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Note

Determination of cytosine arabinoside triphosphate in leukemic cells by isocratic high-performance anion-exchange column chromatography

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Cytosine arabinoside (ara-C, 1-β-D-arabinofuranosylcytosine) is a cytostatic drug, effective in the treatment of acute leukemia [1,2]. In vivo, extracellular ara-C is deaminated rapidly to the inactive metabolite uracil arabinoside (ara-U, 1-β-D-arabinofuranosyluracil) [3]. Ara-C is also taken up by leukemic cells and phosphorylated to cytosine arabinoside triphosphate (ara-CTP) [4, 5], a metabolite interfering with DNA synthesis [5, 6]. The extent of ara-C phosphorylation is one of the main determinants of its cytocidal property [7, 8]. Decreased phosphorylation of ara-C is associated with drug resistance in human and animal tumors [7, 9—14].

Hitherto, phosphorylation of ara-C has been studied by means of radioactive-labeled drug. Consequently, the investigations have been limited to in vitro experiments [11, 12, 14]. Since a method for the determination of small amounts of unlabeled ara-CTP was not available, data concerning intracellular amounts of ara-CTP in vivo are lacking.

Recently, some methods have been developed for ara-CTP determination by means of high-performance liquid chromatography (HPLC) [15, 16]. These methods include gradient elution and laborious sample preparation, making them time-consuming.

In this paper a simple method for cell extraction in combination with a rapid and sensitive HPLC procedure, using isocratic elution, is described. The method is illustrated by data obtained in two patients treated with ara-C.

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MATERIALS AND METHODS

Chemicals

Ara-CTP, cytidine-5′-triphosphate (CTP), uridine-5′-triphosphate (UTP), deoxythymidine-5′-triphosphate (dTTP), inosine-5′-triphosphate (ITP), adenosine-5′-triphosphate (ATP) and guanosine-5′-triphosphate (GTP) were purchased from Brunschwig Chemie, deoxycytidine-5′-triphosphate (dCTP) was from Hoechst and deoxyadenosine-5′-triphosphate (dATP) and deoxyguanosine-5′-triphosphate (dGTP) were from Boehringer. All other chemicals were supplied by E. Merck. All chemicals were of analytical grade.

Preparation of cell extracts

Bone marrow aspirates and peripheral blood samples are collected in ice-cold buffered acid citrate dextrose solution (ACD, formula A, pH 7.4) and immediately placed on ice. Cells are washed once with ACD to prevent fibrin formation, and 10^7 to 10^6 nucleated cells are layered on a Percoll cushion (d = 1.080 g/ml), and centrifugated for 20 min at 800 g and 4°C to remove erythrocytes. The cells are collected from the interface and washed in ice-cold ACD. Cell counting is performed with a Coulter Counter; 2×10^7 to 4×10^7 cells are spun down (10 min, 800 g, 4°C). A 500-μl sample of an ice-cold ethanol—HPLC eluent solution (1:1, v/v) is added to the cell pellet and the intracellular nucleotides are extracted in 15 min at 0°C.

Precipitated protein is removed from the extract by centrifugation (4 min, 8000 g, 0°C). Of these extracts, 100 μl are used for analysis.

HPLC equipment

A Pye-Unicam LC-3-XP pump, equipped with an automatic injection valve (Valco Houston AH 60), a 100-μl loop, and a column (250 × 4.6 mm I.D., Whatman) packed with Partisil-10-SAX anion-exchange resin (particle size 10 μm) is used for separation of the nucleotides. A guard column (30 × 4.6 mm I.D., Partisil-10-SAX) is incorporated in the system to protect the analytical column. Two UV detectors are used: an UV III monitor LDC 1203 (10-μl flowcell, wavelength 280 nm) and a Pye-Unicam LC-UV (8-μl flowcell, wavelength 254 nm).

HPLC procedure

Samples of 100 μl of cell extract are injected into the column. Elution is carried out at a constant temperature (35°C) and at a constant flow-rate of 2.5 ml/min with 0.125 M potassium dihydrogen phosphate solution, containing 0.075 M trisodium citrate as counter-ion, adjusted to pH 4.6 (at 35°C) with phosphoric acid. Helium gas is led through the eluent to prevent development of air bubbles at the low pressure side. Detection of ara-CTP is optimal at 280 nm. Peak heights are used for quantification, whereas ratios of the absorbance at 280 nm and at 254 nm are used for purity control of the peaks.

RESULTS

Chromatographic separation, accuracy, and calibration curves

Separation of ara-CTP from naturally occurring analogues is achieved within 17 min (Fig. 1a). A chromatogram of a blank leukemic cell sample is shown in Fig. 1b. Leukemic cell extracts did not contain compounds which interfere with the ara-CTP peak, as could be checked by the 280/254 nm ratio. The lowest detectable amount in biological samples is about 20 pmol. A chromatogram of a leukemic cell extract containing 40 pmol is shown in Fig. 1c. The calibration curve (not shown) is linear over three decades (from 20 pmol to 20 nmol) and passes through the origin. The coefficient of variation ranges from 3% (1 nmol, n=6) to 11% (50 pmol, n=6).

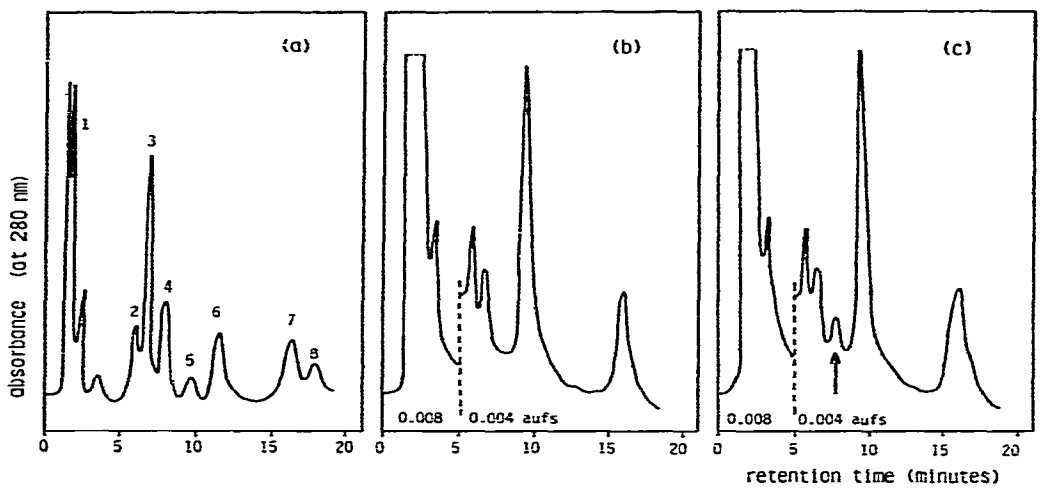

Fig. 1. (a) Chromatogram of a mixture of nucleotides. Detection at 280 nm. Conditions: see under Methods. Peaks: 1 = mono- and diphosphates; 2 = UTP; 3 = dCTP, CTP and dTTP; 4 = ara-CTP; 5 = ATP; 6 = dATP; 7 = GTP; 8 = dGTP. (b) Chromatogram of a blank leukemic cell extract. Detection at 280 nm, 0.004 a.u.f.s. (c) Chromatogram of a leukemic cell extract. The sample was taken 15 min after starting a 1-h intravenous infusion of ara-C (200 mg/m^2). Detection at 280 nm, 0.004 a.u.f.s. The injection sample (100 μl) represents 5.4 x 10^6 cells and contains 40 pmol of ara-CTP. Arrow indicates ara-CTP peak.

Up to 4 X 10^7 cells can be extracted quantitatively with 500 μl of extraction solution (Fig. 2a). To demonstrate linearity of the calibration curve, including the extraction procedure, leukemic cells incubated in vitro with ara-C were mixed with non-incubated cells in different proportions before extraction. This calibration curve appears linear (Fig. 2b).

Application

Ara-CTP has been measured in the leukemic cells of two patients after administration of ara-C. Ara-C (200 mg/m^2 body surface) was administered either as a bolus injection (patient A) or as a constant-rate infusion during 1 h (patient B). At several time intervals blood samples were taken for the determination of the ara-C plasma concentration and for quantification of ara-CTP

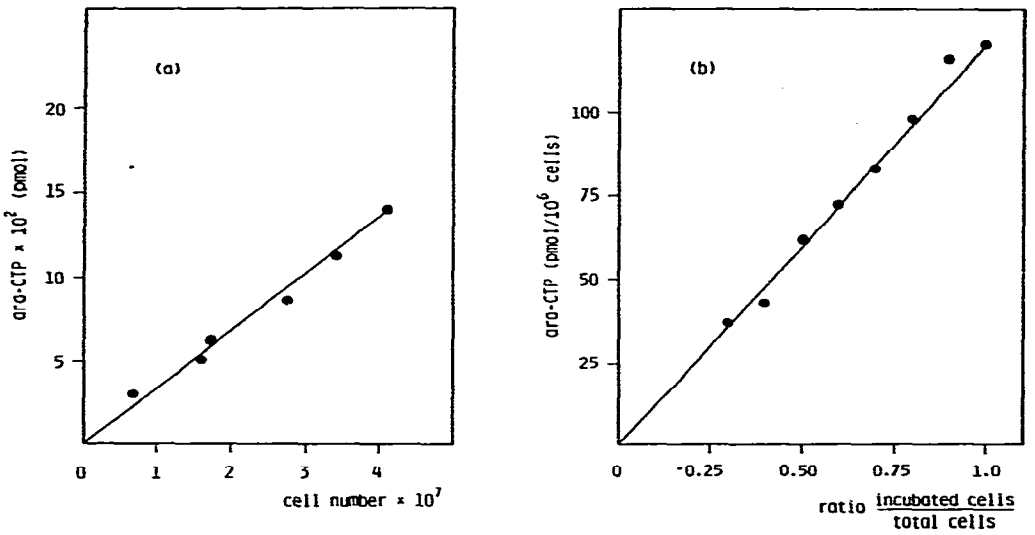

Fig. 2. (a) Calibration curve of the extraction of increasing cell numbers. Cells are incubated with ara-C in vitro and extracted with 500 µl of extraction solution. Chromatographic conditions: see under Methods. (b) Calibration curve of the determination of ara-CTP, including the extraction procedure. Leukemic cells, incubated with ara-C in vitro, are mixed with non-incubated leukemic cells in varying ratios.

accumulated in the leukemic cells. Determination of ara-C was performed as previously described [17].

Five minutes after ara-C administration, ara-CTP was already detected in considerable amounts in the leukemic cells. During the constant-rate infusion

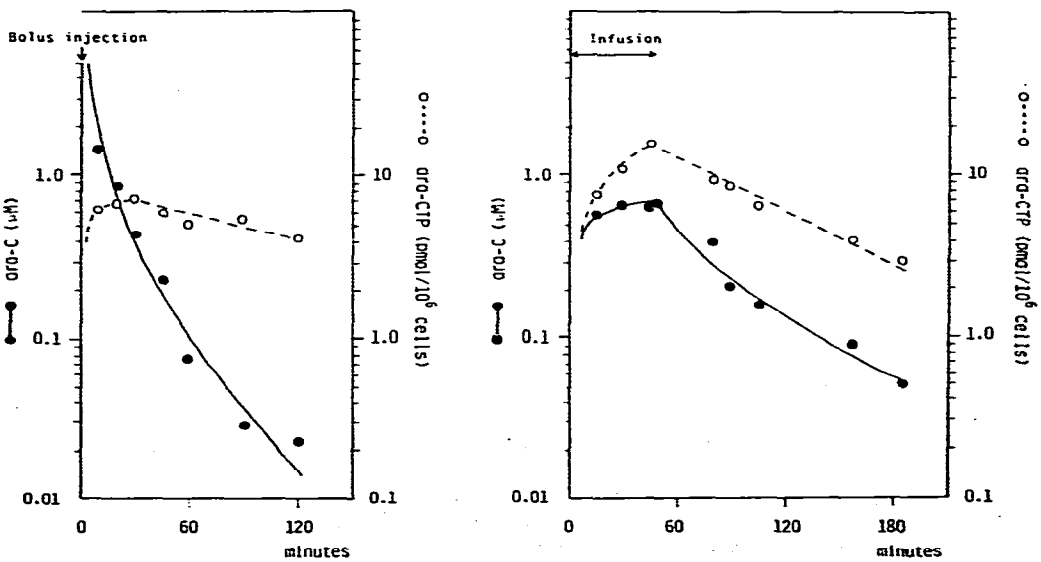

Fig. 3. Time curves of ara-C plasma concentration (●——●) and of amount of ara-CTP in leukemic cells (o — — — o) after administration of 200 mg/m² ara-C as a bolus injection and as a constant-rate infusion of 1 h.

ara-CTP showed an almost linear increase. After the bolus injection the intracellular amount of ara-CTP increased during 15 min.

Elimination of ara-CTP from the leukemic cells showed first-order kinetics with half-life times of 60 and 120 min in the two patients studied.

Time curves of ara-C in plasma and of ara-CTP in the leukemic cells are depicted in Fig. 3.

DISCUSSION

Pharmacokinetic studies are performed generally to relate variations in pharmacokinetic parameters to individual therapeutic and toxic effects [18, 19]. In all previous studies of the pharmacokinetics of ara-C, only plasma concentrations have been taken into account. A correlation between the half-life of ara-C and the therapeutic response has been reported [20, 21], but this was not confirmed by others [22]. Since the active compound is not ara-C but the intracellular metabolite ara-CTP, variations in the therapeutic efficacy of ara-C may be related more closely to differences in the pharmacokinetics of intracellular ara-CTP. This hypothesis is supported by the wide variation in the phosphorylating activity of leukemic cells [12, 23].

The intracellular pharmacokinetics of ara-CTP can be monitored by means of the method described in this paper. Extraction of the intracellular nucleotides is performed with an ethanol solution. This procedure is more suitable for application to small samples, since neutralization is not needed. When using perchloric acid careful neutralization is necessary, otherwise ara-CTP will be hydrolyzed. Separation of ara-CTP from the other nucleotides was achieved with the use of an isocratic eluent. This makes the procedure less time-consuming in comparison to methods using gradient elution. In conclusion, this method is sensitive and rapid, and can be readily applied for clinical pharmacological studies. This has been demonstrated in two examples of patients receiving ara-C therapy. The results indicate that after an intravenous bolus injection of ara-C maximal ara-CTP levels are reached after about 15 min, whereas ara-CTP increased linearly during the time period of a constant-rate infusion. Elimination of intracellular ara-CTP in vivo showed first-order kinetics. The observed half-lives of 60 and 120 min are in good agreement with those measured in vitro [14, 23].

REFERENCES

1 R.P. Gale, N. Engl. J. Med., 300 (1979) 1189.
2 J.A. Whittaker, Brit. Med. J., 281 (1980) 960.
3 D.H.W. Ho and E.J. Freireich, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Part 2, Springer Verlag, Berlin, 1975, p. 257.
4 R.L. Momparler, Cancer Res., 34 (1974) 1775.
5 W.A. Creasey, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Part 2, Springer Verlag, Berlin, 1975, p. 232.
6 R.L. Momparler, Mol. Pharmacol., 8 (1972) 362.
7 D. Kessel, T.C. Hall and I. Wodinsky, Science, 156 (1967) 1240.
8 T. Lincoln, P. Morrison, J. Aroesty and G. Carter, Cancer Treatm. Rep., 60 (1976) 1723.
9 A.W. Schrecker and M.J. Urshel, Cancer Res., 28 (1968) 793.

10 M.B. Meyers and W. Kreis, Cancer Res., 38 (1978) 1105.
11 T.C. Chou, Z. Arlin, B.D. Clarkson and F.S. Philips, Cancer Res., 37 (1977) 3561.
12 D. Kessel, T.C. Hall and D. Rosenthal, Cancer Res., 29 (1969) 459.
13 J.S. Hart, D.H. Ho, S.L. George, P. Salem, J.A. Gottlieb and E. Frei, Cancer Res., 32 (1972) 2711.
14 Y.M. Rustum and H.D. Preisler, Cancer Res., 39 (1979) 42.
15 W. Plunkett, V. Hug, M.J. Keating and S. Chubb, Cancer Res., 40 (1980) 588.
16 M.G. Pallavicini and J.A. Mazrimas, J. Chromatogr., 183 (1980) 449.
17 P. Linssen, A. Drenthe-Schonk, H. Wessels and C. Haanen, J. Chromatogr., 223 (1981) 371.
18 G. Tognoni, C. Bellantuono, M. Bonati, M. D'Incalci, M. Gerna, R. Latini, M. Mandelli, M.G. Porro and E. Riva, Clin. Pharmacokin., 5 (1980) 105.
19 J.R. Trounce, Brit. J. Clin. Pharmacol., 8 (1979) 205.
20 H.C. van Prooijen, E. van der Kleijn and C. Haanen, Clin. Pharmacol. Ther., 21 (1977) 744.
21 B.C. Baguley and E.-M. Falkenhaug, Cancer Chemother. Rep., 55 (1971) 291.
22 A.L. Harris, C. Potter, C. Bunch, J. Boutagy, D.J. Harvey and D.G. Grahame-Smith, Brit. J. Clin. Pharmacol., 8 (1979) 219.
23 A. Drenthe-Schonk, Thesis, Nijmegen, 1982.