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TWO SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR SIMULTANEOUS DETERMINATION OF 2'-DEOXYCYTIDINE 5'-TRIPHOSPHATE AND CYTOSINE ARABINOSIDE 5'-TRIPHOSPHATE CONCENTRATIONS IN BIOLOGICAL SAMPLES

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SUMMARY

Cytosine arabinoside (ara-C) has been used in the treatment of leukemia, but its exact mechanism of cytotoxicity is not yet known. One of the proposed mechanisms for the effectiveness of this drug in treating leukemias suggests that a metabolite of ara-C, i.e., 2'-deoxycytidine 5'-triphosphate (araCTP), competes with cytosine arabinoside 5'-triphosphate (dCTP) for binding to DNA polymerase. The ratio of the drug metabolite to the endogenous nucleotide (araCTP/dCTP) may, therefore, be important in determining the effectiveness of ara-C therapy. This ratio may also play a role in drug resistance. Previously published methods have focused on either araCTP or dCTP, along with metabolites and analogues of one of these compounds. The methods presented here provide two simple, sensitive ways to measure dCTP and araCTP in the same biological sample.

INTRODUCTION

It has been proposed that metabolites of cytosine arabinoside (ara-C), used in the treatment of leukemia, inhibit cell proliferation by several different mechanisms [1—3]. One of these mechanisms, i.e. competition by cytosine arabinoside 5'-triphosphate (araCTP) for the binding of 2'-deoxycytidine 5'-triphosphate (dCTP) to DNA polymerase, suggests that the araCTP/dCTP ratio may play an important part in determining the effectiveness of the drug. Until now, direct measurement of the levels of dCTP and araCTP in human white blood cells has been limited. Because the amount of sample obtained from patients is usually small, most studies have been done with cultured cell lines [4] and with cells from bone marrow aspirates, incubated with radioactive compounds [5]. With the advent of high dose ara-C therapy (HDARA-C) in leukemia [6], however, it has become advantageous to measure

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directly the levels of dCTP and araCTP in human white blood cells from bone marrow samples. Simultaneous measurement of these two compounds permits assessment of the importance of the ratio of araCTP/dCTP in the effectiveness of ara-C therapy, and minimizes the problem of small sample size.

Methods are available for determining concentrations of either araCTP or dCTP and related compounds [5, 7]; but a simple method for the simultaneous detection and quantitation of both compounds has not been reported. This paper reports two high-performance liquid chromatographic (HPLC) methods by which the concentrations of dCTP and araCTP can be measured in the same biological sample.

MATERIALS AND METHODS

HPLC grade $NH_4H_2PO_4$ was purchased from Fisher Scientific (St. Louis, MO, U.S.A.). Methanol was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Lymphoprep was purchased from Pharmacia (Piscataway, NJ, U.S.A.). All nucleotides and nucleosides used as chromatographic standards, and all other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Clinical samples

Refractory pediatric leukemic patients were treated with HDARA-C, i.e., 3.5 g/m^2/day by constant infusion over four consecutive days. Bone marrow aspirates or peripheral blood samples were taken prior to, 24 h after, and 72 h after the infusion of HDARA-C was begun. Heparin was used as anticoagulant in the collecting tubes. Mononuclear cells and blasts were then separated by the Ficoll—Hypaque method [8]. The number of cells in suspension was determined on a Model ZBI Coulter Counter. The cell samples were treated with perchloric acid (0.5 *N*), neutralized with potassium hydroxide, and then centrifuged to remove the potassium perchlorate precipitate [9]. The acid soluble supernatant fractions were stored at —70°C.

Preparation for chromatography

Immediately before analysis, the samples were treated by the periodate oxidation method of Garrett and Santi [10] to remove ribonucleotides which interfere with the resolution of dCTP and araCTP. This method quantitatively removed peaks of CTP and UTP in standard solutions from this area of the chromatogram.

HPLC

The high-pressure liquid chromatograph used was a Beckman (Altex) Model 332, with a Partisil SAX 10 anion-exchange column (25 cm × 4.6 mm).

Method I. The mobile phase was 500 m*M* $NH_4H_2PO_4$ (pH 3.5) with 2% methanol; the flow-rate was 0.5 ml/min. The methanol was added to the buffer at the beginning of each day and was used for one day only, because the concentration of methanol was critical. The solution was filtered and kept under vacuum for at least 30 min if the most sensitive scale, 0.005 absorbance units full scale (a.u.f.s.), was used, or baseline drift made interpreta-

tion of the graphs difficult. The difference in retention times of araCTP and dCTP was approximately 1.5 min.

Method II. The method used a gradient system and separated dCTP and araCTP peaks by greater than 2 min. The buffers used were: Buffer A, 150 m*M* $NH_4H_2PO_4$ (pH 3.5); Buffer B, 750 m*M* $NH_4H_2PO_4$ (pH 3.5) with 5% methanol. The flow-rate was 0.5 ml/min. The gradient was programmed into an Altex Microprocessor as follows:

Step	Time (min)	%B Buffer
1	0—10	0 → 50
2	10—20	50 → 10
3	20—50	10 → 100
4	50—60	100 → 0

If the descending gradient in Step 2 was omitted, dTTP coeluted with araCTP. After the program was completed, the baseline was allowed to stabilize with 100% Buffer A for 15 min before injection of the next sample.

The elution profiles obtained by both methods were monitored at 254 nm and 280 nm and the peak areas of the output of the 254-nm detector were quantitated by a Shimadzu Model E1B integrator. Because the detectors are connected in series, the peak corresponding to absorbance at 254 nm precedes the peak at 280 nm by several seconds. Quantitation of dCTP and araCTP by standard calibration curves based on measurements of peak heights and calculated peak areas agreed with the values obtained with the integrator at 0.01—0.16 a.u.f.s. sensitivity settings. Peak height measurement was a more reliable criterion for accurate measurements at the 0.005 a.u.f.s. sensitivity setting.

RESULTS

Figs. 1 and 2 illustrate the separations obtained by Method I with a standard solution of deoxyribonucleoside triphosphates (dNTP) and with a patient sample, equivalent to $4 \cdot 10^6$ cells, after treatment with ara-C. The sensitivity setting for each of these tracings was 0.01 a.u.f.s. As shown in Fig. 2, when the intracellular level of araCTP is extremely high as compared to dCTP, separation between these two compounds appears less complete than with standard solutions. This does not occur when concentrations of these compounds are more equal (as has been the case with most samples in our study); even when resolution appears less than optimal, quantitation by peak height measurement and computer calculation is reproducible and gives the same value (± 10%) as Method II.

The separations obtained with the gradient system of Method II are shown in Fig. 3 with an aliquot of the same standard solution as in Fig. 1, and in Fig. 4 with an aliquot of the same patient sample as in Fig. 2, equivalent to $5 \cdot 10^7$ cells. The sensitivity setting for each of these tracings was 0.08 a.u.f.s.

Retention times for dNTP standard solutions (Table I) were the same as retention times for these compounds when patient samples were injected and were reproducible ± 0.21 min for dCTP and araCTP for all patient samples.

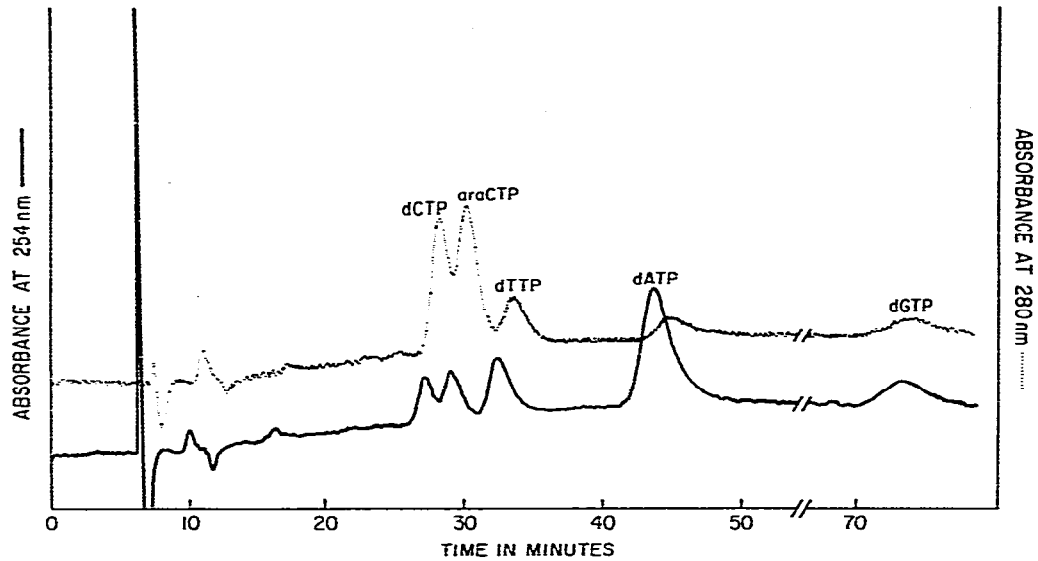

Fig. 1. Chromatogram (Method I) of a standard solution of deoxyribonucleoside triphosphates and araCTP. Peaks represent 300 pmole of each compound.

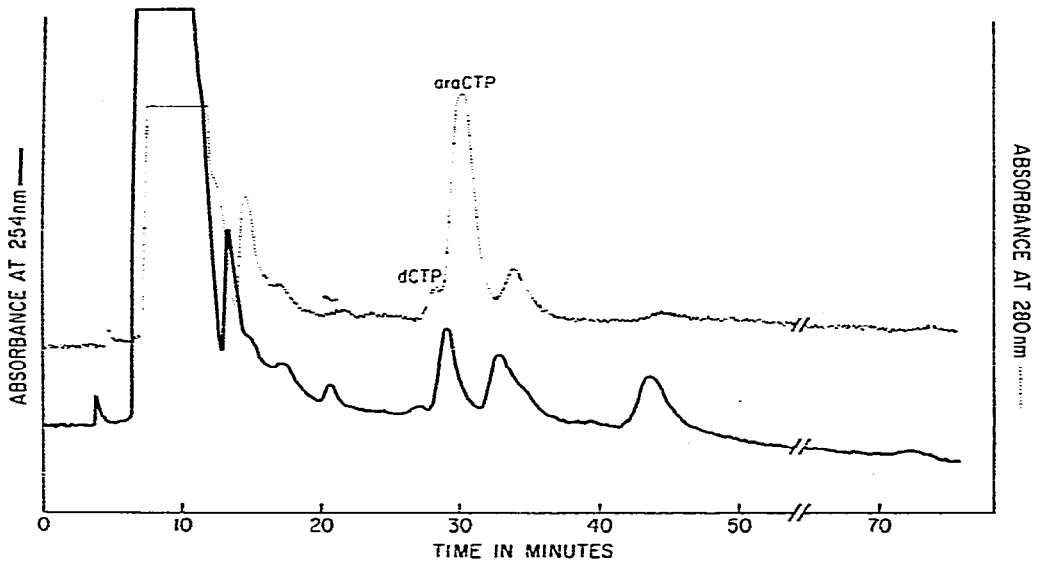

Fig. 2. Chromatogram (Method I) of acid-soluble fraction of the equivalent of $4 \cdot 10^6$ cells of a patient treated with HDARA-C therapy.

All other known metabolites of dCTP and araCTP elute within the first 20 min of the chromatogram by both methods [11—13]. While it is not impossible that unknown metabolites of these compounds are formed within the cell, it is unlikely that retention times and 254/280 absorbance ratios of both compounds in each system would remain constant and give equivalent results if significant contamination were present. These observations indicate that

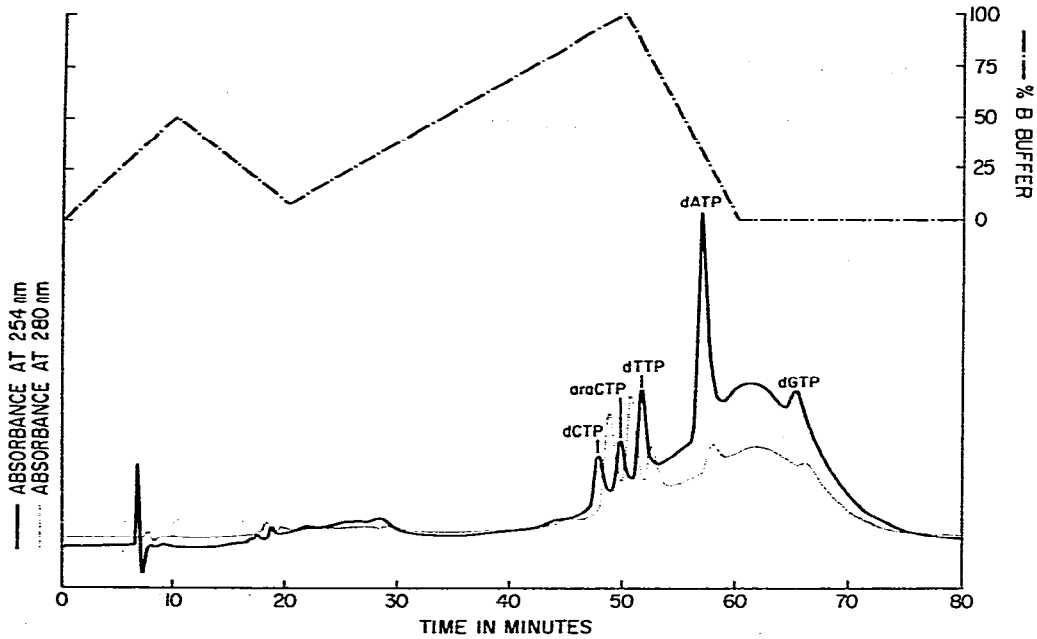

Fig. 3. Chromatogram (Method II) of a standard solution of deoxyribonucleoside triphosphates and araCTP. Peaks represent 2 nmole of each compound.

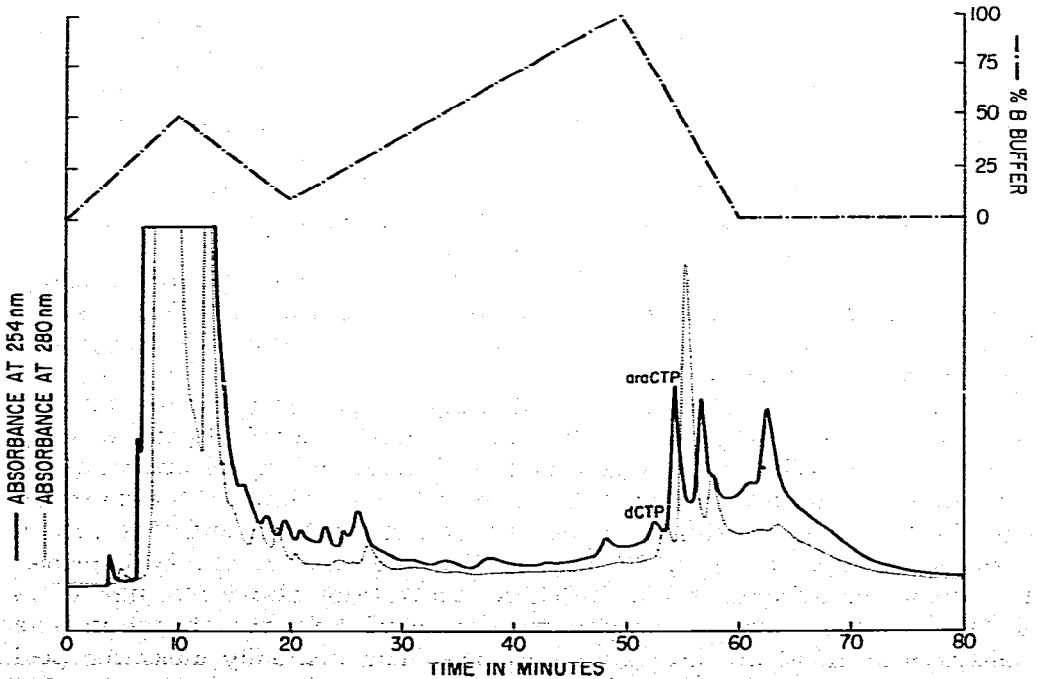

Fig. 4. Chromatogram (Method II) of acid-soluble fraction of the equivalent of $5 \cdot 10^7$ cells of a patient treated with HDARA-C therapy.

TABLE I

RETENTION TIMES OF dNTPs AND araCTP

	Compound	Retention time (min)
Method I	dCTP	26.81
	araCTP	28.63
	dTTP	32.25
	dATP	43.25
	dGTP	72.24
Method II	dCTP	47.82
	araCTP	51.96
	dTTP	53.82
	dATP	61.96
	dGTP	66.06

neither dCTP nor araCTP coeluted with other unidentified compounds. (The dTTP peak in patient samples appeared to coelute with another compound, possibly araUTP, which was not identified.) The dCTP concentrations were in the same range as those reported in cultured cells (ca. 25 pmole/10^6 cells) [4].

DISCUSSION

Both methods outlined in this paper gave quantitative, reproducible, simultaneous measurements of dCTP and araCTP in all samples studied. The number of cells required to give detectable levels of dCTP and araCTP by these methods will undoubtedly be related to dose and schedule of administration of ara-C as well as the time at which the tissue sample is taken. With the HDARA-C protocol given at this institution, dCTP and araCTP were both measureable in the 0.005 a.u.f.s. sensitivity range when the equivalent of $1.5—5.0 \cdot 10^6$ cells was injected onto the column.

Method I has the advantage of being an isocratic system, which allows instrument settings of maximum sensitivity with no baseline drift. This method has been useful in detecting the low levels of dCTP in the cells of patients prior to treatment with ara-C.

Method II has been most useful in evaluating samples of cells from patients who have been given HDARA-C. Using this method, the dCTP peak was separated from the araCTP peak by greater than 2 min, and so resolution of the two compounds was better than with Method I. Alterations of the methanol gradient, buffer concentrations, pH of one or both buffers, and flow-rate all gave poorer separations of dCTP and araCTP than the methods described. Baseline drift was evident at $NH_4H_2PO_4$ concentrations above 500 m*M* (Figs. 3 and 4). The integrator was programmed to correct for the change in baseline, and results based on integrated area units and manually measured peak heights and peak areas were equivalent. Quantitation of araCTP and dCTP concentrations was not impaired by the baseline drift. When standards were run in order to graph peak height or area vs. concentration of nucleotide,

the correlation coefficients of the lines generated were all between 0.989 and 0.999 for each compound for Method I and Method II. Further, if a known amount of standard was added to a patient sample as a tracer, and the sample and standard were injected onto the column together, the appropriate peak was increased by the predicted amount.

Each method outlined here gives adequate separation of dCTP and araCTP and has the added advantage of delaying elution of each of these peaks long enough to allow quantitation of these compounds in biological samples. When the equivalent of 10^5—10^7 cells is first oxidized and then injected onto an HPLC column, cell debris may obscure peaks which elute in the first 8—15 min of the run. (The exact time to reestablish the baseline after elution of debris will depend upon solvent, flow-rate, etc.) With each method reported here, elution is delayed long enough to circumvent this problem.

Additionally, Method II has the advantage of allowing quantitation of the deoxynucleoside diphosphates in the cell (data not shown). This is done simply by lengthening the 10-min gradient in Step 1 to a total of 30 min. In this way, deoxynucleoside diphosphates are separated from each other by a greater time interval than with an initial 10-min gradient, and separation and resolution of dCTP and araCTP are not adversely affected. Cell debris usually obscures the peaks of some of the deoxynucleoside monophosphates. While quantitation of deoxynucleoside diphosphates, as well as triphosphates, offered no advantage for our HDARA-C study, it may be useful in assessing other drug protocols.

Other possible methods for separating dCTP and araCTP include reversed-phase techniques with ion-pairing and anion-exchange techniques with either a $Na_2B_4O_7$—NH_4Cl gradient or a sodium citrate gradient [14—16]. Reversed-phase methods have usually been unsatisfactory because nucleotides elute very quickly and peaks of dCTP and araCTP are obscured by cell debris [17]. The method of Knox and Jurand [14] might possibly be adapted to measure araCTP and dCTP; but the approximate 30-min retention time (vs. a 26-min retention time for dCTP for Method I) of nucleotides offers no obvious advantage over the methods described here. Alternatively, other anion-exchange methods are available which could probably be adapted to separate dCTP and araCTP [15, 16]. If either of these methods could be adapted to give good resolution of araCTP and dCTP, it would have the benefit of simultaneous detection of bases, nucleosides, and nucleotides in the same sample. It would also have the disadvantage of taking over 2 h to run a single sample.

Obviously, each method has advantages and disadvantages which are related to time and equipment requirements, as well as compounds of interest other than dCTP and araCTP. This paper provides two simple, relatively short, reliable methods for quantitating dCTP and araCTP in biological samples.

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