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A simple isocratic ion-pair high-performance liquid chromatographic determination of 1-β-D-arabinofuranosylcytosine 5′-triphosphate for intracellular drug-monitoring and *in vitro* incubation assays

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Abstract: A sensitive isocratic ion-pair HPLC method using a reversed-phase C_{18} column and phosphate buffer at pH 6 for the determination of the cytotoxic intracellular anabolite 1-β-D-arabinofuranosylcytosine-triphosphate (Ara-CTP) of the antineoplastic drug cytarabine in leukaemic cells *in vivo* is described. Simultaneous determination of the physiological nucleotide deoxycytidine-triphosphate is possible. The recovery from cells is >90%, the limit of detection is 25 ng ml^{-1} (51 nM l^{-1}) and about 10 pM mg^{-1} cell protein. Extraction procedure and pitfalls are discussed. The method enables intracellular drug monitoring of Ara-CTP with standard HPLC equipment.

Keywords: *Arabinofuranosylcytosine-triphosphate; leukaemia; intracellular drug monitoring; cytarabine metabolism.*

Introduction

1-β-D-Arabinofuranosylcytosine (cytarabine, Ara-C) is one of the most important cytostatic drugs in the treatment of acute myelogenous leukaemia [1–3]. Dosing and scheduling are still a point of intensive discussion [4–10].

Ara-C is an isomer of cytosine (Fig. 1). *In vivo* deamination by cytidine deaminase leads rapidly to the major metabolite uracil arabinoside (Ara-U; Fig. 1) [11, 12]. Studies of pharmacokinetic parameters of Ara-C in plasma did not show any significant correlation with response to therapy [13–15].

Cellular uptake of the pro-drug Ara-C by facilitated diffusion [16] and three-step phosphorylation resulting in the active anabolite 1-β-D-arabinofuranosylcytosine-triphosphate (Ara-CTP) [17, 18] are the preconditions of cytotoxic efficacy. Ara-CTP inhibits the DNA-polymerase by competing with the binding of deoxycytidine-triphosphate (d-CTP). Incorporation of Ara-CTP into DNA induces single strand breakage [19, 20]. The accumulation and duration of retention of this major intracellular metabolite as parameters of intracellular pharmacokinetics *in vitro*, as well as *in vivo*, are important determinants of therapeutic activity [21–26]. A correlation was found between Ara-CTP exposure of cells and

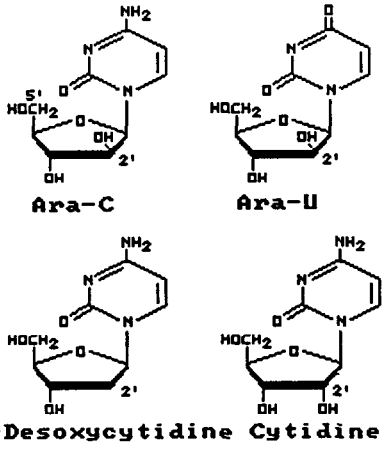

Figure 1
Structure of Ara-C and related nucleosides.

Ara-C incorporation into DNA [27, 21]. Therefore, intracellullar determination of Ara-CTP is gaining growing importance in the design of therapeutic schedules and optimizing dose intensity of Ara-C therapy [10, 22, 23, 28, 29]. The methods of determination previously described are almost done by HPLC on anion-exchange columns [30–34] and require a gradient-system [31–34], elevated column temperature [30, 35], detection at two UV wavelengths [32] or controlled valve-switching for backflush-procedure [35].

*Dedicated to Prof. Dr. med. G. Schellong for his 65th birthday.

Retention of the physiological nucleotide d-CTP is not described [31–33] or d-CTP co-eluates with other nucleotides [30]. The presented method requires only basic HPLC equipment to determine Ara-CTP in leukaemic blasts *in vitro* and *in vivo*. Additionally, it offers the possibility of measuring d-CTP levels.

Experimental

Materials and reagents

Ara-CTP and other nucleotides, anthranilic acid (A-ac) and tartaric acid were all purchased from Sigma (St. Louis, USA). Tetrabutylammoniumhydrogen sulphate (TBA), Na_2HPO_4, KH_2PO_4 (all from Serva, FRG), tetrahydrofurane (Merck, FRG), Lymphoprep™ (Nycomed, Oslo), RPMI-medium with 1-glutamine (Gibco, UK) fetal calf serum (Boehringer, FRG) and Millipore Ultrafree-MC (Millipore-Waters, FRG) for membrane filtration were used.

Apparatus

The HPLC system consisted of a LKB 2150 double piston pump, a Rheodyne 7125 injection valve (LKB 2154), a variable UV-detector (LKB 2140) and the integration system Nelson Analytical Software 4.1.

The stainless steel column (3.9 mm × 15 cm) packed with 4 μm reversed-phase C_{18} material (NOVA-PAK™, No. 86344) as well as the corresponding guard columns (Waters 15220 Guard Pak™ with NOVA-PAK™ material) were purchased from Millipore-Waters (FRG).

Cell specimens

The method was established and optimized with the promyelocytic cell lines K562, HL60 and nucleated cells from patients. These were extracted from heparinized bone marrow aspirates and peripheral venous blood samples for diagnostic punctures by standard Ficoll-Hypaque density centrifugation.

Preparation of cell extracts

At all steps of the extraction procedure tubes and solutions were kept on ice. After density centrifugation cells were washed three times, resuspended in a definite volume of isotonic phosphate buffer (PBS, pH 7.2) to perform cell counting in a Neubauer counter chamber and once more centrifuged (400 *g*, 10 min). Ten microlitres of an 1 M tartaric acid solution were added to the cell-pellet to enhance recovery.

The cells were then extracted with a definite volume (normally 200 μl) of a solution of 0.01 M TBA and 100 mg l^{-1} A-ac in 0.04 M phosphoric acid. Vortex-mixing and centrifuging were followed by neutralization of the supernatant with 1 M potassium hydroxide solution (KOH) (to pH 6–7) and the determination of the cellular protein (according to Bradford using Coomassie-Blue [36]). After membrane-filtration the sample extract was injected into the HPLC system.

HPLC procedure

Fifty microlitres of the probe were injected into the HPLC system. The mobile phase consisted of 0.09 M phosphate buffer (Na_2HPO_4–KH_2PO_4) adjusted to pH 6.0 with 0.35% tetrahydrofuran and 0.01 M tetrabutylammoniumhydrogen sulphate as ion-pair reagent. The buffer was continuously de-areated by helium gas. The flow rate was 1.4 ml min^{-1}.

The detector was set at 270 nm which was the maximum of the UV-spectrum in the mobile phase. Peak heights were used for quantification. Calibration curves with three concentrations of standard solution were made daily for external quantification. A-ac was

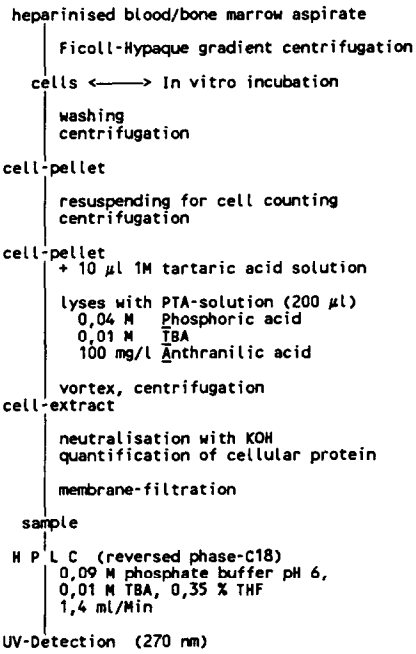

Figure 2
Assay procedure.

added as internal standard to estimate the total volume of the sample (pellet of known cell count + PTA-solution for lysis) and to calculate the extracted number of cells per ml sample. By this way the exact number of the injected cells was known without loss of cells by taking a definite volume of the (inhomogenous) cell-pellet.

Results and Discussion

The separation of CTP, Ara-CTP and d-CTP was expected to be the main analytical problem. Therefore, a mixture of these nucleotides in the mobile phase was used as standard sample. Figure 3 shows the separation of 1 μg ml^{-1} of each nucleotide under the prescribed conditions. Neither in extracts of HL60 and K562, cells nor in pretherapeutic samples from patients blood or bone marrow cells ($n > 10$) any interference at the retention time of Ara-CTP could be observed (Fig. 4). Figure 5 shows a mixture of extract of the same K562 celline and a standard solution at a final concentration of 0.25 μg ml^{-1} of Ara-CTP and d-CTP. Co-elution with the mono-, di- and triphosphates of uridine, guanosine, inosine, thymidine and adenosine could be excluded.

Standard curves

The limit of detection for all compounds was 25 ng ml^{-1} = 51 nM or 2.6 pM on column (50 μl sample loop).

The standard curve was tested to be linear between 3 pM on column (29 ng ml^{-1}) and >512 pM on column (6 μg ml^{-1}). Calibration curves obtained from peak height gave correlation coefficients of >0.998. The linear regression equitations were: amount (x) = 0.0545 × height (y) − 0.746 for CTP, x = 0.0555 × y − 0.872 for Ara-CTP and x = 0.0679 × y = 1.918 for d-CTP.

Reproducibility

To test the reproducibility the daily calibration curves (n = 12) with the concentrations of 0.2, 1 and 2.5 μg ml^{-1} of each nucleotide were evaluated and the relative standard deviation (RSD) according to peak height was <10% for these concentrations of the three nucleotides. Taking the daily calibration curves as a basis, the RSD of standard samples diminished to <4% at 1 μg Ara-CTP ml^{-1} (n = 12) and 7% at 0.2 μg Ara-CTP ml^{-1} (n = 6).

The correlation coefficient of the 3-point calibration curves forced through origin were always >0.996 for CTP, Ara-CTP and d-CTP (n = 12).

Calibration curves with two concentrations of A-ac were also performed daily. The day-to-day variation of a standard solution (33.3 μg ml^{-1}, i.e. one-third of the concentration of PTA-solution) injected at 14 days was 5.9% (RSD).

To determine the reproducibility of HPLC assay in cells, HL60 cells incubated in 1 μg Ara-C ml^{-1} RPMI-medium for 60 min were extracted, divided into three parts and injected. Determination on the same day led to intracellular concentrations with mean of 1493 pM/10 million cells (≡ sample concentration 0.53 μg ml^{-1}) and a RSD of 1%.

Intracellular concentration in cells incubated in Ara-C-containing medium were not influenced by additional (up to six) washing steps.

To determine the reproducibility of extraction procedure and HPLC assay cumulatively, K562 cells were incubated on 2 separate days. After 1 h incubation cells were split and three parallel extraction procedures were performed each day. The RSD of these six determinations of two incubations was 10.7% (1251 ± 134 pM 10^{-7} cells).

Recovery

The recovery was determined with suspensions of a pellet of 12.5 million cultured cells per ml, spiked to concentrations of 0.406 μg ml^{-1} (recovery 96.8 ± 5.6%), 1.35 μg ml^{-1} (86.2 ± 5.0%) and 2.03 μg ml^{-1} (95.9 ± 9.2%). Additionally, in 26.7 million cells separated from normal bone marrow and spiked to 0.3 μg ml^{-1} 98.8 ± 5.7% were recovered. Overall, this led to a mean recovery of 94.4% with a standard deviation of 8.2% and a RSD of 9%.

Influence of tartaric acid on the recovery

During the evaluation of the method low and variable recoveries were observed. Chromatograms of identical cells showed variable peak heights and patterns. Loss of Ara-CTP following repeated freezing and thawing was observed earlier [37] and did occur rapidly even at 4°C when denaturation of proteins with perchloric acid was not carried out [38].

Therefore, this variation was thought to be caused by enzymatic phosphatase activity.

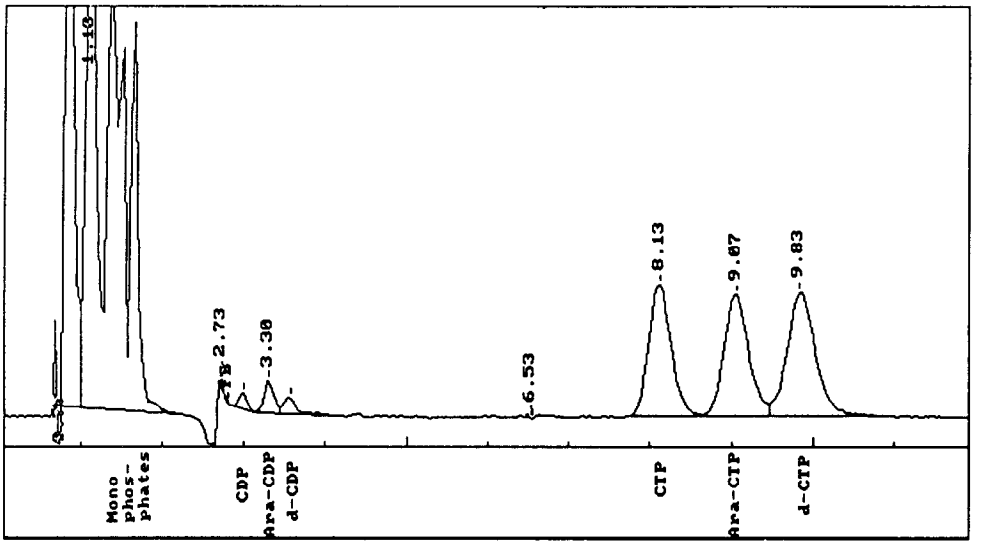

Figure 3
Chromatogram of the standard sample, 1 μg ml^{-1} of each nucleoside (full range 6 mV, offset −5 mV).

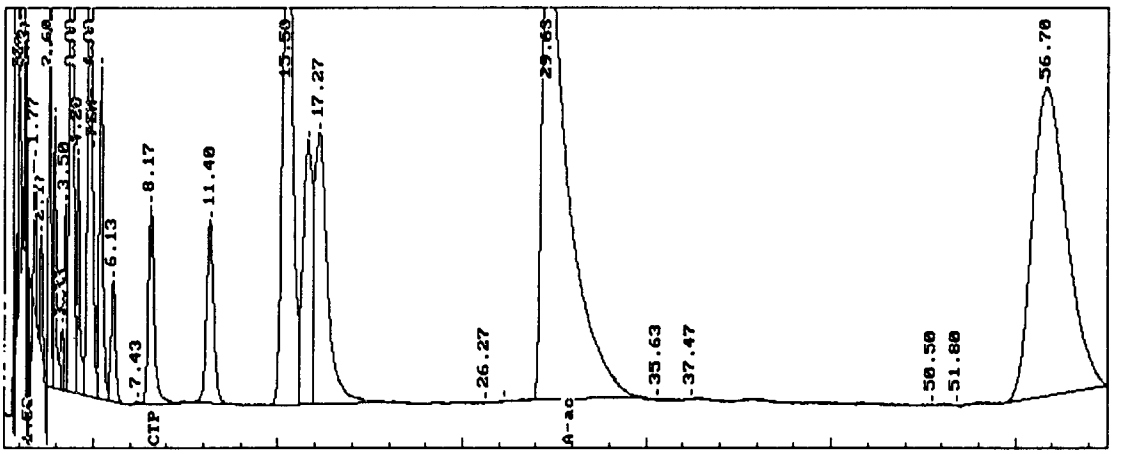

Figure 4
Chromatogram of the extract of K562 cells without Ara-CTP (full range 6 mV, offset −5 mV) ≈360,000 cells on column.

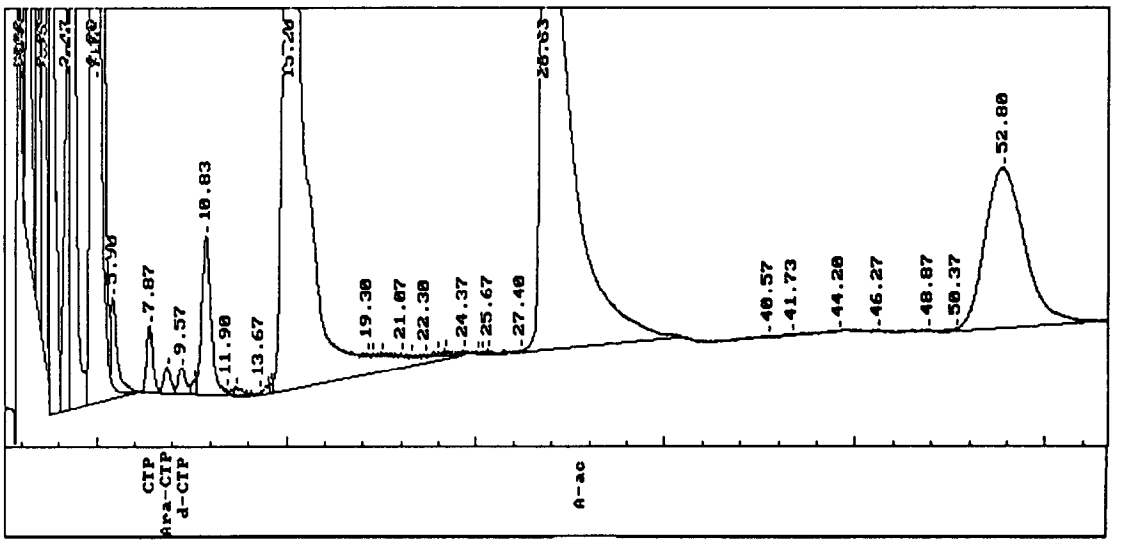

Figure 5
Chromatogram of K562 cells (Fig. 4) mixed with standard solution (0.25 μg ml^{-1} Ara-CTP and d-CTP final concentration).

These problems could principally be overcome by working on ice and adding tartaric acid solution to the cell-pellets before lysis. To evaluate both steps, cell pellets have been mixed with Ara-CTP solution and extracted. (Final concentrations were 1.3 μg Ara-CTP ml^{-1} and 12.5 million cells ml^{-1}.)

To show the potential inhibition of enzymatic processes by tartaric acid, cells were first extracted rapidly at room temperature. The recovery was 63 and 53% without tartaric acid. Adding tartaric acid 86% ± 8.7 (n = 5) of Ara-CTP could be recovered. Extraction procedure on ice without tartaric acid resulted in 81 and 79.8% recovery and adding tartaric acid resulted in 93 and 96% recovery, respectively.

The lower part of Fig. 6 shows the chromatogram of a K562 extract with addition of tartaric acid. The peaks with the retention times of ATP (52 min), inosine-triphosphate (16.3 min) and CTP (8.17 min) were high. Extracting an identical pellet without tartaric acid resulted in the upper chromatogram. The ATP, ITP and CTP peaks were lower and the height of adenosine-diphosphate with a retention time of 15.2 min was increased. The amount of cell-pellet was comparable, indicated by the equivalent protein content of the two samples.

The loss of triphosphates stops with separation of the supernatant from the cell membranes.

Therefore, there is evidence that enzymatic activity localized at the cell membranes was the reason for the irreproducible results in the beginning. This phenomenon has to be considered when working with methods without strong acids like trichloracetic acid or perchloric acid which destroy the cells and simultaneously denature the proteins.

Inhibition of this activity by tartaric acid and working on ice results in recoveries over 90%.

The described method to determine the intracellular Ara-CTP levels offers the possibility of quantitating the contents in relation to the cell count (pM/10 million cells) [25, 30] or the cellular protein (pM/mg protein) [28]. A third calculation as μM l^{-1} cell volume was used by Plunkett *et al.* [31].

Differences in cell volume, protein and nucleotide content result in difficulties to define sensible and comparable units. The limit of detection is variable in different cell specimens. In our studies with *in vitro* incubations of myeloid and lymphoblastic leukaemic blasts and *in vivo* intracellular drug monitoring in children with acute myeloblastic leukaemia the limit of detection was in order of ≈10 pM mg^{-1} cell protein and ≈10 pM 10^{-7} cells.

The method enables us to detect Ara-CTP as active anabolite of cytarabine in the leukaemic blasts under continuous infusion with 100 mg m^{-2}/24 h (data not shown). Dependent on cellularity, acceptable small volumes of blood (1–5 ml) are needed.

Requiring only standard HPLC equipment, this simple HPLC method may be useful for cooperative studies with a greater number of patients to monitor and study intracellular Ara-CTP kinetics.

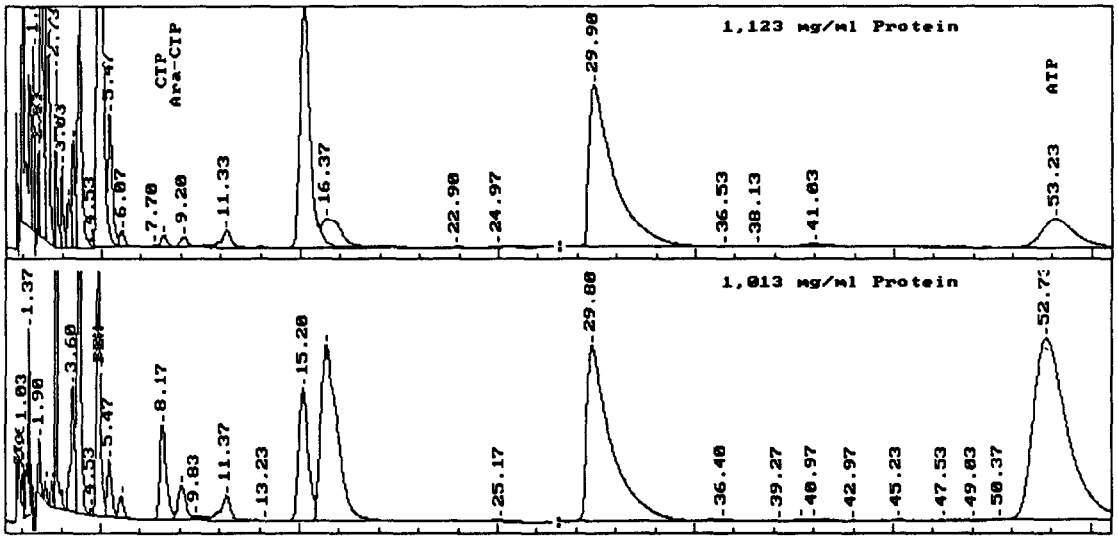

Figure 6
Extraction of K562 cells without (upper part) and with (lower part) tartaric acid solution.

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