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Simultaneous determination of cytosine arabinoside and its metabolite uracil arabinoside in human plasma by LC-MS/MS: application to pharmacokinetics-pharmacogenetics pilot study in LAM patients

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Abstract:

Purine analogs like aracytine (Ara-C) are a mainstay for treating acute myeloid leukemia (AML). There are marked differences in drug dosing and scheduling depending on the protocols when treating AML patients with Ara-C. Large inter-patient pharmacokinetics variability has been reported, and genetic polymorphisms affecting cytidine deaminase (CDA), the liver enzyme responsible for the conversion of Ara-C to inactive uracil arabinoside (Ara-U) could be a culprit for either life-threatening toxicities or poor efficacy related to substantial changes in plasma exposure levels among patients. The quantitative determination of Ara-C in plasma is challenging due the required sensitivity because of the short half-life of this drug (i.e., less than 10 minutes) and the metabolic instability in biological matrix upon sampling possibly resulting in erratic values. We developed and validated a liquid chromatography tandem mass spectrometry method (UPLC-MS/MS) for the simultaneous determination of Ara-C and Ara-U metabolite in human plasma. After simple and rapid precipitation, analytes were successfully separated and quantitated over a 1-500 ng/ml range for Ara-C and 250-7500 ng/ml range for Ara-U. The performance and reliability of this method was tested as part of an investigational study in AML patients treated with low dose cytarabine and confirmed marked differences in drug exposure levels and metabolic ratio, depending on the CDA status of the patients. Overall, this new method meets the requirements of current bioanalytical guidelines and could be used to monitor drug levels in AML patients with respect to their CDA phenotypes.

Keywords: Cytarabine; LC-MS/MS; Pharmacokinetics; Acute myeloid leukemia patients; Cytidine deaminase.

1.Introduction:

Cytarabine or cytosine arabinoside (or arabinofuranosyl cytidine – Ara-C) was first synthesized the late 50's. It has been approved by the United States Food and Drug Administration (FDA) in June 1969 for treating hematological disorders. Since then, Cytarabine has been a mainstay for treating acute non lymphoblastic leukemia [1] and AML [2,3]. In general, cytarabine treatment for AML is based upon an induction phase at low dose (i.e., 100 to 200 mg/m² over 24h for 7 days) followed by a high-dose consolidation phase (i.e., 1 to 3 g/m² over 3h Q2D for 3 to 5 days). As most nucleoside analogs, cytarabine is characterized by extremely short plasma half-life (i.e., 10 minutes and 1-2 hours for the first and the second elimination phase, respectively) and erratic pharmacokinetics with large inter-patient variability. The inter-patient variability is much probably related to genetic polymorphisms affecting liver metabolism and the conversion between cytarabine and inactive metabolite Ara-U [4]. Consequently, measuring plasma exposure of cytarabine and its metabolite, plus predicting its pharmacokinetics is clinically relevant but requires sensitive methods to monitor circulating drug levels. Therefore our aim was to develop a technique suitable to the simultaneous determination of both Ara-C and Ara-U plasma concentrations, after either administration of low or high dose of cytarabine. To this end, LC-MS/MS techniques are usually considered as more sensitive and time-effective than most analytical methods. Importantly, cytarabine is unstable in whole blood or plasma once sample has been withdrawn, most probably because of residual activities or deoxycytidine kinase (dCK) and cytidine deaminase (CDA). Consequently, sample preparation must be as rapid as possible to avoid loss of the analytes during the pre-analytical steps. Numerous analytical methods have been published over the last decades to assay cytarabine in plasma, such as microbiological assay [5], radioimmunoassay [6], gas-chromatography [7], paper chromatography [8] or thin-layer chromatography methods [9]. Similarly, several HPLC-UV methods have been published over the last 30 years[10]. Of note, ultra-filtration system is often required to deproteinization of plasma samples but usually the claimed sensitivity fails to meet the requirements of drug monitoring when low dose cytarabine is administered [11–15]. Recently, some new hydrophilic interaction liquid chromatography (HILIC) methods have been tested as part of HPLC-UV [16] or tandem mass spectrometric methods for assaying cytarabine [16], but still with

sensitivity levels not low enough to detect cytarabine administered at low dose. Sensitivity has been improved recently by using s new C18 columns, however time-consuming SPE procedure could lead to loss of the analytes because of *in silico* degradation of cytarabine [17]. Many studies have developed HPLC techniques using mass spectrometry (MS) or MS/MS detection for simultaneous determination of low concentrations of Ara-C and Ara-U in blood [18–20]. For instance, an assay in rat plasma was developed with concentration ranging from 2-100 ng/ml for both Ara-C and Ara-U [18]. In human, sensitive methods mostly focus on Ara-C only with concentration ranges of 20-2500 ng/ml or 0.5-500 ng/ml [19]. However, these methods were not suitable to assay Ara-U. Despite their respective performances, none of these methods meet all the requirements (i.e., time- and cost-effectiveness) to be applied in routine for monitoring both Ara-C and Ara-U in patients treated with a wide variety of dosing. Consequently, here, our aim was to develop and validate a sensitive, specific and rapid LCMS/MS method for the simultaneous quantification of Ara-C and Ara-U in human plasma that would be suitable for routine therapeutic drug monitoring and pharmacokinetic studies of low-dose Ara-C in adult patients with AML.

2.Material & Methods

2.1 Chemicals and reagents

Ara-C, ARA-U and two internal standards (IS, $^{13}C,^{15}N_2$-Cytarabine and 5-methylcytidine) were purchased from Sigma-Aldrich (Munich, Germany). Acetonitrile, methanol and formic acid were obtained from VWR International France**.** All solvents were of LC-MS/MS grade. Dilutions were prepared in Milli-Q water (Millipore France). Pooled human plasma for calibration standards and quality control (QC) samples was obtained from the Etablissement Français du Sang (EFS, Marseille France).

2.2 Apparatus and MS conditions

The LC-MS/MS system used was an ACQUITY TQD, ultra-compact, tandem-quadrupole, atmospheric pressure ionization (API), mass detector-based system designed for routine UPLC-MS/MS analyses (Waters France). The probe capillary was set at 3.00 kV and the extractor at 3.00 V; The source temperature was set at 130 °C, and desolvation temperature was set at 400 °C. Desolvation gas flow

was1000 L/h with a collision gas (i.e., argon) flow of 0.25 mL/min to reach a pressure of 3e-3 mbars. Cone voltages were 100.0V, 18.0V, 24.0V and 20.0 V (positive ion mode) for Ara-C, Ara-U and IS (i.e. ^{13}C, $^{15}N_2$-Cytarabine and 5-methylcytidine) respectively. Ara-C was measured with a specific transition *m/z* 244.18 (precursor ion) and *m/z* 112.08 (qualifier). Ara-U was measured with two specific transitions m/z 245.04 (precursor ion) and between 132.96 and 112.96 (qualifier, Ara-U [1]). The chromatographic separation was performed on ACQUITY UPLC C18 HSS T3 column, 100Å, 100 × 2.1mm (Waters France).

The mobile phase consisted of solvent A as a mixture of purified water using Milli-Q system (Millipore France) and formic acid (0.05 %) and solvent B as a mixture of methanol and formic acid (0.05%). Two-microliters were injected into the column eluted with the following gradient program: 100% solvent A (0-2.0 min), from 100 to 20% solvent A (2.0-4.0 min), from 20 to 100% solvent A (4.0-4.5 min), and 100% solvent A (4.5-6.0 min). The column temperature and flow rate were 50°C and 0.4 ml/min, respectively. Retention times for Ara-C, Ara-U, ^{13}C, $^{15}N_2$-Cytarabine and 5-methylcytidine were 1.15 min, 2.55 min, 1.15 min and 1.65 min. The MassLynx software (Waters France) was used for MS control and data analysis (i.e., measuring peak area, regression analysis of standard curves and calculation of concentrations).

2.3 Standard and sample preparation

Stock solutions were prepared by dissolving Ara-C, Ara-U and IS in Milli-Q water (1:1, v:v) at concentrations of 1 mg/ml. Aliquots of stock solutions were then stored at -80°C for Ara-C, Ara-U and two IS for no more than 12 months. Ara-C and Ara-U stock solutions were further serial-diluted in Milli-Q water the day of extraction, to obtain working solutions of 100, 10, 1 and 0.1 µg/ml.

The concentration range was 1-500 ng/ml for Ara-C and 250-7500 ng/ml for Ara-U. Quality Control (QC) samples containing Ara-C/Ara-U at 2/300, 25/800 and 250/4000 ng/ml were obtained by spiking known quantities of diluted stock solutions into human plasma.

Internal standard solution was diluted with Milli-Q water and spiked to standards and samples with a final concentration (IS solution) of 5 ng/µL for ^{13}C, $^{15}N_2$-Cytarabine and 10 ng/µl for 5-methylcytidine.

Plasma sample (100 µl) was mixed with 400 µL of acetonitrile with 1% formic acid and 10 µL of IS solution. The mixture was vortexed for 2 min. The solution was centrifuged at 15000 tr/min for 15 min. Thereafter, the supernatant was evaporated at 47°C under nitrogen flow and reconstituted with 400 µl of Milli-Q water. The mixture was centrifuged at 15000 rpm for 10 min. The resulting solution was transferred into 96-well plates (Waters®). Two microliters were injected into the column for analysis.

2.4 Validation

Validation of the assay was performed in accordance with current FDA guidelines for Bioanalytical Methods Validation for Human Studies [21].

2.4.1 Linearity and sensitivity

Calibration standards were prepared and analyzed in 6 replicates as 6 independent runs. Calibration curves were best fitted using a quadratic regression. In order to obtain acceptable linearity, deviation of the mean calculated concentrations over 6 runs had to be within ± 15% of nominal concentrations for the non-zero calibration standards, except for the limit of quantification (LOQ) level where a deviation of ± 20% was allowed [21]. The LOQ was determined by analyzing 6 replicates in the same run. A precision of ± 20% and a accuracy of 80-120% were allowed for the LOQ [21].

2.4.2 Precision and accuracy:

Intra-assay precision and accuracy were determined by analyzing 6 replicates of each spiked QC sample on a single assay. Inter-assay precision and accuracy were determined by analyzing one QC sample per day at each concentration over 6 different days. Intra- and inter-assay precisions were expressed as the coefficient of variation (CV) at each QC concentration and should not exceed ± 15% [21]. Accuracy was calculated as the percent deviation from the nominal concentration and had to be within ± 15% [21].

2.4.3 Selectivity and specificity

Interferences from endogenous plasma compounds were investigated by analyzing 9 patients plasma hospitalized in different clinical Units and treated with different

agents (i.e., antiretrovirals, antivirals, antifungals, tyrosine kinase inhibitors, aminoglycosides, antimetabolites, antiepileptics, glycopeptides).

2.4.4 Matrix effect:

Matrix effect was evaluated on 7 different extracts of blank plasma samples spiked at 5/500 ng/ml and 100/5000 ng/ml for Ara-C and Ara-U respectively, and injected in duplicate. Peak areas obtained from the plasma extract was compared with peak areas obtained from the diluted stock solution at the same concentration in Milli-Q water.

2.4.5 Stability and dilution studies:

To test the post-preparative stability of Ara-C and Ara-U, the processed samples were analyzed, then left unattended in the autosampler for 20h at 20°C, and then analyzed again. The analyte was considered stable in the matrix when the differences in concentration between the two measures was < 20%.

Dilution procedures were validated by spiking blank plasma with stock solutions to a concentration corresponding to 1000/60 (Ara-C/Ara-U) times the LOQ and then diluted as following: 1/10, 1/5 and 1/2. The diluted plasma was analyzed (6 replicates) and the limit of acceptance was ± 15%.

2.5 Method application:

Ara-C and Ara-U concentrations were determined in plasma samples using the above described method. Blood samples were withdrawn from 7 patients treated for AML with Ara-C as part of a study approved by the institutional review board of the Conception Hospital (Marseille, France) registered as # 2017-A00070-53. Patients were phenotyped for CDA status prior to starting the infusion following a spectrophotometric method previously describe [22] and categorized as Poor Metabolizer (PM) or Extensive Metabolizer following CDA activity[23]. Patients were sampled at the end of the administration, then 5 min, 10 min, 1H, 2H and 6H after the end of the infusion. Blood samples were centrifuged immediately, separated and plasma stored at -80°C until analysis. Plasma Area Under Curve (AUC) were calculated using standard trapezoidal rule both for Ara-C and Ara-U. Metabolic ratio was defined as the AUC ratios between Ara-U and Ara-C, the higher the ratio, the

higher the metabolic conversion. Statistics were performed using Sigma Stat Software (Jandel Scientific, Germany).

3.Results:

Ara-C, Ara-U and their IS (^{13}C, $^{15}N_2$-Cytarabine and 5-methylcytidine, respectively) were detected and quantified over a total run of 6 min. Detection of all analytes was optimized prior to their quantification by LC-MS/MS. For each selected transition of Ara-C (m/z 244.18 → 112.08), Ara-U (m/z 245.04 → 132.98, 112.96), Ara-U [1] (m/z 245.04 → 112.96), ^{13}C, $^{15}N_2$-Cytarabine (m/z 247.17 → 115.03) and 5-methylcytidine (m/z 258.1 → 126.0). Retention times were 1.15 min, 2.55 min, 1.15 min and 1.65 min, respectively. Figure A shows representative chromatograms for the compounds (Ara-C, Ara-U and Ara-U [1], respectively) and internal standards (13C, 15N2-Cytarabine and 5-methylcytidine, respectively) in blank matrix.

3.1 Method development

The LC-MS/MS parameters were optimized for determination of Ara-C, Ara-U and IS. Different columns, mobile phase gradient and several extraction techniques were tested to optimize both retention times and overall running time (data not shown). Protein precipitation with acetonitrile and 1% formic acid proved to be a simple and rapid method for sample preparation with reduced background noise and higher sensitivity as compared with other methods.

3.2 Method validation

3.2.1 Linearity, sensitivity and carry-over:

The quadratic regression analysis yielded the following correlation coefficient (r^2) for Ara-C, Ara-U (m/z m/z 245.04 → 132.98) and Ara-U [1] (m/z 245.04 → 112.96): 0.99918, 0.996912, and 0.999054 respectively. A precision ranging from 0.12% to 13.93%, from 1.69 % to 11.20 %, and from 0.72 % to 7.11 % for Ara-C, Ara-U and Ara-U [1] was obtained. A deviation ranging from -5.07 % to 4.09 %, from -4.30 % to 4.28 %, and from -1.65 % to 1.46 % for Ara-C, Ara-U and Ara-U [1] respectively were observed across the 7 calibrations levels (6 replicates). Mean calculated concentrations over 6 runs did not deviate by more than ± 15 % from nominal concentrations for the non-zero calibration standards. The limit of quantification

(LOQ) was of 1 ng/ml and 250 ng/ml for Ara-C and Ara-U, respectively, with a precision of 12.85 % and 5.41 %. Therefore, the response at LOQ was identifiable, discrete and reproductible with a precision of ± 20 % and accuracy of 80-120% fulfilling the FDA guidelines [21]. The ratio (%) of blank samples signals to the LOQ-signals was 0.00% for Ara-C, 0.96% for Ara-U, and 1.22% for Ara-U [1].

3.2.2 Precision and accuracy

The results of assay performance, assessed at three different QC concentrations, are summarized in Table A and B and were in line with validation guidelines [21].

3.2.3 Selectivity and specificity

After injection of 9 plasma samples collected from patients treated for several diseases, no significant chromatographic interference from co-administered drugs was observed with respect to Ara-C and Ara-U.

3.2.4 Matrix effect

The calculated ratio and typical matrix effect (mean ± standard deviation, SD) and precision (CV %) is presented in Table C.

3.2.5 Stability and dilution

The stability of Ara-C/Ara-U in plasma was assessed at 3 QC concentrations injected after 20 hours at 10 °C. The differences between the two assays did not exceed 15 %. The concentrations measured after dilution relative at 1/10, 1/5 and 1/2 for Ara-C/Ara-U/Ara-U [1] are summarized in Table D.

3.3 Application and relevance of the method

Using the validated method, plasma samples collected from 7 patients were assayed. Figure B shows chromatograms for Ara-C, Ara-U and Ara-U[1], respectively and internal standard (13C, 15N2-Cytarabine and 5-methylcytidine, respectively) in a representative patient. Four patients were CDA deficient (i.e., CDA ≤ 2 U/mg, aka PM), and 3 patients were CDA no-deficient (i.e., CDA > 2 U/mg, aka EM) [23]. These AML patients were all treated with 200 mg/m^2 Ara-C at as part of induction phase. Cytarabine and Ara-U plasma concentrations are summarized in Figure C and D. For Ara-C, AUCs were 3312 ± 326 ng/ml.min and 1502 ± 497 ng/ml.min, for PM and

EM patients, respectively. The difference was statistically different (p=0.024, t test). For Ara-U, AUCs were $7.3.10^5 \pm 2.1.10^5$ ng/ml.min and $4.8.10^5 \pm 0.8.10^5$ ng/ml.min, for PM and EM patients, respectively. This difference was not statistically different (p>0.05, t test). Metabolization ratio between AUC of Ara-C and AUC of its metabolite Ara-U was calculated. The mean metabolization ratio was 255 ± 103 and 460 ± 218 for PM and EM patients, respectively (Figure E). This 1.8-fold difference was not statistically different (p>0.05, t test).

4. Discussion

Cytarabine remains the backbone of the vast majority of protocols for treatment of acute myeloid leukemia as well as many other myeloid or lymphoid malignancies (including lymphoma, myelodysplasia, acute lymphoblastic leukemia) We have previously demonstrated that clinical outcome with cytarabine was markedly influenced by genetic polymorphisms affecting CDA, the enzyme responsible for its detoxification to Ara-U in the liver [23]. Interestingly, AML patients with CDA PM phenotype exhibited both high risk of severe/lethal toxicities upon cytarabine treatment, but a trend towards longer progression-free and overall survival as well. Because of the lack of pharmacokinetics support until we could only hypothesize that PM patients had probably higher circulating cytarabine plasma levels and lower circulating plasma levels of inactive Ara-U. To confirm this hypothesis, monitoring drug and main metabolite level was mandatory. With respect to its pharmacokinetics profile, especially a plasma half-life of 10 minutes for the first phase of its biphasic elimination, monitoring cytarabine concentrations in plasma required highly sensitive bioanalytical methods coupled to simple and rapid sample preparation, especially during induction phase with low dose cytarabine. To this end, we have developed a new LC-MS/MS method that meets these requirements. Two transitions have been identified for Ara-U, but only the *m/z* 245.04 → 112.96 was selected eventually.

Prior validation, the method was optimized, i.e., by using ^{13}C, $^{15}N_2$-Cytarabine which performed better in limiting the matrix effect as compared with Maraviroc-d6 (data not shown). Thereafter, different techniques such as solid phase extraction, and protein precipitation with many solvents (e.g., methanol, acetonitrile, acetic acid and sulfosalicylic acid) were tested. Protein precipitation with acetonitrile and 1% formic acid provided the best purification. Secondly, retention times of Ara-C were further

optimized by testing a variety of columns (i.e., Kinetex F5 Core-shell LC columns (Phenomenex®), Hilic XBridge BEH amide column (Waters®)) using different mobile phases (i.e., 95% acetonitrile 5% ammonium formate (5%) at different pH, 0.05 % formic acid and methanol with or without 10mM ammonium formate, to name but a few (data not shown)). This is critical when assaying Ara-C and Ara-U because they are isomers of physiological cytidine and uracil, thus calling for optimal separation. In addition, large inter-individual variability reported in patients (e.g., age, co-morbidities, polypharmacy) can lead to marked changes in plasma as a biological matrix [24], advocating again for optimal sample preparation, chromatographic separation and injection volume to limit erratic matrix effect. Of the different columns tested, the HSS T3 column eluted with a 0.05% formic acid in water-methanol gradient performed best and therefore was selected. Lastly, injection volumes ranging from 2 to 5 µl were evaluated - 2 µl was finally retained so as to reduce the matrix effect. In addition, total run time was 5 minutes, i.e. longer than the longer retention time of the last analyte (2.55 min) so as to allow possible interfering peaks to be eluted and a better column equilibration before the next injection starts.

Pre-analytical sample preparation was kept as simple and minimal as possible, both to meet the requirements of future routine analysis and because of metabolic instability of the analytes in biological matrix. Moreover, only a small volume of plasma for analysis was required to quantify Ara-C and Ara-U (100 µL, i.e., less than 0.5 ml of whole blood), making this method suitable in the future in frail patients with limited sampling possibilities, such as children with AML and dry-spot sampling. Our method was fully validated following current guidelines in bioanalysis and was used next to measure drug and metabolite levels in a small subset of AML adult patients treated by low dose cytarabine. No chromatographic interferences were observed, despite the fact that patients were treated following standard care in our institute, i.e. with multiple co-administered drugs. It was possible to monitor Ara-U metabolite over 6 hours, regardless the CDA status of the patient. Regarding Ara-C, we found that plasma levels fall below our limit of quantification 1 hour after the end of the infusion in EM patients, whereas it remained fully quantifiable in PM patients. However, data from EM patient suggest that future PK investigations should be based upon sampling times comprised over the first 60 minutes following the end of cytarabine administration so as to better picture the first part of the elimination phase. Despite

this limitation, our method was sufficient to evidence marked differences between PM and EM patients both in terms of cytarabine AUCs (3312 ng/ml.min *vs.* 1502 ng/ml.min respectively, $p<0.05$) and metabolic ratio (255 *vs.* 460 respectively, n.s.). The observed differences are fully in line with the CDA status (i.e., PM patients display higher plasma exposure than EM patients with lower metabolic ratio) and consistent with differences in pharmacodynamics endpoints (i.e., toxicity, survival) evidenced between PM and EM patients treated with cytarabine [23]. Conversely, data on Ara-U were not consistent because mean exposure was higher in PM patients as well. Of note, this difference on Ara-U AUCs (i.e, $7.3.10^5$ *vs.* $4.8.10^5$ ng/ml.min) was not statistically significant, mostly because the large intra-group variability in the PM subset (i.e., 30%) and the fact that a single outlier patient with very high Ara-U levels has a strong impact on the final mean value of the subgroup. Because of the small number of patients, this outlier patient probably prevents statistical analysis to be convincing with Ara-U. However, and although preliminary, our pilot clinical data suggest that this bioanalytical method could help to decipher the mechanisms underlying differences in clinical outcome between CDA PM and CDA EM patients because strong differences in both exposure levels in Ara-C and metabolic ratio were observed. Because the method was developed to be time-effective with simple and rapid pre-analytical steps, it could be used as part of routine drug monitoring of Ara-C, a pivotal drug to treat a variety of hematological disorders.

5.Conclusion:

A sensitive, accurate and rapid procedure based on LC-MS/MS has been developed and validated for the determination of Ara-C and Ara-U metabolite in human plasma. A good quadratic regression was obtained over the concentration range 1-500 ng/ml and 250-7500 ng/ml of Ara-C and Ara-U, respectively, covering plasma exposures observed in AML patients. This method was successfully applied to determine the pharmacokinetic profile of Ara-C and Ara-U in 7 patients and evidenced marked differences in both drug levels and metabolic ratio depending on patient's CDA status. Evaluation of Ara-C full pharmacokinetics as part of a prospective clinical trial is currently ongoing in our institute using this new analytical method.

Declarations of interest: none.

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Tables and figures

Table A: Intra-day precision and accuracy for Ara-C, Ara-U and Ara-U [1] (n=6)

Intra-day	*Ara-C*			*Ara-U*			*Ara-U [1]*		
QC concentrations (ng/ml)	2	25	250	300	800	4000	300	800	4000
Mean (ng/ml)	2.20	27.0	270	283	690	3404	243	692	3519
SD (ng/ml)	0.140	1.73	18.0	45.8	48.1	112	14.3	16.0	50.3
Precision (CV %)	6.29	6.30	6.68	16.2	6.97	3.29	5.87	2.32	1.43
Accuracy (%)	9.83	9.87	7.83	-5.54	-13.8	-14.9	-19.0	-13.5	-12.0

Table B: inter-day precision and accuracy for Ara-C, Ara-U and Ara-U [1] (n=6)

Inter-day	*Ara-C*			*Ara-U*			*Ara-U [1]*		
QC concentrations (ng/ml)	2	25	250	300	800	4000	300	800	4000
Mean (ng/ml)	2.00	26.0	247	320	784	3886	287	784	3892
SD (ng/ml)	0.190	2.70	20.0	46.4	60.5	474	32.5	67.0	463
Precision (CV %)	9.80	10.3	8.10	14.5	7.72	12.2	11.3	8.54	11.9
Accuracy (%)	-2.08	3.85	-1.12	6.62	-2.04	-2.85	-4.34	-2.02	-2.70

Table C: results of matrix effect with Ara-C, Ara-U and Ara-U [1]

Ext/SP	***Ara-C***	***Ara-U***	***Ara-U [1]***
Concentration (ng/ml)	5	50	50
Mean	1.12	1.55	1.14
SD	0.160	0.230	0.05
CV (%)	14.5	15.0	4.50
Ext/SP	**Ara-C**	**Ara-U**	**Ara-U [1]**
Concentration (ng/ml)	100	5000	5000
Mean	1.34	1.15	1.10
SD	0.110	0.110	0.068
CV (%)	8.20	9.50	6.2

Table D: Mean, SD, precision and accuracy for several composites after dilution at 1/10, 1/5, and ½.

Dilution: 1/10	***Ara-C***	***Ara-U***	***Ara-U [1]***
Mean (ng/ml)	99.0	1519	1466
SD (ng/ml)	6.00	96.3	52.6
Precision, CV (%)	5.60	6.34	3.59
Accuracy (%)	-1.00	1.23	-2.24
Dilution: 1/5	**Ara-C**	**Ara-U**	**Ara-U [1]**
Mean (ng/ml)	197	3069	2881
SD (ng/ml)	10.0	217	86.9
Precision, CV (%)	4.90	7.05	3.02
Accuracy (%)	-1.34	2.31	-3.98
Dilution: 1/2	**Ara-C**	**Ara-U**	**Ara-U [1]**
Mean (ng/ml)	489	7434	7197
SD (ng/ml)	13	376.98	147
Precision, CV (%)	2.72	5.07	2.05
Accuracy (%)	-2.30	-0.880	-4.04

FIGURE A: Representative chromatograms for Ara-C and ^{13}C, $^{15}N_2$-Cytarabine (top), Ara-U and 5-methylcytidine (middle) and Ara-U [1] and 5-methylcytidine (bottom) in spiked blank plasma.

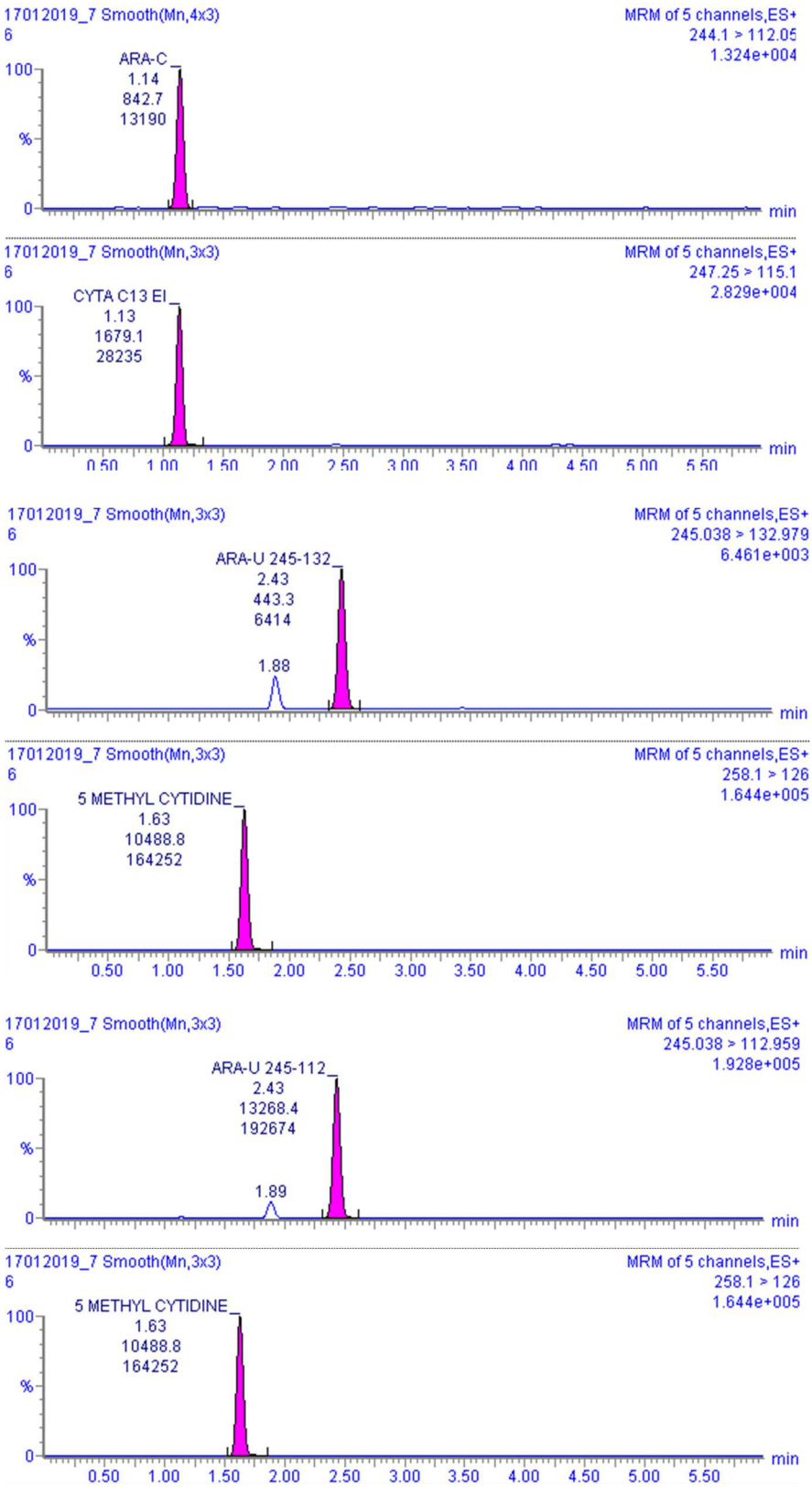
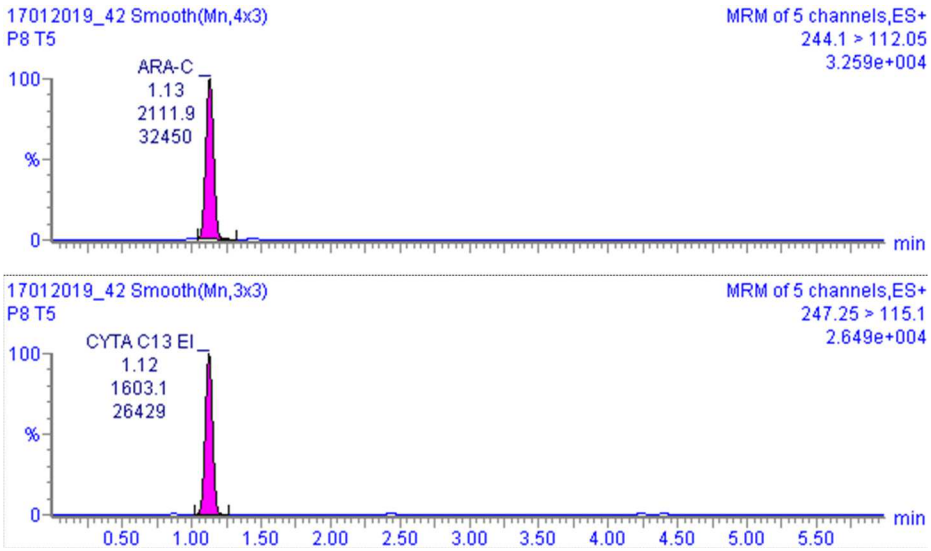


FIGURE B: Example of chromatograms in patients for Ara-C and ^{13}C, $^{15}N_2$-Cytarabine (top), Ara-U and 5-methylcytidine (middle) and Ara-U [1] and 5-methylcytidine (bottom) from a representative patient.



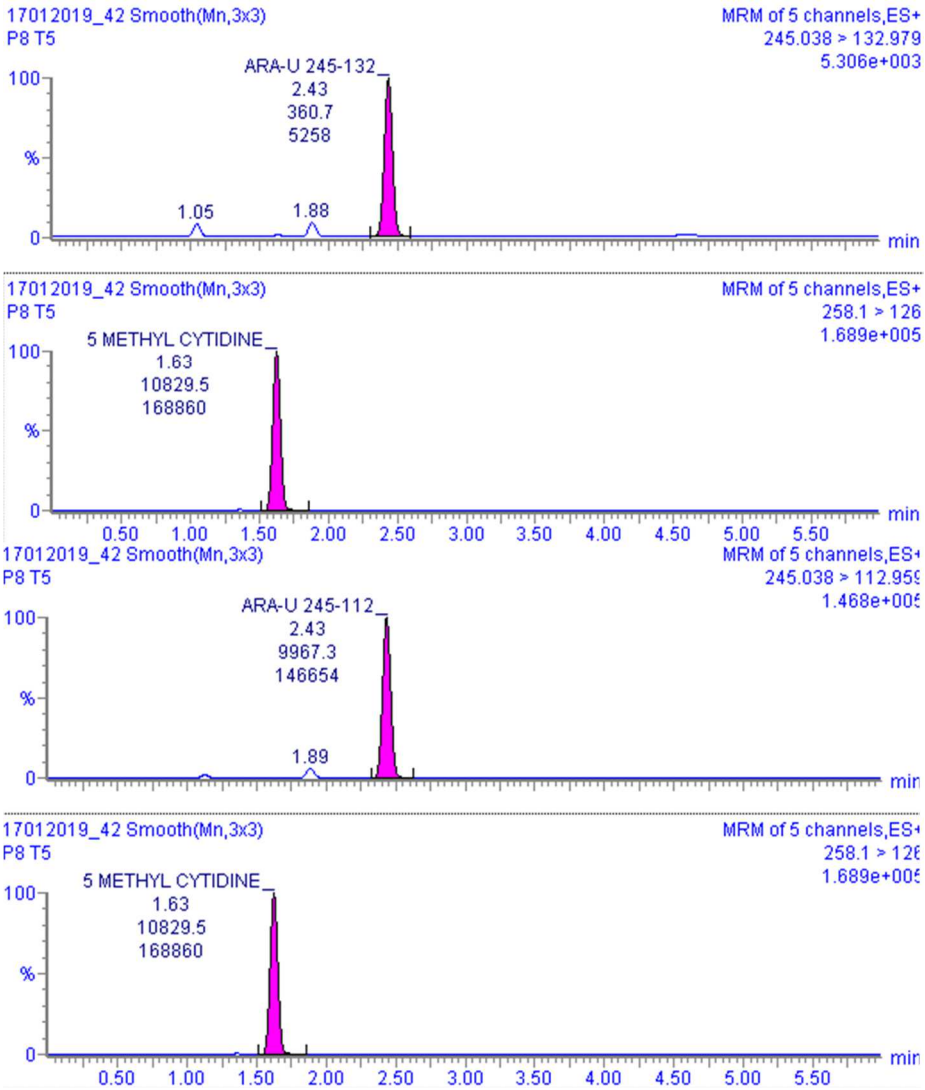

17012019_42 Smooth(Mn,3x3)
P8 T5
MRM of 5 channels,ES+
245.038 > 132.979
5.306e+003
ARA-U 245-132
2.43
360.7
5258
1.05
1.88
100
%
0
min
17012019_42 Smooth(Mn,3x3)
P8 T5
MRM of 5 channels,ES+
258.1 > 126
1.689e+005
5 METHYL CYTIDINE
1.63
10829.5
168860
100
%
0
min
0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50
17012019_42 Smooth(Mn,3x3)
P8 T5
MRM of 5 channels,ES+
245.038 > 112.959
1.468e+005
ARA-U 245-112
2.43
9967.3
146654
1.89
100
%
0
min
17012019_42 Smooth(Mn,3x3)
P8 T5
MRM of 5 channels,ES+
258.1 > 126
1.689e+005
5 METHYL CYTIDINE
1.63
10829.5
168860
100
%
0
min
0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50

FIGURE C: Pharmacokinetic profiles for Ara-C in PM (red, 4 patients) and EM (blue, 3 patients) patients.

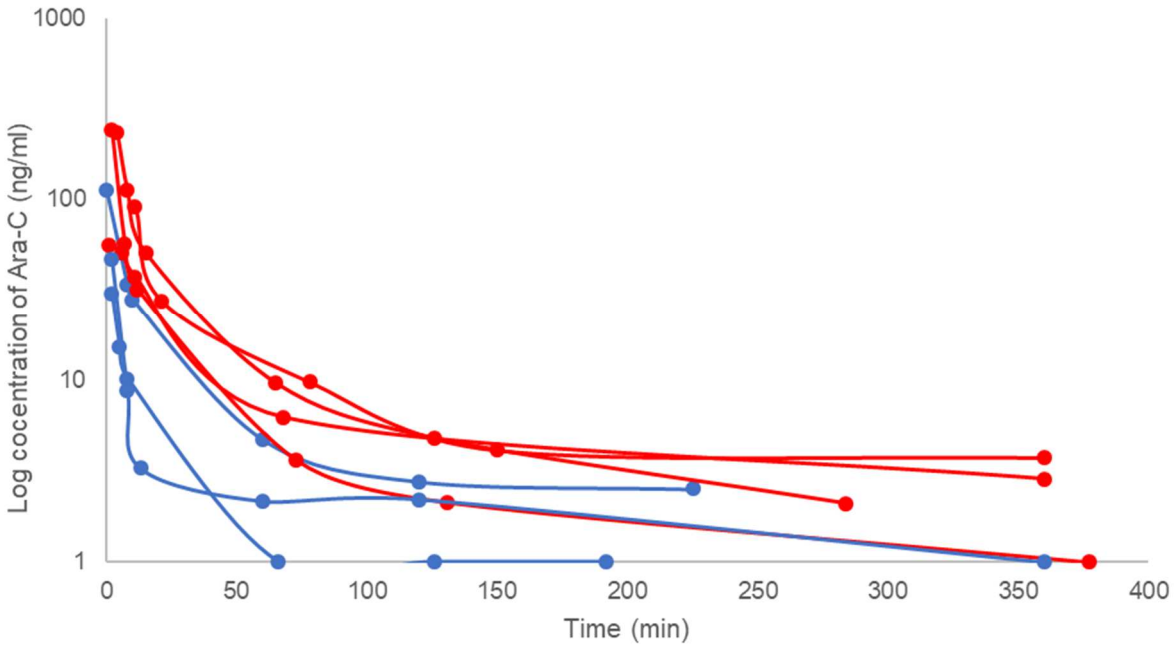


FIGURE D: Pharmacokinetic profiles for Ara-U in PM (red, 4 patients) and EM (blue, 3 patients) patients

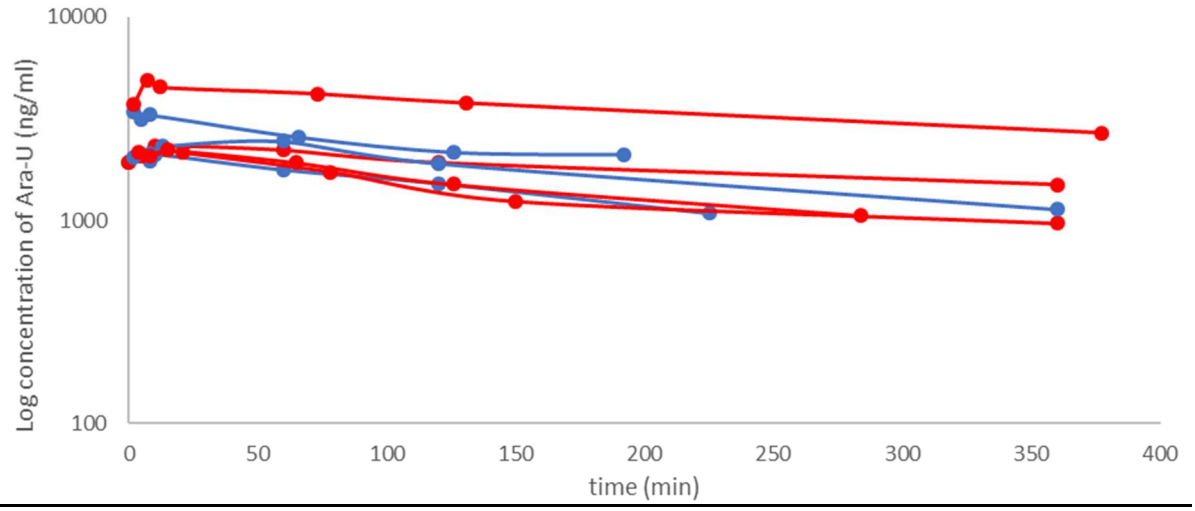


FIGURE E: Metabolic ratio (Ara-U/Ara-C) in PM (red and EM (blue) patients.