Journal of Chromatography, 221 (1980) 425—430
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 670

Note

Simultaneous determination of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and 3-deazauridine 5′-triphosphate in human leukemia cells by high-performance liquid chromatography

WILLIAM PLUNKETT*, SHERRI CHUBB and BARTHEL BARLOGIE

Department of Developmental Therapeutics, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030 (U.S.A.)

(First received March 19th, 1980; revised manuscript received July 17th, 1980)

A firm knowledge of the pharmacologic behavior of the active metabolite of a drug is critical to the evaluation of the biochemical basis of its therapeutic efficacy. Antileukemic drugs such as 1-β-D-arabinofuranosylcytosine (ara-C) [1] and 3-deazauridine (deazaUrd) [2] require intracellular phosphorylation to their respective 5′-triphosphates, ara-CTP and deazaUTP, for activity. Thus, determination of the active nucleotide forms of these nucleoside analogs in tumor and host tissues is crucial to the biochemical appraisal of such clinical parameters as tumor reduction and resistance to therapy.

We have described sensitive chromatographic methods for the detection of ara-CTP and deazaUTP in bone marrow and tissue samples obtained from patients receiving therapy with either ara-C [3] or deazaUrd [4]. Recent observations in preclinical systems [5—7] suggest that these drugs may express synergistic activity when used together in the treatment of acute leukemia. However, it has been our experience that bone marrow aspirates from patients with leukemia may contain too few cells to permit the performance of multiple determinations of the metabolites of different drugs. This report describes a high-performance liquid chromatographic (HPLC) method for the simultaneous detection and quantitation of ara-CTP, deazaUTP, and CTP, the normal cellular nucleotide affected by the action of deazaUTP, in leukemia cells in vitro and in the leukemic cells of patients being treated with a combination of ara-C and deazaUrd. The assay has the same sensitivity as those previously described for each nucleotide analog alone and represents an improvement in that it is readily adapted to automated analysis.

0378-4347/80/0000—0000/$02.25 © 1980 Elsevier Scientific Publishing Company

MATERIALS AND METHODS

Chemicals

Reagent grade $NH_4H_2PO_4$ was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) and used without purification. Both ara-C (Upjohn, Kalamazoo, MI, U.S.A.) and deazaUrd (Division of Cancer Treatment, National Cancer Institute) were provided by the pharmacy of this institution. Normal nucleotides, used as chromatographic standards, were purchased from Sigma (St. Louis, MO, U.S.A.), as was ara-CTP. The [6-^{14}C]deazaUrd, used as a marker for deazaUTP after metabolism by CCRF-CEM cells, was kindly supplied by Dr. A. Bloch and Dr. P. Creaven of Roswell Park Memorial Institute.

Cell culture

CCRF-CEM cells, originally cultured from the peripheral blood of a patient with acute lymphocytic leukemia [8], were grown in agitated suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum (Grand Island Biological, Grand Island, NY, U.S.A.).

Bone marrow specimens

The treatment protocol (77—73) involving the combination of ara-C and deazaUrd treatment in patients with acute myelogenous leukemia in relapse was approved by the office of Research and the Surveillance Committee of this institution. One goal of this regimen was to evaluate the hypothesis that prior treatment with deazaUrd promotes sensitivty to ara-C in previously resistant cell populations. All patients volunteered informed consent for treatment and for bone marrow aspiration for diagnostic and laboratory investigation. Bone marrow aspirated from patients treated with ara-C and deazaUrd were drawn into syringes containing heparin as an anticoagulant and transported to the laboratory in an ice bath. Nucleated bone marrow cells were separated by standard Ficoll—Hypaque gradient centrifugation procedures [9].

Nucleotide extraction

Single cell suspensions of either CCRF-CEM cells or leukemic bone marrow cells were counted and their mean volumes were determined by an electronic particle counter (Coulter Electronics, Hialeah, FL, U.S.A.). Perchloric acid-soluble material containing cellular nucleotides was extracted and neutralized by standard procedures [3, 4] and stored at —20°C prior to chromatographic analysis.

High-performance liquid chromatography

A Waters Assoc. (Milford, MA, U.S.A.) high-pressure liquid chromatograph Model ALC-204 equipped with two Model 6000A pumps and a Model 660 gradient programmer was used to analyze nucleotides in perchloric acid-soluble cell extracts. Samples of 0.01—2.0 ml were injected onto a column of Partisil-10 SAX anion-exchange resin (Whatman, Clifton, NJ, U.S.A.) with a Model U6K injection system. Optimal separation of ara-CTP and deazaUTP from normal cellular constituents was obtained by the following gradient scheme.

Starting with the initial conditions of 65% Buffer A, 0.005 *M* $NH_4H_2PO_4$, pH 2.8, and 35% Buffer B, 0.75 *M* $NH_4H_2PO_4$, pH 3.7, a concave gradient described by curve 9 on the gradient programmer was run at 3.0 ml/min for 30 min to the final condition of 100% Buffer B. The eluted compounds were detected by their absorbance at 280 nm by the Model 440 monitor or a Schoeffel Instruments (Westwood, NJ, U.S.A.) Model 770 Spectroflow monitor. Absorbance was recorded by a Model 9126 strip chart recorder (Varian, Palo Alto, CA, U.S.A.) attenuated to 10 mV, and peak areas were quantitated with a Model CDS-111 electronic integrator (Varian). Peak areas were converted to absolute quantities using predetermined calibration curves. Baseline drift during gradient elution was minimized if the Partisil column was purged before use with 50 ml 2.0 *M* $NH_4H_2PO_4$, pH 4.2, equilibrated with 60% Buffer A and 40% Buffer B, and treated with 0.10 ml 0.1 *M* disodium EDTA. Following nucleotide analysis, the column was purged with 20 ml of the initial conditions mixture before injection of a subsequent sample.

When desired, this elution scheme and the subsequent analysis was directed by an automatic injector system (Waters Assoc. Model 710A automatic sample processor). The elution scheme described represents an improvement in this respect since schema previously reported for the separation of either ara-CTP [3] or deazaUTP [4] from the cellular ribonucleoside triphosphates consisted of isocratic elution then gradient elution steps in series. These requirements exceeded the capabilities of the automatic injector.

RESULTS AND DISCUSSION

The ability of this elution scheme to separate the major ribonucleoside 5′-triphosphates extracted from CCRF-CEM cells is illustrated in Fig. 1. A parallel culture was incubated with 100 μM deazaUrd for 4 h before addition of 2.0 μM ara-C for one additional hour (Fig. 2). Three major changes are evident in the chromatogram of nucleotides extracted from drug treated cells. First, a new peak eluting between CTP and UTP has been identified as ara-CTP by its UV absorbance characteristics, radioactivity after incubating cells with [^{3}H]ara-C, resistance to oxidation with $NaIO_4$, and cochromatography with authentic ara-CTP. Standards indicate that the incubation procedure resulted in the accumulation of 0.59 nmol of ara-CTP per $2 \cdot 10^6$ cells. A cellular concentration of 292 μM ara-CTP was calculated after the average cell volume (1010 μm^3) was considered and uniform cellular distribution of the nucleotide was assumed. These calculations indicate the ability of cultured human leukemia cells to accumulate ara-CTP to cellular concentrations over 100 times that of the exogenous nucleoside. Second, a new peak eluting between ATP and GTP has been identified as deazaUTP by its UV absorbance characteristics, cochromatography with the majority of radioactivity in extracts after incubating cells with [^{14}C] deazaUrd and sensitivity to incubation with both phosphatase and $NaIO_4$. DeazaUTP was present in drug-treated cells at a concentration of 2.33 nmol per $2 \cdot 10^6$ cells, or 1150 μM. Finally, the CTP peak in drug-treated cells is only 12% that of controls, 0.23 nmol per $2 \cdot 10^6$ cells versus 1.90 nmol per $2 \cdot 10^6$ cells. This suggests the inhibitory action of deazaUTP on the CTP syn-

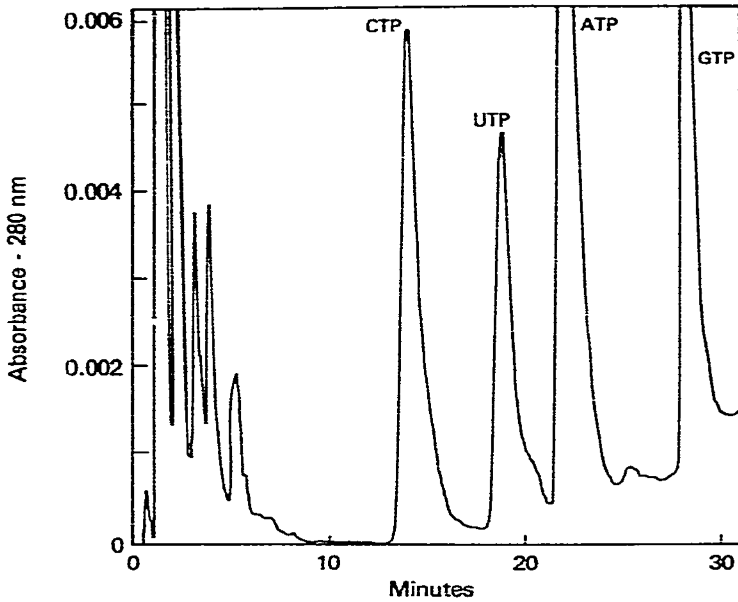

Fig. 1. Separation of perchloric acid-soluble material extracted from the equivalent of $2 \cdot 10^6$ CCRF-CEM cells by HPLC.

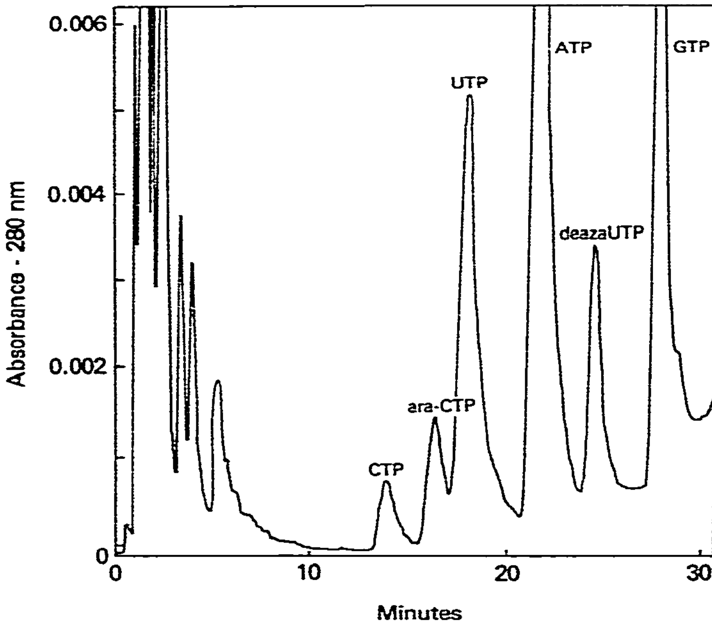

Fig. 2. Separation of perchloric acid-soluble material extracted from the equivalent of $2 \cdot 10^6$ CCRF-CEM cells that had been incubated 4 h with 100 μM deazaUrd before the addition of 2 μM ara-C for one additional hour.

thetase [10] of these cells, leading to a predictable decrease in CTP [4]. Serial dilutions of cell extracts containing deazaUTP and addition of known amounts of ara-CTP to cell extracts indicate the lower limits for quantitation by electronic integration of the peak areas of deazaUTP and ara-CTP to be 250 and 25 pmol, respectively, values similar to those obtained in assays for each nucleotide separately [3, 4] in cell extracts.

The reproducibility of the retention time of each nucleotide separated in this manner is shown in Table I. The maximum variation in the elution times

TABLE I

REPRODUCIBILITY OF RETENTION TIME AND QUANTITATION OF NUCLEOTIDES EXTRACTED FROM CCRF-CEM CELLS

Cells were incubated with deazaUrd and ara-C as described in the legend for Fig. 2. Samples from the equivalent of $1 \cdot 10^6$ cells were injected and analyzed under the direction of the Model 710A automatic sample processor ($n = 8$).

Nucleotide	Retention time (min)	Quantity (nmol/$1 \cdot 10^6$ cells ± S.E.M.)
CTP	13.50 ± 0.05	0.17 ± 0.005
ara-CTP	16.14 ± 0.09	0.36 ± 0.01
UTP	18.35 ± 0.04	3.78 ± 0.07
ATP	20.90 ± 0.03	6.05 ± 0.11
deazaUTP	25.35 ± 0.02	1.45 ± 0.05
GTP	28.21 ± 0.03	2.04 ± 0.06

was 0.6% (ara-CTP). In addition, the maximum variation in quantitation when the six nucleotides in this cell extract were analyzed was less than 3% (CTP).

This chromatographic technique for the simultaneous detection and quantitation of ara-CTP, deazaUTP, and the affected normal nucleotide, CTP, is directly applicable to biochemical pharmacology studies in the cells of patients receiving ara-C and deazaUrd chemotherapy. Chromatographic analysis of a perchloric acid-soluble extract from nucleated bone marrow cells from a patient who had been treated with ara-C (50 mg/m^2/day) for 2 days then simultaneously with deazaUrd (1000 mg/m^2/day) for 1 day is shown in Fig. 3. Both ara-CTP and deazaUTP are clearly present in addition to the four ribonucleoside 5′-triphosphates. Similar peaks have never been observed in extracts from lymphocytes, leukemic cells from the peripheral blood, red blood cells or solid tumors from patients who had not been treated with either ara-C or deazaUrd. Using the determined mean cell volume of 283 μm^3, the nucleotide concentration in these cells was calculated as follows: CTP, 280 μM;UTP, 1560 μM; ATP, 2230 μM; GTP, 476 μM; ara-CTP, 15 μM; deazaUTP, 150 μM. The last two figures indicate that the nucleoside analogs entered the bone marrow cells, were phosphorylated by the active triphosphates, and accumulated to values considerably in excess of the K_i value for the respective target enzymes [10, 11]. This is significant for the interpretation of the therapeutic effect of these drugs, since this patient's disease had previously failed to respond to treatment regimens containing ara-C or to deazaUrd alone. In a separate publication [12] we present the results of serial determinations on the leukemic cells of patients treated on this protocol that indicate this assay may be useful in establishing the biochemical basis for drug scheduling, interaction, and resistance.

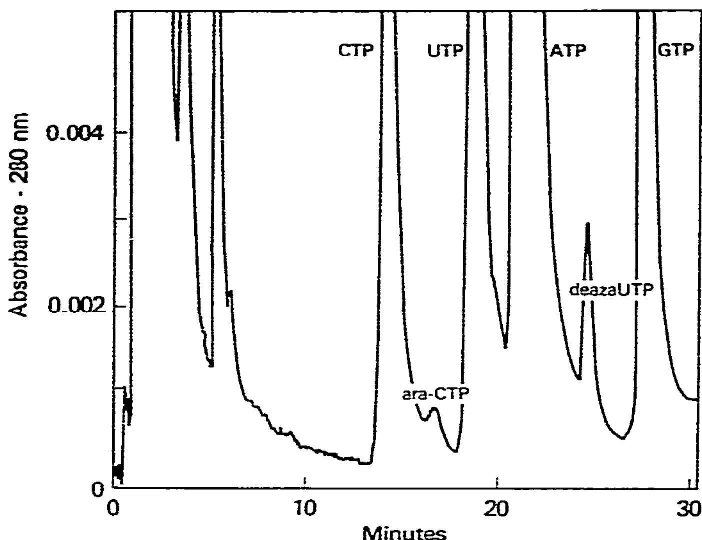

Fig. 3. HPLC separation of perchloric acid-soluble material extracted from the equivalent of $3 \cdot 10^7$ nucleated bone marrow cells from a patient receiving combination chemotherapy with ara-C and deazaUrd.

This rapid and sensitive assay for the simultaneous detection of ara-CTP, deazaUTP, and the affected nucleotide, CTP, should be useful for evaluating the biochemical and pharmacological bases of the therapeutic efficacy of treatment regimens that combine ara-C and deazaUrd. The elution scheme required to achieve this separation is within the capability of automated injection systems currently available.

ACKNOWLEDGEMENTS

This work was supported by NIH Grants CA-11520, CA-14528, and CA-05831, National Cancer Institute Contract CM-87185, and American Cancer Society Grant CH-130.

REFERENCES

1 D. Kessel, T.C. Hall and D. Rosenthal, Science, 156 (1967) 1240.
2 M.C. Wang and A. Bloch, Biochem. Pharmacol., 21 (1972) 1063.
3 W. Plunkett, V. Hug, M.J. Keating and S. Chubb, Cancer Res., 40 (1980) 588.
4 W. Plunkett, J.A. Benvenuto, D.A. Stewart and T.L. Loo, Cancer Treat. Rep., 63 (1979) 415.
5 K. Hande, B. Lewis and B. Chabner, Proc. Amer. Assoc. Cancer Res., 19 (1978) 149.
6 C. Mills-Yamamoto, G.J. Lauzon and A.R.P. Paterson, Biochem. Pharmacol., 27 (1978) 181.
7 A.R.P. Paterson, E.S. Jakobs, C.J. Lauzon and W.M. Weinstein, Cancer Res., 39 (1979) 2216.
8 G.E. Foley, H. Lazarus, S. Farber, B.G. Uzman, B.A. Boone and R.E. McCarthy, Cancer, 18 (1965) 522.
9 A. Boyum, Scand. J. Clin. Invest., 21 Suppl. 97 (1968) 77.
10 R.P. McPartland, M.C. Wang, A. Bloch and H. Weinfeld, Cancer Res., 34 (1974) 3107.
11 J.J. Furth and S.S. Cohen, Cancer Res., 28 (1968) 2061.
12 B. Barlogie, W. Plunkett, M. Raber, J. Latreille, M. Keating and K. McCredie, Cancer Res., in press.