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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 743–751
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Effects of alkali and simulated gastric and intestinal fluids on danazol stability

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Received 17 June 2002; accepted 23 October 2002

Abstract

The degradation kinetics of methanolic solution of danazol (0.020% w/v) in aqueous buffers and sodium hydroxide was investigated using stability-indicating HPLC method. The drug degrades in alkaline medium through a base-catalysed proton abstraction rather than via an oxidative mechanism involving oxygen species. The degradation followed pseudo-first-order kinetics. The rates pH-profile exhibited specific base catalysis. The stability of the drug was found to be dependent on pH, buffer concentration, buffer species (acetate, borate, phosphate) and temperature. The ionic strength did not affect the stability of the drug. The energy of activation according to Arrhenius plot was estimated to be 22.62 kcal mol^{-1} at pH 12 and temperatures between 30 and 60 °C. The effect of simulated gastric and intestinal fluids on the drug stability was also investigated. Two major hydrolytic degradation products were separated and identified by IR, NMR and mass spectrometry and the degradative pathway suggested.

Keywords: Danazol; HPLC; Degradation product; Base-catalysis

1. Introduction

Danazol, [pregna-2,4-dien-20-ynol[2,3-d]-isoxazole-17-ol, (17α) (**I**, Fig. 1), is a synthetic androgen derived from ethisterone. It suppresses the pituitary gonadotropins [1].

In a previous study, we investigated the photostability of danazol using a developed stability-indicating HPLC method [2]. In this study, we investigated the effect of pH, buffer (concentration and species), ionic strength and temperature on the stability of the drug using the previously described HPLC method [2]. Such accelerated (stress) testing helps to determine the intrinsic stability characteristics of the drug molecule by establishing degradation pathways and to identify likely degradates. These studies were initiated as a part of preformulation studies to characterize physico-chemical properties of danazol and to aid in the development of possible formulations of the drug other than the available solid dosage form.

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(02)00660-X

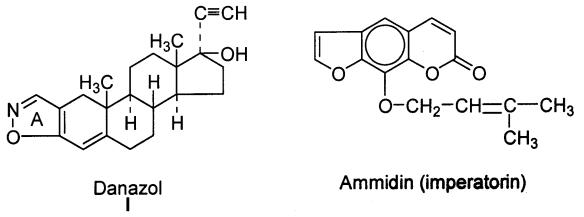


Fig. 1. Danazol and ammidin (imperatorin).

2. Materials and methods

2.1. Materials

Danazol was obtained from Sanofi Winthrop, France. Ammidin (imperatorin) (internal standard) was obtained from the Memphis chemical Company, Cairo, Egypt. Methanol (Spectrosol®, BDH, Poole, England). Acetonitrile (Hipersolv™, BDH, Poole, England). Water (Chromosolv®, Riedel-de Haën, Germany). Lichrosphere 100 RP-18 (5 μm) 125×4.0 mm^2 (E. Merck). Oxygen and nitrogen of high purity grade ($>99\%$) were purchased from local market.

2.2. Kinetic studies

2.2.1. Apparatus

A Waters Liquid Chromatograph consisted of a 600 E System Controller, U6K Injector, tunable absorbance detector 486 and 746 data module. The column was Lichrosphere 100 RP-18 (5 μm) 125 × 4.0 mm i.d. The mobile phase was acetonitrile: water (55:45 v/v) filtered through Millipore filter (0.45 μm) and degassed by bubbling helium (20 ml min^{-1}) into the solvent reservoir. It was pumped isocratically at a flow rate of 1.3 ml min^{-1}. The UV detector was set at 287 nm.

A Shimadzu UV 1601 PC spectrophotometer (Kyoto, Japan) was used to follow the pattern of degradation of danazol solution in sodium hydroxide.

2.2.2. Reagents and solutions

Britton-Robinson buffer (pH 2–12) [3]. Sodium hydroxide (0.2, 0.5, 1.0 molar) solutions in water. Simulated gastric and intestinal fluids USP [4]. Stock solutions of danazol (100 μg ml^{-1}) and ammidin (300 μg ml^{-1}) were freshly prepared in methanol. All the solutions for the stability studies were prepared by diluting 2 ml of the stock solution of danazol to 10 ml with the appropriate buffer and stored at 60 °C for the pH-profile study and at 30, 40, 50 and 60 °C for the study of the influence of temperature on the drug at pH 12.0 in a thermostatically temperature controlled water bath using a Thermostep® temperature controller, Yellow Springs Co, Inc, Ohio (YSI) model 74. Samples were withdrawn at suitable time intervals, immediately cooled in an ice bath and analysed by the HPLC method.

The effect of NaOH concentrations 0.2, 0.5 and 1.0 molar on the stability of danazol at room temperature (25 °C ± 1) was carried out by transferring 2 ml of the danazol stock solution in a number of 10 ml volumetric flasks; 1 ml of the appropriate molarity of sodium hydroxide was added. The reaction was stopped at suitable time intervals by acidifying with hydrochloric acid. Two milliliter of the internal standard solution were added to the solutions intended to be analysed by the HPLC method before completing to volume with methanol. In another set, danazol solutions were reacted similarly with sodium hydroxide, acidified with hydrochloric acid and volume completed with methanol without addition of internal standard solution. These solutions were then monitored by UV scanning between 340 and 215 nm.

To study the effect of dissolved oxygen on the drug stability in solution, solutions of danazol (20 μg ml^{-1}) in 20% v/v methanol in BRB, pH 11.0 were saturated by bubbling with either oxygen or nitrogen for 30 min. Both sets of solutions, together with control solutions not treated with either oxygen or nitrogen, were heated at 60 °C for 30 min, cooled and analysed by the HPLC method. Another set of danazol solutions was prepared similarly but using 0.5 M sodium hydroxide instead of the buffer at room temperature and left for 15 min. The percentage drug remaining in these sets was calculated relative to their respective solutions at zero time.

To determine the effect of artificial gastric and intestinal fluids on danazol stability, solutions of danazol (20 μg ml^{-1}) in 20% v/v methanol diluted

with either media, prepared with the enzyme (test solutions) and without the enzyme (control solutions) were incubated at 37 °C for 6 h. Samples were taken at suitable equal time intervals, and analysed by the HPLC method.

2.2.3. Preparation and the identification of the alkaline hydrolysis products of danazol

Fifty milligrams of danazol were dissolved in 1N NaOH in methanol and allowed to stand overnight at room temperature. The reaction mixture was poured into 50 ml water and mixed and the slowly formed pale yellow crystalline product was filtered off, washed with water and was left to dry in a desicator over phosphorous pentoxide. The filtrate was allowed to evaporate at room temperature. The resultant residue was dissolved in methanol, filtered and acidified with HCl and allowed to dry in the desicator to give a pale yellow sticky material.

3. Results and discussion

The formulation of a drug substance into a specific pharmaceutical form(s) depends on previous preformulation studies that cover physicochemical properties and stability studies of the drug substance. These stability studies are also extended to the formulated drug product. Detailed different stability studies that can be conducted on drug substances or drug products are presented in the 1987 Guidelines and the 1993 International Conference on Harmonisation (ICH) [5]. One of the requirements of these stability studies is the development of a validated stability-indicating assay method that can distinguish the active ingredient from its degradation products [5]. The HPLC method used in this study was validated before as stability-indicating method, and the details of the developed method were reported in our previous work [2]. The regulations of ICH regarding stability testing requirements also provide the stress testing conditions with the aim of assessing the effect of severe conditions on the drug product.

Table 1
K_{obs}, $t_{\frac{1}{2}}$ and $t_{90\%}$ values for the degradation of danazol at 60 °C in BRB at different pHs

pH	K_{obs} (h^{-1})[a]	$t_{\frac{1}{2}}$ (h)	$t_{90\%}$ (h)
12.0	10.40	0.067	0.010
11.0	1.28	0.54	0.082
10.0	0.32	2.17	0.33
9.6	0.14	4.95	0.75
9.0	0.036	19.25	2.93
8.0	0.023	30.13	4.58
6–7	0.019	36.5	5.55

[a] Values are an average of two determinations with RSD values within 6%.

3.1. Degradation of danazol in solution

Many drugs in solution are subject to chemical degradative reactions. Various factors that may

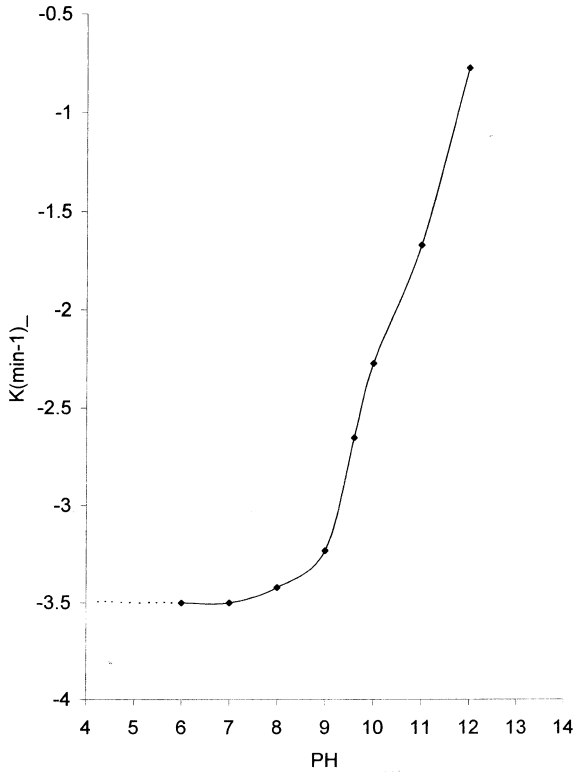


Fig. 2. Effect of pH on degradation rate of danazol in alkaline media at 60 °C.

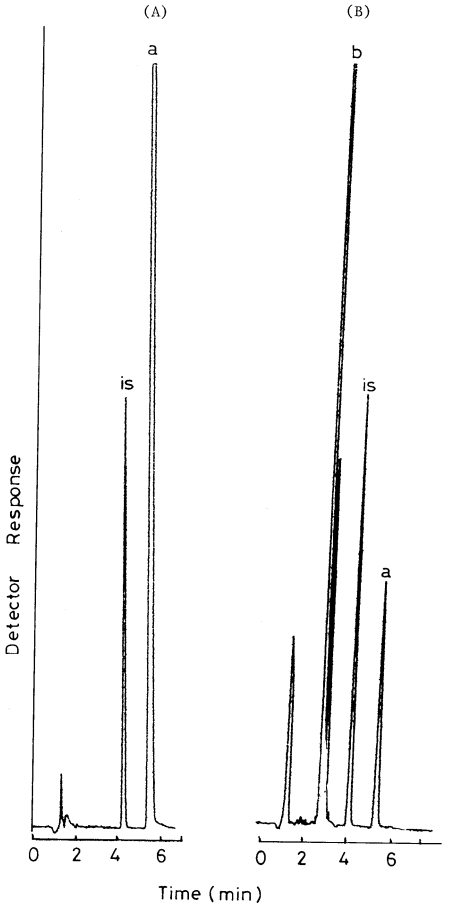


Fig. 3. Typical chromatogram of danazol solution (20 μg ml^{-1}) and the internal standard (6 μg ml^{-1}) (A); sodium hydroxide degraded danazol solution (20 μg ml^{-1}) (20 min) in presence of the internal standard (6 μg ml^{-1}) (B).

affect the reaction rates include pH, buffer concentrations, temperature and ionic strength.

3.1.1. *Effect of pH on degradation of the drug*

The disappearance of intact **I** was monitored by the HPLC method over the pH range 6–12 at 60 °C. The degradation rate-constant (K_{obs}) was calculated by using linear regression analysis obtained from at least six time intervals. The degradation half life ($t_{\frac{1}{2}}$) and the shelf-life (t_{90}) were calculated as $0.693/K_{obs}$ and $0.1054/K_{obs}$, respectively (Table 1). These results indicate that the drug degrades faster along the basic pH side especially at pHs above 9.0. The drug was found

Table 2
K_{obs}, $t_{\frac{1}{2}}$ values for the degradation of danazol at room temperature (25 °C ± 1) in different sodium hydroxide concentrations

Molarity	K_{obs} (min^{-1})[a]	$t_{\frac{1}{2}}$ (min)
0.2	0.0163	42.5
0.5	0.0417	16.6
1.0	0.0817	8.5

[a] Calculated from regression data of log [peak area ratio at time t (Pt) divided by peak area ratio at $t_0(P_0)$] vs time in minutes i.e. log P_t/P_0 vs time for at least two determinations with RSD values within 2%.

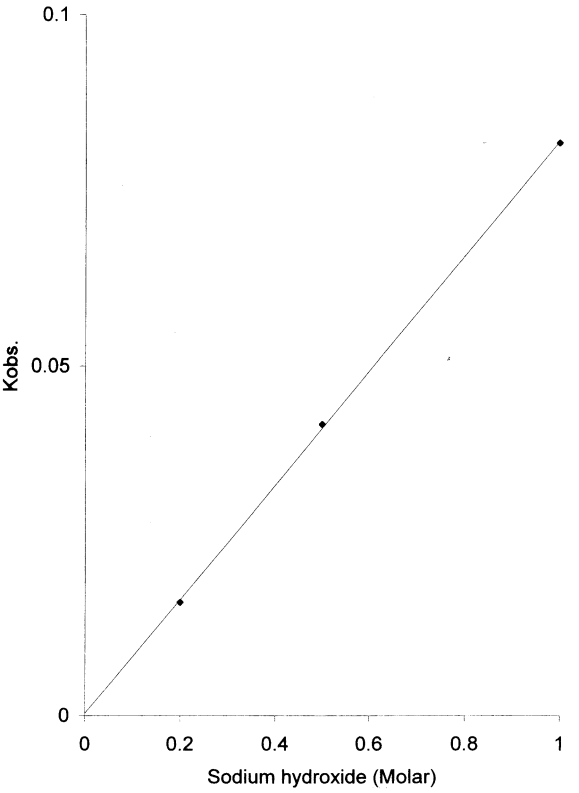


Fig. 4. Effect of sodium hydroxide concentration on the degradation constant (k) of danazol at pH > 12 and room temperature (25 °C ± 1).

stable along the acidic pH side even at temperatures above 60 °C. The pH-profile plot obtained (Fig. 2) resembles subtype BCD in the generalized pH-profile polygon (5).

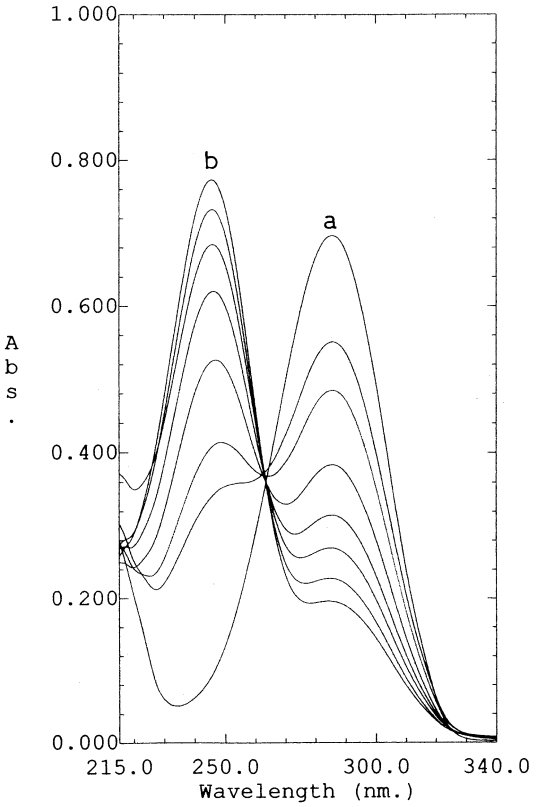


Fig. 5. UV-scan of degradation of danazol with 0.5 M sodium hydroxide: *Key:* a, Danazol peak; b, Degradation product peak.

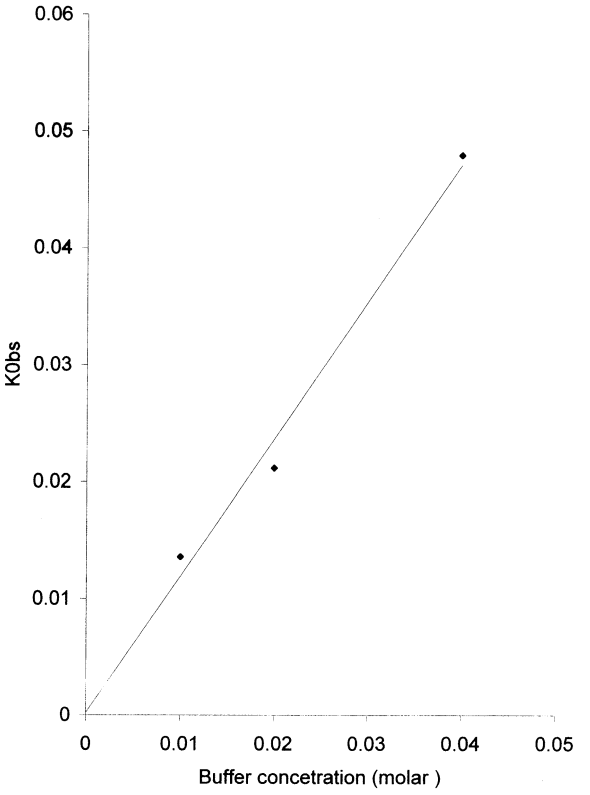


Fig. 6. Effect of buffer concentration on the degradation constant as (log K_{obs}) of danazol at pH 11.0 and 60 °C.

3.1.2. *Reaction of the drug with sodium hydroxide at different concentrations*

3.1.2.1. HPLC method. The disappearance of the intact drug under the influence of sodium hydroxide at different concentrations was monitored by the HPLC method using the internal standard (Fig. 3). The degradation rate constant (K_{obs}) was calculated by using linear regression analysis data of log [peak area ratio at time (t) over peak area ratio at time zero (t_0)] vs time for at least six time intervals. Table 2 and Fig. 4 summarize the results obtained. The degradation rate increases and subsequently $t_{\frac{1}{2}}$ decreases with increasing concentrations of sodium hydroxide.

3.1.2.2. UV-spectrophotometry. The spectral changes of danazol solution treated with 0.5 M sodium hydroxide at room temperature, reflect the decrease of danazol peak at its λ_{max} at 287 nm and the subsequent formation of degradation product(s) with λ_{max} at 242 nm. Although the λ_{max} values of danazol and the degradation product(s) are apart, an interference on the assay of danazol by UV in presence of the degradation product(s) is possible through residual absorption of the products at the λ_{max} of danazol (Fig. 5).

3.1.3. *The effect of oxygen on the stability of danazol*

The results obtained in this study showed no significant difference in the percentage drug remaining for the different sets of studied solutions (see Section 2.2.2). The average percentage remaining was $58 \pm 3\%$ ($n = 2$) for the buffer solutions (oxygen-treated and controls) and $54 \pm 2\%$ ($n = 2$) for the sodium hydroxide solution (oxygen-

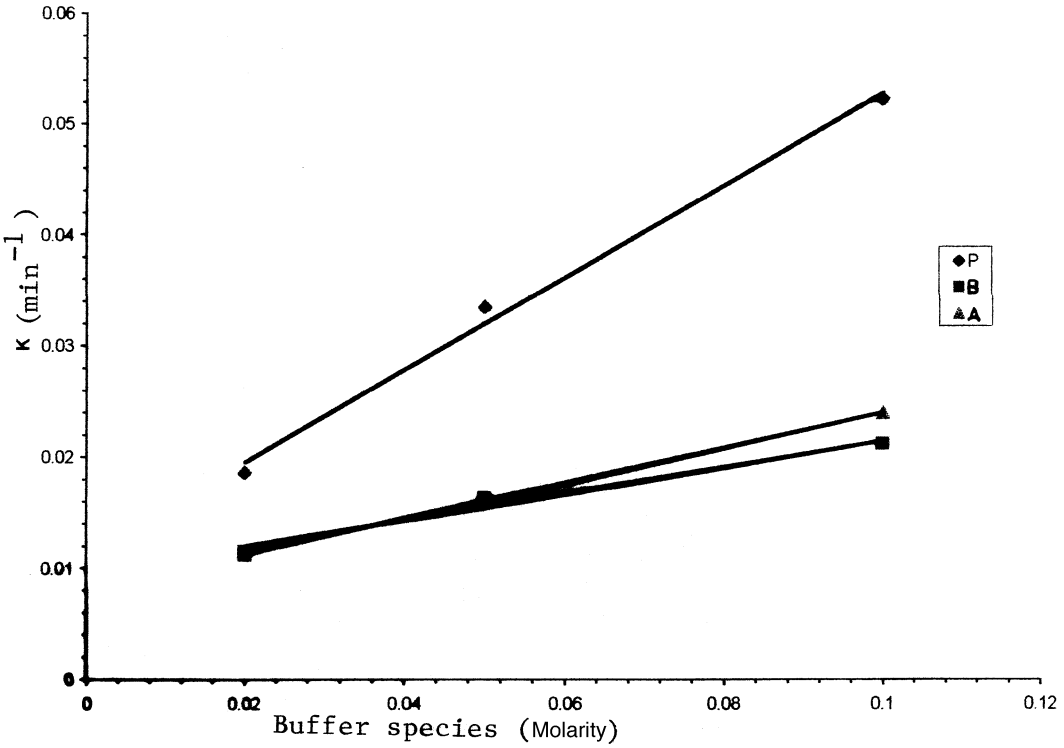


Fig. 7. Effect of buffer species on the degradation constant (k) of danazol at pH 11.0 and 60 °C. *Key:* P, Phosphate; A, Acetate; B, Borate.

treated and controls). These results exclude an oxidative reaction playing a role in the degradation process. The selected times in this study (30 min for the buffer solution and 15 min for the sodium hydroxide reaction) depended on the previously calculated $t_{\frac{1}{2}}$ values in these media to obtain about 50% reduction for good comparison.

3.2. Catalytic effects of buffer concentrations, buffer components and ionic strength on the degradation of danazol

The decomposition rate was hastened with increase of buffer concentrations (Fig. 6) or buffer species (acetate, borate, phosphate; Fig. 7). The phosphate species seems to have more impact on the catalytic process. The effect of ionic strength on the degradation of danazol was determined by keeping the pH (at 11), buffer concentration (0.02 M) and temperature (60 °C) constant, and varying the ionic strength by addition of different amounts of KCl to obtain ionic strength of 0.3, 0.5, 0.75 and 1.0 μ. No kinetic salt effect was observed for the degradation of the drug.

3.3. Effect of temperature on reaction rate of the drug

The Arrhenius plot (Fig. 8) derived from the time-course decomposition of danazol in BRB (pH 12) at temperatures 30, 40, 50 and 60 °C (Fig. 9) demonstrates the effect of temperature in the degradation of the drug. The linearity obtained reflected the dependence of the decomposition on temperature as a determining factor. The calculated activation energy was 22.62 kcal mol^{-1}, a value which lies within the typical 10–30 kcal mol^{-1} for hydrolysis reactions [6].

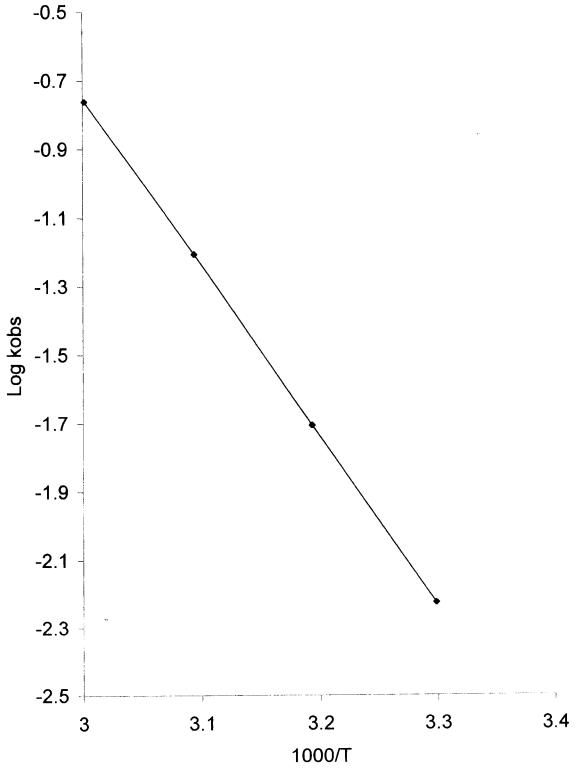


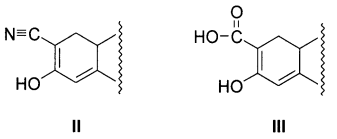
Fig. 8. Arrhenius plot for degradation of danazol in BRB buffer, pH 12 and temperatures 30, 40, 50 and 60 °C.

4. Effect of gastric and intestinal fluids on the stability of danazol

No effect was observed on danazol solutions incubated in the gastric or intestinal fluids, indicating that the drug passes these media without being subjected to chemical or enzymatic reactions.

5. Identification of the degradative products

The alkaline degradation products of danazol were isolated as described before and identified as the cyanohydrin **II** and the hydroxyacid (**III**) derivatives.



II III

The structures were elucidated using IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopic techniques. All spectra were consistent with the assigned structures. The characteristic features of the IR spectra of **II** are the appearance of strong C≡N stretch at 2125 cm^{-1} and a broad (hydrogen bonded) OH stretch at 3370 cm^{-1}. The IR spectrum of **III** showed a dimer that displayed an intense O–H stretching absorption in the region of 3500–3400 cm^{-1} and a C=O stretching band at 1680 cm^{-1}) together with the disappearance of the characteristic C≡N absorption of **II**. The mass spectrum of **II** and **III** showed different fragmentation pattern from that of danazol. The molecular ion peak of **II** was distinct at *m/z* 337 with an $M^{\bullet+}+I$ peak and a base peak at *m/z* 185 assigned to fragment $[C_{12}H_{11}NO]^{\bullet+}$. Spectrum of **III** showed a characteristic base peak at *m/z* 204 of fragment $[C_{12}H_{12}O_3]^{\bullet+}$. All other major fractions of **II** and **III** were also assigned to their respective fragments. The ^{1}H-NMR of **II** showed the disappearance of the singlet (8.11 ppm) of the protonated carbon of the isoxazole ring in danazol. The resulting OH group of **II** caused an upfield shift of neighbouring CH proton of the cyclohexadiene ring. The chemical shift of the CH of isoxazole (148.5 ppm) disappeared in the ^{13}C-NMR spectrum of **II** with the appearance of a new chemical shift at 126.4 ppm attributed to the CN carbon.

5.1. Mechanism and proposed degradation pathway

Mechanistically, the degradative process seems to involve initial proton abstraction, by the base OH, of the isoxazole ring to give the cyanohydrine (**II**) (Scheme 1). The latter (**II**) is envisaged as an intermediate which is further hydrolysed by the base forming the hydroxyacid, **III**. It is noteworthy that **II** was converted to **III** with NaOH followed by acidification under the same conditions of the

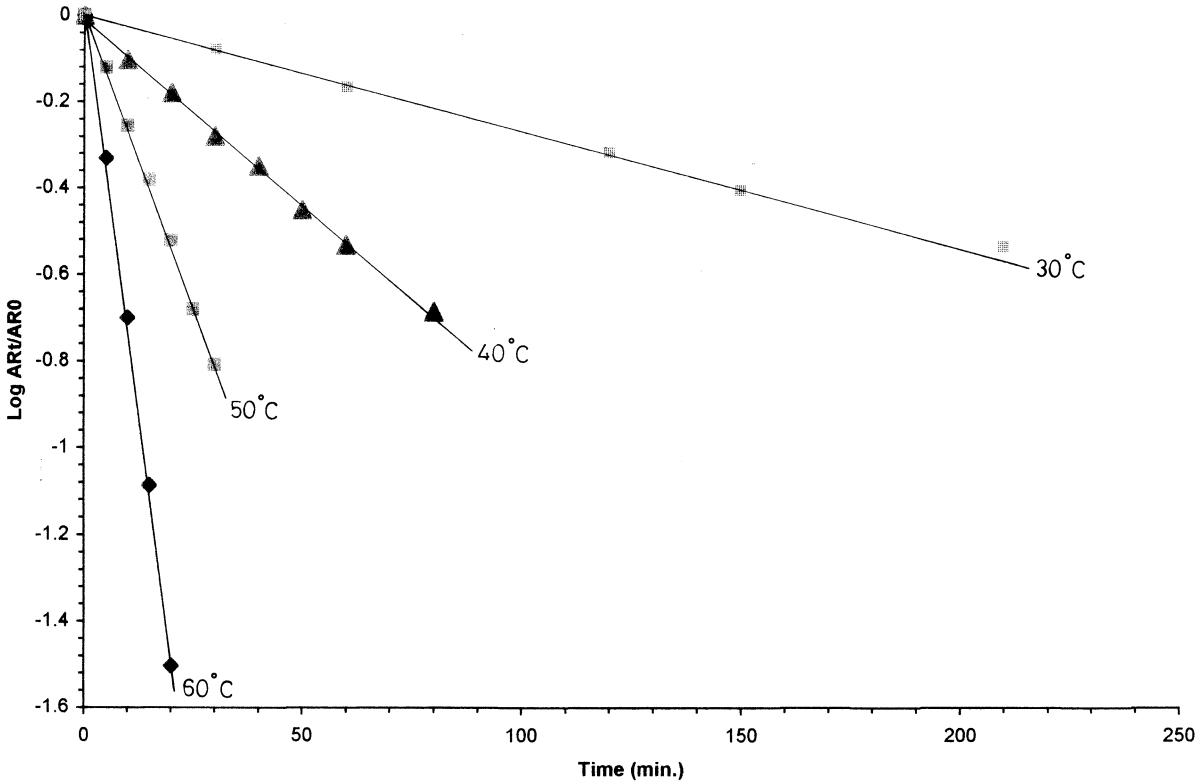
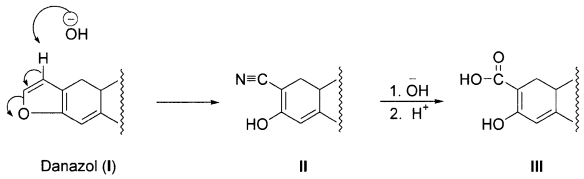


Fig. 9. Time-course of the decomposition of danazol in BRB, pH 12 at various temperatures.



Scheme 1.

alkali degradation of danazol. Fig. 10 compares the UV-spectrum of **II** and **III** in methanol.

6. Alkali vs the photodegradation products of danazol

The main photodegradation product (DP_1) observed in our previous paper (2) was compared and found to share physical properties with the danazol cyanohydrin (II) characterized in this work as the major alkaline degradate. The two compounds have identical retention times ($\simeq 3$ min) whether injected separate or spiked using the specified HPLC method. The R_f value of 0.2 (USP TLC method) and the λ_{max} (242 nm) were also identical. These observations suggest, but do not confirm, that the photodegradation product, DP_1 is probably the danazol cyanohydrine, **II**. Further confirmatory studies are underway.

7. Conclusion

Danazol solution underwent a base-catalysed reaction that lead to the opening of the isoxazole ring. Abstraction of the acidic methine proton of the isoxazole ring resulted in the formation of danazol cyanohydrin as the major alkaline degra-

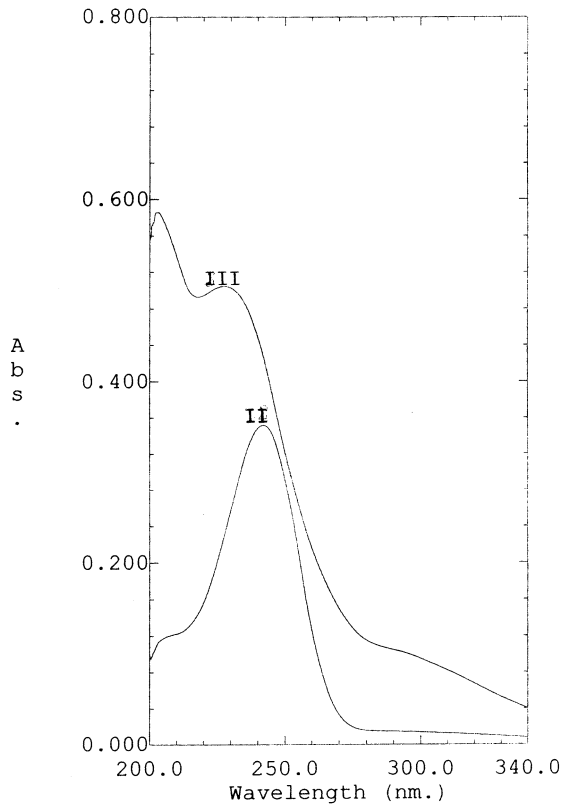


Fig. 10. UV-spectrum of isolated alkali degradation products of danazol. *Key:* II Cyanohydrin, $\lambda_{max} = 242$ nm; III Hydroxy acid, $\lambda_{max} = 227$ nm.

dation product. Basic hydrolysis of the cyanohydrin lead to the formation of the corresponding danazol hydroxyacid. Identification of the degradates allowed a suggestion of the degradative mechanism and a proposal for the pathway of the process. Comparative study on the alkali- and photo-degradative processes suggested that the danazol cyanohydrin could be a major common intermediary product for both reactions.

It is hoped that these preformulation studies, along with others, could contribute to the development of other dosage forms of the drug.

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