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Detection and determination of the major metabolites of [^{3}H]cytosine arabinoside by high-performance liquid chromatography

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Abstract

An ion-pair high-performance liquid chromatographic assay involving solid-phase scintillation detection was established for the rapid identification and determination of all major metabolites of tritium-labelled cytosine arabinoside (Ara-C) in an in vitro system. In a single run of 50 min, Ara-C, Ara-CMP, Ara-CDP-choline, Ara-CDP, Ara-U, Ara-UMP, Ara-CTP, Ara-UDP and Ara-UTP can be measured. The method is fast, sensitive, with limits of detection ranging from 40 to 200 pg (absolute), and highly reproducible.

Keywords: Solid-phase scintillation detection; Cytosine arabinoside

1. Introduction

Cytosine arabinoside (Ara-C) is a widely used cytotoxic agent in the treatment of various haematological malignancies and forms the background of therapy especially in acute myeloid leukaemias (AML). In order to elucidate its mechanism(s) of action and to define the optimum dosage and schedule, the pharmacokinetics of this drug and some aspects of its intracellular metabolism have been extensively investigated [1,2]. Besides the kinetics of plasma concentrations, most investigations have been focused on the triphosphorylated metabolite of cytosine arabinoside (Ara-CTP), which is considered to constitute the major determinant of cytotoxicity. Several assays for the detection and determination of this substance have been published [3–5] and used to analyse Ara-CTP pharmacokinetics in relation to clinical response. These studies indicated that, unlike the parent drug Ara-C itself, the intracellular concentration and/or retention of Ara-CTP was correlated with therapeutic response [6]. From these studies it was also concluded that doses of 0.5–1.0 g/m^2 of Ara-C are sufficient to saturate the phosphorylation-regulating enzyme deoxycytidine kinase (dCK) and that further dose escalation would only increase the toxicity to other organ systems and not enhance the antileukaemic ac-

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tivity. In contrast to these data, a prospective comparative evaluation of 1.0 vs. 3.0 g/m^2 of Ara-C clearly indicated a superior response to a high-dose therapy in selected patients with AML refractory to conventional therapies and strongly suggested that additive mechanisms must be involved in Ara-C-mediated cytotoxicity [7–11]. One potential mode of action could be the impairment of cell membrane integrity through the interaction with phosphatidylcholine formation [12,13]. Hence deeper insights into the intracellular metabolism of Ara-C are needed to elucidate further its mechanisms of action.

In order to provide a more exact and more specific evaluation of the intracellular metabolism of Ara-C, a highly sensitive and specific high-performance liquid chromatographic (HPLC) assay involving tritium-labelled Ara-C ([^{3}H]Ara-C) was developed for the determination of all major intracellular metabolites of Ara-C. An in vitro system employing radioactivity was used in order to ensure sufficient specificity and sensitivity concerning up to nine different intracellular substances (including Ara-C itself). Using this assay, the individual profiles of HL60 and Raji cells regarding their formation of intracellular metabolites of Ara-C at a clinically relevant concentration of 1 μg/ml were evaluated.

2. Experimental

2.1. Materials

Tritium-labelled cytosine arabinoside ([^{3}H]Ara-C) was purchased from Amersham (Braunschweig, Germany). All nucleotides used as standards (purity ranging from 98 to 99%) and RPMI medium (supplemented with 10% foetal calf serum) were provided by Sigma (Deisenhofen, Germany). Water, salts and reagents were of analytical quality and were supplied by Merck (Darmstadt, Germany). All solvents (HPLC quality) were obtained from Baker (Gross-Gerau, Germany). Tetrabutylammonium phosphate (PIC A low UV) was purchased from Waters (Erkrath, Germany).

2.2. HPLC apparatus

The chromatographic system consisted of a Type 501 HPLC pump (Waters, Erkrath, Germany), a Type LP2 pulsation reducer (Macherey–Nagel, Düren, Germany), a 500-μl injection loop (Macherey–Nagel), a Model 5011 low-pressure mobile phase selection valve (Macherey–Nagel), a Rheodyne Model 7125 injection valve (Macherey–Nagel) and 1/16-in. HPLC capillaries. As stationary phases a 30 $\times$ 5 mm I.D. 5-μmC_{18} precolumn and a 250 4.6 mm I.D. 5-μm C_{18} reversed-phase column as the analytical column were used (both from Macherey–Nagel). A column heater (Jasco, Gross Baunstadt, Germany) was used to maintain the analytical column at 21°C. Detection of the radioactive signal was performed with a Ramona 92 scintillation counter (Raytest, Germany). For this application, a detector cell with a volume of 250 μl containing CaF_2 as a solid scintillator was chosen instead of a liquid scintillation cell. Analysis and quantification were performed on a personal computer (AT 286, Viking, Germany) employing the chromatography evaluation software NINA by Nuclear Interface (Münster, Germany), which also allowed automatic control of the flow gradient. While establishing the respective calibration graphs for each metabolite, Mark III liquid scintillation counter (Searle Analytic, Indianapolis, IN, USA) was also used.

2.3. HPLC conditions

Following injection of a given sample into the injection loop, switching of the injection valve enabled the pump to transport the substances across the precolumn on to the analytical column, which was maintained at room temperature by a column heater set at 21°C. Initially a weak eluent containing water with a concentration of 0.0025 *M* PIC A (also providing a 0.0001 *M* phosphate buffer) and 0.2% acetonitrile at pH 3.0 was employed at a flow-rate of 0.8 ml/min. A 5-μm filter was fitted between the injection valve and the analytical column in order to prevent microscopic particles from affecting the latter. After 18 min, the flow-rate was gradually in-

creased to 1.5 ml/min in the course of 1 min. This flow-rate was maintained for 27 min, resulting in a total of 45 min for one analysis. After 13.5 ml of the first eluent had passed through the system, it was followed by a stronger eluent consisting of water with concentrations of 0.1 *M* KH_2PO_4, 0.005 *M* PIC A and 0.5% acetonitrile at pH 2.7. For convenient handling of this procedure, the plastic tubes connecting the pumps to the bottles containing the eluents were adjusted to the exact length providing this volume. Therefore, switching of the eluents could be performed right at the start of the run with the strong eluent being preceded by exactly 13.5 ml of its weaker counterpart. Nine minutes before the end of the analysis the weak eluent was reconnected by switching the low-pressure valve. By these means, the 13.5-ml volume in the plastic tubing was filled with the weak eluent at the end of analysis and was available for the 10 min of re-equilibration of the initial chromatographic conditions at a flow-rate of 1.5 ml/min. After reduction of the flow-rate to 0.8 ml/min, a new measurement could be started.

2.4. Solid scintillation HPLC detector

[^{3}H]Ara-C and its metabolites were detected by a solid scintillation detector. The number of tritium-labelled molecules is decisive for the anticipated signal. Therefore, ideally a calibration graph for [^{3}H]Ara-C would be proportional to the calibration graphs of all its derivatives and need only be corrected for the different molecular masses.

This statement ignores the fact that some of the negatively charged molecules will most likely interact with CaF_2 (the solid scintillator) and thus be retained in the detector cell and contribute to the measured radioactivity for a longer time than a molecule that is able to pass through the cell without delay. This effect would produce some tailing of the chromatographic peaks of substances that are retained by CaF_2. This "tailing problem" is solved by the use of a liquid scintillation counter which is not influenced by this particular chemical interaction.

2.5. Liquid scintillation counter

In the case of the Mark III 6880 liquid scintillation counter, a fluid sample featuring β-emission (such as tritium) is mixed with an organic phase (Inst Gel II Plus; Packard) which will respond to excitation by β-particles by emitting flashes of light, which can then in turn be detected by the counter itself. Corresponding to the situation involving the solid scintillation detector, the signal of the counter is proportional to the number of emitting particles in the sample. Therefore, a certain number of tritium-labelled Ara-C molecules will result in the same signal as the same number of labelled Ara-CTP molecules. Considering the relative molecular masses of these compounds the calibration graphs for liquid scintillation detection can therefore be interchanged. Quantification of a known substance in a sample is therefore feasible.

When establishing this assay, calibration graphs for each substance using solid scintillation detection were needed in order to allow the quantification of the respective metabolites. In the case of Ara-C which is available as a radioactive compound, a regular calibration graph could be derived from measurements of aqueous standards using the solid scintillation detector. In the case of the other compounds, an alternative method had to be used. During measurements of cell samples following incubation with tritium-labelled Ara-C, regular chromatograms via the solid scintillation detector were taken and at the same time on-line sampling of the eluent containing the respective front of the substance was performed, the cut-off point being the time when the baseline was reached again. In this scenario, all derivatives of [^{3}H]Ara-C were formed intracellularly during the incubation process. Peaks were first identified via the chromatogram but then quantitated using liquid scintillation detection of the on-line samples. The amounts of substance found during liquid scintillation detection were then correlated with their respective areas under the peaks (AUP) during solid scintillation detection, producing excellent correlations for all substances involved in more than twenty such experiments. The tailing problem

was thus "circumvented" since liquid scintillation is not influenced by any possible interaction between the different metabolites and CaF_2.

2.6. Peak identification

In order to determine the order of elution and to establish the exact retention times of Ara-C and its metabolites, a UV detector was installed immediately prior to the solid scintillation detector when developing this assay. With Ara-C, Ara-CTP and Ara-U being available as aqueous non-radioactive (and in the case of Ara-C also as tritium-labelled) standards, the retention times of Ara-CMP, Ara-CDP and Ara-CDP-choline were approximated by their cytidine analogues CMP, CDP and CDP-choline. Previous investigations had already shown [5] that the cytidine derivatives only slightly precede the respective Ara-C derivatives under nearly identical conditions. These findings could be reproduced in this setting. The relative positions of Ara-UMP, Ara-UDP and Ara-UTP in the order of elution were established by their respective uridine analogues UMP, UDP and UTP, which are commercially available. Measurement of this solution using the UV detector will demonstrate the order of elution of all the substances involved with only a slight distortion of the situation shown for the ^{3}H measurement due to the cytidine derivatives preceding their Ara-C counterparts. These differences in the respective retention times because of the minute chemical differences in the measured molecules have to be differentiated from the mere delay of an eluted front of a given substance that will result from its passage from the UV detector to the solid scintillation detector. This delay, which is identical for all substances, can be demonstrated as the difference between the retention times in the two respective channels for the three molecules of which there are both non-radioactive and tritium-labelled representatives, viz., Ara-C, Ara-U and Ara-CTP.

In order to present all these findings in a single graph, a complex measurement involving a mixture of an aqueous solution (containing Ara-C, CMP, CDP-choline, Ara-U, CDP, UMP Ara-CTP, UDP and UTP, plus uridine and dCTP as further markers) and a prepared sample of cells following incubation with [^{3}H]Ara-C (therefore containing the tritium-labelled equivalents of these substances) was performed.

2.7. Cell incubation

Raji, Hl60 and K562 cells were used for validation of this assay; $0.5 \cdot 10^{6}$ cells/ml RPMI (cell culture medium) were exposed to a concentration of 1 μg/ml of [^{3}H]Ara-C for 5 h. A temperature of 37°C and a pCO_2 of 5% were maintained as standard conditions.

2.8. Sample preparation

Following incubation, the cells were centrifuged at 400 *g*, the supernatant was removed and the cell pellet was resuspended in 0.9% NaCl. After this cleaning procedure, the cells were again centrifuged with consecutive removal of the aqueous solution. Cells were lysed by adding 120 μl of the weak eluent [containing amphoteric tetrabutylammonium phosphate (pH 3.0)] to the pellet. Under these conditions, cell membranes were disrupted and enzymatic degradation of Ara-C metabolites was inhibited. After vigorous vortex mixing and final centrifugation of the sample, the eluent was separated from the cell debris and was then filtered through a Millipore 5-μm filter in order to prevent microscopic particles from affecting the HPLC apparatus. The sample was then frozen at −20°C until analysis.

3. Results

3.1. Specificity

With detection depending on radioactivity, no co-eluting substances but metabolites of [^{3}H]Ara-C were detected. [^{3}H]Ara-C was shown to have a retention time of 5.24 min and that of deaminated metabolite [^{3}H]Ara-U 13.34 min. The phosphorylated metabolites [^{3}H]Ara-CMP, [^{3}H]Ara-CDP and [^{3}H]Ara-CTP had retention times of 7.04, 14.68 and 23.50 min, respectively.

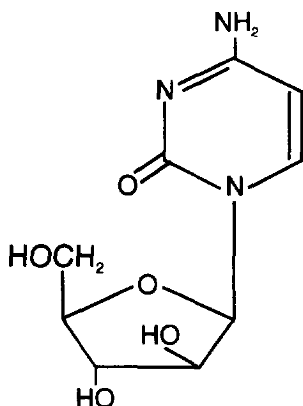


Fig. 1. Structure of cytosine arabinoside (Ara-C).

[^{3}H]Ara-CDP-choline was detected at 7.78 min, which is 0.52 min later than its cytidine equivalent CDP-choline. A similar finding was observed for [^{3}H]Ara-UMP, which was preceded by UMP also by 0.52 min. The delay between the detection of an eluted substance by UV detection and by solid scintillation detection was calculated via the differences in the retention times of Ara-C by UV detection and [^{3}H]Ara-C by solid scintillation detection and amounted to 0.40 min. Nearly identical delays could be demonstrated for Ara-U (0.46 min) and Ara-CTP (0.48 min). A representative chromatogram illustrating these findings is shown in Fig. 1.

3.2. Intra- and inter-day precision and recovery

Following incubation, an aliquot of the cells was first washed and then measured by liquid scintillation counting without any prior sample preparation, thereby representing the total radioactivity contained in the cells. Sample preparation was then performed as described above and an aliquot taken for measurement of the amount of tritium-labelled molecules. An average of 85% of the total radioactivity was thus shown to remain in the cell extract, which means that an average of 15% of the initial radioactivity was lost during the sample preparation process.

In order to demonstrate the intra- and inter-day precision of the assay, ten cell incubations and measurements were performed on a single day for the former and another ten on ten consecutive days for the latter. Following incubation, the cells were pooled and then again separated into ten distinct samples in order to differentiate the variances resulting from sample separation and from the HPLC assay as such from variations due to the incubation conditions. The coefficients of variation representing both intra-day and inter-day precision are given in Table 1 and range from 9.8% as intra-day precision for Ara-CDP-choline to 21.4% for Ara-C.

3.3. Limit of detection

A signal twice the noise was defined as the limit of detection (LOD) for substances producing narrow peaks, i.e. Ara-C, Ara-CMP, Ara-CDP-choline, Ara-U and Ara-UMP. The LODs were 40, 80, 70, 90 and 100 pg, respectively. For substances producing moderate tailing under the present conditions, i.e., Ara-CDP, a signal four times the noise was taken as the lower limit,

Table 1
Characteristics of the individual substances under the conditions of the described HPLC assay

Parameter	[^{3}H]Ara-C	[^{3}H]Ara-CMP	[^{3}H]Ara-CDP-choline	[^{3}H]Ara-U	[^{3}H]Ara-CDP	[^{3}H]Ara-UMP	[^{3}H]Ara-CTP
LOD (pg absolute)	40.0	80.0	70.0	90.0	100.0	100.0	110.0
AUP/pg	5.02	2.93	3.18	3.50	16.09	1.96	50.48
Correlation coefficient (r)	0.98	0.99	0.98	0.92	0.96	0.73	0.98
Intra-day precision (n = 10), C.V. (%)	21.4	16.4	9.8	17.7	13.2	11.4	12.0
Inter-day precision (n = 10), C.V. (%)	23.5	18.3	11.2	22.0	18.7	13.8	14.5
t_R (min)	5.24	7.04	7.78	13.34	14.68	20.28	23.50

resulting in an LOD of 100 pg for this particular molecule. In the case of Ara-CTP, which exhibited the most significant tailing, a signal-to-noise ratio of 6:1 was chosen, producing an LOD of 110 ng. Ara-UDP and Ara-UTP were detected in a small number of measurements and at very low concentrations. Since in these measurements they demonstrated chromatographic behaviour (tailing) nearly identical with that of their respective counterparts (Ara-CDP and Ara-CTP), similar LODs of 150 pg for Ara-UDP and 200 pg for Ara-UTP were defined.

3.4. Quantification

In the case of [^{3}H]Ara-C, a calibration graph using aqueous standards was combined with results of multiple cell incubations wherein [^{3}H]Ara-C had not only been detected by HPLC with solid scintillation detection but also had been determined via sampling and consecutive liquid scintillation counting. The excellent correlation ($r = 0.98$) of these results is shown in Fig. 2. A slope of 5.02 AUP/pg was found. Calibration graphs for the other substances (Fig. 3) were obtained in an identical fashion (initial quantification by liquid scintillation counting) resulting in slopes (AUP/pg) of 2.93 for Ara-CMP, 3.18 for Ara-CDP-choline, 3.50 for Ara-U, 16.09 for Ara-CDP, 1.96 for Ara-UMP and 50.48 for Ara-CTP. The correlation coefficients and variances for each substance are given in Table 1. No calibration graphs are given for Ara-UDP and Ara-UTP, which could only be detected at low concentrations in a small number of measurements. Quantification of these metabolites is therefore restricted to the determination of whether intracellular formation of Ara-UDP and Ara-UTP has resulted in concentrations above or below 1.5 ng/ml $\cdot 10^7$ cells and 2.0 ng/ml $\cdot 10^7$ cells according to their respective LODs.

3.5. Linearity

Since a linear relationship between the incubation concentration of Ara-C and the formation of

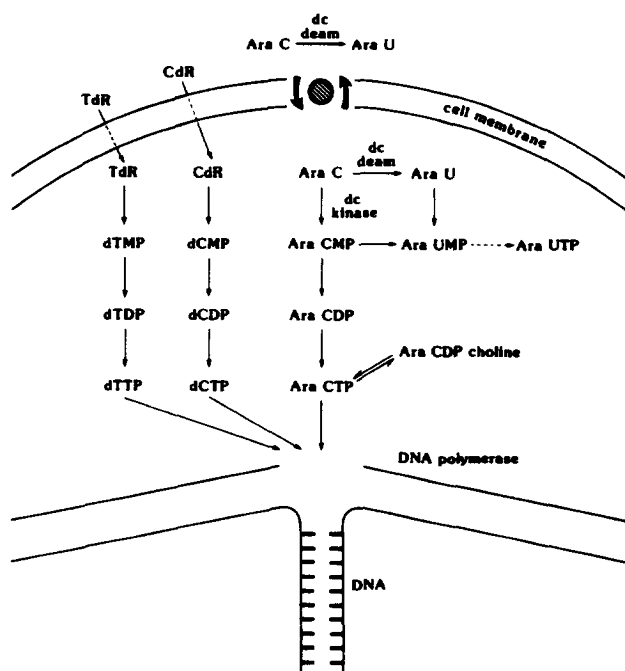
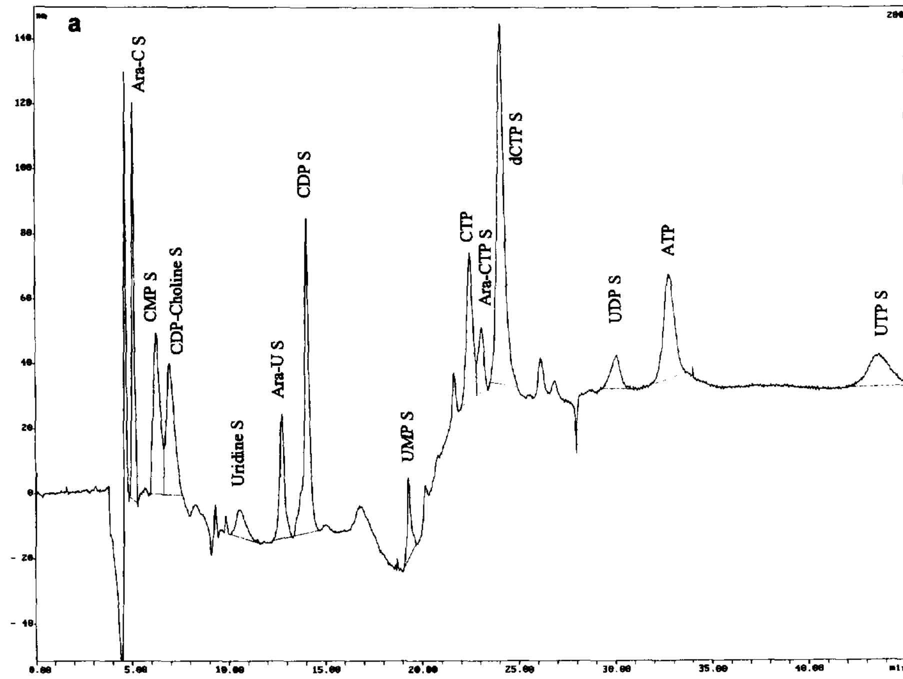


Fig. 2. Metabolism of Ara-C. Dc deam = deoxycytidine deaminase; Ara-U = uracil arabinoside; TdR = thymidine; CdR = cytidine; MP = monophosphate; DP = diphosphate.



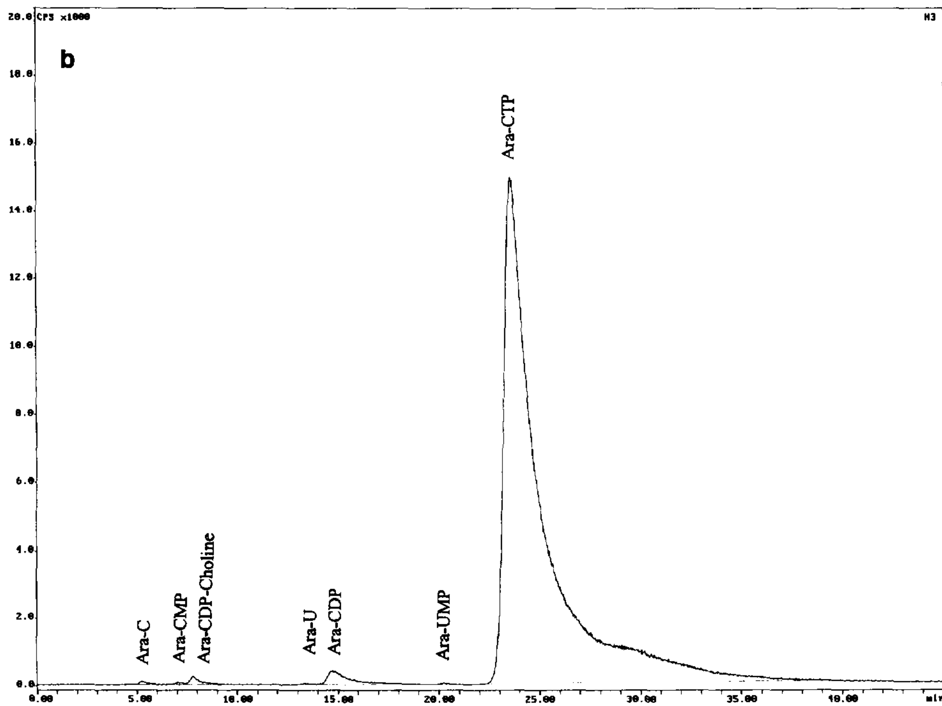


Fig. 3 (*continued on next page*)

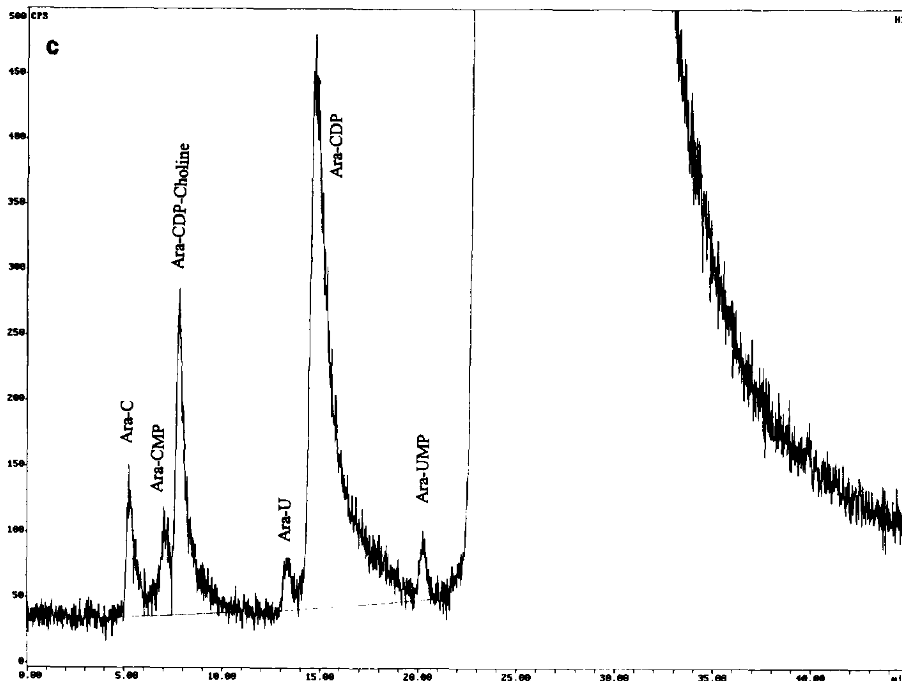


Fig. 3. (a) Chromatogram (UV detection at 280 nm) of $0.5 \cdot 10^6$ HL60 cells incubated with 1 μg/ml [^{3}H]Ara-C for 5 h spiked with Ara-C (3 μg), CMP (4.5 μg), CDP-choline (4.0 μg), uridine (1.0 μg), Ara-U (20.0 μg), CDP (25 μg), UMP (2.0 μg), CTP (5.0 μg), Ara-CTP (1.0 μg), UDP (2.0 μg) and UTP (2.0 μg), marked with S. (b) Simultaneous ^{3}H chromatogram with an ordinate ranging from 0 to 20 000 counts/s. (c) Simultaneous ^{3}H chromatogram with an ordinate ranging from 0 to 500 counts/s.

its metabolites does not necessarily exist, linearity of the HPLC analysis was demonstrated for each metabolite by defined dilutions (100, 50, 12.5 and 5% of the original sample) of two pooled samples (1 μg/ml [^{3}H]Ara-C for 5 h). Linear relationships between the measured amounts of the metabolites and their respective dilutions were found with correlation coefficients ranging from 0.98 to 0.99 [slopes (ng/percentage of initial amount): Ara-C 0.031, Ara-CMP 0.007, Ara-CDP-choline 0.042, Ara-U 0.009, Ara-CDP 0.029, Ara-CTP 0.166].

3.6. Intracellular Ara-C metabolism in HL60 and RAJI cells

The intracellular formation of [^{3}H]Ara-C metabolites is demonstrated in Figs. 4 and 5. For both cell lines measurements were made in triplicate. In HL60 cells an average of 93 ng/ml $\cdot$ 10^7 cells (range 85–102) of Ara-CTP were found with Ara-C mono- and diphosphates only reaching levels of 6 and 22 ng/ml $\cdot$ 10^7 cells (range 3–9 and 17–25), respectively. Ara-CDP-choline levels are of a similar order at 17 ng/ml $\cdot$ 10^7 cells (range 15–19).

In the case of Raji cells, moderately higher levels of Ara-CTP (139 ng/ml $\cdot$ 10^7 cells range 125–147) are found. In relation to the triphosphate, the mono- and diphosphates are distinctly lower at 7 (range 5–9) and 26 (range 22–29) ng/ml $\cdot$ 10^7 cells. Ara-CDP-choline levels are higher at 68 (range 57–74) ng/ml $\cdot$ 10^7 cells, reaching nearly 50% of the total amount of Ara-CTP. In both cell lines only minute amounts of Ara-UMP were found, with Ara-UDP and Ara-UTP remaining below the detection limit.

4. Discussion

Quantification of Ara-CTP via HPLC measurements has led to valuable insights into the

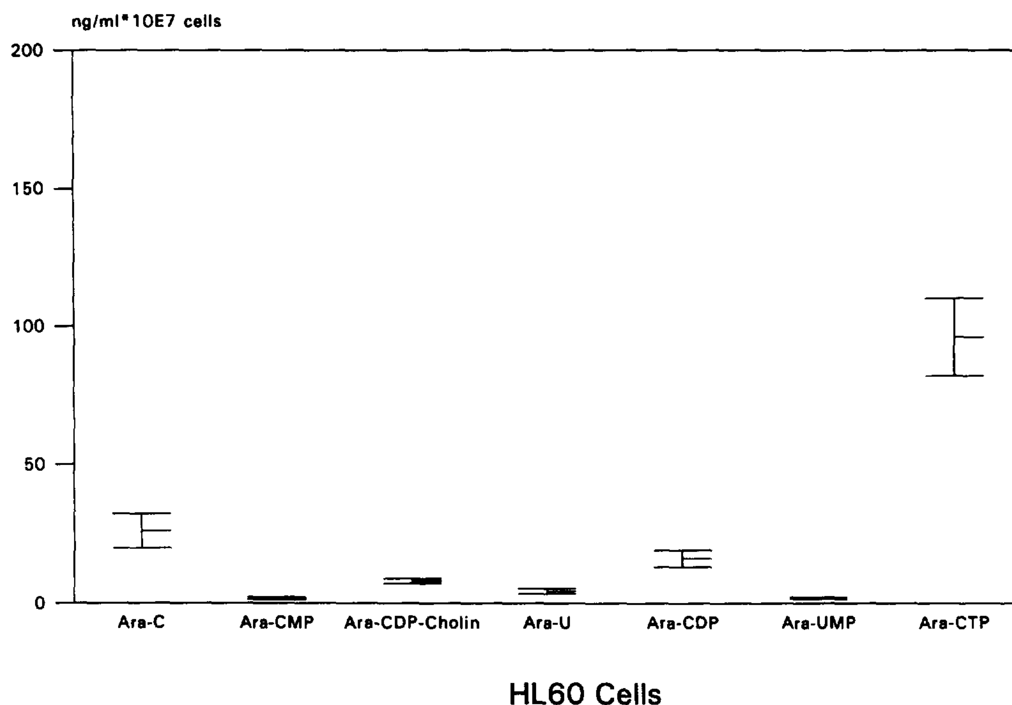


Fig. 4. Amounts (mean and standard deviation) of [^{3}H]Ara-C and [^{3}H]Ara-C metabolites in HL60 calls after incubation with 1 μg/ml [^{3}H]Ara-C for 5 h.

kinetics and mechanism of Ara-C cytotoxicity. Nevertheless, major issues such as the reason for the efficacy of high-dose Ara-C regimens and the membrane alterations during Ara-C treatment probably mediated by Ara-CDP-choline remain unclear. To address these topics, a more specific evaluation of the intracellular metabolism of Ara-C is required. The present assay was developed in order to provide a sensitive and exact quantification of all major Ara-C metabolites in an in vitro system using tritium-labelled molecules. Under these conditions, Ara-C and eight metabolites (Ara-CMP, Ara-CDP, Ara-CTP, Ara-CDP-choline, Ara-U, Ara-UMP, Ara-UDP and Ara-UTP) can be separated and determined in a single run. Two eluents of different strengths and a technically simple step gradient restrict the time needed for analysis to 45 min and an additional period of 10 min for re-equilibration. The limits of detection range from 40 pg (absolute) for Ara-C to 110 pg for Ara-CTP. Sample preparation is easy and yields a high recovery of 85%. The measuring process is convenient and robust, resulting in coefficients of variation between 9.8% for the intra-day precision for Ara-CDP-choline and 23.5% for the inter-day precision for Ara-C. The use of an HPLC scintillation detector facilitates the measurements and avoids the burdensome on-line sampling necessary in other assays [4,5]. The reason for the choice of a solid-phase scintillation detector was that early experiments had shown a higher sensitivity of this mode of detection compared with liquid scintillation detection when considering the peak dilution due to the large amounts of scintillation liquid needed for the latter technique. Interaction of the solid scintillator CaF_2 with the negatively charged phosphate moieties of Ara-CDP and especially Ara-CTP resulted in marked chromatographic tailing of these molecules. The increased AUP due to this certain amount of tailing was easily corrected by on-line sampling and quantification by liquid scintillation. Overall on-line sampling was only needed initially during this study in order to provide the presented calibration graphs for each substance which will enable any user of this method to quantitate his or her own measured results.

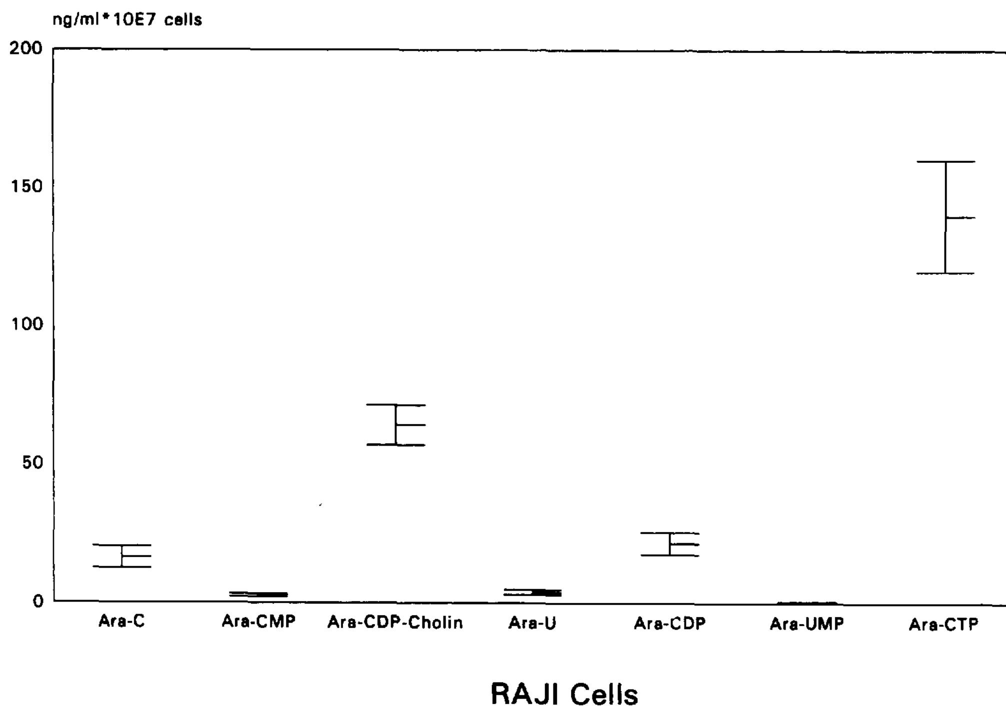


Fig. 5. Amounts (mean and standard deviation) of [^{3}H]Ara-C and [^{3}H]Ara-C metabolites in Raji cells after incubation with 1 μg/ml [^{3}H]Ara-C for 5 h.

The only other HPLC assay attempting to detect several Ara-C metabolites simultaneously, proposed by Schilsky and Ordway [4], lacks substantial validation, involves a cumbersome sample preparation and does not include the metabolite Ara-CDP-choline. So far, no other successful attempts for measurement of such a comprehensive range of intracellular Ara-C metabolites have been published. The present assay, therefore, represents a tool that will provide new scope in the investigation of the intracellular metabolism of Ara-C. Such a detailed and quantitative approach will be vital in addressing the issues of Ara-C cytotoxicity in a high-dose Ara-C regimen when deoxycytidine kinase is saturated and in establishing the role of Ara-CDP-choline in the perturbation of cell membranes during Ara-C treatment.

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