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DETERMINATION OF ^{3}H-LABELLED CYTOSINE ARABINOSIDE AND EIGHT METABOLITES IN CELL EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SCINTILLATION SPECTROMETRY

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SUMMARY

We have developed a method for the separation and quantitation of radiolabeled cytosine arabinoside and its eight metabolites in cell extracts by anion-exchange gradient high-performance liquid chromatography. Baseline separation of cytosine arabinoside and uracil arabinoside and their respective 5′-mono-, di- and triphosphates, as well as cytosine arabinoside diphosphocholine was obtained with the shortest interval between peaks being 3 min. This degree of separation was found to be essential for quantitation of ^{3}H-labeled metabolites by scintillation counting of 1-min fractions. Application of this procedure to the quantitation of [^{3}H] cytosine arabinoside and its metabolites from HL60 human leukemia cells is demonstrated.

INTRODUCTION[a]

Cytosine arabinoside (ara-C), an isomer of cytidine (cytosine riboside) with the reversed configuration of the 2′-hydroxyl group (Fig. 1), is one of the most

[a]Abbreviations used are: ara-C, cytosine arabinoside, 1-β-D-arabinofuranosylcytosine; ara-CMP, ara-C 5′-monophosphate; ara-CDP, ara-C 5′-diphosphate; ara CTP, ara-C 5′-triphosphate; ara-CDP-choline, ara-C diphosphocholine; ara-U, uracil arabinoside; ara-UMP, ara-U 5′-monophosphate; ara-UDP, ara-U 5′-diphosphate; ara-UTP, ara-U 5′-triphosphate; CDP, cytidine 5′-diphosphate; CTP, cytidine 5′-triphosphate; CDP-choline, cytidine diphosphocholine; UMP, uridine 5′-monophosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; dUTP, deoxyuridine 5′-triphosphate.

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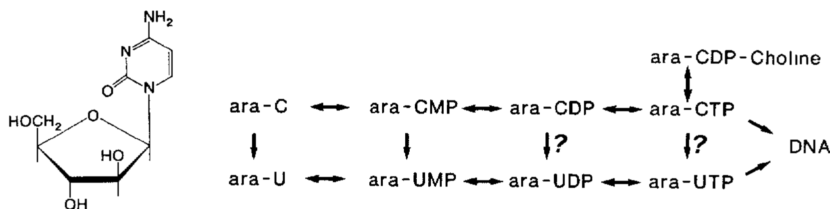

Fig. 1. Structure of ara-C and its reaction pathways.

effective drugs in the treatment of acute leukemia [1–3]. Ara-C is anabolized to the triphosphate, ara-CTP, by the pathway for salvage for deoxycytidine. Ara-CTP is a substrate and an inhibitor of DNA polymerase, and its incorporation into DNA results in inhibition of DNA synthesis [4,5]. The level and half-life of ara-CTP in leukemia cells has been shown to correlate with their sensitivity to ara-C [6], but other ara-C metabolites may also have important effects. Ara-CDP-choline, an analogue of CDP-choline, may alter phospholipid metabolism [7] and lead to changes in membrane structure [8]. Resistance to ara-C correlates with cellular ara-C deaminase activity [9,10] and other ara-C metabolites may also be deaminated [11,12] to ara-U derivatives. These deaminated metabolites, which until recently have been considered only as detoxification products, may play an important role in the cellular pharmacology of ara-C. Ara-U treatment causes cytostasis, and pretreatment of cells with ara-U enhances their sensitivity to ara-C [13].

Satisfactory methods exist for the direct determination of ara-CTP in cells exposed to high levels of ara-C by high-performance liquid chromatography (HPLC) with UV detection [14]. The levels of intracellular ara-C and other metabolites are much lower, and these compounds have been more difficult to separate from the naturally occurring ribo- and deoxyribonucleotides in cell extracts. Procedures have been described for the determination of certain radiolabeled metabolites after incubation of cells with [^{3}H]ara-C [13,15]; however, improved methods are necessary for analysis of quantitatively minor derivatives of ara-C, particularly the deaminated derivatives of [^{3}H]ara-C. This report describes a method for the simultaneous separation and quantification of [^{3}H]ara-C and all eight of its metabolites.

EXPERIMENTAL

Chemicals

HPLC-grade KH_2PO_4 and orthophosphoric acid were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). HPLC-grade acetonitrile was from Burdick and Jackson (Muskegon, MO, U.S.A.). Nucleotide standards were purchased from Sigma (St. Louis, MO, U.S.A.). Tritiated ara-C (specific activity 1000 GBq/mmol) was from Amersham (Arlington Heights, IL, U.S.A.).

Incubation with [^{3}H]ara-C and preparation of cell extracts

HL60 cells were cultured in RPMI-1640 media with 10% (v/v) fetal bovine serum and harvested in log phase. The cells were resuspended in the same media at $2 \cdot 10^6$–$3 \cdot 10^6$ cells per ml and exposed to 0.4–1.0 μM [^{3}H]ara-C (74–370 KBq/ml) for 3 h. Cells were lysed and ara-C metabolites were extracted essentially as previously described by White and co-workers [16,17]. Samples of the cell suspension (1.0 ml) were transferred to 1.5-ml centrifuge tubes containing 100 μl of 10% (w/v) trichloroacetic acid overlaid with 300 μl silicone oil (d=1.03), then centrifuged at 9000 g for 30 s to separate the cells from the radiolabel. After aspiration of the media and oil layers, the trichloroacetic acid was removed from the cell lysate by the method of Khym [18]. Briefly, the lysate was transferred to a 400-μl microcentrifuge tube and vigorously mixed on a vortex with 150 μl of 0.5 M tri-n-octylamine in freon (1,1,2-trichlorotrifluoroethane). The trichloroacetic acid enters the dense organic phase leaving a neutral aqueous extract above. Although nucleoside 5′-triphosphates are somewhat unstable in acid, [^{3}H]ara-C and all of its metabolites are stable at $-20\,^{\circ}$C after removal of the trichloroacetic acid. A 50-μl aliquot of the aqueous layer plus 5 μl of nucleotide standards (0.1–0.5 μmol each of ara-U, ara-C, ara-CMP, CDP-choline, ara-UMP, CDP, UDP, ara-CTP and dUTP) were injected into the HPLC system.

HPLC analysis

Analysis of ara-C metabolites was performed on a Varian Model 5000 liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.). The column was a Partisil SAX anion-exchange column (10 μm particle size, 25 cm$\times$4.6 mm I.D., Whatman, Clifton, NJ, U.S.A.). The system included a guard column packed with pellicular anion-exchange silica (Whatman) and a solvent conditioning pre-column, located in-line before the injector, packed with pre-column gel (Whatman). Effluent was monitored with a Hewlett-Packard 1040-A HPLC detection system (Hewlett-Packard, Corvallis, OR, U.S.A.) set to monitor 254 nm.

The mobile phase consisted of three buffers: buffer A: 2 mM KH_2PO_4, pH 2.85; buffer B: 500 mM KH_2PO_4, pH 3.40; buffer C: acetonitrile–2 mM KH_2PO_4, pH 2.85 (80:20, v/v). The flow-rate was 1 ml/min; the temperature was 40$\,^{\circ}$C. The gradient program is described in Table I.

Fractions were collected at 1-min intervals. A 4-ml volume of AQS scintillation fluid (Fisher) was added to each 1-ml fraction. Radioactivity was determined on a Beckman LS 1800 liquid scintillation counter with automatic quench correction (Beckman Instruments, Columbia, MD, U.S.A.).

The column was treated with 30 ml of 0.5 M orthophosphoric acid after every thirty analyses of cell samples to maintain column integrity. When unaccept-

TABLE I

HPLC MOBILE PHASE GRADIENT PROGRAM

The mobile phase consisted of three buffers: buffer A, 2 m*M* KH_2PO_4, pH 2.85; buffer B, 500 m*M* KH_2PO_4, pH 3.40; buffer C, acetonitrile–2 m*M* KH_2PO_4, pH 2.85 (80:20, v/v). The flow-rate was 1 ml/min. The temperature was 40°C.

Step	Time (min)	%A	%B	%C
1	0	0	0	100
2	5	100	0	0
3	25	100	0	0
4	50	0	100	0
5	60	0	100	0
6	70	100	0	0

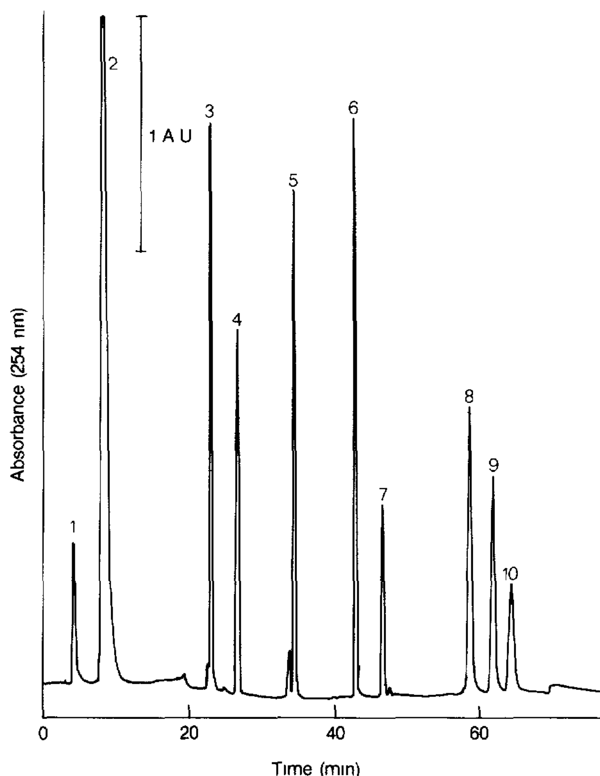

Fig. 2. HPLC separation of a mixture (20 μl) of nucleotide standards (0.5 μmol of each). Peaks: 1 = ara-U; 2 = ara-C; 3 = ara-CMP; 4 = CDP-choline; 5 = UMP; 6 = CDP; 7 = UDP; 8 = CTP; 9 = ara-CTP; 10 = dUTP.

able baseline drift occurred, 100 μl of 0.1 M Na_2-EDTA was injected followed by 40 ml of buffer B to restore the column.

RESULTS AND DISCUSSION

A mixture of ten standards (0.1–0.5 μmol) was completely separated by our method as shown in Fig. 2. Since ara-CDP, ara-CDP-choline, ara-UMP, ara-UDP and ara-UTP standards were not commercially available, we used the corresponding cytosine and uracil nucleotides to locate and identify the radiolabeled peaks. The ribonucleotides, which have the same base and charge as the arabinosyl analogues, eluted with the radiolabeled peaks, with the exception of CTP which eluted slightly before ara-CTP.

The retention times of the standards are listed in Table II. The smallest interval between any two peaks was 3.5 min. This separation was essential in order to obtain baseline separation between ^{3}H-labeled metabolites when 1-min fractions were collected.

A representative assay of ara-C metabolites for the HL60 cell line is shown in Fig. 3. HL60 cells ($2 \cdot 10^6$ cells per ml) were treated with 0.4 μM ara-C and 370 KBq/ml [^{3}H]ara-C for 3 h. A 50-μl volume of cell extract plus 5 μl of standard were injected into the system. The elution of the standards is shown in Fig. 3A; Fig. 3B is a plot of the radioactivity found in each fraction collected. Ara-C and eight ara-C metabolites were completely separated; the radioactive peaks corresponded well with the retention times of their equivalent standards (Table II). In the HL60 cell line, ara-C and ara-C nucleotides made up 85% of the total; and ara-U and ara-U nucleotides comprised 15% (Table III). With these experimental conditions the smallest peak, representing ara-UDP, con-

TABLE II

RETENTION TIMES OF ARA-C, ARA-C METABOLITES AND REPRESENTATIVE NUCLEOTIDES

Compound	Retention time (min)
Ara-U	4.5
Ara-C	8.5
Ara-CMP	23.8
CDP-choline	27.3
UMP	34.8
CDP	43.3
UDP	47.1
CTP	59.1
Ara-CTP	62.4
dUTP	65.1

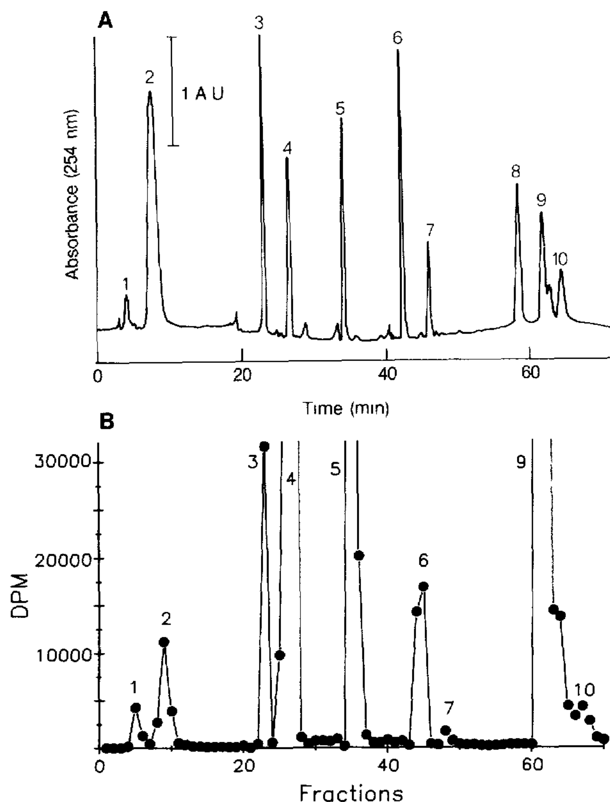

Fig. 3. HPLC analysis of radiolabeled metabolites of ara-C. Logarithmic phase HL60 cells were incubated for 3 h at $2 \cdot 10^6$ cells per ml in medium containing 0.4 μM [^{3}H]ara-C (1000 KBq/pmol), then the cells were centrifuged through a layer of oil into 100 μl of 10% (v/v) trichloroacetic acid. After extraction of the acid as described in the Experimental section 50 μl of extract, with 5 μl of mixed standards, were injected into the chromatograph. Seventy 1-ml fractions were collected and counted for radioactivity. (A) UV profile; peaks. 1=ara-U; 2=ara-C; 3=ara-CMP; 4=CDP-choline; 5=UMP; 6=CDP; 7=UDP; 8=CTP; 9=ara-CTP; 10=dUTP. (B) Radioactivity profile; peaks: 1=ara-U; 2=ara-C; 3=ara-CMP; 4=ara-CDP-choline; 5=ara-UMP; 6=ara-CDP; 7=ara-UDP; 9=ara-CTP; 10=ara-UTP.

tained 3211 dpm (55 Bq). At the specific activity used here of 1000 Bq/fmol (60 000 dpm/fmol), this peak contained about 0.045 pmol of ara-UDP. Since a peak of about 300 dpm (5 Bq) could have easily been detected, the limit of sensitivity would be about 5 fmol. Thus, this assay could have been performed with a ten-fold lower cell density, sample volume or specific activity.

The concentration of acetonitrile in buffer C was critical for the separation of ara-C and ara-U. With no acetonitrile in buffer C (e.g. buffer A only), ara-C eluted before ara-U at pH 2.80–3.80 with a difference in retention time of 1 min or less (Fig. 4). We found that the retention time of ara-C was increased

TABLE III

METABOLISM OF [^{3}H]ARA-C IN HL60 CELLS

HL60 cells were incubated for 3 h at $2 \cdot 10^{6}$ cells per ml in medium containing 0.4 μM [^{3}H]ara-C (1000 KBq/pmol). A 1-ml volume of the cell suspension was layered over silicone oil and the cells were centrifuged into 100 μl of 10% (v/v) trichloroacetic acid as described in the Experimental section. After neutralization, 50 μl of the cell extract, equivalent to $1 \cdot 10^{6}$ cells, plus 5 μl of a mixture of nucleotide standards were injected onto the column. Seventy 1-ml fractions were collected and counted. Recovery of radiolabel from the column was 99%.

Compound	Peak No. (fraction)	dpm	pmol per 10^{6} cells	Percentage of total
Ara-U	5	6 247	0.07	0.40
Ara-C	9	18 721	0.21	1.20
Ara-CMP	24	32 945	0.38	2.12
Ara-CDP-CL	28	281 299	3.20	18.07
Ara-UMP	35	211 086	2.40	13.56
Ara-CDP	45	31 479	0.36	2.07
Ara-UDP	48	3 211	0.04	0.21
Ara-CTP	62	959 226	10.91	61.63
Ara-UTP	67	12 141	0.14	0.78
Total of ara-C derivatives			15.03	85.04
Total of ara-U derivatives			2.68	14.95
Total		1 556 355	17.71	100.00

with the addition of acetonitrile (Fig. 5); 80% (v/v) acetonitrile was optimal for our purposes and resulted in ara-C eluting 4 min after ara-U.

Separation of ara-CMP, CDP-choline and UMP was pH-dependent (Fig. 5). Increasing the pH of buffer A from 2.80 to 4.0 caused UMP elution to be delayed, but there was no effect on the elution of either ara-CMP or CDP-choline. Delaying the onset of buffer B would also increase the retention time interval between ara-CMP and UMP.

The method described here for the separation of ara-C and its eight known metabolites will be useful in future studies of the mechanism of action of this important antileukemia drug. Several published methods are available for a more rapid separation and determination of ara-CTP, the major metabolite. Although ara-CTP has been identified as the key metabolite of ara-C that is responsible for inhibition of DNA synthesis, the effects of other metabolites also contribute to the cellular pharmacology of ara-C. For example, ara-CDP-choline is likely to be the metabolite responsible for the effects of ara-C on phospholipid metabolism [7]. The major value of this new separation method will likely be in studies of the role of the deaminated products, ara-U, ara-UMP, ara-UDP and ara-UTP. Prior to this study, there has been no chromatographic method available for the separation and quantification of these de-

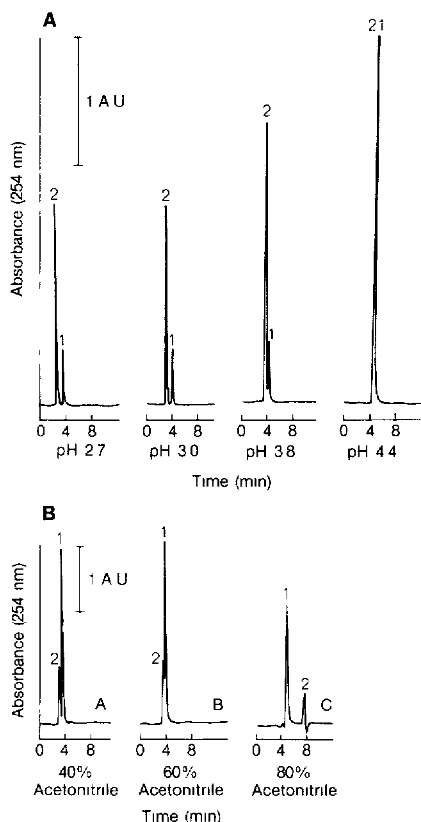

Fig. 4. Effect of buffer pH and acetonitrile concentration on the separation of ara-C and ara-U. A mixture of ara-C and ara-U was injected onto a Partisil SAX column and eluted at 1 ml/min with 2 mM KH_2PO_4. (A) Effect of buffer pH (2.7–4.4) without acetonitrile; (B) effect of varying the acetonitrile concentration from 40 to 80% (v/v).

aminated metabolites simultaneously. The deamination of ara-C to ara-U by cytidine deaminase and the deamination of ara-CMP to ara-UMP by deoxycytidylate deaminase have usually been considered solely as reactions that detoxify ara-C. Indeed, resistance to ara-C has been correlated with high ara-C deamination activity [9,10], and deamination of ara-CMP appears to be the key reaction in the net catabolism of ara-CTP in leukemia cells from patients [19]. Recent studies have shown, however, that exposure of cells to ara-U causes cytostasis and enhances their sensitivity to ara-C [13]. When cells are exposed to high levels of [^{3}H]ara-U, small quantities of ara-U nucleotides slowly accumulate [20]. Since ara-U is a very poor substrate for thymidine kinase its initial conversion to ara-UMP is very slow. The demonstration here that relatively high levels of ara-UMP plus smaller but quite possibly significant amounts of ara-UDP and ara-UTP are formed in HL60 cells when exposed to

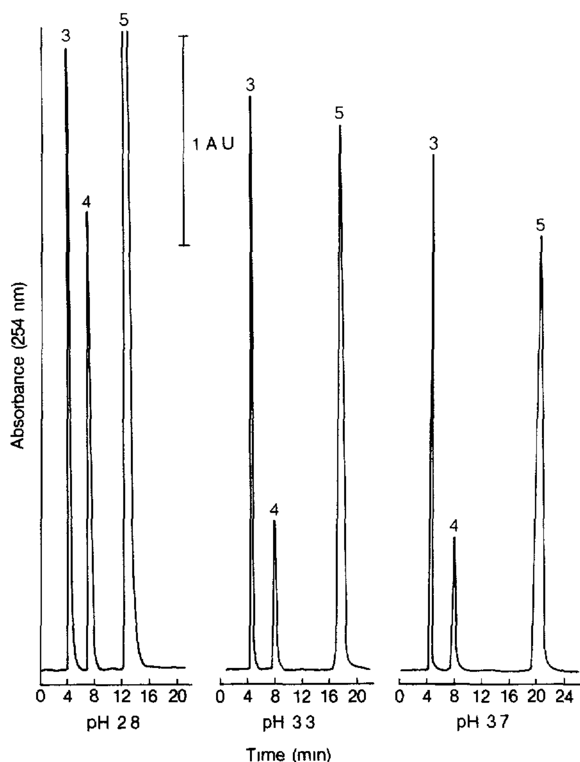

Fig. 5. Effect of buffer pH on retention time of UMP. A mixture of ara-CMP, CDP-choline and UMP was injected onto a column. Buffer A was 2 m*M* KH_2PO_4 with pH varying from 2.80 to 3.70, buffer B was 500 m*M* KH_2PO_4 with pH 3.40. The gradient was linear over 25 min.

ara-C is of considerable interest. Studies are in progress to assess the interactions of these deaminated products with ara-CTP in producing cytotoxicity.

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