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DETERMINATION OF THE ANTILEUKEMIA AGENTS CYTARABINE AND AZACITIDINE AND THEIR RESPECTIVE DEGRADATION PRODUCTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase high-performance liquid chromatography (HPLC) system was developed for the determination of the antineoplastic agents cytarabine and azacitidine. Separations were performed on an octadecylsilane column with a mobile phase of methanol–phosphate buffer pH 7.0 (5:95). The assay methods are suitable for bulk drugs and sterile powder formulations of the agents. Specificity in the presence of analogues and decomposition products was demonstrated. UV spectra of the components of interest were obtained in the HPLC effluent, and appropriate wavelengths were employed for the various analytes. Samples of azacitidine in various solutions were analyzed as a function of time by HPLC to determine the three first-order rate constants associated with its decomposition.

INTRODUCTION

Cytarabine (Ara-C; cytosine arabinoside; 1-β-D-arabinofuranosyl cytosine) and azacitidine (5-AC; 5-azacytidine; 4-amino-1-β-D-ribofuranosyl-*s*-triazin-2(1H)-one) are nucleoside analogues which have antitumor activity. Cytarabine is formulated as a freeze-dried powder and is marketed for induction and maintenance of remission in acute myelocytic leukemia (Cytosar-U®, The Upjohn Company). A new drug application was made in the U.S.A. for a freeze-dried powder containing equal amounts of 5-AC and mannitol (Mylosar®, The Upjohn Company) for induction of remission in acute non-lymphocytic leukemia.

This report describes a reversed-phase high-performance liquid chromatographic (HPLC) method for quantitative determination of these antineoplastic agents in bulk drugs and pharmaceutical formulations. The sample preparations and chromatographic conditions are simple and rapid. The determinations are specific for these agents in the presence of analogues and products of hydrolytic decomposition.

Although Ara-C and 5-AC are stable in the solid state, they are degraded by hydrolysis in aqueous solutions. Hydrolytic deamination of Ara-C (I) results in the elimination of ammonia and the formation of uracil arabinoside (II), as shown in Fig. 1[1,2]. In common infusion solutions of Ara-C, degradation is less than 1% in five days[3]. In neutral and basic solution, the hydrolysis of 5-AC (III) occurs via nucleo-

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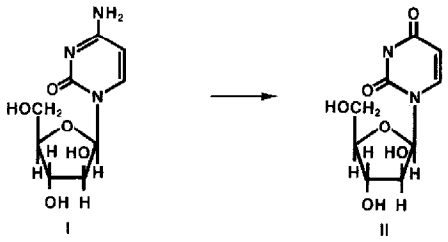

Fig. 1. Hydrolytic deamination of cytarabine (I) to uracil arabinoside (II).

philic attack, opening the triazine ring at the 5,6-position to form N-(formylaminine)-N'-β-D-ribofuranosylurea (IV), as shown in Fig. 2[4,5]. The N-formyl group of IV is eliminated to form 1-β-D-ribofuranosyl-3-guanylurea (V). In strongly acidic solutions, the glycoside bond is hydrolyzed to produce 5-azacytosine and D-ribose. Hydrolysis of 5-AC in infusion solutions is much more rapid than that of cytarabine. Over 10% of 5-AC is degraded in common infusion solutions within 4 h[6]. The kinetics of 5-AC decomposition have been studied previously by UV spectroscopy[4], HPLC[6], and NMR spectroscopy[7].

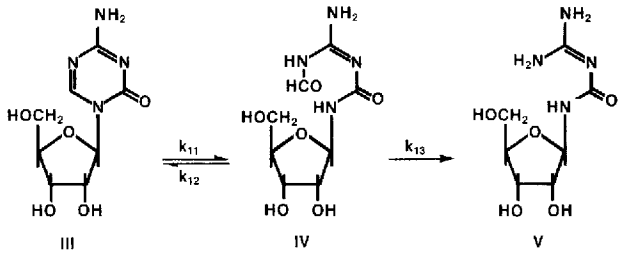

Fig. 2. Stepwise hydrolysis of azacitidine (III) to N-(formylaminine)-N'-2β-ribofuranosylurea (IV), then 1-β-D-ribofuranosyl-3-guanylurea (V).

EXPERIMENTAL

Chromatographic system

The mobile phase was delivered by an Altex Model 110A pump (Altex Scientific, Berkeley, CA, U.S.A.). Sample injection was performed with a WISP 710B autosampler (Waters Assoc., Milford, MA, U.S.A.) or a manual Model 7010 loop injector (Rheodyne, Cotati, CA, U.S.A.). Detection was generally performed at 254 nm with a LDC Model 1203 detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). A Tracor (Austin, TX, U.S.A.) Model 970A variable-wavelength detector was also employed. Some full UV chromatograms were obtained with HP Model 1040A diode-array detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). Separations were performed on a 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc). The mobile phase, water–methanol (95:5) containing 1.34 g disodium hydrogen phosphate heptahydrate, 0.71 g sodium dihydrogen phosphate monohydrate per liter, was pumped at a rate of 1 ml/min. The apparent pH of the mobile phase was 7.0.

Reagents

Methanol was distilled-in-glass grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Disodium hydrogen phosphate heptahydrate and sodium dihydrogen phosphate monohydrate were analytical-reagent grade. The *p*-toluic acid was obtained from Crescent Chemical (Hauppauge, NY, U.S.A.). Cytosine, D-(−)-ribose, and D-(−)-arabinose were obtained from Sigma (St. Louis, MO, U.S.A.). A sample of 5-azacytosine was provided by Ash-Stevens (Detroit, MI, U.S.A.).

Cytarabine preparations and procedure

Internal standard solution. A 1.4-mg/ml solution of *p*-toluic acid was prepared in methanol.

Standard preparation. A 0.02-mg/ml solution of uracil arabinoside in water was prepared. Approximately 3 mg of Ara-C was accurately weighed and 5.0 ml of the uracil arabinoside solution, 5.0 ml of internal standard solution, and 20 ml of mobile phase were added.

Bulk drug preparation. Cytarabine (3 mg) was accurately weighed and 5.0 ml of internal standard solution and 25 ml of mobile phase were added.

CYTOSAR-U® sterile powder preparation. The contents of the vial were quantitatively diluted with water to prepare a 1-mg/ml solution of Ara-C. A 3.0-ml portion of this Ara-C solution was combined with 5.0 ml of internal standard solution and 20 ml of mobile phase.

Procedure. Portions of 10 μl of the preparations were injected. The cytarabine and uracil arabinoside content of the samples were calculated by comparing the ratio of the peak response relative to the internal standard to the ratio of the standards.

Azacitidine preparations and procedure

Internal standard solution. A 2-mg/ml solution of *p*-toluic acid was prepared in water–methanol (20:80).

Standard and bulk drug preparations. A 1-mg/ml solution of 5-AC was prepared in internal standard solution.

MYLOSAR® sterile powder preparation. The contents of the vial were quantitatively diluted with internal standard solution to prepare a 1-mg/ml solution of 5-AC.

Procedure. Exactly 15 min after addition of the internal standard solution to the 5-AC, a 2-μl portion of the preparation was chromatographed. The 5-AC content of the sample was calculated by comparing the ratio of the peak responses to the internal standard to the ratio of the standards.

Azacitidine decomposition

Decomposition of 5-AC in several solutions was monitored by performing from 50–120 HPLC assays as a function of time. Large-volume parenteral (LVP) solutions in 1-l glass bottles and plastic bags were obtained from Travenol Labs. (Deerfield, IL, U.S.A.) and Abbott Labs. (Chicago, IL, U.S.A.). The pH of the solutions was adjusted to the desired value with hydrochloric acid or sodium hydroxide. Solution administration sets with particulate filters were obtained from Travenol Labs. Direct injection with a 50-μl injection loop and no internal standard was used to analyze the dilute solutions. The time range monitored was two to three days, except for at 4°C, where the sample was monitored for 3 weeks.

RESULTS AND DISCUSSION

Since Ara-C and 5-AC are polar molecules, reversed-phase HPLC must be performed with a mobile phase containing a low concentration of organic modifier. Although 5-AC has been assayed by reverse-phase HPLC without any organic modifier in the mobile phase[5], the addition of 5% methanol to the aqueous mobile phase improved the reproducibility of retention times.

Tailing of some of the peaks, *e.g.* of 5-AC, was observed with unbuffered and acidic mobile phases. The irreversible adsorption which resulted in peak tailing is assumed to be the result of bonding of the primary amines of the analytes with residual silanol group on the surface of the stationary phase. The neutral pH of the phosphate buffer in the mobile phase was an effective compromise to assure symmetrical peaks and resonable column life. Retention times of the analytes varied from column to column and slowly decreased with time. Relative retention behavior of the components of interest were reproducible on all columns. With this mobile phase, a small, approximately 2 mm, void would form at the head of the column and result in reduced chromatographic efficiency. To maintain acceptable chromatographic performance, it was necessary to repack the head of the column after *ca.* 40 h of operation. The mobile phase was not bacteriostatic, and the column would be ruined if stored with the mobile phase for extended time periods.

Chromatographic specficity

Selectivity of the chromatographic system is demonstrated for Ara-C, 5-AC,

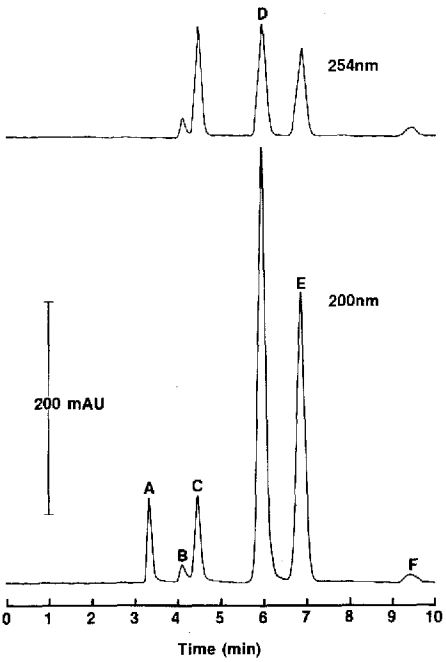

Fig. 3. Chromatograms recorded at 200 and 254 nm for cytarabine, azacitidine, and their analogues. Peaks: A = 0.45 μg V; B = 0.20 μg cyclocytidine; C = 0.90 μg IV; D = 2.6 μg azacitidine; E = 2.0 μg cytarabine; F = 0.20 μg uracil arabinoside. See Figs. 1 and 2 for full names and structures.

and their analogues in Fig. 3. A pro-drug, cyclocytidine, which is hydrolyzed to form Ara-C[8], is separated from Ara-C and uracil arabinoside. Cleavage of the glycosidic bond in Ara-C and 5-AC would result in the formation of D-arabinose and D-ribose, respectively. These sugars elute at the column void volume. The bases which result from cleavage of the glycosidic bond elute earlier than the corresponding nucleoside analogues, with retention times of 4.1 and 3.6 min for cytosine and 5-azacytosine, respectively. None of the analogues or products of decomposition interfered with the peaks for Ara-C or 5-AC.

A chromatogram of the analysis of a Cytosar-U sterile powder sample is shown in Fig. 4. Although Ara-C has an absorbance maximum at wavelengths higher than 254 nm, the common HPLC detection wavelength of 254 nm was employed. The additional sensitivity which could be obtained at the wavelength of maximum absorbance was not necessary for pharmaceutical samples. Extrapolation from elevated temperatures yields a decomposition rate constant at 25°C of $2.2 \cdot 10^{-5}\ h^{-1}$ (ref. 9). Thus, only 0.05% of the Ara-C will be degraded in the sample preparation in one day. A small amount of uracil arabinoside is expected to form in the product during the freeze-drying process. A small peak for uracil arabinoside is present in the chromatogram of Cytosar-U sterile powder that corresponds to 0.05% of the Ara-C content.

Chromatograms at 230 and 254 nm are shown in Fig. 5 for a sample prepared from Mylosar sterile powder. No detector response is observed for mannitol, which is present at an amount equal to the 5-AC in Mylosar, because it does not absorb UV radiation at these wavelengths. Small amounts of the primary decomposition

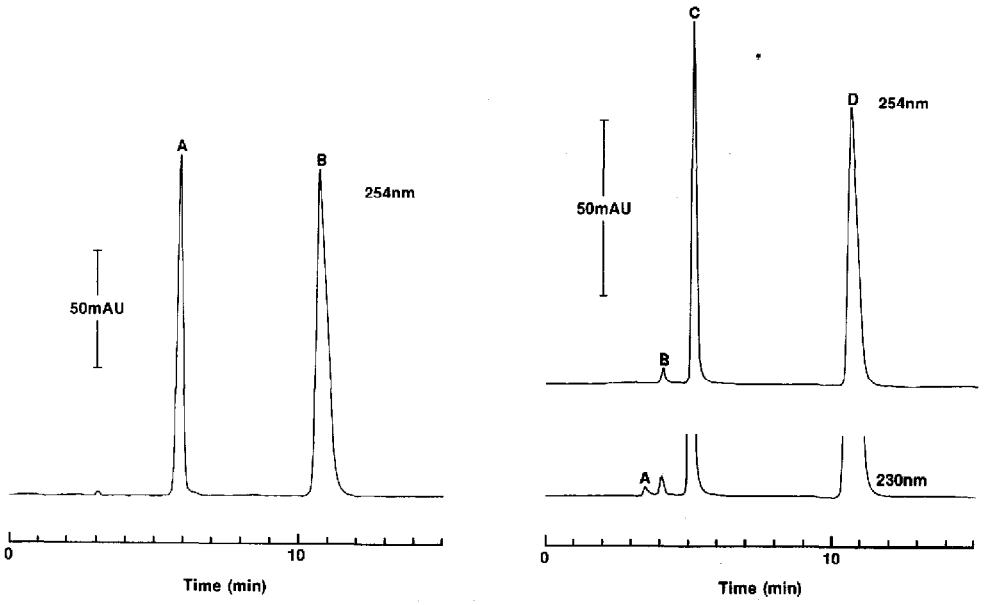

Fig. 4. Chromatogram recorded at 254 nm of a 100-mg Cytosar-U sterile powder sample. Peaks: A = cytarabine; B = *p*-toluic acid.

Fig. 5. Chromatograms recorded at 230 and 254 nm of a 100-mg Mylosar sterile powder sample. Peaks: A = V; B = IV; C = azacitidine; D = *p*-toluic acid.

product (IV, Fig. 2) and the secondary decomposition product (V, Fig. 2) can be detected in the Mylosar sample. The decomposition products of 5-AC are formed during the freeze-drying process and in the sample preparation, prior to injection. The peak for the primary decomposition product corresponds to 2.1% of the 5-AC content, of which 1.7% is present in the freeze-dried cake and 0.4% formed in the sample preparation. To minimize decomposition of 5-AC in the sample and standard preparations, methanol–water (80:20) is used as a solvent for dilution of the preparations. Hydrolysis rates in the aqueous alcohol are much slower than in purely aqueous solution. The preparations are injected 15 min after addition of the alcoholic internal standard solution to reproduce the extent of decomposition. Use of the lower wavelength (230 nm) was necessary to obtain a response for the secondary decomposition product.

UV spectra

UV spectra obtained with the diode-array detector for Ara-C, 5-AC, and their analogues are shown in Fig. 6. The spectra were obtained as the analytes eluted from the chromatograph. In the neutral pH of the mobile phase, Ara-C absorbs light from 200 through 300 nm with a small maximum at about 275 nm. Cytosine, the base portion of Ara-C, has an absorbance spectra that closely parallels Ara-C. The sugar

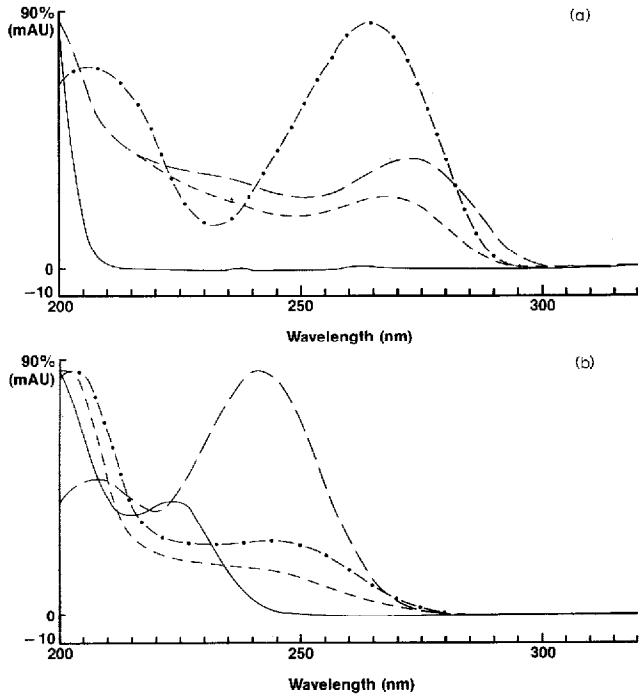

Fig. 6. Spectra of (a) cytarabine and analogues, and (b) azacitidine and analogues. (a) ——, D-arabinose (λ_{max} = 200 nm, max. abs. = 30 mAU); ---, cytosine (λ_{max} = 200 nm, max. abs. = 137 mAU); – – –, cytarabine (λ_{max} = 200 nm, max. abs. = 314 mAU); – · – · –, uracil arabinoside (λ_{max} = 262 nm, max. abs. = 12 mAU). (b) ——, V (λ_{max} = 200 nm, max. abs. = 115 mAU); ----, 5-azacytosine (λ_{max} = 200 nm, max. abs. = 321 mAU); ——, IV (λ_{max} = 242 nm, max. abs. = 216 mAU); · · · · · · ·, azacitidine (λ_{max} = 202 nm, max. abs. = 506 mAU).

portion of the molecule, D-(−)-arabinose, only shows a weak end absorbance above 200 nm. The primary decomposition product of Ara-C, uracil arabinoside, has a broad peak with a maximum at 270 nm. Except for the sugar, the common 254 nm HPLC detectors provide good sensitivity for the analysis of these components.

The UV spectra of 5-AC and 5-azacytosine have broad bands, which are similar to those of Ara-C and cytosine. The primary decomposition product of 5-AC has a maximum at *ca.* 254 nm. Maximal absorptivity for the second decomposition product of 5-AC (V) is at lower wavelengths, and sensitivity for it can be increased by changing from the normal analytical wavelength of 254 nm to 230 nm. Ribose, the sugar portion of the 5-AC molecule, and mannitol, the sugar which is present in an amount equal to the 5-AC in the Mylosar formulation, are analogous to D-(−)-arabinose in that they contain no significant ultraviolet chromophore.

Linearity and precision

Linearity of response was investigated by injecting nine samples of Ara-C, ranging from 0.3 to 1.5 μg. These amounts correspond to 30–150% of the amount specified in the assay procedure. Linear regression analysis of the amount determined by peak height and peak area *versus* the amount added resulted in slopes of 1.006 and 1.012, respectively, and intercepts which were less than the assay variation. Correlation coefficients (r) for these lines were greater than 0.9999. An Ara-C sample was assayed a total of eight times on six different days with a relative standard deviation (R.S.D.) of 1.1 and 0.9% by peak height and peak area, respectively.

Nineteen spiked sample preparations of Ara-C were prepared with 0–15% of its decomposition product, uracil arabinoside. Regression analysis of the uracil arabinoside amount determined by peak height *versus* the amount added resulted in a slope of 0.975 with $r > 0.99$. The amounts of uracil arabinoside determined in the presence of large amounts of Ara-C are slightly below theory (2.5%), because of the limited resolution from the major component. Peak-area quantitation tended to be less accurate than peak-height quantitation of uracil arabinoside on the tailing side of the Ara-C peak. A total of eight assays on six different days were performed on a sample that contained 0.5% uracil arabinoside with a resulting R.S.D. of 3.7% by peak height.

Linearity for 5-AC was established by injecting seven samples ranging from 0.8 to 2.8 μg. These amounts correspond to 40–140% of amount specified in the assay procedure. Peak-height and peak-area responses were linear over this range with $r > 0.999$ and intercepts which were less than the assay variation. Precision of the procedure has been established by a relative standard deviation of 1.0% for twenty determinations performed on a solid sample over a period of a year.

Azacitidine decomposition kinetics

The kinetics of the two-step decomposition route of 5-AC can be fit to the following equation: $[III] = Ae^{-\alpha t} + Be^{-\beta t}$ (refs. 7 and 9). Iterative best-fit calculations were performed on [III] *versus* time (t) profiles to determine the coefficients (A, B) and exponential terms (α, β). From the terms of the biexponential equation, the respective first-order kinetic rate constants were calculated[10].

The values for the three first-order rate constants associated with the decomposition of 5-AC are listed in Tables I and II. Kinetic rate constants were not sig-

TABLE I

FIRST-ORDER DECOMPOSITION RATE CONSTANTS FOR 0.1 mg/ml AZACITIDINE SOLUTIONS

Solvent	*Temperature (°C)*	k_{11} *(min^{-1})*	k_{12} *(min^{-1})*	k_{13} *(min^{-1})*
Water	25	0.000692	0.00180	0.000336
Water	30	0.00120	0.00312	0.000542
Phosphate (0.0067 *M*)	30	0.00135	0.00333	0.000842
Phosphate (0.067 *M*)	30	0.00214	0.00558	0.00185
Phosphate (0.2 *M*)	30	0.00562	0.0139	0.00366

nificantly different for solutions made from the bulk drug and Mylosar formulation, indicating that the small amount of mannitol does not affect the solution stability of 5-AC.

An example of the profiles of 5-AC and its two decomposition products as a function of time in a lactated Ringer's injection solution USP is shown in Fig. 7. These profiles were calculated from the values of the biexponential equation which was fit to the experimental data for 5-AC. Within the 1% R.S.D. of the HPLC assay, values for 5-AC which were calculated in an iterative fashion from the first-order kinetic rate constants equaled the experimental values.

The thermal dependence of the three first-order rate constants fit the Arrhenius

TABLE II

FIRST-ORDER DECOMPOSITION RATE CONSTANTS FOR AZACITIDINE (0.1 mg/ml) IN VARIOUS THERAPEUTIC SOLUTIONS OF MYLOSAR

Solvents: water = purified water USP; BWFI = bacteriostatic water for injection; LRI = lactated Ringer's injection USP; dextrose = 5% dextrose injection USP; saline = 0.9% sodium chloride injection USP. Suppliers of solvents: A = laboratory purified water USP; B = The Upjohn Company, C = Travenol Labs., D = Abbott Labs.

Solvent	*Solvent supplier*	*pH*	*Temperature (°C)*	*Container type*	k_{11} *(min^{-1})*	k_{12} *(min^{-1})*	k_{13} *(min^{-1})*	*IV set*
Water	A	7.0	30	Glass	0.00121	0.00334	0.000498	
Water	A	7.0	40	Glass	0.00343	0.00938	0.00157	
BWFI	B	5.7	25	Glass	0.000526	0.00170	0.000385	
LRI	C	7.0	4	Glass	0.0000619	0.000136	0.0000158	
LRI	C	6.0	25	Glass	0.00149	0.00413	0.000355	
LRI	C	6.5	25	Glass	0.00100	0.00258	0.000341	
LRI	C	7.0	25	Glass	0.000878	0.002501	0.000462	
LRI	C	7.0	25	Plastic	0.000769	0.00178	0.000348	Yes
LRI	C	7.0	25	Plastic	0.000897	0.00224	0.000370	
LRI	D	7.0	25	Plastic	0.000730	0.00181	0.000354	Yes
LRI	C	7.5	25	Glass	0.000991	0.00268	0.000428	
Dextrose	D	4.0	25	Plastic	0.00304	0.00829	0.00131	Yes
Dextrose	C	6.0	25	Glass	0.000608	0.00115	0.00147	
Saline	C	4.5	25	Glass	0.00296	0.0106	0.00168	
Saline	C	4.5	25	Plastic	0.00296	0.00810	0.000436	
Saline	D	5.8	25	Plastic	0.00172	0.00451	0.00332	Yes

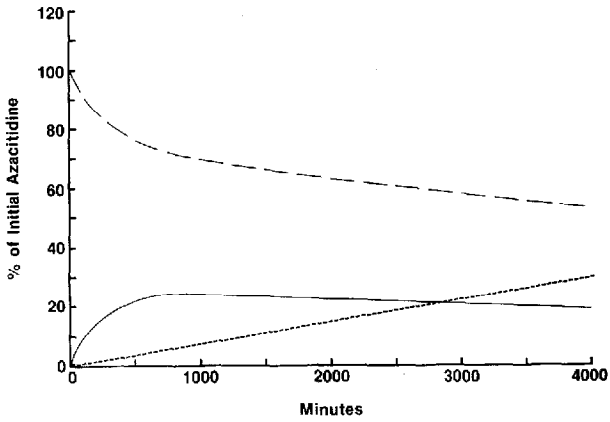

Fig. 7. Calculated profiles for azacitidine and its decomposition products in a solution of Lactated Ringer's injection USP, pH 6.5, 25°C.

equation for neutral solutions (pH 7) over the temperature range of 4–40°C. The calculated activation energies for k_{11}, k_{12}, and k_{13} were 8.33, 8.76, and 9.58 kcal/mol, respectively. For pharmaceutical preparations of 5-AC, the stability is greatly enhanced by reducing the temperature. After 10 h, *ca.* 20% decomposition occurs at 25°C, while *ca.* 4% decomposition occurs at 4°C.

The rate constants in Table I show the decrease in stability of 5-AC with increasing ionic strength of pH 7 phosphate buffered solutions. The negative effect of ionic strength on the stability of 5-AC has been observed by other researchers[4]. Infusion solutions of low ionic strength provide optimal stability for administration of Mylosar®. Of the pharmaceutical solutions, degradation of 5-AC is slowest in the bacteriostatic water for injection USP solution. Electrolytes in each of the LVP solvents have a destabilizing effect on the 5-AC. Because of the lower pH of the saline a· 1 dextrose solutions, the decomposition of the drug is much more rapid than the nearly neutral lactated Ringer's injection USP solution. Even within the specified range of 6.0–7.5 for the pH of lactated Ringer's injection USP, differences in the decomposition rates are detectable. The maximum for stability in this infusion solution is obtained at pH 7.0. Use of an intravenous (i.v.)-solution-administration set with particulate filter did not affect the decomposition kinetics.

Without isolating the decomposition products of 5-AC, IV and V, their response factors can be calculated from the observed responses and the kinetic factors. Responses for the decomposition products in solution are a result of the initial level of the component and the amount formed as a function of time. After subtracting the initial response, the response factor can be calculated from the amount predicted from the kinetic factors. Reproducible values for the response factors were observed for the time window from 700 to 4200 min. Response factors calculated from early time points were not reproducible because only a small amount of the decomposition products had formed. For data from three different solutions using the 254-nm mercury line source detector, an average response factor for IV *versus* 5-AC on a mass basis was 2.14, with an R.S.D. of 3%. For data from three different solutions using a variable-wavelength detector set at 254 nm, the factor was 2.20, with an R.S.D. of

15%. Better precision for the relative response factor is expected with the line source detector, because of the reproducibility of the source wavelength. With the photodiode-array detector, a relative response factor for V *versus* 5-AC on a mass basis at 230 nm was found to be 0.56, with an R.S.D. of 7%.

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