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2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone II: Isolation and Characterization of Degradation Products

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Abstract □ A direct high-performance liquid chromatographic (HPLC) method was applied to monitor 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (I) and its degradation products in pharmaceutical vehicles at 25 ± 0.1°. At the optimal pH for stability of I, an increase in buffer concentration [phosphate and tris(hydroxymethyl)-aminomethane] or ionic strength accelerated degradation. The reaction rate in the solutions studied followed pseudo-first-order kinetics. Degradation products were characterized by mass spectrometry after isolation by semipreparative HPLC. Different degradation pathways prevailed in acidic and basic media. The acid-catalyzed reaction resulted in consecutive aziridine ring opening, while the base-catalyzed reaction led to nucleophilic displacement of the aziridine ring(s).

Keyphrases □ 2,5-Diaziridinyl - 3,6 - bis(carboethoxyamino)-1,4-benzoquinone—isolation and characterization of degradation products □ Degradation—2,5-diaziridinyl -3,6- bis(carboethoxyamino)-1,4-benzoquinone, isolation and characterization of degradation products □ High-performance liquid chromatography—analysis, 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone and degradation products

The chemical behavior of a new drug in solution as well as the number and nature of degradation products and any impurities present in such a solution is always important. 2,5-Diaziridinyl-3,6 - bis(carboethoxyamino)-1,4-benzoquinone (I), a potential central nervous system (CNS) antitumor agent administered intravenously, is currently in Phase I of clinical trial. As part of a continuing study concerning the reactivity of I (1), the high-performance liquid chromatographic (HPLC) assay was applied to assess the influence of various pharmaceutical parameters (nature and concentration of the buffer, ionic strength, and storage conditions) on the stability of I in aqueous solution.

This report also describes the semipreparative HPLC isolation and the mass spectrometric structure determination of the major impurity as well as the degradation products of I generated in various pharmaceutical media. A combination of other methods (UV and NMR spectroscopy) and comparison with authentic synthetic materials also were used when sufficient sample was available.

This investigation defined the degradation behavior of I in solution with various pharmaceutically useful reagents to optimize its formulation and proper pharmaceutical use. In addition, the information accumulated here will serve as a foundation for the delineation of degradation products and metabolites of I in future pharmacokinetic studies.

EXPERIMENTAL

Reagents—Compound I (NSC 182986)[1] and the internal standard, 5-chloro-2-pyridinol[2], were used as supplied. 2-Aziridinyl-5-(2′-hydroxyethanolamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone (II) and 2,5 - di(2′-hydroxyethylamino)-3,6-bis(carboethoxyamino) - 1,4-benzoquinone (III) were isolated by semipreparative HPLC from partially hydrolyzed I and were characterized as will be described. Acetonitrile[3] (distilled in glass) and distilled water were filtered through 0.45-μm

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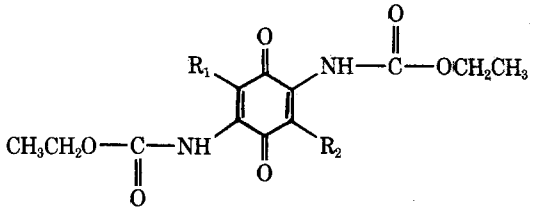

I: $R_1 = R_2 = -N\triangleleft$

II: $R_1 = NHCH_2CH_2OH$, $R_2 = -N\triangleleft$

IIa: $R_1 = NHCH_2CH_2OCOCH_3$, $R_2 = -N\triangleleft$

III: $R_1 = R_2 = NHCH_2CH_2OH$

IIIa: $R_1 = R_2 = NHCH_2CH_2OCOCH_3$

V: $R_1 = NHCH_2CH_2OH$, $R_2 = NHCH_2CH_2Cl$

VI: $R_1 = -N\triangleleft$, $R_2 = NHCH_2CH_2Cl$

VII: $R_1 = R_2 = NHCH_2CH_2Cl$

VIII: $R_1 = R_2 = OH$

IX: $R_1 = -N\triangleleft$, $R_2 = OH$

X: $R_1 = NH_2$, $R_2 = OH$

solvent-resistant filters[4]. All other chemicals were reagent grade.

Preparation of Solutions—Solutions of I (2.75×10^{-4} *M*) were prepared as described previously (1) in appropriate media at the optimum pH[5] (pH 6.5) and stored at 25 ± 0.1° under oxygen, nitrogen, or air under fluorescent lighting or in the dark. Experiments also were conducted in different concentrations (0.01, 0.1, and 0.5 *M*) of pH 6.5 phosphate buffer or tris(hydroxymethyl)aminomethane hydrochloride buffer as well as in solutions of various ionic strengths (0.016–0.70 μ) obtained by the addition of sodium chloride or sodium nitrate.

For isolation of the desired benzoquinone decomposition products, solutions of I in pH 3–8 buffer were subjected to degradation at ambient temperature. Different major products were obtained in different pH solutions. Compound I also was allowed to degrade in pH 6.5 tris(hydroxymethyl)aminomethane buffer, sodium chloride solution, and at alkaline pH.

HPLC—A modular high-pressure liquid chromatograph consisting of a constant-flow pump[6] coupled with a rotary valve injector[7], a 10-μl injection loop, and a 5-μm reversed-phase column[8] (250 × 3 mm i.d.) was used to deliver the eluent at a rate of 1 ml/min. A variable-wavelength UV detector[9] set at 345 nm and 0.1 aufs deflection was used to detect eluted compounds. The output signals were recorded with a strip-chart recorder[10]. All chromatographic operations were performed at ambient temperature. Chromatographic conditions in the analytical mode were described previously (1).

Semipreparative separation and isolation of I and its degradation products were carried out on a 10-μm octadecylsilane column[11] (500 × 9.20 mm i.d.). The chromatographic profile of the sample was determined first on the analytical column[8]. Then an analytical injection was made on the semipreparative column, using 15% acetonitrile[3] in distilled water as the eluent at a flow rate of 5 ml/min. A concentrated solution of the sample was diluted to the proper concentration with the mobile phase and clarified through a 0.45-μm solvent-resistant filter[4] prior to injection of 250 μl onto the semipreparative column. Acetonitrile was removed under reduced pressure[12] from each collected fraction, and the residual aqueous solution was frozen immediately in dry ice and lyophilized under low temperature in a conventional freeze drier[13]. The resulting solid was subjected to further analysis.

Mass Spectrometry—All derivatization reactions were carried out in glass screw-capped vials[14] (3.5 ml) or Reacti-vials[14] (1.0 ml) equipped with polytef-lined rubber septums. Samples (0.3–2 mg) were trimethylsilylated by reaction with 0.45 ml of a 2:1 mixture of redistilled acetonitrile[3] and bis(trimethylsilyl)trifluoroacetamide[2] for 1–2 hr at room temperature. Acetylation was effected by reaction with 0.3 ml of a 1:2 mixture of redistilled acetic anhydride[15] and redistilled pyridine for 3 hr at room temperature. Aliquots of the derivatization mixtures were subjected to mass spectral analysis after transfer to glass capillary sample holders and *in vacuo* removal of excess reagents.

All mass spectra were obtained by direct-probe introduction of the sample into the ion source of a mass spectrometer[16] operated under data system[17] control. When the sample was known to be a mixture of compounds, fractional vaporization was used to obtain spectra of the individual components. Typical mass spectrometer (electron-impact) operating conditions were: resolution, 1000 (10% valley); ion source, 265°; electron energy, 75 ev; ionizing current, 250 μamp; and scan speed, 2 sec/decade. Accurate mass measurements (I, VIII, and X) were obtained under data system control at a scan rate of 10 sec/decade and a dynamic resolution of 5000 using perfluorokerosene as an internal reference. Probable elemental compositions of the various fragment ions were calculated using the commercially supplied data system software.

Table I—Mass Spectra of Acetylated II and III

IIa Mass (Relative Intensity)	IIIa Mass (Relative Intensity)	Probable Assignment
424 (3)	484 (18)	M^+
378 (2)	438 (8)	$M - CH_3CH_2OH$
351 (4)	411 (11)	$M - CH_2OOCCH_3$
332 (1)	392 (6)	$M - 2\,CH_3CH_2OH$
318 (4)	378 (2)	$M - CH_3CH_2OH - CH_3COOH$
305 (4)	365 (4)	$M - CH_3CH_2OH - CH_2OOCCH_3$
272 (10)	332 (2)	$M - 2\,CH_3CH_2OH - CH_3COOH$
—	305 (6)	$M - CH_3CH_2OH - CH_3COOH - CH_2OOCCH_3$
87 (10)	87 (96)	$C_4H_7O_2$
60 (25)	—	CH_3COOH
45 (79)	45 (21)	$C_2H_5O^+$
43 (100)	43 (100)	$CH_3C{\equiv}O^+$
31 (84)	31 (40)	$CH_2{=}O^+H$
29 (10)	29 (50)	$C_2H_5^+$

General Methods—Melting points[18] were determined in open glass capillaries and are uncorrected. UV spectra were obtained with a scanning UV–visible spectrophotometer[19]. NMR spectra were recorded[20] in deuterated dimethyl sulfoxide (containing 1% tetramethylsilane)[21]. Microanalyses[22] for carbon, hydrogen, and nitrogen were within 0.4% of the calculated values.

2,5-Di(2-hydroxyethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone (III)—Compound I (0.5 g, 1.37 mmoles) was dissolved in *N,N*-dimethylacetamide (20 ml), and the resulting solution was diluted with water (225 ml). The solution was adjusted to pH 3 with 0.1 *N* aqueous HCl and stirred at ambient temperature for 24 hr. The reaction was followed to completion by HPLC, and the resulting solution was freeze dried. Recrystallization of the solid from benzene gave III as red crystals, 0.45 g (82%), mp 182–184°; UV (water–acetonitrile, 85:15): λ_{max} 343 (log ϵ 4.27) and 237 (3.94) nm; NMR (dimethyl sulfoxide-d_6): δ 1.20 (t, 6H, 2 CH_3), 3.40–3.70 (m, 8H, 2 NCH_2CH_2O), 4.05 (q, 4H, 2 CH_2CH_3), 4.05 (s, 2H, 2 OH), 7.30 (s, 2H, 2 NH), and 7.90 (s, 2H, 2 NH of amide) ppm. The mass spectral data (acetylated) are given in Table I.

Anal.—Calc. for $C_{16}H_{24}N_4O_8$: C, 48.00; H, 6.04; N, 13.99. Found: C, 48.20; H, 6.08; N, 13.69.

2,5 - Dihydroxy - 3,6 - bis(carboethoxyamino) - 1,4 - benzoquinone (VIII)—Compound I (0.18 g, 0.5 mmole) dissolved in *N,N*-dimethylacetamide (8 ml) was diluted with 50 ml of 0.02 *N* aqueous NaOH. The resulting solution was stirred overnight at room temperature and filtered. The filtrate was acidified with dilute hydrochloric acid and extracted into chloroform. The extracts were dried (sodium sulfate), filtered, and con-

[4] Millipore, Bedford, Mass.
[5] Beckman Zeromatic pH meter, Beckman Instruments, Irvine, Calif.
[6] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[7] Valco Instruments Co., Houston, Tex.
[8] Spherisorb ODS (5 μm), Spectra-Physics, Santa Clara, Calif.
[9] Model SP 770, Spectra-Physics, Santa Clara, Calif.
[10] OmniScribe, Houston Instruments, Austin, Tex.
[11] Partisil M9, Whatman Inc., Clifton, N.J.
[12] Buchi Rotavapor-R, Brinkmann Instruments, Westbury, N.Y.
[13] 10-MRSA Freezemobile, Virtis Co., Gardiner, N.Y.
[14] Pierce Chemical Co., Rockford, Ill.
[15] B & A, Morristown, N.J.
[16] DuPont 21-492B, DuPont Instruments, Monrovia, Calif.
[17] VG 2040, VG Data Systems, Altrincham, Cheshire, England.
[18] Thomas-Hoover Uni-Melt apparatus, A. H. Thomas, Philadelphia, Pa.
[19] Cary 15 recording spectrophotometer, Varian Associates, Monrovia, Calif.
[20] T-60 spectrometer, Varian Associates, Palo Alto, Calif.
[21] Silanor, Merck & Co., Rahway, N.J.
[22] National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md.

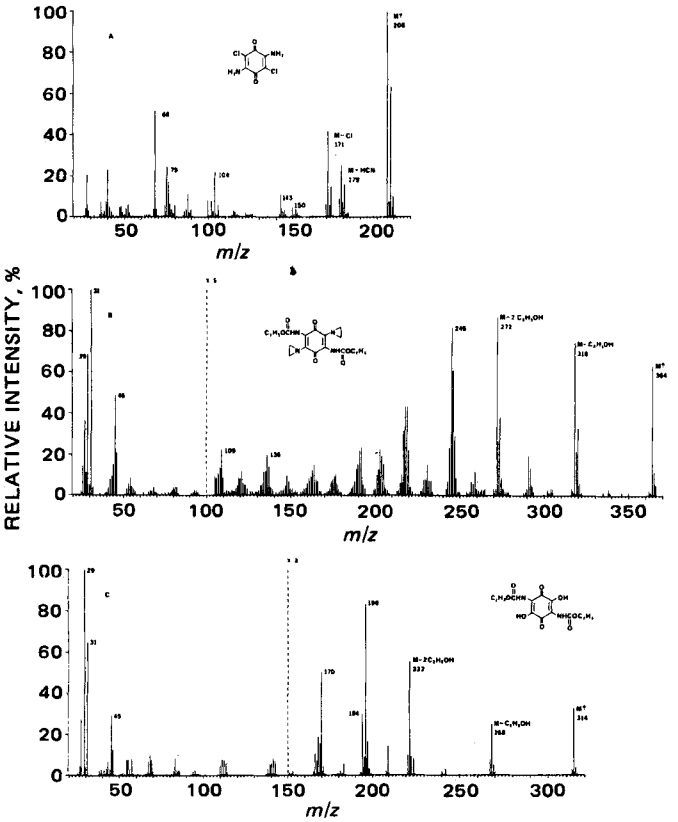

Figure 1—*Direct-probe electron-impact mass spectrum of IV (A), I isolated by HPLC (B), and VIII (C).*

centrated under reduced pressure. Recrystallization of the residue from methanol–water yielded VIII as reddish crystals, 0.125 g (80%), mp 197–200° dec.; UV (methanol): λ_{max} 304 (log ϵ 4.06) nm; NMR (dimethyl sulfoxide-d_6): δ 1.25 (t, 6H, 2 CH_3), 4.03 (q, 4H, 2 CH_2CH_3), 4.58 (broad, 2H, 2 OH), and 8.10 (s, 2H, 2 NH) ppm. The mass spectrum is shown in Fig. 1C.

Anal.—Calc. for $C_{12}H_{14}N_2O_8$: C, 45.86; H, 4.49; N, 8.91. Found: C, 46.10; H, 4.68; N, 8.62.

RESULTS AND DISCUSSION

Separation and Identification of Hydrolysis Products of I—The isolation and identification of the major impurity associated with I and the degradation products generated in various pharmaceutical media were accomplished by semipreparative HPLC and mass spectrometry. The retention order of I and its degradation products on the semipreparative column was identical to that of the analytical column. When desired, the retention times were adjusted by varying the proportion of the organic component of the eluent.

Molecular ion peaks were present in the mass spectra of all compounds examined, and the mass spectral fragmentation patterns allowed determination of the various functional groups present. When needed, derivatization by acetylation or silylation was employed to increase the volatility of selected degradation products for direct-probe analysis. Acetylation was preferred over silylation, even though the persilylated derivatives were much more volatile, because the former procedure added much less mass to the molecular weight of the derivative and the structural features were delineated more clearly in the mass spectrum.

The mass spectrum of the most abundant HPLC-isolated impurity of I[23] indicated an apparent molecular weight of 206 and the 9:6:1 isotopic pattern indicative of two chlorine atoms (Fig. 1A). Comparison of this mass spectrum with that of an authentic sample of 2,5-diamino-3,6-dichloro-1,4-benzoquinone[24] (IV), a synthetic intermediate of I, confirmed the identity. The mass spectrum of IV was unusual in that the successive losses of two molecules of carbon monoxide were not as prominent as in other 1,4-benzoquinones (2). These ions were observed at *m/z* 178 (M − CO) and 150 (M − 2 CO), but the loss of hydrocyanic acid from the intact molecule (*m/z* 179) was more pronounced. There was no fission into two halves, as is commonly observed with other 1,4-benzoquinones, but fission with hydrogen transfer did occur to give an ion at *m/z* 104 containing one chlorine atom. Loss of hydrogen chloride from this fragment produced an ion of *m/z* 68. The peak at *m/z* 75 probably was due to elimination of 1-amino-2-chloroacetylene with concomitant charge retention.

The mass spectrum of I (Fig. 1B) served as a model for interpreting the mass spectra of the degradation products. In addition to a molecular ion (M^+) peak of moderate intensity at *m/z* 364, the most noticeable feature of this spectrum was the successive loss of two ethanol molecules at *m/z* 318 (M − CH_3CH_2OH) and 272 (M − 2 CH_3CH_2OH), an indication of *N*-substituted ethyl carbamates (3). Compound I was not very volatile and required probe temperatures at or greater than its 230° melting and decomposition point (4) before a recognizable spectrum could be obtained. Thus, the successive losses of ethanol probably occurred thermally as well as during electron impact. Metastable peaks were observed for the transformation, but the low mass portion of the spectrum (below *m/z* 50) was identical to the spectrum of ethanol (5).

When the amount of I was low and there was appreciable water or another hydrogen radical source in the mass spectrometer inlet system or ionization chamber, an appreciable M + 2 peak was observed. This peak has been commonly observed in the mass spectra of other 1,4-benzoquinones and represents formation of the analogous hydroquinone (2). Therefore, the moderately intense A + 2 peaks observed for both *m/z* 318 and 272 probably were due to hydroquinone formation from the isocyanoquinones resulting from thermal decomposition of I with use of the resultant ethanol as a hydrogen radical source. Thus, the peaks at *m/z* 318 and 272 probably represented fragment ions as well as molecular ions produced by thermal decomposition. The other peaks in the spectrum then could be interpreted as resulting from further fragmentation of these ions. Thus, *m/z* 136 would represent fission of the bis(isocyano)quinone of mass 272 into halves, while *m/z* 109 might result from loss of an ethoxy radical from the acetylene formed by 1,3-cleavage of I.

Compound III, the major and ultimate degradation product of I in acidic media, was isolated by HPLC and characterized as 2,5-di(2′-hydroxyethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone. The acetylated derivative (III*a*) was more volatile and exhibited less thermal decomposition than the parent compound. The mass spectral analysis of III*a* clearly indicated its molecular weight (*m/z* 484) as well as all pertinent structural features (Table I). Successive losses of ethanol indicated intact *N*-substituted ethyl carbamate moieties (3), while loss of 73 amu (*e.g.*, *m/z* 411, M − CH_2OCOCH_3) was characteristic of an acetate of a primary alcohol. The high mass end of the spectrum (*m/z* > 300) could be explained by the appropriate combination of the losses of ethanol (46 amu), acetic acid (60 amu), and ·CH_2OCOCH_3 (73 amu). As with I, peaks from ethanol were superimposed on the low mass portion of the spectrum (*m/z* < 50). NMR and elemental analyses of the synthetically obtained III (see *Experimental*) supported the mass spectral data.

Compound II was the second major degradation product of I detected in acidic to neutral media. The concentration of II was pH dependent and had a maximum at pH ~ 5. Mass spectrometry suggested that II was 2-aziridinyl-5-(2′-hydroxyethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone, the intermediate hydrolytic product. Acetylation was employed to make a more volatile derivative, and the mass spectrum of II*a* corroborated the proposed structure (Table I). A molecular ion was evident at *m/z* 424, and most of the structural features present in III*a* also were indicated.

Buffer Effect—The analytical HPLC method described previously (1) was applied to evaluate the effect of various pharmaceutical media at the optimal pH on the degradation rate of I. The pseudo-first-order rate constants for the disappearance of I in aqueous solutions of pH 3.0–8.0 (1) do not necessarily reflect the hydrolysis rate of aziridine rings since anions present in the solution may compete with water molecules for the protonated aziridines. Water is sufficiently acidic to provide a very low concentration of aziridinium ion, which then undergoes reaction with the various nucleophilic reagents present at rates corresponding to the nucleophilicity of the reagent.

The catalytic effect of the buffer systems was determined at constant temperature (25 ± 0.1°), pH (6.5 ± 0.05), ionic strength (1.0), and concentration of I (2.75×10^{-4} *M*) in various strengths (0.01, 0.1, and 0.5 *M*) of phosphate or tris(hydroxymethyl)aminomethane buffer. The disappearance rate of I followed pseudo-first-order kinetics with respect to

[23] The amount of IV was 1.5% (w/w); λ_{max} (acetonitrile–water, 25:75) 338 (log ϵ 4.45).

[24] Provided by Dr. J. S. Driscoll, National Cancer Institute, National Institutes of Health, Bethesda, Md.

Table II—Pseudo-First-Order Rate Constants for Loss of I (2.75×10^{-4} *M*) in Various Concentrations of pH 6.5 Phosphate and Tris(hydroxymethyl)aminomethane Buffer at 25 ± 0.1°

Total Buffer Concentration, *M*	$K_{obs} \times 10^2$, day^{-1}	
	Phosphate Buffer	Tris(hydroxymethyl)aminomethane Buffer
0.01	3.07	3.20
0.1	5.07	5.23
0.5	12.64	13.36

Table III—Retention Volume (V_R), Capacity Factor (k'), Separation Factors (α), and Resolution (R_s) of Various Solutes [a]

Compound	V_R, ml	k'	α	R_s
I	8.50	7.50		
			1.23	2.53
V	10.20	9.20		
			1.96	6.96
VI	19.00	18.00		
			2.28	8.22
VII	42.00	41.00		

[a] Retention data of various solutes from a 250 × 3-mm i.d. stainless steel Spherisorb ODS (5-μm particles) column; the mobile phase was 15% acetonitrile in water at a flow rate of 1 ml/min; UV detection was at 345 nm.

its concentration. The reaction rate constants were computed by the least-squares method. Plots of the observed rate constant of the reactions *versus* buffer concentration showed a direct increase in the disappearance rate of I with an increasing concentration of either buffer (Table II).

Examination of the chromatogram of degraded solutions of I indicated that the reaction is partially a function of phosphate ion. Increased buffer concentration intensified the hydrophilic peak at the void volume while it decreased peaks corresponding to the hydrolytic products (II and III) in the chromatogram. The catalytic effect of phosphate buffer on unsubstituted aziridines (6–9) and its derivatives (10, 11) was observed previously. The present study confirmed similar observations. Increasing the pH of the 0.01 *M* phosphate buffer (*i.e.*, dibasic ion concentration of the phosphate buffer) increased the intensity of the unretained peak in the chromatogram. At pH 8.0, only I and this peak were observed.

In spite of their corresponding rates for the disappearance of I (Table II), the nature and relative proportion of the degradation products of I in similar concentrations of tris(hydroxymethyl)aminomethane and phosphate buffer systems were noticeably different. In tris(hydroxymethyl)aminomethane buffer solutions, beside the hydrolysis products (II and III), additional decomposition products (V–VII) were detected, which increased with increasing buffer concentration. Table III summarizes the chromatographic retention volumes (V_R in milliliters), capacity factors (k'), separation factors (α), and resolution (R_s) of V–VII. These products also were observed in sodium chloride solution and will be described further.

Aziridinylbenzoquinone (I) in tris(hydroxymethyl)aminomethane buffer (pH 6.5 ± 0.05) led, apart from the cleavage reactions, to the displacement of one or both aziridine residues and the formation of 2,5-dihydroxy-3,6-bis(carboethoxyamino)-1,4-benzoquinone (VIII) or 2-aziridinyl-5-hydroxy-3,6-bis(carboethoxyamino)-1,4-benzoquinone (IX). Both compounds were detected as a single peak in the void volume of the chromatogram. Only partial separation of these compounds could be achieved, even with water as the mobile phase. Mass spectral analysis employing controlled fractional vaporization indicated that the mixture consisted of VIII and IX in approximately equal proportions. The NMR and elemental analyses of VIII (see *Experimental*) confirmed the structure suggested by mass spectrometry.

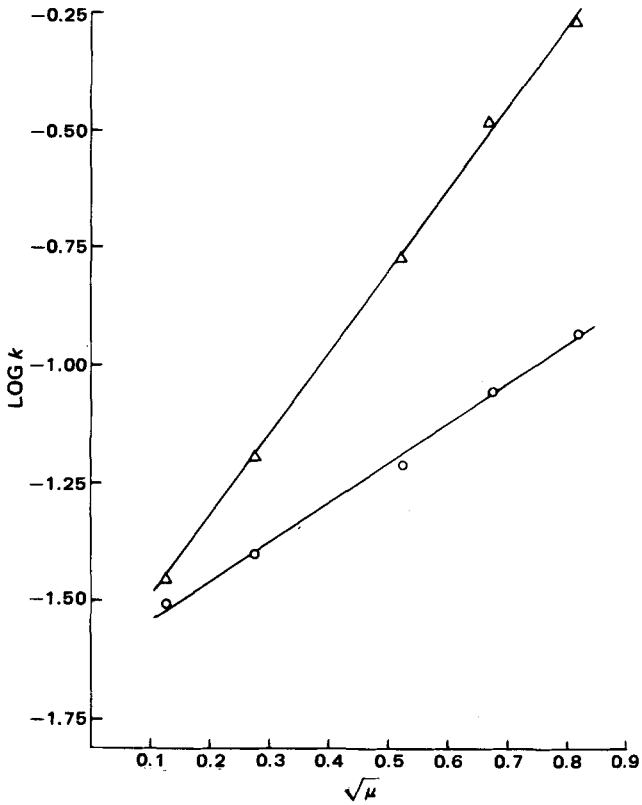

Figure 2—*Bronsted–Bjerrum plot of the logarithm of the observed rate constants* versus *μ. The ionic strength was controlled with sodium chloride (△) or sodium nitrate (○).*

The generation of the degradation products under mild conditions prompted an investigation of the reactivity of I with basic nucleophiles. Compound I in the presence of dilute sodium hydroxide generated a similar peak in the high-performance liquid chromatogram. HPLC monitoring of this reaction showed an instantaneous decrease of >90% in the concentration of I upon addition of sodium hydroxide. The molecular weight of this new compound appeared to be 314, 50 amu less than I (Fig. 1C). Accurate mass measurement of the major peaks in this spectrum allowed generation of a self-consistent set of fragment ion elemental compositions (Table IV), which indicated that this compound was VIII. Similarly, treatment of I with equimolar ammonium hydroxide followed by lyophilization resulted almost exclusively in 2-amino-5-hydroxy-3,6-bis(carboethoxyamino)-1,4-benzoquinone (X). This compound possessed a mass spectrum that exhibited the same neutral losses as that of VIII and in which most of the major peaks were shifted 1 amu lower (Table IV). Direct replacement of the aziridine groups in similar 1,4-benzoquinone derivatives was observed after treatment with alkylamines (12–15) or sodium hydroxide (16). The ease of replacement depended on the basicity of the reagent and the nature of the other substituents present in the quinone (17). Thus, the primary mode of decomposition of I under basic conditions involves direct displacement of one or both aziridine moieties.

Primary Salt Effect—An investigation of the disappearance rate of I as a function of ionic strength was carried out to determine the influence of electrolytes. To conduct these studies, solutions were prepared at constant pH (6.5 ± 0.05), concentration of I (2.75×10^{-4} *M*), phosphate

Table IV—Accurate Mass Measurement of Selected Ions in the Mass Spectra of the Basic Degradation Products of I

Structural Assignment	VIII			X		
	Nominal Mass	Accurate Mass	Elemental Composition	Nominal Mass	Accurate Mass	Elemental Composition
M^+	314	314.078	$C_{12}H_{14}N_2O_8$	313	313.081	$C_{12}H_{15}N_3O_7$
$M - CH_3CH_2OH$	268	268.035	$C_{10}H_8N_2O_7$	267	267.045	$C_{10}H_9N_3O_6$
$M - 2\,CH_3CH_2OH$	222	221.992	$C_8H_2N_2O_6$	221	221.004	$C_8H_3N_3O_5$
$M - C_2H_4 - CO_2 - CH_3CH_2OH$	196	196.007	$C_7H_4N_2O_5$	195	195.018	$C_7H_5N_3O_4$
$M - 2\,CH_3CH_2OH - CO$	194	193.992	$C_7H_2N_2O_5$	193	193.009	$C_7H_3N_3O_4$

Table V—Mass Spectra of Ring-Opened One-Armed Mustards

VI Mass (Relative Intensity)	VII Mass (Relative Intensity)	Probable Assignment
400 (13)	436 (13)	M^+
364 (29)	400 (4)	M − HCl
354 (7)	390 (7)	M − CH_3CH_2OH
351 (14)	387 (25)	M − CH_2Cl
—	364 (21)	M − 2 HCl
305 (13)	341 (9)	M − CH_2Cl − CH_3CH_2OH
318 (37)	318 (26)	364 − CH_3CH_2OH
272 (21)	272 (17)	364 − 2 CH_3CH_2OH
245 (28)	245 (17)	272 − C_2H_3
45 (41)	45 (37)	$C_2H_5O^+$
36 (65)	36 (100)	HCl^+
31 (76)	31 (71)	$CH_2{=}O^+H$
29 (100)	29 (85)	$C_2H_5^+$

buffer concentration (0.01 *M*), and temperature (25 ± 0.1°) but at varying ionic strengths. The ionic strength was controlled by the addition of sodium chloride or sodium nitrate.

The results are shown in Fig. 2. The logarithm of K_{obs} is plotted *versus* $\sqrt{\mu}$ according to the method of Bronsted and Bjerrum (18). These plots resulted in straight lines ($r > 0.970$), indicating that the reaction was dependent on the ionic strength ($\sqrt{\mu}$). However, the disappearance rate of I was 1.1- to 4.6-fold faster in the presence of sodium chloride than in sodium nitrate due to the nucleophilic attack of the chloride ion on the aziridine ring(s) (19), producing, in addition to the hydrolysis products, the one-armed mustards (V–VII) in significant proportions (Table III).

Compound VII was identified as 2,5-di(2′-chloroethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone by mass spectral analysis (Table V). Obvious losses of ethanol (*m/z* 390) and the chloromethylene radical (*m/z* 387) from the molecular ion (*m/z* 436) plus the characteristic isotopic pattern of two chlorine atoms (*e.g.*, *m/z* 436) allowed definition of the key structural features of VII. Comparison with the mass spectrum of an authentic sample[24] confirmed this structural assignment.

Degradation product VI was identified as the derivative of I in which only one aziridine ring was opened by chloride-ion attack, namely, 2-aziridinyl-5-(2′-chloroethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone. The mass spectral fragmentation is similar to that of VII, except for the molecular ion peak (*m/z* 400) and the presence of only one chlorine atom (Table V).

The mass spectral analysis of V suggested the presence of 2-(2′-chloroethylamino) - 5-(2′-hydroxyethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone. This degradation product was identified tentatively on the basis of an apparent molecular ion peak at *m/z* 418, displaying the characteristic isotope pattern of a single chlorine, as well as fragment peaks at *m/z* 387 (M − CH_2OH) and 369 (M − CH_2Cl).

Oxygen and Light Effect—Identical solutions of I (2.75×10^{-4} *M*) were stored for 3 weeks in 0.01 *M* phosphate buffer (pH 6.5) under oxygen, nitrogen, and air at 25 ± 0.1°. Similar solutions were exposed to fluorescent light or kept in the dark at the same temperature. When assayed periodically, no difference was observed among the pseudo-first-order rate constants for the disappearance of I in these solutions as well as among the proportions of its degradation products. These observations, coupled with the reproducibility of the observed rate constants at various times and their strict adherence to pseudo-first-order kinetics, suggest nucleophilic (hydrolytic) rather than oxidative breakdown of I in aqueous solution.

Degradation Patterns of I—Although this study does not permit definitive judgments on the detailed mechanisms of degradation of I throughout the pH range studied, it does demonstrate that I is subject to both general acid and base catalysis. Several reactions appear to be responsible for the degradation of I, the relative importance of each being dependent on both the buffer species and the form of I present in solution.

At pH 3, the diaminoalcohol (III) was the major hydrolytic product; in solutions between pH 3 and 7, both II and III were present in varying amounts. Once the ethyleneimine was protonated, it became the primary target of nucleophilic attack by water or other nucleophiles to produce the respective degradation products. Ring opening by water or halogen acids is among the oldest and most often reported reaction for aziridines (20). At pH 3.0, the chromatogram of I at various time intervals provides conclusive evidence that hydrolysis constitutes the only degradative pathway through a two-step sequential mechanism since a total of 1 mole of II plus III appears for each mole of I consumed.

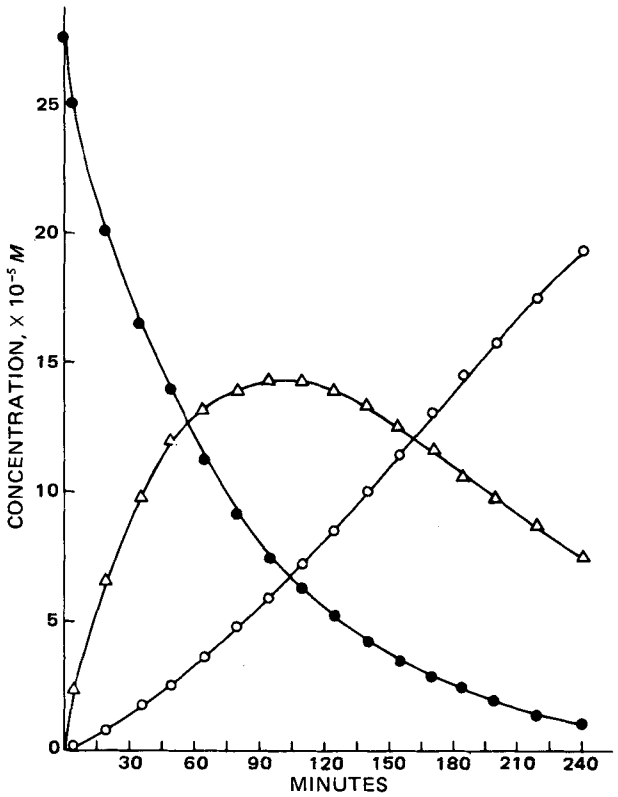

Figure 3—*Concentration–time curves for I (●), II (△), and III (○) at 25 ± 0.1° and pH 3.0.*

A typical plot of the hydrolysis of I and the appearance of II and III at pH 3.0 and 25 ± 0.1° is presented in Fig. 3. The rapid decrease of I is accompanied by an increase in II, which reaches a maximum value after 100 min and then decreases in an approximately linear fashion. After a lag period of 20 min, the concentration of III rises gradually. This pattern suggests two consecutive first-order reactions:

$$I \xrightarrow{K_1} II \xrightarrow{K_2} III$$

where K_1 and K_2 are pseudo-first-order rate constants. Several methods (21, 22) have been described for the determination of the second rate constant of consecutive first-order reactions using dimensionless parameters and variables. With the method of Jensen and Lamb (22), K_2 was 1.11×10^{-3} (min^{-1}) while K_1 was 5.29×10^{-3} (min^{-1}).

The data obtained from the degradation of I in solutions ranging from weakly acidic to weakly alkaline cannot be treated likewise. The pseudo-first-order disappearance rates of I in these solutions are not conclusive evidence that ring-cleavage hydrolysis constitutes the only degradative pathway since new compounds are generated as the pH of the solution is raised. This additional degradation mode of I in solution, unlike acid-catalyzed solvolysis, involves the displacement of one or both aziridine moieties by nucleophiles. This decomposition pattern of I becomes its major degradation route in basic media.

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Simultaneous GLC Analysis of Aspirin and Nonaspirin Salicylates in Pharmaceutical Tablet Formulations

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Abstract □ The analysis of aspirin and nonaspirin salicylates in buffered and plain tablet formulations employing nearly nonaqueous extraction is described. The results obtained compare favorably with those obtained from USP procedures. A simultaneous assay for aspirin and nonaspirin salicylates in buffered tablets involves the use of an acidified chromatographic siliceous earth column for the separation of the aspirin and nonaspirin salicylates from various buffers or antacids. The column is eluted with chloroform, and the aspirin and nonaspirin salicylates are analyzed by GLC as their trimethylsilyl derivatives. The methods described here have definite advantages over USP XX procedures, and the buffered aspirin tablet procedure also is adaptable to aspirin formulations containing codeine, acetaminophen, propoxyphene, caffeine, and many antihistamines.

Keyphrases □ Aspirin—simultaneous GLC analysis with nonaspirin salicylates in tablet formulations □ Salicylates, nonaspirin—simultaneous GLC analysis with aspirin in tablet formulations □ GLC—analysis, aspirin and nonaspirin salicylates in tablet formulations

The GLC analysis of aspirin and nonaspirin salicylates in solid pharmaceutical dosage forms and biological fluids has been reported (1–18). The extraction of nonaspirin salicylates from various buffers is accomplished by the current USP procedure; however, the USP procedure for the analysis of aspirin does not effectively free aspirin from aged tablets containing calcium, aluminum, and magnesium buffers. Complexation of aspirin and nonaspirin salicylates with buffers has been reported (19–22). The current USP spectrophotometric procedures for aspirin tablets may not separate impurities such as acetylsalicylsalicylic acid and acetylsalicylic acid anhydride (23–27). Analysis of aspirin and nonaspirin salicylates by high-pressure liquid chromatography (HPLC) also has been reported (28–35). Mobile phases and extractions employing methanol and water, even in small quantities, result in unavoidable hydrolysis of aspirin, thereby giving variable and nonreproducible quantitation of salicylic acid.

The buffered aspirin tablet procedure described here enables the extraction of both aspirin and nonaspirin salicylates from excipients and buffers in a virtually nonaqueous procedure, thereby keeping hydrolysis of aspirin to a minimum.

EXPERIMENTAL

Materials—Aspirin USP reference standard (GLC purity 99.95%), salicylic acid USP reference standard (GLC purity 100.0%), chromatographic siliceous earth prepared by the USP procedure, propylparaben USP, and *N,O*-bis(trimethylsilyl)acetamide were used. Chloroform and hydrochloric acid were reagent grade.

Simultaneous GLC Analysis of Aspirin and Nonaspirin Salicylates in Buffered Tablets—*Internal Standard Solution*—About 425 mg of propylparaben USP was diluted to 100 ml with chloroform and mixed.

Aspirin Standard Preparation—About 125 mg of aspirin USP reference standard was weighed accurately, transferred to a 50-ml volumetric flask, diluted to volume with chloroform, and mixed. The standard preparation was derivatized within 2 hr of preparation.

Salicylic Acid Standard Preparation—About 15 mg of salicylic acid USP reference standard was weighed accurately, transferred to a 200-ml volumetric flask, diluted to volume with chloroform, and mixed.

Assay Preparation—The average tablet weight of 20 tablets was determined, and the tablets were ground to a fine powder. Without delay, a portion of the ground tablets equivalent to 500 mg of aspirin was transferred to a small beaker containing 3.0 g of acid-washed chromatographic siliceous earth. The powders were mixed with a glass rod, 2.0 ml of 6 *N* HCl was added, and the powders were mixed again with the glass rod. The mixture was transferred to a 20 × 2.5-cm chromatographic column, and the beaker was dry washed with 1.0 g of siliceous earth (glass wool was used at both ends of the column).

The column was packed uniformly and eluted with successive portions of chloroform *via* the sample beaker at the rate of ~10 ml/min. About 150 ml of the eluate was collected in a 200-ml volumetric flask. The tip

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