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Kinetics of hydrolysis of fetindomide (NSC-373965), bis-*N*,*N*′-phenylalanyloxymethyl prodrug of mitindomide (NSC-284356); an unexpected catalytic effect of generated formaldehyde

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Summary

The degradation kinetics of fetindomide, a potential prodrug of mitindomide, was studied in aqueous solution at 25 °C. The hydrolysis of dilute fetindomide solutions maintained between pH 2.5 and 4.5 followed apparent first-order kinetics over several half-lives. At higher pH values (5.0–8.5) the kinetics of degradation were more complex even in dilute solutions. Above pH 8.5, the degradation of fetindomide was too rapid to measure by HPLC without quenching. In addition to these observations, it was found that various buffers showed general base catalysis. The degradation of fetindomide results in the production of formaldehyde which was found to accelerate the degradation of fetindomide. Analysis of the kinetic data obtained from experiments in which formaldehyde was added to aqueous fetindomide solutions, indicated that fetindomide reacts with formaldehyde to form an unstable intermediate, possibly a carbinolamine. It is proposed that the intermediate degrades more rapidly than fetindomide which would account for the non-first-order kinetics seen at pH values greater than 4.5. HPLC chromatograms indicated that the degradation products of fetindomide, irrespective of whether formaldehyde was added, were the same under all conditions.

Introduction

Fetindomide (NSC-373965, bis-*N*,*N*′-phenylalanyloxymethylmitindomide dihydrochloride hemihydrate) is a potential prodrug of mitindomide (NSC-284356) with improved aqueous solubility (Haugwitz et al., 1987). Although mitindomide itself has been shown to be active against a variety of tumor models (Narayanan, 1983), it is poorly soluble in water and in most pharmaceutically acceptable solvents. Although the disodium salt of mitindomide has adequate solubility in water (Vishnuvajjala and Cradock, 1986), an unstable solution of unacceptably high pH results when a freeze dried formulation is

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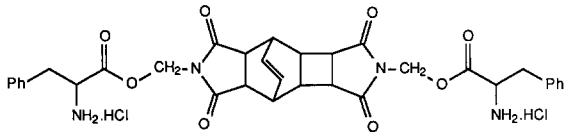
reconstituted with water to a concentration of 20 mg/ml.



Fetindomide

Fetindomide was synthesized (Haugwitz et al., 1987) in an attempt to improve the solubility of the parent compound so that a pharmaceutically acceptable formulation could be developed. The prodrug was designed so that the mitindomide is released in vivo by the loss of two molecules of phenylalanine and formaldehyde. Previous studies in our laboratory (Umprayn et al., 1987) resulted in the development of a stability-indicating assay for fetindomide using HPLC. These studies (Umprayn et al., 1987) showed that the release of mitindomide from the prodrug in aqueous solution occurs via two parallel pathways each involving two isomeric intermediates of mono-*N*-phenylalanyloxymethylmitindomide (Scheme 1). The purpose of the present study was to investigate the aqueous stability of fetindomide and to determine whether phenylalanine and formaldehyde, decomposition products of fetindomide, influence the rate of degradation since apparent product catalysis kinetics were observed in the hydrolysis of fetindomide at pH values greater than 4.5.

A number of prodrugs have been described in which an aldehyde (generally formaldehyde) is released during the reconversion to the active drug substances. In fact, Johansen et al. (1983) monitored the hydrolysis of such compounds by measuring the amount of aldehyde released. According to Johansen et al. (1983), the aldehyde is generally produced in stoichiometric amounts. However, the yield of aldehyde can be decreased when the aldehyde released reacts with an amino group, which can occur, for example, during the conversion of pivampicillin to ampicillin. In the case of pivampicillin, the reaction of ampicillin and formaldehyde produces an unstable Schiff base which rearranges to a 4-imidazolidine (Johansen et al., 1983; Bundgaard and Klixbull, 1985).

Experimental

Apparatus

High-performance liquid chromatography (HPLC) used an Altex 110A pump, a Rheodyne 7125 injector (20 or 50 μl loop), a Waters 440 UV detector (240 nm) and a Shimadzu C-R3A integrator. A 150×4.6 mm reversed-phase column packed with 5 μm C_8MOS Hypersil was used. The column was protected with a 60×4.6 mm pre-column also packed with C_8 MOS Hypersil.

Chemicals

Fetindomide (NSC-373965) and mitindomide (NSC-284356) were used as received from the National Cancer Institute (Bethesda, MD). The L-phenylalanine and formaldehyde (37% aqueous solution) were obtained from J.T. Baker (Phillipsburg, NJ) and Sigma (St. Louis, MO), respectively, and were used without further purification. The HPLC grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ). All the other chemicals or solvents used were of reagent grade and were obtained from various standard sources.

Kinetic measurements

All the kinetic studies were performed, at least in duplicate, in aqueous buffer solutions at 25.0 ± 0.1° C. The buffers used were acetate (pH 3.5, 4.5 and 5.5), citrate (pH 4.0), phosphate (pH 6.5, 7.0 and 7.5) and Tris (pH 8.5). The total buffer concentrations were 0.05 M except in experiments where the effects of varying buffer concentration were studied specifically. The ionic strength of the buffer solutions was adjusted to 0.5 with sodium chloride. The rates of degradation of fetindomide in the presence or absence of phenylalanine (6.4×10^{-4} M) or formaldehyde (4×10^{-5}–3.2×10^{-3} M) were determined using the stability indicating assay described previously (Umprayn et al., 1987). The mobile phase was 5% acetonitrile in a 0.1 M acetate buffer containing 0.04 M tetrabutyl ammonium hydrogen sulfate, adjusted to pH 2.0 with sodium hydroxide. The elution was at ambient

temperature at a rate of 2.0 ml/min. Quantification was by peak area (Shimadzu C-R3A chromatopac, obtained from Delta Instruments, Overland Park, KS).

The reactions were initiated by adding 400 μl of a stock solution of fetindomide hydrochloride in dimethylsulfoxide (DMSO) to buffer solutions producing an initial concentration of 8×10^{-5}–3.2×10^{-4} M fetindomide. The final DMSO concentration was maintained at 4% throughout. The reaction solutions were thermostated at 25.0 $\pm$ 0.1° C, aliquots were removed at suitable intervals and analyzed for fetindomide. The appearance of two intermediates, mitindomide, and phenylalanine was also noted. No other significant peaks were observed in the chromatograms.

Results and discussion

Kinetics of hydrolysis of fetindomide

The kinetics of hydrolysis of fetindomide were studied in aqueous solution at 25° C over a pH range of 1–8.5. The degradation of fetindomide at pH values < 4.5 displayed strict first-order kinetics for several half-lives, while in the pH range 5.5–7.0, the loss of fetindomide showed apparent first-order kinetics for only the first half-life. The total time profile at higher pH values was suggestive of product catalysis kinetics. Representative plots of the degradation of fetindomide at pH 3.5 and 5.5 are shown in Figs. 1 and 2, respectively. The pseudo-first-order rate constants (k_{obs}) are given in Table 1. For these reactions in which first-order kinetics were not observed at later time points, i.e. above pH 5.5, k_{obs} was calculated from the initial slopes of the plots of the natural logarithm of fetindomide concentration or HPLC peak areas against time. In all cases, phenylalanine, mitindomide, and two other products attributed to the isomeric intermediates, mono-*N*-phenylalanyloxymethylmitindomide (II and III in Scheme 1) were observed by HPLC in degraded solutions of fetindomide, as reported previously (Umprayn et al., 1987). Phenylalanine and mitindomide had retention times identical to those of authentic samples.

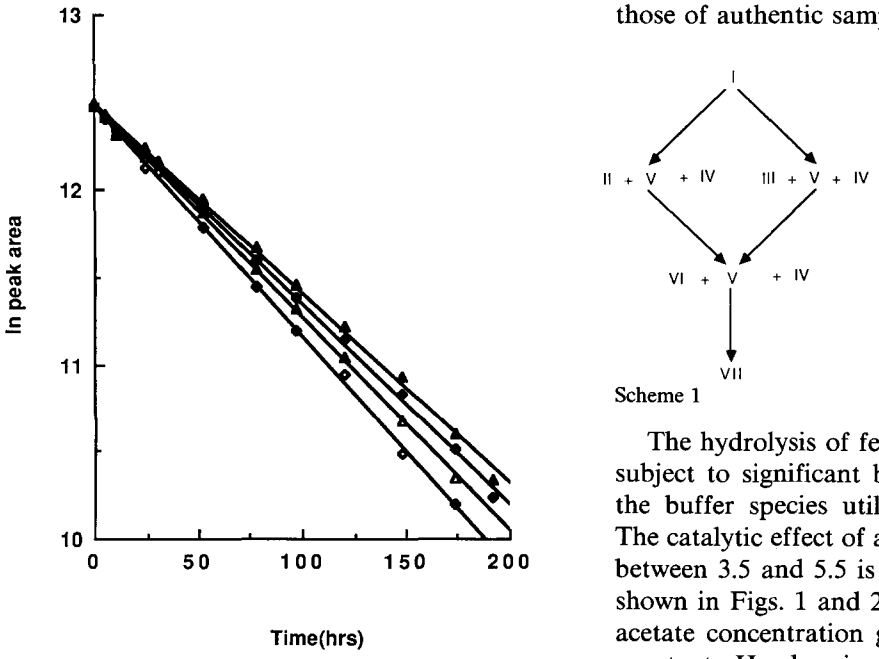
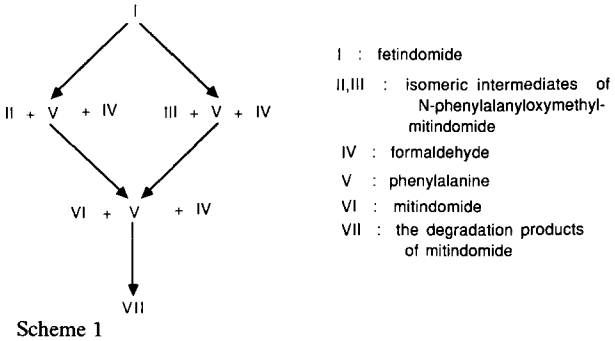


Fig. 1. Time-dependent loss of fetindomide in acetate buffers of different concentrations at pH 3.5 (25° C, μ = 0.5). ▲, 0.025 M acetate buffer; ◆, 0.05 M acetate buffer; △, 0.075 M acetate buffer; ◇, 0.1 M acetate buffer.



Scheme 1

The hydrolysis of fetindomide was found to be subject to significant buffer catalysis by most of the buffer species utilized in the present study. The catalytic effect of acetate buffers at pH values between 3.5 and 5.5 is readily seen from the data shown in Figs. 1 and 2. Plots of k_{obs} versus total acetate concentration gave linear relationships at constant pH values in all cases (Fig. 3). Extrapolation of such plots, for all the buffer systems, to zero concentration provided the buffer-indepen-

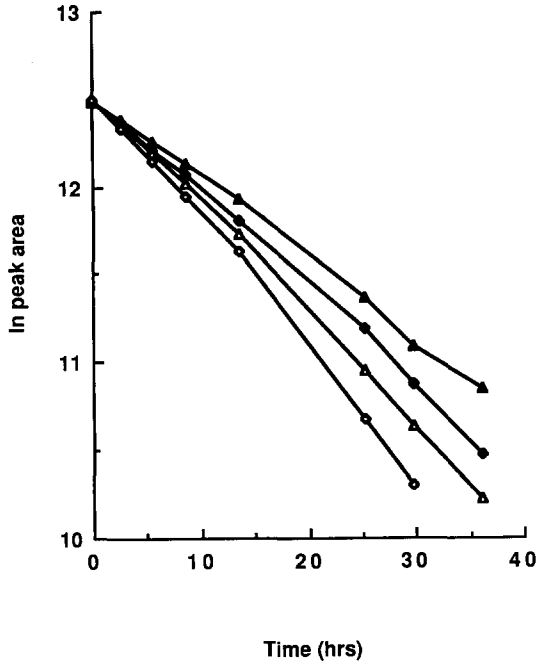


Fig. 2. Time-dependent loss of fetindimide in acetate buffers of different concentrations at pH 5.5 (25 °C, $\mu = 0.5$). ▲, 0.025 M acetate buffer; ◆, 0.05 M acetate buffer; △, 0.075 M acetate buffer; ◇, 0.1 M acetate buffer.

dent apparent first-order rate constants, k'_{obs}. Analysis of the data shown in Fig. 3 by plotting the slopes of the buffer plots against the fraction of acetate in its acid form, f_{AcOH}, provided a second-order catalytic rate constant of 3.35×10^{-1} $M^{-1} \cdot h^{-1}$ for acetate ion and no significant catalysis by acetic acid (Fig. 4). The reaction, therefore, is more susceptible to general base catalysis than general acid catalysis in this pH range. Phosphate and citrate buffers also affected the rate of degradation of fetindomide; however, no attempt was made to evaluate the catalytic constants for these buffer components because of the complication of the non-linear kinetics.

The pH–rate profile for the hydrolysis of fetindomide is shown in Fig. 5. The profile shape is similar to that of a series of aminoacyl esters of 3-hydroxymethylphenytoin (Varia et al., 1984). The profile (solid line in Fig. 5) can be adequately described by Scheme 2 and Eqn. 1

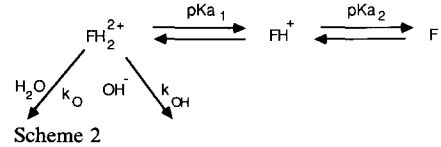
$$k'_{obs} = k_0 f_{FH_2} + k_{OH}[OH] f_{FH_2} \quad (1)$$

TABLE 1

A summary of apparent first-order rate constant (k_{obs}) for the degradation of fetindomide in various buffer solutions ($\mu = 0.5$ with NaCl) at 25 °C

pH	Buffer	k_{obs} (h^{-1})	$t_{1/2}$ (h)
1.0	0.100 M HCl	8.20×10^{-3}	84.6
1.5	0.032 M HCl	8.00×10^{-3}	86.6
2.0	0.010 M HCl	8.31×10^{-3}	85.3
2.5	0.0032 M HCl	8.39×10^{-3}	82.7
3.5	0.025 M acetate	1.09×10^{-2}	63.6
3.5	0.050 M acetate	1.14×10^{-2}	60.8
3.5	0.075 M acetate	1.22×10^{-2}	56.8
3.5	0.100 M acetate	1.32×10^{-2}	52.5
4.0	0.010 M citrate	1.29×10^{-2}	54.0
4.0	0.025 M citrate	1.56×10^{-2}	44.4
4.0	0.035 M citrate	2.03×10^{-2}	34.1
4.0	0.050 M citrate	2.53×10^{-2}	27.4
4.5	0.025 M acetate	1.66×10^{-2}	41.7
4.5	0.050 M acetate	2.15×10^{-2}	32.2
4.5	0.075 M acetate	2.55×10^{-2}	27.2
4.5	0.100 M acetate	3.02×10^{-2}	23.0
5.5	0.025 M acetate	4.09×10^{-2}	17.0
5.5	0.050 M acetate	5.04×10^{-2}	13.8
5.5	0.075 M acetate	5.63×10^{-2}	12.3
5.5	0.100 M acetate	6.42×10^{-2}	10.8
6.5	0.025 M phosphate	3.42×10^{-1}	2.2
6.5	0.050 M phosphate	4.20×10^{-1}	1.7
6.5	0.075 M phosphate	4.62×10^{-1}	1.5
6.5	0.100 M phosphate	6.60×10^{-1}	1.1
7.0	0.050 M phosphate	8.58×10^{-1}	0.81
7.5	0.050 M phosphate	1.28	0.54
8.5	0.050 M Tris	2.50	0.28

where f_{FH_2} is the fraction of fetindomide present in its diprotonated form, k_0 is a spontaneous water catalyzed hydrolysis constant, and k_{OH} is the hydroxide ion catalysis rate constant. The solid line in Fig. 5 was drawn assuming values of 8.12×10^{-3} h^{-1} for k_0, 7.2×10^6 $M^{-1} \cdot h^{-1}$ for k_{OH}, and a value of unity for f_{FH_2}. From the profile the deviation of the experimental points from the solid line seen at pH > 7 probably indicates the approach to the pK_{a_1} and pK_{a_2} of fetindomide, i.e. f_{FH_2} becomes less than unity.



Scheme 2

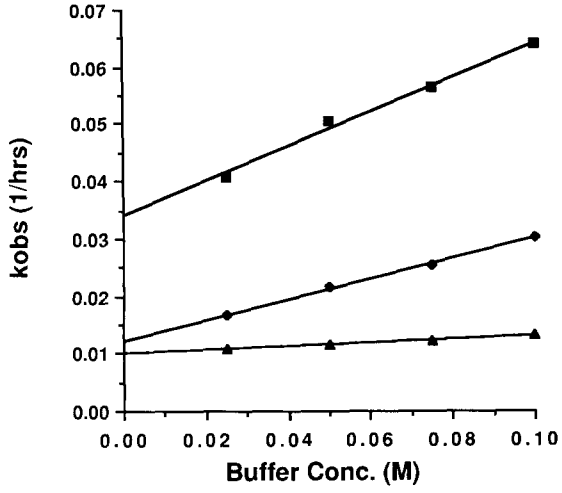


Fig. 3. The effect of acetate buffer concentration on the observed rate constants for the degradation of fetindomide at various pH values (25 °C, $\mu = 0.5$). ▲, pH 3.5 acetate buffer; ◆, pH 4.5 acetate buffer; ■, pH 5.5 acetate buffer.

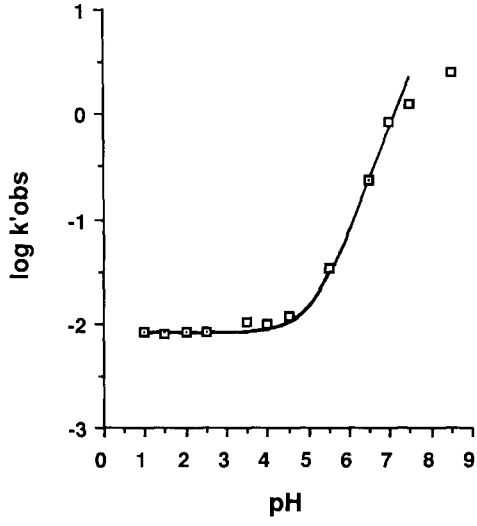


Fig. 5. pH–rate profile for the degradation of fetindomide in aqueous solution at 25 °C ($\mu = 0.5$).

Normally it is possible to estimate the dissociation constants from the shape of the pH–rate profile; however, in the present case there is insufficient data, as well as confidence in the data, at pH values approaching the pK_a values of fetindomide. Because of solubility and stability limitations, it was also not possible to estimate pK_{a_1} and pK_{a_2} using other standard techniques. Qualitatively, it appears that fetindomide has a pK_a of ≈ 7.5.

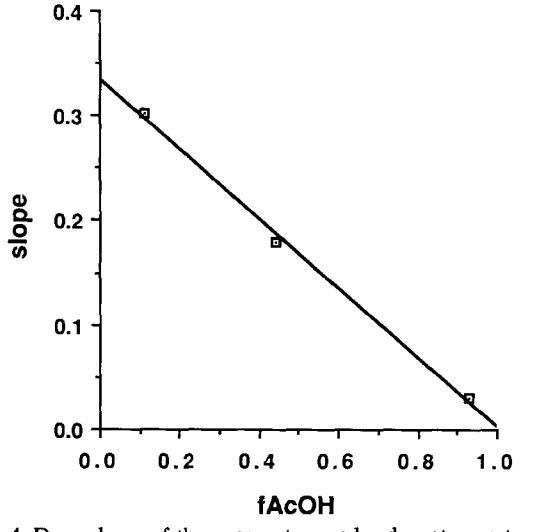


Fig. 4. Dependence of the apparent second order rate constant for acetate buffer-catalyzed degradation of fetindomide at 25 °C on the fraction of acetic acid in the buffers. See Fig. 3 for other conditions.

Reaction of fetindomide with its own decomposition products

The degradation of fetindomide in the pH range 5.5–7.0 shows apparent first-order kinetics for only the first half-life. Also the loss of fetindomide was dependent on its initial concentration (Fig. 6). That is, at low fetindomide concentration better adherence to first-order kinetics was observed. This behavior was consistent with fetindomide decomposition being catalyzed by one of its own decomposition products. To identify the reason for the change in apparent reaction order, the reactivity of fetindomide in the presence of its decomposition products, phenylalanine and formaldehyde, was studied in 0.05 M acetate buffer (pH 5.5) at 25 °C. As seen in Fig. 7, phenylalanine had no effect on the degradation of fetindomide, while formaldehyde dramatically accelerated the loss of fetindomide. The number of peaks and their retention times seen by HPLC were identical in both the presence and absence of phenylalanine and formaldehyde. This suggested that, in the presence of added formaldehyde, no new degradation products were produced in substantial

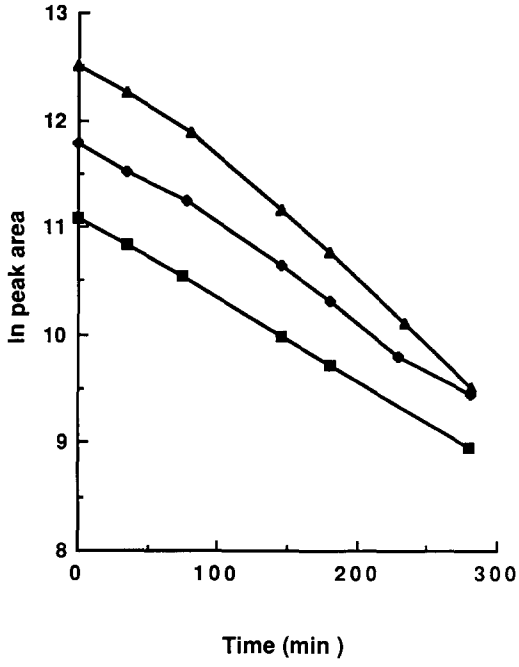


Fig. 6. Time-dependent loss of fetindomide with different initial concentrations in 0.075 M acetate buffer (pH 6.5, μ = 0.5) at 25 °C: (■), 50 μg/ml fetindomide; (◆), 100 μg/ml fetindomide; (▲), 200 μg/ml fetindomide.

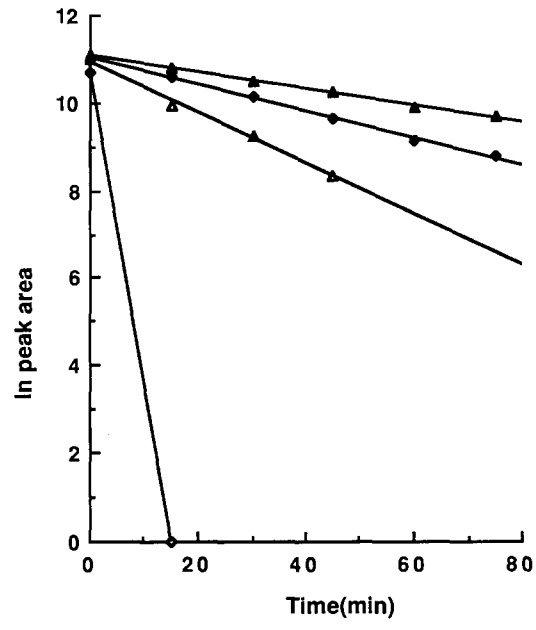


Fig. 8. Time-dependent change in ln(peak area) of fetindomide (0.08 mM) with different initial concentration of formaldehyde at pH 5.5, 25 °C. ▲, 0.02 mM formaldehyde; ◆, 0.04 mM formaldehyde; △, 0.08 mM formaldehyde; ◇, 0.5 mM formaldehyde.

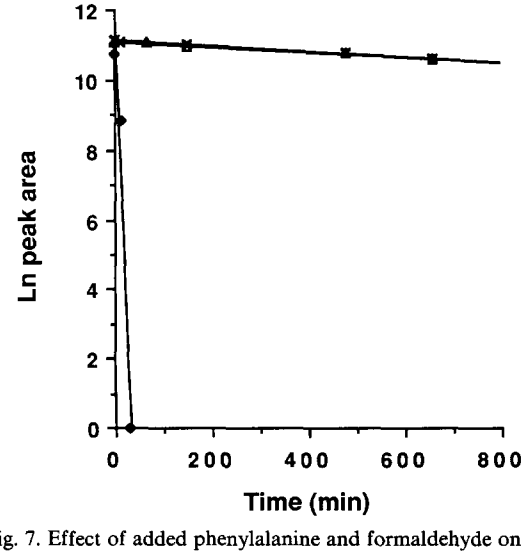


Fig. 7. Effect of added phenylalanine and formaldehyde on the degradation of fetindomide at pH 5.5, in 0.05 M acetate buffer 25 °C (μ = 0.5). ×, 0.08 mM fetindomide alone; △, 0.08 mM fetindomide plus 0.64 mM-phenylalanine; ◆, 0.08 mM fetindomide plus 0.64 mM formaldehyde.

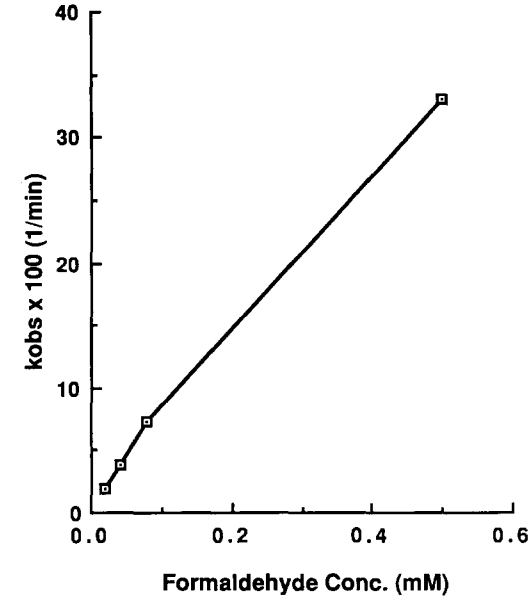
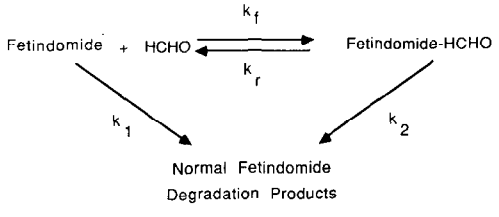


Fig. 9. Effect of formaldehyde concentration on the observed first-order rate constant of the degradation of fetindomide (0.08 mM) at pH 5.5, 25 °C.

amounts. A representative example of the time course of fetindomide loss in the presence of varying formaldehyde concentration at pH 5.5 is shown in Fig. 8. With formaldehyde in excess, fetindomide degradation followed pseudo-first-order kinetics. If formaldehyde was simply acting as a catalytic species, linear plots of k_{obs} versus added formaldehyde concentration should be observed.

Figs. 9 and 10 show the effect of initial added formaldehyde concentration on the degradation of fetindomide (0.08 mM) at pH 5.5 and 3.5 at 25 °C. It can be seen that k_{obs} is not linearly related to formaldehyde concentration. This suggests a mechanism whereby fetindomide reacts reversibly with formaldehyde to produce an unstable intermediate, as described by Scheme 3, which then rapidly degrades to the normal fetindomide degradation products.



Scheme 3

It is known that formaldehyde reacts with amines in aqueous solution to form carbinolamines and Schiff bases (Kallen and Jencks, 1966). The reaction between uracil and formaldehyde produces *N*-hydroxymethylated uracil and the rate of *N*-hydroxymethylation is dependent on the concentration of formaldehyde (Bansal et al., 1981). In addition, ampicillin reacts with aldehydes reversibly to form an unstable intermediate which rearranges to a 4-imidazolinone (Johansen et al., 1983; Bundgaard and Klixbull, 1985). In this reaction, a Schiff base is considered to be the intermediate formed between the carbonyl compound and primary amine of ampicillin (Bundgaard and Klixbull, 1985). Another example of this type of reaction is that reported by Kallen (1971) who showed the formation of a thiazolidine from L-cysteine and formaldehyde. The mechanism of this reaction was believed to

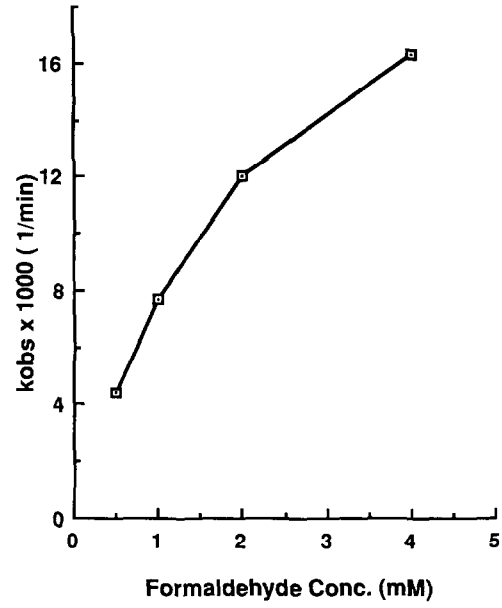


Fig. 10. Effect of formaldehyde concentration on the observed first-order rate constant of the degradation of fetindomide (0.08 mM) at pH 3.5, 25 °C.

involve both carbinolamine and Schiff base intermediates. The apparent first-order rate constants for thiazolidine formation increased linearly with increasing formaldehyde concentration at neutral or acidic pH values. However, in alkaline pH values and at higher formaldehyde concentration the rate of reaction became independent of formaldehyde concentration.

With and without formaldehyde, the decomposition products of fetindomide were identical when assessed from HPLC chromatograms. Therefore, the kinetics of fetindomide hydrolysis in the presence of formaldehyde appears to be reasonably represented by Scheme 3 where k_1 is equal to k_{obs} in the absence of endogenous or exogenous formaldehyde. Higuchi et al. (1967) have presented a similar scheme for reversible formation and hydrolysis of phthaloyl monophosphates in aqueous solution. According to Scheme 3, the rate of degradation of fetindomide is given by:

$$-d[F]_T/dt = k_1[F] + k_2[Int] \quad (2)$$

where $-d[F]_T/dt$ is the overall loss of fetindomide

species, [F] and [Int] refer to the concentration of fetindomide, state of ionization not defined, and the unknown intermediate respectively, and k_1 and k_2 represent the apparent first-order constants for the degradation of F and Int, respectively. Assuming that the equilibrium reaction defined by k_f and k_r in Scheme 3 is fast relative to the other rate processes, it follows that:

$$k_f/k_r = [\text{Int}]/[\text{F}][\text{HCHO}] = K \quad (3)$$

and that

$$-\text{d}[\text{F}]_T/\text{d}t = (k_1/\{1 + K[\text{HCHO}]\} + k_2 K[\text{HCHO}] /\{1 + K[\text{HCHO}]\}) \times ([\text{F}] + [\text{Int}]) \quad (4)$$

Since Int is probably present only in small quantities under the conditions employed here, it follows that the overall loss of fetindomide in the presence of excess formaldehyde follows first-order kinetics with an observed rate constant, k_{obs}, defined by Eq. 5.

$$k_{obs} = k_1/\{1 + K[\text{HCHO}]\} + k_2 K[\text{HCHO}] /\{1 + K[\text{HCHO}]\} \quad (5)$$

Rearrangement of Eq. 5 gives

$$k_1/(k_{obs} - k_1) = [k_1/K(k_2 - k_1)] \times 1/[\text{HCHO}] + k_1/(k_2 - k_1) \quad (6)$$

According to Eqn. 6, a plot of $k_1/(k_{obs} - k_1)$ against 1/[HCHO] should be linear with an intercept equal to $k_1/(k_2 - k_1)$ and the intercept at the abscissa equal to negative K. This double-reciprocal plot has also been described previously by Connors and Mollica (1966) for the analysis of kinetic data involving 1 : 1 complexation. Representative plots are shown in Figs. 11 and 12. These plots allow the estimation of K and k_2. It should be noted that the constant K represents an observed equilibrium constant that could be pH-dependent in that it will be affected by the state of ionization of fetindomide. Formaldehyde exists in aqueous solution largely in a hydrated state. In the pH range in question, the state of hydration of formaldehyde is pH-independent. Other aldehydes, such as acetaldehyde, exist primarily in their non-hydrated state. Since fetindomide is

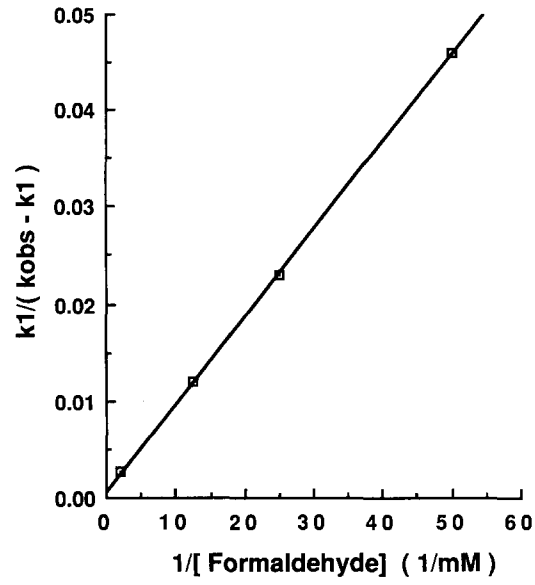


Fig. 11. Double-reciprocal plot of the rate constants for the degradation of fetindomide (0.08 mM) at pH 5.5, 25 ° C against formaldehyde concentration according to Eqn. 6.

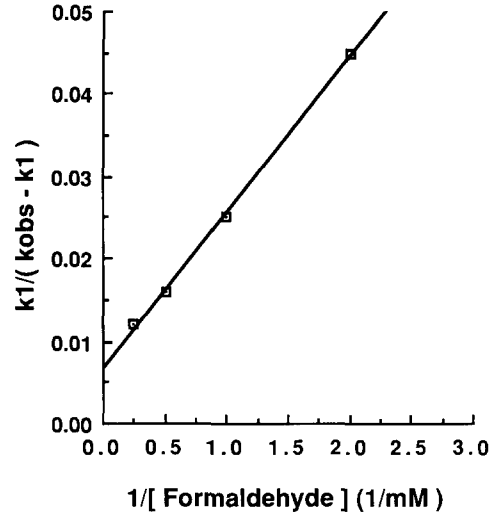


Fig. 12. Double-reciprocal plot of the rate constants for the degradation of fetindomide (0.08 mM) at pH 3.5, 25 ° C against formaldehyde concentration according to Eqn. 6.

TABLE 2

Kinetic parameters for fetindomide degradation in the presence of formaldehyde according to Scheme 3, as a function of pH in 0.05 M acetate buffer, 25 °C ($\mu = 0.5$)

	k_1	k_2	K	k_2/k_1
pH 3.5	1.10×10^{-2}	1.71	0.35	150
pH 4.0	1.19×10^{-2}	4.24	0.37	356
pH 4.5	2.15×10^{-2}	21.6	0.20	1004
pH 5.0	3.01×10^{-2}	25.4	0.61	843
pH 5.5	5.04×10^{-2}	75.3	0.74	1974

likely to only add to the non-hydrated formaldeyde, K will also depend on the hydration state of the aldehyde. Therefore, in comparing the catalytic effects of various aldehydes, (ongoing studies), the hydration state of the various aldehydes will have to be considered.

Scheme 3 was selected as the simplest system that satisfies the experimental observations and the kinetic parameters obtained at different pH values were given in Table 2. Semilog plots of k_2 and K versus pH are given in Fig. 13. Within the limitations imposed by the precision of the data, a number of conclusions can be drawn from the data in Table 2 and Fig. 13. The equilibrium constant K appears to be relatively insensitive to pH. A priori one might expect K to increase with increasing pH since formation of the intermediate, probably the carbinolamine, is likely to occur by reaction of the free base form of fetindomide with formaldehyde. However, breakdown of the intermediate, given by the rate constant k_r, as well as the states of ionization of various intermediates would also be pH-sensitive possibly cancelling out the expected pH effects on k_f. The pH-dependency of k_2, ignoring the pH 4.5 data point, suggests that k_2 is base-sensitive. The solid line in Fig. 13 is the linear regression line for log k_2 versus pH ($r = 0.998$, slope = 0.81), ignoring the pH 4.5 data point. The slope of 0.81 for this data suggests that the reactivity of the intermediate, as defined by Scheme 3, possibly follows specific base catalysis.

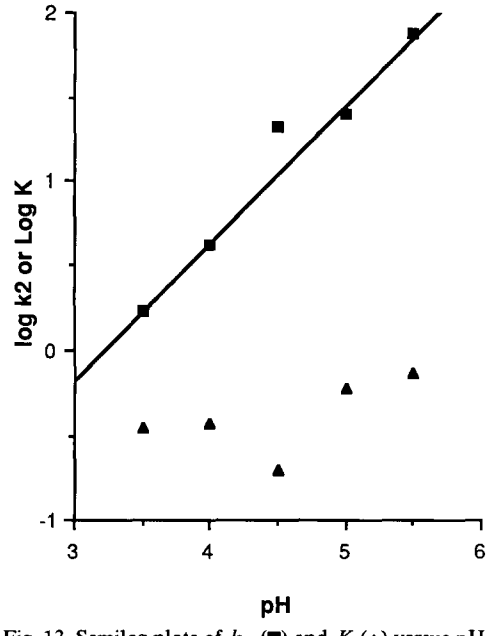
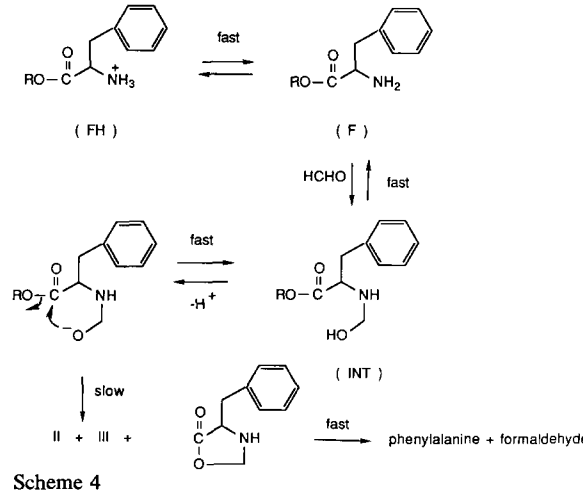


Fig. 13. Semilog plots of k_2 (■) and K (▲) versus pH.

Based on the data presented above, the reaction of fetindomide in the presence of formaldehyde appears to be described by Scheme 4, where the actual reaction, defined by k_2, involves an intramolecular attack of the carbinolamine anion on the ester function to produce an unstable product. Instability of the cyclic product formed between formaldehyde and phenylalanine is assumed since no additional peaks were seen by HPLC, and in those cases where formaldehyde was present in catalytic quantities, the formaldehyde must have



Scheme 4

been regenerated to account for the observed kinetics. The proposed mechanism would account for both the large acceleration of fetindomide hydrolysis by formaldehyde (compare k_2, and intramolecular nucleophilic mechanism, to k_1, an intermolecular nucleophilic mechanism) and the apparent pH-dependency of k_2. The fraction of the carbinolamine in its anionic form will increase by an order of magnitude for each unit increase in pH. To our knowledge, there is no precedent for the intramolecular reaction pathway outlined in Scheme 4, although formaldehyde addition to amines has been clearly implicated in other rearrangement reactions (Jencks, 1969), and it is similar to the mechanism proposed by Kovach et al. (1975) for the carbon dioxide-catalyzed hydrolysis of amino acid esters of acetaminophen.

Formaldehyde is often used as a spacer group in preparing various types of prodrugs. In many cases, formaldehyde is released during the reconversion to parent drug. The results presented here suggest that care should be taken in the design of prodrugs using formaldehyde, if the formed formaldehyde is capable of undergoing similar reactions to those seen in the present study.

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