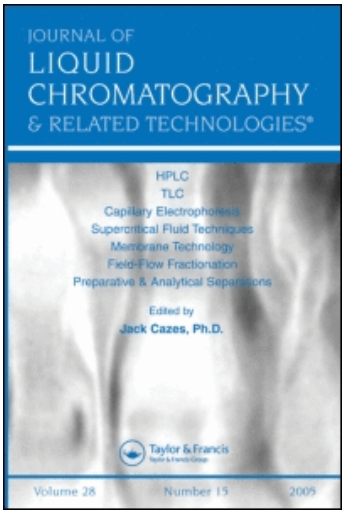

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A RAPID STABILITY-INDICATING HPLC ASSAY FOR THE ARABINOSYLCYTOSINE PRODRUG, CYCLOCYTIDINE

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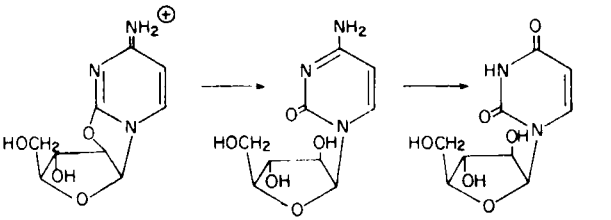
ABSTRACT

Hydrolysis of cyclocytidine in aqueous solutions produced arabinosylcytosine which, in some cases, further reacted to form arabinosyluracil. No other degradation products were detected. A rapid isocratic reverse-phase HPLC assay for all three components in mixtures arising from cyclocytidine hydrolysis was developed. The analysis employs a 4.6 cm column together with a low methanol mobile phase containing 1-heptane sulfonic acid at pH 2.9. The ion-paring of cyclo-C, a cation, was independent of pH. However, ion-paring of arabinosylcytosine was controlled by adjusting the pH to 2.9 which is below its pKa of 4.2. The retention time of neutral arabinosyluracil (pKa = 9.2) was not affected by either the pH or the ion-pairing agent. Its separation was achieved by using a primarily aqueous mobile phase with the minimum methanol required for the other components. The time courses for cyclocytidine and its hydrolysis products were successfully defined under a variety of aqueous conditions.

INTRODUCTION

Arabinosylcytosine (ara-C) is one of the most active chemotherapeutic agents for the treatment of acute myelogenous leukemia

in adults. In man, the prodrug cyclocytidine (cyclo-C) is partially converted to ara-C which is primarily metabolized to arabinosyluracil (ara-U) as shown in Scheme I (1). Although



Cyclocytidine (cyclo-C) | Arabinosylcytosine (ara-C) | Arabinosyluracil (ara-U)

Scheme I

the hydrolytic deamination of ara-C is catalyzed by pyrimidine nucleoside deaminase, the biotransformation of cyclo-C appears to be due solely to chemical hydrolysis (2). Therefore, hydrolysis represents a potential stability problem when storing aqueous solutions of cyclo-C. The deamination of cyclo-C to form cyclouridine has not been reported.

In aqueous buffered solutions ara-C undergoes chemical hydrolysis to inactive ara-U (3,4). The goal of this research was to develop a stability-indicating assay for cyclo-C, ara-C and ara-U in mixtures arising from the hydrolysis of cyclo-C in aqueous solutions.

Resolution was complicated by the fact that cyclo-C is a cation, ara-U is extremely polar and ara-C has a pKa of 4.2. Both ara-C and cyclo-C required ion-pairing and high methanol

to give practical resolution times on normal length columns. Conversely, ara-U appeared with the solvent front in the presence of any significant methanol concentration. Rapid separation was achieved by using a 4.6 cm column with a low methanol mobile phase containing an ion-pairing agent at controlled pH.

MATERIALS AND METHODS

Chemicals and Reagents

Arabinosyluracil and 2,2'-anhydro(1-β-D-arabinofuranosyl) cytosine HCl (cyclo-C) (ICN Nutritional Biochemicals, Cleveland, OH), arabinofuranosyl cytosine HCl (ara-C) (The Upjohn Co., Kalamazoo, MI) and thymidine (P-L Biochemicals, Inc., Milwaukee, WI) were used as reference standards. The methanol (Burdick and Jackson Labs, Muskegon, MI) was HPLC grade. Filtered double distilled water was used in the preparation of all solutions. The monobasic sodium phosphate (monohydrate), dibasic sodium phosphate (dodecahydrate) and glacial acetic acid (J.T. Baker, Co., Phillisburg, NJ) were analytical grade.

Mobile Phase

One liter of 0.005 M aqueous 1-heptane sulfonic acid was filtered under vacuum through a type HA 0.45 μm membrane filter (filter and all-glass filtration apparatus, 47 mm, Millipore Corp., Bedford, MA). After addition of 30 ml of methanol the solution was degassed and adjusted to pH 2.9 with glacial acetic acid.

Neither the degassing nor the pH adjustment altered the volume significantly.

Apparatus

An isocratic separation was employed using one pump of a gradient liquid chromatograph (Model 332), having a 20 µl loop on the injection port (Model 210) and a 254 nm UV detector with 8 µl analytical cuvet and pump (Model 110A) (Altex, Berkeley, CA). An all-glass 250 µl syringe (2001, Becton-Dickinson, NJ) was used to fill the loop throughout the study. A 4.6 cm x 4.2 mm I.D. column was packed with Spherisorb®, 5 µ, ODS, C_{18} (Altex, Berkeley, CA) under 4000 psi using a Haskell Pump (Tracor Inc., Austin, TX).

Preparation of Calibration Plots

A 0.3 ml aliquot of methanolic 2.42×10^{-3} M thymidine was pipeted into each glass test tube using a syringe microburet (Micro-Metric Instrument Co., Cleveland, OH). The methanol was then evaporated in a vacuum desicator and the residual thymidine later served as the internal reference standard after the addition of the aqueous samples. Simulated mobile phase, containing all but the 1-heptane sulfonic acid, was used as the diluent. Reference standard solutions were prepared by adding 1 ml of buffered solution containing the compound for assay to 10 ml of diluent in the tubes containing thymidine to give final concentration ranges of $0.3\text{-}3.3 \times 10^{-4}$ M (cyclo-C), $0.3\text{-}3.9 \times 10^{-4}$ M (ara-C)

and 0.1-1.0 x 10^{-4} M (ara-U) and 6.6 x 10^{-5} M thymidine. These standard solutions were analyzed by injecting approximately 200-250 µl to fill the 20 µl loop. Calibration plots were based on the ratio of the peak height of the reference standard to that of the peak height for the constant thymidine concentration as a function of concentration of the reference standard. The column was periodically cleaned by increasing the amount of methanol in the mobile phase.

Analysis of Cyclo-C Hydrolysis Reactions

Buffered aqueous solutions of cyclo-C were maintained at constant temperature. As a function of time, 1 ml aliquots were removed, added to tubes containing 10 mls of diluent and thymidine and assayed as described above.

RESULTS AND DISCUSSION

A preliminary screen using TLC showed that cyclo-C was stable in acid pH at room temperature as was already reported for ara-C (3,4). Cyclo-C hydrolysis reactions could therefore be quenched in acid pH and stored for analysis. Both TLC and HPLC data verified that cyclo-C hydrolyzes according to Scheme I at neutral and alkaline pH. Within the temperature range of 40° - 90° and pH 5-10 there did not appear to be products other than those in Scheme I.

These three reaction components proved difficult to separate. Ara-U was found to pass through reverse-phase columns

with the solvent front if methanol was in excess of 4-5% of the mobile phase. At low methanol concentrations both cyclo-C and ara-C remained on the columns. Cyclo-C, a cation, requires both ion-pairing agent and high methanol (40-50%) for practical retention times. Ara-C, which has a pKa of 4.2 (2), gave best results when ion-paired (pH < 5) at similar high methanol concentrations. In order to reduce both the methanol concentration and the cyclo-C and ara-C retention times a column of the length normally employed as a pre-column was employed.

After selecting a 4.6 cm column, three properties of the mobile phase were adjusted in order to achieve resolution of cyclo-C, ara-C and ara-U: polarity, pH and ion-pairing agent. It was found that 3% was the highest methanol percentage which allowed separation of ara-U from the solvent front. At neutral pH, 3% aqueous methanol did not resolve ara-C and cyclo-C which also had very long retention times. The ara-U retention time was insensitive to ion-pairing agent and pH in the range 3-7. Cyclo-C retention time was decreased by methanol and ion-pairing but not by pH since it is a cation. Ara-C (pKa=4.2) retention time was decreased by methanol, ion-pairing and low pH in the presence of ion-pairing agent. A 0.005 M 1-heptane sulfonic acid solution in 3% methanol at pH 2.9 was found to provide excellent separation for all three reaction components and the internal standard, thymidine (Fig. 1). Table I summarizes the capacity factors and the peak height ratios (PHR) relative to the thymidine.

TABLE 1

Capacity factors (k')[a] and relative peak height ratios (PHR)[b] for reaction components (Scheme I) and thymidine (I.S.) at a 2 ml/min flow rate.

Compound	k'	Relative PHR
ara-U	1.1	2.3
thymidine (I.S.)	3.5	1
cyclo-C	8.2	0.57
ara-C	17	0.20

[a]Calculated from $k' = (V-Vo)/Vo$

[b]Relative $(PHR)_x = PH_x/PH_{I.S.}$ calculated at equimolar concentrations

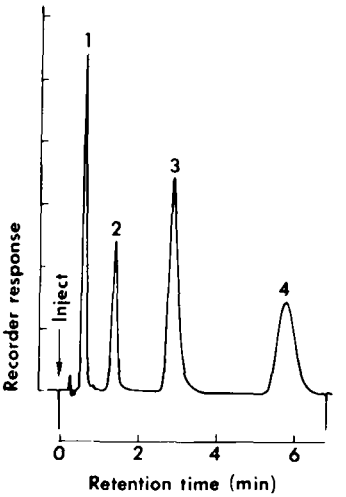


FIGURE 1. Chromatogram from a 20 µl sample mixture containing: (1) ara-U, 6.57×10^{-5} M. (2) thymidine, 6.44×10^{-5} M. (3) cyclo-C, 17.6×10^{-5} M. and (4) ara-C, 18.7×10^{-5} M. using a 2 ml/min flow rate of 3% methanol in water, pH 2.9, containing 0.005 M. 1-heptane sulfonic acid with 254 nm UV detector attenuation of 0.08 X.

The assay was applied to solutions of cyclo-C which were hydrolyzing as a function of time. In each case a 1 ml aliquot of reaction was added to 10 ml of simulated mobile phase containing all but the ion-pairing agent to quench the reaction. For convenience, these solutions were stored in the refrigerator until each set of assays could be carried out in a single day. Although the storage period was normally a few days, no change was observed in a 2-week period which was the maximum tested. Calibration plots were run on the day of assay using four solutions of known mixtures of ara-U, cyclo-C, ara-C and a fixed concentration of thymidine. The thymidine peak height did not vary to any detectable degree within a given set. Therefore the peak height data could have been used directly without conversion

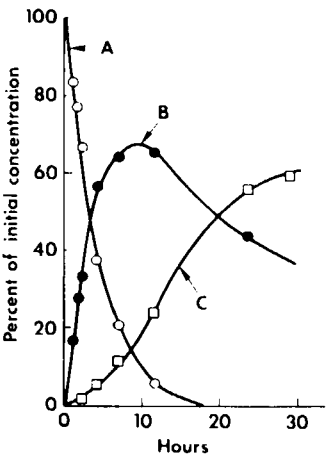


FIGURE 2. Percent of initial 2.5 x 10^{-4} M. cyclo-C concentration in phosphate buffer, pH 5.5 at 80°, as a function of time as determined by the HPLC assay where (A) is cyclo-C, (B) is ara-C and (C) is ara-U.

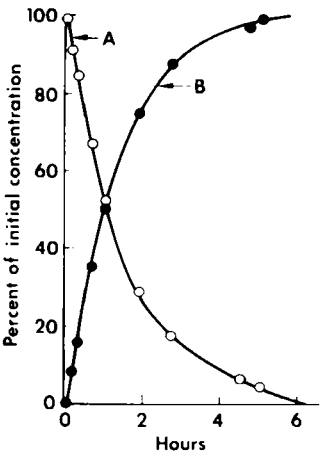


FIGURE 3. Percent of initial 2.5×10^{-4} M. cyclo-C concentration in phosphate buffer, pH 7.2 at 60°, as a function of time as determined by the HPLC assay where (A) is cyclo-C and (B) is ara-C.

to peak height ratios. The assay was successfully applied to a variety of degrading solutions of cyclo-C. Figure 2 illustrates a typical case wherein all three components were detected. Figure 3 shows an example wherein conversion of cyclo-C to ara-C was complete without detectable deamination of ara-C to ara-U. Detailed studies on the kinetics and mechanisms of the hydrolysis of ara-C have been reported (3,4) and those on cyclo-C are in progress.

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