°C. In Fig. 7, the observed rate constants were plotted according to the Arrhenius equation (Connors et al., 1986; Carstensen, 1995). The apparent activation energies ($E_a$) obtained from the slope of the lines are 17.33 and 11.49 kcal $mol^{-1}$ at pH 3.0 and 8.0, respectively.

## 4. Conclusions

This investigation shows that the degradation of col-3 follows first order kinetics. A rate expression covering the degradation of the various ionic forms of the drug was derived. Under basic conditions, the degradation of col-3 involved oxidation, which is catalyzed by various phosphate species and metal ions. EDTA and Sodium bisulfite decreased the base-catalyzed degradation rate of col-3.

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