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Effects of pH and Temperature on the Stability and Decomposition of *N,N′N″*-Triethylenethiophosphoramide in Urine and Buffer[1]

Brian E. Cohen, Merrill J. Egorin, M. S. Balachandran Nayar, and Peter L. Gutierrez[2]

Division of Developmental Therapeutics, University of Maryland Cancer Center, Baltimore, Maryland 21201

ABSTRACT

N,N′,N″-Triethylenethiophosphoramide (thiotepa) was dissolved at 100 μg/ml in urine or in 0.1 M sodium acetate buffer and incubated at 37° or 22°. After 0, 15, 30, 60, 90, and 120 min of incubation, 0.1-ml samples were extracted into ethyl acetate and analyzed by gas-liquid chromatography (1.8-m x 2-mm column packed with 3% OV225 on 100/120 Supelcoport; oven at 180°; injection port and nitrogen-phorphorus detector at 230°). Thiotepa was more stable at 22° than at 37° and at pH 6 to 7 than at pH 4 to 5.5. After 2 hr of incubation at 37°, thiotepa concentrations decreased by 40% at pH 5.0 but only 10% at pH 6 or 7. Although thiotepa concentrations declined as described above, alkylating activity, as assessed by *p*-nitrobenzyl pyridine reactivity, was stable at all temperatures and pHs tested. Partition coefficients of thiotepa degradation products into toluene, ethyl acetate, diethyl ether, and hexane were determined after 0 and 120 min of incubation in urine at pH 4.0. The extractability of alkylating activity into these organic solvents decreased dramatically after 120 min. Thiotepa degradation products were extracted from urine at pH 4.0 after 0, 30, 60, and 120 min incubation at 37° and were separated by thin-layer chromatography. In addition to thiotepa (R_f 0.15), 3 degradation products possessing *p*-nitrobenzyl pyridine alkylating activity (R_f 0.35, 0.52, and 0.60) were observed during the course of incubation. The structures of the materials with R_f 0.35 and 0.52 were identified by mass spectrometry and indicated that thiotepa degradation occurs by successive addition of HCl molecules with opening of the aziridine rings and conversion to 2-chloroethyl moieties.

INTRODUCTION

Thiotepa[3] is an alkylating agent the clinical activity of which has been known for approximately 30 years (21) and which is used presently in the chemotherapy of metastatic carcinoma of the breast (10, 18), carcinomatous meningitis (9, 23), and superficial carcinoma of the bladder (6, 11, 16, 24, 25). Reflecting the era of its development and initial clinical evaluation, chemical and pharmacological studies of thiotepa utilized methods, such as radiolabeled drug (1, 3, 5, 14, 20), paper chromatography (3, 5), and fluorometric (15) or spectrophotometric assays (1, 4, 19, 20, 22), that were relatively insensitive, cumbersome, and often nonspecific.

The paucity of precise pharmacological data concerning thiotepa raises the issue of whether the drug is used currently in an optimal fashion. More specifically, the use of thiotepa to treat superficial carcinoma of the bladder must be considered in light of conflicting reports on the stability of thiotepa in solution (11, 12, 14, 15, 26), the absorption of thiotepa from the bladder (12, 17), and the structures of degradation products of thiotepa (2, 26). Several investigators have described the stability of thiotepa in various media (11, 12, 14, 15, 26). To date, however, there has been no systematic definition of the stability of the drug in urine. Moreover, although previous investigators have documented the presence of alkylating activity in the plasma of patients receiving intravesical instillations of thiotepa (12, 17), the exact nature of this material remains undefined. It may be that decomposition of thiotepa in urine results in alkylating compounds that are absorbed differently than is parent compound. As such, the behavior of thiotepa in an individual patient's urine of given pH and ionic strength may produce variations in the systemic exposure to active cytotoxic compounds and the subsequent systemic manifestations of local antineoplastic therapy. Alternatively, decomposition of thiotepa to inactive compounds may reduce the exposure of bladder mucosa to effective drug. The pursuit of this concept has been further impeded by uncertainty as to the exact structures of degradation products of thiotepa (2, 26). The recent development by Grochow *et al.* of a highly sensitive and specific GLC assay for thiotepa (8) and the availability of mass spectrometric technology for structure identification allowed us to undertake a systematic description of the behavior of thiotepa in urine and buffer. This study was to provide a foundation for subsequent clinical investigations at our institution.

MATERIALS AND METHODS

Thiotepa was graciously provided by Lederle Laboratories (Pearl River, NY) and proven to be >99% pure by the GLC system described below. Thiotepa was dissolved to a final concentration of 100 μg/ml in freshly voided urine or 0.1 M sodium acetate buffer that had been adjusted to a pH between 4.0 and 7.0 with HCl or NaOH. These solutions were incubated at 22° or 37°. At 0, 15, 30, 60, 90, and 120 min after addition of thiotepa, triplicate 0.1-ml samples of the solution were placed into separate 1.5-ml Eppendorf microtubes and immediately extracted with 0.4 ml of ethyl acetate (Fisher Scientific Co., Fairlawn, NJ) containing diphenylamine (50 μg/ml) (Sigma Chemical Co., St. Louis, MO) as an internal standard. The samples were centrifuged at 1000 × *g* for 5 min at 4° in a Beckman Microfuge 12 (Beckman Instrument Co., Palo Alto, CA). One μl of the ethyl acetate supernatant was injected into a Hewlett Packard 5840A gas chromatograph (Hewlett Packard Co., Palo Alto, CA) fitted with a 1.8-m x 2-mm (internal diameter) glass column containing 3% OV 225 on 100/120 mesh Supelcoport (Supelco Inc., Belfonte, PA). The oven and injection port were maintained at 180° and 230°, respectively. Nitrogen, at a flow rate of 30 ml/min, was utilized as a carrier gas. Detection was accomplished with a nitrogen-phosphorus detector that was maintained at 230° with an air flow rate of 100 ml/min, a hydrogen

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: thiotepa, *N,N′,N″*-triethylenethiophosphoramide, GLC, gas-liquid chromatography; TLC, thin-layer chromatography; NBP, *p*-nitrobenzyl pyridine.

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flow rate of 7 ml/min, and a bead voltage of 18 to 22 V. Peaks were traced and integrated with a Hewlett Packard 5840A GC terminal, and Thiotepa concentrations were calculated by comparison with the area of the internal standard peak. Thiotepa decay was calculated based on the concentration of Thiotepa present in each solution at the start of the incubation.

In subsequent experiments, Thiotepa was dissolved to a final concentration of 100 μg/ml in urine adjusted to pH 4.0 with HCl, and solutions were incubated at 37°. At 0, 15, 30, 60, 90, and 120 min after the addition of thiotepa, triplicate samples were analyzed by GLC as described above, and duplicate 1-ml samples were immediately frozen in a dry ice/methanol bath and stored frozen until analyzed for alkylating activity. Alkylating activity was assessed with NBP by the method of Friedman and Boger (7) and compared to simultaneously performed standard curves of thiotepa and nor-nitrogen mustard. When assessed by this colorimetric method, alkylating activity is expressed in terms of reactivity with NBP and may be different for different nucleophiles.

To elucidate further the nature of alkylating compounds generated from thiotepa, urine, at pH 4.0 and initially containing thiotepa (100 μg/ml), was incubated at 37° for 0 or 120 min. At each time, aliquots were extracted with 4 volumes of ethyl acetate, toluene, diethyl ether, or hexane. One-ml samples of the aqueous phase were immediately frozen in a dry ice:methanol bath and kept frozen until assayed for alkylating activity. Four ml of the organic phase were evaporated to dryness under nitrogen and frozen until alkylating activity could be assessed. Immediately prior to analysis of alkylating activity, which was always performed within 12 hr of sample preparation, the evaporated organic phases were reconstituted in 1 ml of distilled, deionized water, and aqueous samples were thawed. All samples were then assayed simultaneously for alkylating activity, and the ratio of organic to aqueous absorbance was determined.

TLC was used to monitor the time course and nature of alkylating species generated at 37° from solutions of thiotepa (100 μg/ml) in urine or saline at pH 4.0. After 0, 30, 60, or 120 min of incubation, 1-ml aliquots of the incubation mixtures were extracted with 4 ml of toluene. Samples of the incubation mixtures and the toluene extracts were spotted onto 250-μm Silica Gel 60 TLC plates (E. Merck, Darmstadt, Germany). Plates were developed in an ascending fashion to a solvent front of 15 cm in ethyl acetate:toluene (1:1). Alkylating species were identified by spraying the plates with NBP (1 g/100 ml) in 50% acetone:50% water containing 0.1% glacial acetic acid, heating the plates at 110° for 20 min, and then spraying them with 1 N NaOH. With this method, compounds with alkylating activity appeared as blue spots which faded rapidly.

For final structural identification, areas of TLC plates containing individual alkylating species were scraped from the plates and then extracted with chloroform. The extracts were dried under nitrogen, redissolved in ethyl acetate, and analyzed by direct-probe, electron-impact analysis on a VG Micromass 30F mass spectrometer (VG Analytical, Altrincham, UK). The mass spectometer was operated under VG Data Systems 2040 computer control with 200° source temperature, 70 eV electron energy, 4 kV accelerating voltage, and 170 μa trap current.

RESULTS

The stability of thiotepa in urine and acetate buffer was highly dependent upon pH, with increasingly more rapid decay observed at progressively lower pHs (Table 1, Chart 1). Moreover, the acid lability of thiotepa was enhanced at body temperature, there being a more rapid decay in all solutions at 37° (Table 1; Chart 1) than at 22° (data not shown). The decay of thiotepa in acetate buffer was similar to that in urine at the same pH. The pH of buffered urine and acetate solutions of thiotepa was stable for the duration of these experiments.

Although there was clearly a loss of thiotepa at various clinically relevant pHs and temperatures, the GLC system utilized to characterize the decay was specific for parent compound only and could not, by itself, define whether there was a loss of biologically relevant alkylating activity. This possibility was evaluated by assaying concomitant urine samples for thiotepa by GLC and for alkylating activity by the NBP spectrophotometric assay. Control studies demonstrated that urine and buffer without thiotepa had no intrinsic alkylating activity and that the NBP assay was linear in the range of concentrations of alkylating activity used in these studies. The results of matched samples analyzed for both thiotepa and alkylating activity are presented in Table 1. In urine at pH 7.0, there was no loss of thiotepa or alkylating activity. After 120 min in urine at pH 5.5, there was a 20% loss of parent compound but no loss of alkylating activity. This discrepancy was even greater in urine at pH 4.0 and in buffer at pH 4.2, where there was 98 and 69% loss of thiotepa, respectively, but no loss of alkylating activity.

In an effort to gain more information about the nature of the breakdown products of thiotepa, which obviously possessed alkylating activity, the extractability of alkylating activity from

Table 1

Decay of thiotepa and alkylating activity in urine and sodium acetate buffer

Thiotepa was dissolved to a final concentration of 100 μg/ml in urine or 0.1 M sodium acetate buffer which had been adjusted to the specified pH. After 0, 15, 30, 60, 90, and 120 min of incubation at 37°, 1-ml samples were immediately frozen in a dry ice:methanol bath and stored frozen. Also, 0.1-ml samples were extracted with 0.4 ml of ethyl acetate containing diphenylamine (50 μg/ml) as an internal standard. These were centrifuged, and the supernatant was analyzed by GLC. The frozen samples were thawed and analyzed for alkylating activity with NBP by the method of Friedman and Boger (7).

		% remaining after:													
		0 min		15 min		30 min		60 min		90 min		120 min		k (min^{-1})	
Medium	pH	Thiotepa[a]	AA[b,c]	Thiotepa	AA	Thiotepa	AA	Thiotepa	AA	Thiotepa	AA	Thiotepa	AA	Thiotepa	AA
Urine	7.0	100	100	101.9[d] ±0.1	107.0[e] ±5.4	98.8 ±0.7	112.2 ±3.3	99.1 ±0.9	111.3 ±9.7	99.0 ±1.8	117.9 ±1.2	97.9 ±1.6	111.6 ±2.9	0.0000	0.0000
Urine	5.5	100	100	98.9 ±0.1	104.5 ±1.7	91.9 ±2.7	96.9 ±3.5	90.8 ±1.4	105.8 ±0.4	85.9 ±2.8	93.5 ±7.2	81.6 ±2.4	105.2 ±4.7	0.0015	0.0000
Urine	4.0	100	100	66.1 ±3.0	6.2 ±4.9	40.6 ±0.6	119.8 ±22.5	15.2 ±0.5	96.2 ±8.1	5.21 ±0.3	92.9 ±15.7	2.1 ±0.2	110.0 ±26.3	0.0316	0.0000
Buffer	4.2	100	100	87.0 ±1.4	96.9 ±4.3	74.6 ±1.9	95.6 ±2.7	54.3 ±1.7	94.7 ±2.0	42.1 ±0.1	86.0 ±1.5	31.0 ±0.6	89.0 ±3.7	0.0098	0.0011

[a] Analysis of thiotepa by GLC.
[b] AA, alkylating activity.
[c] Analysis of alkylating activity by reaction with NBP.
[d] Mean ± S.E. of 3 experiments, each done in triplicate.
[e] Mean ± S.E. of 3 experiments, each done in duplicate.

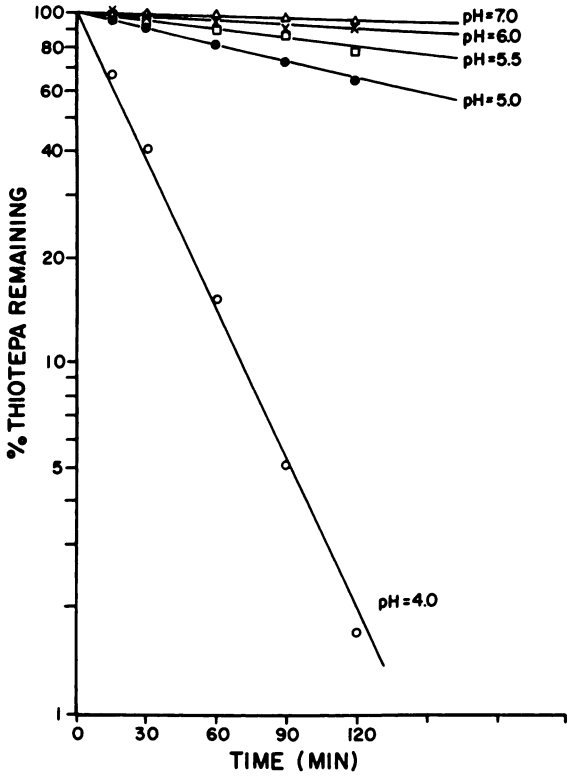

Chart 1. Decay of thiotepa at 37° in urines of varying pH. Thiotepa was dissolved in urine at a concentration of 100 μg/ml and incubated at 37°. At indicated times, thiotepa was extracted from urine into ethyl acetate. Thiotepa concentrations were determined by GLC as described in "Materials and Methods." *Points*, means of 3 experiments, each done in triplicate; *bars*, S.E.

Table 2

Extraction by organic solvents of alkylating activity from urine initially containing thiotepa

Thiotepa was dissolved at a final concentration of 100 μg/ml in urine, pH 4.0 at 37°. After 0 and 120 min of incubation, duplicate samples were extracted with 4 volumes of the specified organic solvent. Four ml of the organic layer were blown to dryness under nitrogen and frozen until alkylating activity could be assessed. One ml of the aqueous phase was immediately frozen in a dry ice:methanol bath until alkylating activity could be assessed. Alkylating activity was determined with NBP by the method of Friedman and Boger (7).

Solvent	Organic/aqueous (t = 0 min)	Organic/aqueous (t = 120 min)
Toluene	14.45[a]	0.12
Ethyl acetate	4.19	0.14
Diethyl ether	1.15	0.15
Hexane	0.36	0.06

[a] Mean of 2 determinations.

urine at pH 4.0 into several organic solvents was assessed after either 0 or 120 min of incubation at 37° (Table 2). Immediately after the addition of thiotepa, most of the alkylating activity was extractable into organic solvents. However, by 120 min, alkylating activity was much less extractable into organic solvents. Toluene had the greatest ability to extract alkylating activity at the start of the experiment, containing approximately 14.5 times what remained in the aqueous layer. After 120 min, however, only 10% of the alkylating activity was extracted into the toluene layer. This same pattern occurred, but to a lesser extent, with ethyl acetate and diethyl ether. Hexane extracted the alkylating activity poorly both at the start (approximately 26%) and at the end (approximately 6%) of the experiment but still demonstrated a decrease in extractable materials after 120 min.

When the nature of thiotepa breakdown products was further characterized by TLC, 5 spots with alkylating activity were identified. These materials had R_fs of 0, 0.15 (A), 0.35 (B), 0.52 (C), and 0.60 (D), and their relative prominence varied with time, implying conversion of one species to the next. The spot at the origin did not extract into toluene. It appeared by 30 min and was present as a faint spot at all later times. Spots A, B, C, and D did extract into toluene. Thiotepa standard had an R_f of 0.15, and it cochromatographed with Material A. At the onset of incubation, most of the alkylating material resided in Compound A, and a small amount of Compound B was present. At 30 min, there was a small amount of Compound A and moderate amounts of Compounds B and C. By 60 min, there were small amounts of Compounds A and B with moderate amounts of Compounds C and D. Finally, by 120 min, all alkylating activity resided in 2 light spots corresponding to Materials C and D.

Final characterization of acid-induced thiotepa breakdown products required mass spectrometric analysis. The mass spectrum of Compound A, which cochromatographed with thiotepa (R_f 0.15), was identical to that of a thiotepa standard (Chart 2*A*). Specifically, there was a molecular ion at m/z 189 and major ions at m/z 147 and 115 which corresponded to the sequential loss of an aziridine ring and sulfur atom from the molecular ion. The mass spectrum of Compound B (R_f 0.35) had a molecular ion at m/z 225, corresponding to 36 atomic mass units, greater than thiotepa (Chart 2*B*). The fragmentation pattern of Compound B further indicated that this material, like thiotepa, underwent loss of an aziridine ring, producing the ion m/z 183 $(M-42)^+$, and the subsequent loss of a sulfur atom, producing the ion m/z 151. In addition, the ratio of ion m/z 183 to ion m/z 185

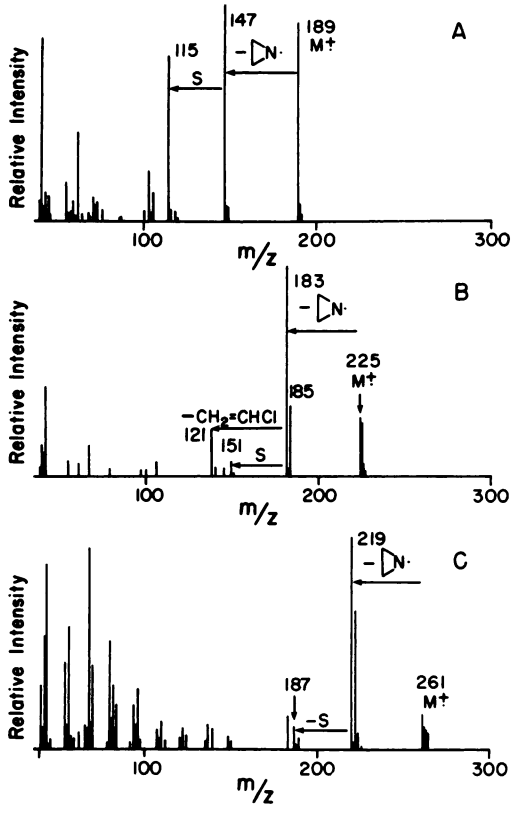

Chart 2. Mass spectra of a thiotepa standard (*A*) and urinary alkylating compounds with R_f 0.15 (*A*), R_f 0.35 (*B*), and R_f 0.52 (*C*).

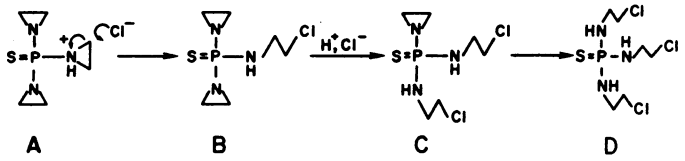

Chart 3. Proposed decomposition scheme of thiotepa in acid urine. The structures of Compounds B and C were confirmed by mass spectrometry. The structure of Compound D was inferred from the chromatographic properties and temporal behavior of thiotepa decomposition products.

was 3:1. This ratio indicates the presence of one chlorine atom in accordance with the relative isotopic abundance of ^{35}Cl and ^{37}Cl. Because of partial chemical ionization present when samples are obtained from preparative TLC plates, the 3:1 ratio of ion *m/z* 225 to ion *m/z* 227 is not clearly seen. Finally, ion *m/z* 183 was capable of losing vinyl chloride via a McLafferty rearrangement to form the ion *m/z* 121. The mass spectrum of Compound C (R_f 0.52) indicated a molecular ion at *m/z* 261 (Chart 2*C*), corresponding to 36 atomic mass units, or one molecule of HCl greater than the molecular ion of Compound B. As with Compounds A and B, the fragmentation pattern of Compound C indicated sequential loss of an aziridine group and sulfur atom with ions at *m/z* 219 and 187, respectively. The 3:2 ratio of intensity of M+ ions at 261 and 263 and $(M-42)^+$ ions at 219 and 221 implied the presence of 2 chloride atoms in Compound C. Quantities of Compound D sufficient to obtain a mass spectrum could not be isolated during the course of these studies.

DISCUSSION

Although the clinical utility of thiotepa was demonstrated more than a quarter century ago (21) and it represents one of the oldest chemotherapeutic agents still used in clinical oncology, there has been sustained interest in defining clinical roles for thiotepa (6, 9–11, 16, 18, 23–25). On the other hand, there has not been a corresponding effort to utilize modern analytical methodology to delineate the pharmacological and pharmacokinetic properties of thiotepa and to resolve the numerous conflicting reports concerning them (2, 11, 12, 14, 15, 17, 26). The study presented here was prompted by our interest in the utility of intravesical instillation of thiotepa to treat superficial carcinoma of the bladder and our belief that optimal utilization of thiotepa required adequate knowledge of its pharmacological and pharmacokinetic behavior.

Our studies, utilizing clinically relevant concentrations of thiotepa, clearly document the decomposition of this drug in urine at body temperature and at physiological pH and show progressively greater decomposition occurring at progressively lower urinary pHs. On the other hand, thiotepa was far more stable in acetate buffer at pH 4.2 than was reported previously by Mellett and Woods (15). Although thiotepa can decompose in urine under physiological conditions, this process may not produce a loss of antineoplastic drug, since there was no corresponding decrease in the urinary concentration of alkylating activity. Rather, a series of compounds with alkylating potential was generated when thiotepa was incubated in acidified urine, and the temporal aspects of the process implied sequential conversion of one material to another. Mass spectral analyses of these materials confirmed thiotepa as the major alkylating species present at the start of the incubation and implied successive conversion of the aziridine rings of thiotepa to 2-chloroethyl moieties (Chart 3). Although only *N,N′*-diethylene,*N″*-2-chloroethylthiophosphoramide (Chart 3*B*) (R_f 0.35) and *N*-ethylene,*N′,N″*-bis-2-chloroethylthiophosphoramide (Chart 3*C*) (R_f 0.52) were identified, the temporal and chromatographic properties of the urinary alkylating material with R_f 0.60 (Chart 3*D*) argue for its identity as *N,N′,N″*-tri-2-chloroethyl thiophosphoramide. These results are compatible with earlier studies of Maxwell *et al.* (13), who reported the conversion of thiotepa in acidified NaCl to materials which cochromatographed with *N,N′*-diethylene,*N″*-2-chloroethylthiophosphoramide and, possibly, *N*-ethylene,*N′,N″*-bis-2-chloroethylthiophosphoramide. The alkylating activity remaining in the aqueous phase, *i.e.*, not extractable into toluene, may reflect P—N bond cleavage with ring opening or aziridine liberation as reported by Zon *et al.* (26) in studies using nuclear magnetic resonance spectrometry. On the other hand, we found no evidence of rearrangement of thiotepa by intramolecular alkylation as reported by Benckhuijsen (2).

As stated previously, the results of this systematic study of the behavior of thiotepa in urine may provide a basis for a better understanding of thiotepa as it is currently used in the treatment of superficial carcinoma of the bladder. Under current practices, no attempt is made to adjust a patient's urinary pH when thiotepa is instilled into the bladder. Moreover, the formulation of thiotepa provides little buffering capacity. As a result, there is likely to be variation in the decomposition of thiotepa within a population of patients with urinary pHs between 5 and 7. The initial impression that increased decomposition of thiotepa might reduce the effective dose of drug to which the bladder mucosa is exposed is contradicted by the preservation of alkylating activity and the characterization of thiotepa degradation products as active alkylating compounds containing 2-chloroethyl moieties. On the other hand, the conversion of thiotepa to active alkylating compounds with altered polarity may affect absorption of drug from the bladder and result in altered myelosuppression, the major toxicity of intravesical thiotepa therapy. With these data and concepts in hand, we are attempting to evaluate prospectively the relationship between urinary pH, drug decay, and absorption and subsequent clinical effects in patients receiving intravesical thiotepa therapy.

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