2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone I: Kinetics in Aqueous Solutions by High-Performance Liquid Chromatography

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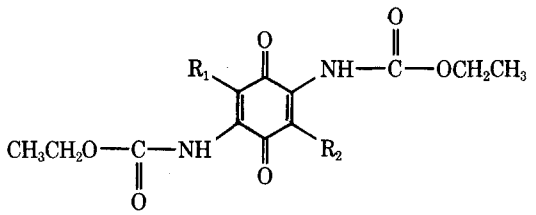
Abstract □ The application of a rapid, selective, and sensitive reversed-phase high-performance liquid chromatographic method to the analysis of 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (I) and its degradation products is described. The method was used to study the kinetics of degradation of I over pharmaceutically useful pH ranges. The overall reaction rate followed pseudo-first-order kinetics. The pH–rate profile demonstrated optimal stability between pH 6.0 and 6.5. The degradation behavior suggests the existence of multiple pathways. The temperature dependence of the disappearance of I also was evaluated from the regression equation derived from the Arrhenius plot.

Keyphrases □ 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone—degradation kinetics, monitoring by high-performance liquid chromatography □ Degradation—2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone, kinetics, monitoring by high-performance liquid chromatography □ High-performance liquid chromatography—analysis, 2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone, degradation kinetics

The antitumor properties of aziridinylbenzoquinone derivatives have been recognized for some time (1, 2), and these compounds were shown to induce cross-links with DNA (3). Interest in this class of compounds was renewed due to the recent demonstration of wide spectrum antitumor activity in experimental tumor systems, particularly against central nervous system (CNS) neoplasms (4, 5). 2,5-Diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone (I) was selected from a series of potential CNS antitumor agents for extensive preclinical studies and clinical trial (5). However, there have been no systematic studies reporting qualitative or quantitative data pertaining to the hydrolytic stability of this class of compounds and of this potentially useful pharmaceutical agent in particular.

The present investigation involved development of a rapid, simple, selective, and sensitive stability-indicating high-pressure liquid chromatographic (HPLC) assay for the simultaneous quantification of I and its decomposition products. Methods used previously to study the hydrolytic behavior of polyfunctional substituted aziridines were based only on the disappearance of the total aziridine moieties (6–8). The HPLC method was applied to evaluate the stability of I over a wide pH range and under various pharmaceutically applicable conditions. Decomposition products were isolated by semipreparative HPLC and characterized chemically; they are described in a separate report (9).



I: $R_1 = R_2 =$ –N◁

II: $R_1 =$ –N◁, $R_2 = NHCH_2CH_2OH$

III: $R_1 = R_2 = NHCH_2CH_2OH$

EXPERIMENTAL

Reagents—Compound I (NSC 182986)[1] and the internal standard, 5-chloro-2-pyridinol[2], were used as received. 2-Aziridinyl-5-(2′-hydroxyethylamino)-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 a semipreparative HPLC method from partially hydrolyzed samples of I, and their identities were verified by comparison of their retention times with those of authentic samples as well as by mass spectrometry (9). Acetonitrile[3] (distilled in glass) and distilled water were filtered through 0.45-μm solvent-resistant filters[4].

Infusion fluids were obtained commercially[5] and were utilized as received. All other chemicals were reagent grade. Initially, the influence of pH on the stability of I was studied between pH 3.0 and 8.0 using 0.01 *M* citrate–phosphate buffer (10) or 0.01 *M* phosphate buffer (11). When necessary, the pH was adjusted[6] to within 0.05 pH unit of the nominal value with either the basic or acidic component of the buffer.

HPLC—A modular high-pressure liquid chromatograph equipped with a constant-flow pump[7] was used to deliver the eluent at a rate of 1 ml/min to a 5-μm reversed-phase column[8] (250 × 3 mm i.d.). No attempt was made to control the column temperature. Injections were made with a rotary valve injector equipped with a 10-μl injection loop[9]. A variable-wavelength UV detector[10], 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[11].

The chromatographic mobile phase was 15% (v/v) acetonitrile in distilled water. The column pressure at a flow rate of 1 ml/min was ~2100 psi. Separations were effected isocratically at room temperature, and the chart speed was 1 cm/min. The chromatographic parameters, *i.e.*, the capacity factor (k'), the separation factor (α), and the resolution (R_s), of various solutes were calculated from the adjusted retention volumes (12).

Peak heights were used to quantitate I and its hydrolysis products. Standard curves comparing the ratios of the peak heights of I–III to 5 μg of the internal standard exhibited linear responses ($r > 0.999$) in the working concentration range of 5–50 μg/ml. The peak heights of I–III were converted to concentrations by comparison with standard curves.

One milliliter of a freshly prepared 2.75×10^{-2} *M* stock solution of I in *N,N*-dimethylacetamide was added to ~95 ml of the appropriate medium, and the volume was rapidly brought to 100.0 ml with the same medium. The resulting solution was shaken and filtered[4], and a zero-time

[1] Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, Md.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] Burdick & Jackson Laboratories, Muskegon, Mich.
[4] Millipore Corp., Bedford, Mass.
[5] Sodium chloride injection USP (lot 64-856-DE-2), 5% dextrose injection USP (lot 51-688-DE-2), and lactated Ringer's injection USP (lot 62-458-DE-2), Abbott Laboratories, North Chicago, Ill.
[6] Beckman Zeromatic pH meter, Beckman Instruments, Irvine, Calif.
[7] Model 3500B, Spectra-Physics, Santa Clara, Calif.
[8] Spherisorb ODS (5 μm), Spectra-Physics, Santa Clara, Calif.
[9] Valco Instruments Co., Houston, Tex.
[10] Model SP 770, Spectra-Physics, Santa Clara, Calif.
[11] OmniScribe, Houston Instruments, Austin, Tex.

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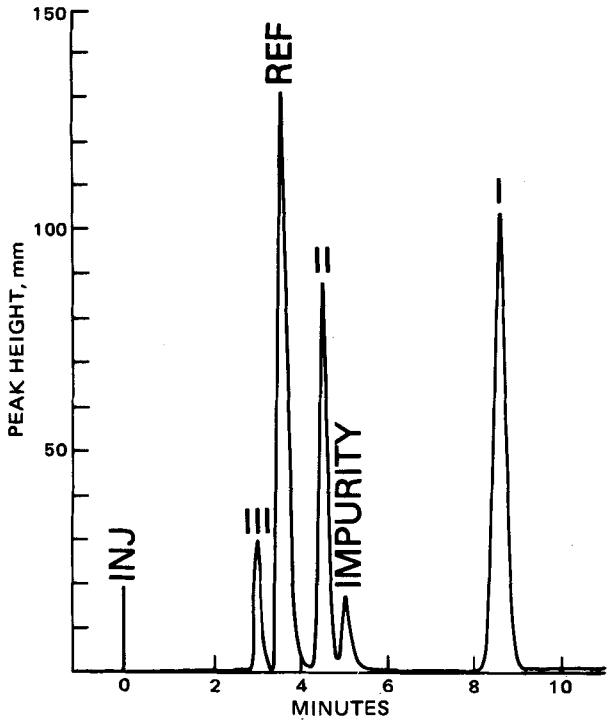

Figure 1—*High-performance liquid chromatogram of I–III and the internal standard (REF). The column conditions are given in the text.*

sample was assayed immediately. The remaining solution was promptly dispensed (3.0 ml) into 5-ml clean flint ampuls, sealed, and stored at 5 ± 0.1, 25 ± 0.1, 37 ± 0.1, and 50 ± 0.1° for a period not exceeding 30 days. The pH of each sample was determined prior to dilution for the assay[6].

At designated time intervals, duplicate 1.0-ml samples were removed, diluted to 2.0 ml with 15% (v/v) acetonitrile in water containing 1.0 mg of the internal standard/ml, and immediately subjected to analysis. Disappearance of I was followed beyond its second half-life. Samples were removed for analysis at a minimum of 10 different time intervals per experimental condition during each experiment.

RESULTS AND DISCUSSION

Solubility Studies—Since I has poor aqueous solubility (~200 μg/ml), mixed solvent systems are required to achieve the desired concentration for intravenous injection. Although I has better solubility in ethanol (2 mg/ml), a 10% ethanolic solution did not improve its solubility. However, solutions of I (2.75×10^{-3} *M*) were readily prepared by dissolving 20 mg in 1 ml of *N,N*-dimethylacetamide and then diluting the solution with 19 volumes of water or buffer. Antitumor data (4) and preliminary toxicological studies[12] indicated that this concentration was adequate for preclinical and clinical studies.

Assay Sensitivity—Colorimetric (6, 7) and titrimetric (8) methods were reported for the quantitative determination of aziridinyl compounds. The colorimetric method is based on a comparison between a reference and a partially decomposed sample in which some aziridine moieties were destroyed. However, in the case of I, the method does not distinguish quantitatively between intact, partially reacted, or fully reacted species. The titrimetric method is based on a reaction of the ethyleneimine ring with thiosulfate ion and provides a measure of intact aziridine moieties. Likewise, no distinction can be made between a mixture of unreacted, fully decomposed polyfunctional aziridines and a mixture of partially reacted species.

In a compound with polyfunctional ethyleneimine moieties, it was expected that ring opening may not proceed simultaneously but rather in discrete steps. Development of a simple, rapid, selective, and sensitive quantitative HPLC–UV assay for I and its various decomposition products confirmed this supposition. The response curve of I was linear ($r > 0.999$) in the concentration range examined (5–50 μg/ml). Good reproducibility of the described HPLC procedure using UV detection was demonstrated when six 10-μl aliquots of a standard solution of I in the presence of II, III, and internal standard were chromatographed. The coefficient of variation of I was 0.48%, and the minimum detectable limit of I was 3 ng.

During the study, there was no noticeable loss in column performance. As a precaution, the column was reconditioned according to the manufacturer's suggestions when not in use for more than a few days.

The order of elution was III, the internal standard, II, and I (Fig. 1). A representative chromatogram of a synthetic mixture of I–III and the internal standard is shown in Fig. 1. The retention data for these substances are listed in Table I.

The retention volumes (V_R in milliliters), k', α, and R_s for I and various decomposition products were identical to those reported for the synthetic mixture. The impurity found in the original sample of I was identified as 2,5-diamino-2,6-dichloro-1,4-benzoquinone (IV) (9).

Order of Reactions—The hydration of 1-aziridinylbenzoquinones in acid solutions (13) was reported to be more rapid than that of 1-unsubstituted basic aziridines (14, 15). Such substituents on an aziridine nitrogen are expected to increase markedly the reactivity of the protonated ring; hence, these compounds more closely resemble activated aziridines than basic aziridines in their reactions. Such an activated aziridine, in addition to being sensitive to acidic reagents, also may undergo ring opening with nucleophilic reagents.

The results of the present investigation confirmed the high reactivity of I in aqueous solution. Semilog plots of residual concentrations of I *versus* time exhibited excellent linearity ($r > 0.99$) for experiments at all temperatures examined and pH 3.0–8.0. The disappearance of I followed pseudo-first-order kinetics under the conditions studied and was in accord with previous observations on aziridines (16). The degradation rate constants of I were computed by the least-squares linear regression method. Table II presents the calculated pseudo-first-order rate constants at fixed concentrations of I (2.75×10^{-4} *M*) and buffer (0.01 *M*) and constant temperature (25 ± 0.1°) over the pH range of 3.0–8.0. Duplicate experiments were performed for certain experimental conditions to verify the results. Rate constants from duplicate runs in these studies differed by <5%.

Influence of pH—The pH–rate profile for the degradation of I was constructed from the logarithm of the pseudo-first-order rate constants and the pH values at 25 ± 0.1° (Fig. 2). The buffering capacities of all of the solutions were sufficient to maintain constant pH values (±0.05) throughout the experiment, with the exception of the pH 8.0 solution. In this case, values as low as pH 7.4 were recorded.

The pH–rate profile showed a degradation minimum between pH 6.0 and 6.5 (Fig. 2). The observed results are in accord with those reported

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

Compound	V_R, ml	k'	α	R_s
III	3.00	2.00		
			1.33	2.70
Internal standard	3.65	2.65		
			1.32	2.83
II	4.50	3.50		
			1.16	1.66
IV	5.05	4.05		
			1.85	6.08
I	8.50	7.50		

[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.

Table II—Pseudo-First-Order Rate Constants for Loss of I (2.75×10^{-4} *M*) in Solutions at Different pH Values and 25 ± 0.1°

pH	$k_{obs} \times 10^2$, day^{-1}
3.0	763
5.0	62.6
Unbuffered	5.10
6.0	3.63
6.5	3.07
7.0	4.22
8.0	12.97

[12] R. Davis, Laboratory of Toxicology, National Cancer Institute, Bethesda, Md., personal communication.

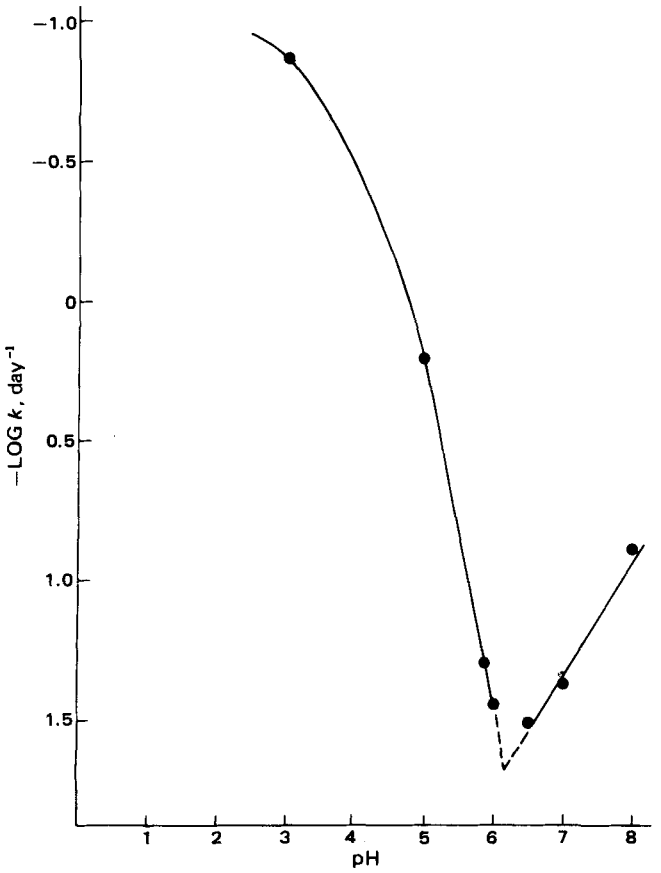

Figure 2—*Log* k versus *pH profile for the decomposition of I (2.75 × 10^{-4} M) at 25 ± 0.1°.*

for ethyleneiminoquinones, in which the hydrolysis rate of the ethyleneimino group increased with decreasing pH (2). Moreover, I in unbuffered solution (pH ~ 6) degraded faster than in pH 6.0 buffer (Table II). This accelerated decomposition of I undoubtedly was caused by the inability of the unbuffered solution to maintain a constant pH throughout the reaction (maximum decrease in pH = 0.5 unit).

The influence of the solution pH on the degradation pattern of I was dramatic. Based on the present findings, several degradation reactions appeared to be responsible for the decomposition of I in phosphate buffer (0.01 *M*). The relative importance of each reaction was dependent on the solution pH. In strongly acid solutions (pH = 1), I apparently degraded instantaneously to yield the dialcohol (III). Under alkaline conditions, the high-performance liquid chromatograms showed the presence of a hydrophilic peak at the void volume, which is characterized in a separate report (9). The intensity of this peak increased gradually as the solution pH was raised (>6), and this peak was the only degradation peak detected in the chromatogram in strongly alkaline solution. In weakly acidic solution (pH ≤ 5.0), I underwent simple, stepwise, consecutive hydrolytic cleavage of the aziridine rings to produce the monoalcohol (II) and eventually the dialcohol (III) (9). The concentration of II in such solutions of I also was pH dependent and showed a concentration maximum at ~pH 5.

Influence of Temperature—An Arrhenius plot of the pseudo-first-order rate constants (k) at varying temperatures (5 ± 0.1, 25 ± 0.1, 37 ± 0.1, and 50 ± 0.1°) in the presence of a constant phosphate buffer concentration (0.01 *M*), constant concentration of I (2.75 × 10^{-4} *M*), and a constant pH (6.5) is shown in Fig. 3. The apparent Arrhenius parameters for the disappearance of I were calculated from the regression equation ($r > 0.99$). The apparent activation energy was 14.0 kcal/mole, and the frequency factor (A) was 7.70 × 10^3 sec^{-1}.

Table III—Stability of I in Reconstituted Solution and in Various Infusion Fluids at Ambient Temperature

Medium	Admixture Solution pH	T_{90}, hr[a]
Reconstituted solution[b]	6.50	84
Lactated Ringer's injection USP[c]	6.40	60
Sodium chloride injection USP[c]	6.30	44
5% Dextrose injection USP[c]	5.60	36

[a] Time for 10% of the original concentration of I to disappear. [b] 2.75 × 10^{-3} *M* I. [c] 5.50 × 10^{-5} *M* I.

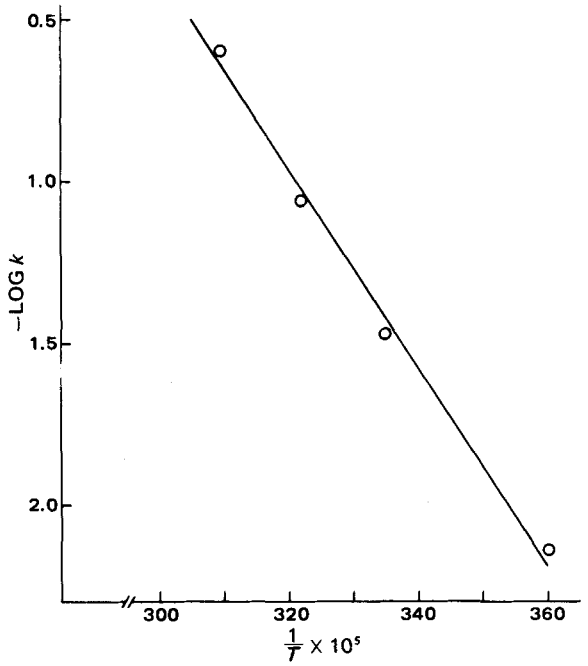

Figure 3—*Arrhenius plots for apparent pseudo-first-order rate constants for the disappearance of I in pH 6.5 phosphate buffer solution.*

Stability of I in Various Infusion Fluids—To test the compatibility of I, its degradation was followed in several commercially available infusion fluids: 5% dextrose injection USP, sodium chloride injection USP, and lactated Ringer's injection USP. A 0.5-ml portion of the stock solution of I was diluted 10-fold with 0.01 *M* phosphate buffer (pH 6.50). The resultant solution was filtered and diluted with 245 ml of infusion fluid to yield a 5.50 × 10^{-5} *M* solution. The admixture solutions were kept at ambient temperature (20 ± 0.5°), and the residual concentration of I and the pH were determined for at least 72 hr. The investigations usually were discontinued when the initial concentration of I had decreased by 25%.

The observed T_{90} values (time for 10% decomposition of original concentration of I) at ambient temperature determined from the respective plots of concentration *versus* time are summarized in Table III. Important factors influencing the stability of I in these infusion fluids were the pH of the admixture solution, which, in turn, was dependent on the pH of the respective fluid, and the chloride concentration in the fluids (9). The chromatographic analyses of both sodium chloride and lactated Ringer's injection containing I indicated, in addition to the hydrolytic products II and III, a trace amount (<3% at T_{90}) of a compound identified by mass spectrometry as 2-aziridinyl-5-(2′-chloroethylamino)-3,6-bis(carboethoxyamino)-1,4-benzoquinone (VI) (9).

Formulation Studies—Data from the solubility and hydrolysis experiments were used to develop a stable injectable formulation of I. The hydrolysis rate of I, even within the optimal pH range (6.0–6.5), was too rapid for formulation as an aqueous solution. Instead, I was prepared as a sterile, dry product using low-temperature vacuum drying (17). Specifically, solutions of I in ethanol (3.7 × 10^{-3} *M*) are sterilized by filtration through 0.22-μm polytef membranes[4], dispensed into vials, and dried aseptically under low temperature in a conventional freeze drier. This product is supplied as one part of a three-component package, which also includes a sterile solution of *N,N*-dimethylacetamide and pH 6.5 sodium phosphate buffer (0.01 *M*). At the time of use, the vial contents are dissolved completely in sterile *N,N*-dimethylacetamide (0.5 ml) and then diluted with phosphate buffer (9.5 ml). Reconstituted solutions are chemically and physically stable for 84 hr at 25 ± 0.1° (Table III). This method is necessary for the convenient solution preparation since dissolution of intact I in 10% *N,N*-dimethylacetamide solution is very slow.

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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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Received December 11, 1979, from the **Pharmaceutical Resources Branch and the* ‡*Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, MD 20205.* Accepted for publication April 4, 1980.

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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[2] Aldrich Chemical Co., Milwaukee, Wis.

[3] Burdick & Jackson Laboratories, Muskegon, Mich.

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