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DETECTION AND SEPARATION OF INTRACELLULAR 1-β-D-ARABINOFURANOSYLCYTOSINE-5-TRIPHOSPHATE BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An ion-pair high-performance liquid chromatographic method, using a reversed-phase C_{18} column, was developed to provide an isocratic, sensitive, fast and reproducible separation of intracellular 1-β-D-arabinofuranosylcytosine-5-triphosphate and its measurement at a low limit of 5 pmol by ultraviolet absorbance at 280 nm with a coefficient of variation lower than 10%. A rapid separation is achieved by using a backflush procedure at 16 min and the retention time is 14 min.

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INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine, Ara-C) is a well-established antimetabolite drug in the treatment of acute leukaemia. Its antileukaemic activity depends on the intracellular phosphorylation to 1-β-D-arabinofuranosylcytosine-5-triphosphate (Ara-CTP), which acts as a DNA polymerase inhibitor and by incorporation into the DNA molecule. These mechanisms lead to the inhibition of DNA synthesis and chromosome damage with subsequent cell death. Recent investigations by Plunkett et al. [1] revealed a close correlation between the intracellular Ara-CTP metabolism and the clinical response to Ara-C treatment in patients with acute leukaemia. Prolonged Ara-CTP retention was found to indicate a higher rate of complete remission and longer remission duration [2,3]. Similar findings were also reported by other groups, emphasizing the impact of Ara-CTP measurements in antileukaemic therapy with Ara-C [4,7]. Investigations of Ara-C metabolism are also carried out to identify possible mechanisms of drug resistance [8–13].

In order to make these findings applicable to the clinical management of patients with acute leukaemia, rapid, reliable and sensitive methods are needed especially for the measurement of intracellular Ara-CTP concentrations. Current high-performance liquid chromatographic (HPLC) methods for Ara-CTP determination are hampered by several limitations: (1) they are sufficiently sensitive only when radiolabelled Ara-CTP is used [14,15]; (2) they require gradient elution for the measurement of non-tritiated Ara-CTP [12,13,15,16]; (3) they are time-consuming [16].

In view of these disadvantages it was the aim of the present study to develop a sensitive, isocratic, reproducible and fast technique for the separation and measurement of Ara-CTP in biological material. The sample clean-up was designed to be simple and the resolution of the Ara-CTP peak to be greater than 1.5, in order to achieve a reproducible identification and quantitation without using radiolabelled material. Stimulated by recent advances in the separation of nucleotides by ion-pair chromatography, the attempt was made to apply ion-pairing for the separation of Ara-CTP [17].

EXPERIMENTAL

Materials

Ara-CTP, CTP, dCTP and all other nucleotides were obtained from Sigma Chemie (Deisenhofen, F.R.G.), water, acetonitrile (HPLC quality), KH_2PO_4 and H_3PO_4 from Baker (Gross-Gerau, F.R.G.) and Unisep Mini-cent-10 ultrafiltration cartridges from Bio-Rad (Munich, F.R.G.).

HPLC apparatus

The chromatographic system consisted of a Waters M 510 solvent-delivery system, a Model 710A sample processor (Wisp), an M 441 UV absorbance detector (adjusted to 280 nm) and a data module M 730 recorder. For the backflush procedure a Rheodyne valve 7000 with a sample loop from 1 to 5 (Model 7040) was used. The Rheodyne valve was driven by a 5701 pneumatic actuator, applying a 12 VDC solenoid valve kit for the automatic control.

Separation was obtained with a Macherey & Nagel Nucleosil 120 Å, 5 μm, octadecyl column (250 mm$\times$4.6 mm I.D.). A cartridge system (30 mm$\times$6 mm$\times$4 mm) from Macherey & Nagel was used as a guard column, with the same stationary phase. In addition, a precolumn was inserted between the pump and the injector, to increase the life of the analytical column [18]. A 250 mm$\times$4.6 mm I.D. column, dry-filled with 40 μm C_{18} material (Baker) was used. The three columns and the Rheodyne valve were heated at 40°C with an incubator.

Chromatographic conditions

Optimal results were obtained under the following conditions: the mobile phase was an aqueous solution containing 0.1 *M* KH_2PO_4, 0.005 *M* tetrabutylammonium phosphate (Pic A low UV; Waters) and 0.5% acetonitrile (pH 2.7, adjusted with H_3PO_4). The column temperature was 40°C. The optimal flow-rate was chosen by means of the Van Deemter equation and was 0.8 ml/min for the stationary phase used. The duration of this flow-rate was 16 min; the flow direction was then reversed through the analytical column with the Rheodyne valve (backflushing) and the flow-rate was increased to 1.4 ml/min till the end of separation at 25 min, in order to elute strongly retained substances, such as ATP, GTP and UTP, in a short time.

Sample clean-up procedures

The cells used for the respective HPLC measurements were leukaemic blasts from patients or cells from the leukaemic cell line K562. Although the latter cells did not require separation or enrichment, blasts from patients were subjected to a density gradient separation over Ficoll (400 g) for purification and enrichment. After Ficoll separation, blasts were washed three times in isotonic sodium chloride. Then a known volume of isotonic sodium chloride (200–300 μl) was added to the cell pellet. From this solution 20 μl were removed for the determination of the cell count. For extraction, the same amount of eluent (mobile phase) was added to the cells corresponding to the isotonic sodium chloride, which was removed. For 10 min the cells were repeatedly vortex-mixed and subsequently centrifuged for 5 min at 1000 *g*. The supernatant was removed and membrane-ultracentrifuged with Mini-cent-10 ultrafiltration cartridges. These cartridges were pre-rinsed with 300 μl of HPLC water. The injected volume varied between 10 and 200 μl, depending on the amount of cells.

Quantitative analysis

Quantitation was performed according to the external standard method by plotting the peak height against known concentrations of standards. The peak heights of known Ara-CTP concentrations in the sample were shown to correspond to those in aqueous standards, so aqueous standards were used for the calibration, which was adjusted with five data points every day.

Specificity

The specificity of the method was checked by injecting the eluent-soluble fraction of peripheral lymphocytes from three healthy volunteers and bone marrow from one healthy person. Additionally, peripheral blasts from four patients with acute myelocytic leukaemia, K562 cells as well as an aqueous solution of CTP, dCTP, ATP, ITP, UTP, GTP, GDP were tested as candidates for co-elution with Ara-CTP.

Recovery

The extraction recovery was determined by incubation of K562 cells with 50 ng/ml [^{3}H]Ara-C. After 1 h the cells were washed six times to remove all extracellular [^{3}H]Ara-C, and the activity of labelled intracellular Ara-C metabolites was measured by liquid scintillation counting. Subsequently, the intracellular [^{3}H]Ara-C metabolites were extracted and the activity of the supernatant was measured. The extraction recovery was defined as the ratio of activity in the supernatant to the cellular activity.

Recovery of sample clean-up and HPLC separation was determined by adding five different known Ara-CTP concentrations to $30 \cdot 10^6$ K562 cells. Each sample was split into three equal portions. Each portion was extracted and measured separately. The ratio of the average measured concentration and added concentration was defined as the recovery.

Detection limit

A peak with twice the height of the average noise level was taken as the detection limit.

Linearity

The linearity of the method was proven from 5 to 1200 pmol in samples and standards at eight known concentrations and evaluated by the linear regression analysis method.

Precision

To determine the measurement precision, ten analyses at four different concentrations of Ara-CTP in K562 cells were made once. Furthermore, we calculated the average, the S.D. and the coefficient of variation (C.V.) of the retention time of Ara-CTP in those forty separations on four different days.

To evaluate the day-to-day variation of Ara-CTP measurements a K562 cell sample with 1 μg/ml Ara-CTP was analysed on ten consecutive days, and the average S.D. and C.V. were calculated.

Identification of Ara-CTP

To identify Ara-CTP, UV absorbance ratios were used. In addition, fractions of the eluate of K562 cells incubated with [^{3}H]Ara-C were analysed.

Pharmacokinetic calculation

From patients with relapsed acute leukaemia under high-dosage Ara-C therapy, 20-ml blood samples were collected before and at 2, 3, 4, 5, 6, 7 and 12 h after the start of infusion. Peripheral blasts were separated by Ficoll centrifugation and treated as described above. AUC, $t_{1/2}$ and maximal Ara-CTP concentration values were calculated with the Topfit pharmacokinetic computer program [19,20].

RESULTS

Separation parameters

Under the conditions described, the following chromatographic parameters for the separation of Ara-CTP were obtained for a standard mixture containing CTP, Ara-CTP and dCTP (Fig. 1): k' (capacity factor) = 3.04; R (resolution) = 2.08 (CTP/Ara-CTP) and 1.67 (Ara-CTP/dCTP); N (effi-

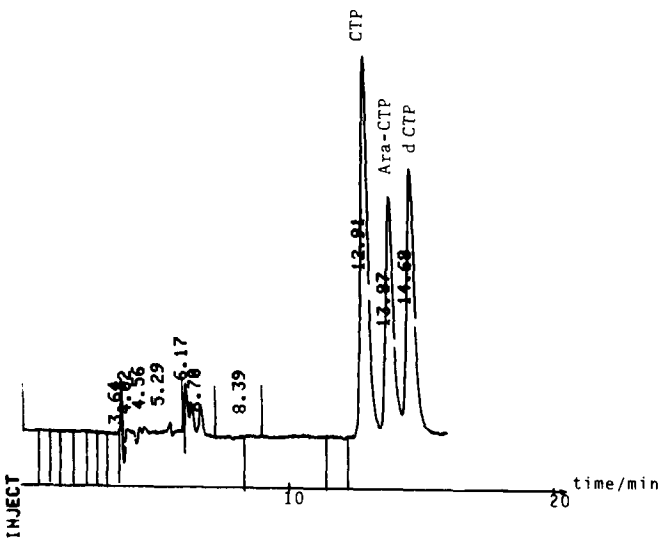

Fig. 1. HPLC separation of a standard mixture of CTP, Ara-CTP and dCTP (10-μl injection, 10 μg/ml Ara-CTP, 0.02 a.u.f.s.).

ciency) = 15 675; H (HETP) = 16 μm; T (tailing factor) = 1.6; u (linear velocity) = 0.12 cm/s.

These values were obtained using the following expressions:

$$k' = \frac{t_{R1} - t_0}{t_0}$$

$$R = 1.198 \frac{t_{R2} - t_{R1}}{w_{1/2(1)} + w_{1/2(2)}}$$

$$N = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2$$

$$H = \frac{L}{N}$$

$$T = \frac{B_{0.1}}{A_{0.1}}$$

$$u = \frac{L}{t_0}$$

where t_{R1} = retention time of Ara-CTP; t_{R2} = retention time of CTP or dCTP; t_0 = deadtime of guard and analytical columns; $w_{1/2(1)}$ = peak width at half peak height of Ara-CTP; $w_{1/2(2)}$ = peak width at half peak height of CTP or dCTP; L = length of column; $B_{0.1}$ = peak width at 10% peak height (to the right with respect to the perpendicular); $A_{0.1}$ = peak width at 10% peak height (to the left with respect to the perpendicular).

Specificity

No substance coeluted with Ara-CTP in any of the samples. After the addition of Ara-CTP to the samples a new peak, which corresponded to the retention time of Ara-CTP, was detected. Incubation of K562 cells with 10 μM Ara-C also showed a new peak at the same retention time (Figs. 2 and 3).

Recovery

Table I shows the recovery data of sample clean-up and HPLC separation at five different Ara-CTP concentrations, indicating an almost complete recovery at all tested concentrations.

Table II lists the recovery data for the extraction of intracellular metabolites of [^{3}H]Ara-C. The activity in the native cells was 283 640 cpm and in the supernatant extracted from the cells 271 588 cpm. The remaining extracellular [^{3}H]Ara-C activity of the isotonic sodium chloride was 322 cpm and the corresponding activity in the extracted cell pellet amounted to 296 cpm. This means that 96% of intracellular Ara-C metabolites were extracted.

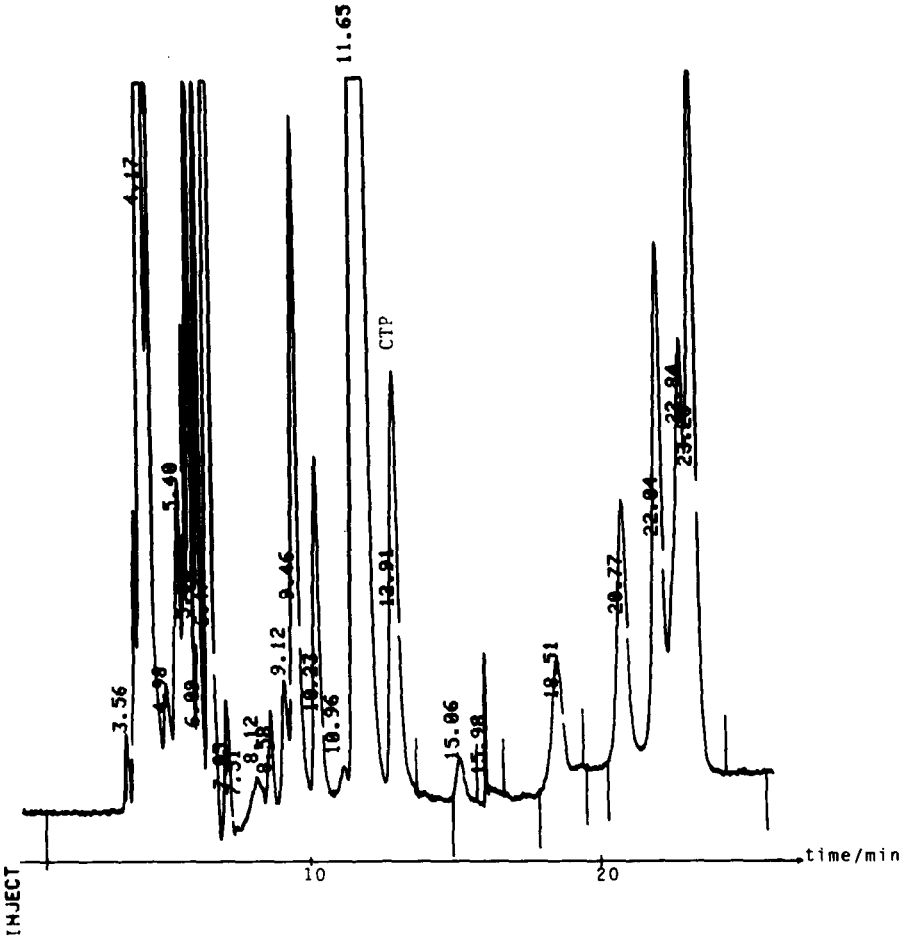

Fig. 2. HPLC separation of eluent-soluble material extracted from the equivalent of $5 \cdot 10^6$ K562 cells (0.01 a.u.f.s.).

Detection limit

The detection limit of intracellular Ara-CTP is 5 pmol. This peak is 5 mm high at 0.005 a.u.f.s. and is thus more than twice the height of the average noise level.

Linearity

Linearity was demonstrated in the range 5–1200 pmol with a correlation coefficient of 0.99. The regression analysis of these measured data resulted in a linear function of $y = 0.952x + 0.0286$.

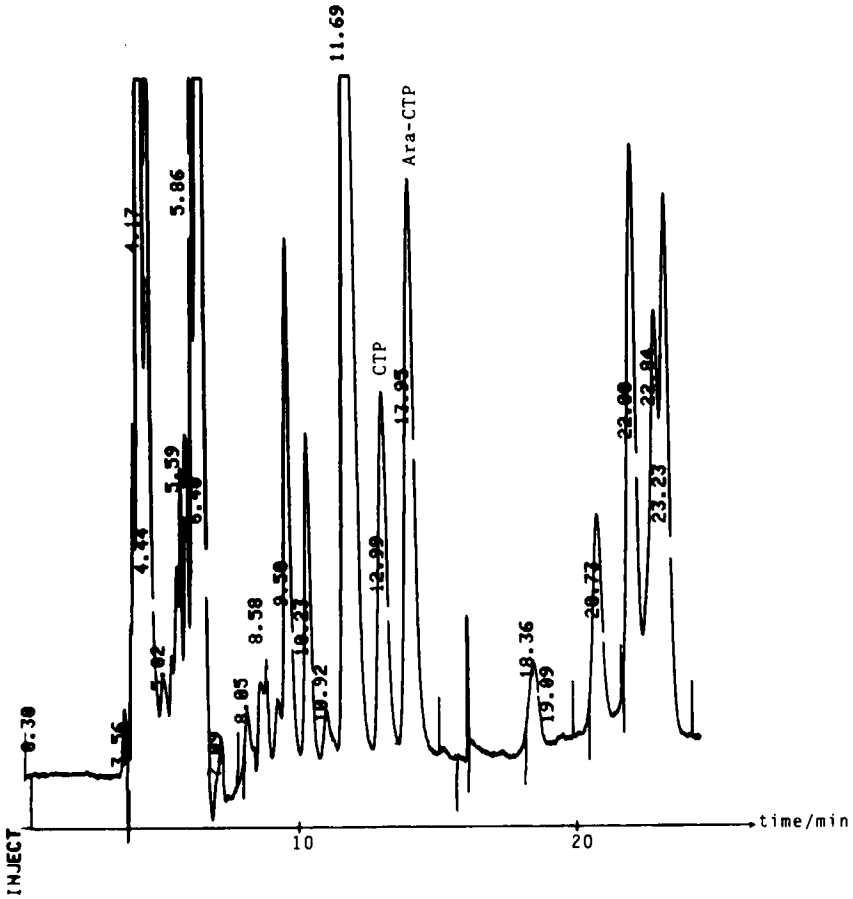

Fig. 3. HPLC separation of eluent-soluble material extracted from the equivalent of $5 \cdot 10^6$ K562 cells incubated with Ara-C (0.01 a.u.f.s.).

TABLE I

RECOVERY DATA OF SAMPLE CLEAN-UP AND HPLC SEPARATION AT FIVE DIFFERENT Ara-CTP CONCENTRATIONS

Concentration (μg per 10^7 cells)	n	Recovery (%)	C.V. (%)
10	3	101.0	0.75
5	3	102.0	7.0
0.5	3	99.2	3.4
0.1	3	99.8	20.0
0.0025	3	98.7	28.5

TABLE II

RECOVERY OF EXTRACTION PROVED BY [^{3}H]Ara-C-INCUBATED K562 CELLS

Ratio of the activity of [^{3}H]Ara-C metabolites in supernatant and pellet is defined as the recovery of extraction.

Sample	Activity of [^{3}H]Ara-C metabolites (cpm)
Pellet	283 640
Supernatant	271 588
Isotonic sodium chloride	322
Pellet after extraction	296

TABLE III

PRECISION OF Ara-CTP DETERMINATION AT FOUR DIFFERENT CONCENTRATIONS ($n=10$)

Concentration found (mean ± S.D.) (μg per 10^7 cells)	C.V. (%)
0.52 ± 0.04	7.6
1.32 ± 0.04	3.0
0.17 ± 0.01	7.0
4.25 ± 0.03	0.7

Precision

The within-day precision of measurements is indicated in Table III. The average retention time of Ara-CTP in these forty analyses was 14.19 min with S.D. = 0.14 min and C.V. = 1.0%.

The day-to-day measurement of the variation at the average Ara-CTP concentration of 1.048 μg/ml gave S.D. = 0.061 μg/ml and C.V. = 5.84%.

Identification of Ara-CTP

The UV absorbance ratio of Ara-CTP measured at 256 and 280 nm was 0.33 for the standard, as well as for pre-incubated K562 samples. The distribution of activity in the fractionated eluate of [^{3}H]Ara-C-incubated K562 cells was equivalent to the retention times of Ara-CMP, Ara-CDP and Ara-CTP (Fig. 4).

Intracellular Ara-CTP pharmacokinetics

Table IV and Fig. 5 show the pharmacokinetic data of intracellular Ara-CTP in blasts of two patients with acute leukaemia under high-dosage Ara-C therapy (1 g/m^2).

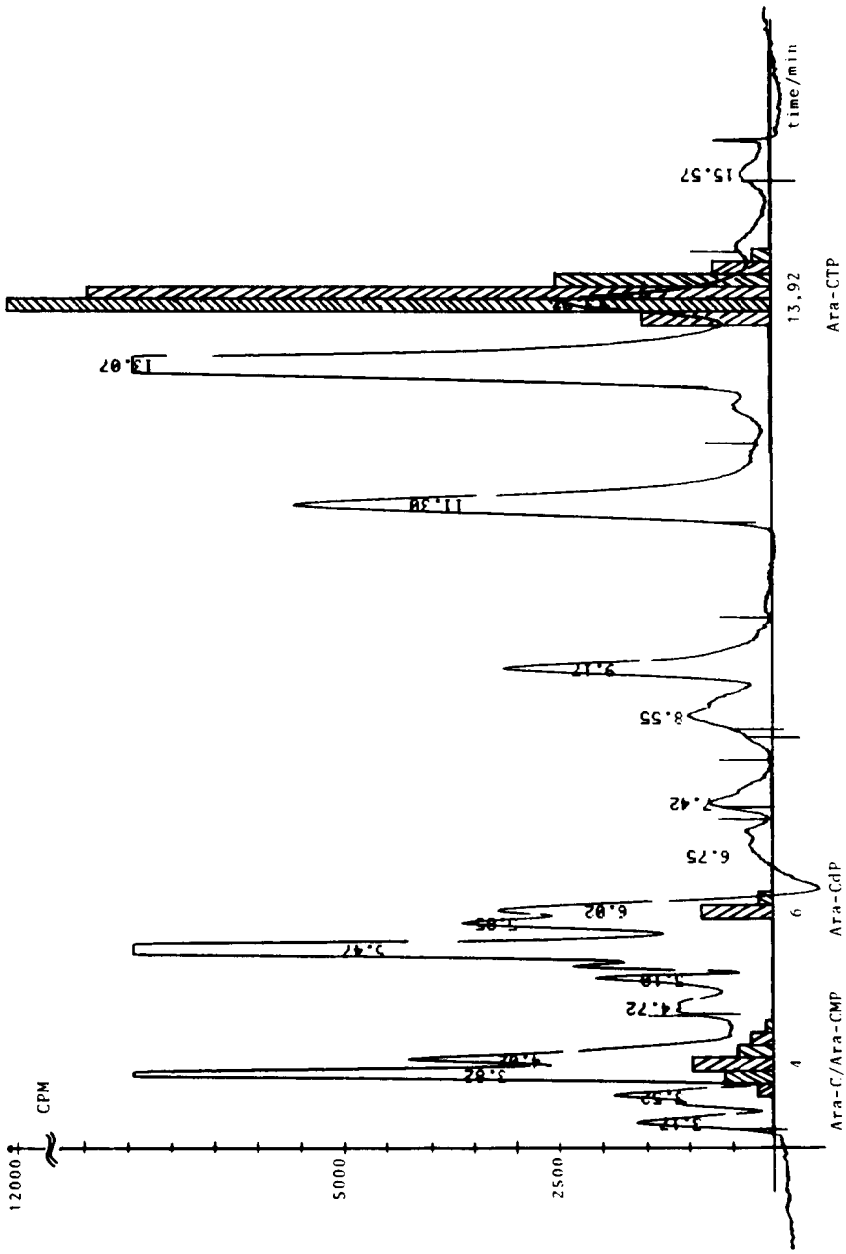

Fig. 4. HPLC separation of eluent-soluble material extracted from the equivalent of $5 \cdot 10^6$ K562 cells incubated with [^{3}H]Ara-C. The eluate was fractionated and measured in a liquid scintillation counter. The columns show the retention of the [^{3}H]Ara-C metabolites (0.01 a.u.f.s.).

TABLE IV

PHARMACOKINETIC DATA OF TWO PATIENTS

Dosage, 1 g/m^2 Ara-C by a 3-h infusion.

Patient	AUC (μg h/10^8 cells)	$t_{1/2}$ (h)	Max. Ara-CTP (μg per 10^8 cells)
1	32.1	7.63	2.66
2	27.71	2.22	4.93

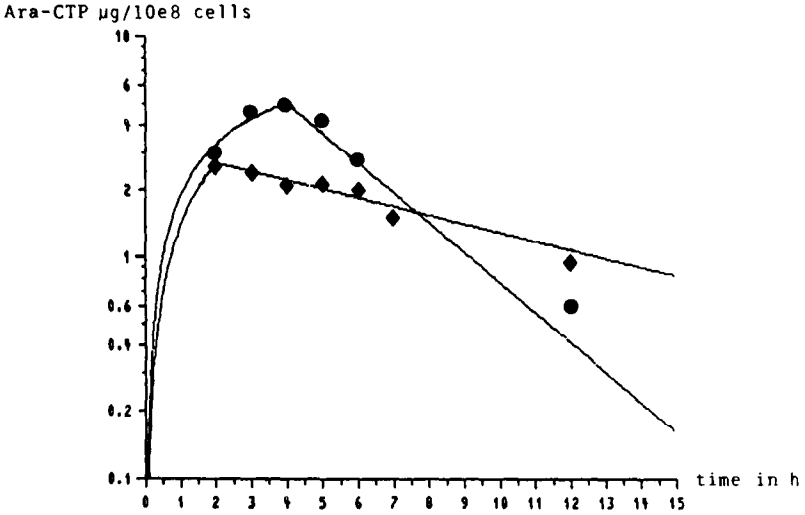

Fig. 5. Computer-fitted curves of intracellular Ara-CTP in leukaemic blasts for patients 1 (♦) and 2 (●).

DISCUSSION

HPLC separation and quantitative measurement of Ara-CTP has enhanced our understanding of the pharmacological mechanism of the cytostatic agent Ara-C. Recent data suggest that the kinetics of intracellular Ara-CTP have prognostic significance in the treatment of acute leukaemia.

In the present study we describe an ion-pair HPLC method for the separation of Ara-CTP. Our newly developed HPLC method for the measurement of Ara-CTP has a high sensitivity with a short analysis time, a simple clean-up procedure and high reproducibility. The isocratic elution does not require any re-equilibration time, and less technical equipment is necessary. Moreover, it is possible to detect [^{3}H]Ara-CTP, [^{3}H]Ara-CDP and Ara-CMP in in vitro experiments by fractionating the eluate. The method is suitable for the clinical

measurement of Ara-CTP during antileukaemic therapy, as well as for in vitro experiments with cell cultures in the laboratory.

The commonly used anion-exchange methods usually suffer from low sensitivity and a longer analysis time, and some of them require gradient elution. Isocratic anion-exchange methods have the disadvantage of a low resolution for the separation of Ara-CTP [5,15]. An ion-pairing chromatographic method introduced by Schilsky and Ordway [16] separates Ara-C, Ara-CDP and Ara-CTP but requires gradient elution and a long analysis time as well as a difficult sample clean-up procedure. Thus, the novel methodology described here represents a significant improvement over the established current techniques and should allow rapid routine monitoring of patients during Ara-C therapy.

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