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SIMULTANEOUS DETERMINATION OF CYTOSINE ARABINOSIDE, ITS NUCLEOTIDES AND METABOLITES BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Currently available high-performance liquid chromatographic assays for cytosine arabinoside (ara-C) and its metabolites suffer from two major shortcomings: inability to resolve both ara-C and its nucleotides in a single chromatographic step and/or inadequate sensitivity to allow quantitation of intracellular cytosine arabinofuranoside-5′-triphosphate (ara-CTP) without the use of radiolabelled drug. In this paper, we describe a new ion-pairing high-performance liquid chromatographic assay for ara-C in biological samples that can separate ara-C from its nucleotides, metabolites, and naturally occurring ribonucleotides in a single chromatographic step with a lower limit of quantitation of 5 pmol for ara-C and 10 pmol for ara-CTP. Examples of the utility of this assay are shown in studies of intracellular pharmacokinetics of ara-C in cultured human breast cancer cells and in analysis of plasma nucleoside levels in patients receiving high-dose thymidine chemotherapy. We conclude that this assay provides a rapid and versatile system that can be applied to the study of both cellular and plasma nucleoside pharmacokinetics.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine, ara-C) is a drug of established value in the treatment of acute non-lymphocytic leukemia [1]. Its active metabolite, cytosine 1-β-D-arabinofuranoside-5′-triphosphate (ara-CTP), inhibits DNA synthesis both by inhibition of DNA polymerase and by incorporation into the DNA molecule [2—4]. Key elements in the cellular sensitivity to ara-C are the extent of ara-CTP formation, the activity of the

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deaminase pathways that inactivate ara-C and the duration of drug exposure [5].

Studies of the biochemical and clinical pharmacology of ara-C have generally focused on two major areas; intracellular ara-CTP formation and the plasma pharmacokinetics of ara-C and its major metabolite, uracil arabinoside (ara-U). The development of specific high-performance liquid chromatographic (HPLC) assays for ara-C and ara-CTP has resulted in a rapid expansion of our knowledge of the pharmacology of this drug. Yet, currently available HPLC assays suffer from two major shortcomings: inability to resolve both ara-C and its nucleotides in a single chromatographic step and/or inadequate sensitivity to allow quantitation of intracellular ara-CTP without the use of radiolabelled drug. Other necessary attributes of a useful HPLC assay for ara-C are a capacity to separate ara-C and its nucleotides from naturally occurring pyrimidines as well as a capacity to separate and quantitate the uracil arabinoside metabolites of ara-C.

The most commonly used HPLC assays for ara-CTP employ anion-exchange columns and are able to detect unlabelled drug with a sensitivity as low as 25 pmol [6—11]. These assays, however, fail to accurately resolve free bases and nucleosides. Separation of nucleosides from nucleotides can be accomplished using a reversed-phase HPLC system to resolve nucleosides and an anion-exchange system to separate nucleotides but this procedure is cumbersome and time-consuming. Simultaneous separation of ara-C nucleosides and nucleotides has been reported using a C_{18} amine column but baseline separation between all peaks was not achieved and the sensitivity of the assay was not reported [12]. Recently, Crowther et al. [13] demonstrated that the use of tetrabutylammonium phosphate as an ion-pairing agent allows rapid and complete resolution of naturally occurring nucleosides and nucleotides. Using this ion-pairing agent, we have developed a new HPLC assay for ara-C that allows resolution of parent drug, its nucleotide derivatives and its metabolites in a single chromatographic step. This assay is applicable to the study of both intracellular and plasma ara-C pharmacokinetics.

MATERIALS AND METHODS

Chemicals

Cytosine 1-β-D-arabinofuranoside (ara-C), cytosine 1-β-D-arabinofuranoside 5′-monophosphate (ara-CMP), cytosine 1-β-D-arabinofuranoside 5′-triphosphate (ara-CTP), uracil-1-β-D-arabinofuranoside (ara-U), cytidine 5′-monophosphate (CMP), cytidine 5′-diphosphate (CDP), cytidine 5′-triphosphate (CTP), uridine 5′-monophosphate (UMP), uridine 5′-diphosphate (UDP), and uridine 5′-triphosphate (UTP) were purchased from Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium phosphate (TBAP) (Kodak Chemical), potassium phosphate monobasic and HPLC-grade acetonitrile were purchased from Fisher Scientific (St. Louis, MO, U.S.A.) and [5-^{3}H]cytosine 1-β-D-arabinofuranoside ([^{3}H]ara-C), specific activity 11.2 Ci/mmol, was purchased from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals used were of analytical grade.

Cell culture supplies

Earles minimum essential medium without L-glutamine (MEM), L-glutamine, non-essential amino acids, penicillin and streptomycin and heat-inactivated fetal bovine serum were purchased from KC Biological (Lenexa, KS, U.S.A.). Sterile pipets and 75-cm^2 tissue culture flasks were purchased from Fisher Scientific.

Instrumentation and HPLC columns

A Waters Assoc. HPLC system (Milford, MA, U.S.A.) consisting of two Model M6000A pumps, a U6K injector, and a Model 440 absorbance detector with 280-nm filter is used in this assay. The mobile phase is prepared by a Model 660 solvent programmer mixing the effluents of these two pumps. Mobile phase A consists of 100 m*M* potassium dihydrogen phosphate with 5 m*M* TBAP, pH 4.5 and mobile phase B is 100% acetonitrile. Authentic standards in a volume of 25 μl or cell or plasma extracts are injected on to a Radial-Pak 5-μm C_{18} cartridge in an RCM 100 radial compression module connected to a Z module radial compression system also containing a 5-μm C_{18} cartridge. Elution is carried out at room temperature at a flow-rate of 2 ml/min using curve No. 8 on the Model 660 solvent programmer. The gradient is run from initial conditions of 0% pump B to 10% pump B for 30 min following which elution is continued with 10% acetonitrile for an additional 14 min. A 15-min re-equilibration period using initial conditions is necessary before injection of the next sample.

Ultraviolet absorbance is monitored at 280 nm and standard chromatograms are recorded on a Houston Instruments Omniscribe recorder. Fractions of cell extracts prepared after incubation with [^{3}H]ara-C are collected every 0.2 min for the first 14 min of the assay and then every 0.5 min for the next 30 min for determination of radioactive peaks. Radioactivity is determined using a Beckman LS-6800 scintillation counter.

Propagation of cells in culture

MCF-7 human breast cancer cells are grown in MEM supplemented with 10% fetal bovine serum, L-glutamine at 584 μg/ml, penicillin at 124 μg/ml and streptomycin at 270 μg/ml under 5% carbon dioxide at 37°C. The human derivation, hormonal responsiveness and growth characteristics of the cells have been described [14].

Preparation of cell extracts

Following in vitro incubation with the desired concentration of ara-C, half the drug-containing medium is removed from the flask and set aside and the cells are scraped off the flask surface with a rubber policeman into the remaining medium. The cell suspension is centrifuged at 400 *g* for 2 min at 4°C, the supernatant is removed and the cell pellet is immediately resuspended in 500 μl of the drug-containing medium set aside earlier. This concentrated cell suspension is then layered over a silicone oil—mineral oil (84:16) interface in a 1.5-ml polypropylene microfuge tube and immediately centrifuged at 500 *g* for 1 min in a table-top centrifuge. The cells, but not the medium, travel to the tip of the tube which contains 500 μl of 1 *M* perchloric acid. The tubes are then quickly frozen and cut at the oil—perchlorate interface. The perchlorate

extract is transferred to another tube and neutralized with 8 *M* potassium hydroxide following which the potassium perchlorate is removed by centrifugation and the supernatant is stored at −80°C until injection into the HPLC system.

Preparation of plasma samples for analysis

Plasma nucleoside levels are determined by addition of 1 ml of 1 *M* perchloric acid to 1 ml plasma, removal of the precipitate by centrifugation and injection of the supernatant on a Sep-Pak C_{18} cartridge previously prepared by injection of 10 ml acetonitrile followed by 10 ml of 1 m*M* perchloric acid. The cartridge is then washed with 10 ml of water and the nucleosides are eluted with 4 ml of acetonitrile, evaporated to dryness under nitrogen and resuspended in the HPLC mobile phase A prior to injection.

RESULTS

HPLC analysis

The separation of ara-C, its nucleotides and metabolites from naturally occurring ribonucleotides is shown in Fig. 1. Following injection of a standard mixture of authentic nucleosides and nucleotides, baseline separation is

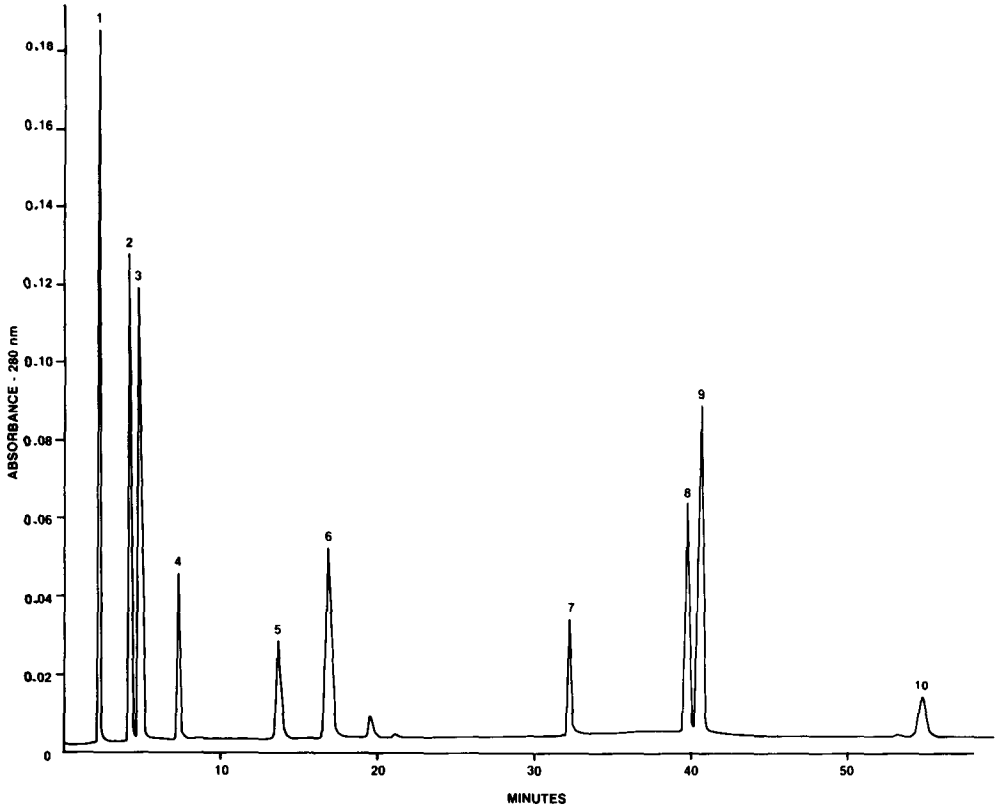

Fig. 1. HPLC separation of a standard mixture of cytosine arabinoside and its metabolites from naturally occurring ribonucleotides. Peaks: 1 = ara-C; 2 = CMP; 3 = ara-CMP; 4 = ara-U; 5 = UMP; 6 = CDP; 7 = UDP; 8 = CTP; 9 = ara-CTP; 10 = UTP.

achieved for all compounds and R factors for all peaks exceed 0.90. Variation in retention time for all peaks is less than 10%. Ara-C is well separated from both its nucleotides and its deamination products and quantitation can be achieved to levels as low as 10 pmol for ara-CTP and 5 pmol for ara-C.

Intracellular ara-C pharmacokinetics in vitro

This assay method can easily be applied to the study of intracellular ara-C pharmacokinetics in vitro. MCF-7 human breast cancer cells were incubated with 1 μM [^{3}H]ara-C and harvested at various times as described above. The time course of ara-C uptake and metabolism in these cells is shown in Fig. 2. Total intracellular drug increases linearly during a 60-min incubation with [^{3}H]ara-C. This increase is due entirely to the formation of phosphorylated derivatives of ara-C, i.e., intracellular levels of ara-C itself remain constant during the period of drug exposure. Ara-CTP is the first nucleotide derivative to accumulate in the cell. As ara-CTP levels increase and eventually plateau, ara-CMP and ara-CDP begin to accumulate suggesting that phosphorylation to the triphosphate proceeds very rapidly initially then subsequently slows allowing accumulation of the mono- and diphosphate derivatives. Neither ara-U nor its phosphorylated derivatives were detected intracellularly during this period of drug exposure.

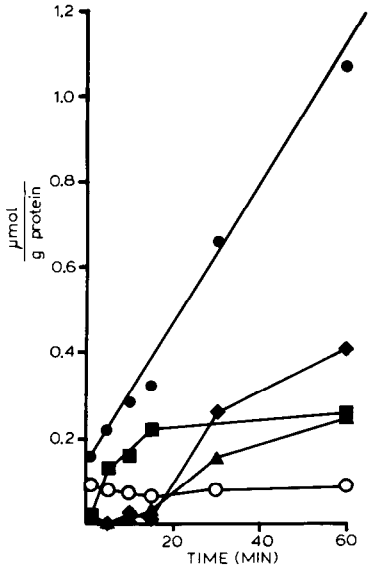

Fig. 2. Time course of [^{3}H]ara-C uptake and metabolism in MCF-7 cells. MCF-7 human breast cancer cells were incubated with 1 μM [^{3}H]ara-C and harvested at various times for HPLC analysis of cellular contents. (●) Total drug; (○) ara-C; (◆) ara-CMP; (▲) ara-CDP; (■) ara-CTP.

Fig. 3 depicts the dose—response relationship for accumulation of ara-C and its metabolites in human breast cancer cells during a 15-min incubation with ara-C. Total intracellular drug increases rapidly with increasing extracellular drug concentration and this is due, again, largely to the formation of ara-C nucleotides, particularly ara-CTP. As the extracellular drug concentration is

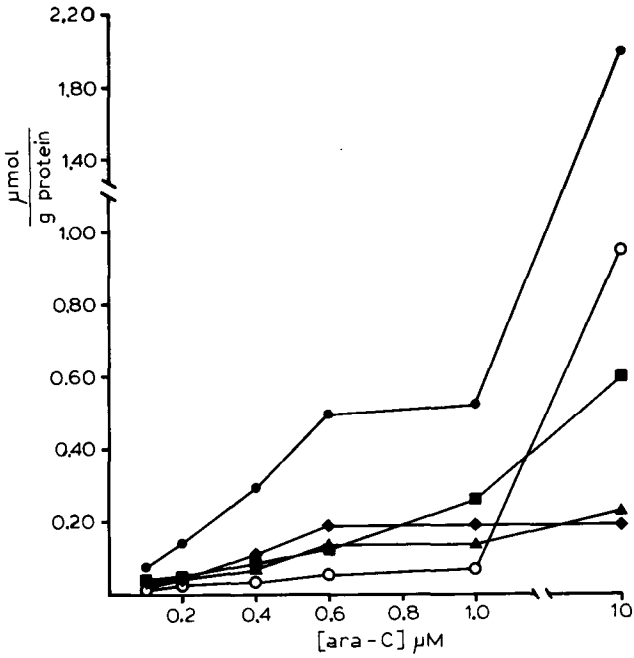

Fig. 3. Uptake and metabolism of [^{3}H]ara-C in MCF-7 cells. MCF-7 human breast cancer cells were incubated with varying concentrations of [^{3}H]ara-C for 15 min then harvested for HPLC analysis of cellular contents. (●) Total drug; (○) ara-C; (◆) ara-CMP; (▲) ara-CDP; (■) ara-CTP.

increased from 1 to 10 μM however, further increases in intracellular drug are due primarily to a marked (twelve-fold) increase in intracellular ara-C, while ara-CMP and ara-CDP levels remain relatively constant and ara-CTP levels increase by only two-fold.

Analysis of plasma nucleosides

This assay can also be easily modified to allow rapid analysis of plasma nucleoside concentrations. As an example of its utility in this area, we have used the assay to measure plasma thymidine levels in patients receiving high doses of this nucleoside as part of an investigational treatment protocol. Plasma extracts are injected on to a 5-μm C_{18} cartridge in an RCM 100 radial compression system. Initial buffer conditions (buffer A) are 100 mM potassium phosphate with 5 mM TBAP and elution is done isocratically for 4 min. A step gradient is then used to immediately bring final buffer conditions to buffer A + 12% acetonitrile and elution is carried out for an additional 11 min. Total assay time is 15 min at a flow-rate of 2 ml/min. Fig. 4 depicts a representative chromatogram of plasma obtained from a patient receiving high-dose thymidine. Deoxyinosine was added during the extraction procedure to serve as an internal standard. A chromatogram of a pretreatment plasma sample is shown for comparison and demonstrates a small peak which co-chromatographs with authentic thymine. No absorbing materials are detected at 6 min or 8 min, the retention times of authentic deoxyinosine and thymidine, respectively. To determine plasma thymidine concentrations, a standard curve is constructed by

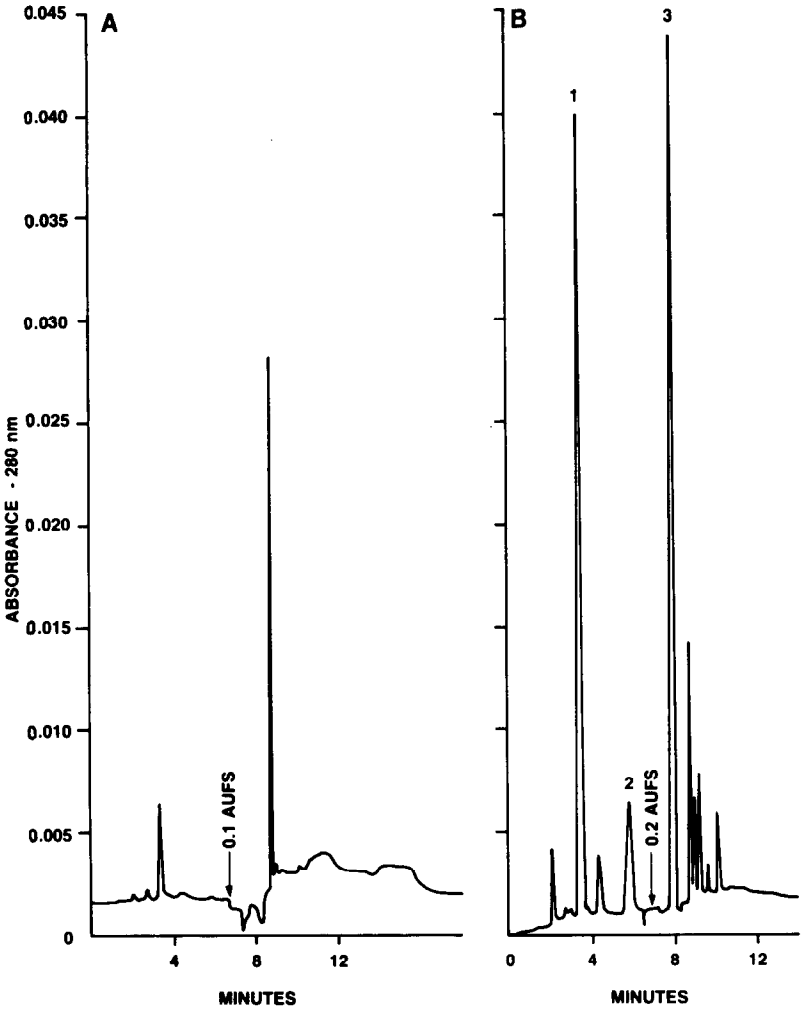

Fig. 4. HPLC analysis of plasma thymidine concentrations. (A) Patient plasma prior to treatment and (B) patient plasma during continuous infusion of thymidine at 24 g/m²/day. Peaks: 1 = thymine; 2 = deoxyinosine (internal standard); 3 = thymidine.

plotting the ratio of peak heights for thymidine and deoxyinosine versus known thymidine concentrations. Coefficients of variation for these ratios are 7.4% and 10.2% at thymidine concentrations of 25 μM and 250 μM, respectively. A similar standard curve is constructed for determination of plasma thymine levels. In the example shown in Fig. 4, the patient was receiving 24 g/m² thymidine as a continuous intravenous infusion. Steady-state plasma thymidine levels were 160 ± 10 μM and steady-state thymine levels were 406 ± 147 μM. In another patient receiving 40 g/m² thymidine, steady-state plasma thymidine levels rose to 381 ± 85 μM. Plasma thymidine concentrations as low as $5 \cdot 10^{-8}$ M can be detected using this assay. The isocratic portion of this assay can also be used to determine plasma ara-C levels with a lower limit of quantitation of 5 pmol injected. The retention time for ara-C in this system is 2 min.

DISCUSSION

The development of specific and sensitive HPLC assays for many antineoplastic drugs has significantly enhanced our understanding of the biochemical and clinical pharmacology of these agents. We have previously applied the concept of ion-pairing in the development of an HPLC assay for methotrexate and its polyglutamate derivatives [15] and, in the present study, we describe a new ion-pairing HPLC assay for nucleosides and nucleotides which is particularly valuable for study of the biochemical and clinical pharmacology of cytosine arabinoside. Unlike the commonly used HPLC assays for ara-C, this ion-pairing assay provides a method for separation of ara-C, its nucleotides and deamination products in a single chromatographic step. This capability provides a unique opportunity for the study of the intracellular pharmacokinetics of ara-C in vitro since ara-C and its mono-, di- and triphosphates can all be determined reliably with a single-extraction procedure and HPLC run thereby eliminating the variability inherent in using separate HPLC assays to quantitate these materials as is commonly done.

The sensitivity of this assay (5 pmol for ara-C and 10 pmol for ara-CTP) compares favorably to that reported for other HPLC assays used to quantitate intracellular ara-CTP without the use of radiolabelled drug. Application of this technique thus offers the potential to accurately determine cellular ara-CTP levels in patients receiving ara-C therapy and to correlate these levels with other important parameters such as plasma ara-C pharmacokinetics and clinical response to treatment.

As an example of the utility of the assay in the study of cellular ara-C pharmacokinetics, human breast cancer cells were incubated in vitro with [^{3}H]ara-C and the time course and dose response of ara-C uptake and metabolism were studied. As shown in Figs. 2 and 3, use of this HPLC assay allows simultaneous analysis of the intracellular distribution of ara-C, ara-CMP, ara-CDP and ara-CTP over time and following exposure to varying extracellular ara-C concentrations. Of particular interest is the finding that ara-CTP is the first nucleotide derivative to accumulate in the cell with ara-CMP and ara-CDP appearing only as ara-CTP levels plateau suggesting that phosphorylation to the triphosphate proceeds rapidly initially but slows as ara-CTP accumulates in the cell then resulting in the accumulation of the mono- and diphosphate derivatives of ara-C. Fig. 3 depicts the dose-dependent nature of ara-C nucleotide formation but reveals that, at high extracellular ara-C concentrations, increases in total intracellular drug are due primarily to accumulation of ara-C rather than to increases in ara-CTP formation. Information such as this might be useful in assessing the potential clinical utility of high dose ara-C therapy in the treatment of solid tumors.

This ion-pair HPLC assay can also easily be modified for rapid determination of plasma nucleoside levels. As shown in Fig. 4, we have successfully applied this assay to measurement of plasma thymidine and thymine concentrations in patients receiving high-dose thymidine as part of an experimental treatment protocol. The assay can be used with equal facility for determination of plasma ara-C and ara-U levels and thus provides a rapid and versatile system which can be applied to the study of both cellular and plasma nucleoside pharmacokinetics.

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