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## Note

# High-performance liquid chromatographic assay for cytosine arabinoside and uracil arabinoside in cerebrospinal fluid and plasma

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1-β-D-Arabinofuranosylcytosine (ara-C) is one of the most effective drugs for the treatment of acute myeloid leukaemia, and it is also employed for treatment of acute lymphoblastic leukaemia and other haematological malignancies [1,2]. Ara-C is an analogue of the physiologically occurring nucleoside deoxycytidine. In order to exert its antineoplastic activity, ara-C must be intracellularly phosphorylated to cytosine 1-β-D-arabinofuranoside 5′-triphosphate (ara-CTP). Ara-CTP blocks DNA synthesis both by inhibition of DNA polymerase and by incorporation into DNA [3]. In humans the drug is deaminated by cytidine deaminase, primarily in the liver, spleen and gastrointestinal mucosa, to an inactive compound, uracil arabinoside (ara-U). Pharmacokinetic studies in humans have shown that ara-C disappears rapidly from plasma (mean half-life 12 min) [4,5].

Several methods are available for measuring ara-C in biological specimens, including microbiological [6,7], radioimmunological [8], radioenzymic [9] and gas chromatographic [10,11] techniques. High-performance liquid chromatography (HPLC) has also been used for this purpose. Some of the methods that have been described are quite sensitive for ara-C but are unable to measure ara-U [12] or are unable to separate it from endogenous interfering substances [13]. Other techniques are not sensitive enough to detect low levels of ara-C [14] or require a dual-column HPLC system [15] or involve time-consuming

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procedures for the separation of arabinose compounds from naturally occurring nucleosides [16].

This paper describes an isocratic, reversed-phase HPLC technique that is simple, sensitive and relatively rapid. Using this method we performed pharmacokinetic studies on cerebrospinal fluid (CSF) and plasma of patients undergoing both conventional and high-dose chemotherapy with ara-C.

## EXPERIMENTAL

### *Chemicals*

Ara-C (Upjohn, Kalamazoo, MI, U.S.A.) was the drug used for the treatment of patients. Tetrahydrouridine (THU) was purchased from Calbiochem (San Diego, CA, U.S.A.). Ara-U, deoxyinosine (DXI), uric acid, cytosine, deoxycytidine, cytidine, hypoxanthine, xanthine, uridine, and deoxyuridine were purchased from Sigma (St. Louis, MO, U.S.A.) and 6-mercaptopurine (6-MP) was obtained from Wellcome (London, U.K.). Methotrexate (MTX) and folinic acid came from Lederle (Wayne, NJ, U.S.A.) and potassium dihydrogenphosphate and methanol were purchased from Merck (Darmstadt, F.R.G.). All chemicals were of analytical grade.

### *Chromatography*

The HPLC equipment consisted of a Beckman (Berkeley, CA, U.S.A.) Model 112 solvent-delivery module, a Rheodyne injection valve equipped with a 100-$\mu$l loop, an Ultrasphere ODS column (250 mm$\times$4.6 mm I.D., particle size 5 $\mu$m, Altex, Berkeley, CA, U.S.A.) and a $C_{18}$ guard column (Brownlee). Two UV detectors were used: a fixed-wavelength Beckman Model 160 at 280 nm for detection of ara-C and a variable-wavelength Beckman Model 163 at 264 nm for detection of ara-U and the internal standard DXI. HPLC assays were carried out at room temperature at a constant flow-rate of 1.0 ml/min (140 bar), using a mobile phase composed of 3.5 m*M* $KH_2PO_4$ and methanol (98:2, v/v) pH 3.3. The peak areas were measured using a two-channel integrator 745 data module (Waters Assoc., Milford, MA, U.S.A.).

### *Assay procedure*

Blood samples (2 ml) were collected in heparinized tubes containing the deaminase inhibitor, THU (final concentration $10^{-4}$ *M*) to prevent deamination of ara-C. After centrifugation (800 *g* for 10 min), plasma was collected and frozen at $-20$°C until analysis. CSF samples (1 ml), also collected in tubes containing THU ($10^{-4}$ *M*), were immediately stored at $-20$°C.

After addition of the internal standard DXI (1 and 100 $\mu$g/ml, respectively, for conventional- and high-dose samples), 0.5 ml of plasma and CSF samples were filtered with the MPS-1 micropartition system (cut-off 10 000) (Amicon,

Lexington, MA, U.S.A.) for 30 min at 1000 *g* in order to remove proteins; 100 $\mu$l of ultrafiltrate were injected directly into the HPLC system.

## RESULTS AND DISCUSSION

Fig. 1A shows a chromatogram of a blank plasma sample from a normal donor, and Fig. 1B the same plasma spiked with 100 ng/ml ara-C, 500 ng/ml ara-U and 1 $\mu$g/ml DXI at 280 nm. Retention times for ara-C, ara-U and DXI were 13.0, 14.3 and 30.0 min, respectively. Blank plasma samples appeared to contain no endogenous compounds that would elute with ara-C, ara-U or DXI. Fig. 2 shows chromatograms of CSF (A) and plasma (B) samples from a leukaemic patient treated with 75 mg/m$^2$ ara-C per 24 h. Plasma and CSF levels were 60 and 12 ng/ml, respectively. The limits of detection for ara-C and ara-U were 15 ng/ml in plasma samples and 5 ng/ml in CSF. We detected ara-U and DXI at 264 nm, but, owing to the large amount of ara-U, it is also possible to detect them at 280 nm, using a single detector. Compared with the peak areas of the pure compounds in standard aqueous solutions, the mean recoveries after ultrafiltration were 98 ± 5% for ara-C, 96 ± 6% for ara-U and 92 ± 10% for DXI. The percentage recovery was determined at concentrations varying from 20 ng/ml to 10 $\mu$g/ml for ara-C, and 100 ng/ml to 100 $\mu$g/ml for ara-U.

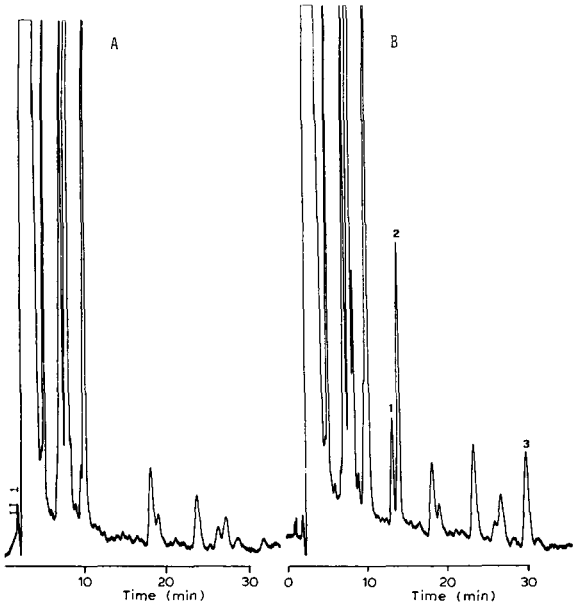


Fig. 1. High-performance liquid chromatograms of (A) blank plasma sample and (B) the same plasma sample spiked with ara-C (100 ng/ml), ara-U (500 ng/ml) and DXI (1 $\mu$g/ml). Peaks: 1 = ara-C; 2 = ara-U; 3 = DXI.

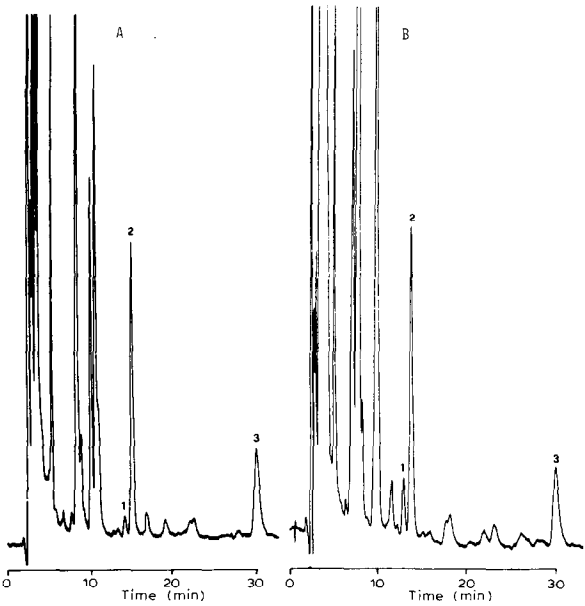


Fig. 2. (A) Chromatogram of CSF from a leukaemic patient treated intravenously with 75 mg/m$^2$ ara-C per 24 h (12 ng/ml ara-C, 540 ng/ml ara-U). (B) Chromatogram of plasma sample from the same patient (60 ng/ml ara-C, 645 ng/ml ara-U). Plasma and CSF samples were collected after 72 h continuous intravenous infusion with ara-C. Peaks: 1 = ara-C; 2 = ara-U; 3 = DXI.

The mean recovery of ara-C was 95.5% at 20 ng/ml and 99.6% at 10 μg/ml; the mean recovery of ara-U was 91.3% at 100 ng/ml and 100.8% at 100 μg/ml. Three to eight assays were carried out at each concentration. Calibration curves for ara-C and ara-U (ratios of the peak areas ara-C/DXI or ara-U/DXI to concentrations) were linear from 10 ng/ml (a.u.f.s. = 0.005) to 100 μg/ml (a.u.f.s. = 2.0); at least three points were used for each curve. The average values for slope, intercept at the $y$-axis, and coefficients of correlation for low-dose and high-dose calibration curves are shown in Table I.

The within-day coefficients of variation for ara-C and ara-U calculated from ten repeated injections were, respectively, 7.4 and 7.0% at 0.1 μg/ml and 3.1 and 2.9% at 100 μg/ml. The between-day variability ($n = 7$) was 5.6% for ara-C (100 ng/ml) and 9.9% for ara-U (500 ng/ml).

Lyophilization or injection of a larger amount of ultrafiltrate did not improve the limit of detection for ara-C. The stability of ara-C after sample collection was tested by measuring the mean recovery of 200 ng/ml in plasma at different concentrations of THU (final concentration $10^{-3}$ and $10^{-4}$ $M$) and at different storage temperatures (−20°C and −70°C). After storage for six months with different methods, no loss of the parent compound was noted. Samples were thus stored at −20°C with final THU concentrations of $10^{-4}$

TABLE I

DATA FOR THE LOW-DOSE AND HIGH-DOSE CALIBRATION CURVES

Values are mean ± S.D.

| Compound | *n* | Slope | Intercept at the *y*-axis | Correlation coefficient |
|---|---|---|---|---|
| *Low doses*[a] | | | | |
| Ara-C | 16 | 0.7 ± 0.09 | 0.007 ± 0.01 | 0.9991 ± 0.001 |
| Ara-U | 16 | 0.16 ± 0.04 | 0.007 ± 0.02 | 0.9994 ± 0.0009 |
| *High doses*[b] | | | | |
| Ara-C | 7 | 0.0394 ± 0.001 | 0.01 ± 0.02 | 0.9994 ± 0.0004 |
| Ara-U | 5 | 0.0325 ± 0.002 | 0.0002 ± 0.012 | 0.9994 ± 0.0001 |

[a] 75 mg/m$^2$ per 24 h and 40 mg/m$^2$ per 2 h.
[b] 2–3 g/m$^2$ per h.

TABLE II

PLASMA AND CSF ARA-C LEVELS WITH DIFFERENT DOSES AND SCHEDULES

CSF and plasma samples, at the dosage of 75 mg/m$^2$, were collected at steady state; with the other doses, plasma and CSF samples were taken immediately before the end of the infusion.

| Dose and schedule | *n* | Concentration (mean ± S.D.) (ng/ml) | |
|---|---|---|---|
| | | Plasma | CSF |
| 75 mg/m$^2$ per 24 h | 14 | 44 ± 17 | 9 ± 3 |
| 40 mg/m$^2$ per 2 h | 5 | 153 ± 42 | 19 ± 8 |
| 2 g/m$^2$ per 2 h | 2 | 8240 | 1000 |
| 3 g/m$^2$ per 1 h | 5 | 30 210 ± 7230 | 1980 ± 1070 |

*M*. This stability was confirmed also in samples from patients with lower ara-C concentrations (15–30 ng/ml) tested again after six months.

Tests for the detection of possible interfering peaks were done with endogenous compounds and drugs commonly administered to patients with acute leukaemia. No interference was found with uric acid, hypoxanthine, xanthine, cytidine, cytosine, deoxycytidine, uridine, deoxyuridine, MTX, folinic acid or routinely prescribed antiemetics (alizapride, chlorpromazine, promazine). Allopurinol and its metabolite oxypurinol elute with ara-U and ara-C, respectively. For this reason, we substituted uricozyme for allopurinol in patients at risk for renal damage being treated with ara-C. In order to eliminate interference by 6-MP, which elutes at the same time as ara-U, samples were collected at least 8 h after 6-MP administration.

With continuous infusion, ara-C reaches a steady-state level in plasma and CSF ca. 30 min and 1 h, respectively, after the start of infusion [17]. Ara-C

seems to cross the blood–brain barrier easily [5,18] and CSF-to-plasma ratios ranging from 0.1 to 0.6 have been reported [5,17,18].

We used the technique described here to measure CSF levels of ara-C in leukaemia patients treated with different doses of the drug and according to different schedules. Table II shows the mean CSF ara-C levels found with doses ranging from 75 mg/m$^2$ per 24 h to 3 g/m$^2$ per h. These findings show that, when 3 g/m$^2$ ara-C are infused over 1 h, the mean CSF drug concentration attains the level of 1.98 $\mu$g/ml (8.15 $\mu M$).

These levels are close to the maximum desirable ara-C concentration in plasma; the capability of ara-CTP formation is, in fact, saturated in leukaemia blasts at ca. 10 $\mu M$ [19]. In addition, ara-C has a considerably longer half-life in the CSF (ca. 2 h) than in plasma [5]. It is, therefore, possible that even lower doses of ara-C given by rapid infusion may achieve an area under the drug concentration versus time curve in the CSF adequate to exert a therapeutic effect. However, combined clinical and pharmacokinetic studies in a larger group of patients are needed to confirm this hypothesis.

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