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Kinetics of degradation of methotrexate in aqueous solution

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Summary

The chemical stability of methotrexate (I) in aqueous solution protected from light was studied utilizing an HPLC procedure. The investigation of the kinetics of degradation took place in aqueous buffer solutions at 85°C over the pH-range of 0–12. The pH–rate profile obtained from first-order kinetic plots showed maximum stability at about pH 7. An Arrhenius-type plot indicated a shelf-life at pH ~ 8.5 of about 4.5 years at 25°C.

At pH above 6.5 N^{10}-methyl-folic acid (II) was the only degradation product while in acidic solution several compounds are formed.

Introduction

The major pharmaceutical formulation of the antileukaemic agent, methotrexate (MTX), exists as a freeze-dried preparation to be reconstituted with water before use. For several reasons, among them potential health hazards for those handling the compound, a liquid formulation is desirable.

As only limited studies on the degradation of methotrexate are reported (Chatterji and Gallelli, 1978) the present study was undertaken to obtain detailed knowledge on the kinetics of degradation of methotrexate in aqueous solution as a function of

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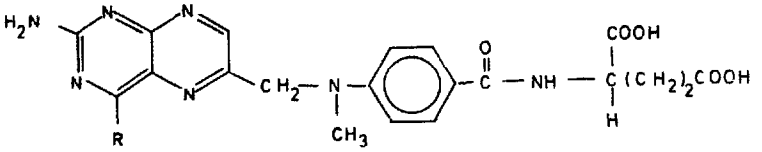
pH in the range of 0–12, nature of buffer substances and of temperature.



I R = NH_2

II R = OH

Materials and Methods

Chemicals and apparatus

Methotrexate, BP 80 (purity 99.5% calculated with reference to the dried substance), 4-amino-4-deoxy-N^{10}-methyl-pteroic acid and 2,4-diamino-6-hydroxymethyl-pteridine were supplied by Niels Clauson-Kaas, Chemical Research Laboratory, Farum, Denmark and were used as received. A sample of N^{10}-methyl-folic acid (purity 93% as is) was obtained from Lederle, U.S.A. All chemicals used were of analytical grade.

Ultraviolet and visible spectra were recorded using a Perkin-Elmer, model 552 spectrophotometer and 1 cm quartz cells. The pH measurements were made at the temperature of study using a Radiometer, type PHM 83 Autocal instrument. High-performance liquid chromatography (HPLC) was carried out on an instrument composed of a Waters Associates, model 6000 A pump, a Rheodyne, model 7125 injection valve with a 20 µl loop, a Perkin Elmer, model LC-15 detector operated at 254 nm, and a Pharmacia Electronics, model BD 41 recorder. The column used, 120 mm long and 4.5 mm i.d., was packed with Nucleosil 5 C_8.

Buffer solutions

The following buffer substances were used: pH < 2.4 hydrochloric acid; pH 2.4–3.7 phosphate; pH 3.8–5.8 acetate; pH 6.0–7.5 phosphate; pH 8.0–9.5 borate; pH 9.6–11.0 carbonate; and pH > 11 sodium hydroxide. The ionic strength of the solution was adjusted to 0.5 using potassium chloride, except where the concentration of hydrogen or hydroxide ions exceeded 0.5 M.

Preparation of N^{10}-methyl-folic acid

5.6 g of methotrexate, dissolved in 400 ml of a 1 M solution of sodium hydroxide, was heated to 85°C for 60 min, protected from light. After cooling pH of the solution was adjusted to 3–4 using a 5 M solution of hydrochloric acid. The yellow precipitate was filtered off, washed with water and dried at vacuo at 30°C for 24 h. The yield was 5.5 g. The compound contained 2.9% of water and exhibited IR and ^{13}C-mr spectra identical to those of an authentic sample.

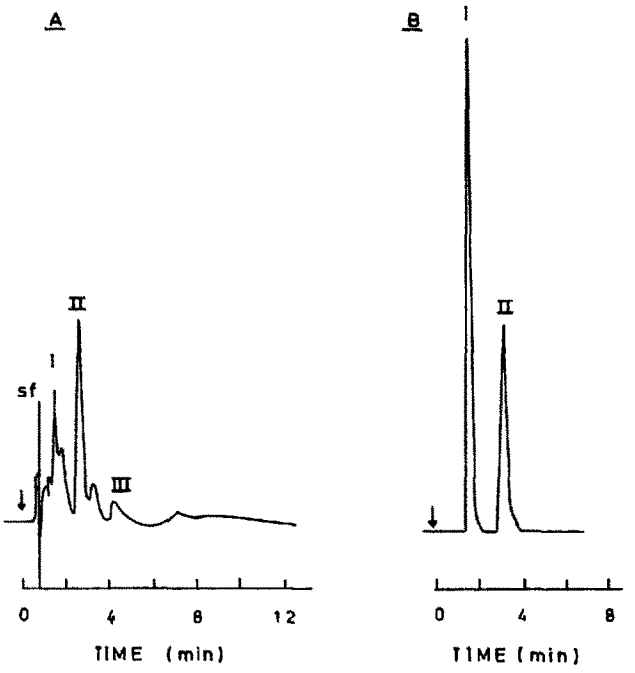


Fig. 1. HPLC-chromatograms of partially degraded solutions with about 30% residual methotrexate. For chromatographic conditions, see Experimental. A: 0.1 M hydrochloric acid. B: 0.1 M sodium hydroxide. s.f. = solvent front; I = N^{10}-methyl folic acid; II = methotrexate; III = 4-amino-4-deoxy-N^{10}-methyl-pteroic acid.

Kinetic measurements

Aqueous buffer solutions with a concentration of methotrexate at about 2×10^{-5} M were kept in screw-capped test tubes in a thermostated oil bath at 85 ± 0.5°C. Samples were withdrawn at appropriate intervals and analyzed for methotrexate using HPLC. The mobile phase used was 0.1 M phosphate buffer (pH 6.7)–methanol (8 : 2), and the flow was 2.0 ml/min. Peak height measurements were used and compared to those obtained with reference solutions. The relative standard deviation of the HPLC method is 0.6%. Typical chromatograms of partly degraded methotrexate solutions are shown in Fig. 1.

Determination of ionization constants

The macroscopic acidic dissociation constants for methotrexate were determined using the solubility technique and the spectroscopic technique (Albert and Serjeant, 1962).

When utilizing the solubility technique, excess methotrexate was equilibrated in the appropriate buffer solutions at 22°C. After a filtration of the samples the apparent solubility was calculated from HPLC analysis of the filtrate.

The spectroscopic determination of ionization constants was done at 22°C and using 258 and 308 nm as the analytical wavelengths.

Results and Discussion

The degradation of methotrexate was studied over the pH range of 0–12 at 85°C and ionic strength of 0.5. At a constant pH and temperature the overall loss of methotrexate displayed a first-order kinetic behaviour over more than 3 half-lives.

At pH above 6.5 the only degradation product observed was N^{10}-methyl-folic acid which was formed in quantitative yield. This is in accordance with Chatterji and Gallelli (1978). At pH below 6.5 the route of degradation was found to be more complex.

The observed pseudo-first-order rate constants (k_{obs}) for the overall degradation of methotrexate were calculated by a linear regression from the slope of linear plots of the logarithm of residual methotrexate against time. Typical first-order plots for the degradation at various pH-values are shown in Fig. 2 and values of k_{obs} for several buffer solutions are listed in Table 1.

General acid–base catalysis

The degradation of methotrexate was found to be subject to general acid–base catalysis by most of the buffer substances used in this study. The catalytic effect was determined by measuring the rate of degradation at constant pH, ionic strength and temperature, varying only the buffer concentration at a specific pH. This was done

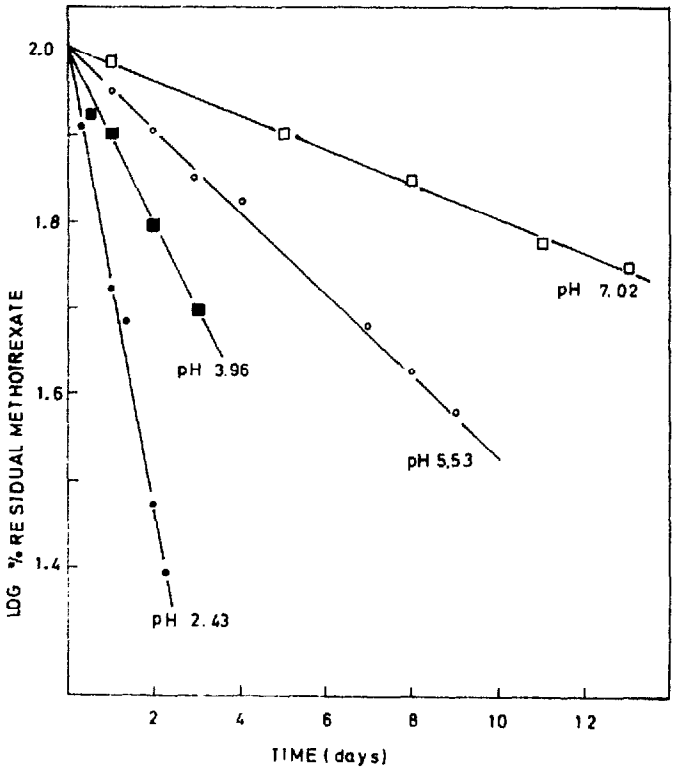


Fig. 2. Typical apparent first-order plots for the degradation of methotrexate in various aqueous buffer solutions at 85°C.

TABLE 1

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS (k_{obs}) FOR THE DEGRADATION OF METHOTREXATE AND TIMES FOR A 10% DEGRADATION ($t_{10\%}$) IN AQUEOUS BUFFER SOLUTIONS AND VARIOUS pH-VALUES (85°C, $\mu = 0.5$)

Buffer		pH	$k_{obs} \times 10^2$ ($days^{-1}$)	$t_{10\%}$ (h^{-1})
Hydrochloric acid	0.1 M	1.17	540	0.47
Phosphate	0.05 M	2.43	65	3.9
Acetate	0.05 M	3.96	22	11.5
Acetate	0.05 M	5.53	11	22.9
Phosphate	0.05 M	7.02	3.8	66.3
Borate	0.05 M	8.20	7.3	34.5
Carbonate	0.05 M	9.45	77	3.3
Sodium hydroxide	0.1 M	11.32	3260	0.08

at several pH-values within the effective range for each of the buffers studied.

The catalytic effect of acetate buffers in the pH-range of 3.9–5.6 on the rate of degradation of methotrexate is shown in Fig. 3.

Table 2 shows how the variation (0.05–0.2 M) in the concentration of phosphate

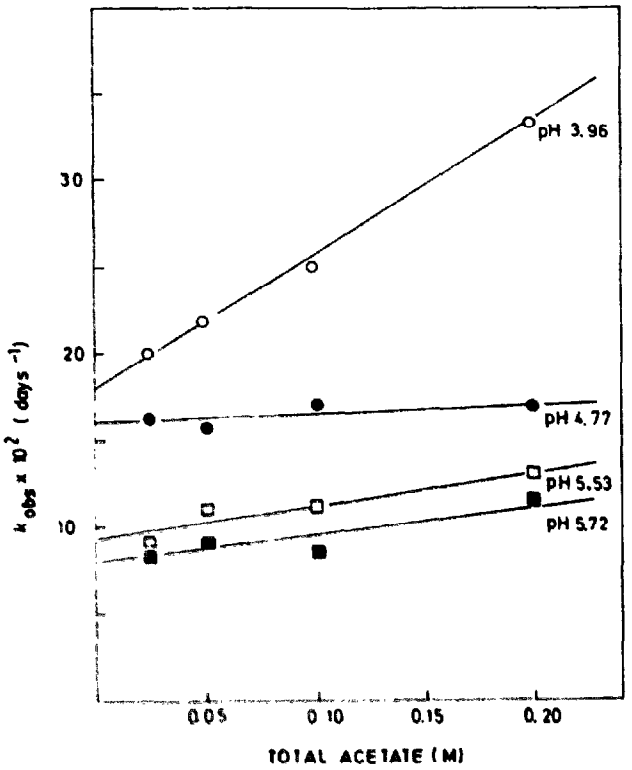


Fig. 3. The effect of acetate buffer concentration on the observed rate constants for the degradation of methotrexate at various pH-values (85°C, $\mu = 0.5$).

TABLE 2

OBSERVED PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF METHOTREXATE IN VARIOUS BUFFER SOLUTIONS ($\mu = 0.5$) AT 85°C

Buffer			pH	$k_{obs} \times 10^2$ (days^{-1})
Phosphate	0.2	M	2.48	66.5
Phosphate	0.1	M	2.48	60.7
Phosphate	0.05	M	2.48	65.1
Phosphate	0.025	M	2.48	64.1
Phosphate	0.2	M	7.02	5.7
Phosphate	0.1	M	7.02	4.2
Phosphate	0.05	M	7.02	3.8
Borate	0.2	M	8.20	7.3
Borate	0.1	M	8.20	6.2
Borate	0.05	M	8.20	5.1
Borate	0.025	M	8.20	4.9

and borate buffers used to maintain a constant pH influenced the degradation of methotrexate at 85°C.

A catalysis of the degradation of methotrexate was also found with a carbonate buffer. But whereas the acetate, phosphate and borate catalysis increased linearly with increasing catalyst concentration, a non-linear variation of rate with increasing carbonate buffer concentration was observed (Fig. 4).

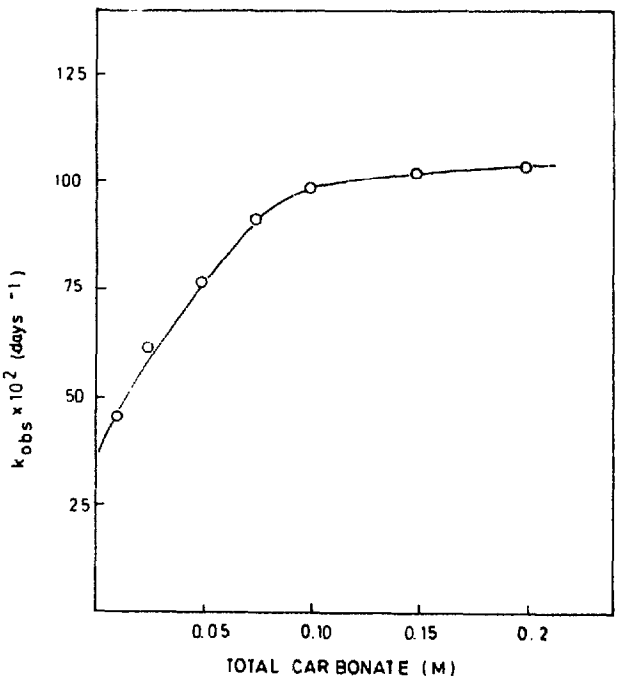


Fig. 4. The effect of carbonate on the pseudo-first-order rate constant for a degradation at 85°C ($\mu = 0.5$).

TABLE 3

THE SOLUBILITY OF METHOTREXATE IN 0.05 M BUFFER SOLUTIONS AT 22°C

pH	Buffer	Solubility of methotrexate (mg/ml)
4.51	Acetate	0.116
3.92	Acetate	0.079
3.41	Phosphate	0.043
3.08	Phosphate	0.030
2.49	Phosphate	0.040
2.03	Phosphate	0.061
1.34	Hydrochloric acid	0.201

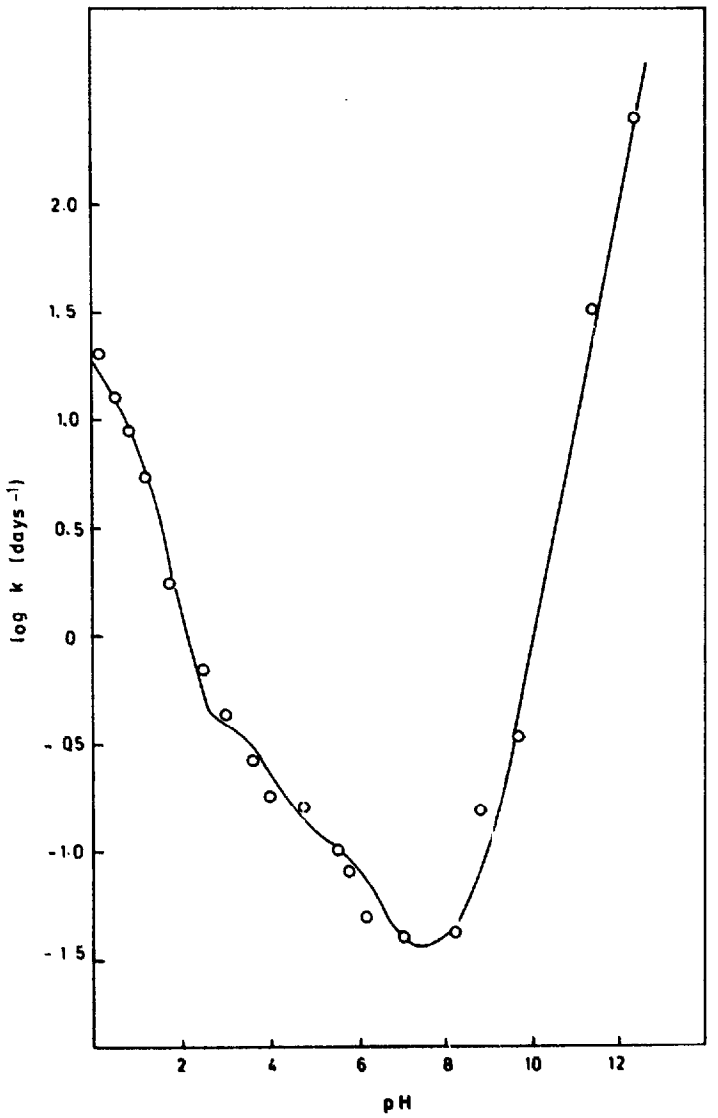


Fig. 5. Log k_{obs}–pH profile for the degradation of methotrexate in aqueous solution at 85°C ($\mu = 0.5$), where k_{obs} is the apparent first-order rate constant for a degradation in buffer-free solutions or in buffer showing no effect on the rate of degradation. The points are experimental and line theoretical, calculated by employing Eqn. 14 and the values of rate constants and ionization constants given in Scheme 2.

Ionization constants

Results of the apparent solubility at 22°C of methotrexate as a function of pH is shown in Table 3. From these data pK_a-values are calculated to 2.15 ± 0.03 and 3.8 ± 0.2. As no spectral changes are observed at these pH-values, the ionization constants observed are attributed to the glutamic acid parts of the molecule. The literature values for glutamic acid are 2.19 and 4.25 (Merck Index, 1976).

The spectroscopic method yielded pK_a-values of 0.65 ± 0.06 and 5.60 ± 0.05. Based on similar measurements using 2,4-diamino-6-hydroxy-methyl-pteridine the above values are attributed to the two pteridine amino groups.

pH–rate profile

The pH-dependence on the overall rate of degradation of methotrexate at 85°C and an ionic strength of 0.5 is shown in Fig. 5. The rate constants used in the construction of the graph were obtained from extrapolated rate constants at zero buffer concentrations for the various buffer solutions studied.

At pH < 2 the runs were performed in dilute hydrochloric acid solutions and at pH > 11 in dilute sodium hydroxide solutions of known molarity. In these solutions the pH-values were calculated from the following equations (Harned and Hamer, 1933):

$$pH = 0.17 - \log[H^+] \tag{1}$$

$$pH = 12.32 + \log[OH^-] \tag{2}$$

In the pH-range studied the compound is suggested to exist in 5 different ionic forms: MTX^{++}, MTX^{+}, MTX^{+-}, MTX^{+--}, MTX^{--} (neglecting microscopic ionization).

The slope of the pH–rate profile suggests that the overall degradation rate represents a summation of several separate reactions. It is not possible from the experimental data to establish the correct reactions because several of these are kinetically indistinguishable from other reactions. However, the following proposed reactions would be in qualitative agreement with the pH–rate profile experimentally determined.

Reactions			Rate constants
(1) MTX^{++}	$+H^+$	→ Products	k_1
(2) MTX^{+}	$+H^+$	→ Products	k_2
(3) MTX^{+-}	$+H_2O$	→ Products	k_3
(4) MTX^{+--}	$+H_2O$	→ Products	k_4
(5) MTX^{--}	$+H_2O$	→ Products	k_5
(6) MTX^{--}	$+OH^-$	→ Products	k_6

Scheme 1

The overall velocity of the degradation of methotrexate is expressed by:

$$v = \frac{-d[MTX]_T}{dt} = k_{obs}[MTX]_T \tag{3}$$

where $[MTX]_T$ is the total concentration of methotrexate:

$$[MTX]_T = [MTX^{++}] + [MTX^{+}] + [MTX^{+-}] + [MTX^{+--}] + [MTX^{--}] \quad (4)$$

At pH < 1.5 methotrexate exists as the MTX^{++} and MTX^{+} species and the overall rate of degradation may in this pH-region be described by the sum of reactions 1 and 2:

$$k_{obs} \cdot [MTX]_T = k_1 \cdot [H^+] \cdot [MTX^{++}] + k_2 \cdot [H^+] \cdot [MTX^{+}] \quad (5)$$

or

$$\frac{k_{obs}}{[H^+]} = (k_1 - k_2) \cdot f_{MTX^{++}} + k_2 \quad (6)$$

where $f_{MTX^{++}}$ is the fraction of the cationic species

$$f_{MTX^{++}} = \frac{[H^+]}{[H^+] + K_a^1} \quad (7)$$

Eqn. 6 predicts that a plot of $k_{obs}/[H^+]$ versus $f_{MTX^{++}}$ should be linear with intercept k_2 and a slope $(k_1 - k_2)$. Fig. 6 shows this curve from which k_1 and k_2 are calculated to be 5.1 $M^{-1} \cdot days^{-1}$ and 74 $M^{-1} \cdot days^{-1}$, respectively.

At pH 3.5–4.8 the overall degradation mainly consists of water-catalyzed reactions (reactions 3 and 4). The apparent first-order rate constant for the methotrexate degradation in this region may thus be formulated:

$$k_{obs} = k_3 \cdot f_{MTX^{+-}} + k_4 \cdot f_{MTX^{+--}} \quad (8)$$

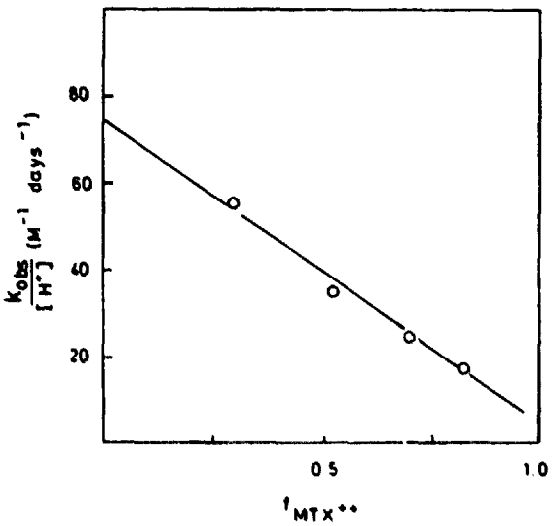


Fig. 6. Plot of $k_{obs}/[H^+]$ versus the fraction of MTX^{++} providing k_2 as the intercept and $(k_1 - k_2)$ as the slope.

or

$$k_{obs} = (k_3 - k_4) \cdot f_{MTX^{+-}} + k_4 \tag{9}$$

where $f_{MTX^{+-}}$ is:

$$f_{MTX^{+-}} = \frac{K_a^{II}[H^+]}{[H^+]^2 + K_a^{II}[H^+] + K_a^{II} \cdot K_a^{III}} \tag{10}$$

According to Eqn. 9, k_3 and k_4 may be derived from the intercept and slope, respectively, of a plot of k_{obs} against $f_{MTX^{+-}}$. The values of k_3 and k_4 derived therefrom were 0.28 $days^{-1}$ and 0.14 $days^{-1}$, respectively.

From the data obtained in the pH-range 7–7.5, the values of k_5 was estimated to be 0.04 $days^{-1}$.

Above pH 8.5 a linear increase of log k_{obs} with increasing pH to give a slope close to unity indicates a specific hydroxide ion-catalyzed reaction of dianionic methotrexate (MTX^{--}) (reaction 6). The apparent first-order rate constant for the methotrexate degradation in the pH-region above 7 may thus be formulated:

$$k_{obs} = k_5 \cdot f_{MTX^{--}} + k_6[OH^-] \cdot f_{MTX^{--}} \tag{11}$$

where $f_{MTX^{--}}$ is the fraction of the anionic species:

$$f_{MTX^{--}} = \frac{K_a^{IV}}{[H^+] + K_a^{IV}} \tag{12}$$

The k_6 value was determined from the portion of the pH-profile above 8.5. The value obtained was: $k_6 = 490\ M^{-1} \cdot days^{-1}$.

By replacing $[OH^-]$ with $K_w/[H^+]$ and inserting Eqns. 5, 8 and 11 in Eqn. 3, the following equation for the overall degradation of methotrexate was obtained:

$$k_{obs} = k_1[H^+] \cdot f_{MTX^{++}} + k_2[H^+] \cdot f_{MTX^{+}} + k_3 \cdot f_{MTX^{\pm}} + k_4 \cdot f_{MTX^{-}} + k_5 \cdot f_{MTX^{--}} + k_6 \cdot \frac{K_w}{[H^+]} \cdot f_{MTX^{--}} \tag{13}$$

or

$$k_{obs} = k_1 \frac{[H^+]^2}{[H^+] + K_a^I} + k_2 \frac{[H^+]^2 \cdot K_a^I}{[H^+]^2 + K_a^I[H^+] + K_a^I \cdot K_a^{II}}$$

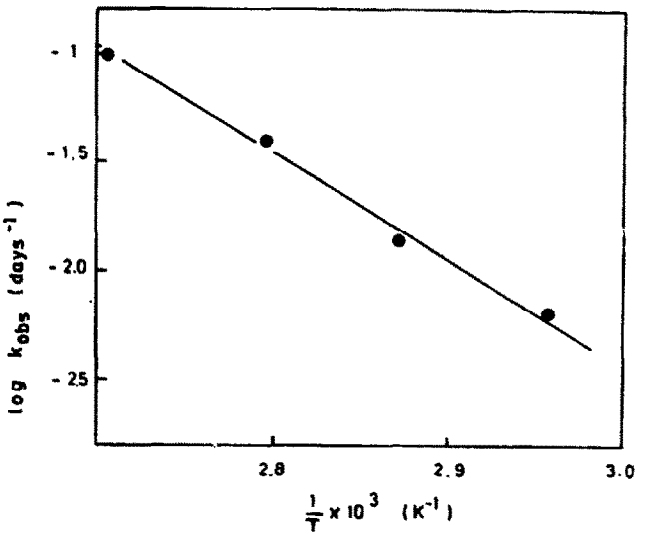


Fig. 7. Arrhenius-type plot of the logarithm of the observed pseudo-first-order rate constants against the reciprocal of absolute temperature for the overall degradation of methotrexate in an isotonic buffer-free solution at pH 8.50 (initial).

$$+k_3\frac{[H^+]K_a^{II}}{[H^+]^2+K_a^{II}[H^+]+K_a^{II}\cdot K_a^{III}}+k_4\frac{[H^+]K_a^{III}}{[H^+]^2+K_a^{III}[H^+]+K_a^{III}\cdot K_a^{IV}}$$

$$+k_5\frac{K_a^{IV}}{[H^+]+K_a^{IV}}+k_6\frac{K_w}{[H^+]}\cdot\frac{K_a^{IV}}{[H^+]+K_a^{IV}} \qquad (14)$$

The first estimates of k-values have then been fitted to Eqn. 14 using a non-linear curve-fitting program, which gives improved estimates of k- and K_a-values.

In Fig. 5 the full line represents the theoretical curve computed using Eqn. 14 and the final estimates of the parameters given in Scheme 2, while the points show the experimental results.

	Rate constants		Ionization constants	
k_1	5	$M^{-1}\cdot days^{-1}$	K_a^{I}	1.3×10^{-1}
k_2	110	$M^{-1}\cdot days^{-1}$	K_a^{II}	6.3×10^{-3}
k_3	0.52	$days^{-1}$	K_a^{III}	4.0×10^{-4}
k_4	0.12	$days^{-1}$	K_a^{IV}	1.3×10^{-6}
k_5	0.03	$days^{-1}$		
k_6	490	$M^{-1}\cdot days^{-1}$		

Scheme 2

The good agreement observed shows that the 6 reactions described (Scheme 1) adequately account for the total degradation of methotrexate in aqueous solution at pH of 0–12. At 85°C the minimum degradation rate occurred at pH of 6.6–8.2.

Effect of temperature on degradation rate

The temperature dependence on the degradation of methotrexate was investigated in isotonic buffer-free solution (pH 8.5 initially) over the range 65–95°C. From the slope and intercept of a plot of the logarithm of the observed pseudo-first-order rate constants against the reciprocal of absolute temperature an activation energy of 96.8 $kJ \cdot M^{-1}$ and a frequency factor of 6×10^{12} $days^{-1}$ were calculated. From the Arrhenius-type plot shown in Fig. 7, the stability of an isotonic buffer-free solution of methotrexate at a pH of 8.5 (initial) may be predicted at various temperatures. This gives at 25°C $k_{obs} = 6.4 \times 10^{-5}$ $days^{-1}$; and at 4°C $k_{obs} = 3.3 \times 10^{-6}$ $days^{-1}$. From these values the time in which 10% of the methotrexate had degraded is estimated to be 54 months and 1064 months, respectively.

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