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A systematic study on the chemical stability of ifosfamide*

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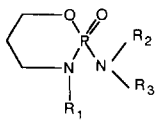
Abstract: The degradation kinetics of ifosfamide in aqueous solution have been investigated over the pH region 1–13 at 70°C. A stability indicating high-performance liquid chromatographic assay with UV detection was used to separate degradation products from the parent compound. The degradation kinetics were studied as related to pH, buffer composition, ionic strength, temperature and drug concentration. A pH-rate profile at 70°C, obtained from (pseudo) first-order kinetic plots, was constructed after corrections for buffer effects were made. The degradation reactions of ifosfamide were found to be largely independent of pH, although proton or hydroxyl catalysis occurs at extreme pH values. Ifosfamide shows maximum stability in the pH region 4–9, corresponding to a half-life of 20 h.

Keywords: *Ifosfamide; chemical stability; degradation kinetics; RP–HPLC; capillary GLC.*

Introduction

Ifosfamide (IF), like cyclophosphamide (CP) and trofosfamide (TF) (Fig. 1), belongs to the oxazaphosphorines, which are strongly alkylating compounds used in cancer chemotherapy. Ifosfamide is inactive *per se* and needs to be activated *in vivo* to 4-hydroxyifosfamide which is in equilibrium with aldofosfamide. Via various metabolic pathways these compounds may be transformed to inactive or cytotoxic products [1].

IF is a very effective agent against a diverse array of neoplastic diseases including small cell lung cancer, ovarian, endometrial, breast and testicular cancer, Hodgkin's disease and soft tissue sarcoma [2]. Inherent to its still growing clinical importance, IF is currently experiencing increasing attention in the field of pharmaceutical and biomedical analysis.



	R_1	R_2	R_3
Ifosfamide	CH_2CH_2Cl	CH_2CH_2Cl	H
Cyclophosphamide	H	CH_2CH_2Cl	CH_2CH_2Cl
Trofosfamide	CH_2CH_2Cl	CH_2CH_2Cl	CH_2CH_2Cl

Figure 1
Structures of ifosfamide, cyclophosphamide and trofosfamide.

The analysis of IF by high-performance liquid chromatography (HPLC) [3, 4] and capillary gas-liquid chromatography (GLC) [5–13] has been described by various authors. Data concerning the chemical stability of IF, however, are scarce. Apart from the study by Zon *et al.* [14] dealing mainly with the degradation products of IF and CP, and some rather limited studies [15–17], no systematic study about the degradation of IF in aqueous solution is available in the literature. On the other hand CP, a structural isomer of IF, has been the object of some stability studies in the past [18–22].

The present study was undertaken to extend an insight into the degradation kinetics of IF and the influence of external factors (pH, buffers, ionic strength, temperature and drug concentration) on these processes. In a subsequent report the identity and stability of the degradation product(s) will be presented.

Experimental

Chemicals

IF and TF were kindly donated by Asta Pharma AG (Frankfurt, Germany). All chem-

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icals used were of analytical grade and were used without further purification. Deionized water was used throughout.

Buffer solutions

For the kinetic studies the following aqueous buffer solutions were used: $1 \leq pH < 3$, perchloric acid; $3 \leq pH < 5.5$, acetate; $5.5 \leq pH < 8.5$, phosphate; $8.5 \leq pH < 11.5$, carbonate; and $pH \geq 11.5$, sodium hydroxide. The pH values were measured at the study temperature using an Ingold Semimicro combination pH electrode (Ingold Electrodes Inc., Wilmington, MA, USA) and a pH meter (Consort P514, Turnhout, Belgium). pH values over 12 were calculated from the equation $pH = pK_w + \log[OH^-]$, where $pK_{w(70°)} = 12.82$.

A constant ionic strength (μ) of 0.3 M was maintained for each solution by addition of an appropriate amount of sodium chloride. This was not the case for solutions used in the experiments where the ionic strength was varied. No significant changes in pH were observed throughout the degradation studies, except for the experiments at buffer concentrations lower than 0.005 M.

Kinetic measurements

The influences of various external factors on the degradation process were studied. To construct the pH-rate profile, the pH was varied between 1 and 13. Values for k_{obs} were calculated at constant pH, ionic strength and temperature, but at different buffer concentrations to determine the effect of the buffer ions on the rate of degradation. The influence of ionic strength on the chemical stability of IF was investigated with 0.01 M buffer solutions at pH 2.5, 7.0 and 11.0, at a temperature of 70.0(±0.2)°C. The ionic strength was varied between 0.05 and 1.0 M by adding various amounts of sodium chloride.

Degradation of IF was investigated at 50.0, 60.0, 70.0, 80.0 and 90.0(±0.2)°C in 0.01 M carbonate buffer, pH 11.0; 0.01 M phosphate buffer, pH 7.0; and in 0.01 M perchloric acid, pH 2.5. The concentration of IF was varied between 40 and 400 $\mu g\ ml^{-1}$ in 0.01 M buffer solutions of pH 2.5, 7.0 and 10.0 at a temperature of 70.0(±0.2)°C and $\mu = 0.3$ M.

The degradation reactions were initiated by adding a 1 ml sample of an aqueous IF solution to 5 ml of the buffered solution to obtain an initial IF concentration of 200 $\mu g\ ml^{-1}$ (7.7×10^{-4} M). Before adding IF, the buffer solutions were preheated to the temperature of study. The stock solution of IF in water, kept at 4°C, was found to be stable for at least 4 weeks. Reaction solutions were kept in crimp-capped glass vials in a thermostatically controlled water bath in the dark. All kinetics experiments have been performed in duplicate.

Analytical procedures

HPLC. At appropriate time intervals 200 μl samples were withdrawn from the reaction solutions and added to 200 μl of a precooled (4°C) 0.1 M phosphate buffer solution of pH 7. When the degradation of IF in buffer solutions of various molarities was studied, 200 μl of a 0.5 M phosphate solution was used to bring the reaction mixtures in 0.1 and 0.2 M buffer solutions to a neutral pH. Until they were analysed (within 2–3 days), the samples were kept at 4°C which stabilized the samples for this period of time. All samples belonging to the same experiment were analysed on the same day by a stability indicating HPLC assay.

GLC. For the gas chromatographic determination of IF and the degradation product a sample of 100 μl was taken from the 200 μl samples neutralized with 200 μl of 0.1 M pH 7 phosphate buffer. Fifty μl of a 1 M sodium hydroxide solution and 25 μl of a solution of the internal standard TF (200 ng μl^{-1}) were added. This solution was extracted with 500 μl ethyl acetate. After centrifugation at 3000*g*, the organic layer was separated and evaporated. The residue was dissolved in 50 μl ethyl acetate. One μl was injected into the gas chromatograph using a split ratio of 1:100.

UV. UV spectra were measured for solutions which were processed in the same way as those used for HPLC analysis.

Instruments

HPLC. The liquid chromatographic system consisted of a model 6000A solvent delivery system, a U6K septumless injection device (both from Waters Assoc., Milford, MA, USA) and a Spectroflow 773, variable wavelength detector (ABI Analytical, Kratos Division, Ramsey, NJ, USA) operated at 205 nm. The stainless steel analytical column (125 × 3.9 mm) was filled with Nova-Pak C18 (particle size 5 μm) (Waters Assoc., Milford, MA, USA). For the degradation

studies in alkaline solutions (pH >10) a mobile phase (I) was used consisting of acetonitrile–water–phosphate buffer (pH 7, 0.1 M) (15:85:1,% v/v/v). For degradation studies at other pH values, a mobile phase (II) was used with the same composition as eluent I except for having components in the ratio (20:80:1,% v/v/v). The mobile phases were used at a flow rate of 1.0 ml min^{-1} with the column at ambient temperature. Quantitation of ifosfamide and the degradation product was based on peak area measurements using a SP4270 integrator (Spectra Physics, Santa Clara, CA, USA).

GLC. The gas chromatographic system consisted of a gas chromatograph (Carlo Erba Instruments HRGC 5300, Milan, Italy) provided with a split/splitless injector and nitrogen/phosporus selective flame ionisation detection (NP-FID). Separation was achieved on a capillary column (CP-Sil 8 CB, 25 m, i.d. 0.32 mm, f.d. 0.12 μm, Chrompack, Middelburg, The Netherlands) with the application of the following temperature program: the starting temperature of the column was 120°C which was raised to 200°C at a rate of 40°C min^{-1}. The column temperature was kept at 200°C for 8 min. Cooling down from 200 to 120°C took 2 min, bringing the total analysis time to 12 min.

The chromatograph operated under the following conditions: injector: 175°C; detector: 275°C; carrier gas: He; flow: 3 ml min^{-1}; detector gas flow: air: 300 ml min^{-1}; hydrogen: 30 ml min^{-1}; make-up gas (He): 30 ml min^{-1}; split injection, where the split depends on concentration in the sample. The detector was connected to a Model DP 700 integrator (Carlo Erba Instruments, Milan, Italy).

UV. UV spectra of IF were recorded on a Lambda 5 UV–vis spectrophotometer (Perkin–Elmer, Norwalk, USA).

Results and Discussion

From the few studies on the chemical stability of IF it is evident that aqueous solutions of IF are reasonably stable at room temperature. Trissel *et al.* [15] reported a stability of at least 1 week at room temperature and 6 weeks in the refrigerator (2–8°C). Zon *et al.* [14] reported a half-life for IF of 6.8 min in deuterated water at room temperature and pD 0 and of 120 days at pD 7. This last value of k_{obs} was twice as low as that found in the present study where the extrapolated value for pH 0 at room temperature was 1 min.

Radford *et al.* [17] performed a study on the stability of IF in aqueous solution in order to test its suitability for continuous 7-day infusion using an ambulatory pump. The authors concluded that IF in aqueous solution is stable for at least 9 days at body surface temperature. No kinetic parameters, nor the order of the degradation reaction, were determined in that study [17].

Analytical procedures

UV spectrophotometric measurements indicate that IF degradation products possess similar chromophores to the parent drug. Consequently, direct UV spectrophotometry is an inadequate technique for the quantitation of the degradation reactions. UV measurements at 205 nm are not very selective, moreover, because of the interferences of buffer components. HPLC and GLC methods were used to resolve the degradation product from the parent IF. Typical chromatograms are shown in Figs 2 and 3.

Measurements of k_{obs} by means of an HPLC and GLC assay were compared. The difference between these values was not statistically significant, reflecting stability indicating properties for each.

HPLC was chosen to perform the kinetics measurements since it can be used at ambient temperature, whereas the very high temperatures in GLC may cause on-column degradation of IF or its degradation product(s). The disadvantage of the HPLC method is the difficulty in detecting degradation product existing in neutral solution because of the rather low sensitivity of the method. Calibration curves of standard IF solutions in 0.1 M pH 7 phosphate buffer showed good linearity ($r > 0.999$) in the concentration range of interest (5–400 μg ml^{-1}). The precision of the k_{obs} measurement was determined by repeatedly ($n = 6$) performing a degradation experiment. The relative standard deviation of the determination of k_{obs} was found to be 5%.

Mobile phases with two different organic modifier concentrations were used. In solutions of high pH the amount of degradation product was sufficient to be detected. It was necessary, however, to use mobile phase I to separate the degradation product peak from those at the dead volume. In neutral solutions

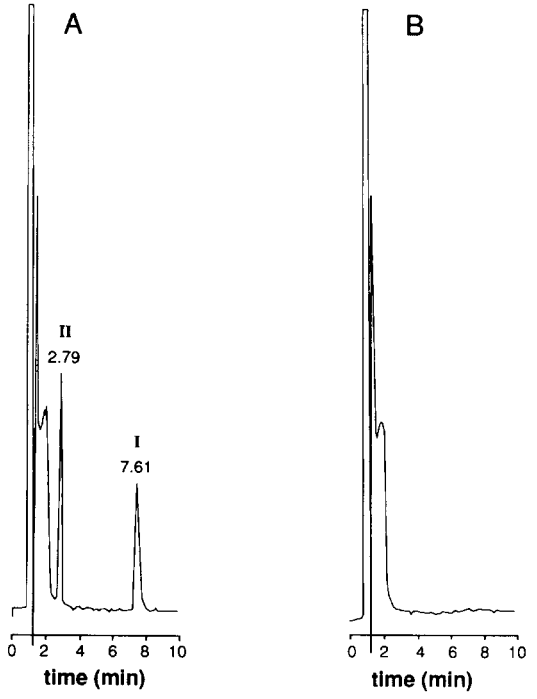

Figure 2
HPLC chromatogram of ifosfamide (I) and degradation product (II) resulting from 3 h at pH 11.5 (A) and a blank sample containing no IF (B).

no degradation product could be detected. By using mobile phase II the analysis time became much shorter [Rt(IF) = 6 min].

Degradation products

GC studies revealed the existence of only one degradation product of IF in alkaline and neutral solutions. In neutral solutions only a small amount of degradation product could be detected, which never exceeded 2% of the initial IF content (calculated as IF). In solutions of high pH the amount of degradation product was as high as 50% of the initial IF amount (Fig. 4).

Degradation kinetics

The disappearance of IF in buffered media follows (pseudo) first-order kinetics. This is indicated by linearity in plots of the natural logarithm of residual IF concentration against time at various pH values (Fig. 4 shows a plot at pH 12). The observed rate constants (k_{obs}) for the overall degradation have been extracted from the slopes of these plots.

The pH-rate profile at 70°C is depicted in Fig. 5. The profile shows three different regions corresponding to proton catalysed (pH 1–4), solvent catalysed (pH 5–9) and hydroxyl

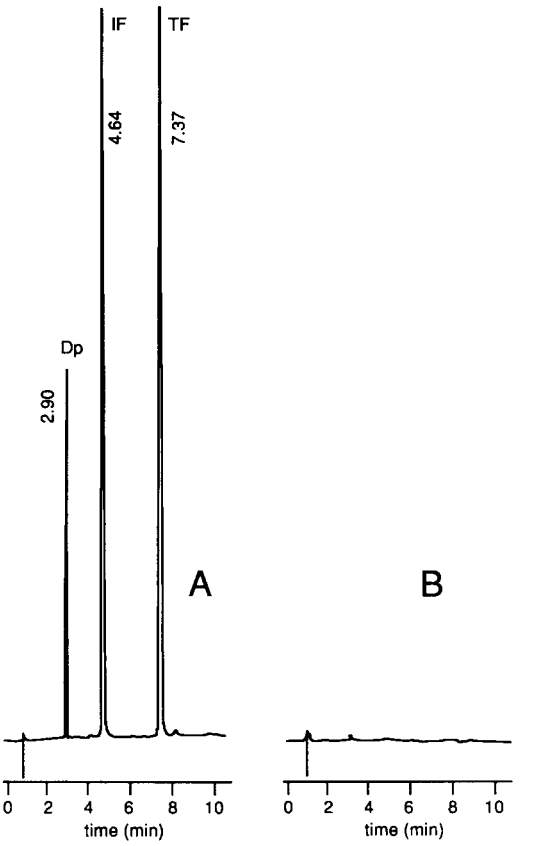

Figure 3
GC chromatogram of ifosfamide (IF), internal standard (TF) and degradation product (DP) (A) and a blank sample (B).

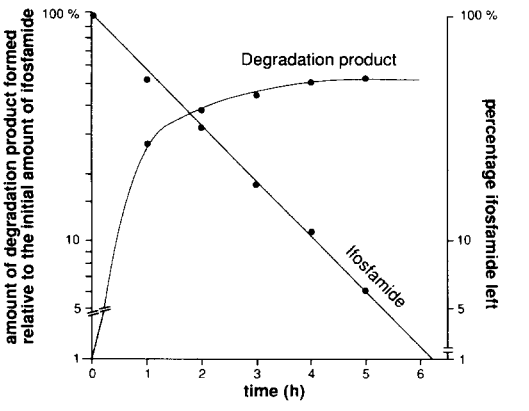

Figure 4
Disappearance of IF and appearance of the degradation product in time, measured in a NaOH solution pH 12, 70°C.

ion catalysed (pH 10–12) degradation reactions, according to the following formula:

$$k_{obs} = k_o + k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_Q[Q]. \quad (1)$$

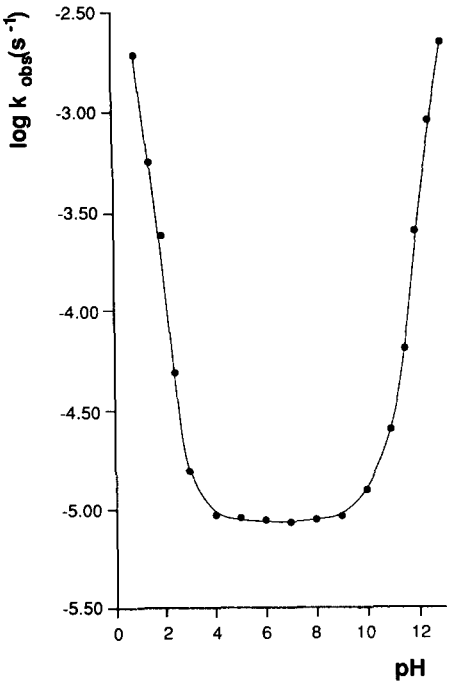

Figure 5
pH-rate profile of ifosfamide at 70°C obtained by extrapolation to zero buffer concentration.

Table 1
Influence of carbonate and phosphate concentration on k_{obs} of the degradation reactions of ifosfamide

	[Concentration] (M)	k_{obs} (s^{-1}) × 10^{-5}
Carbonate pH 11.5	0.005	7.6
	0.01	8.7
	0.05	16.6
	0.1	20.7
	0.2	21.9
Phosphate pH 11.0	0.005	4.0
	0.01	5.1
	0.05	12.1
	0.1	17.2
	0.2	18.3
Carbonate pH 10.0	0.005	2.9
	0.01	3.6
	0.05	4.6
	0.1	5.0
	0.2	5.7

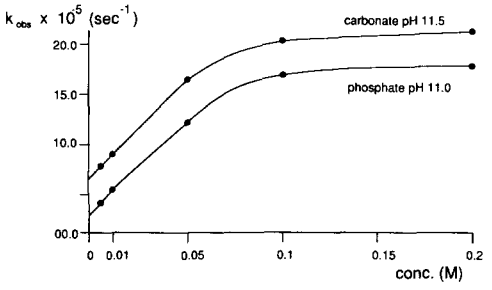

Figure 6
Influence of carbonate concentration (pH 11.5) and of phosphate concentration (pH 11.0) on k_{obs} for the degradation of ifosfamide at 70°C.

In which k_o is the rate constant of the solvent catalysed reaction; k_{H^+} the rate constant of the proton catalysed reaction; k_{OH^-} the rate constant of the hydroxyl ion catalysed reaction and k_Q the rate constant of the protonated and deprotonated buffer molecule catalysed reaction.

Carbonate and phosphate buffers only, from among all buffers studied, show a statistically significant effect on k_{obs} in the pH region 10–11.5 (Table 1). Apparently a proton transfer takes part in the degradation mechanism in this pH region. In the buffer concentration region <0.005 M a linear relationship between k_{obs} and buffer concentration is presumed. At higher buffer concentrations (>0.1 M) the curves level (Fig. 6). In order to delete the influence of buffer ions on k_{obs}, all k_{obs} values in the pH region 9–11.5 have been extrapolated to zero buffer concentration, resulting in the corrected k^1_{obs} and the pH-rate profile which is plotted in Fig. 5. After the correction for buffer catalysis has been made, the k_{H^+} can be calculated as the intercept on the y-axis from the portion of the pH-rate profile between pH 1–4. The slope of −1 shows that specific proton catalysis applies.

From the graph in the region pH 5–9, k_o can be calculated, because in this region log k_{obs} = k_o. The k_{OH^-} can be calculated from the straight line in the pH region 10–12 with pK_w of 12.82 at 70°C. The observed slope of +1 indicates that specific hydroxyl ion catalysis occurs.

The specific rate constants were calculated with the use of a non-linear least-squares curve fitting program and are shown in Table 2. It appears that k_{obs} is not influenced by a change in the ionic strength of the solution, indicating that a reaction between at least two charged particles does not play an important role in the degradation mechanism of IF.

Table 2
Specific rate constants of the degradation of ifosfamide at 70°C

k_{OH^-}	= 1.32 × 10^{-3} M^{-1} s^{-1}
k_o	= 8.13 × 10^{-6} s^{-1}
k_{H^+}	= 1.41 × 10^{-2} M^{-1} s^{-1}

The influence of temperature on the degradation reaction at the pH values 2.5, 7.0 and 11.0, corresponding to the three different parts of the pH-rate profile could be described by the Arrhenius equation:

$$\ln k_{obs(T)} = \ln A - E_A/R \times 1/T,$$

in which A is the frequency factor, E_A the activation energy, R the gas constant (8314 J mol^{-1} K^{-1}) and T the temperature (K). The calculated parameters are shown in Table 3.

Table 3
Parameters from the Arrhenius equation

pH	A (s^{-1})	E_A (J mol^{-1})	r
2.5	1.62×10^6	6.73×10^4	−0.9990
7.0	3.12×10^9	1.01×10^5	−0.9995
11.5	3.21×10^{12}	1.08×10^5	−0.9995

The experiments of Hirahata *et al.* [19] on CP for comparison led to an activation energy of approximately 1×10^5 J mol^{-1} at pH 2.0, 7.0 and 9.5. In the present study the difference between the activation energies at pH 7.0 and 11.0 is not statistically significant, whereas the activation energy at pH 2.5 significantly differs from those at pH 7.0 and 11.0. A possible inference from this phenomena may be that the reaction mechanisms of the degradation at pH 7 and 11, although, respectively, solvent and hydroxide ion catalysed, do not differ widely, whereas the degradation of IF at pH 2.5 may involve another mechanism. However, at neutral pH only a maximal 2% of IF was converted into the degradation product, whereas at pH 12, 50% of IF may degrade to this compound. A general presumption is that compounds may be more stable under the mild conditions at pH 7 than under the relatively severe conditions at pH > 10. Consequently, when the degradation follows the same mechanism in solutions of pH 7 and 11 a larger amount of the degradation product would be expected at pH 7. Apparently, the observed degradation product is not the only compound formed at pH 7, indicating different reaction mechanisms at pH 7 and 11 despite the equal activation energies.

By means of the Arrhenius equation theoretical pH profiles could be constructed at 20 and 4°C (Fig. 7), resulting in a t_{90} of 39 and 344 days, respectively.

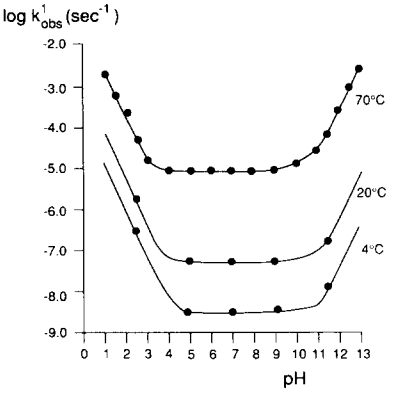

Figure 7
pH-rate profiles of IF in aqueous solution extrapolated to 20 and 4°C using the Arrhenius equation.

The concentration of IF did not have any influence on k_{obs} in the range 40–400 μg ml^{-1}.

Conclusions

Despite some earlier studies about the chemical stability of IF in aqueous solution, information about the kinetics of the degradation reactions of IF is scarce. To study these reactions and their kinetics, HPLC and GLC are suitable stability indicating methods.

Degradation of IF follows (pseudo) first-order kinetics. The pH-rate profile shows three distinctive regions corresponding to a proton, solvent and hydroxide ion catalysed degradation reaction. The minimum in the pH-rate profile appears in the pH range 5–9, corresponding to a half-life of 20 h at 70°C, of 254 days at room temperature and of almost 6 years at 4°C. Therefore, aqueous solutions of IF are reasonably stable even at room temperature.

In neutral and alkaline solutions the same degradative compound of IF is formed, although not via the same reaction mechanism. At neutral pH the observed degradation product is believed to be accompanied by another product, which was not detected by the employed HPLC system.

Unlike CP, the activation energy for the degradation reaction of IF in acid media differs from the activation energies measured at pH > 7, indicating yet another degradation mechanism. The identity of the observed degradation product, the exact mechanism by which it is formed and its kinetics are still under study.

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