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Stability studies on diloxanide furoate: effect of pH, temperature, gastric and intestinal fluids

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Abstract

The degradation of the amoebicide diloxanide furoate in alkaline medium at different temperatures was investigated using both a spectrophotometric and a developed HPLC method. In solutions, the drug was found to undergo decomposition, i.e., temperature and pH dependent. The pH-rate profile at pH between 7.6 and 9.6 indicated a first-order dependence of K_{obs} on [$^{-}$OH]. Arrhenius plot obtained at pH 8 was linear between 40 and 63 °C. The estimated activation energy of hydrolysis was found to be 18.25 kcal degree.mol^{-1}. The effect of simulated gastric and intestinal fluids on the drug was also investigated. A new thin-layer chromatographic (TLC) procedure for the fractionation of the drug and its alkaline hydrolysis products has been developed and was found to compare favorably with that of the British Pharmacopoeia. Three hydrolysis products of a basic methanolic solution of the drug, namely furoic acid, diloxanide and methylfuroate could be identified by the use of TLC, HPLC, infrared and mass spectrometry.

Keywords: Diloxanide furoate; HPLC; UV spectrophotometry; TLC; Kinetics; Stability

1. Introduction

During the pharmaceutical development of a new drug, it is necessary to select as soon as possible the formulation with the best stability characteristics. Regulations regarding stability testing for registration application are provided by current International Commission for Harmonisation (ICH), which emphasizes the stress testing conditions with the aim of assessing the effect of severe conditions on the drug [1]. In practice, the effects of pH and temperature changes on drug stability are often used in such studies. The well-known Arrhenius theory is still used to allow a rapid stability prediction. The preformulation pharmacist uses the stress testing to provide knowledge on the drug's degradation products, degradation pathways and mechanisms. The results of such studies are of vital importance in the estimation of a drug product shelf life during early stages of its pharmaceutical development. The results may also serve as guides for better drug design, drug formulation and drug analysis.

Diloxanide furoate (DF): 4-[*N*-methyl-2,2-dichloroacetamido] phenyl-2-furoate is a luminal amoebicide. The drug is orally administered and is hydrolyzed in the gut to release diloxanide, which is considered as the active drug [2]. Methods described for the assay of DF in bulk and/or dosage forms are compiled in a review by Al-Majed et al. [3]. Procedures for the quantification of the drug are also reported [4,5]. Recently, we developed a stability-indicating HPLC method suitable for the assay of the drug in presence of its photodegradation products [6]. In this piece of work, we investigated the effects of pH, temperature and simulated gastric and intestinal fluids on the stability of DF making use of the stability-indicating power of the developed HPLC procedure.

2. Experimental

2.1. Materials

A pure drug sample of DF (Batch No. CDF/DF/175/97) was kindly provided by Eipico Pharmaceutical Company, Cairo, Egypt. Ammidin, the internal standard, was obtained from Memphis Chemical Company, Cairo, Egypt. 2-Furancarboxylic acid was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade reagents.

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2.2. Reagents

Phosphate and alkaline borate buffers (pH range 7–10) and simulated gastric and intestinal fluids were prepared according to USP 1995 [7]. Aqueous sodium bicarbonate ($NaHCO_3$) (1% w/v) solution was prepared. Acetonitrile (Hipersolv™, BDH, Poole, England) and water (Chromosolv®, Ridel-de Häen, Germany) were also used. Other solvents were of analytical grade and obtained from BDH, Poole, England.

2.3. Standard solutions

Fresh standard solution of DF (200 μg ml^{-1}) in methanol was prepared and was further diluted with the mobile phase (30:70 v/v, water/acetonitrile) to the appropriate concentrations of the working solutions.

A stock solution of the internal standard ammidin (300 μg ml^{-1}) was prepared in methanol and was further diluted as appropriate.

2.4. Apparatus

A Waters Liquid Chromatograph 600E, equipped with Rheodyne 7161 injector, Waters 486-tunable absorbance detector and Waters-746 data module, was used. The column used was Lichrosphere 100 RP-18 (5 μm) 150 × 4.6 mm i.d. (Phase Separation Ltd.). The mobile phase consisted of water/acetonitrile (30:70 v/v) pumped isocratically at a flow rate of 1 ml min^{-1}. Degassing of the mobile phase was carried out by purging pure helium into the solvent reservoir at a rate of 20 ml min^{-1}; ultraviolet (UV) setting was at 258 nm and 20 μl volumes were injected onto the column at room temperature.

UV spectrophotometric studies were carried out on a Shimadzu UV 1601 PC Spectophotometer (Kyoto, Japan). The EI mass spectra were obtained on a Shimadzu GC/MS spectrometer (Shimadzu Corporation, Japan) operated with an electron energy of 70 eV and injector temperature of 250 °C. Thin-layer chromatography (TLC) was conducted on precoated silica gel sheets 60F254, 5 × 10 cm with 0.2 mm thickness (Riedel-de Häen, Germany). Solvent systems were; dichloromethane/methanol (24:1 v/v) [8] and cyclohexane/ethylacetate (3:2 v/v). Visualization was accomplished under UV light (254 nm).

2.5. Procedures

All the solutions for stability studies were prepared by diluting 200 μg ml^{-1} of the DF solution in methanol with the appropriate aqueous buffers (with pH values of 7.6, 7.8, 8.0, 8.2, 8.4, 8.8, 9.2 and 9.6) to obtain a final solution containing 20 μg ml^{-1} DF and 10% v/v methanol. The study was carried out at 40 °C. The kinetics of the decomposition of the drug was monitored adopting the HPLC method [6]. The rate constant for each pH was calculated from the plot of log [remaining drug] vs. time.

2.5.1. Effect of $NaHCO_3$ on the stability of DF

Two solutions were prepared as follows:

Solution (a): To 3 ml of the DF solution (200 μg ml^{-1}) in methanol in a 25-ml volumetric flask, 2.5 ml of 1% w/v aqueous $NaHCO_3$ solution were added before completing to volume with methanol.

Solution (b): Three milliliters of the DF solution (200 μg ml^{-1}) in methanol were transferred into a 25-ml volumetric flask and completed to volume with 1% $NaHCO_3$ aqueous solution.

Both solutions were monitored by UV scanning between 320 and 200 nm and by the HPLC method [6] at 5 min intervals.

2.5.2. Effect of temperature on the stability of DF solution

Five milliliters aliquots of the DF solution (200 μg ml^{-1}) were placed into four 50-ml volumetric flasks. Each flask was completed to volume with borate buffer (pH 8.0) equilibrated at the appropriate temperatures of the study (40, 50, 55 and 63 °C). Twenty microliters volumes of each solution were injected onto the column at appropriate time intervals ranging between 5 and 20 min, depending on the temperature of incubation of the solution.

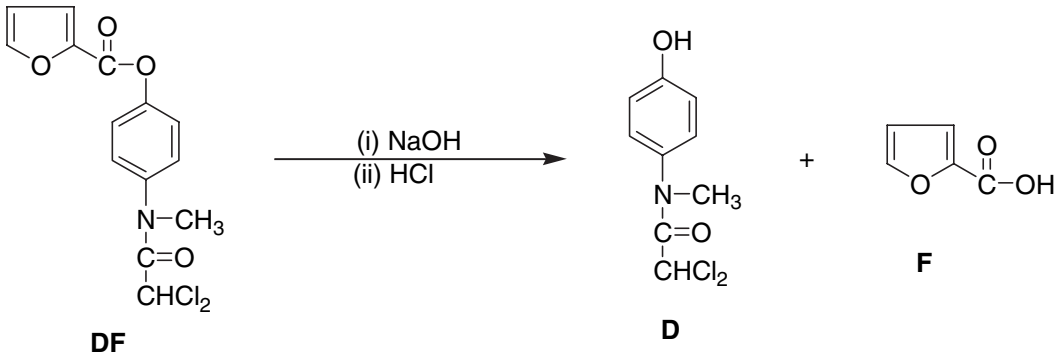
2.5.3. Effect of simulated gastric and intestinal fluids on DF

Five milliliters aliquots of DF solution (200 μg ml^{-1}) were placed into two 25-ml volumetric flasks. The contents of one flask was completed to volume with the simulated gastric fluid and the other with the simulated intestinal fluid; both being equilibrated at 37 °C. After thorough mixing, the flasks were incubated at 37 °C. One milliliter aliquots of each solution were withdrawn at 10 min intervals into 10-ml volumetric flasks; mixed with 1 ml of the internal standard solution (60 μg ml^{-1}) and completed to volume with the mobile phase (duplicates). Twenty microliters volumes were injected onto the column. Two blanks for each medium were used, one containing the medium without the enzyme and the other consisting of the medium containing the boiled inactivated enzyme. The data were treated for log peak area ratio with time.

2.5.4. Isolation of the hydrolysis products in the basic methanolic solution of DF

Fifty milligrams of DF reference material were dissolved in about 15–20 ml of 0.1 M sodium hydroxide in methanol and allowed to stand for about 15 min. Complete hydrolysis was checked by both TLC and HPLC methods.

The base-hydrolyzed drug mixture prepared above was acidified with concentrated HCl (≅0.5–1.0 ml) and 20 ml of chloroform were added. The solution was then concentrated to precipitate out sodium chloride. The mixture was filtered and the filtrate was allowed to evaporate slowly in the dark in a fume cupboard. The separated white crystals were characterized by infrared (IR) and mass spectrometry. The absorption spectrum of the substance was recorded in the UV region and its λ_{max} was determined.



Scheme 1.

3. Results and discussion

The structure of DF contains both an ester and a substituted amide linkage; both linkages are liable to hydronium or hydroxide ion-catalyzed hydrolysis; however, the rate of [$^{-}$OH] catalyzed hydrolysis of esters is known to proceed faster than that of the [H^{+}]-catalyzed process. Amides are generally much more stable towards alkaline hydrolysis than esters [9]. Accordingly, the ester group in the structure of DF is expected to undergo alkaline hydrolysis to yield diloxanide and furoic acid sodium salts which on acidification give diloxanide (D) and furoic acid (F) as shown in Scheme 1.

3.1. UV spectral changes in alkali-treated DF solution

The absorption spectrum of DF solution shown in Fig. 1, exhibited a major peak at 258 nm. Treatment of this solution with alkali resulted in the reduction of its absorption peak indicating the occurrence of structural changes in its molecule. Further changes in the spectrum were also observed when the solution containing the alkaline degradation products was acidified with HCl solution.

3.2. TLC of the acidified basic hydrolysates of DF

Using the TLC method of the British Pharmacopoeia (BP), the reaction mixture gave three spots. One spot coincided with the parent compound (R_f value ≅ 0.90), the other spots had R_f values of about 0.70 and ≅0.01; suggesting incomplete hydrolysis (see Scheme 1). The spots were then isolated from the TLC plates and further analyzed by UV spectrometry and HPLC. The results revealed that the spot with R_f value of 0.9 which, using the BP method, coincided with that of the parent compound, was actually a composite of DF and another alkaline degradation product of the methanolic DF solution. It is clear that the TLC system of the BP failed to resolve the latter alkaline degradate from the parent drug. A mixed spot of the drug and the degradation products confirmed this finding. In an attempt to separate this product from the drug, we developed a TLC method using a mobile phase consisting of cyclohexane/ethylacetate (3:2) with running developing time of about 20 min for 8-cm distance. The method successfully separated the drug from its degradation products. The R_f value for the drug was 0.6 and those for the degradation products were ≅0.75 (volatile), 0.5 and 0.01.

3.3. Characterization of the isolated degradation product

The isolated white crystalline powder obtained from the acidic methanolic solution (Section 2.5.4) was confirmed by IR and mass spectrometry to be diloxanide. The IR spectrum showed the loss of the ester C=O stretch at 1703 cm^{-1} and the appearance of O–H stretch at 3300 cm^{-1}. The amide C=O remained at 1650 cm^{-1}. Table 1 shows the mass spectral assignments of the product; the presence of two chlorine

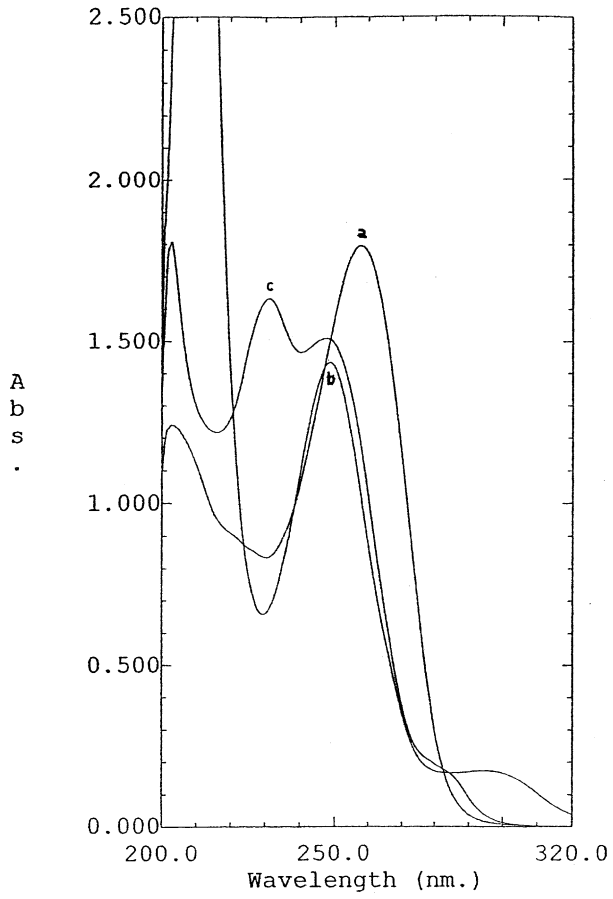


Fig. 1. UV spectra for DF solution (24 μg ml^{-1}): (a) before alkaline hydrolysis; (b) after alkaline hydrolysis and (c) acidified alkaline hydrolysate.

Table 1
Mass spectral assignments of diloxanide

m/z	Species
234	M^+
233	$[M–H]^+$
122 (100%)	$C_7H_8NO^+$
150	$C_8H_8NO_2^+$
94	$[C_6H_7N]^+$
65	$[C_5H_5^+]$
41	$C_2H^+O \rightarrow C_2HO^+$

atoms was evident by the distinct M + 2 and M + 4 peaks (not shown) due to molecular ions and (M–H) ion at 233 containing two atoms of the heavy isotope ^{37}Cl. Fig. 2 shows the UV spectra of the isolated crystals in neutral and acidic methanol to be identical (λ_{max} at 277 and 228 nm) while that in alkaline medium exhibited λ_{max} at 247 and 305 nm showing a bathochromic shift possibly due to the formation of the phenoxide ion. In TLC (Section 3.2), the degradation product with R_f value = 0.5 corresponded to diloxanide which was isolated and characterized as a hydrolytic product of DF. The presence of furoic acid was also confirmed by TLC where the degradation product of R_f = 0.01 corresponded to that of authentic furoic acid. The third degradate (R_f = 0.75) was first thought to be formed through the cleavage of the amide linkage. However, studies revealed the product to be methylfuroate formed through the alcoholysis of DF by the solvent methanol resulting in the transesterification reaction shown in Scheme 2. This was confirmed by the preparation of methylfuroate by refluxing a mixture of methanol, furoic acid and sulfuric acid and extracting the product in chloroform. The R_f, RT and UV spectrum (λ_{max} at 253 nm) of the degradate corresponded to those of the prepared methylfuroate. The use of ethanol or propanol instead of methanol lead to the formation of ethylfuroate or propylfuroate having different chromatographic peaks, which were also resolved from the drug peak. As expected, when the experiment was conducted using CHCN/H_2O as the solvent, only furoic acid and diloxanide were identified as the degradation products.

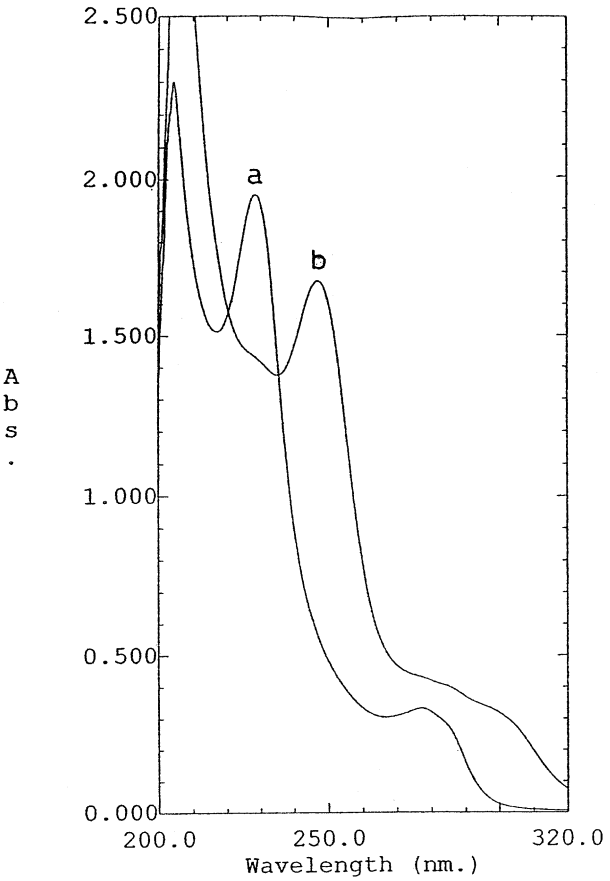


Fig. 2. UV spectra of isolated hydrolysis product: in methanol (a) and in alkaline medium (b).

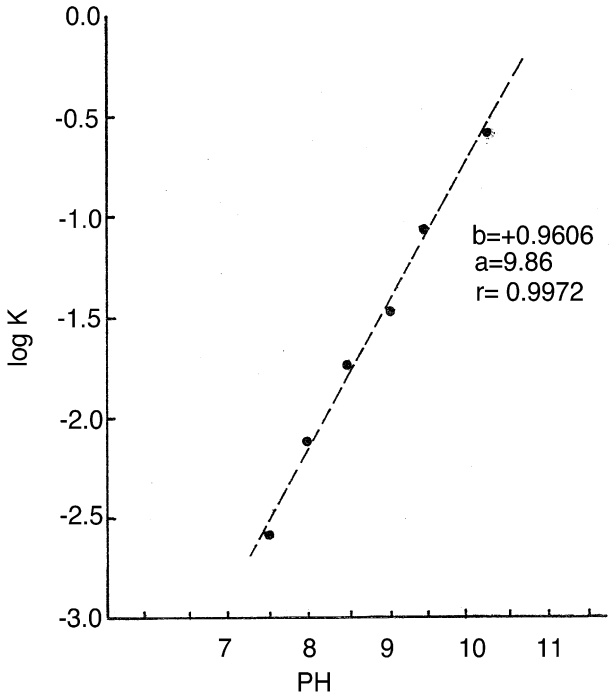
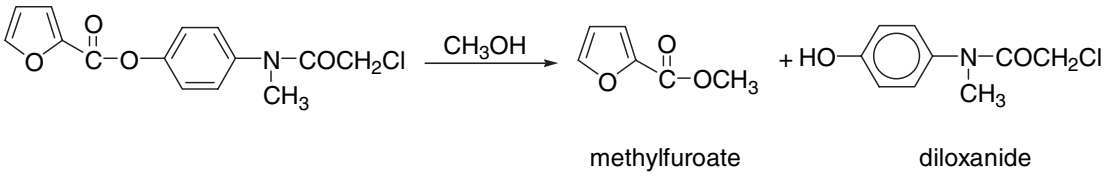


Fig. 3. pH-rate profile for alkaline hydrolysis of DF at 40 °C (pHs 7.6, 8.0, 8.4, 8.8, 9.2 and 9.6).



Scheme 2.

Table 2
Values of K_{obs} of DF at alkaline pHs at 40 °C and their equivalent $t_{1/2}$ values

pH value	K_{obs} (min^{-1})	$t_{1/2}$ (min)
7.6	2.526×10^{-3}	274.35
8.0	7.448×10^{-3}	92.50
8.4	1.776×10^{-2}	38.98
8.8	3.333×10^{-2}	20.80
9.2	8.485×10^{-2}	8.17
9.6	2.533×10^{-1}	2.74

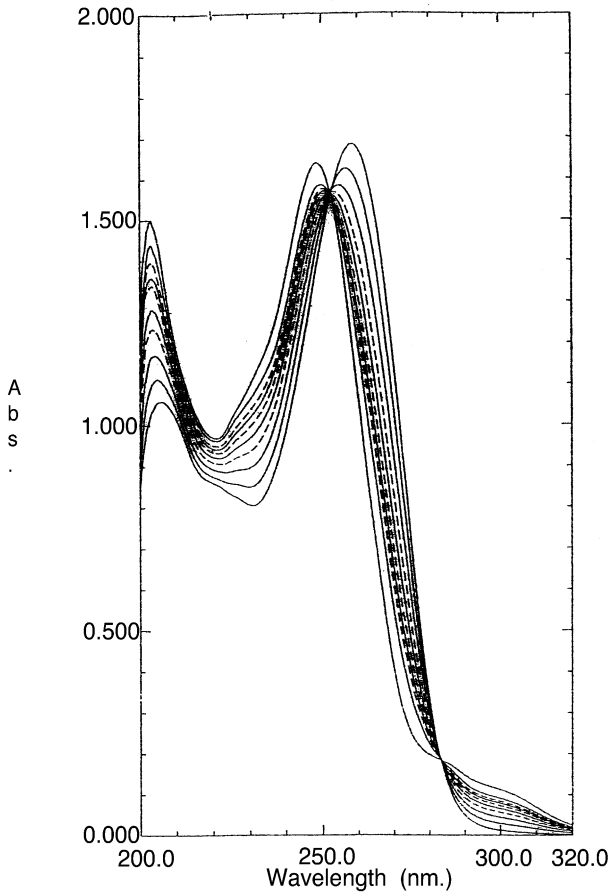


Fig. 4. Pattern of the change of UV absorption of DF solution (24 μg ml^{-1}) with the hydrolysis process in 0.1% w/v, $NaHCO_3$ in methanol.

3.4. pH-rate profile of the alkaline hydrolysis of DF

The hydrolysis of esters undergoes specific base-catalysis, and consequently the observed apparent first-order rate constant is expected to vary with the hydroxide ion concentration [9]:

(1) $K_{obs} = K_{OH}[^{-}OH]$

where K_{OH} is the rate constant for specific base-catalysis and:
$\log K_{obs} = \log K_{OH} + \log [^{-}OH]$

(2) i.e., $\log K_{obs} = \log K_{OH} + \log k_w + pH$

where k_w is the ion product of water.

This indicates that log K_{obs} should increase linearly with increase of pH on the alkaline side giving slope of +1.0. Fig. 3 shows a plot of log K_{obs} vs. pH at pHs > 7.4. The linear

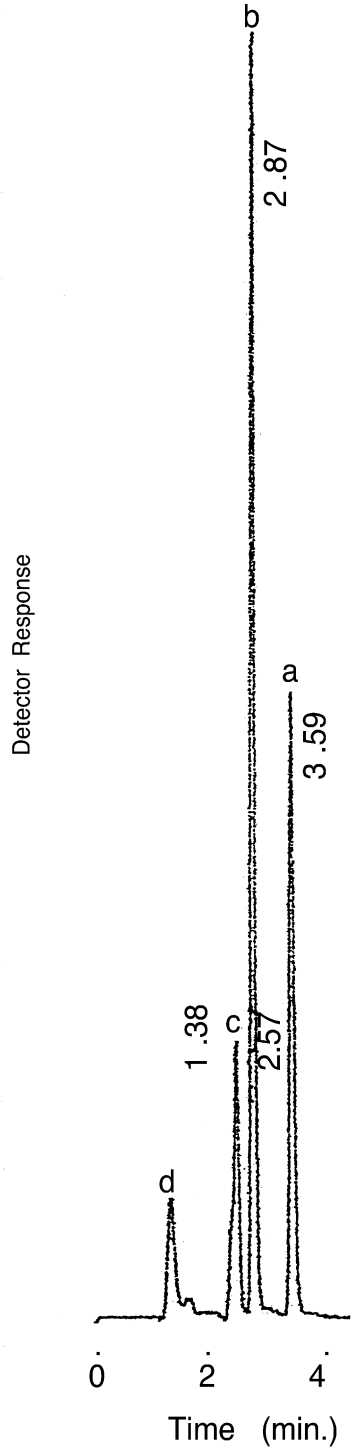


Fig. 5. Specimen chromatogram of the remaining drug in presence of its hydrolysis products in 0.1% w/v, $NaHCO_3$ in methanol: (a) DF intact drug; (b) methylfuroate; (c) diloxanide and (d) furoic acid.

plot gave a slope with a value ≅1.0, suggesting a first-order dependence of K on [$^{-}$OH]. This is clearly evident from the results of Table 2 which point out that the magnitude of the observed rate constant (K_{obs}) increased rapidly at higher pH values and it is also reflected by the short $t_{1/2}$ values obtained at these media. At pHs > 10, fast hydrolysis can take place even at room temperature. This observation was confirmed

by the fact that NaOH solutions caused immediate and complete hydrolysis of the drug compared to solutions of $NaHCO_3$, which required either a longer time to achieve complete hydrolysis at room temperature, or heating the solution at elevated temperatures.

3.5. Rate-controlled alkaline hydrolysis of the drug with $NaHCO_3$

A study was conducted using the weak base, $NaHCO_3$, to obtain a rate-controlled hydrolysis reaction. This goal was achieved by the addition of 2.5 ml of 1% (w/v) $NaHCO_3$ solution to a solution of DF (24 μg ml^{-1}) contained in 25-ml volumetric flasks and completing to volume with methanol. The hydrolysis of the drug was followed by both the UV spectroscopy and the HPLC methods. The follow up of the hydrolysis of DF with the UV method conducted with time revealed a decrease of the intact drug absorbance intensity with time coupled with change of λ_{max}. An equilibrium state seems to be reached after about 20 min, beyond which an increase in absorbance occurred, indicating the existence in the system of more hydrolytic products other than the intact drug. Fig. 4 shows the pattern of the change in UV absorption during the hydrolysis process. These UV results emphasize that the absorbance measurements at a specific wavelength may not be adequate for these types of determination if the degradation products also absorb in the same region. This is confirmed by the resolved hydrolysis products of the same alkaline methanol solution monitored by the HPLC method (Fig. 5). Peak d was identified as furoic acid (RT = 1.38 min) based on the RT value observed for authentic furoic acid and peak c was assigned to diloxanide (RT = 2.57 min) corresponding to that of the hydrolysis product isolated and characterized earlier in this study as diloxanide. Similarly, peak b was identified as methylfuroate (RT = 2.87 min) based on the RT value observed for the prepared methylfuroate. The hydrolysis kinetics of the alkaline methanolic solution as monitored by the HPLC method, gave a first-order reaction data for the plot of log [remaining drug] vs. time. The regression data obtained was: $y = -0.025t + 6.184$, $r = 0.999$. where y = log area of the [remaining drug] at time t in minutes.

The hydrolysis rate constant was found to be 5.756×10^{-2} min^{-1} and $t_{1/2}$ = 12.04 min. This high rate of hydrolysis was unexpected for solutions in $NaHCO_3$ at room temperature, as the expected pH of the solution should remain between 8 and 8.5. However, the pH of the final solution was found to be 10.6. This experiment was then repeated using the same concentration of the drug (24 μg ml^{-1}) in methanol but completing the solution with 1% w/v aqueous solution of $NaHCO_3$, i.e., the solution contained 12% methanol and 88% v/v $NaHCO_3$ (1% w/v). A considerably slow rate of hydrolysis was then observed; and the pH of this solution was found to be 8.7 (pH of 1% w/v $NaHCO_3$ in water was 8.3). Fig. 5 shows high level of methyl furoate, suggesting a driving effect by the transesterifiction on the DF degradation. Thus, the unexpected high rate of hydrolysis in $NaHCO_3$ solution (0.1% w/v in methanol) could be ascribed to the pH and the transesterification reaction. This aspect is of practical importance in view of the DF handling and storage.

3.6. Effect of temperature on rate of hydrolysis of DF

The influence of temperature (40, 50, 55 and 63 °C) on the hydrolysis rate was studied by the HPLC method using alkaline borate buffer of pH 8. The hydrolysis process followed first-order kinetics at all temperatures (Fig. 6). Using the Arrhenius plot (Fig. 7), the calculated energy of activation was 18.25 kcal degree.mol^{-1} which lies within the accepted limits for hydrolysis of esters (8–20 kcal degree.mol^{-1}) [9].

3.7. Effect of simulated gastric and intestinal fluids on the hydrolysis of DF

DF, being an ester, is considered as a pro-drug, that is predicted to pass the acidic gastric juice without hydrolysis

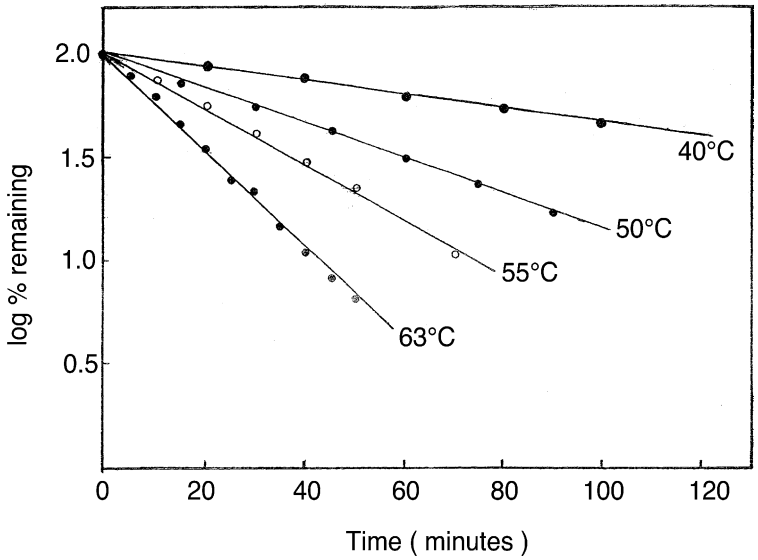


Fig. 6. First-order kinetics plots of degradation of DF in alkaline borate buffer, pH 8.0, at various temperatures.

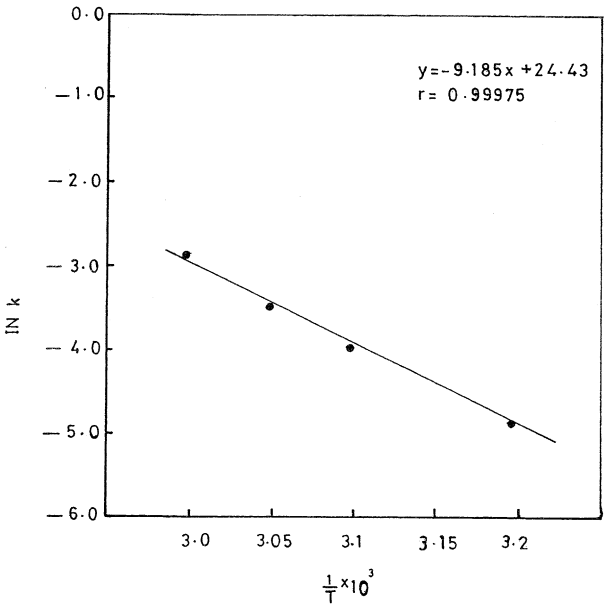


Fig. 7. Arrhenius plot for DF at pH 8.

and reaches the intestinal fluid where it undergoes a hydrolytic process, releasing the active drug, diloxanide [2]. In this study, we confirmed the stability of DF in simulated gastric fluid as no degradation products were detected by the HPLC method when the drug solution was incubated in the simulated gastric fluid at 37 °C for more than 2 h. On the other hand, the incubation of DF in the simulated intestinal fluid resulted in a pseudo first-order hydrolysis reaction as monitored by the HPLC method. Linear regression analysis of the data gave the following formula: $P = -1.514 \times 10^{-2}t + 0.081$, $r = 0.9987$ with K value of 3.49×10^{-2} min^{-1} and $t_{1/2} = 20$ min; where P is the log peak area ratio of the [remaining drug] relative to the internal standard at time t in minutes. The pH of the intestinal fluid is 7.4 in which the drug proved to be stable based on the pH-rate profile studies presented in this work. To confirm the effect of the enzymes incorporated into the intestinal fluid, two experiments were conducted as blanks, one using a solution of DF containing only phosphate buffer of pH 7.4 and another consisting of a solution of DF containing a boiled intestinal fluid (to destroy the enzyme); both experiments showed slight hydrolysis, indicating that the process is mainly achieved through an enzyme-catalyzed reaction.

4. Conclusion

The results of this study showed that DF solution is degraded via a hydrolysis process, which appears to be temperature and [$^-$OH] dependent at constant methanol content. Three major degradation products were obtained: two were identified as diloxanide (D) and furoic acid (F), which were the results of the predicted hydrolysis of the ester group. The third product was identified as methylfuroate formed as a result of transesterification.

The TLC procedure developed in this study was successfully used to separate the alkaline degradation products from the parent compound and is recommended to substitute the official BP TLC method, which failed to accomplish such separation.

The results of the stability study of DF in simulated gastric and intestinal fluids confirmed its hydrolysis in the intestines to its active form diloxanide and furoic acid. It is also established that the hydrolytic reaction is an enzyme-catalyzed process that takes place in the intestines.

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