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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-

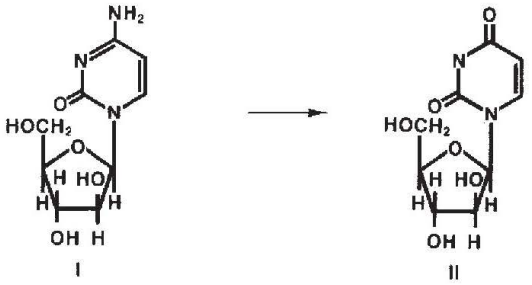

Fig. 1. Hydrolytic deamination of cytarabine (I) to uracil arabinoside (II).

philic attack, opening the triazine ring at the 5,6-position to form N-(formylamine)-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].

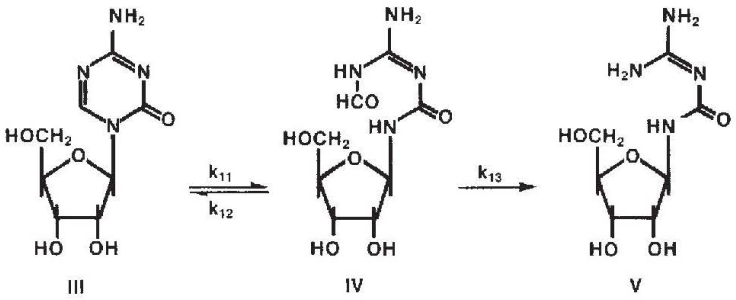

Fig. 2. Stepwise hydrolysis of azacitidine (III) to N-(formylaminine)-N′-2β-ribofuranosylurea (IV), then 1-β-D-ribofuranosyl-3-guanylurea (V).

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,

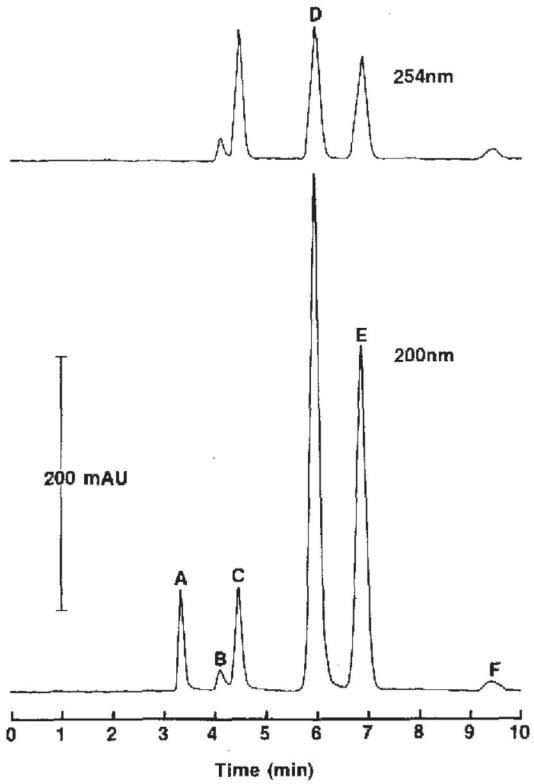

Fig. 3. Chromatograms recorded at 200 and 254 nm for cytarabine, azacitidine, and their analogues. Peaks: A = 0.45 μg V; B = 0.20 μg cyclocytidine; C = 0.90 μg IV; D = 2.6 μg azacitidine; E = 2.0 μg cytarabine; F = 0.20 μg uracil arabinoside. See Figs. 1 and 2 for full names and structures.

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

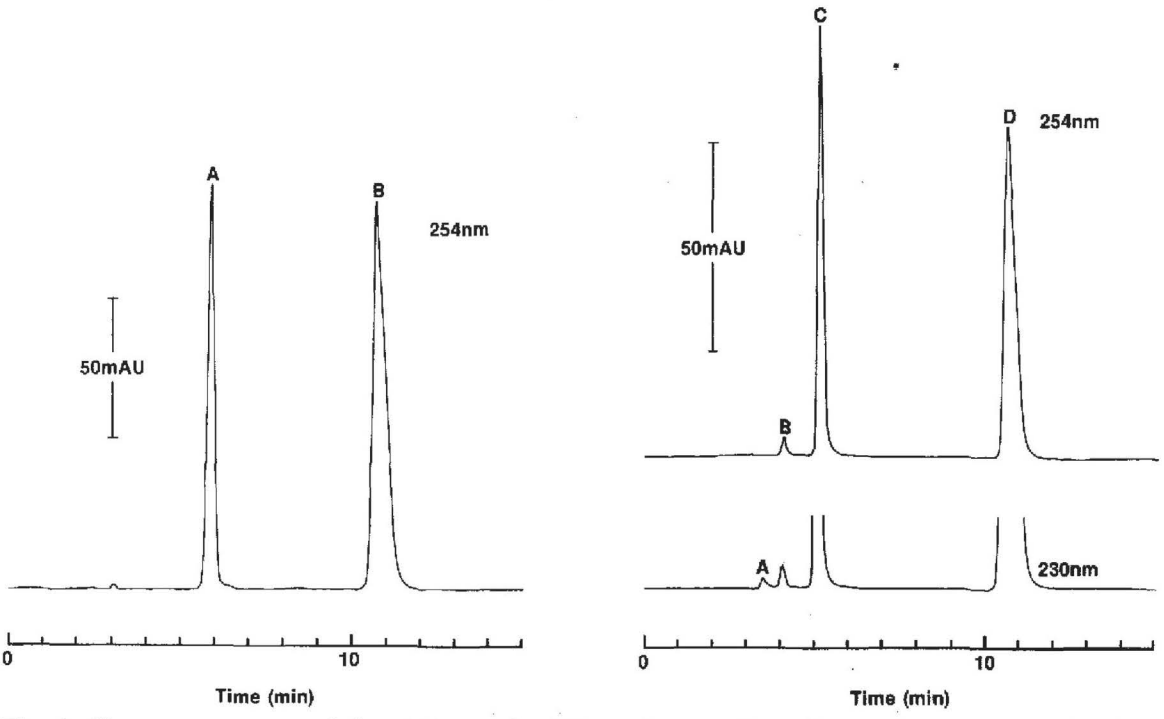

Fig. 4. Chromatogram recorded at 254 nm of a 100-mg Cytosar-U sterile powder sample. Peaks: A = cytarabine; B = *p*-toluic acid.

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.

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