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Degradation study of thiotepa in aqueous solutions

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Abstract

The degradation of *N*,*N*′,*N*″-triethylenethiophosphoramide (thiotepa) in aqueous solutions has been investigated over the pH range 1–14. Samples were analyzed using a high-performance liquid chromatographic system with UV detection. The degradation kinetics were studied as a function of pH, sodium chloride concentration and temperature. The degradation of thiotepa follows pseudo first order kinetics. The pH–log k_{obs} profile shows that thiotepa is most stable in the pH range 7–11. At pH > 11 chloride has no influence on the degradation rate. The degradation products were isolated and the structures identified by mass spectrometry. Chloro adducts of thiotepa are generated in the presence of sodium chloride and in acidic medium. In the pH range 7–11 only the mono-chloro adduct of thiotepa could be found. No detectable degradation products were formed at pH > 11.

Keywords: Chemical stability; Degradation kinetics; HPLC; Sodium chloride-catalyzed degradation; Thiotepa

1. Introduction

The alkylating agent *N*,*N*′,*N*″-triethylenethiophosphoramide (thiotepa) has been used in cancer therapy for more than 40 years (Sykes et al., 1953). Because of the broad spectrum of antitumor activity and manageable toxicities, thiotepa is currently being employed in many high-dose chemotherapy regimens for, for example, breast cancer, ovarian cancer and other solid tumors (Antman et al., 1990; Dimopoulos et al., 1993; Vaughan et al., 1994; Van der Wall et al., 1995a,b; Rodenhuis et al., 1996). Despite its many years of application, little is known about the metabolic profile of thiotepa. The appearance of the main metabolite *N*,*N*′,*N*″-triethylenephosphoramide (TEPA) was first reported by Mellet and Woods (1960). Other studies showed the presence of other alkylating metabolites of thiotepa in urine as determined by the alkylating activity of samples. Thiotepa and TEPA excretion repre-

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sented only 5% of the administered dose, while the total alkylating activity accounted for 25% of the administered dose (Cohen et al., 1986; Hagen et al., 1990). As the metabolic and chemical reactivity of thiotepa is largely unknown, we started to study this in a systematic manner. More insight into the chemical reactivity of thiotepa may support us in the search for thiotepa metabolites. Results from the sparse studies on thiotepa degradation, however, are contradictory. During the hydrolysis of thiotepa the formation of an SH group after intramolecular rearrangement was described by Benckhuijsen (1968), whereas others could not detect this product (Pyatigorskaya et al., 1987). Most of the studies showed that, during the degradation of thiotepa in acidic media and in the presence of chloride, chloro adducts of thiotepa were found (Maxwell et al., 1974; Pyatigorskaya et al., 1987; Murray et al., 1997), but Zon et al. (1976) disputed these findings. Degradation of thiotepa has also been established in acidified urine (Cohen et al., 1984) and whole blood (Mellet and Woods, 1960).

This article describes a systematic degradation study of thiotepa in acidic and alkaline media in the presence and absence of sodium chloride.

2. Experimental

2.1. Chemicals

Thiotepa was obtained from Cyanamid Benelux (Etten-Leur, The Netherlands). All other chemicals used were of analytical grade unless otherwise specified.

2.2. Buffer solutions

For the kinetic studies the following buffer solutions were used: $1 \leq pH < 3$, perchloric acid; $3 \leq pH \leq 5$, acetate; $5 < pH \leq 8$, phosphate; $8 < pH \leq 11$, carbonate; $pH > 11$, sodium hydroxide. For the degradation studies of thiotepa in the presence of sodium chloride, 1 M sodium chloride was added to the buffer solution and if necessary the pH was adjusted with sodium hydroxide or hydrogen chloride. The pH was measured with a Slim-trode pH electrode (Hamilton, Darmstadt, Germany) and a pH meter (Consort P514, Turnhout, Belgium) at the temperature of study. pH values over 12 were calculated from the equation $pH = pK_w - pOH$.

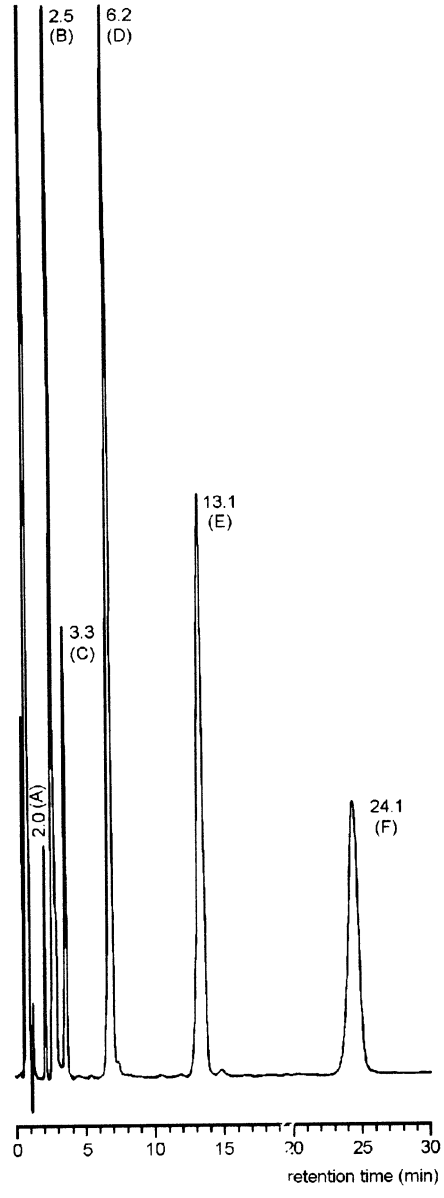

Fig. 1. HPLC of a degradation sample of thiotepa. Thiotepa was degraded for 10 min at pH 3.0 in the presence of 1 M sodium chloride at ambient temperature. The peak eluting at 2.5 min originates from thiotepa; the other peaks are decomposition products.

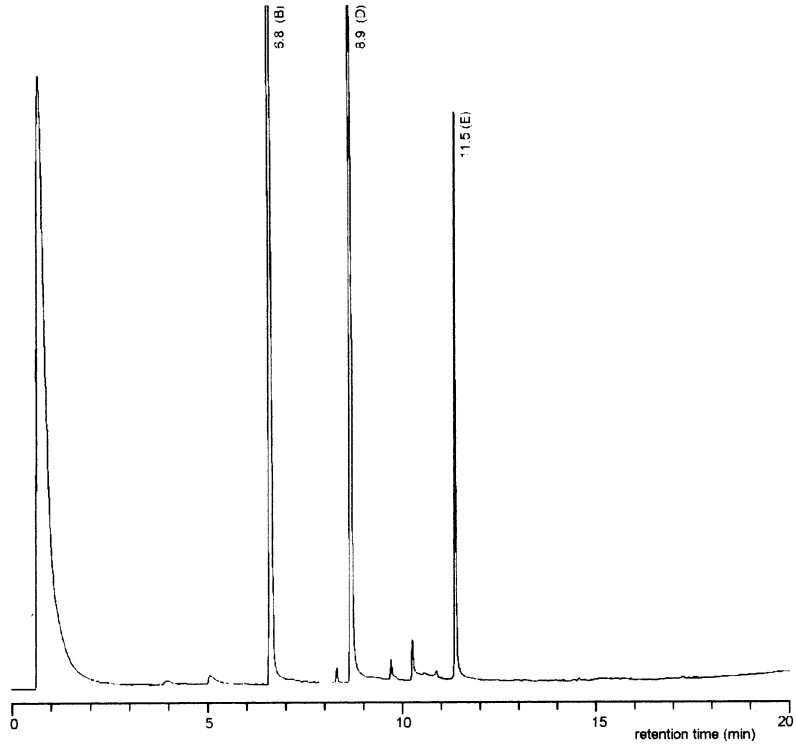

Fig. 2. GC of a degradation sample of thiotepa. Thiotepa was degraded for 10 min at pH 3.0 in the presence of 1 M sodium chloride at ambient temperature. The peak eluting at 6.8 min originates from thiotepa; the other peaks are decomposition products.

2.3. Instrumentation

2.3.1. High-performance liquid chromatography (HPLC)

An HPLC system consisting of a P100 isocratic pump, an AS300 autosampler and a UV100 variable wavelength detector operating at 205 nm (all from Thermo Separation Products, Fremont, CA, USA) was used. The HPLC system was equipped with a LiChrospher® 100 RP-18 (5 μm) column with a length of 12.5 cm (Merck, Darmstadt, Germany). A mobile phase of 35% (v/v) acetonitrile in water at a flow rate of 1 ml/min was used for the kinetic studies. For the isolation of the degradation products a mobile phase comprising 30% (v/v) acetonitrile in water was used. Quantitation of thiotepa and its degradation products was based on peak area measurements using an SP4400 integrator (Thermo Separation Products, Fremont, CA, USA). Mass spectrometry (MS) measurements were performed on a VG Platform, equipped with an electronspray interface operating in the positive mode (Fisons Instruments, Beverly, MA, USA). Nitrogen was used as drying gas and as nebulizing gas at a flow rate of 25 l/h and 20 l/h, respectively. The source temperature was set at 80°C and the cone voltage at 35 V.

2.4. Kinetic measurements

Influences of several factors on the degradation of thiotepa were measured. The pH–K_{obs} profile was constructed from pH 2 to 14, in the absence and presence of 1 M sodium chloride. Degradation experiments were executed at ambient temperature for $2 \leq pH \leq 6$ and at 80°C for $pH > 6$. To determine the influence of sodium chloride on the degradation its concentration was varied between 0.1 and 1.2 M at pH 3 and 5. The influence of the temperature on the degradation was measured at 25, 30, 40 and 50°C in a 25 mM buffer with a pH of 4.

The degradation reactions were initiated by adding 120 μl of a 5 mg/ml thiotepa solution in water to 2.88 ml of the buffer solution to obtain a concentration of 200 μg/ml. The stock solution of thiotepa in water was stored at 4°C and was stable for at least 1 month.

The reaction solutions were kept in glass vials for the degradation experiments at ambient temperature. For degradation at higher temperatures, the solutions were kept in ampullae in a thermostatically controlled water bath. All experiments were performed in duplicate.

2.5. Analytical procedures

2.5.1. HPLC

At appropriate time intervals 200-μl samples were withdrawn from the reaction solutions and allowed to achieve room temperature. Next, 200 μl of a 0.2 M phosphate buffer pH 8 was added to bring the solutions to a neutral pH. Samples were stored at 4°C and were analyzed within 1 week. The samples were stable during this storage period as verified by HPLC analysis.

2.5.2. MS

Flow injection analysis–mass spectrometry (FIA–MS) was used for the characterization of the degradation products of thiotepa. Thiotepa was degraded during 15 min at pH 3 as described in Section 2.4 and samples were analyzed by FIA–MS after isolation with HPLC. HPLC separation was achieved with a mobile phase of 30% (v/v) acetonitrile in water and various eluates were collected. The acetonitrile was then evaporated under a stream of nitrogen at ambient temperature. The remaining aqueous solution was extracted with a mixture of 10% (v/v) 1-propanol in chloroform. The organic layer was separated and evaporated to complete dryness and the residue was dissolved in methanol.

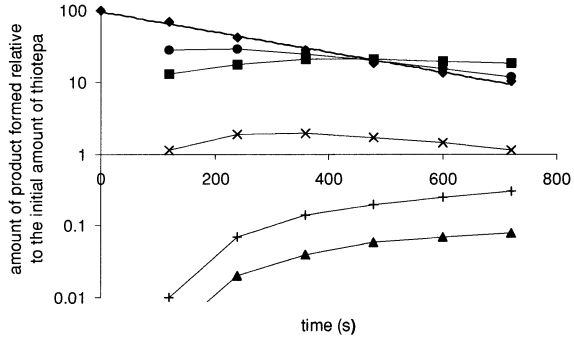

Fig. 3. Formation of the intermediate products during the degradation of thiotepa as a function of time at pH 3 in the presence of sodium chloride (×, product A, t_r 2.0 min; ◆, thiotepa, t_r 2.5 min; ▲, product C, t_r 3.3 min; ●, product D, t_r 6.2 min; ■, product E, t_r 13.1 min; +, product F, t_r 24.1 min). The amount of degradation product was calculated (as thiotepa) relative to the amount of thiotepa at the start of the degradion.

3. Results

3.1. Chromatography

HPLC and gas chromatography (GC) were used to separate thiotepa from its degradation products. Using HPLC, five degradation products were detected (Fig. 1). In the same sample only two products could be tracked down, after extraction with chloroform and GC analysis (described in van Maanen et al., 1997) (Fig. 2). The high temperature of the injector and oven can lead to conversion of the formed degradation products, therefore HPLC was used to perform the analysis of the degradation kinetics of thiotepa. In the absence of sodium chloride no detectable products were seen using the HPLC system described above. Variation of the amount organic modifier in the mobile phase from 0% to 20% to separate putative degradation products from compounds in the dead volume gave also no detectable compounds.

Using a mobile phase of 35% (v/v) acetonitrile in water, baseline separation was obtained and was applied (Fig. 1).

3.2. Degradation products

HPLC analysis showed the formation of five degradation products when thiotepa was decomposed in the presence of sodium chloride at pH $\leq$ 6. Using buffer solutions with pH $\geq$ 7 only one degradation product was formed while at pH $\geq$ 13

no degradation products were detected. At pH ≥ 7 chloride ions had no effect on the degradation pattern of thiotepa. In the absence of sodium chloride no detectable products were formed at all pH levels. In Fig. 3 the formation of degradation products of thiotepa at pH 3 in the presence of 1 M sodium chloride as a function in time are plotted. As can be seen, the products with retention times of 2.0 (product A), 6.2 (product D) and 13.1 min (product E) are intermediates. Products at 3.3 (product C) and 24.1 min (product F) can be considered stable and end-products of the degradation under the test conditions. Characterization of the products was performed with GC–MS and FIA–MS. In Fig. 4 the mass spectra with corresponding molecular formulae of all products are depicted. For product A an m/z value of 266 was found with a chloro isotope at $m/z = 268$ (Fig. 4A), representing the sodium adduct of 1-chloro, 2-hydroxyl, 3-ethylenthiophosphoramide. The ion at $m/z = 301$ is background signal. A molecular ion at $m/z = 190$ is seen in the mass spectrum of product B (Fig. 4B) corresponding with thiotepa. In the mass spectrum of product C (Fig. 4C) an ion at m/z of 280 was seen. The ratio of ion m/z 280 to ion m/z 282 and 284 was 9:6:1,

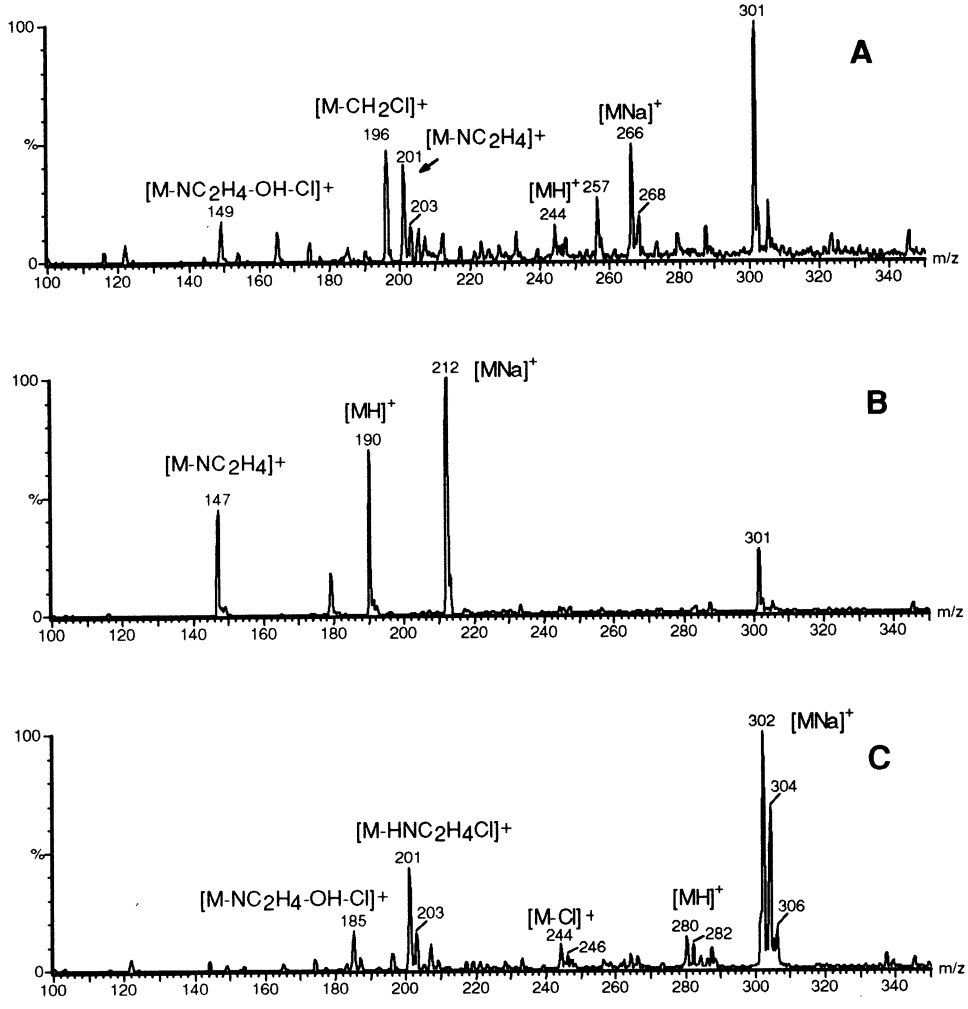

Fig. 4. Mass spectra of thiotepa (B) and its degradation products with t_r 2.0 min (A), t_r 3.3 min (C), t_r 6.2 in (D), t_r 13.1 min (E) and t_r 24.1 min (F).

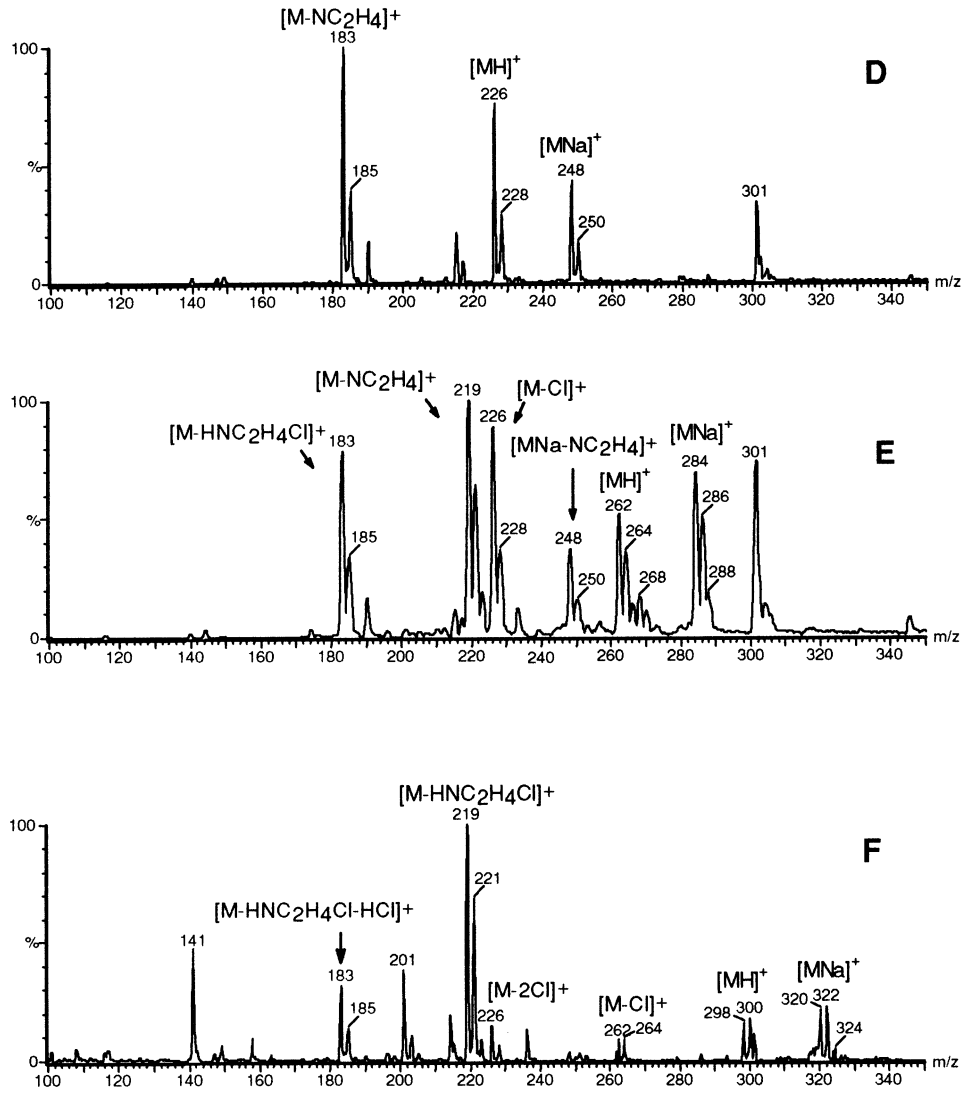

Fig. 4. (*Continued*)

indicating the presence of two chloro atoms. This mass corresponds to 1,2-dichloro, 3-hydroxylethylenthiophosphoramide. Product D has a molecular ion at m/z 226 with a mono-chloro isotope at 228, derived from the mono-chloro adduct of thiotepa (Fig. 4D). Fig. 4E depicts the mass spectrum of product E consistent with the di-chloro derivative of thiotepa. A molecular ion with $m/z = 262$ was found with two chloro isotopic peaks of 264 and 266. For product F an m/z of 320 was found with isotopic peaks at 322, 324 and 326, which is the sodium adduct of 1,2,3-trichloroethylthiophosphoramide (Fig. 4F).

Additional information on the degradation products was obtained by isolating each product by HPLC and studying consecutive degradation processes at pH 3 with or without sodium chloride. The results of these experiments are summarized in Table 1. As can be seen, 1,2-dichloro, 3-hydroxylethylthiophosphoramide (product C) and 1,2,3-trichloroethylthiophosphoramide (product F) are the end-products formed during

Table 1
Further degradation of isolated degradation products of thiotepa

Isolated product	Formed products	
	pH 3	pH 3+sodium chloride
A	–	C
C	–	–
D	A	C, E, F
E	C	C, F
F	–	–

thiotepa degradation in an acidic environment and in the presence of sodium chloride, corresponding with the degradation–time profiles (Fig. 3). Degradation in acidic medium (in the absence of chloride) only leads to the formation of a hydroxylethyelenimine when the aziridine moiety if present. Under these conditions 1,2,3-trihydroxylethylthiophosphoramide can be considered as the end-product of the degradation of thiotepa in acidic solutions. As all hydroxyl compounds showed a more polar character compared to the non-hydroxyl-containing equivalent, 1,2,3-trihydroxylethylthiophosphoramide probably elutes in the dead volume and gave therefore no traceable peaks on HPLC. In Fig. 5 the proposed degradation scheme of thiotepa in acidic environment in the presence or absence of sodium chloride is presented. Products A, C–F have been identified while the identities of the other compounds are hypothesized.

3.3. Kinetic studies

The degradation rate of thiotepa at various pH values with or without sodium chloride can be described with pseudo first-order kinetics. This can be concluded by the linear character of the plots of the natural logarithm of the residual thiotepa concentrations against time (Fig. 6). The observed rate constants (k_{obs}) for the overall degradation have been extracted from the slopes of these plots.

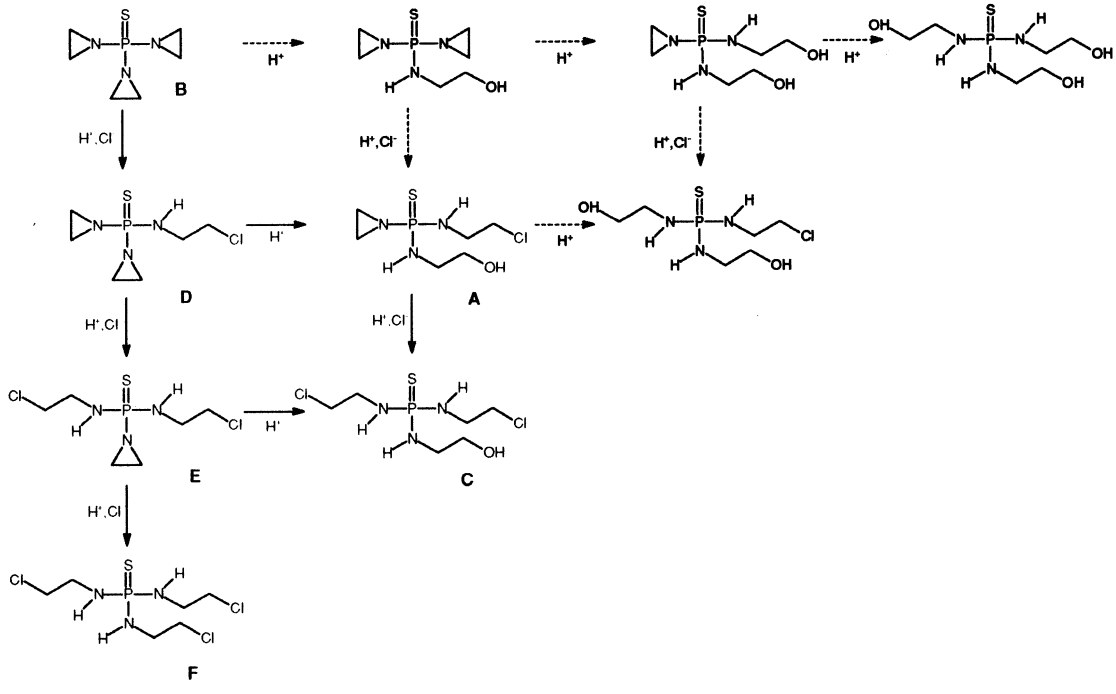

Fig. 5. Proposed degradation scheme (dotted arrow, putative; solid arrow, proven) of thiotepa in the presence or absence of sodium chloride.

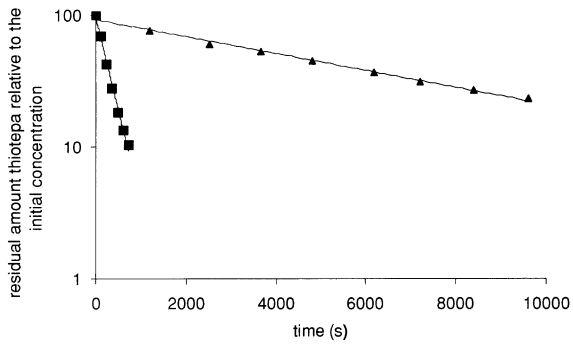

Fig. 6. Disappearance of thiotepa during the degradation in the presence (■) and absence (▲) of sodium chloride at pH 3.

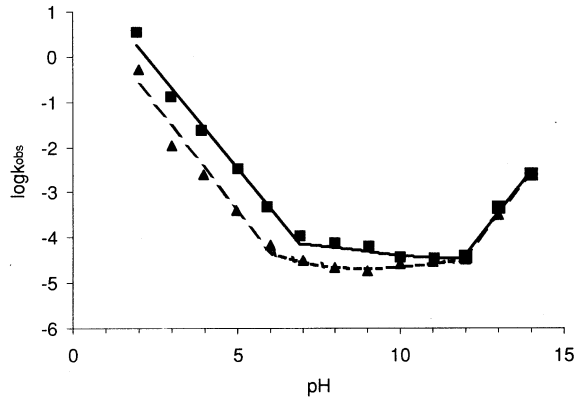

Fig. 7. pH profiles of the degradation of thiotepa in the presence (■) and absence (▲) of sodium chloride.

Degradation experiments at pH < 6 without sodium chloride and at pH < 7 in the presence of sodium chloride were performed at ambient temperature. At higher pH degradations were performed at 80°C. To construct the overall log k_{obs}–pH profile, reaction rate constants determined at ambient temperature were recalculated at 80°C using the Arrhenius equation:

$$\ln k_{obs(T)} = \frac{\ln A - E_a}{RT}$$

in which A is the frequency factor (s^{-1}), E_a is the energy of activation (J/mol), R is the gas constant (8.31 J/K/mol) and T is the absolute temperature (K).

A and E_a (Table 2) are calculated from Arrhenius plots in which the k_{obs} of the degradation of thiotepa at pH 4 with or without sodium chloride were plotted against the inverse of the absolute temperature. The log k_{obs}–pH profiles of the thiotepa degradation at 80°C in the presence and absence of sodium chloride are depicted in Fig. 7. The linear character of the plot at pH < 7 indicates no change in reaction mechanism within this pH range.

Table 2
Parameters calculated from the Arrhenius plot

pH 4	r^2	E_a (J/mol)	A (s^{-1})
With sodium chloride	0.9900	7.5×10^4	3.39×10^9
Without sodium chloride	0.9967	6.87×10^4	3.72×10^7

The pH profiles can be divided in three parts: a proton-catalyzed part (2 < pH < 7), an hydroxyl-catalyzed part (pH > 12) and a solvent-catalyzed part (6 < pH ≤ 12), according to the following formula:

$$k_{obs} = k_0 + k_{H^+}[H^+] + k_{OH^-}[OH^-] + k_{NaCl}[NaCl]$$

in which k_0 is the rate constant of the solvent catalyzed reaction, k_{H^+} is the rate constant of the proton-catalyzed reaction, k_{OH^-} is the rate constant of the hydroxyl-catalyzed reaction and k_{NaCl} is the rate constant of the sodium chloride-catalyzed reaction. For pH < 7 and pH > 11 the slope was calculated from the pH–log k_{obs} profile. In the absence of chloride the slopes were −0.92 and +0.95, indicating a specific proton- and hydroxyl-catalyzed reaction, respectively. In the presence of chloride the slope at pH < 7 was −0.87, due to the influence of chloride. At pH > 11 the slope was 0.92, showing no influence of chloride on the degradation reaction, with no indications for the formation of any chloro adducts. In Fig. 8 the influence of the sodium chloride concentration on the k_{obs} at pH 3 and 5 is depicted. The reaction rate constant of the chloride-catalyzed reaction (k_{Cl}) can be calculated from the slope of this plot. The k_{Cl} is 1.30×10^{-2}

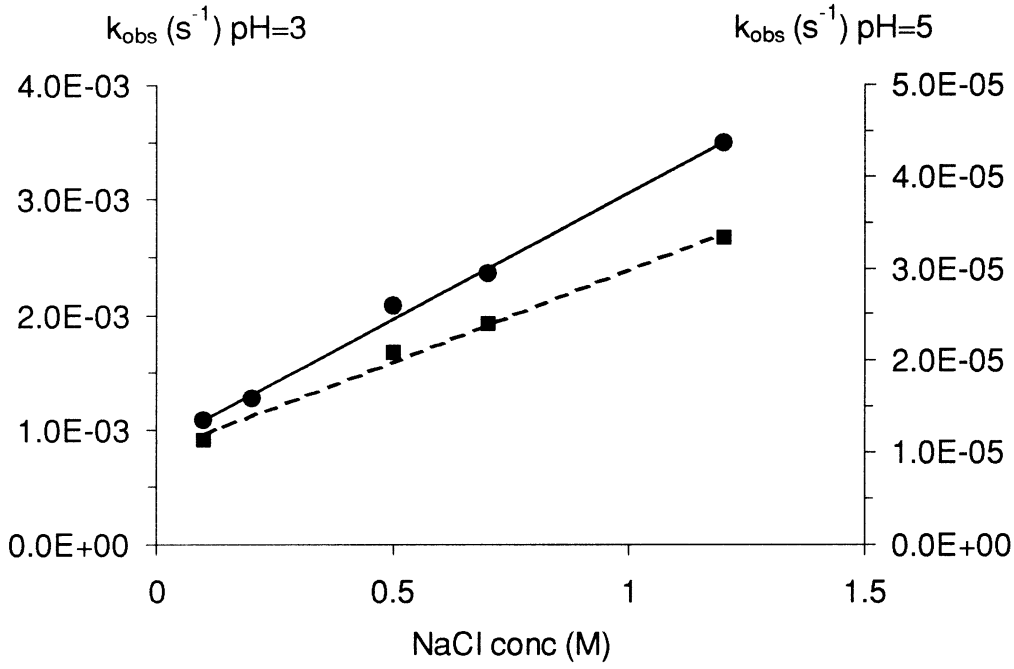

Fig. 8. Influence of the sodium chloride concentration on the observed reaction rate constant (■, pH 3; ●, pH 5).

$M^{-1}\,s^{-1}$ for pH 3 and $2.74 \times 10^{-5}\,M^{-1}\,s^{-1}$ for pH 5.

4. Discussion

Several studies on the chemical degradation of thiotepa in buffered media have been published (Mellet and Woods, 1960; Benckhuijsen, 1968; Maxwell et al., 1974; Zon et al., 1976; Cohen et al., 1984; Pyatigorskaya et al., 1987; Murray et al., 1997). Qualitative analyses were generally performed using thin-layer chromatography (Maxwell et al., 1974; Cohen et al., 1984; Pyatigorskaya et al., 1987), which gave no insight into the quantities of the products formed. In this study an HPLC system with UV detection was used which allowed both the quantification of thiotepa and the degradation products.

Apart from the mono- and di-chloro adducts of thiotepa (Maxwell et al., 1974; Pyatigorskaya et al., 1987; Murray et al., 1997) we have identified 1,2,3-trichloroethylthiophosphoramide after degradation in acidic environment in the presence of sodium chloride. The formation of chloro adducts is in agreement with the chemical properties of the aziridine moiety in thiotepa. In acidic environment in the presence of a halogenide, it is coupled to the aziridine to form a 2-haloethylamine (Bestian, 1950; Dermer and Ham, 1969). Thiotepa, classified as an activated aziridine, will also undergo ring opening with nucleophilic reagents, even in the absence of acid (Dermer and

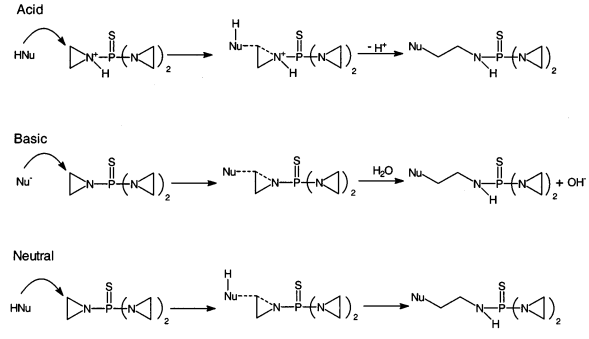

Fig. 9. Ring opening reactions of thiotepa with a nucleophilic reagent in acidic, basic and neutral media (Dermer and Ham, 1969).

Ham, 1969). In Fig. 9 the ring opening reactions of thiotepa with a nucleophilic reagent in acid, basic and neutral media is given (Dermer and Ham, 1969). This reaction scheme can explain the absence of degradation products during the degradation in alkaline media. When thiotepa reacts with a hydroxyl as nucleophil, hydroxyl adducts are formed with more polar properties than thiotepa. The adducts will elute in the dead volume and will therefore not be adequately detected.

The sparse reported kinetic data of the degradation of thiotepa are in contradiction. Mellet and Woods (1960) reported a loss of 95% thiotepa within 30 min at 38°C in 0.1 M acetate buffer pH 4.2, while Cohen et al. (1984) found a decrease of 25% under the same conditions. Calculated from the k_{obs} profile and the Arrhenius plot in this study, an amount of 90% thiotepa should be present after 30 min.

This systematic study of the degradation of thiotepa has provided additional information to previous studies and the results can be a guide for the search for new metabolites of thiotepa.

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