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Note

High-performance liquid chromatographic determination of 5-azacytidine in plasma

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5-Azacytidine, because it is a nucleoside analogue of the pyrimidine derivative cytidine, affects the synthesis of RNA. It has been shown to be effective in treating certain kinds of malignant tumors [1–3] and it has been used as an antileukemic drug [4]. 5-Azacytidine can be highly toxic [5]. An accurate method for determining this drug could permit monitoring its plasma levels so as to make them maximally therapeutic but minimally toxic.

A microbiological and a radioactive carbon labeling assay have been used to determine 5-azacytidine in biological samples [6,7]. High-performance liquid chromatography (HPLC) has been used for determination in aqueous solution but not for biological samples [8,9]. A significant problem in determining 5-azacytidine is its instability in solution. In this paper, we wish to report an HPLC method for determining 5-azacytidine in plasma and some studies of its stability in this medium.

EXPERIMENTAL

Apparatus

A Waters M-6000 pump (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.), a Rheodyne 7125 injector (Berkeley, CA, U.S.A.), a Kratos 773 variable-wavelength UV–visible detector (ABI Analytical Kratos Division, Ramsey, NJ, U.S.A.), and a Houston Instrument TM 4500 strip chart recorder (Houston Instrument, Austin, TX, U.S.A.) were used. A 10 cm $\times$ 4.6 mm column of 10-μm spherical reversed-phase packing (styrene–divinylbenzene polymer, Hamilton PRP-1, Hamilton, Reno, NV, U.S.A.) was used. A C-130B Upchurch

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guard column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with 10-μm PRP-1 was used. It was replaced when a rapid pressure rise occurred (after 20 to 30 injections of treated plasma samples). The analytical column was packed with a Micromeritics packer (Norcross, GA, U.S.A.). The mobile phase was 10 m*M* sodium octanesulfonate/sulfonic acid (pH 2.36)–methanol (85:15, v/v). The flow-rate was 1.0 ml/min and the detection wavelength was 252 nm. The working range was 0.20 to 0.002 a.u.f.s.

Materials

5-Azacytidine (Azacitidine) was obtained from the National Cancer Institute, National Institutes of Health (Bethesda, MD U.S.A.). Chemicals were reagent grade or better. Mobile phase solvents were HPLC grade: water was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and methyl alcohol from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Calibration standards

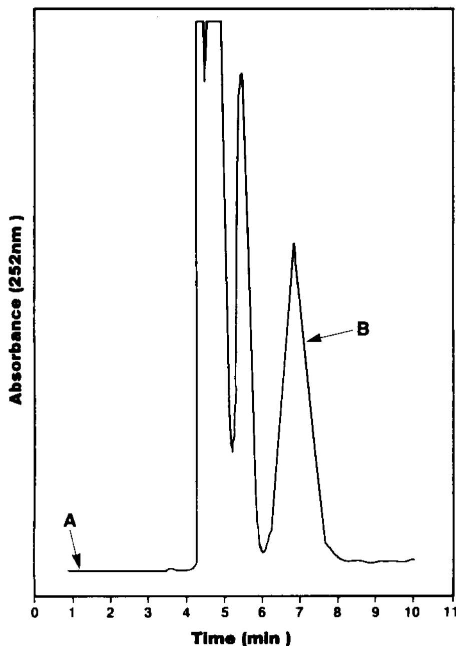
A stock solution of 5-azacytidine was prepared by dissolving 50.0 mg of it in 100 ml of HPLC water and taking 20.0 ml of this and again diluting to 100 ml. This solution was made fresh for each calibration because of the instability of 5-azacytidine in water. Standards were made by adding to 1.0 ml of water or plasma enough stock solution to give concentrations of 0.00, 0.25, 0.50, 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 μg/ml.

Procedure

To precipitate proteins, 200 μl of acetonitrile were added to 1.0 ml of a patient's plasma or control plasma. The mixture was vortex-stirred for 1 min. To further precipitate proteins and reduce the number of constituents in the sample, 50–100 mg of zinc sulfate were added. Again, the sample was vortex-stirred for 1 min. The mixture was then centrifuged for 5 min at 3000 *g*. The clean supernate was further purified by filtering through a 0.45-μm Nylon-66 membrane filter tip (Rainin Instrument, Woburn, MA, U.S.A.) of a gas-tight syringe. From the total volume of filtered solution, 20 μl were injected into the chromatograph.

Stability of 5-azacytidine in plasma

A plasma solution containing 20 μg/ml 5-azacytidine was transferred in approximately 2-ml volumes into borosilicate test tubes. Immediately, 1.0 ml was analyzed and this was considered zero time, and it was assumed that no decomposition occurred. Periodically, another test tube's contents was analyzed. The determinations were continued beyond 20% loss of 5-azacytidine. A similar procedure was used for samples stored at −10 and −60°C. However, time was required, about 3 h, for the samples to thaw to room temperature. The 5-azacytidine determination was performed as soon as room temperature was reached. The study was not a highly accurate kinetic one, and, therefore, this period was considered an approximately constant offset for each time that would not change the shape of the stability curve.



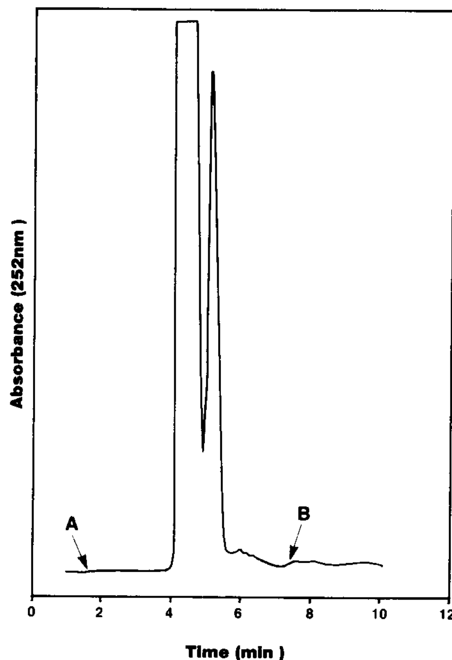


Fig. 1. Chromatogram of a plasma extract, detector: 0.002 a.u.f.s. B = Azacytidine; A = injection point.

Fig. 2. Chromatogram of a plasma extract containing no 5-azacytidine, detector: 0.003 a.u.f.s. B = Azacytidine; A = injection point.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of plasma containing 5-azacytidine pretreated by the acetonitrile and zinc sulfate procedure used in this work. The drug peak is well resolved from nearby peaks. Fig. 2 is a chromatogram of a plasma containing no 5-azacytidine. The retention time region for 5-azacytidine is clearly free of interfering peaks.

Peak heights were used for 5-azacytidine determination. Calibration curves were straight lines ($r = 0.999$) with virtually zero intercepts. Calibration curve slopes for plasma and water were the same. This identity showed that plasma constituents were not affecting the prechromatography treatment results. The calibration curve for water standards chromatographed directly had the same slope as a curve with those standards after they received the acetonitrile–zinc sulfate treatment. This identity showed that these reagents did not reduce or enhance the 5-azacytidine peak heights.

The reproducibility of the concentration of 5-azacytidine expressed as relative standard deviation over the range of spiked plasma concentrations of 0.21–20.6 μg/ml was 3.6–5.2% ($n = 8$ for each of six concentrations). The limit of detection was 80 ng/ml in plasma at a signal-to-noise ratio of 2. A recovery experiment in which 5-azacytidine was spiked into a patient's plasma to double the originally

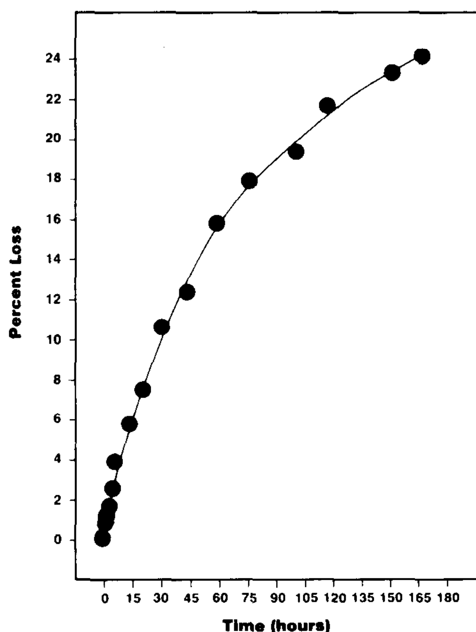


Fig. 3. Stability of 5-azacytidine in plasma at -60 °C at a concentration of 20 μg/ml.

determined concentration of 270 ng/ml gave 96±10% recovery (n=3) after redetermination.

The volume injected into the chromatograph was determined by studying peak height and resolution as a function of volume. To obtain the greatest peak height without destroying resolution from nearby peaks, 20 μl were found to be optimum.

Exploratory initial studies showed that the most likely method for successful resolution of 5-azacytidine was the use of ion-pair chromatography because of the low retention in ordinary reversed-phase. The pH of the mobile phase was reduced to a low value to produce the positive ion of 5-azacytidine. Octanesulfonate was used for ion pairing. Evidence that ion pairing had actually occurred was the increase in capacity factor with concentration increase of octanesulfonate and a steady drop in capacity factor as sodium chloride was added to the mobile phase from 0 to 30 m*M*. Methyl alcohol content of the mobile phase was chosen so as to give good resolution with short retention time. Acetonitrile gave equally good results.

Fig. 3 shows the results of a study of the stability of 5-azacytidine in plasma. In the case illustrated, the temperature was -60 °C. Even at this low temperature, the drug is unstable. In 30 h, there was a 10% loss when the initial concentration was 20 μg/ml. As expected, as the temperature rose, the disappearance rate increased. At -10 °C, a 10% loss occurred in 2.5 h and in only 0.5 h at room temperature.

Thus, low-temperature storage of samples immediately after drawing is essential. In our work, samples were refrigerated at -80 °C. Obviously samples should be analysed as soon as possible after drawing. No delays can be tolerated during the determination of 5-azacytidine.

5-Azacytidine was determined in ten leukemic patient plasma samples. The samples were handled as stated above. The range of values was 320 ng/ml to 1.3 μg/ml. These values were consistent with the clinical dosages used.

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