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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CYTOSINE ARABINOSIDE AND METABOLITES IN BIOLOGICAL SAMPLES

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SUMMARY

A rapid, non-radioactive method to quantitate therapeutically realistic levels of 1-β-D-arabinofuranosylcytosine (Ara-C) and its metabolites would be useful both in the clinic, for monitoring drug levels, and in the laboratory for correlating drug levels with cellular and molecular perturbations. Liquid chromatographic analysis of arabinose-nucleoside analogs in biological samples is complicated by the presence of interfering nucleosides and nucleotides. We report the development of two analytic procedures to measure Ara-C and metabolite levels in biological samples. One method uses a quaternary ammonium type anion-exchange resin to achieve isocratic separation in less than one hour. The second method utilizes a boronate-derivatized polyacrylamide column which binds *cis*-diols to selectively retain cytosine and uridine, while arabinose compounds are eluted with recovery approaching 100%. The eluted compounds are then easily quantitated on a reversed-phase C_{18} column. The sensitivity of both procedures was sufficient to obtain pharmacokinetic data on Ara-C and uracil-arabinose levels in serum and urine and on Ara-C triphosphate levels in tumor cells.

INTRODUCTION

The potential of pharmacokinetic parameters as predictors of response to cancer chemotherapeutic agents is becoming more apparent [1—4]. Information about drug levels, tissue distribution, and the persistence of drug metabolites in biological samples can be used to monitor drug effects at both the clinical and experimental level. Clinically, variation in drug metabolism and excretion patterns is believed to be one of the factors responsible for differences in drug response and toxicity between individual patients. In addition, since it has been shown that drug metabolism may change during a multiple dose therapeutic schedule [5], which is the common treatment regime for many cancers,

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it is important to be able to monitor drug levels during a course of therapy. Such information would allow drug dose adjustments to be made to accommodate inter- and intra-patient variation. Experimentally, pharmacokinetic information is needed to better understand the extent and duration of drug-induced biochemical and cellular perturbations.

1-β-D-Arabinofuranosylcytosine (Ara-C) is a pyrimidine nucleoside analog which is currently a component of several therapeutic drug combination protocols. It is metabolized to an active metabolite, Ara-C triphosphate (Ara-CTP) and to an inactive compound, 1-β-D-uracil arabinoside (Ara-U). High-performance liquid chromatography (HPLC) offers a rapid method to monitor levels of Ara-C and metabolites in tissues. However, chromatographic analysis of arabinose-nucleoside analogs in biological samples is complicated by interference of naturally occurring nucleosides and nucleotides. Most of the previously described chromatographic procedures cannot separate arabinose analogs from the ribose or deoxyribose compounds of cytosine and/or uridine [1, 6—9]. Therefore, in the past it has been necessary to use radioactively-labeled Ara-C, chromatography and subsequent liquid scintillation counting to separate and quantitate drug levels in biological samples. Rustum [6] described an HPLC technique to separate arabinose nucleosides; however, baseline separation was not achieved with all potentially contaminating compounds. Rustum and Preisler [3] used a different HPLC column to separate arabinose and ribose metabolites in biological samples; however, their technique still required a radioactively-labeled precursor since several of the separated compounds appeared as shoulders on major peaks, and the baseline showed considerable upward drift. These techniques tend to be time consuming and the expense and radioactivity of radiolabeled Ara-C limits their usefulness in obtaining pharmacokinetic information in animals and patients.

We have developed two methods to separate and quantitate Ara-C and its metabolites in biological tissues using HPLC. One method utilizes a quaternary ammonium anion-exchange resin (Aminex) to separate isocratically arabinose-containing analogs from all other interfering compounds. The usefulness of Aminex columns in nucleoside and nucleotide quantitation has been discussed in detail by Khym [10]. The second method utilizes a boronate-derivatized polyacrylamide resin to selectively retain cytosine, uridine and other *cis*-diols, while the arabinose-containing analogs are eluted. The eluted compounds are then easily quantitated on a reversed-phase C_{18} column. These methods were used to measure drug levels in mouse serum and urine and in tumor cells.

MATERIALS AND METHODS

Reagents and standards

Ara-C was purchased from Upjohn (Kalamazoo, MI, U.S.A.). Other arabinose standards, Ara-U and Ara-CTP, were obtained from Sigma (St. Louis, MO, U.S.A.). Cytidine, uridine and their triphosphate derivatives were obtained from P. & L. Biochemicals (Milwaukee, WI, U.S.A.). Ammonium acetate (Mallinckrodt, St. Louis, MO, U.S.A.), formic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.), sodium citrate (Mallinckrodt) and sodium tetraborate decahydrate (Matheson, Cole and Bell, Norwood, OH, U.S.A.) were used in preparing

buffers and aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (0.45 μm) prior to use.

Instrumentation and HPLC columns

We used a Beckman chromatographic unit (Model 330) with a variable-wavelength ultraviolet detector (Model 450, Waters Assoc., Milford, MA, U.S.A.) set at 270 nm to detect Ara-C and metabolites. Aminex A-27 or A-29 (particle size 13.5 μm and 9.0 μm, respectively), strong anion-exchange resins (Bio-Rad Labs., Richmond, CA, U.S.A.) were used for isocratic separation of Ara-C and its metabolites from naturally occurring compounds. For nucleoside quantitation the A-27 material was slurry-packed at 140 bar into a stainless-steel column (500 mm × 4 mm I.D.) and nucleosides were eluted with a 0.025 *M* sodium citrate and 0.08 *M* sodium tetraborate buffer, pH 9.3 at a pressure of 105 bar, a flow-rate of 0.7 ml/min and at a temperature of 65°C. Nucleosides can also be eluted on a reversed-phase μBondapak (10 μm) C_{18} column (300 mm × 3.9 mm I.D.) (Waters Assoc.) with 0.01 *M* potassium phosphate, pH 5.6 at a flow-rate of 0.6 ml/min. A guard column (7 cm) packed with Co:pell ODS (Whatman, Clifton, NJ, U.S.A.) was used when analyzing biological samples to protect the analytical column from absorbing compounds. Nucleotides were eluted from a slurry-packed Aminex A-29 resin (200 mm × 4 mm I.D.) (see above) using 0.25 *M* sodium citrate, pH 8.2 at a flow-rate of 0.7 ml/min and at a temperature of 65°C.

Boronate affinity gel

In order to quantitate Ara-C and its metabolites on a reversed-phase C_{18} column, we found it necessary to use Affi-gel 601 to separate arabinose compounds from naturally occurring nucleosides. Affi-gel 601 (1.0 mequiv./g) was obtained from BioRad Labs. A slurry was made in 0.1 *M* sodium chloride and the resin (approximately 0.2 g) placed in a glass column (5 mm I.D.) to a height of 30 mm. The resin was then rinsed with 20 ml of 0.25 *M* ammonium acetate (pH 8.8) followed by 0.1 *M* formic acid, as described by Gehrke et al. [11]. Prior to application of the sample, the column was re-equilibrated with the ammonium acetate buffer. After the sample was applied to the resin, the arabinose-containing compounds were eluted with 8.0 ml of ammonium acetate. To remove the bound *cis*-diols, the column was washed with 8.0 ml of formic acid.

Biological samples

Arabinose-nucleoside levels were measured in mouse urine and plasma at various time intervals after administration of Ara-C in vivo. Ara-C (10 mg/ml) was dissolved in sterile saline and injected intraperitoneally into female C3H mice (8—12 weeks) at a dose of 100 mg/kg. Samples from 3—5 animals were pooled for each analysis. Urine samples were collected from ether-anaesthesized animals by bladder puncture and blood was withdrawn from the abdominal aorta with a heparinized syringe with care being taken to minimize red blood cell lysis. The plasma was removed from blood by centrifugation at 1000 *g* for 10 min at 4°C. Urine and plasma samples were stored at 0°C until subsequent cleanup procedures and analysis.

Prior to HPLC analysis, urine samples (200 μl) were passed through the Affi-

gel column to remove the *cis*-diols. The ammonium acetate urine eluate was then injected directly onto the C_{18} reversed-phase column. Plasma samples were deproteinized by ultracentrifugation (Amicon conical ultrafilters, 2500 GE, Amicon, Lexington, MA, U.S.A.) and the ultrafiltrate then analyzed on the Aminex anion-exchange column (A-27).

Nucleotide levels were measured in KHT tumor cells exposed to Ara-C in vitro. These mammalian cells were derived from the solid murine KHT tumor [12] and adapted in our laboratory to grow in monolayers. Ara-C was added to KHT cells (approximately $2 \cdot 10^7$ cells) in exponential growth phase and aliquots of the culture removed at specified intervals after drug administration. These samples were washed twice with cold phosphate-buffered saline, pH 7.2 and protein precipitated overnight in 60% methanol at 0°C. After centrifugation (700 *g*) the supernatant was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μl of 0.25 *M* sodium citrate buffer, pH 8.2. Ara-CTP levels were then quantitated on the Aminex (A-29) column.

Concentrations of standards and drugs in biological samples were quantitated using peak height measurements. Maximum sensitivity for peak height quantitation was considered to be five times the background noise level. Samples were analyzed at 0.10 a.u.f.s.

RESULTS

Analytical conditions

Fig. 1a illustrates the separation of a standard pyrimidine nucleoside mixture achieved in less than one hour by isocratic elution from the A-27 anion-exchange column. Aproximately 2.5 ng Ara-C and 10 ng Ara-U can be detected minimally in this system. Ara-C and Ara-U are also separated on a reversed-phase C_{18} column in less than 20 min as shown in Fig. 1b. Although the C_{18} column is unable to resolve Ara-C and Ara-U from cytosine and uridine, respec-

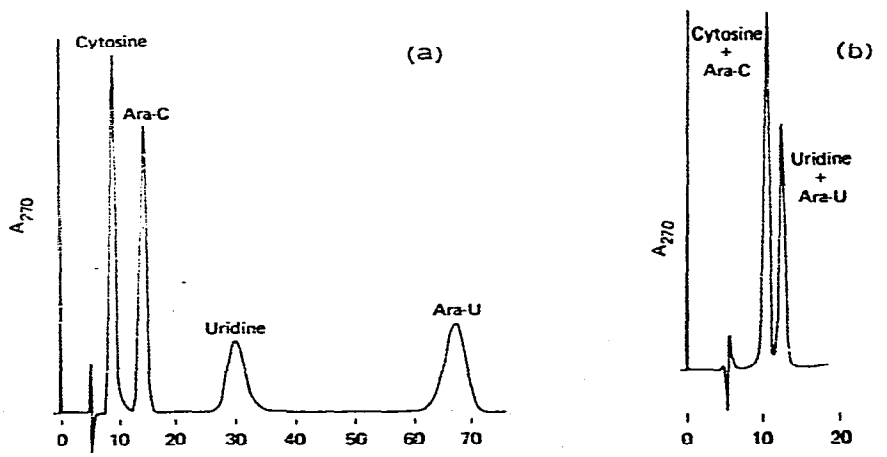

Fig. 1. Chromatogram of a nucleoside mixture containing Ara-C, Ara-U, cytosine and uridine on the ion-exchange (A-27) resin (a) and on the reversed-phase C_{18} column (b). Column operating conditions are described in Materials and methods.

tively, this problem is circumvented by prior treatment of the sample with a boronate affinity gel, as described below. The minimum detection limit of Ara-C and Ara-U on the C_{18} column is 0.32 and 0.44 ng, respectively. The reproducibility of sample injection was greater than 97%. Both analytical HPLC methods gave linear response curves for all nucleosides over a 1000-fold nmole range. This wide range of linearity is sufficient for quantitation of Ara-C and Ara-U levels in biological samples.

Fig. 2 shows the separation of nucleoside triphosphates on the A-29 anion-exchange column. Pyrimidine nucleotide triphosphates were eluted isocratically with sodium citrate buffer in less than 40 min. Ara-CTP is well separated from CTP and UTP and approximately 10 ng Ara-CTP and 20 ng Ara-UTP can be detected minimally at 270 nm.

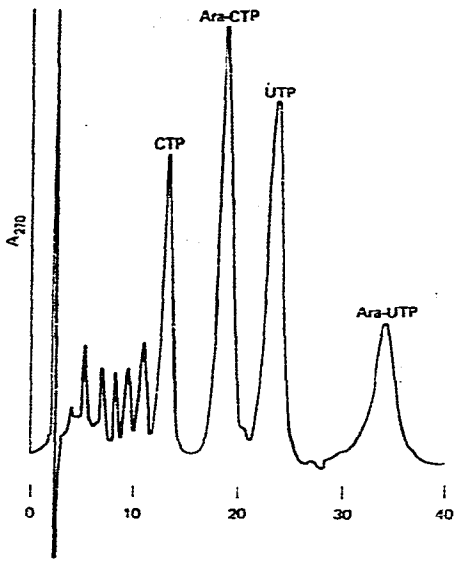

Fig. 2. Chromatogram of a nucleotide mixture of Ara-CTP, Ara-UTP, CTP and UTP on the ion-exchange (A-29) column. Temperature 65°C; flow-rate 0.7 ml/min.

Boronate affinity resin

We used a boronate affinity gel (Affi-gel 601) to selectively bind *cis*-diols as an aid to separating ribose and arabinose compounds. A mixture of nucleoside standards, Ara-C, Ara-U, cytosine and uridine was applied to the Affi-gel 601 column. The column was washed with ammonium acetate and formic acid as described in Materials and methods. The eluates were then analyzed on the A-27 ion-exchange resin (Fig. 3). Ara-C and Ara-U were found only in the ammonium acetate wash, whereas cytosine and uridine were found in the formic acid eluate. Recovery of the eluted compounds was greater than 98%. Thus, pretreatment of samples with Affi-gel allows quantitative measurements of Ara-C and Ara-U levels with the commonly available C_{18} column.

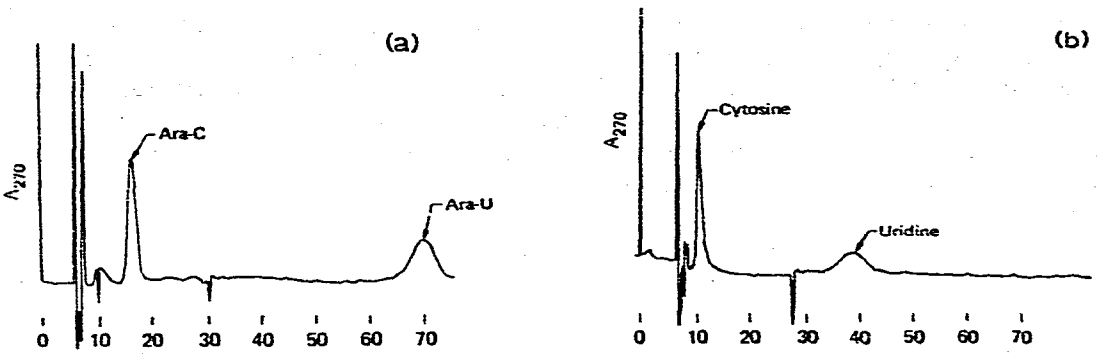

Fig. 3. Profiles of ribose and arabinose compounds after elution from Affi-gel 601. A standard pyrimidine nucleoside mixture (Ara-C, Ara-U, cytosine and uridine) was passed through Affi-gel 601 and the ammonium acetate eluate (a) and formic acid eluate (b) analyzed on the A-27 column.

Biological samples

As was discussed previously, one of the major problems in analyzing biological tissues using HPLC is that naturally occurring compounds may coelute with the compound of interest. Therefore, with each analytical system, it is necessary to obtain nucleoside and nucleotide profiles of the tissues to be analyzed to determine if the region of interest is free of contaminating peaks. Urine and plasma from untreated animals were analyzed on both the anion-exchange column (A-27) and on the reversed-phase C_{18} column. KHT tumor cell extracts were analyzed on the A-29 Aminex resin. These chromatograms, as well as those obtained from drug-treated animals are shown in Fig. 4. It is evident that at the attenuation (0.10 a.u.f.s.) necessary to analyze drug levels there are no major peaks which interfere with the quantitation of Ara-C and Ara-U in either urine or plasma.

We measured Ara-C and Ara-U levels in plasma and urine at various times after administration of Ara-C (100 mg/kg). This dose level of Ara-C was chosen since it is minimally toxic to C3H mice and previous studies in our laboratory indicated that it induces marked cell cycle kinetic perturbations in solid tumors (unpublished results). Ara-C was injected intraperitoneally and samples were collected at 0.5—24 h after drug administration. Fig. 5a illustrates Ara-C and Ara-U levels in plasma ultrafiltrates. The disappearance curve of Ara-C was biphasic with a half-time ($t_{1/2}$) of 20 min, as estimated from the first phase of the decay curve. Ara-U reached a maximum level of 12 ng/μl over a period of 30—60 min and decayed exponentially thereafter. Both Ara-C and Ara-U were excreted in the urine (Fig. 5b). Data in Fig. 5b are expressed as the ratio of Ara-C and Ara-U concentration in order to normalize variation in urinary output between individual animals. The ratio value of 1.0 is equivalent to 186 ng nucleoside per μl urine. These urinary data indicate that initially Ara-C was the major arabinose analog excreted; however, at two hours, equivalent amounts of Ara-C and Ara-U were eliminated. At later times after Ara-C administration a large percentage of the excreted arabinose nucleosides was due to Ara-U. Neither Ara-C or Ara-U were detected in the urine and plasma 24 h after drug injection. To verify that Ara-U was not simply a result of chemical breakdown

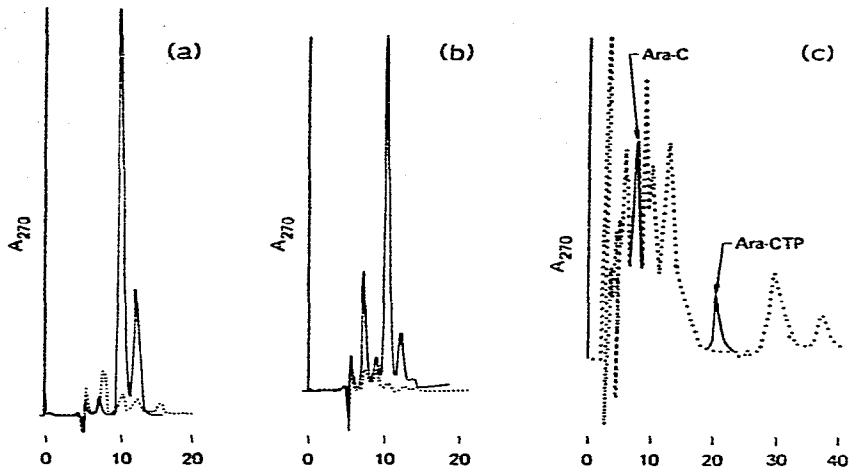

Fig. 4. Representative chromatograms of biological samples obtained from untreated (...) and treated (—) animals. Identical attenuation settings were used for each set of control and drug-treated samples. (a) Urine was obtained from mice 30 min after injection of Ara-C (100 mg/kg) or saline. Samples were passed through the Affi-gel resin prior to analysis on the reversed-phase C_{18} column. (b) Plasma was obtained from drug-treated and saline-treated animals 30 min after injection. Samples were analyzed on the ion-exchange (A-27) column. (c) Location of Ara-CTP peak in KHT tumor cells extracts. Samples were analyzed on the ion-exchange (A-29) column.

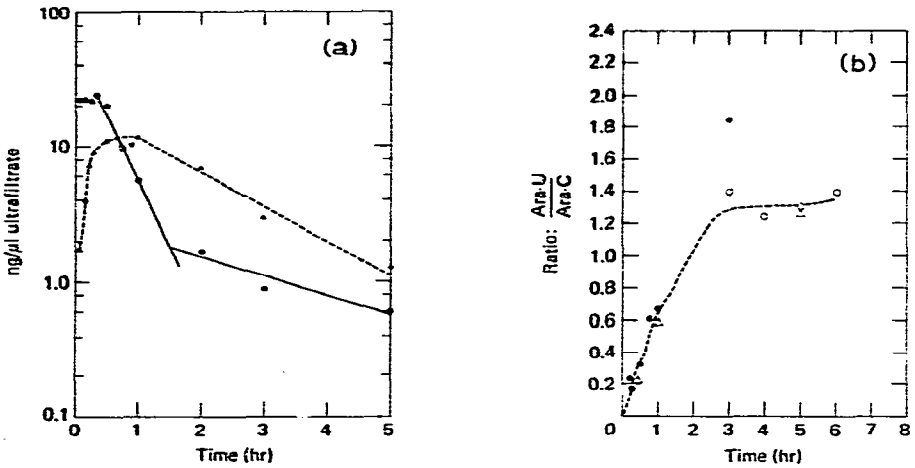

Fig. 5. Arabinose-nucleoside levels in plasma ultrafiltrates (a) (●, Ara-C; ▲, Ara-U) and urine pretreated with Affigel (b). Animals received Ara-C (100 mg/kg) and samples were collected at specific intervals thereafter. Plasma samples were analyzed on the ion-exchange (A-27) column and urine samples on the reversed-phase C_{18} column. In (b) each symbol represents an individual experiment.

of Ara-C, the parent drug was added to both urine and plasma ultrafiltrates and stored at room temperature for 18 h. These samples were analyzed on the reversed-phase C_{18} column. During this period, Ara-U was not detected in either sample, indicating that catalytic breakdown occurring after sample collection was not responsible for the observed Ara-U levels.

We also measured Ara-CTP levels in KHT tumor cells exposed to Ara-C ($2 \cdot 10^{-6}$ *M*) in vitro. A 50% cell kill is achieved during a 30-min exposure to this dose level of Ara-C (data not shown). Ara-C was added to cultures of KHT cells and aliquots of the suspension taken at 30-min intervals for a 2-h period. The cells were washed twice, protein precipitated with 60% methanol, and after evaporation the extract was reconstituted in 100 μl buffer and analyzed by HPLC using the ion-exchange column (A-29). These data, shown in Table I, are expressed as pmoles Ara-CTP per 10^7 cells. Ara-CTP levels ranged from 80—320 cells and from 60—410 pmoles per 10^7 cells in two experiments.

TABLE I

ARA-CTP LEVELS IN KHT CELLS EXPOSED TO ARA-C IN VITRO

KHT cells were incubated for various time intervals at 37°C in α-media plus Ara-C ($2 \cdot 10^{-6}$ *M*)

Time (min) in Ara-C	Ara-CTP (pmole/10^7 cells)	
	Exp. 1	Exp. 2
30	80	60
60	240	310
90	260	410
120	320	160
150	80	140

DISCUSSION

We have described two HPLC analytical methods to separate and identify arabinose-containing pyrimidine analogs from naturally occurring compounds. Both separation methods are isocratic, thereby avoiding the drift in baseline observed with gradient elution, and can detect nanogram quantities of Ara-C and its metabolites. The anion-exchange resins (A-27 and A-29) were used to quantitate Ara-C, Ara-U and Ara-CTP levels directly from biological tissues in less than 40 min. Ara-C and Ara-U were also quantitated on a reversed-phase C_{18} column after pre-treatment of samples with a boronate affinity gel. The use of the C_{18} column for drug quantitation offers the advantages of speed (Ara-C and Ara-U are eluted in approximately 20 min), less expense, greater sensitivity and is more common in clinical laboratories than the ion-exchange resin.

The use of a boronate affinity gel for HPLC analysis of nucleosides in biological materials has previously been described [11, 13]. These workers used the affinity gel as a sample clean-up procedure to remove deoxyribonucleosides and other contaminating material prior to analysis of ribonucleosides. Their biological sample was placed on the column and the column washed with ammonium acetate and formic acid buffers. The formic acid eluate containing *cis*-diols was then analyzed by HPLC. We used the capabilities of affinity gel in an alternative manner to achieve separation of arabinose and ribose nucleosides; we collected and analyzed the ammonium acetate eluate instead of the formic acid eluate. Thus, it was possible to eliminate contamination with the interfering *cis*-diols, cytosine and uridine, which are not separated from arabinose-containing nucleosides on the reversed-phase C_{18} column. The recovery of the arabinose nucleoside compounds from the affinity gel was excellent and biological levels were sufficiently high that drug and metabolite dilution with the ammonium

acetate buffer did not pose problems of detection. However, if lower drug levels are to be measured, the ammonium acetate buffer can easily be evaporated or lyophilized with no detrimental effects on drug recovery.

We applied both the ion-exchange and the reversed-phase C_{18} methods to the measurement of Ara-C and Ara-U levels in plasma and urine. The plasma disappearance curve of Ara-C was biphasic and the $t_{1/2}$ was estimated to be 20 min. This value is in agreement with estimates obtained by other techniques [14]. Plasma Ara-U levels increased slowly over a 60-min period after Ara-C administration and disappeared exponentially thereafter. Approximately equivalent amounts of Ara-C and Ara-U were excreted at 2 h after drug administration (see Fig. 5b). These data are similar to those reported by Creasey et al. [15] using radioactively-labeled Ara-C.

The measurement of Ara-CTP levels in biological samples is somewhat more difficult than quantitating arabinose-nucleoside analogs. Ara-CTP, the active metabolite of Ara-C, is present in extremely low levels. In addition, the lability of nucleotide triphosphates in extraction procedures [16] requires careful experimental controls to ensure that measurements are indeed accurate. We found that extraction of nucleotides after protein precipitation with 60% methanol yielded good recoveries of Ara-CTP. This extraction procedure was used to recover nucleotides and nucleosides after exposure of KHT tumor cells to Ara-C in vitro (Table I). Ara-CTP concentrations ranged from 60—410 pmoles per 10^7 cells during a 150-min incubation period. These levels are similar to those reported by Rustum and Preisler [3] in leukemic cells using the radioactive form of Ara-C.

The rates and extent of phosphorylation and deamination of Ara-C in vitro to active and inactive metabolites, respectively, are believed to correlate with drug response [1, 4, 9, 17]. However, the tedious nature and expense of the studies using the radiochemical form of Ara-C has limited the detailed investigation of Ara-C pharmacokinetics in animals and patients. Such studies are needed to determine patient and tumor variation in drug metabolism and to better understand the extent and duration of drug effects at the cellular and molecular levels in both normal and tumor tissues.

We are applying the described analytical procedures to analyze Ara-C and metabolite levels in vivo in solid tumors and normal tissue. These pharmacokinetic data can then be related to observed drug-induced biochemical and cellular perturbations in an effort to rationally design multiple treatment schedules of Ara-C with improved therapeutic indices.

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