Simple Sensitive Microbioassay for Adenine Arabinoside and Hypoxanthine Arabinoside in Human Plasma

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Previous methods using low- or high-pressure liquid chromatography and UV absorbance for quantitation of arabinosides in plasma can practically detect only ≥ 200 ng of adenine arabinoside and ≥ 100 ng of hypoxanthine arabinoside per ml, and they require expensive equipment and expert technical assistance. We describe in this report a simple quantitative microbioassay for arabinosides in human plasma based on their ability to inhibit the cytopathic effect of vaccinia virus in an adenosine deaminase-free cell culture system. Using prior separation of nucleosides in plasma by thin-layer chromatography, followed by the microbioassay, we quantitated adenine arabinoside with a sensitivity of ~ 4 ng/ml and hypoxanthine arabinoside at ~ 625 ng/ml. This assay method is simple, sensitive, and reproducible, requires small plasma samples, measures biological activity, and is adaptable to routine use. It is an important tool for evaluating the pharmacology of adenine arabinoside and Ara-AMP in patients in current clinical trials.

Adenine arabinoside (Ara-A) has significant *in vitro* and *in vivo* antiviral activity against *Herpesvirus hominis* types 1 and 2, varicella zoster virus, cytomegalovirus and vaccinia virus (VV) (1, 5). Ara-A has been shown to have efficacy in the treatment of herpes simplex encephalitis (6). Further blinded clinical trials to evaluate the therapeutic efficacy of Ara-A in other severe and life-threatening infections due to *Herpesvirus hominis* and varicella-zoster virus are underway.

Recently we have examined, by high-pressure liquid chromatographic methods and UV absorbance (3), the plasma levels of arabinosyl compounds from patients treated with Ara-A. This procedure is lengthy and requires sophisticated and expensive equipment and expert technical assistance. The ≥ 200 -ng/ml quantitative limit of the high-pressure liquid chromatography method is near or below plasma levels of Ara-A resulting from present dosage schedules. A simple, more sensitive quantitative assay of Ara-A in plasma and other body fluids based upon biological (antiviral) activity is the subject of this report.

MATERIALS AND METHODS

Cells. The horse skin fibroblast line was obtained from the American Type Collection (ATCC CCL57, E. Derm [NBL-6]) and propagated as monolayers in Eagle minimal essential medium with 10% fetal bovine serum. Maintenance medium (MM), consisting of minimal essential medium with 2% fetal bovine serum and

1% of both penicillin G and streptomycin, was used for all virus-infected cultures. Plaque assays were performed with confluent cell monolayers in 16-mm-diameter wells of 24-well plates (Falcon no. 3008). For the microassay, confluent cell monolayers in Microtest II plates (Falcon) were employed.

Drugs. Ara-A (9- β -D-arabinofuranosyladenine), Ara-Hx (9- β -D-arabinofuranosylhypoxanthine) and adenosine deaminase inhibitor {ADAI, (R)-3-[2-deoxy- β -D-erythro-pentofuranosyl]-3,6,7,8-tetrahydroimidazo[4,5-d]-[1,3]diazapin-8-01} (7) were gifts of Parke Davis & Co. Ara-A and Ara-Hx were made up in 500- μ g/ml stock solution; ADAI was diluted for use from a 1-mg/ml stock concentration.

Virus. VV (calf lymph vaccine) was obtained from Wyeth Laboratory, reconstituted in minimal essential medium with 20% skim milk (Difco), and stored at -70°C in 0.5-ml aliquots until use.

Plaque assay. Confluent CCL57 cell monolayers were infected with 0.2 ml of VV per well diluted in MM sufficient to yield approximately 60 plaque-forming units per 16-mm well. Virus was adsorbed for 1 h at 37°C in 5% CO_2 . Serial twofold dilutions of Ara-A or Ara-Hx were prepared in MM. ADAI (final concentration, 0.83 $\mu\text{g/ml}$) was added to medium containing Ara-A to prevent deamination. Residual virus was then removed, and 1 ml of medium containing serial drug dilutions was added to each well. The plates were incubated for 48 h at 37°C , fixed with methanol, and stained with 1% crystal violet. Plaques were scored under a dissecting microscope at $\times 10$ magnification. The average number of plaques in triplicate wells of drug-treated cultures was expressed as percentage of plaques developing in virus-infected control cultures.

Microassay. Confluent microwell cultures were infected by adding 50 μl of VV per well diluted in MM

sufficient to result in complete cytopathic effect (100% CPE) during a 48-h period of incubation. After virus adsorption at 37°C in 5% CO₂ for 1 h, residual virus was removed and replaced with 100 μ l of MM containing ADAI (0.83 μ g/ml) per well. A 100- μ l portion of each sample to be tested was then added to the first well in each of two rows, and serial twofold dilutions were prepared in the plate with a micropipette, allowing adequate mixing in each well prior to transfer. Uninfected cell controls were included to determine that the cultures were intact throughout the test period, and virus-infected, drug-free controls were included to confirm sufficient infectivity of challenge inocula. The microtest plates were then incubated for 48 h at 37°C in 5% CO₂, fixed, stained, and scored for viral CPE. Scoring was graded from 0 to 4+; 4+ indicated 100% CPE as in the virus control, and 0 indicated complete protection. End points of drug inhibition were defined as the last well in a row to show 0 to 25% CPE.

Plasma samples. Whole blood (5 ml) was obtained aseptically from laboratory volunteers in heparinized tubes containing 25 μ l of ADAI. Plasma was separated by centrifugation to perform reconstruction experiments. Quantities of Ara-A or Ara-Hx were added to plasma samples in various starting concentrations so that a standard dilutional gradient could be observed in control experiments, and end points calculated. Plasma samples were deproteinized with a 1:1 volume of 95% ethanol and centrifuged at 1,200 $\times$ *g* to pellet the precipitate; supernatants were saved for bioassay or for separation of arabinosides by thin-layer chromatography (TLC), followed by bioassay. In later experiments specimens were deproteinized by Amicon filtration. For determination of total antiviral activity, samples of the deproteinized plasma supernatants were dried under nitrogen and reconstituted in MM before bioassay.

Separation of Ara-A and Ara-Hx by chromatography. Separation of nucleosides for individual quantitation in the bioassay was accomplished by modification of the TLC method described by Drach and Novack (4) as follows: 50- μ l samples of deproteinized plasma were spotted in 1-cm streaks on silica gel plates (EM Reagents 60 F-254). Plates were developed with the lower phase of the solvent chloroform-methanol-3% acetic acid in 3:2:1 proportions, until the solvent front reached 11 to 15 cm, which usually required 1 to 1.5 h. Average *R_f* values for Ara-A and Ara-Hx were 0.32 and 0.17, respectively, allowing adequate separation. Plates were then dried, and, whenever possible, spots were visualized under UV light, compared with drug standards, and scraped for elution and recovery.

When the drug concentration of the sample was below the limit of visibility under UV light, duplicate areas of the gel parallel to drug controls were scraped. Gels were eluted with 2 ml of distilled water and centrifuged at 1,200 $\times$ *g* for 5 min to pellet the silica. Aliquots of these eluates were lyophilized in preparation for quantitation in the bioassay.

RESULTS

Plaque assay. The plaque assay was employed to determine the susceptibility of VV

plaque development to the inhibitory activity of Ara-A and Ara-Hx in horse fibroblasts. The average number of plaques in triplicate wells of drug-treated cultures was expressed as a percentage of controls. In Fig. 1, percentage of control plaque development is plotted as a function of drug concentration on a log axis. The points are fitted to a least-square line as indicated. ADAI alone had no intrinsic antiviral effect. It may be noted that 50% inhibition of VV plaque development occurred at a concentration of 2.5 ng of Ara-A per ml and 120 ng of Ara-Hx per ml and that the ratio of antiviral activity of these compounds is approximately 50:1 as previously reported (1, 2). This cell line and VV provided an exquisitely sensitive system for quantitation of antiviral activity of Ara-A in biological fluids.

Microassay. To simplify the biological assay and to conserve materials, the horse fibroblast-VV system was adapted to microtest plates (Fal-

FIG. 1. Inhibition of VV plaque development (as percentage of control plaque number) by Ara-A and Ara-Hx. Confluent horse cell fibroblasts (CCL57) were infected with 50 to 60 plaque-forming units of VV and incubated postinfection in MM containing serial dilutions of arabinosides, and plaques were scored 48 h postinfection as described in the text. ADAI (0.83 μ g/ml) was included with the Ara-A. Linear regression lines calculated from the points and drug concentrations for 50% plaque inhibition are indicated.

con no. 3008) using viral CPE rather than plaque development as a measure of viral infectivity and test end points. To compare the sensitivity of this assay with that of the plaque assay, we first determined standard end points for Ara-A and Ara-Hx prepared in MM. Microcultures in 96-well plates were infected with VV, residual virus was removed, and 100 μ l of MM was replaced per well (see Materials and Methods). An additional 100 μ l of MM containing either Ara-A (1,000 ng/ml) + ADAI or Ara-Hx (5,000 ng/ml) was then added to the first well of each of two rows, and serial twofold dilutions were made in the plate as previously described. Table 1 summarizes six initial experiments that demonstrated the susceptibility of VV to Ara-A and Ara-Hx by the microassay. Using the last well with confluent microculture or the first with 25% CPE (1+) as end points, it may be observed that 4 to 8 ng of Ara-A per ml and 625 ng of Ara-Hx per ml protected the microcultures from VV challenge. This experiment was repeated a minimum of 20 times, and, on the average, the dilutional method varied 1 dilution greater or 1 dilution less between individual tests using drug control standards.

Reconstruction experiments of arabinosides in plasma. Repeated experiments were designed to see if preparation and deproteinization of plasma samples resulted in loss of arabinoside or if plasma itself was cytotoxic.

Briefly, drugs were added to heparinized plasma, which was then deproteinized and lyophilized as before. Controls of plasma alone and plasma with ADAI were prepared in a similar manner. After infection of microcultures with VV, 100 μ l of reconstituted plasma sample was added to the first well in each of two rows, and serial twofold dilutions were made in the plates.

Figure 2 is a photograph of a microtest plate in which one of these experiments was performed. Crystal violet staining of the plates readily allowed differentiation by naked eye between destroyed and protected microcultures. Preliminary examination of plates was confirmed by reading them at $\times 10$ magnification under a dissecting microscope. In this plate all well cultures were infected with VV except the single vertical row on the extreme right of the plate, which contained uninfected cell controls. The next-to-last vertical row contained virus controls, which showed 100% CPE. Each of the four samples was run in duplicate and diluted serially twofold from left to right.

Plasma or plasma with ADAI neither showed antiviral effect nor was cytotoxic to the cells. Note that Ara-A was protective through well 7 of rows 3 and 4 and Ara-Hx through well 3 of rows 1 and 2 (Fig. 2). In this test plate, these end points corresponded to 4 ng of Ara-A per ml and 625 ng of Ara-Hx per ml and were the same as those of arabinosides prepared in control MM. Thus, complete recovery of antiviral activity occurred through these procedures. On the basis of these experiments, repeated a minimum of 10 times, and other similar experiments, it is possible to conclude that the bioassay may be used as a standard measure of Ara-A with a sensitivity of ~ 4 ng/ml or of Ara-Hx with a sensitivity of ~ 625 ng/ml in plasma. Since Ara-A is constantly deaminating *in vivo* to Ara-Hx, clinical plasma samples may contain either or both of these arabinosides in different concentrations and ratios. This assay may be used also as described for determination of total antiviral activity of plasma. However, for individual quantitation of arabinosides, the compounds must be separated prior to bioassay.

Previous experiments (results not shown) us-

TABLE 1. Inhibition of CPE of VV in horse cell fibroblast microcultures by arabinosides^a

Ara-A expt no.	CPE score ^b at drug concn (ng/ml of MM):											
	500	250	125	62.5	31.2	15.6	7.8	3.9 ^c	1.9	0.97	VC ^d	CC ^e
1	0	0	0	0	0	0	0	1+	2+	4+	4+	0
2	0	0	0	0	0	0	0	0	2+	4+	4+	0
3	0	0	0	0	0	0	0	0	3+	4+	4+	0

Ara-Hx expt no.	CPE score ^b at drug concn (ng/ml of MM):										
	2,500	1,250	625 ^c	315	156	78	39	19	9.7	VC ^d	CC ^e
1	0	0	0	3+	4+	4+	4+	4+	4+	4+	0
2	0	0	1+	3+	4+	4+	4+	4+	4+	4+	0
3	0	0	0	3+	4+	4+	4+	4+	4+	4+	0

^a Summary of initial experiments to standardize microassay.

^b CPE score: 0, confluent microculture; 1+, 25% CPE; 2+, 50% CPE; 3+, 75% CPE; 4+, 100% CPE.

^c End point concentration of drug in last well showing 0 to 1+ CPE.

^d VC, Virus control (VV).

^e CC, Cell control.

FIG. 2. Microtest plate containing confluent cultures of horse cells (CCL57). All well cultures were infected with VV except the vertical row marked cell control (CC). Virus controls (VC) showed 100% CPE. Four plasma samples, containing Ara-A, Ara-Hx, plasma and ADAI, and plasma alone, were assayed in duplicate and serially diluted twofold from left to right. The darkly stained wells show complete protection of cultures (0 CPE) (see text for details).

ing radioactive Ara-A (adenine [2^3 -H](N)- β -D-arabinofuranoside) showed ~85% recovery of radioisotope from silica gel TLC plates at concentrations as low as 7 ng/ml. The next experiments were designed to see the recovery of antiviral activity after preparation and separation of arabinosides by thin-layer chromatography. This involved the reconstitution of plasma, collected with ADAI and either Ara-A (1,000 ng/ml) and Ara-Hx (10,000 ng/ml) alone or in combination. These plasma samples were then deproteinized, and nucleosides were separated by TLC (see Materials and Methods). The eluted specimens were lyophilized, dissolved in MM, and bioassayed for antiviral activity. Results for Ara-A (Table 2) were identical to those for plasma reconstituted with individual arabinosides without separation by TLC, showing complete recovery in the procedure. The end points were ~3.9 ng/ml for Ara-A and ~625 ng/ml for Ara-Hx. Plasma alone or plasma with ADAI had no antiviral activity. Eluted areas without arabinosides from TLC plates showed no nonspecific toxicity or antiviral activity in the assay. Unknown samples can be assayed in parallel with these standards, and the end points can then be determined as the last well showing 0 to 25%

CPE. A simple calculation based on twofold dilutions is then made: $2^n \times Y =$ plasma concentration of the unknown, where Y equals the concentration in end point wells of the standards and n equals the number of wells with 0 to 25% CPE. Therefore, $2^n \times 3.9$ ng/ml = concentration of Ara-A in the unknown sample, and, likewise, $2^n \times 625$ ng/ml = concentration of Ara-Hx in the unknown sample.

A final experiment was designed to measure antiviral activity of Ara-A (at concentrations of 2,000 to 32 ng/ml) in MM, deproteinized plasma, and plasma that had been separated by TLC, to evaluate recovery over a wide dilutional gradient under conditions simulating tests of clinical specimens (Table 3). Samples were prepared as in previous experiments, with identical drug concentrations in the first wells with corresponding letters (i.e., A, B, C, D). Results of the bioassay were virtually identical regardless of preparation or starting concentration, again demonstrating accuracy of the test methodology.

DISCUSSION

Previous methods for quantitation of Ara-A and Ara-Hx in plasma and biological fluids used in our lab and others (3) have utilized low- and

TABLE 2. Recovery of antiviral activity from silica gel TLC plates and deproteinized plasma

Ara-A sample	CPE score ^a at drug concn (ng/ml):												
	1,000 ^b	500	250	125	62.5	31.2	15.6	7.8	3.9 ^c	1.9	0.97	VC ^d	CC ^e
Ara-A eluates of TLC gel	0	0	0	0	0	0	0	0	2+/1+	4+	ND ^f	4+	0
Deproteinized Ara-A plasma	ND	0	0	0	0	0	0	0	1+	4+	4+	4+	0
Ara-A in MM	ND	0	0	0	0	0	0	0	1+	4+	4+	4+	0
Deproteinized plasma alone	ND	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	0

Ara-Hx sample	CPE score ^a at drug concn:												
	10,000	5,000 ^b	2,500	1,250	625 ^c	312	156	78	39	19	9.7	VC ^d	CC ^e
Ara-Hx eluates of TLC gel	ND	0	0	0	1+	3+	4+	4+	4+	4+	4+	4+	0
Ara-Hx in MM	ND	0	0	0	1+	3+	4+	4+	4+	4+	4+	4+	0

^a CPE score: 0, confluent culture; 1+, 25% CPE; 2+, 50% CPE; 3+, 75% CPE; 4+, 100% CPE.^b Calculated drug concentration in first well after TLC, dilution, concentration, lyophilization, and reconstitution.^c End point concentration of drug in last well showing 0 to 1+ CPE.^d VC, Virus control.^e CC, Cell control.^f ND, Not done.

TABLE 3. Comparison of recovery of antiviral activity of Ara-A in various starting concentrations in MM, in deproteinized plasma, and after TLC procedure

Ara-A prepn	Expt	Starting concn (2n) ^a	CPE score ^b at dilution:											VC ^c	CC ^d
			n/1	n/2	n/4	n/8	n/16	n/32	n/64	n/128	n/256	n/512			
Ara-A deproteinized plasma	A	2,000	0	0	0	0	0	0	0	0 ^e	2+	4+	4+	0	
			0	0	0	0	0	0	0	0	3+	4+	4+	0	
	B	500	0	0	0	0	0	0	3+	4+	4+	4+	4+	0	
			0	0	0	0	0	1	4+	4+	4+	4+	4+	0	
	C	125	0	0	0	1+	2+	4+	4+	4+	4+	4+	4+	0	
			0	0	0	0	2+	4+	4+	4+	4+	4+	4+	0	
	D	31.2	0	0	2+	4+	4+	4+	4+	4+	4+	4+	4+	0	
			0	0	2+	4+	4+	4+	4+	4+	4+	4+	4+	0	
Ara-A in MM	A	2,000	0	0	0	0	0	0	1+	1+ ^e	3+	4+	4+	0	
			0	0	0	0	0	0	0	1+	2+	4+	4+	0	
	B	500	0	0	0	0	0	1+	2+	4+	4+	4+	4+	0	
			0	0	0	0	0	0	2+	4+	4+	4+	4+	0	
	C	125	0	0	0	0	2+	4+	4+	4+	4+	4+	4+	0	
			0	0	0	0	2+	4+	4+	4+	4+	4+	4+	0	
	D	31.2	0	0	2+	4+	4+	4+	4+	4+	4+	4+	4+	0	
			0	0	3+	4+	4+	4+	4+	4+	4+	4+	4+	0	
Ara-A TLC eluates	A	2,000	0	0	0	0	0	0	1+	1+ ^e	3+	4+	4+	0	
			0	0	0	0	0	0	0	1+	2+	4+	4+	0	
	B	500	0	0	0	0	0	1+	2+	4+	4+	4+	4+	0	
			0	0	0	0	0	0	2+	4+	4+	4+	4+	0	
	C	125	0	0	0	0	2+	4+	4+	4+	4+	4+	4+	0	
			0	0	0	0	2+	4+	4+	4+	4+	4+	4+	0	
	D	31.2	0	0	2+	4+	4+	4+	4+	4+	4+	4+	4+	0	
			0	0	3+	4+	4+	4+	4+	4+	4+	4+	4+	0	

^a 2n, Calculated starting concentration of Ara-A (ng/ml) prior to doubling dilutions.^b CPE score: 0, confluent culture; 1+, 25% CPE; 2+, 50% CPE; 3+, 75% CPE; 4+, 100% CPE.^c VC, Virus control.^d CC, Cell control.^e End points defined as the concentration of drug in last well showing 0 to 1+ CPE.

high-pressure liquid chromatography UV absorbance. The practical limits of detection for Ara-A and Ara-Hx by these methods are 200 ng/ml and 100 ng/ml, respectively, using two different analytical systems. A combination of these systems can be used in a research situation to increase the sensitivity of Ara-A detection to ≥ 10 ng/ml, at great expense of money and time with a low volume yield. Using such a double-column system, analysis by high-pressure liquid chromatography of 99 plasma specimens from patients on present Ara-A dosage schedules (5 to 20 mg/kg per 12 h) revealed no detectable Ara-A in 75. Twenty specimens contained 22 to 350 ng of Ara-A per ml, with the majority of values below 100 ng/ml. Ara-Hx, the deaminated metabolite of Ara-A produced in vivo, was detected in patients' plasma in concentrations from 1 to 10.5 μ g/ml.

In this report we have described a simple, sensitive biological assay for quantitation of the arabinosides Ara-A and Ara-Hx in human plasma, based upon inhibition of VV CPE in an adenosine deaminase-free cell culture system.

Combining prior separation of arabinosides by TLC with the bioassay should allow convenient, inexpensive pharmacological analysis of Ara-A and its active metabolites in body fluids.

We have previously reported an in vitro system for determination of the susceptibility of DNA viruses, including VV, to Ara-A, where deamination is prevented by the use of ADAI in human diploid fibroblasts and in monkey kidney cells (LLCM K₂) (1, 2). We employed an adenosine deaminase-deficient line of horse fibroblasts (CCL57) which is permissive for cytopathic infection by herpes simplex virus types 1 and 2 and VV. Prior experiments in our laboratory measuring the metabolism of Ara-A by these cells showed a half-life of greater than 5 days as compared to 13 h in human diploid fibroblasts. These experiments showed that ADAI in this system completely inhibited the deaminase produced by these cells and present in the 2% fetal bovine serum of the MM for up to 7 days. In this cell line, plaque formation by VV showed the greatest susceptibility to Ara-A with 50% inhibition of VV plaques by 2.5 ng/ml, or 120 ng of Ara-Hx per ml. To simplify the procedure and make it more clinically applicable by allowing the use of small concentrated plasma samples and multiple assays to be run simultaneously, we adapted the CCL57 VV system to a microassay using CPE as a measure of viral infectivity. In this system 75 to 100% viral CPE was prevented by approximately 4 ng of Ara-A or 625 ng of Ara-Hx. Unknown plasma samples can be run in parallel with these known stan-

dards, end points can be determined, and calculations of plasma arabinoside concentrations may be made. Our experiments using this assay have been reproducible and show good recovery of antiviral activity throughout. This bioassay method can be utilized in several ways. Individual quantitation of arabinosides in plasma can be accomplished by prior separation of these nucleosides using TLC. Total antiviral activity in plasma can be determined without prior separation.

The bioassay method described has the advantage of a ~ 50 -fold greater sensitivity for detection of plasma Ara-A than high-pressure liquid chromatography methods. It is simple and does not require expensive equipment and therefore is more easily adaptable for routine use. Only small plasma samples are required (~ 0.2 ml), and many specimens can be assayed simultaneously. As opposed to the high-pressure liquid chromatography assay, the bioassay clearly determines that TLC-separated nucleosides have antiviral activity.

Determinations of concentrations of Ara-A nucleosides in plasma and other biological fluids and correlation with clinical outcome are important in understanding the metabolism and antiviral activity of Ara-A in vivo. We have recently used this assay successfully to measure antiviral activity in plasma of patients receiving Ara-A or Ara-AMP; this will be the subject of a future report. We predict that this assay will be a useful tool in evaluating the pharmacology and establishing doses of these antiviral agents now in clinical trials.

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