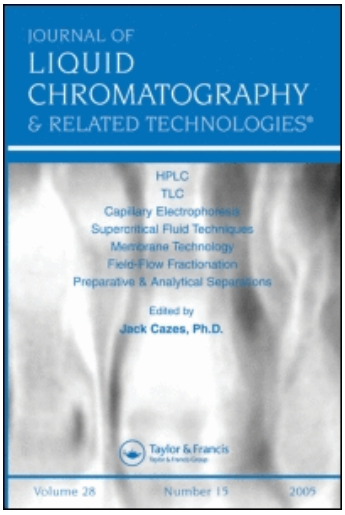

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Reverse Phase HPLC Determination and Murine Pharmacokinetics of Arabinosyl-5-azacytosine

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REVERSE PHASE HPLC DETERMINATION AND MURINE PHARMACOKINETICS OF ARABINOSYL-5-AZACYTOSINE

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ABSTRACT

A sensitive and specific reverse phase HPLC assay has been developed to measure the new antitumor agent arabinosyl-5-azacytosine (ara-AC) in biological fluids at concentrations as low as 50 ng/ml (0.2 μM). This assay also detects arabinosyl-N-formylguanylurea (AGU-CHO), the initial hydrolytic metabolite of ara-AC. 2'-Deoxy-5-azacytidine, an analogue with similar chemical stability, is used as an internal standard. Chromatographically interfering plasma ribosides are removed by solid phase extraction on a phenyl boronic acid cartridge. Separation of ara-AC, AGU-CHO and internal standard is then accomplished isocratically (1% CH_3CN in 10 mM pH 6.8 phosphate buffer) on fully carbon loaded and end-capped C_8 and C_{18} columns connected in tandem. The compounds of interest are detected by UV absorption at 240 nm and total analysis time is 20 min. This assay has been used to determine bolus dose plasma kinetics in male BDF_1 mice given 200 mg/kg ara-AC as a tail vein injection. Plasma elimination of the ara-AC is triphasic with a terminal phase half-life of 52 min and the elimination of the

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AGU-CHO metabolite parallels that of the parent drug. Analysis of ara-AC in human plasma indicates that this method is suitable for determining drug disposition and pharmacokinetics in human subjects.

INTRODUCTION

Arabinosyl-5-azacytosine (ara-AC, Fazarabine, NSC 281272) is a recently synthesized antineoplastic agent which is currently undergoing Phase I clinical trials. Structurally, ara-AC is a hybrid molecule which contains elements of two known antitumor agents, arabinosyl cytosine (ara-C) and 5-azacytidine (5-AC, Figure 1) [1]. It possesses the s-triazine base of 5-AC that is isosteric to cytosine and the arabinose sugar of ara-C. Chemically, ara-AC is similar to 5-AC in that it undergoes facile hydrolytic decomposition in aqueous solutions at ambient temperature to ring-opened products (Figure 2) [2-4]. The initial product of nucleophilic attack by water, arabinosyl-N-formylguanylurea (AGU-CHO, 2), is in equilibrium with ara-AC (1). However, 2 irreversibly decomposes to guanylurea 3 which is devoid of antitumor activity. Biochemically, ara-AC more closely resembles ara-C, since both agents require intracellular activation by deoxycytidine kinase to nucleotide triphosphates. Like ara-C, ara-AC inhibits DNA synthesis and is readily incorporated into DNA in a dose dependent manner [5]. The cytotoxic effect of ara-AC appears to result from DNA fragmentation and strand breakage secondary to ring opening of the unstable s-triazine base [5]. In addition, like 5-AC, ara-AC is also capable of producing *in vitro* differentiation of HL-60 promyelocytic leukemia cells [6].

This potential new antitumor agent shows a much broader spectrum of activity and greater antitumor effects than either ara-C or 5-AC in murine model tumor systems [7]. Like many other nucleoside antimetabolites, the antitumor effect of ara-AC is markedly schedule dependent. Enhanced effectiveness against murine *in vivo* L1210 on a frequent dosing schedule or after extended continuous infusion suggests Phase I clinical evaluation of this

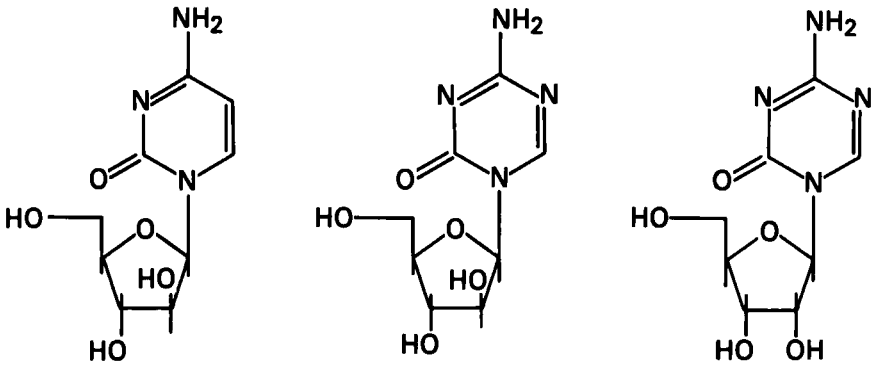


FIGURE 1. Structure of Arabinosyl Cytosine, Arabinosyl-5-azacytosine and 5-Azacytidine.

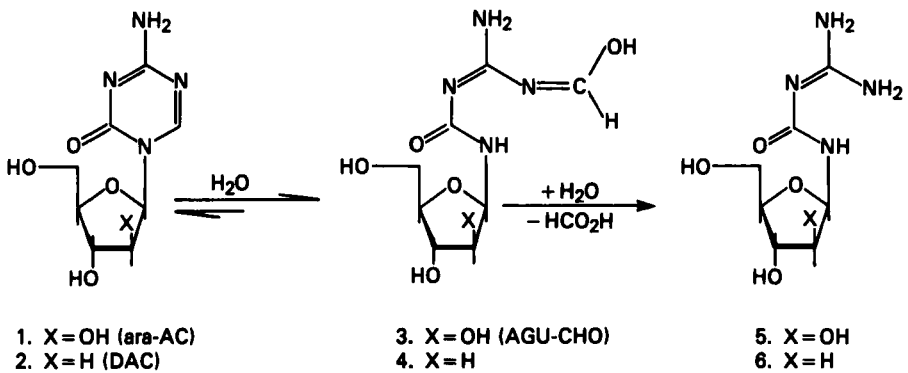


FIGURE 2. Hydrolytic Decomposition of 5-Azacytosine Nucleosides.

agent on an extended continuous infusion schedule [6,7]. Thus, especially at the lower starting doses of the Phase I clinical trials [8], determination of ara-AC pharmacokinetics in humans will require both a sensitive and specific analytical method to measure this compound and its metabolite in physiological fluids. The following report details the development of a reverse phase HPLC assay for ara-AC and its initial hydrolytic metabolite. The suitability of this method for studying the clinical pharmacokinetics of ara-AC in humans is demonstrated in a murine model.

MATERIALS

Ara-AC (4-amino-1-β-D-arabinofuranosyl-1,3,5-triazine-2(1H)-one, Fazarabine, NSC 281272) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI as a white crystalline powder of 98% purity. This material (Lot AP-02-157) showed no evidence of hydrolytic decomposition products when analyzed by HPLC. The 2'-deoxy-5-azacytidine (DAC) internal standard was a generous gift of Pharmachemie B.V., Haarlem, Holland. Anhydrous HPLC-grade dimethyl sulfoxide (DMSO) was obtained from Aldrich Chemical Co. (Milwaukee, WI) while HPLC-grade acetonitrile and methanol were purchased from Fisher Chemical Co. (Fairlawn, NJ). Distilled water was vacuum filtered through a Norganic cartridge (Millipore Corp., Bedford, MA) while acetonitrile was filtered through a 0.45 μm solvent resistant filter (Millipore) before mixing to make the mobile phase. Monobasic sodium phosphate and sodium hydroxide (Fisher) were employed to make pH 6.8 to pH 8.0 buffers.

METHODS

Separations were accomplished at ambient temperature (20-22°C) on a cartridge column system (Brownlee MPLC, Brownlee Labs, Inc., Santa Clara, CA) containing a 4.6 X 30 mm 5 μm C_{18} Spheri-5 cartridge as a guard column, a 4.6 X 100 mm 5 μm C_8 Spheri-5 cartridge and a 4.6 X 100 mm 5 μm C_{18} Spheri-5 cartridge. These cartridges

were connected in series and assembled as a single unit in 13 and 10 cm holders connected by a union. A mobile phase of 1% CH_3CN in 0.01 M pH 6.8 phosphate buffer was used at a flow rate of 1.0 ml/min. The remainder of the HPLC system was comprised of a Waters Associates (Milford, MA) Model 6000A solvent delivery system, a U6K injector and a LC-85 variable wavelength detector (Perkin-Elmer, Norwalk, CT) operated at 240 nm. Injections of standards and unknown samples (100 μl) were made using a 100 μl Waters gas tight syringe. Peak areas and heights were determined simultaneously on a SP4200 computing and recording integrator (Spectra-Physics, Santa Clara, CA).

Blood was obtained from normal human volunteers and was collected in rubber stoppered 10-ml glass tubes containing 143 units of USP heparin (Vacutainer, Becton Dickinson and Co., Rutherford, NJ). The heparinized blood was immediately centrifuged at 1100 X g for 3 min on a Dynac table top centrifuge (Clay Adams, Becton Dickinson and Co.) to obtain plasma, which was then placed on ice. Ara-AC standards of 0.05-10 μg/ml concentration were made by adding the appropriate volumes of 0.05 or 1.0 mg/ml ara-AC in DMSO to cold 1 ml aliquots of plasma. Two microliters of 1 mg/ml DAC in DMSO was added to each sample for an internal standard. The sample was vortexed for 15 sec to ensure thorough mixing and then put back on ice until the next step.

Plasma must be processed to remove endogenous ribosides which interfere with the chromatographic analysis when low levels of ara-AC are to be measured. This was done by using an activated 1.0 ml capacity phenylboronic acid (PBA) solid phase extraction cartridge (Bond Elut, Analytichem International, Harbor City, CA). The PBA Bond Elut cartridges were activated just before use by washing with 1 ml methanol followed by 1 ml pH 8, 0.01 M phosphate buffer. The sample was slowly pushed through the PBA cartridge using a disposable 3-ml syringe which was attached by an adaptor, and the eluant collected. A 0.5 ml pH 7.4, 0.01 M phosphate buffer rinse was used to wash the sample completely from the column. This rinse was added to the previous eluant and ultrafiltered in an Amicon Centrifree

system (Amicon Corp., Danvers, MA) by centrifuging at 1100 X g for 45 min at 4°C. The resulting ultrafiltrate was kept on ice until HPLC analysis, which typically began immediately after the above sample processing.

The in vivo murine plasma pharmacokinetics of ara-AC was determined after administering an intravenous (i.v.) bolus injection of 200 mg/kg ara-AC to male BDF_1 mice weighing 21-26 gm via the tail vein. Drug solution at a concentration of 20.3 mg/ml was prepared fresh daily by diluting a DMSO stock solution of ara-AC with 4 volumes of sterile 0.9% NaCl solution. Mice were sacrificed under ether anesthesia at predetermined times to obtain plasma for drug measurement. For each timepoint of 90 min or less, three mice were used; otherwise, five mice were employed. For the 4 and 5 hr points, individual plasma samples of the same time were pooled to provide a sufficient volume of plasma for measuring the anticipated low levels. Since very high concentrations of ara-AC were expected at the early time points, the 2 and 20 min plasma samples were diluted 20X and the 10, 30, 60 and 90 min 10X with pH 7.4, 0.01 M phosphate buffer. A standard curve was constructed daily by processing 0.5 ml aliquots of pooled plasma from untreated mice, which had been spiked with the appropriate amounts of ara-AC. A 0.5 ml aliquot of each diluted or undiluted sample was then subjected to workup and analysis. These samples were analyzed on the same day they were obtained and all samples, both processed and unprocessed, were stored on ice continuously.

The recovery of ara-AC and DAC from spiked human plasma was evaluated by direct comparison with appropriate buffer standards. To 5 ml fresh human plasma was added the appropriate volume of a DMSO solution of ara-AC at either a 1 mg/ml or 10 mg/ml concentration to give a final plasma concentration of 1, 10 or 100 μg/ml. Four 1-ml aliquots of each plasma sample were then taken for processing and HPLC analysis as described above. A higher concentration of 10 μg/ml DAC was used as an internal standard for the 100 μg/ml buffer and plasma aliquots. For these samples a 20 μl injection, rather than one of 100 μl, was employed for analysis.

Both the peak area and peak height ratio of ara-AC to internal standard were calculated to determine assay precision.

Plasma protein binding for human plasma was determined at ara-AC concentrations of 0.25 and 1 μg/ml. Quadruplicate 0.5 ml aliquots of both fresh human plasma and its ultrafiltrate were spiked at the appropriate ara-AC concentrations. Ultrafiltrate was processed directly through the PBA cartridge as described above, while plasma was first ultrafiltered in an Amicon Centrifree system before PBA processing. The ultrafiltration step immediately preceding HPLC analysis was accordingly omitted. Plasma protein binding was defined as the per cent difference between ara-AC concentrations in ultrafiltrate (C_{UF}) and ara-AC in plasma (C_p):

$$\text{Plasma Protein binding (\%)} = 100\ (1 - [C_p/C_{UF}])$$

Initial pharmacokinetic parameters for ara-AC in mice were estimated from the plasma concentration (C_p) *versus* time curve by the method of residuals [9]. The experimental data points were then fit to a triexponential function representing a three-compartment open model ($C_p = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\tau t}$) by using MLAB, an on-line computer modeling laboratory utilizing an interactive, non-linear least squares program [10]. Based on observed assay characteristics, each data point was weighted by $1/(C_p)^2$. The area under the C_p *versus* time curve was also calculated using the trapeziod rule [9] with an extrapolation to infinity from the last data point using the terminal (τ) rate constant.

RESULTS

A variety of reverse phase HPLC columns were found to separate ara-AC, AGU-CHO (3), the DAC internal standard and its analogous hydrolytic decomposition product 4 (Table 1). Good chromatography on these columns was usually obtained with an aqueous mobile phase which was buffered to maintain pH control and which contained a small amount (0-2%) of organic modifier (CH_3CN) to adjust retention. The best single column separation was achieved on an Alltech C_8 Econosphere column (Column B, Table 1). However, a

complicating factor with most of the C_8 columns was insufficient retention of the nucleosides of interest so that AGU-CHO appeared on the tail of the DMSO peak. DMSO was used as the solvent for standard stock solutions; and since it will also be used to formulate the drug for clinical administration [4], it can be anticipated as a potential interference. The separation between DMSO and AGU-CHO was much better on C_{18} columns although the ara-AC/DAC separation was not as good (Column C, Table 1). Therefore, two short (i.e. 10 to 15 cm) C_8 and C_{18} columns were placed in tandem to take advantage of the selectivity of each packing material [11,12]. A Brownlee MPLC Cartridge system was found

TABLE 1

Capacity Factors of 5-Azacytosine Nucleosides on Various Reverse Phase Column Systems

Component	Capacity Factor (k')		
	Column A[1]	Column B	Column C
DMSO	1.07[2]	1.38	0.77
Cytidine	2.04	2.15	2.44
Uridine	3.18	2.88	4.27
2'-Deoxycytidine	3.77	4.20	4.39
5-Azacytidine	2.14	2.03	2.90
AGU-CHO (3)	2.47	2.32	2.81
ara-AC (1)	2.94	2.72	3.93
DAC (2)	3.34	3.27	4.44
dRGU-CHO (4)	4.47	4.31	5.82

[1]The columns and mobile phases used were: A, Brownlee MPLC cartridge system as described in Methods; B, 4.6 X 250 mm Alltech 5 μm C_8 Econosphere eluted with pH 6.8, 0.01 M phosphate buffer; C, 4.6 X 250 mm Altex/Beckman 5 μm Ultrasphere ODS eluted with 0.5% CH_3CN in pH 6.8, 0.01 M phosphate buffer.
[2]Capacity factor values are the means of at least three determinations.

to be the most convenient way to incorporate a guard column and both a C_8 and a C_{18} column into a single unit. Figure 3 depicts a typical chromatogram of a slightly decomposed 1 μg/ml ara-AC standard in buffer on this column system.

The isolation procedure of Scheme I allowed efficient recovery of both ara-AC and the DAC internal standard from human and mouse plasma over the range of ara-AC concentrations expected in biological samples (Table 2). This same procedure almost completely (>95%) removed endogenous levels of uridine [13], so that chromato-

TABLE 2

Recovery and Measurement Precision of ara-AC and DAC in Plasma

	ara-AC[1]	DAC[2]	r_a[3]	r_h[4]
Human Plasma				
1 μg/ml	79 ± 5	95 + 5	0.372 (6.6%)	0.546 (7.2%)
10 μg/ml	89 ± 6	79 ± 5	5.36 (2.0%)	5.76 (1.1%)
100 μg/ml	96 ± 7	99 ± 8	11.4 (3.0%)	11.6 (2.8%)
Mouse Plasma				
1 μg/ml	90 ± 6	94 ± 5	0.460 (3.7%)	0.547 (1.5%)
Rat Plasma				
1 μg/ml (n=5)	90 ± 13	97 ± 7	0.539 (8.0%)	0.536 (3.2%)

[1]Values reported are the means of 4 replicate determinations except where noted.
[2]Internal standard concentration was 2 μg/ml except for ara-AC concentrations of 100 μg/ml, where it was 10 μg/ml.
[3]Peak area ratio of ara-AC to DAC internal standard. The relative standard deviation of this measurement is indicated in parentheses.
[4]Peak height ratio of ara-AC to DAC internal standard.

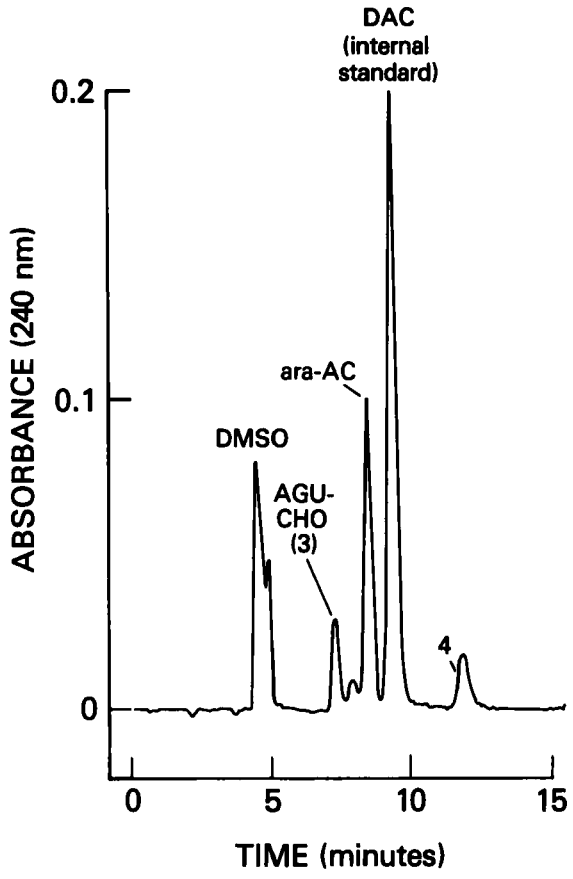
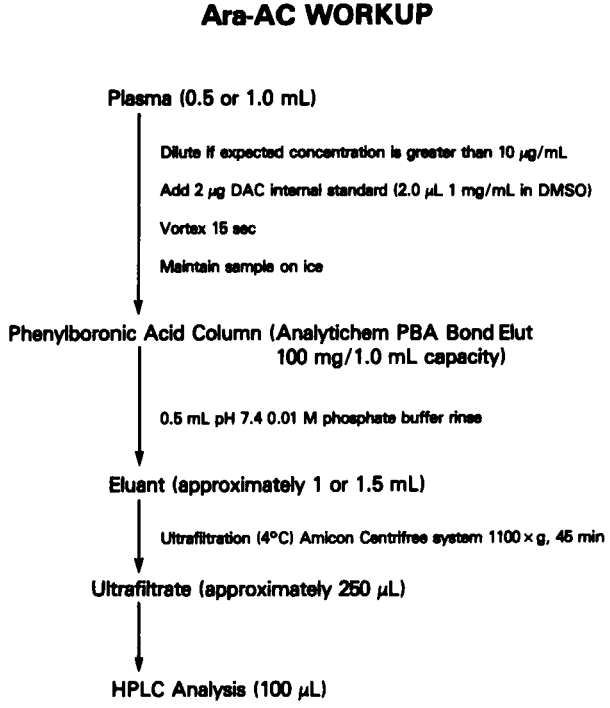


FIGURE 3. HPLC Analysis of a Partially Hydrolyzed Buffer Standard of ara-AC (1 μg/ml) and DAC (2 μg/ml). Chromatographic conditions are as indicated in Methods.

graphic interference (see Table 1) by this nucleoside was eliminated. This is illustrated by the chromatographic blank obtained from pretreatment mouse plasma (Figure 4a). Thus linear ($r \geq 0.999$) and reproducible calibration curves could be constructed from spiked plasma standards at even submicromolar concentrations (Figure 5). For human plasma, the limit of quantitation (S/N > 5) was 0.2 μM (50 ng/ml) although smaller concentrations could be



Scheme I

reliably detected. Because of the smaller plasma sample (0.5 ml) obtained from mice, the corresponding limit of quantitation was only 0.5 μM (125 ng/ml). Figure 4b, the chromatogram obtained from mouse plasma 3 hr after a 200 mg/kg i.v. bolus dose of ara-AC, demonstrates that both ara-AC and its hydrolytic metabolite 3 can be readily detected and measured in biological samples.

Protein binding studies were difficult to carry out, since the concentration of bound ara-AC was always lower or approximately equal to the limit of quantitation. In human plasma binding was minimal (1 ± 13%, n=6) at 4.1 μM (1.0 μg/ml) and low (19 ± 5%, n=7) at 1.0 μM (250 ng/ml). This minimal protein binding could be neglected for most purposes. Even though there was probably some

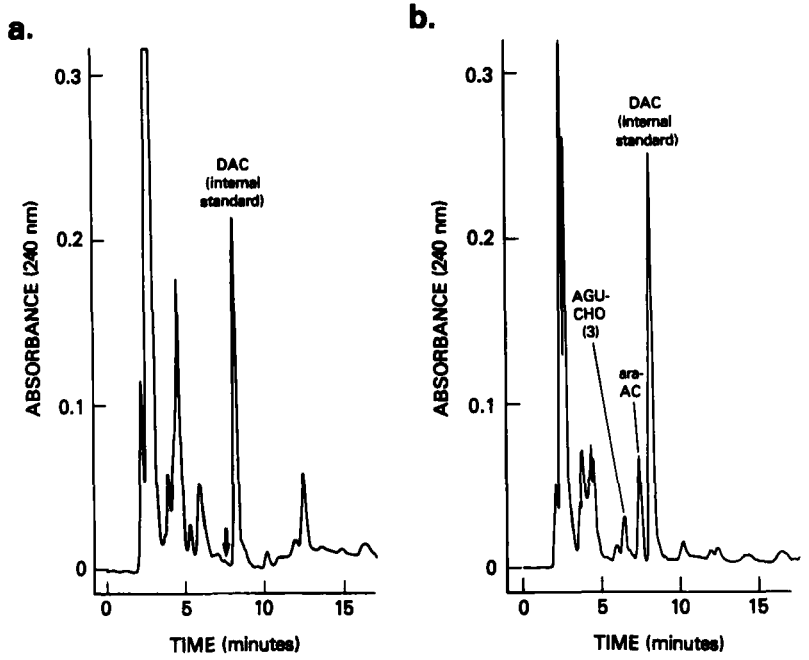


FIGURE 4: HPLC analyses of representative mouse plasma samples. Analysis and experimental conditions are as indicated in Methods. a.) Chromatographic blank from pretreatment mouse plasma. The retention time of ara-AC is indicated by an arrow. b.) Plasma from 3 hr following an i.v. bolus dose of 200 mg/kg. Measured ara-AC concentration is 1.08 μg/ml. Correction for a sample volume of only 0.21 ml gives an actual ara-AC concentration of 2.57 μg/ml.

loss of ara-AC during the ultrafiltration step, it was not noticeable since even low level standard curves were linear at very low concentrations (Figure 5, insert). With such negligible levels of binding it will also usually be unnecessary to make corrections in pharmacokinetic models.

The effect of both refrigeration and freezing on spiked plasma samples was investigated to determine to what extent samples could be stored before analysis. Figure 6 shows that for human plasma

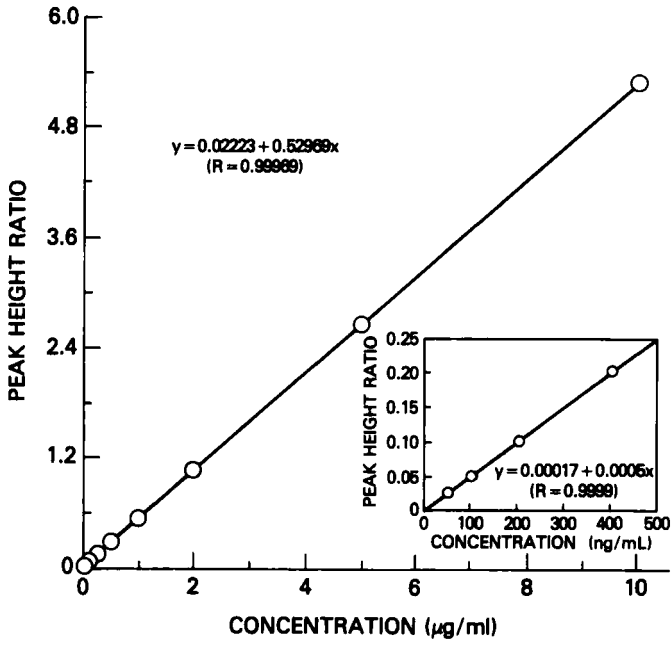


FIGURE 5. Peak height ratio standard curve for ara-AC in human plasma (0.1-10 μg/ml). Insert: Low level (50-400 ng/ml) standard curve for human plasma. The equation for the linear regression is expressed in ng/ml.

spiked at 1 μg/ml, which is approximately the highest concentration measured during Phase I clinical trials [14], storage stability is poor. Unacceptable degradation occurs both in samples which have been processed and refrigerated and in plasma which is immediately frozen after separation of the red blood cells.

DISCUSSION

A sensitive and specific analytical method for the measurement of ara-AC in biological fluids is required for determining the clinical pharmacokinetics of this agent in Phase I trials in humans [14, 15]. As a class of compounds, nucleosides are readily analyzed with good sensitivity and specificity by reverse phase HPLC

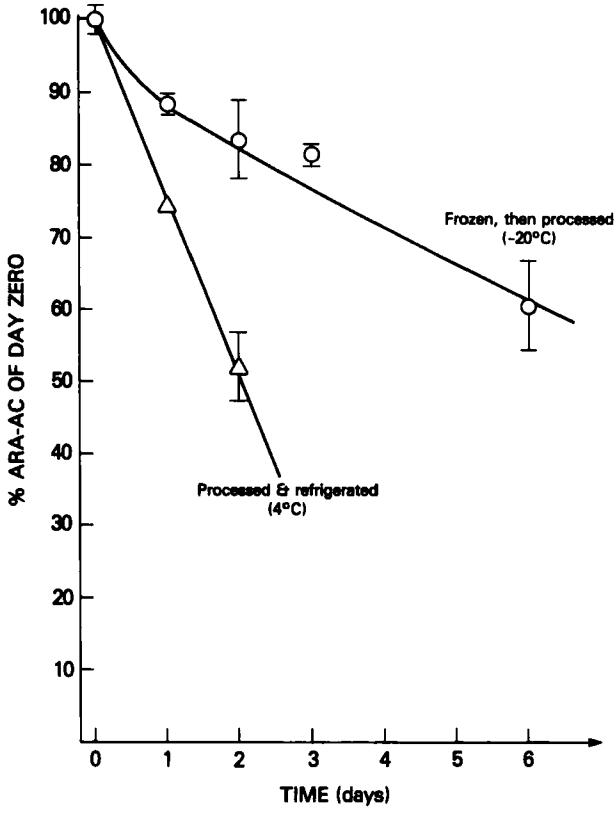


FIGURE 6: Stability of ara-AC in plasma as a function of storage conditions. Human plasma was spiked with ara-AC at a concentration of 1 μg/ml. One milliliter aliquots were taken and frozen in 3.5 ml screw-cap vials at -20°C. After being thawed on the days indicated samples (n=3) were processed and analyzed. Day zero samples were freshly spiked plasma which was immediately worked up, analyzed and then refrigerated at 4°C for subsequent analysis. Key: frozen (o); refrigerated (Δ).

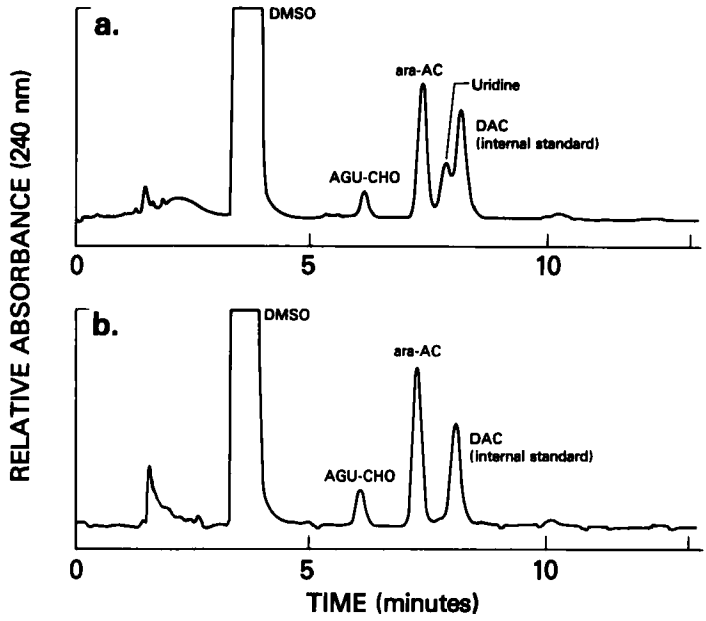


FIGURE 7: Removal of uridine by covalent solid phase extraction. a.) Chromatogram of 2 μM ara-AC, 2 μM DAC and 4.1 μM uridine in 0.1 M pH 6.8 phosphate buffer. b.) Chromatogram of above buffer solution after elution through a PBA cartridge according to the procedure of Scheme I.

[16,17]. Even though previous studies have shown that ara-AC and its hydrolytic degradation products are amenable to HPLC analysis in aqueous solutions and pharmaceutical formulations [1, 4], the sensitivity required for the quantitation of the low drug levels anticipated in the initial human studies is severely limited by endogenous plasma interferences. Uridine, whose normal concentrations in human plasma are quite high (2-8 μM [13]), is a major problem since it produces significant chromatographic interference with either ara-AC or the DAC internal standard (Table 1 and Figure 7a).

Since ara-AC is itself not chemically amenable to simple and selective isolation from a complex biological matrix such as

plasma, the sample preparation approach focused on removal of any endogenous interferences. As indicated above, these were anticipated to be closely related pyrimidine nucleosides such as uridine. Since cis diols, including ribonucleosides, have been successfully isolated from plasma on columns composed of a phenyl boronic acid resin [18-20], this approach for removal of these interferences was investigated with the use of commercially available minicolumns. Typically, the phenyl boronic acid resin is activated at high pH (8-10) to form a covalent complex with any cis-diols (e.g. ribonucleosides) and then washed at neutral pH to remove compounds nonspecifically bound to the resin [21]. The covalent complex is broken and the cis-diols are then eluted at low pH (<5). Our approach, subtractive solid phase extraction, was only concerned with the efficient removal of interfering ribonucleosides and not with the retention of compounds of interest. Because hydrolytic decomposition of the s-triazine ring was accelerated at basic pH [3, 4], conditions for use of the phenyl boronic acid cartridges had to be carefully adjusted. Thus, the lowest possible pH at which covalent binding occurs (pH 8) was used to activate the PBA cartridges. Minimal analyte decomposition and satisfactory (>95%) retention of endogenous plasma uridine were achieved with this procedure. Figure 7b illustrates the chromatographic enhancement for measuring ara-AC that is possible when a buffer solution containing ara-AC and uridine (Figure 7a) was subjected to the workup procedure of Scheme I. Efficient recovery of ara-AC and DAC required a 0.5 ml pH 7.4 buffer rinse column rinse to remove all the non-covalently bound material. Although this did result in sample dilution, the speed of the subsequent ultrafiltration was increased.

Choice of an internal standard for this assay was especially critical because of the hydrolytic instability of ara-AC and the constraints of the isolation procedure. At a minimum, the internal standard had to have similar, yet different, chromatographic properties and not be retained by the phenylboronic acid minicolumn. 2'-Deoxy-5-azacytidine, the deoxyribose analogue of 5-AC

and ara-AC (DAC, Figure 2 and Table 1), met these criteria. Because DAC contained a 2′-deoxyribose sugar, it was not retained by the PBA cartridge and could be added to the plasma when a sample aliquot was taken. In addition, since its hydrolytic stability was comparable to that of ara-AC [22], DAC could be used as a marker for sample decomposition during processing or storage. Presence of dRGU-CHO (4, Figures 2 and 3) in a sample was an indication of this degradation and could be used to determine whether the hydrolytic metabolite AGU-CHO (vide infra) was a sample component or artifact.

The hydrolytic instability of ara-AC was such that plasma samples had to be worked up immediately and analyzed on the same day. Attempts to store plasma by rapid freezing or to store processed extracts at 4°C were unsuccessful in preventing unacceptable sample decomposition (Figure 6). This meant that standards had to be prepared and processed along with samples and that careful planning was required when large numbers of samples had to be analyzed as in a pharmacokinetic study.

This assay was applied to investigate the plasma kinetics of ara-AC in mice given a single IV bolus dose of 200 mg/kg. This dose was chosen because it was a dose known to have little toxicity to mice and also because it would allow direct comparison to previous work using 6-^{3}H-ara-AC and a less sensitive HPLC method [23]. The ara-AC concentration versus time data best fit (r^2=0.9988) a three-compartment open model where the half-life for the terminal phase was 52 min (Figure 8). Less than 1% of the estimated area-under-the-curve was derived from extrapolation of the terminal phase. This experimental data indicated a total body clearance (CL_{TB}) of 17.1 ml/min/kg. These results compare favorably to those of Zaharko and Covey [23], who observed a CL_{TB} of 14.8 ml/min/kg and a similar triphasic elimination of ara-AC in mice given the same bolus dose.

It was also possible to estimate the plasma concentration of AGU-CHO (3) for up to three hours (Figure 4b). Since a pure standard of 3 would be very difficult to handle [2] and was not available, the concentrations of this degradation product are expressed as ara-AC equivalents. The relative concentration of 3

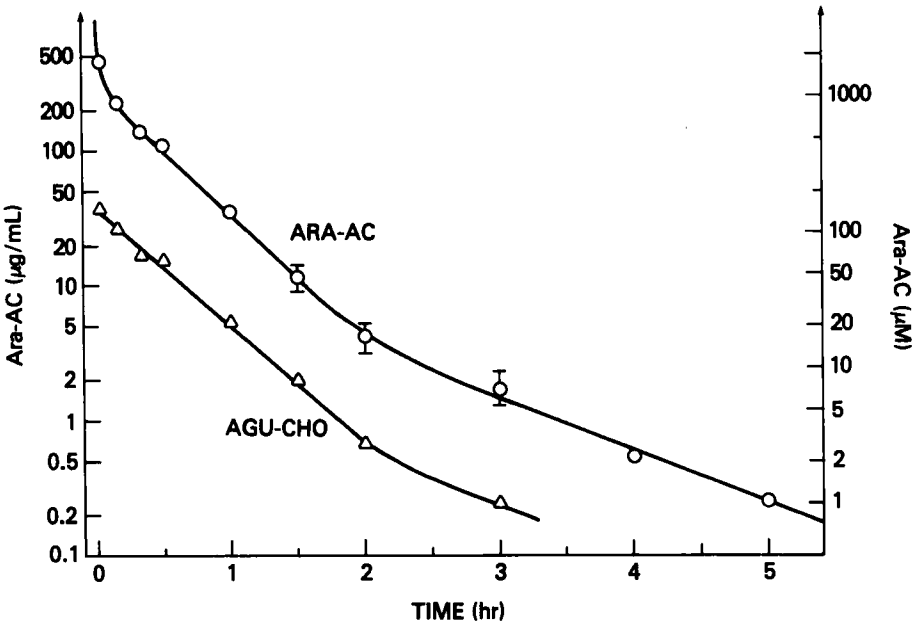


FIGURE 8: Plasma concentration versus time curve (C_p X t) for BDF_1 mice given a 200 mg/kg i.v. dose of ara-AC. Each point represents the mean ara-AC concentration in three or more animals except for the 4 and 5 hr points where the plasma from 5 animals was pooled before analysis. The brackets about each point signify the range of measured ara-AC concentrations. Key: ara-AC (o); AGU-CHO (Δ).

gradually increased to an apparent equilibrium value of about 0.15 ara-AC molar equivalents at 1 hr and then appeared to parallel the plasma elimination of the parent compound. A similar in vivo behavior has been observed for 5-azacytidine and its N-formyl-guanylribosyl urea decomposition product [24].

CONCLUSION

Sample preparation involving covalent solid phase extraction to remove endogenous interferences has permitted development of a specific and more sensitive reverse phase HPLC method for measuring ara-AC and its initial hydrolytic metabolite in plasma. The suit-

ability of this assay for pharmacokinetic studies can be seen from the bolus dose murine kinetics presented here, where plasma drug concentrations ranging from 1 - 1840 μM could be measured. This assay has also been used to define the preclinical pharmacology of ara-AC in rhesus monkeys in order to develop a pharmacokinetic model to predict plasma concentrations in humans [25]. At the present time the analytical method described here is being used to determine ara-AC levels and plasma kinetics in patients receiving the drug as 24- and 72-hr continuous infusions in ongoing Phase I clinical trials [14,15].

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