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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR CYTOSINE ARABINOSIDE, URACIL ARABINOSIDE AND SOME RELATED NUCLEOSIDES

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SUMMARY

A novel, dual-column high-performance liquid chromatographic method for determination of the anti-cancer drug cytosine arabinoside (Ara-C) and its major metabolite uracil arabinoside (Ara-U) has been developed. The analytical procedure is sensitive (25 ng/ml) and specific for Ara-C, Ara-U and the endogenous nucleosides that may influence response to Ara-C therapy, cytidine and deoxycytidine. Conventional and high dose calibration curves were linear and the method precise with the assay coefficient of variation for Ara-C and Ara-U not greater than 9.1% over the range of 0.1—10 μg/ml. Accuracy was determined to be within ± 3 to 9% over this concentration range. Using this method, patient plasma samples from both conventional dose (100—200 mg/m^2 per day) and high dose (3500—6500 mg/m^2 per day) Ara-C can be simultaneously analyzed for Ara-C, Ara-U and nucleosides so that comparative pharmacokinetic and pharmacodynamic studies can be conducted.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine; Ara-C) is a pyrimidine analogue that is effective in the treatment of acute myelogenous leukemia and acute lymphocytic leukemia [1—4].

After activation by deoxycytidine kinase and further intracellular phosphorylation to arabinosyl-CTP [5,6], the anti-metabolite selectively inhibits DNA synthesis.

Ara-C is rapidly deaminated to an inactive metabolite, uracil arabinoside (Ara-U) by cytidine deaminase [7,8]. Due to this rapid deamination, Ara-C has a short plasma elimination half-life (12—200 min) when administered by intravenous bolus injection [9,10]. The short half-life of Ara-C and its S-phase

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(cell cycle-dependent) specificity have led to clinical trials of continuous intravenous or subcutaneous (S.Q.) infusions of conventional dose (CD = 100—200 mg/m^2 per day) or high doses (HD = 1000—6500 mg/m^2 per day) of Ara-C, over a period of several days [10—15]. Clinically, variation in the pharmacokinetics (drug metabolism and disposition) of Ara-C may be a potentially important determinant of its efficacy and toxicity [16]. The interindividual variation in the endogenous nucleoside and nucleotide concentrations may also be an important determinant in cytotoxic effects during Ara-C treatment [17,18]. To investigate the human disposition and biochemical effects after administration (pharmacodynamics) of Ara-C, a sensitive and specific method for quantitating Ara-C, Ara-U, cytidine and deoxycytidine is required.

Several techniques have been employed for the elucidation of Ara-C pharmacokinetics, including microbiology [19,20], radioimmunology [21,22], ultraviolet (UV) spectroscopy [23] and gas chromatography—mass spectrometry [12] or gas—liquid chromatography—flame ionization—mass spectrometry [24]. Some of these techniques are sensitive enough for the detection of Ara-C when administered at conventional dosages. However, the assays may not be specific for quantitation of both Ara-C and Ara-U, or they may require specialized handling of radioactive materials.

High-performance liquid chromatography (HPLC) with UV detection offers a sensitive and specific method to monitor concentrations of Ara-C and its metabolites in biological fluids of patients receiving the drug. There have been numerous publications of HPLC methods for determination of Ara-C [15, 25—29]; however, most of the previously described procedures cannot separate Ara-C, Ara-U and the structurally similar endogenous nucleosides (cytidine and deoxycytidine), which may influence the anti-cancer effects of Ara-C (Fig. 1). Therefore, we have developed a sensitive and specific dual-column HPLC method to monitor plasma, urine and cerebrospinal fluid (CSF) levels of Ara-C,

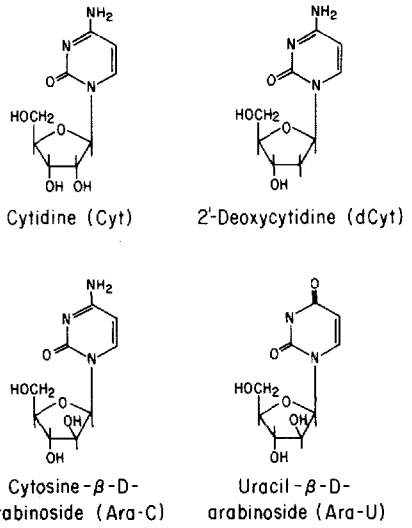

Fig. 1. Comparative structures of cytidine (Cyt), deoxycytidine (dCyt), cytosine arabinoside (Ara-C) and uracil arabinoside (Ara-U).

its major metabolite Ara-U and the endogenous nucleosides, deoxycytidine and cytidine during continuous infusions of conventional and high doses of Ara-C in children with cancer.

EXPERIMENTAL

Materials

Ara-C, Ara-U, adenine arabinoside (Ara-A), tetrahydrouridine (THU), nucleosides and deoxyribonucleosides including cytidine (Cyt) and deoxycytidine (dCyt) were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium phosphate (monobasic) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). HPLC-grade methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was deionized, distilled and filtered for HPLC use. The MPS-1 micropartition system (Amicon, Lexington, MA, U.S.A.) was used to filter plasma samples before HPLC analysis. Other reagents and glassware were of standard laboratory quality.

Instrumentation and chromatography

The chromatographic conditions used a mobile phase of 2.5 m*M* KH_2PO_4 (pH 3.2) with 2.5% methanol, a Waters Assoc. (Milford, MA, U.S.A.) Model M-45 HPLC pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 100-μl sample loop, and a 5-μm reversed-phase Ultrasphere-ODS column, 15 cm × 4.6 mm, Altex, Berkeley, CA, U.S.A.), connected in series with a 10-μm cation-exchange column (Partisil PXS 10/25 SCX; 25 cm × 4.6 mm; Whatman, Clifton, NJ, U.S.A.).

The column effluent was monitored for UV absorbance at 280 nm with a Spectra-Physics (Houston, TX, U.S.A.) 8300 UV monitor. Detector output was integrated by a Spectra-Physics SP4000 chromatography data system with graphical display on a Fisher Recordall strip chart recorder attenuated to 10 mV. The mobile phase flow-rate was about 0.8 ml/min at a pressure of 17 MPa (2500 p.s.i.).

Assay procedure

Blood and CSF samples (2—3 ml) were collected in heparinized tubes containing the deaminase inhibitor, tetrahydrouridine (0.1 mmole) and immediately placed on ice. Urine was collected in four 12-h intervals in sterile containers and refrigerated immediately. After the whole blood was centrifuged (800 *g* for 7 min), the plasma was collected and frozen at −70°C until analyzed. After appropriate addition of internal standard (Ara-A, 1 μg/ml), up to 25 plasma samples (1 ml) were filtered with the MPS-1 Micropartition System for 15—20 min at 800 *g* in a clinical centrifuge. Aliquots (100 μl) of the plasma ultrafiltrates were injected in duplicate. CSF samples (100 μl) were injected directly into the system and urine was appropriately diluted with deionized-distilled water (1:5 or 1:10) before HPLC injection (100 μl). Stability of Ara-C after sample collection was assessed by incubation of Ara-C (0.1 and 5 μg/ml) in fresh plasma, urine and CSF in tubes with and without 0.1 mmole THU. The samples were incubated at 25°C and 0°C (on ice) and the concentration of Ara-C was determined at 0 h and at the end of the incubation (1 h).

Results are expressed as the percent Ara-C remaining relative to the 0-h concentration.

In order to assess the linearity, precision and accuracy at low and high plasma concentrations of Ara-C and Ara-U, multilevel calibration curves were constructed. The peak areas of the conventional and high-dose patient samples were thereafter converted to absolute quantities using the respective predetermined calibration curves. Linear regression analysis was used to determine the best-fit line through the peak area or peak height versus concentration plot. Replicate analysis of low, medium and high calibrators were used to determine the assay precision (intra-assay $n = 10$ and inter-assay, $n = 5$) with three different operators and the same chromatographic system. Accuracy was determined by analysis of four operator-blinded "unknowns" at low to high concentrations of Ara-C, Ara-U, Cyt and dCyt (range of 0.05—10 μg/ml). Potential loss of the five compounds during the filtration process was attained by comparing filtrate of spiked samples in water, mobile phase and plasma to the equivalent amount injected directly in the system.

RESULTS AND DISCUSSION

There have been a number of reversed-phase or cation-exchange HPLC methods published for the determination of Ara-C [15,25—29], however, the ability to quantitate Ara-C, Ara-U, Cyt, dCyt and Ara-A using a novel reversed-phase cation-exchange dual-column method has not been previously described. Figs. 2 and 3 illustrate a patient's plasma ultrafiltrate obtained at 72 h during an intravenous infusion of high-dose Ara-C and at 24 h during a conventional

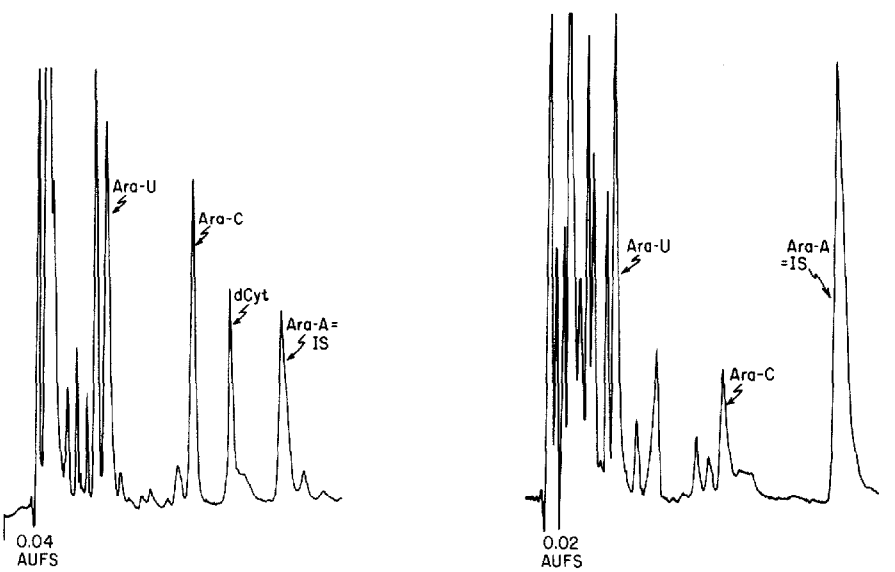

Fig. 2. Patient's plasma sample separation (high-dose Ara-C). High dose = 5 g/m² per day; 72-h during infusion sample. Ara-C = 2.6 μg/ml; Ara-U = 42.6 μg/ml.

Fig. 3. Patient plasma separation (conventional-dose Ara-C). Conventional dose = 100 mg/m² per day; 24-h during infusion sample. Ara-C = 0.045 μg/ml; Ara-U = 0.99 μg/ml.

dose infusion of Ara-C, respectively. The retention times and capacity factors (k' values) of Ara-U, Cyt, Ara-C, dCyt and Ara-A (used as internal standard) are 13.4 min (1.25), 22.0 min (4.5), 25.7 min (5.0), 28.5 min (6.25) and 35.7 min (8.0), respectively. The coefficient of variation for retention times over a 3-day period for all four compounds was less than 1%. The quantitative limit of detection for Ara-C and Ara-U was 20 ng/ml, which is comparable to other HPLC methods [29] but less sensitive than radioimmunoassay methods [21,22]. Loss of any of the five compounds during the filtration process was less than 8% in water, mobile phase and plasma and not considered significant.

Ara-C stability studies verified the need for THU-containing tubes for plasma sample collection. These studies showed no loss of parent drug at room temperature or on ice when the deaminase inhibitor was added. Samples that did not contain THU showed substantial conversion on ice (70% remaining) and at room temperature (only 40% remaining) after 1-h incubation in fresh plasma. Ara-C was stable in urine and CSF for at least 48 h at room temperature and on ice.

The high-dose and conventional-dose calibration curves were linear over their respective concentration range, as illustrated in Figs. 4 and 5. The intra-assay (n = 10) coefficients of variation for Ara-U and Ara-C, respectively, were 8.1% and 9.1% for 0.1 μg/ml samples, 4.4% and 4.8% for 1 μg/ml and 3.4% for 10 μg/ml samples. The inter-assay (day to day, n = 5) variability was less than 8% for all compounds of interest. Accuracy at the lowest concentration (0.05 μg/ml) was within ± 9% (91—109%) and accuracy improved to ± 3% (97—103%) at the higher concentrations (⩾ 5 μg/ml).

Blood samples (2—3 ml) from two patients who received conventional-dose Ara-C and two patients administered high-dose Ara-C were taken daily during an intravenous infusion of the drug in four children with acute myelogenous

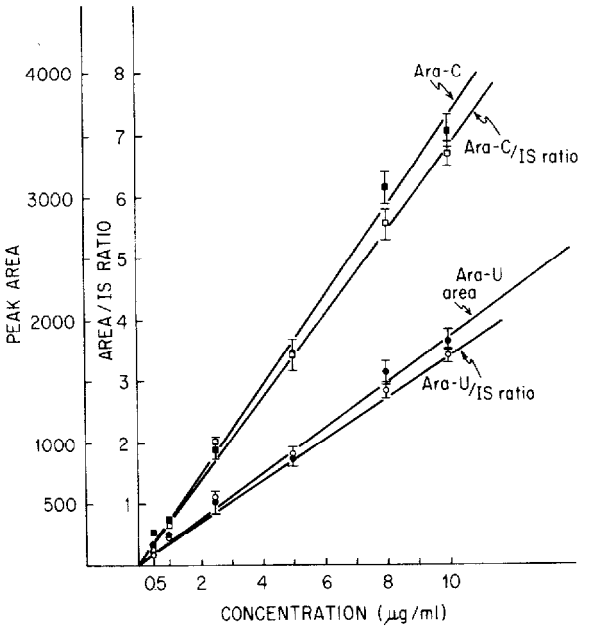

Fig. 4. High-dose calibration curve.

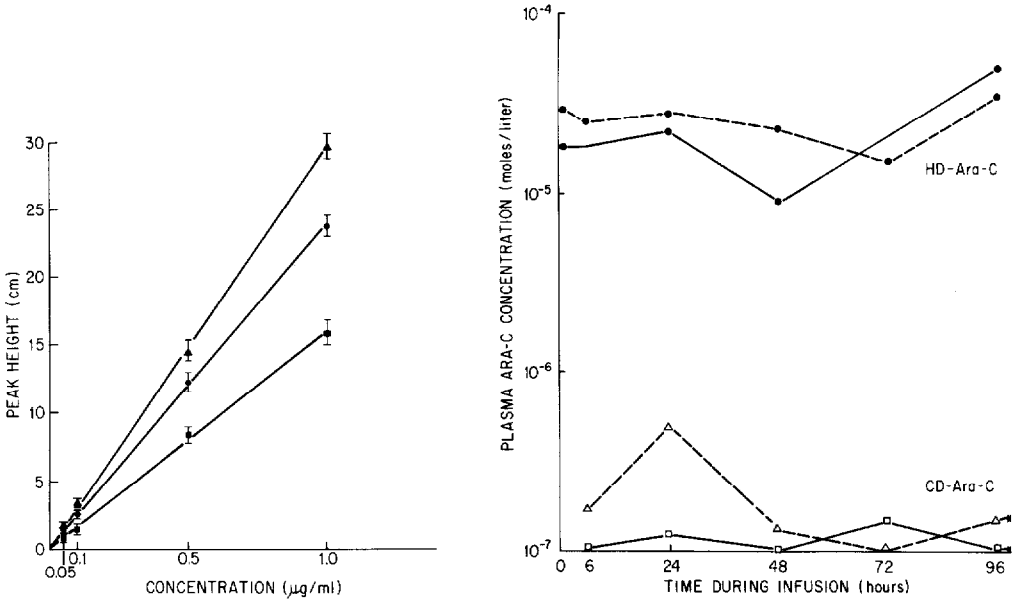

Fig. 5. Conventional-dose calibration curve. ● = Ara-C; ▲ = Ara-U; ■ = dCyt.

Fig. 6. Comparative concentration versus time curves of two patients administered high-dose (HD) Ara-C (3.5 g/m² per day) and two patients administered conventional-dose (CD) Ara-C (100 mg/m² per day.

leukemia (AML). As shown in Fig. 6, there appears to be significant variation in the steady-state plasma concentrations (Cp_{ss}) and the plasma concentration versus time profiles. Plasma clearance of Ara-C (K_0/Cp_{ss}) calculated for the two patients given high-dose Ara-C average 323 ml/min/m², substantially slower than that observed in the two patients given conventional-dose Ara-C (1543 ml/min/m²) or previously reported in adults [13] following conventional-dose Ara-C (942 ml/min/m²). This suggests the potential saturation of Ara-C clearance at the high plasma concentrations of Ara-C and/or Ara-U obtained after continuous infusion of high-dose Ara-C. This observation remains to be confirmed by additional pharmacokinetic studies in a larger number of patients.

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