

An ¹H NMR study of the cytarabine degradation in clinical conditions to avoid drug waste, decrease therapy costs and improve patient compliance in acute leukemia

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Cytarabine, the 4-amino-1-(β-D-arabinofuranosyl)-2(1H)-pyrimidinone, (ARA-C) is an antimetabolite cytidine analogue used worldwide as key drug in the management of leukaemia. As specified in the manufacturers' instructions, once the components—sterile water and cytarabine powder—are unpackaged and mixed, the solution begins to degrade after 6 hours at room temperature and 12 hours at 4°C. To evaluate how to avoid wasting the drug in short-term, low-dose treatment regimens, the reconstituted samples, stored at 25°C and 4°C, were analyzed every day of the test week by reversed-phase HPLC and high-field NMR spectroscopy. All the samples remained unchanged for the entire week, which corresponds to the time required to administer the entire commercial drug package during low-dose therapeutic regimens. The drug solution was stored in a glass container at 4°C in an ordinary freezer and drawn with sterile plastic syringes; during this period, no bacterial or fungal contamination was observed. Our findings show that an cytarabine solution prepared and stored in the original vials retains its efficacy and safety and can, therefore, be divided into small doses to be administered over more days, thus avoiding unnecessary expensive and harmful waste of the drug preparation. Moreover,

patients who require daily administration of the drug could undergo the infusion at home without need to go to hospital. The stability of the aliquots would help decrease hospitalization costs. *Anti-Cancer Drugs* XXX:000–000 Copyright © 2019 The Author(s). Published by Wolters Kluwer Health, Inc.

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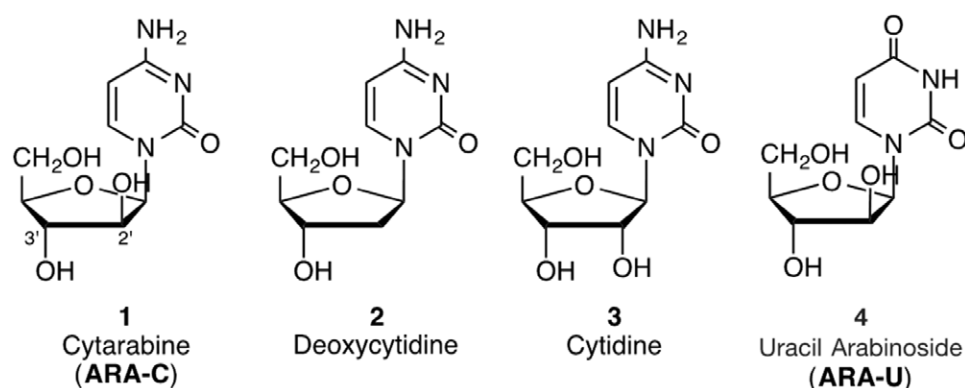
Introduction

Cytarabine (ARA-C) (1, Fig. 1) is used worldwide for the management of leukaemia in children and adults [1]. Particularly, pediatric and adult acute and lymphoblastic myeloid leukaemia, lymphomas, adult myelodysplastic syndromes require ARA-C treatments [2]. The WHO's List of Essential Medicines includes ARA-C among the most important medications needed in a basic health system [3,4], the American Society of Clinical Oncology has

stressed the crucial importance of this drug, while the USA Food Drug Administration recently announced a world shortage of its availability in the near future. Taken together, all these aspects underline the importance of a sparing use of this drug. Nonetheless, a remarkable waste does take place in the treatment of acute lymphoblastic leukaemia in children, where ARA-C is administered subcutaneously in small daily doses. At children's hospitals, during the induction phase (1b) of acute lymphoblastic leukaemia, children undergo four 4-day cycles of ARA-C (75 mg/sqm/d in monoadministration v. or s.c., on days 38–41, 45–48, 52–55, 59–62). Small aliquots of ARA-C must be administered subcutaneously as palliative treatment of acute myeloid leukaemia [5,6] and,

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Fig. 1



Structural formulas of cytarabine, physiological cytarabine-analogues. Deoxycytidine, cytidine and uracil arabinoside, a degradation product of cytarabine.

according to the commercial indications for the correct use of ARA-C, a new solution should be prepared every day [5,6]. Patients should go to hospital for a subcutaneous injection almost everyday, which has a negative impact on their compliance, quality of life, and family organization [7]. Accordingly, the remainder of the unused solution should be discharged and disposed of as hazardous waste, with additional costs. To avoid these drawbacks, optimize drug usage and improve clinical management, a study on the short-term stability (one week, i.e., the maximum time required for the consumption of a whole pack) of ARA-C, that is, was performed.

The rationale of our previous study[4] was to define the stability of the bortezomib molecule beyond an 8-hour interval; the purpose was to avoid discarding the residual drug not administered within the interval specified. It is worth underlining that, in 2003, the cost of a 3.5 mg vial of bortezomib was 1060€, that is, 302.85€/mg. Considering that, on average, patients were given 1.5 mg, the remaining 2 mg were discarded. The analyses carried out in our previous study showed the absence of degradation molecules in bortezomib resuspended for a week after its reconstitution and no evidence of contamination. This allowed us to employ the remaining drug on other patients, beyond the 8-hour interval suggested by the producers, remarkably cutting down costs for bortezomib.

A similar analysis carried out for cytosine arabinoside—the object of this report—was also aimed to define the safety of using the reconstituted residual drug, which should be discarded after the initial use.

Noteworthy, ARA-C used to be provided by the national health system upon medical prescription and was available in pharmacies for home administration to patients who required only small subcutaneous injections. This proved to be feasible and practical for hundreds of patients referring to Italian hematology units, with no evidence in the literature of any drawback. Following the institution

of the so-called Unità Manipolazione Chemioterapici Antitumorali: Antitumor chemotherapy modulation units in Italy, these drugs are now only administered in hospital setting. Patients who, for years, could avail themselves of home treatment must now go to hospital for the administration of ARA-C, obviously overburdening the health care system.

Based on sound experience in several countries, many years may pass before reliable data (in terms of efficacy and safety) in the literature are gathered on large case series of patients treated in a given way, with a given drug administered in a given way, and the instruction label is modified.

Inevitably, in common and sound clinical practice and in the patient's best interest (should he/she reflect the case series described), the physician may disregard some of the indications in the instruction label. The physician's approach, in this case, may be defined as 'off-label.'

As a result of what stated above, this adaptation, in all safety—as it is based on data available in the literature [8,9] or, as in our case, on evidence from reliable laboratory tests—should more properly be defined as 'awaiting label adjustment.'

ARA-C is an antimetabolite analogue of cytidine, a synthetic nucleoside. It differs from the normal nucleosides deoxycytidine (2) and cytidine (3) in that the sugar moiety is arabinose (Fig. 1).

Arabinose plays an important role in the mechanism of action of ARA-C, since the C-2'-hydroxyl group, in trans to the C-3'-hydroxyl group of the sugar, causes steric hindrance to the rotation of the pyrimidine base around the nucleoside bond. Whereas the nucleotide triphosphate (ARA-CTP) is incorporated at the terminal position of a growing DNA chain, it dramatically inhibits DNA synthesis [10–12]. This makes ARA-C a powerful antitumor agent, able to interfere with physiological molecules due to its structural affinity with the cytidine nucleoside and

its antiproliferative activity exerted at nanomolar concentrations (i.e. human leukaemia cell lines CCRF-CEM, $IC_{50} = 6.30 \text{ nm}$). As aminoglycoside, the chemical and microbiological stability of ARA-C in its pharmaceutical form, after reconstitution and storage, could be affected by the hydrolysis of the amine group and the *N*-glycoside bond. The change in the sugar feature could be due to chemical and bacterial factors. As reported in the literature, both the D- and L-enantiomeric forms of the pentose sugar arabinose undergo chemical transformations under acidic condition or in the presence of degradating microorganisms that use some pentoses, hexoses, oligosaccharides, and polysaccharides as sole carbon and energy source [10].

Moreover, some microorganisms as those of the *Lactobacillus* species, during their growth in media, can secrete some metabolites that alter the pH of the medium, contributing to hydrolyze the hemiaminal bond [11,12].

Cytidine is also reported to be largely deaminated to the therapeutically inactive 1- β -D-arabinosyluracil ARA-U (4) merely on recrystallization from water [13–17]. This reaction is explained in terms of an addition-elimination mechanism where the α,β -unsaturated system of the pyrimidine ring would be expected to facilitate water attacks (Fig. 2). The reaction may proceed via the simple addition of water on the N4-protonated base. A similar deamination of guanine and adenine residues in DNA is much slower, occurring at only 2–3% the rate of cytosine deamination [15].

In the light of the available evidence, an in-depth study by reversed-phase HPLC (RP-UHPLC) and high-field ^1H NMR was performed on commercial ARA-C samples to explore whether the pharmaceutical solution prepared is able to retain [18,19] the physical, therapeutic, and microbial properties limitedly to the week required for small-dose therapeutic regimens.

Methods

All chemicals were purchased from Sigma Aldrich (Milan, Italy). ARA-C is commercialized by numerous companies around the world; in Italy, it is distributed by Pfizer under

the tradename Aracytin and is administrated as intravenous bolus or subcutaneous injection. The product information states that reconstituted ARA-C (100 mg of lyophilized ARA-C in 5 ml of sterile water) is stable for 6 hours when stored at 25°C and for 12 hours at 4°C.

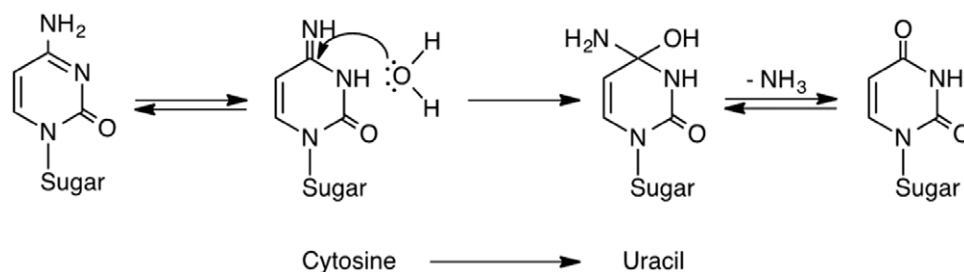
Our experiments were conducted using this product dissolved in the sterile water provided by the commercial company (samples A) and in deuterated water (D_2O , 99.99% Merck) (samples B) to investigate, respectively by RP-UHPLC and NMR, the stability of the solution stored at 4°C. Each experiment was performed in triplicate and the spectral data were recorded every day for one week.

^1H and ^{13}C NMR spectra were recorded at 500 MHz on a Fourier Transform NMR Varian 500 Unity Inova spectrometer. Chemical shifts are reported in δ values (ppm) and J values are reported in Hz.

RP-UHPLC analyses were performed on a Shimadzu Nexera UHPLC system (Shimadzu, Milano, Italy), consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD20A photodiode array detector (equipped with a semi-micro flow cell of 2.5 μl), a CTO-20A column oven, a SIL-30 AC autosampler. The column employed in this study was a Synergi Fusion-RP 80 Å 150 \times 4.6 mm, 4 μm (Phenomenex, Bologna, Italy). For RP-UHPLC analyses, the mobile phase employed was (A) 0.1% TFA in water v/v and (B) 0.1% TFA in ACN v/v. The analysis was performed with the following gradient elution: 0–15 minutes, 0% B; 15–17 minutes, 95% B and 17–20%, 0% B. Column oven temperature was set to 25°C and 5 μl of the sample were injected. The chromatogram was monitored at 245 and 260 nm at the two-absorbance maxima of the compound investigated.

Moreover, after a week, the samples used for the HPLC and NMR measurements underwent ESI-MS analyses by direct infusion (Fig. 3). A hybrid mass spectrometer Q-TOF Premier (Waters) composed of a single quadrupole, a collision cell and a mass analyzer of flight time were used. The nebulizing gas N_2 was set to 300 L/h at

Fig. 2



Hydrolysis of ARA-C cytosine moiety yielding the carbonyl uracil derivative (ARA-U, 4). ARA-C, cytarabine.

a temperature of 210°C, operating in positive mode. The cone gas was set to a flow rate of 100 L/h, while source temperature was set at 90°C. The tensions of the capillary and cone were fixed at 3 kV and 30 V, respectively. The mass range TOF for data collection was set between 80 and 300 m/z. The Q-TOF Premier was set in V mode with an 8500 resolution, a scan time of 0.3 seconds and interscan delay of 0.02 seconds. Mass and composition of the ions were calculated using the MassLynx 4.1 and Elementar Composition.

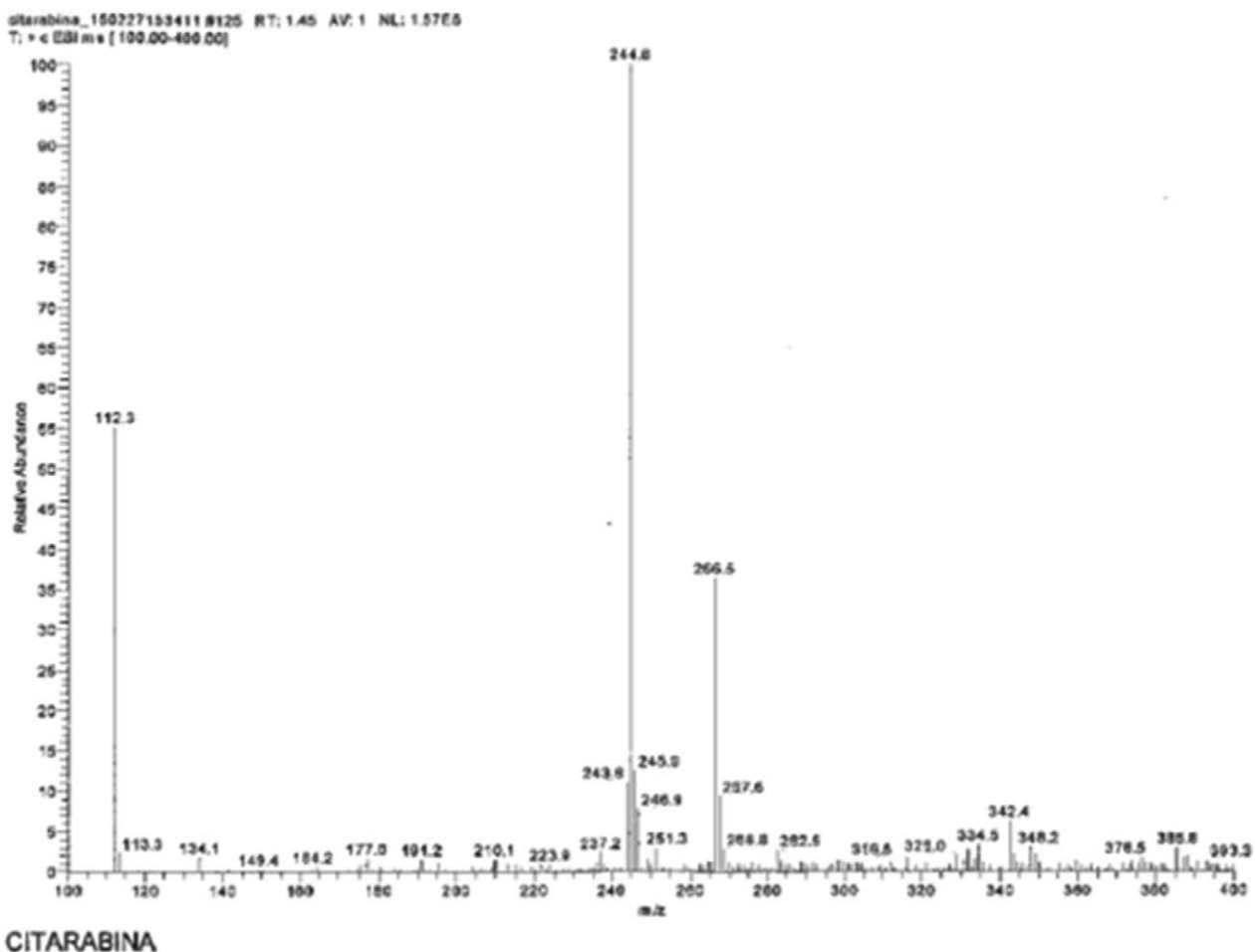
Concerning the microbiological assays sterility was tested using the following culture media: Fluid Thioglycolate Medium (Becton Dickinson) supplemented with hemin and vitamin K1, to grow aerobic microaerophile and anaerobic microorganisms, including strict anaerobes; tryptic soy broth with casein extract (TSB, Becton Dickinson) to grow common aerobic and facultative anaerobic microorganisms[20,21]; solid media on plates: trypticase soy agar supplemented with 5% sheep blood (AS, Becton

Dickinson); agar Schaedler supplemented with vitamin K1 and 5% sheep blood (SCH, Becton Dickinson); agar Sabouraud with dextrose + gentamycin + chloramphenicol (SAB, Becton Dickinson); chocolate agar + growth supplement for *Hemophilus*, *Neisseria*, *Streptococcus pneumoniae* culture (Biomerieux).

Four ARA-C vials from the same lot, stored at 4°C, were reconstituted according to the manufacturers' instructions.

Every day of the test week, 0.5 ml of solution were drawn from each of the four vials, with a sterile syringe. The 2 ml of solution sampled were used to verify its sterility. In detail, 1 ml of sample was inoculated into 5 ml of FTM broth supplemented with vitamin K and hemin, subsequently incubating the solution for 7 days at 35 ± 2°C in aerobic atmosphere; 1 ml of sample was inoculated in 5 ml of TSB broth and the solution incubated for 7 days at 20–25°C in aerobic atmosphere. At the end of the incubation period, 30 µl of each culture in liquid medium

Fig. 3



TOF-MS spectra of Aracytin Pfizer Cytarabine sample after one week.

was inoculated on a solid medium (AS, SAB, SCH, chocolate agar); the subcultures were incubated for 3 days at $35 \pm 2^\circ\text{C}$ under appropriate atmospheric conditions: AS and SAB in aerobiosis, SCH in an anaerobic atmosphere generator pouch (Becton Dickinson), and chocolate agar in atmosphere supplemented with 5% CO_2 .

Results and discussion

Solutions A and B were stored at 4°C and 25°C ; they were tested every day for one week using ^1H NMR and RP-UHPLC (Fig. 4), respectively, and no differences were detected between the initial and the final stages. To provide a complete assignments of the ^1H NMR spectrum, we performed 1D as well as 2D experiments (^1H - ^1H COSY, NOESY); likewise, carbon signals were then attributed by means of one-bond and long-range ^1H - ^{13}C hetero-correlated 2D-NMR spectra (HSQC and HMBC). In details, the ^1H NMR spectrum showed interalia, the H-6 and H-5 protons of the cytosine moiety, as well as the H-1' proton of the arabinose core in the low field region at δ 7.70, 5.92, and 6.06, respectively. The other protons were identified by chemical shifts at higher fields, thus at δ 4.25, 3.98, 3.87, 3.78, and 3.70, respectively, for H-2', H-3', H-4', H-5'a, and H-5'b.

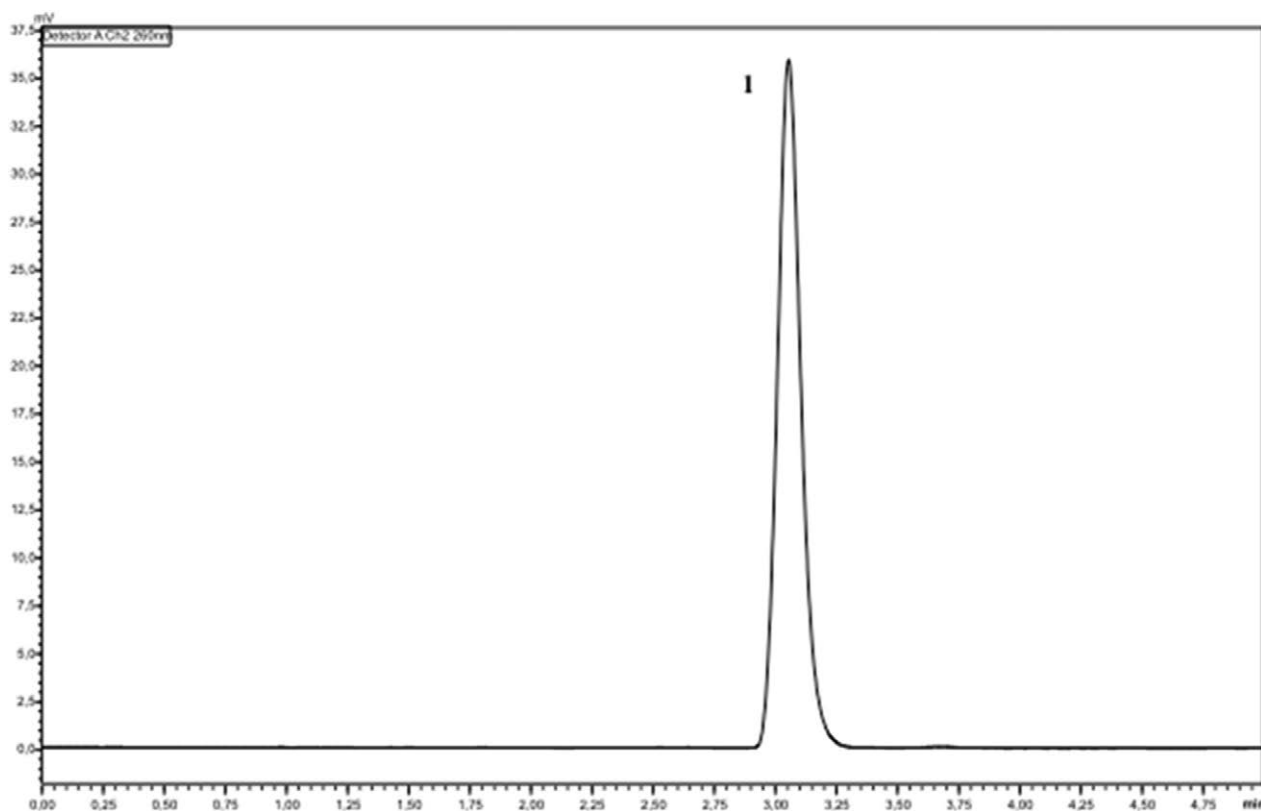
According to this evidence, the stored ARA-C seemed stable at 25°C as well as at 4°C .

At the end of incubation period, the microbiological analyses on the liquid culture medium inoculated with drug aliquots failed to detect any turbidity, and the subcultures on solid culture medium had no evidence of bacterial growth.

Therefore, the results of the sterility tests can be considered satisfactory, given the lack of microbial contamination in the specimen taken from each vial stored a 4°C for seven days.

Our findings provide evidence of an optimal physico-chemical stability and microbiological sterility of ARA-C solution stored for one week at 4°C . This encourages the use of the reconstituted drug for the time required for short-term multidose treatments, avoiding drug waste, patient stress, and hospital crowding. Moreover, it seems possible to leave in the same container the surplus of different ARA-C packages, improving the cost-effectiveness of the treatment without affecting its efficacy and safety. An additional advantage is the fact that patients are able to have the treatment administered at home.

Fig. 4



Chromatograms of Aracytin Pfizer Cytarabine sample at 245 and 260 nm, in water solution after one week.

Our results show that a solution of reconstituted ARA-C could be employed for a longer period than what suggested by the manufacturers. In fact, patients could receive a safe aliquot to be used at home for short-term treatments, thus optimizing the use of aliquot residues and avoiding vial manipulation and the production of special waste material.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement:

Data are available in Dipartimento di Scienze Chimiche, Università di Napoli Federico II, Naples, Italy. Data sharing: Information on the techniques used in the present study are available upon request from the Corresponding Author.

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