ELSEVIER

Journal of Chromatography A, 692 (1995) 59–66

JOURNAL OF CHROMATOGRAPHY A

Alkylsulphonic acid ion pairing with radial compression columns for determining plasma or cerebrospinal fluid 1-β-D-arabinofuranosylcytosine in pediatric pharmacokinetic analysis

M.L. Stout*, Y. Ravindranath

Departments of Pediatrics and Pharmacology, Wayne State University, and Divisions of Clinical Pharmacology/Toxicology and Hematology/Oncology, Children's Hospital of Michigan, 3901 Beaubien Boulevard, Detroit, MI 48201, USA

Abstract

In order to accurately and precisely measure plasma and cerebrospinal fluid (CSF) 1-β-D-arabinofuranosylcytosine (Ara-C) in pediatric samples with adequate sensitivity and without interference, we have developed a reversed-phase ion-pairing technique utilizing the free amino group of the pyrimidine ring of Ara-C. Optimum resolution and separation was achieved utilizing a 4 μm C_{18} radial compression column. Ara-C and the internal standard 8-bromo-cyclic-AMP eluted at 6.5 and 4.6 min, respectively, with complete resolution. The minimum detectable amount is 2.5 pmol in a 50-μl volume. The assay was linear in both plasma and CSF. Intra- and inter-day assay precision were less than 4% and 9%, respectively, for plasma with similar results obtained for CSF. Neither endogenous compounds nor commonly co-administered drugs interfere. Validity for our method was supported by the successful assay of over 400 pediatric plasma and 50 CSF samples for pharmacokinetic analysis. The method offers accuracy, precision, sensitivity and efficiency for plasma or CSF Ara-C determination.

1. Introduction

Several high-performance liquid chromatographic (HPLC) methods have been developed within the last fifteen years to determine both plasma and cerebrospinal fluid (CSF) levels of the antileukemic drug 1-β-D-arabinofuranosylcytosine (Ara-C). Many were developed for adult studies that utilize short-duration (1–3 h) intravenous (i.v.) high-dose infusions. The ensuing infusion, and post-infusion levels, remain elevated well above the sensitivity limit of standard HPLC detection equipment. Also, adequate plasma and CSF samples can be obtained in adults so that sensitivity limits for most HPLC methods are not a serious concern. On the other hand, analysis of plasma and CSF Ara-C in pediatric patients presents special problems, especially when patients are treated on long-duration (2–4 days) i.v. high-dose regimens [1,2]. While adequate concentrations are present within plasma during infusion, these levels may be 5–50 times lower than that seen in the short-duration i.v. high-dose adult regimens [3–9]. In addition, post-infusion determinations are required for up to 4 h for proper calculation of biexponential elimination kinetics [10]. Many HPLC methods available today lack the required sensitivity due to several factors: inadequate extraction recovery techniques [11–14], poor separation of drug from endogenous plasma constituents or from drug metabolites and poor

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)00994-5

baseline resolution and detection at low concentrations [11,12,15–21]. Several methods have been developed and tested on only pure aqueous or buffered samples rather than plasma or CSF extracts [22,23]. A large proportion of these methods extract the drug from plasma but do not utilize an internal standard [4,11,14,15,17–19,21,24], or directly inject plasma or plasma ultrafiltrates, which may shorten column life [3,13,20,25]. Many methods have relatively long chromatographic analysis times (20–50 min) or utilize multiple columns, extractions or gradient systems [4,7,11,12,15,20,21,24].

While these problems are easily addressed and solved in assay systems utilizing adult samples, the same cannot be said for pediatric samples. Because of small sample sizes encountered (≤ 200 μl plasma), increased sensitivity needs due to low post-infusion concentrations, interferences due to combination or adjuvant drug therapy and/or tumor lysis products, and need for rapid analysis to avoid concentrations associated with toxicity, we have developed a reversed-phase ion-pairing method that utilizes the free amino group of the pyrimidine ring of Ara-C and the ion-pairing alkylsulphonic acid, 1-pentanesulphonic acid (PSA). Using our method, and a Radial-Pak column, the required sensitivity, specificity and speed of analysis can be obtained for accurate and precise analysis of this drug in pediatric infusion and post-infusion plasma and CSF.

2. Experimental

2.1. Chemicals

Ara-C (Sigma, St. Louis, MO, USA) was utilized as the free base. Tetrahydrouridine (THU) was purchased from Calbiochem (San Diego, CA, USA). Uracil arabinoside (Ara-U), uracil, uridine, cytosine, cytidine, adenosine, adenine and 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) were all purchased from Sigma. A variety of other drugs were tested for possible interference with Ara-C (see Table 3). Of these compounds only ketorolac (Syntex Labs., Palo Alto, CA, USA) and metoclopramide (A.H. Robins Co., Richmond, VA, USA) were obtained directly from the pharmaceutical manufacturer. All other drug compounds were obtained from Sigma. Potassium phosphate buffer, sodium hydroxide and trichloroacetic acid (TCA) were purchased from Mallinckrodt (Paris, KY, USA). All ion-pairing agents and tri-N-octylamine were obtained from Sigma. Hydrochloric acid was obtained from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile, methanol, dichloromethane and 1,1,2-trichlorotrifluoroethane were purchased from Burdick & Jackson (Muskegon, MI, USA).

2.2. Chromatography

The HPLC system consisted of a Beckman (Berkeley, CA, USA) Model 110B single-piston isocratic solvent-delivery module, a Rheodyne Model 7125 injection valve equipped with a 100-μl loop, a 4-μm C_{18} Nova-Pak (150 × 3.9 mm I.D.) radial compression column and a Nova-Pak C_{18} Guard-Pak precolumn (Waters Millipore, Milford, MA, USA). Radial compression was achieved by a Waters Z module. In order to reduce band broadening and decrease dead space, microbore tubing (0.18–0.23 mm I.D.) was used throughout the system. A Waters Model 441 fixed-wavelength UV–Vis detector (280 nm) was utilized for all detections. All HPLC assays were carried out at ambient temperature with a flow-rate of 1.8 ml/min. Mobile phase consisted of 10 m*M* potassium phosphate buffer and 27.5 m*M* PSA as ion-pairing agent with acetonitrile and methanol (96:3:1, v/v/v). The pH was adjusted to 4.00 with KOH. Both peak area and height were measured using a LCI-100 laboratory computing integrator (Perkin-Elmer, Norwalk, CT, USA).

2.3. Assay procedure

In order to accurately determine extremely low levels of Ara-C in plasma, all background noise should be reduced to a minimum so as to obtain the highest possible signal-to-noise ratio. Therefore, both dichloromethane, and 1,1,2-tri-

chlorotrifluoroethane solvents used in the extraction procedure were further purified of possible UV absorbing impurities by acid/base washes. Using a separation funnel, equal volumes of the solvent and 1 *M* NaOH were shaken together, allowed to separate, and the base removed. This was followed by a wash in distilled deionized water and by a wash in an equal volume of 1 *M* HCl. After removal of the acid, two further washes in distilled deionized water removed all traces of acid. Further purification of these organic solvents was accomplished by adsorption with activated charcoal followed by filtration to remove all charcoal fines. The NaOH and HCl solutions were made in pure distilled deionized water, slurried overnight in activated charcoal, and filtered as above. All organic solvents were stored at −20°C. Enough purified solvent can be prepared at one time for several hundred assays. In order to monitor the purification process, blank plasma extractions were carried out and assayed before and after these procedures. Baseline noise was reduced significantly so that the signal-to-noise ratio increased at least twofold. This allowed detection of Ara-C in plasma at the 10^{-8} *M* level.

Blood (ca. 1 ml) was collected in heparinized tubes containing THU, a deaminase inhibitor (final concentration 100 μM). Whole blood was centrifuged in a microfuge (14 000 *g*) for 5 min at 0–4°C and plasma was collected and stored at −70°C until analysis. CSF (1–2 ml) was also collected in the presence of THU, centrifuged, and stored at −70°C until analysis.

Extraction and centrifugation were performed at 0–4°C at all times. After the addition of 10 μl of the I.S., 8-Br-cAMP (5 μM final concentration) to 190 μl of Ara-C containing plasma, 200 μl of cold 0.6 *M* TCA was added dropwise with continual vortex mixing. Samples were left on ice for 10 min with occasional vortex mixing throughout that period. Precipitated plasma or CSF samples were microfuged 5 min at 14 000 *g*, supernatant was removed to a new 1.5-ml microfuge tube and the volume recorded. To the acidified supernatant was added 1.1 volumes of 0.5 *M* tri-N-octylamine in 1,1,2-trichlorotrifluoroethane and the mixture vortex mixed vigorously (30–45 s) several times and microfuged for 5 min. The lower organic phase was removed and the remaining aqueous extract was again microfuged for three minutes. The aqueous phase was carefully removed to a new 1.5-ml microfuge tube and approximately 1.1 ml of dichloromethane was added, the mixture vortexed for 1–2 min, centrifuged as above and the lower organic phase carefully removed. The remaining aqueous phase was again microfuged for 2–3 min to remove all traces of organic solvent. The final aqueous extract was removed to a new 1.5-ml microfuge tube and dried at 50°C under nitrogen atmosphere. Aqueous extracts were stored at −70°C until chromatography.

At analysis, all aqueous extracts were reconstituted in 100 μl of mobile phase and 50 μl were injected. Plasma standard curves were prepared from healthy pooled plasma of volunteers. Standards ranged from 50 to 0 μM. CSF standard curves were prepared in artificial CSF (Elliotts B solution) and ranged in concentration from 50 to 0 μM. After extraction and analysis, data were analyzed by linear regression methods.

3. Results and discussion

In order to determine the optimal ion-pairing agent and pH for separation, 1-hexanesulphonic acid, 1-heptanesulphonic acid and 1-octanesulphonic acid were also tested in addition to PSA. All tests were performed at 5 μM Ara-C in extracted pooled volunteer plasma. Use of these ion-pairing agents at constant pH (5.0) caused retention times for Ara-C and the internal standard to increase greatly ($\geq$10 min). Reduction of the concentration of these agents reduced retention times but with loss of resolution of drug from normal plasma constituents. The PSA ion-pairing agent gave optimal separation of drug and internal standard from normal plasma constituents within a reasonable analysis time ($\leq$8.0 min). The pH was altered between 3.0 and 7.0 for the PSA ion-pairing agent and optimal separation and analysis time was obtained at pH 4.0. Since this ion-pairing agent is better than 97% ionized (anion) and the amino

function of Ara-C is essentially 100% charged (cation) at pH 4.0, it is not surprising that optimal ion-pairing chromatography is attained at this pH. Decrease in pH would result in reduction of charged ion-pairing anion while increase in pH would reduce the effective cationic drug species. In either case, alterations in pH from 4.0 ultimately reduced chromatographic resolution. The other ion-pairing agents were also examined at pH 4.0 but the same relative changes seen at pH 5.0 were retained at this new pH.

Initial chromatograms of blank plasma revealed a few unknown plasma constituents that were highly retained on this column. Washing the aqueous extracts with dichloromethane removed these late-eluting plasma constituents from all samples. Although these late-eluting constituents were not found in blank CSF, these samples were also treated with dichloromethane. Chromatography and recovery of pure standards in Elliotts B solution or with patient CSF samples was not affected by dichloromethane washes.

Fig. 1a illustrates a typical chromatogram of blank plasma from a pediatric patient prior to chemotherapy with Ara-C. As can be seen there is a resolvable baseline at the retention time for Ara-C (ca. 6.5 min). The internal standard has a retention time of 4.8 min. Overall chromatographic time is under 10 min. Fig. 1b is a chromatogram of the same patient receiving 100 $mg/m^2/h$ for 48 h. This sample was taken before the infusion was terminated and represents a plasma steady state level (hour 47). As seen from this figure both Ara-C and the internal standard resolve from one another and no post-peak shoulders or severe tailing occurs. Peak shape is uniformly symmetrical. Fig. 1c repre-

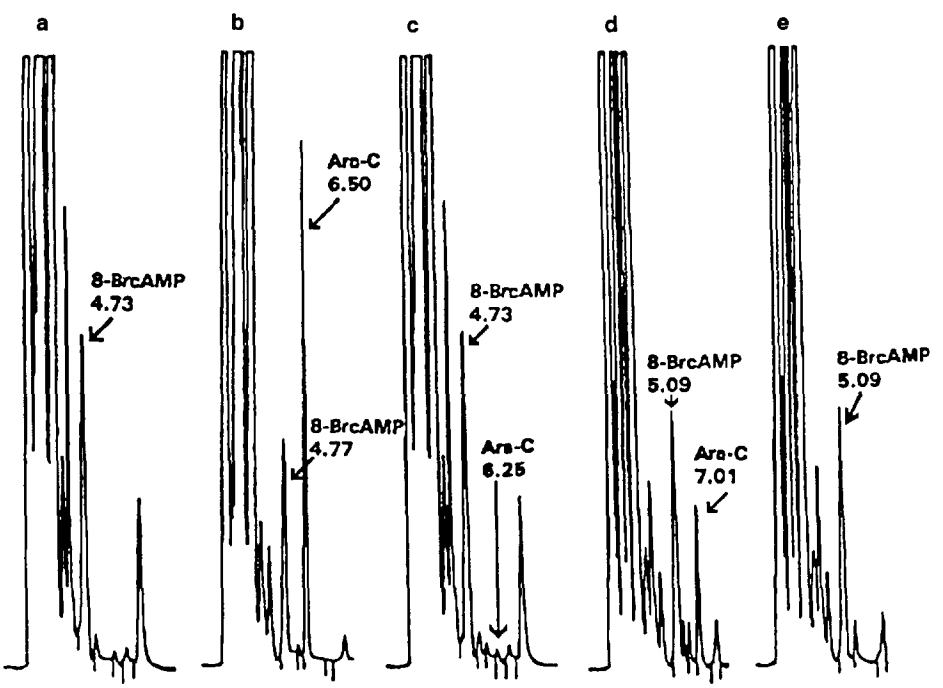

Fig. 1. HPLC of Ara-C in plasma and CSF of a pediatric patient receiving 100 $mg/m^2/h$ Ara-C for two days. (a) Blank preinjection plasma; (b) steady state plasma level (sampled at hour 47 of infusion); (c) 4-h post-infusion Ara-C plasma level; (d) steady state Ara-C CSF level (sampled at hour 47 of infusion); (e) sample of drug-free CSF (taken from diagnostic CSF). This sample is not the same as that seen in (d). Retention times for Ara-C in plasma were 6.25–6.50 min and in CSF 7.01 min. Internal standard retention times were approximately 4.73 min in plasma and 5.09 min in CSF. In order to compensate for an increase in ambient temperature during this assay for CSF, and to keep all pertinent peaks within their window tolerances, flow-rates were decreased to 1.7 ml/min. This caused a slight increase in the retention times for internal standard and Ara-C.

sents a 4-h post-infusion plasma determination. The peak representing Ara-C is easily quantitated even though plasma levels have fallen over 10-fold. Patients also had CSF levels drawn simultaneously with their steady state plasma levels. As seen in Fig. 1d, Ara-C appears in easily detected concentrations in the CSF. Fig. 1e is a chromatogram of CSF in which Ara-C is absent (blank). These results indicate that high-dose long-duration i.v. infusions in children attain detectable CSF levels, and Ara-C can cross the blood brain barrier from plasma. There appear to be no interfering peaks for either Ara-C or the internal standard within CSF. The major plasma metabolite for Ara-C, Ara-U, coeluted within the solvent front and did not interfere with the chromatography of the parent drug or I.S.

The following analytical procedures were followed to validate our chromatographic method for both plasma and CSF. Standard curves for both plasma and CSF were linear ($r^2 \geq 0.998$) and intersected true zero within 1% (relative to the full scale curve). The regressional equation for plasma [$y = 1.0043(\pm 0.0068$ S.E.M.)$x + 0.0386(\pm 0.1297)$] indicated that analytically HPLC determined concentrations for these standards varied in a direct linear 1:1 proportion with respect to the actual prepared concentrations. Also, the validity of these curves can be further verified by the accuracy of blind samples prepared in an independent laboratory. Plasma samples were prepared in a blind fashion in concentrations ranging from 50 to 0.0 μM. The maximum relative percent error for these determinations never exceeded 5.5% and the value determined for a blank sample was zero. (Table 1). Minimum detectable amount in plasma is 0.1 μM and in CSF, 0.08 μM. Standard curves for both plasma and CSF are linear over at least a 2.5 log range. The regressional equation for CSF was similar to that seen for plasma [$y = 0.968(\pm 0.00965)x + 0.1856(\pm 0.1834)$]. Accuracy results for CSF in artificial CSF (Elliotts B solution) were not performed since it was felt this media would have little effect on drug determination and, results would vary minimally from a repeat of the standard curve.

Table 1
Ara-C assay validation data for plasma and CSF analysis

Concentration (μM)	R.S.D. (%)	
	Plasma	CSF
Intra-day		
10	1.33	–
2	–	3.21
1	4.16	5.30
Inter-day		
10	3.69	–
2	–	6.53
1	8.71	7.74

Accuracy for plasma[a]

Known concentration (μM)	Analytically determined (μM)	Error (%)
0.0	0.0	0.0
9.0	8.82	2.00
16.0	16.17	1.06
25.0	23.66	5.36
40.0	39.38	1.55

[a] Samples were prepared in a blind fashion in the laboratory of Dr. G. Dombi, Childrens Hospital of Michigan.

At least six to eight samples at both low (1 μM plasma, 1.0 μM CSF) and high (10 μM plasma, 2.0 μM CSF) concentrations were analyzed during one experiment (intra-day precision assay) and at least one sample was analyzed at each concentration over at least six to eight different assay days (inter-day precision assay). Inter- and intra-day precisions were determined from quantitative data of both peak area and peak height. Table 1 indicates that both intra- and inter-day assay relative standard deviations (R.S.D.s) for plasma at each concentration were excellent. At lower concentrations peak height proved to be the better measure in determining the precision of this method. This may be related to either peak tailing or baseline noise during determination. The intra- and inter-day assay R.S.D.s for artificial CSF (Elliotts B solution) were excellent regardless of the method of peak

quantitation utilized. It must be remembered that this solution is not a true CSF since many constituents within actual CSF are not present (enzymes, carbohydrates, lipids, amino acids, neurotransmitters, certain ions, vitamins, etc.). This would undoubtedly contribute to an increase in the actual determined coefficients.

Recovery of sample was determined by spiking plasma or Elliotts B solution with a known amount of Ara-C, extracting and analyzing the sample repetitively, and comparing this to a pure aqueous sample of equivalent concentration that has not been extracted. Percent recovery is defined as the concentration ratio of extracted spiked plasma or CSF to pure aqueous sample × 100. As can be seen from Table 2, recovery of drug from pooled plasma or from artificial CSF was essentially quantitative at both low and high concentrations. Extraction from lipemic or hemolyzed patient samples did not alter extraction efficiency.

The radial compression columns used for our assay were superior in resolution and decreased overall chromatographic time by 2–3-fold from standard steel columns. Well over 600 assays could be performed on a single column without serious loss of resolution, peak symmetry, or increase in baseline noise.

A variety of other pure compounds were assayed to determine if interference occurs with either the internal standard or drug itself. Table 3 list many agents commonly co-administered with Ara-C during antileukemic therapy. Although many of these compounds possess a free amino group, a substituted amino group, or a ring containing hetero nitrogen, there was no interference produced. Interference was determined by assay of the pure compound in the presence of Ara-C and internal standard. If the retention time of the interfering compound was within ± 0.5 min of either the internal standard or Ara-C, the compound was spiked into plasma and extracted. Of all the compounds tested, only the metabolites of Ara-C and other nucleosides chromatographed close to the retention times of the standard and Ara-C. The antibiotics, antiemetics, antipyretics, sedative agents and other antineoplastics drugs did not interfere. These

Table 2
Ara-C assay validation data for plasma and CSF analysis

	Extraction efficiency (%)[a]
Plasma: 5 μM	96.20 (R.S.D. = 8.37%, $n = 6$)
Plasma: 1 μM	96.88 (R.S.D. = 6.56%, $n = 6$)
CSF: 2 μM	98.2 (R.S.D. = 2.43%, $n = 6$)
CSF: 1 μM	97.8 (R.S.D. = 4.56%, $n = 6$)

[a] Extraction efficiency was determined by comparing extracted "spiked" plasma and CSF samples against pure unextracted samples in water. The ratio of the HPLC-determined spiked plasma vs. water values × 100 equaled the percent extraction efficiency.

Table 3
Ara-C assay validation data for plasma and CSF analysis

Interference analysis	
Drugs	*Plasma drug metabolite*
Thorazine	Uracil arabinoside
Chlorpromazine	
Promazine	*Natural metabolite*
Metoclopramide	Cytosine
Diazepam	Uracil
Oxazepam	Adenosine
Chlordiazepoxide	Cytidine
Phenobarbital	Adenine
Diphenhydramine	Adenosine monophosphate
Cimetidine	Cyclic adenosine monophosphate
Codeine	Uridine
Ibuprofen	
Ceftazidime	
Chloramphenicol	
Penicillin	
Ampicillin	
Gentamycin	
Tobramycin	
Kanamycin	
Dexamethasone	
Acetaminophen	
Hydrocortisone	
Methotrexate	
Daunorubicin	
Cyclophosphamide	
Vinblastine	
Vincristine	
Etoposide	
Teniposide	
Actinomycin D	
Asparaginase	

compounds either were non-retained, or had excessively long retention times. Also, many of these agents had their maximum UV absorbance well above or below that of the optimal absorbance for Ara-C (280 nm) and could not be detected at clinical plasma levels. The various drug and natural metabolites mentioned in Table 3 were all spiked into plasma or artificial CSF, extracted and assayed in the presence of the internal standard and Ara-C. The major plasma drug metabolite of Ara-C, Ara-U, and cytidine, the naturally occurring 1-α isomer of Ara-C, did not interfere with our assay. Adenosine monophosphate, cyclic adenosine monophosphate (cAMP), adenine, uridine and adenosine did not interfere with the internal standard.

Comparison of our method with other HPLC methods analyzing this drug in similar populations and at similar dose and infusion durations, indicate equivalent or better precision than previously published assays. Table 4 lists both intra- and inter-day assay R.S.D.s for Ara-C in several different HPLC assay systems. Coefficients for these assays range from 2.0 to 15.2% (intra) and from 2.7 to 12.3% (inter) over a wide concentration range for plasma. Our method attained intra- and inter-day assay R.S.D.s for plasma ranging between 1.33 and 4.16% (intra) and between 3.69 and 8.71% (inter).

We have successfully used our method to analyze Ara-C steady state plasma and CSF levels and post-infusional elimination pharmacokinetics in pediatric patients receiving high-dose long-duration i.v. infusions. Well over 400 plasma and 50 CSF samples have been measured. Our results [27,28] correlate well in many respects with other HPLC methods used to determine Ara-C plasma pharmacokinetics. Ara-C steady state plasma, CSF, CSF to plasma ratios, and biexponential pharmacokinetic con-

Table 4
Ara-C HPLC plasma assays: precision comparisons

Ref.	Concentration range (μM)	Within R.S.D. (%)	Between R.S.D. (%)
[3]	0.2–492 (within)	≤ 5.0	
	0.82 and 4.92 (between)	–	9.3 and 7.0
[12]	8.2–410 (within)	6–12%	–
[22]	Unknown pure aqueous sample		1.0
[30][a]	0.001–0.025	≤ 10.0	5.2–13.0
[16]	82 (within)	2.0	4.3
	328 (between)	2.7	2.7
[25]	0.41–410 (within)	3.1–7.4	–
	0.41 (between)		5.6
[21]	82	5.2	6.8
	205	2.3	3.2
[17]	2	9.1	12.3
	8	15.2	12.0
Present paper	10	1.33	3.69
	1	4.16	8.71

All samples were prepared in plasma or serum.
[a] Radioimmunoassay for comparison.

stants that we have determined for pediatric long-duration high-dose infusions are all within reasonable values of the current literature for this drug [1,2,19,26,29]. This assay fulfilled all the necessary criteria of specificity, sensitivity, efficiency, accuracy and precision required to monitor this potent antineoplastic agent in pediatric plasma and CSF samples.

Acknowledgements

This work was funded by a grant from Leukemia Research Life Inc., Detroit, MI, USA. The authors gratefully acknowledge the assistance of Ms. Ginni Cosgrove in the preparation of this manuscript.

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