Journal of Chromatography, 223 (1981) 371—378
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 808

DETERMINATION OF 1-β-D-ARABINOFURANOSYLCYTOSINE AND 1-β-D-ARABINOFURANOSYLURACIL IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received September 16th, 1980; revised manuscript received December 2nd, 1980)

SUMMARY

A method is described for the determination of 1-β-D-arabinofuranosylcytosine (Ara-C) and its metabolite 1-β-D-arabinofuranosyluracil (Ara-U) in human plasma. After deproteinization of the plasma sample, separation is performed by reversed-phase liquid chromatography.

For Ara-C concentrations exceeding 0.05 mg/l and for Ara-U concentrations exceeding 1 mg/l, injection volumes of 100 μl are applied. For lower concentrations an injection volume of 500 μl is used. Ara-C is detected at 280 nm with a lowest detection limit of 0.002 mg/l in plasma. Ara-U is detected at 264 nm with a lowest detection limit varying from 0.01 to 0.1 mg/l in plasma. This variation is caused by an unknown substance with the same elution properties as Ara-U and which appears to be present in plasma in variable concentrations. The coefficient of variation of the whole procedure is about 6% for Ara-C concentrations above 0.005 mg/l and for Ara-U concentrations above 0.1 mg/l. For lower concentrations the coefficient of variation is about 14%.

INTRODUCTION

The pyrimidine analogue 1-β-D-arabinofuranosylcytosine (Ara-C) is one of the most effective drugs in the treatment of acute non-lymphocytic leukemia [1,2]. After administration, Ara-C is inactivated rapidly by deamination to 1-β-D-arabinofuranosyluracil (Ara-U). Both compounds are eliminated simultaneously by renal excretion [3,4]. Plasma concentration curves of Ara-C as a function of time vary considerably after the same intravenous dose in different patients. Since treatment results have been related to the plasma concentrations achieved [5—7], a suitable method for the determination of the Ara-C concentration in plasma is required in order to optimize therapy. For detailed pharmacokinetic studies, measurement of both Ara-C and Ara-U is required.

Various methods have been used for the determination of Ara-C. Among them are an enzymatic assay [8], bioassays [7,9,10], radioimmunoassays [11,12], gas chromatography [13], and determination of radioactivity after administration of labeled Ara-C [3,4]. Most of these methods are rather insensitive or not suitable for routine application. Recently, several methods for the determination of Ara-C by high-performance liquid chromatography (HPLC) have been reported [14—16]. At concentrations far below the detection limits of these procedures, Ara-C is still biologically active [17—19]. Therefore these procedures are not adequate for proper pharmacokinetic studies.

This paper describes an HPLC procedure for the determination of very low concentrations of Ara-C and Ara-U in plasma.

EXPERIMENTAL

Chemicals

Cytidine (Cyd), 5-methylcytidine (5-CH_3-Cyd), deoxycytidine (dCyd), uridine (Urd), and deoxyuridine (dUrd) were purchased from Sigma, St. Louis, MO, U.S.A. Ara-C was purchased from Upjohn, Kalamazoo, MI, U.S.A., and Ara-U from Calbiochem, San Diego, CA, U.S.A. Tetrahydrouridine (THU) was generously supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, MD, U.S.A. All other chemicals were purchased from E. Merck, Darmstadt, G.F.R. All chemicals were of analytical grade.

Blood sample preparation

Blood samples of 5 ml were collected in heparinized tubes, containing 50 μg of THU to prevent deamination of Ara-C [20]. After centrifugation (10 min, 2000 *g*, 4°C) plasma was collected and then ultrafiltrated through conical ultrafilters (Centriflo F 25, Amicon) by centrifugation (30 min, 800 *g*, 4°C) in order to remove most of the protein. To remove small molecular weight proteins still present in the ultrafiltrate, 1 ml of ultrafiltrate was mixed with 50 μl of ice-cold 8 *M* perchloric acid and the mixture was placed immediately in an icebath. After 10 min, precipitated protein was removed by centrifugation (10 min, 2000 *g*, 4°C). To 1 ml of the supernatant were added 100 μl of 4 *M* dipotassium hydrogen phosphate. The precipitated potassium chloride was removed by centrifugation. The term blank plasma is used for plasma samples taken before Ara-C administration.

HPLC equipment

The HPLC equipment exists of a Pye Unicam LC 3-XP pump, an injection valve (Rheodyne Model 7120) equipped with a 500-μl loop, and two reversed-phase columns (Nucleosil 10 C-18, 250 × 4.6 mm I.D., particle size 10 μm) linked together to improve separation (see HPLC procedure). Two UV detectors are used: a UV III monitor LDC 1203 with a 10-μl flow cell and a fixed wavelength of 280 nm (Ara-C), and a Pye Unicam LC-UV with an 8-μl flow cell and a variable wavelength set at 264 nm (Ara-U).

HPLC procedure

A 500-μl sample of deproteinized plasma is injected into the two linked reversed-phase columns. Elution is carried out at a constant flow with 0.2 *M* potassium dihydrogen phosphate, adjusted to pH 2.0 with phosphoric acid. Helium gas is led through the eluent to prevent development of air bubbles at the low pressure side. The detection of Ara-C appears to be optimal at 280 nm, whereas for the detection of Ara-U 264 nm is more appropriate (Fig. 1). Peak heights are used for the quantitation of the assay. Ratios of the absorption at 280 nm to that at 264 nm are used to detect impurities underlying the peaks of Ara-C and Ara-U.

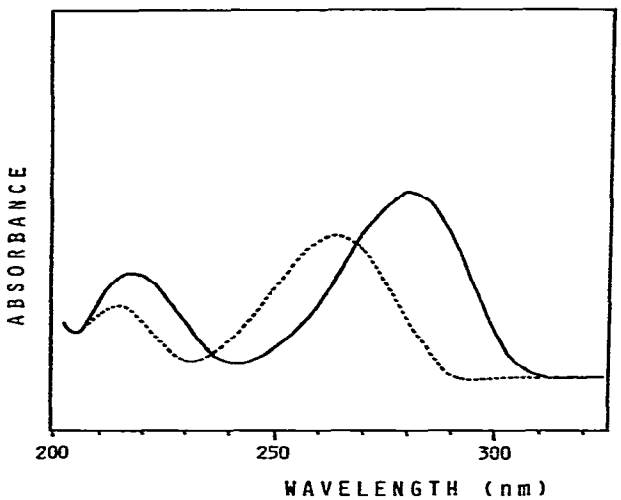

Fig. 1. UV spectrum of Ara-C (——) and of Ara-U (— — —) at pH 2.0

For concentrations of Ara-C above 0.05 mg/l and of Ara-U above 1 mg/l an injection volume of 100 μl was used, and separation on a single column proved to be sufficient. The maximal run-time for the complete separation of a 500-μl sample with the two-column system is 45 min, whereas separation of a 100-μl sample with the one-column system can be carried out in 22 min.

RESULTS

To prevent dilution of the plasma samples, most protein is removed by ultrafiltration. Thus, further precipitation can be carried out with a small volume of perchloric acid. This procedure combines maximal deproteinization with minimal dilution (1 ml of the injection sample equals 0.866 ml of plasma).

Deproteinization of the plasma samples led to a recovery of Ara-C and Ara-U of 92 ± 8% (n=20). This is in agreement with the observation of Van Prooijen et al..[21] that 13 ± 2% of Ara-C is bound to plasma protein.

Chromatographic separation

The chromatogram of a standard mixture of Ara-C, Cyd, dCyd, 5-CH_3-Cyd,

Ara-U, Urd, dUrd and uric acid shows that Ara-C and Ara-U can be separated from their analogues (Fig. 2). Fig. 3 depicts the chromatograms of a blank plasma sample, spiked before deproteinization with Ara-C (final concentration 0.006 mg/l) and with Ara-U (final concentration 0.24 mg/l) for which the detection was at 280 nm (Fig. 3a) and at 264 nm (Fig. 3b), respectively.

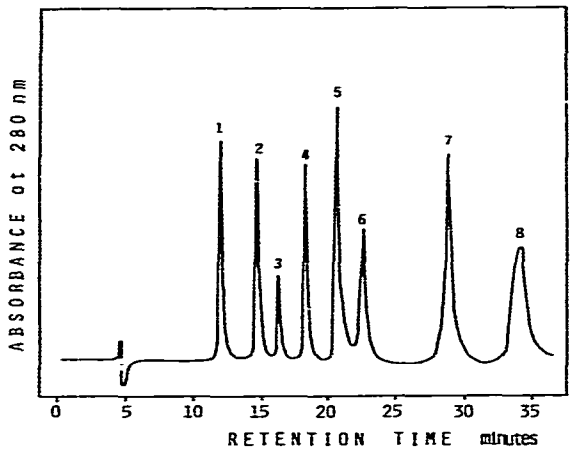

Fig. 2. Separation of a standard mixture of Ara-C and Ara-U and some of their analogues by HPLC. Injection volume 500 µl, separation on two-column system (see Methods), detection at 280 nm. 1=Cyd, 2=Ara-C, 3=dCyd, 4=5-CH_3-Cyd, 5=Urd, 6=uric acid, 7=Ara-U, 8=dUrd.

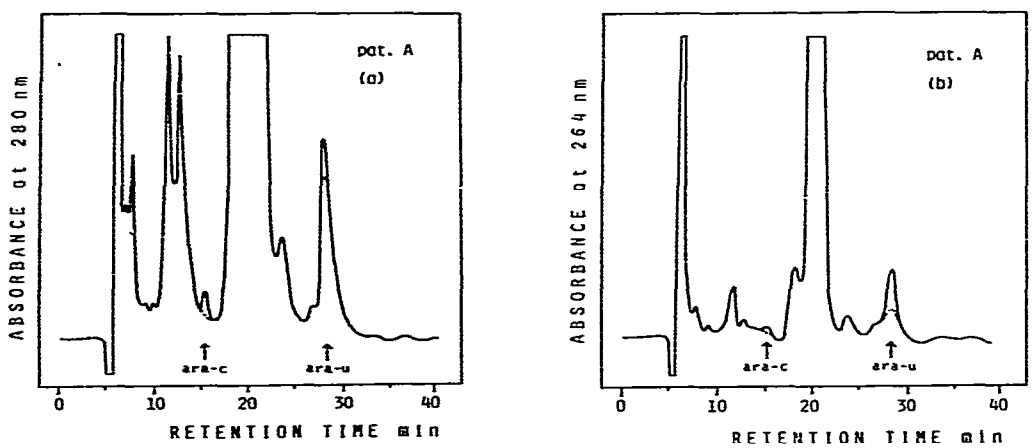

Fig. 3. High-performance liquid chromatogram of blank plasma of patient A spiked with Ara-C (0.006 mg/l) and Ara-U (0.24 mg/l). Injection volume 500 µl, separation on two-column system, detection at 280 nm, 0.002 a.u.f.s. (a) and at 264 nm, 0.005 a.u.f.s. (b). Dotted lines indicate chromatogram without Ara-C and Ara-U.

Blank plasma of patients with acute leukemia reveals great differences. In Fig. 4 the chromatograms of blank plasma of two patients are shown. All plasma samples appeared to be free of compounds interfering with the detection of Ara-C, but several plasma samples contain a substance with the same elution properties as Ara-U. The concentration of this compound in plasma

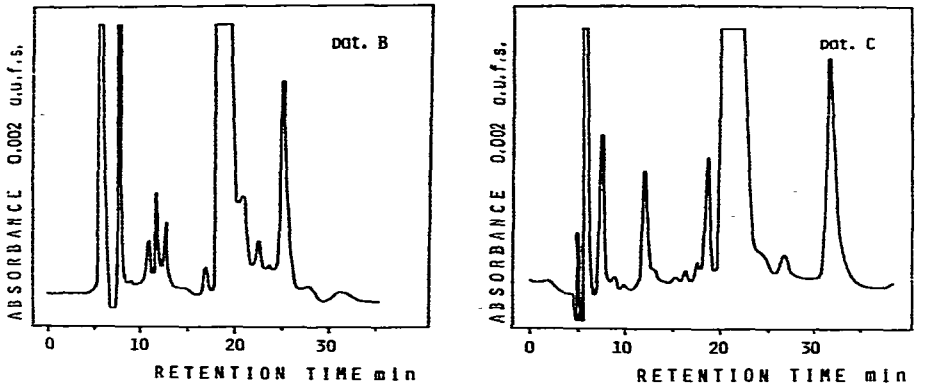

Fig. 4. Chromatograms of blank plasma of patients B and C. Conditions as in Fig. 3. Detection at 280 nm, 0.002 a.u.f.s.

of individual patients varies considerably (see Figs. 3a and 4) and accounts for the variable detection limit of Ara-U in plasma. Since the ratio of the absorption at 280 nm and at 264 nm is different for Ara-U and this compound (see Fig. 3), the influence of this compound on the determination of Ara-U can be eliminated mathematically.

Calibration curves and accuracy

Calibration curves of Ara-C and Ara-U are linear and pass through the origin. Detection limits in plasma are 0.002 mg/l for Ara-C and about 0.1 mg/l for Ara-U. The detection limit of Ara-U is estimated to be 0.01 mg/l when the interfering compound is absent. The relative coefficient of variation for the determination of plasma concentrations, including the deproteinization step, ranges for Ara-C from 14% at the detection limit to 6% at higher concentrations. Detailed information about accuracy at different concentrations is given in Table I.

TABLE I

COEFFICIENT OF VARIATION IN THE DETERMINATION OF ARA-C AND ARA-U

Concentration (mg/l)	*n*	Coefficient of variation (%)
Ara-C		
0.1	10	6
0.02	6	4
0.005	9	14
0.002	8	12
Ara-U		
0.4	5	7
0.1	7	14
0.05	8	12

Application

Fig. 5 illustrates the concentration curves of Ara-C and Ara-U after an intravenous bolus injection of 100 mg/m² in a patient with acute leukemia. Ara-C is eliminated rapidly and Ara-U rapidly reached maximal values. Elimination of Ara-U is a relatively slow process with a half-life time of 360 min. Also the concentrations of Ara-C and Ara-U could be monitored during infusion of a very small amount of Ara-C as is shown in Fig. 6. This patient received 17 mg of Ara-C in a 1-h infusion. This led to a concentration of Ara-C of 0.07 mg/l during infusion. A rapid decline was measured after cessation of the drug administration. Ara-U reached equilibrium within 20 min; elimination after infusion was again much slower than the elimination of Ara-C.

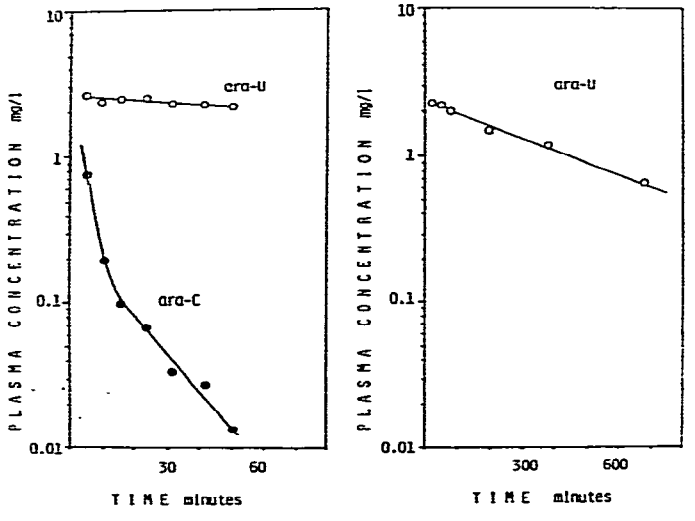

Fig. 5. Plasma concentration—time curves of Ara-C (•) and Ara-U (○) after an intravenous bolus injection of 100 mg/m² in a patient with acute leukemia.

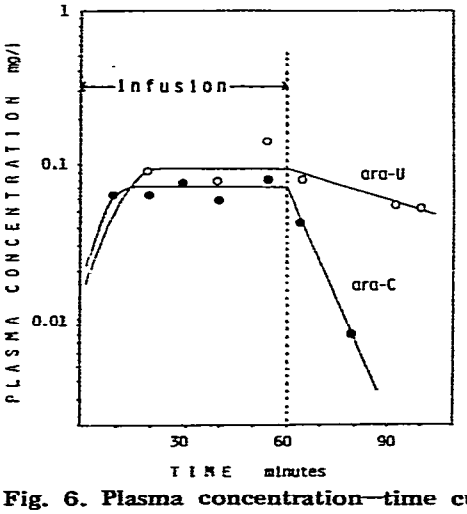

Fig. 6. Plasma concentration—time curves of Ara-C (•) and Ara-U (○) during and after infusion of 17 mg of Ara-C (10 mg/m²).

DISCUSSION

Several studies in patients with acute non-lymphocytic leukemia treated with Ara-C indicate that treatment results are correlated with differences in pharmacokinetics [5—7]. Adjustment of dosages in individual patients requires a rapid and reliable method for the determination of Ara-C and its metabolite Ara-U.

All previously described methods of measuring Ara-C exhibit more or less disadvantages. Radioimmunoassays may be disturbed by endogenous nucleosides, which are sometimes present in the plasma of patients with leukemia in very high concentrations [22]. This objection may also hold for bioassays. Furthermore, the application of bioassays is limited since Ara-C is often used in combination with other cytostatic drugs. Both radioimmunoassays and bioassays require a calibration curve and since these curves are linear over only a small concentration range, appropriate dilutions have to be made. This makes these procedures time-consuming and laborious. Gas chromatography combined with mass spectroscopy, although very sensitive, is difficult to perform routinely. Radioactive drug administration requires special precautions to prevent environmental contamination. All these objections can be overcome by the use of HPLC. However, the detection limits of the HPLC procedures described until now are higher than those of other methods. The HPLC procedure described in this paper offers a rapid and reliable method for the determination of very low concentrations of both Ara-C and Ara-U in plasma.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. J.M. Lintsen for packing the columns, and to Mrs. G. Vierwinden for skilful assistance. This study was supported by a grant from the Queen Wilhelmina Foundation, The Netherlands Organisation for Cancer Research.

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