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Photolytic and oxidative degradation of an antiemetic agent, RG 12915

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Abstract

RG 12915 (I) undergoes dechlorination and substitution in aqueous solutions when exposed to an artificial daylight fluorescent light. The free-radical photodecomposition exhibits apparent first-order kinetics at all concentrations and the reaction proceeds faster in dilute solutions. The drug substance is also susceptible to oxidative degradation. Oxidation occurs on the benzofuran moiety as well as on the quinuclidine moiety. Auto-oxidation on the benzofuran moiety produces a corresponding hydroperoxide. Subsequent decomposition of the hydroperoxide gives rise to the secondary oxidation product, the hydroxy compound. The oxidative free-radical reaction shows an induction period, followed by a period of accelerating degradation and eventual leveling off. The oxidation reaction proceeds faster in concentrated solutions and at lower pH, and is accelerated by cupric ion. EDTA prevents the oxidative degradation of the drug substance. Propyl gallate inhibits the oxidation of the benzofuran moiety but not *N*-oxide formation.

Keywords: Antiemetic agent; Photodecomposition; Oxidation; Degradation; Stability; Kinetics

1. Introduction

RG 12915 (I), *N*-[azabicyclo[2.2.2]octan-3(*S*)-yl]-2-chloro-*cis*-5*a*(*S*)-9*a*(*S*)-5*a*,6,7,8,9,9*a*-hexahydrodibenzofuran-4-carboxamide, is a highly potent antagonist of 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor (Fitzpatrick et al., 1990; Youssefyeh et al., 1992a,b; Martin et al., 1993). The drug attenuates emesis or nausea induced by chemotherapeutic agents and is being developed as an antiemetic agent for patients receiving chemotherapy.

RG 12915 in aqueous solutions is subject to photolytic and oxidative degradation processes that give rise to a number of degradation products. The present work reports on the isolation and identification of the degradation products of the drug and investigates the photolytic and oxidative degradation pathways in aqueous solutions. An understanding of the kinetics and mechanism by which the drug substance photodecomposes or oxidizes enables the more judicious selection of an inhibitor or antioxidant for stable formulations. The present report describes the

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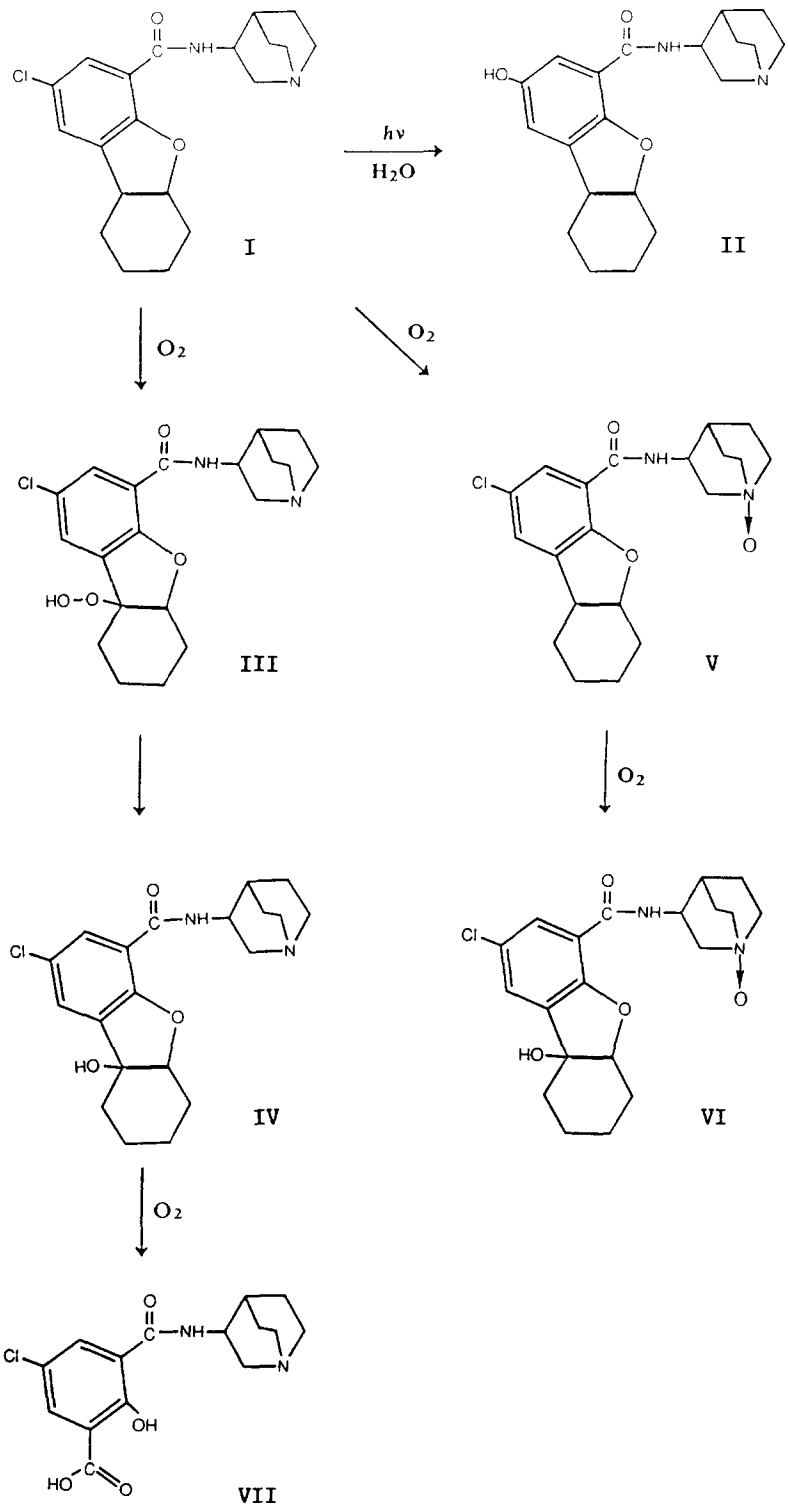

Scheme 1. Scheme showing degradation of RG 12915 (I) to the degradation products II–VII.

use of chelating agents and antioxidants in stabilizing the system.

2. Materials and methods

2.1. Materials

RG 12915A (HCl salt of RG 12915) was prepared by the Chemical Process Research and Development, Rhône-Poulenc Rorer Central Research. The purity of the synthesized compound was greater than 99% as determined by HPLC analysis. Water used was purified by a Milli-Q Plus ultra-pure water system (Millipore, Milford, MA). HPLC grade acetonitrile and trifluoroacetic acid were used to prepare the mobile phase. Standard 1.0 N HCl and NaOH solutions (Fisher, Pittsburgh, PA) were used as received. Buffer reagents, metal salts, propyl gallate (Aldrich, Milwaukee, WI), 3-chloroperoxybenzoic acid (Aldrich) and all other chemicals were of reagent grade. TLC plates were obtained from Analtech (Newark, DE).

2.2. HPLC analysis

The HPLC system consisted of a Perkin Elmer 410 pump, a Perkin Elmer ISS 100 automatic injector, a Perkin Elmer 480 diode array detector and a Waters 860 networking computer system. The HPLC method employed a 300 mm × 3.9 mm i.d. 10 μm particle size, octadecyl-bonded silica stationary phase column (Waters microBondapak C-18) and a mobile phase consisting of acetonitrile/water/trifluoroacetic acid (400:600:1, v/v). The flow rate was 1 ml/min and the detector wavelength for UV absorbance detection was 216 nm for solutions of initial concentrations of 600 μg/ml or lower and 230 nm for solutions of 800 μg/ml or higher.

2.3. Kinetic methods

The photodecomposition of **I** in aqueous solutions was carried out in the presence of air in clear borosilicate glass vials (diameter 10 mm, volume 2 ml) which allow the transmission of wavelengths longer than 310 nm. Stock solutions of 100 μg/ml RG 12915A (Mol. Wt = 397.3) and 0.2 M buffer in water were prepared. In a typical kinetic experiment, 2.5 ml of the RG 12915A stock solution, an appropriate amount of a phosphate buffer (NaH_2PO_4-Na_2HPO_4) stock solution of pH 6.8 and an appropriate amount of 1 M NaCl to maintain an ionic strength of 0.2 were transferred to a 25 ml volumetric flask and filled to volume with water. Aliquots (1 ml) of the solution in the vial were exposed to a 180-foot-candle artificial daylight fluorescent light with UV maximum output at 350–370 nm and maximum visible output at 450–650 nm. The temperature of the solutions was 22–25° C. The influence of the initial drug concentration on the reaction was studied from 2.5 to 100 μg/ml in a phosphate buffer of pH 6.8.

In experiments performed to study oxidative degradation, 20 ml of RG 12915A solutions (0.2–1.0 mg/ml) was placed in a 100 ml volumetric flask and kept in a constant temperature water bath at 60.0 (±0.5) °C and the solution was protected from light. At appropriate time intervals, aliquots (1 ml) were withdrawn and analyzed by HPLC.

For studies of the copper(II) ion effect on the oxidative degradation, various volumes of a cupric sulfate stock solution (5×10^{-4} M) were used to provide Cu^{2+} concentrations ranging from 1.25×10^{-6} to 1.0×10^{-5} M. The influence of pH was examined in HCl (pH 2.1), acetate (pH 4.8) and phosphate (pH 7.6) buffers. The influences of factors other than pH were studied in a phosphate buffer of pH 6.8 at a drug concentration of 1 mg/ml. The phosphate buffer solution contained approx. 1 ppm of heavy metals. The buffers were not purified of trace metals.

2.4. Spectroscopy

The proton and carbon spectra were recorded on a Varian VXR 200 NMR spectrometer using $CDCl_3$ or DMSO-d_6 as the solvent. The electron impact (EI) mass spectra were obtained with a VG 7070 SE mass spectrometer via direct inlet. The ion potential was 75 eV.

2.5. Isolation of II

250 mg of RG-12915A was dissolved in 2000 ml of a phosphate buffer (pH 6.8). The solution was irradiated in a light cabinet with a 295-foot-candle artificial daylight fluorescent lamp for 19 days. HPLC analysis showed a sole degradation product and the reaction was nearly complete. After adjusting the pH to 8–9, 300-ml portions of the solution were shaken with 100 ml of methylene chloride. Upon evaporating the methylene chloride, a white solid was obtained. The solid was recrystallized in acetonitrile and dried at 60° C under vacuum for 2 h.

2.6. Identification of II

The EI mass spectrum of the degradate exhibited a molecular ion at m/z 342 ($C_{20}H_{26}N_2O_3$) and fragments at m/z 215 ($C_7H_{13}N_2$), 217 ($C_{13}H_{13}O_3$) and 125 ($C_7H_{13}N_2$). The mass spectrum did not exhibit the isotopic cluster of one chlorine atom. It was consistent with the structure where the chlorine in the benzofuran moiety of **I** was replaced with a hydroxyl group. The ^{1}H-NMR spectrum of the degradate demonstrated a broad peak at 5.6 ppm corresponding to a phenolic OH group. The ^{13}C-NMR showed an OH-attached carbon at 149.3 ppm in place of a Cl-attached carbon at 125.8 ppm in the spectrum of **I**.

2.7. Isolation of oxidative degradation products in aqueous solutions

800 mg of RG 12915A was dissolved in 20 ml of water and placed in a water bath at 60° C for 2 weeks. After this time period, the solution was evaporated to dryness. The residue was dissolved in a small amount of methanol, streaked across a 1 mm thick silica gel GF preparative TLC plate and developed with a solvent system consisting of methylene chloride, methanol and ammonium hydroxide (90:10:1). Under these conditions, **I** had an R_f of 0.46. The bands corresponding to R_f values of 0.16, 0.18 and 0.20 were scraped off and eluted with methylene chloride.

2.8. Identification of III

The fast atom bombardment (FAB) mass spectrum of the degradate having an R_f of 0.20 showed a molecular ion at 393 (M + H) and 377 (M + H − O), indicating the degradate to be the dioxygenated compound of **I**. The on-line LC-MS technique was used to verify the structure of the peak at 5.2 min (Fig. 1). The same molecular

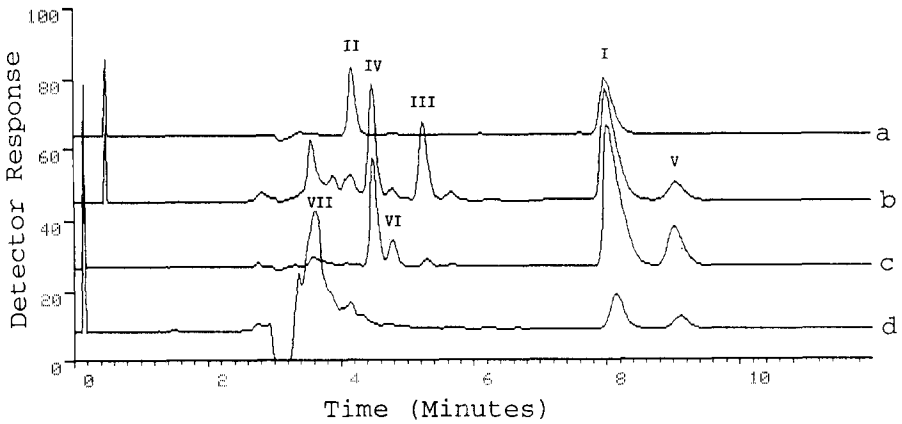

Fig. 1. HPLC chromatograms of degraded **I**. (a) Degraded under 180-foot-candle artificial daylight fluorescent lamp at 0.1 mg/ml, room temperature (22–25° C) and pH 6.8 for 9 h. (b) Degraded in acetate buffer of pH 4.8 at 1 mg/ml and 60° C for 13 days. (c) Degraded in phosphate buffer of pH 6.8 at 1 mg/ml and 60° C for 13 days. (d) Degraded at 40° C/80% RH for 16 days.

weight of the compound was determined by the LC-MS analysis.

2.9. Identification of IV

The EI mass spectrum of the degradate having an R_f of 0.16 showed a molecular ion at m/z 376 and peaks at m/z 267, 251, 233 and 109. The ion of m/z 376.1552 in a high resolution mass spectrum corresponded to $C_{20}H_{25}N_2O_3Cl$ (molecular ion), 251.0481 to $C_{13}H_{12}O_3Cl$ and 233.0368 to $C_{13}H_{10}O_2Cl$. From the mass spectral information, the degradate was identified as a mono-oxygenated product of **I**. The oxidation occurred on the benzofuran moiety of the molecule. The exact location of the oxidation in the benzofuran moiety was not known until a sufficient quantity of the compound was prepared for NMR spectra by oxidizing **I** with potassium permanganate. Valuable information required for the structure elucidation of the compound was obtained by comparing the carbon NMR spectra of **I** and **IV**. The resonance of the tertiary carbon, α to the benzene ring, was shown at 39.4 ppm in the spectrum of **I**. However, this resonance was lacking in the spectrum of **IV**. Instead, the spectrum of **IV** showed a resonance at 77 ppm as a quaternary carbon, indicating that the α-carbon was oxygenated.

2.10. Preparation of IV

300 mg of RG 12915A was dissolved in 300 ml of water. To the solution was intermittently added a saturated aqueous solution of potassium permanganate until the pink color of the potassium permanganate remained for the entire reaction period. The solution was stirred for 5 h at room temperature and then filtered. After pH adjustment to approx. 10, the filtrate was shaken with 200 ml of methylene chloride. The methylene chloride was then removed by evaporation. The resultant residue was dried at 60° C under vacuum for 2 h.

The structure of **IV** was confirmed by comparison of its mass spectrum and HPLC retention time with those of the compound prepared by oxidation of **I** with potassium permanganate.

2.11. Identification of V

The largest fragment found in the EI mass spectrum of the degradate having an R_f 0.18 was m/z 360. It was suspected that the degradate might be the *N*-oxide (**V**) of **I**. The peak at m/z 360 might be a fragment which was formed by splitting oxygen off the *N*-oxide. The authentic *N*-oxide of **I** was prepared by oxidizing the drug substance with *m*-chloroperoxybenzoic acid. The co-injection of the degraded sample and the authentic *N*-oxide resulted in one peak. The LC-MS analysis of the peaks showed identical mass spectra. The degradate was confirmed to be the *N*-oxide (**V**) of **I**.

2.12. Preparation of VI

1 g of RG 12915A was dissolved in 10 ml of water. After pH adjustment to 10–11, the solution was shaken with two 20-ml portions of chloroform. To the chloroform solution was added 1 g of 50–60% *m*-chloroperoxybenzoic acid. The solution was stirred at room temperature for 4 h. After the reaction, the chloroform solution was shaken with 30 ml of water at pH 10–11. The aqueous layer was repeatedly shaken with 50-ml portions of chloroform until an HPLC analysis showed that practically all of the product (**V**) was extracted into the chloroform layer. The combined chloroform solution was evaporated to dryness. The resultant residue was dissolved in 20 ml of 0.01 N HCl and oxidized with potassium permanganate as described in the preparation of **IV**.

2.13. Identification of VI

The FAB mass spectrum of **VI** obtained using nitrobenzyl alcohol as a matrix showed a protonated molecular ion $(M + H)^+$ at m/z 393, and peaks at m/z 375 (loss of H_2O) and 377 (loss of O). The carbon NMR spectrum showed the resonance of the quaternary carbon, α to the benzene ring, at 77 ppm as in the spectrum of product **IV**.

2.14. Isolation of VII

RG 12915A undergoes significant degradation in the presence of moisture. The chromatogram

(Fig. 1d) of the humidity-exposed sample at 40° C/80% relative humidity for 16 days showed one major degradation peak at $t_R = 3.4$ min.

The degraded sample was dissolved in a minimum amount of methanol. Upon adding acetonitrile, a white precipitate formed, which was collected by centrifugation. The solid was redissolved in methanol and precipitated by acetonitrile. The solid material was dried at 60° C under vacuum for 2 h.

2.15. Identification of VII

The FAB mass spectrum of the degradate obtained using glycerol as a matrix exhibited a protonated molecular ion (M + H) at m/z 325. The EI mass spectrum showed a molecular ion at m/z 324. The high resolution mass spectrum of the degradate demonstrated a molecular ion at m/z 324.0865 corresponding to $C_{15}H_{17}N_2O_4Cl$. The ^{1}H-NMR spectrum showed a hydrogen-bonded phenolic proton at 9.4 ppm which disappeared on deuteration with D_2O. The ^{13}C-NMR spectrum exhibited two quaternary carbons at 164.7 and 168.7 ppm consistent with carbonyl carbons and four quaternary aromatic carbons. Based on the spectral information, the structure of the degradate was determined to be **VII**.

3. Results and discussion

3.1. Photolytic degradation

When exposed to artificial daylight fluorescent light, the drug substance in aqueous solutions was found to degrade to a sole degradation product which was different from that formed by oxidation (Fig. 1a). The identity of the degradation product suggests that the photolytic pathway involves a carbon-chlorine bond cleavage. In photolytic degradation, a drug molecule in solution absorbs light and the excited-state electronic energy is dissipated through bond cleavage(s). RG 12915 has a UV maximum absorbance at 215 nm (molar absorptivity = 32 400) and another maximum at 312 nm (molar absorptivity = 5000). The drug shows some UV absorption at approx. 350 nm and may absorb enough light to undergo direct photoreaction. The C-Cl bond cleavage has been reported to account for the photodegradation of many drug molecules. The photolabile nature of chlorine has been demonstrated in many chloroaromatic compounds such as chlorpromazine, prochlorperazine, hydrochlorothiazide and furosemide (Greenhill and McLelland, 1990).

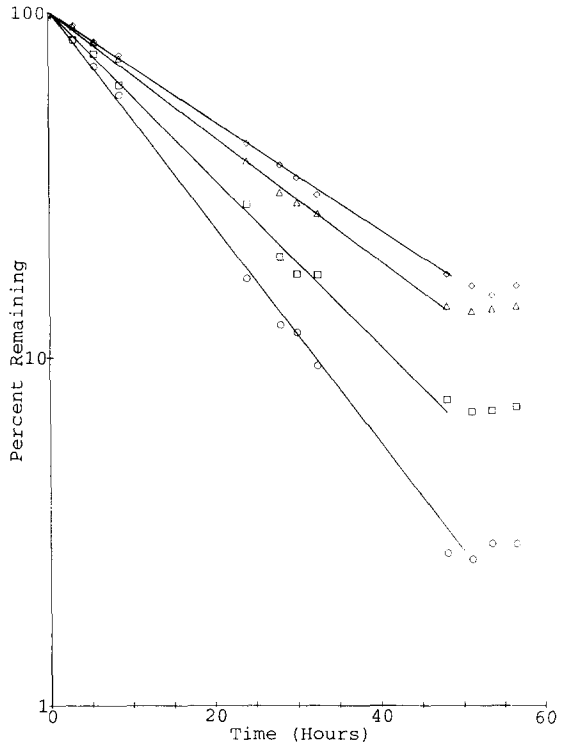

Fig. 2. Semilogarithmic plots of percent drug remaining against time at various initial concentrations in the photolytic degradation of I under 180-foot-candle artificial daylight fluorescent light at room temperature (22–25° C) and pH 6.8. 25 (○); 50 (□); 75 (△); 100 (◇) μg/ml.

A photodegradation reaction approaches pseudo-zero order kinetics in concentrated solutions as the reaction becomes limited by the incident light, but the reaction follows first-order kinetics in dilute solutions (Mendenhall, 1984; Connors et al., 1986). In this study, apparent first-order kinetics was observed over several half-lives as shown by the examples in Fig. 2.

The first-order rate constant appears to be inversely proportional to the initial concentration. Although the concentration effect on the degra-

dation rate in dilute solutions has not been elucidated, a possible explanation may be that the solutions are not actually optically dilute and therefore we are seeing a mixed mechanism between zero- and first-order kinetics. It is also possible that the photodegradation product **II** is competing for the incident light, thereby resulting in the concentration dependent rate and stopping the photoreaction after approx. 50 h.

A similar concentration-dependent first-order degradation rate has been noted in the photochemical decomposition of adriamycin (Tavoloni et al., 1980; Asker and Habib, 1988), dothiepin (Tammilehto and Torniainen, 1989), midazolam (Andersin and Tammilehto, 1989) and metronidazole (Karim et al., 1991).

3.2. Oxidative degradation

The stability-specific HPLC method was used to follow the extent of the oxidative degradation of **I** in aqueous solutions (Fig. 1). Under the experimental conditions, no thermal degradation products were observed. The identities of the degradation products formed indicate the reaction to be auto-oxidation. Many drug compounds have been reported to be subject to free-radical-mediated auto-oxidation (Connors et al., 1986; Carstensen, 1990; Greenhill and McLelland, 1990). The rate of auto-oxidation reactions in solution is influenced by various factors. Hence, the experiments were run under the same conditions so that the rate data could be compared to ascertain the influence of variables on the reaction. Control experiments, therefore, were always included for comparison.

Under the mild conditions in which a reaction can occur, oxygen often attacks hydrocarbons to form alkyl hydroperoxides (Hiatt, 1971). The relative reactivities of hydrocarbons in oxidation are predictable on the basis of the relative ease of hydrogen abstraction from various C-H bonds (Bateman, 1954). The abstraction of a hydrogen occurs more readily if the resulting radical is stabilized by resonance. Consequently, the preferential position of an oxygen attack in RG 12915 molecule is the tertiary carbon, α to the benzene ring, and the corresponding hydroperoxide (**III**)

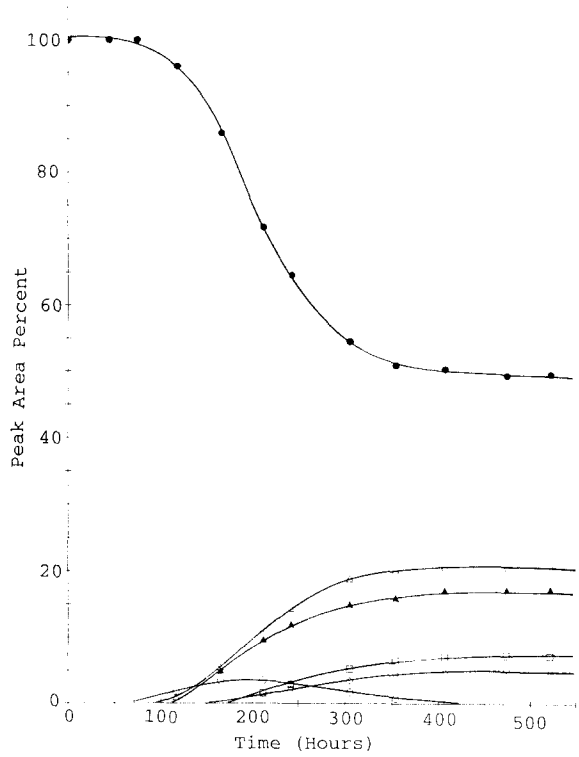

Fig. 3. Time course for **I** (●), **III** (○), **IV** (△), **V** (▲), **VI** (□) and **VII** (◇) during the oxidative degradation of **I** at 1 μg/ml, 60° C and pH 6.8.

appears as the first product of the oxidation reaction.

The kinetics of oxidation of **I** follows the pattern shown in Fig. 3. The reaction shows an induction period, followed by increasingly rapid oxidation and eventual leveling off. The acceleration phase occurs due to free-radical chain branching. The free radical thus formed adds molecular oxygen and propagates the chain mechanism by abstracting hydrogen from another molecule, giving rise to the hydroperoxide (**III**). The decomposition of the hydroperoxide leads to the formation of the secondary oxidation product, the alcoholic compound (**IV**).

In the oxidation of **I**, the hydroperoxide (**III**) appeared first followed by **IV** and **V** (Fig. 3). Product **III** was present in an amount of 3.5% relative to the initial concentration in the acceleration phase. The hydroperoxide concentration having reached a maximum showed a steady de-

cline and was barely detectable in the leveling-off phase. The auto-oxidation levels off when the secondary oxidation products appear in appreciable amounts. The oxidation stops probably because the reaction is inhibited by the oxidation product(s). Being a hindered phenol, product **VII** is a likely antioxidant that could inhibit the reaction. It may also be possible that some of the degradation products are chelating agents capable of sequestering transition-metal ions, thereby stopping initiation. Product **VI** was formed as a minor product and found later in the reaction when there was a considerable accumulation of **V**. This indicated that **V** further oxidized to **VI**. It is not known whether **IV** also undergoes oxidation to **V**.

It has been stated that the rate of auto-oxidation is proportional to the substrate concentration (Bateman, 1954; Betts, 1971; Connors et al., 1986). As shown qualitatively in Fig. 4, the higher concentration oxidizes faster and levels off at a lower percent, whereas the lower one oxidizes slower and levels off at a higher percent. The concentration effect on the leveling-off point may be due to the solvent effect. When the reactant is present in very low concentration, the active free radicals generated will react with, and be rendered inert by, solvent molecules, before the radicals survive to react further.

With ordinary fluorescent laboratory lighting serving as an activator, the oxidation reaction proceeded faster. Under an artificial daylight fluorescent lamp, the photolytic C-Cl bond cleavage was observed as previously described.

Bubbling oxygen into the solutions in the initiation or leveling-off stage was found to exert very little effect on the oxidative degradation of **I**. In most auto-oxidation reactions, even though oxygen is involved, the initiation of the oxidation reaction does not involve molecular oxygen. As long as sufficient oxygen is present in the solution, oxygen has little effect on the oxidation rate (Betts, 1971; Connors et al., 1986).

The effect of pH on the degradation is shown in Fig. 5. The degradation rate increases with an increasing acidity. The lag time also shortens with an increasing acidity. No lag time was observed at pH 2.1.

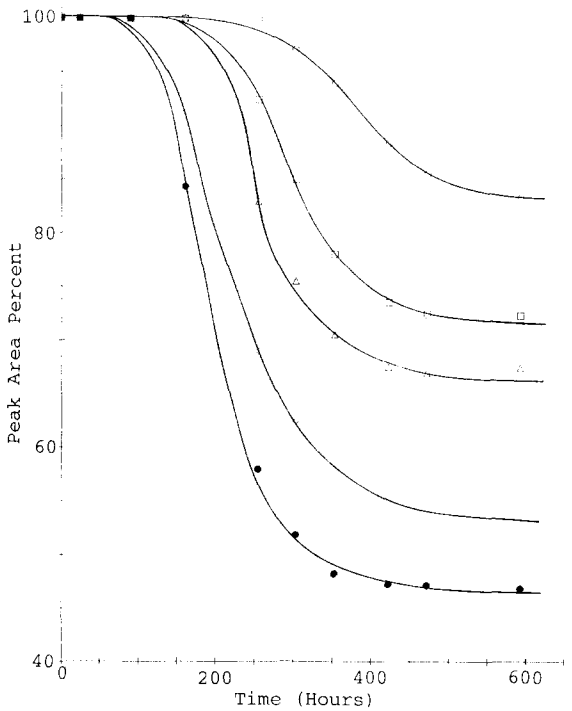

Fig. 4. Effect of initial concentration on the oxidative degradation of **I** at 60° C and pH 6.8. Concentration: 0.2 (○), 0.4 (□), 0.6 (△), 0.8 (◇) amd 1.0 (●) mg/ml.

RG 12915A is stable in the solid state at elevated temperatures. However, when the sample was stored at 40° C/80% relative humidity for 16 days, it became a glaze-like material. An HPLC analysis revealed the humidity-exposed sample to be 42% pure. The chromatogram of the sample showed a major degradation peak which was identified as **VII** (Fig. 1d). Product **VII** was also found to be the final degradation product when the drug substance was left in aqueous media for a long time. The instability of the drug substance in the presence of moisture may be due to the concentration and pH effects in aqueous solutions. The drug exists as a saturated solution around the solid particle when the drug particle is exposed to high humidity. Being a hydrochloride salt of a weak base, the drug molecule in the saturated solution is in an acidic environment of pH approx. 4. Consequently, the oxidation reaction in the acidic and concentrated solution would proceed very rapidly.

Soluble salts of transition-metal ions are known to catalyze the auto-oxidation of hydrocarbons. The active metals are those having two or more valency states, suggesting that an oxidation-reduction process is involved. Addition of iron or nickel ion to the solutions of I did not show a significant effect on the auto-oxidation rate, but copper(II) ion significantly accelerated the degradation rate (Fig. 6). The lag phase was also greatly reduced with the copper ion. The dependency of the reaction rate on the copper-ion concentration is shown in Fig. 7. A copper-ion concentration as low as 1.25×10^{-6} M showed a significant effect on the auto-oxidation rate. The metal ions may act on other auto-oxidation phases but their main effect is to increase the rate of initiation (Twigg, 1962). Higher copper-ion concentrations gave faster reactions but leveled off at slightly higher reactant level. The same observation was made in the cobalt(II)-catalyzed oxidation of cumene (Rouchaud, 1967).

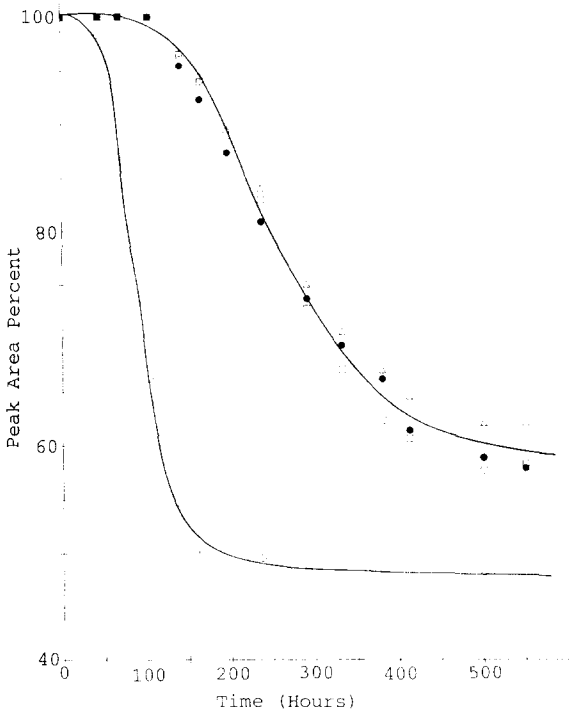

Fig. 6. Effect of metal ions on the oxidative degradation of I at 1 mg/ml, 60° C and pH 6.8. Metal ion concentration was 1.25×10^{-5} M. No metal ion (●), ferric chloride (△) nickel chloride (□) and cupric sulfate (○).

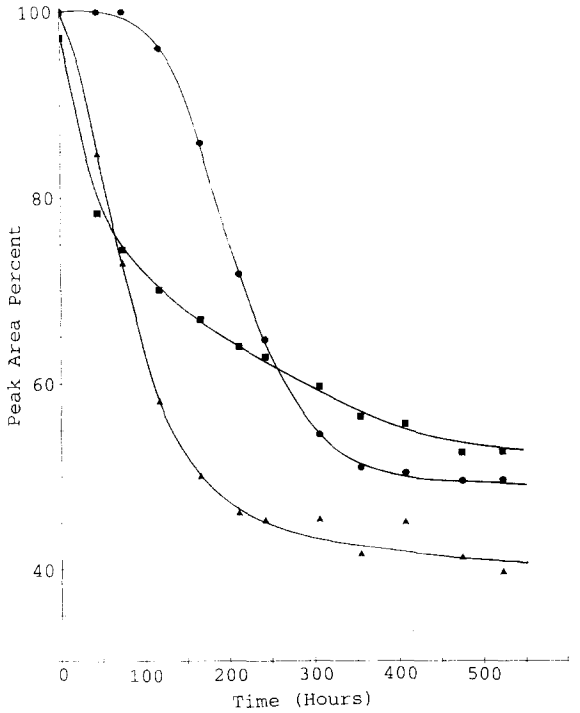

Fig. 5. Effect of pH on the oxidative degradation of I at 1 mg/ml and 60° C. pH 2.1 (■), 4.8 (▲) and 6.8 (●).

The oxidation reaction of I is catalyzed by copper ion and probably by some other heavy metal ions. Inhibiting metal-catalyzed oxidation of drugs by complexing these metal ions with the aid of metal-complexing agents has been attempted by numerous investigators. A variety of chelating agents are available for use in aqueous solutions. Of these, EDTA is by far the most commonly used. The effect of EDTA on the oxidative degradation of I is shown in Fig. 8. Aqueous solutions of I, which contained copper(II) ion as well as those which did not, were completely stabilized in the presence of EDTA. The fact that the sequestering agent stabilizes I in aqueous buffer solutions indicates a catalytic effect of trace metal contaminants in the drug substance and/or buffer reagents.

It is possible to enhance the stability of drug substances through the incorporation of certain antioxidants. The most effective are aromatic

amines and phenols. The inhibitors interfere with the propagation process by providing an alternative and easier reaction for peroxy radicals (Betts, 1971). Interestingly, it has been observed that no oxidative degradates were produced when **I** was degraded to **II** under an artificial daylight fluorescent lamp (Fig. 1a). The degradation product **II**, being a phenol and amine, might have inhibited the oxidation reaction. As expected, incorporation of 0.025% **II** to an aqueous solution of **I** suppressed the degradation (Fig. 8). Addition of 0.025% (w/v) propyl gallate did not completely inhibit the degradation but slowed it down (Fig. 8). An HPLC analysis of the solution degraded in the presence of propyl gallate showed that the antioxidant prevented the formation of the hydroperoxide (**III**) and the alcohol (**IV**). However, it was ineffective against *N*-oxide (**V**) formation.

Adventitious impurities may also play an important role as inhibitors or activators of the free-radical mediated oxidation reaction. One lot (RG 12915A-5) contained a considerable amount (2%) of an unknown impurity and was found to be very stable. Probably because of the nature and amounts of impurities present, there is considerable lot-to-lot variation with respect to the susceptibility of the drug substance to auto-oxidation. The presence of even trace amounts of unusual chemical entities is cause for serious concern.

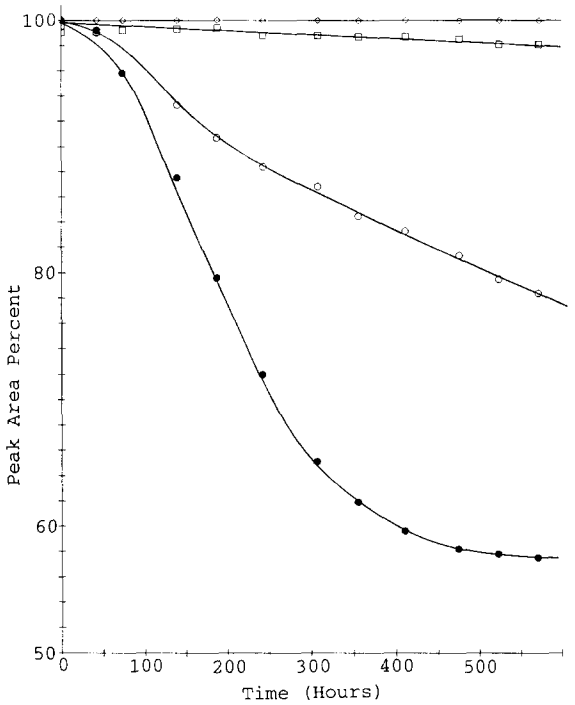

Fig. 8. Effect of additives on the oxidative degradation of **I** at 1 mg/ml, 60° C and pH 6.8. No additive (●), 0.025% (w/v) propyl gallate (○), 0.025% (w/v) **II** (□) and 0.1% (w/v) EDTA with and without 1.25×10^{-4} M cupric sulfate (△).

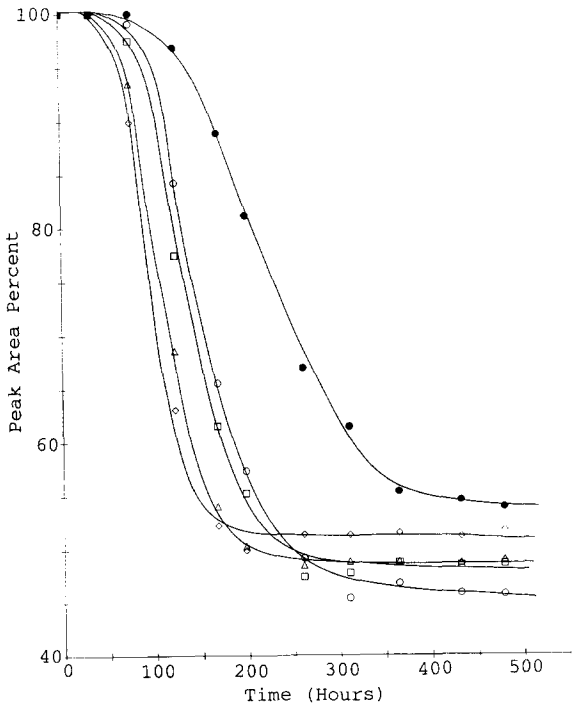

Fig. 7. Effect of cupric sulfate concentration (M) on the oxidative degradation of **I** at 1 mg/ml, 60° C and pH 6.8. No cupric sulfate (●), 1.25×10^{-6} (○), $2.5\times10-6$ (□), 5.0×10^{-6} (△) and 1.0×10^{-5} (◇).

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