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# Development of a sensitive and selective LC/MS/MS method for the simultaneous determination of intracellular 1-beta-D-arabinofuranosylcytosine triphosphate (araCTP), cytidine triphosphate (CTP) and deoxycytidine triphosphate (dCTP) in a human follicular lymphoma cell line

Céline Crauste<sup>a</sup>, Isabelle Lefebvre<sup>a,\*</sup>, Michael Hovaneissian<sup>a</sup>, Jean Yves Puy<sup>a</sup>, Béatrice Roy<sup>a</sup>, Suzanne Peyrottes<sup>a</sup>, Sabine Cohen<sup>b</sup>, Jérôme Guitton<sup>b</sup>, Charles Dumontet<sup>c</sup>, Christian Perigaud<sup>a</sup>

<sup>a</sup> Institut des Biomolécules Max Mousseron, UMR 5247 CNRS-UM1-UM2, Université Montpellier 2, case courrier 1705, place Eugène Bataillon, 34095 Montpellier Cedex 05, France

<sup>b</sup> Hospices Civils de Lyon, Laboratoire de pharmacologie, Hôpital E. Herriot, 5 Place d'Arsonval, 69437 Lyon, France

<sup>c</sup> Hospices Civils de Lyon, Inserm, U590, Lyon F-69008, France

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## ABSTRACT

A method was developed for the quantification of araCTP, CTP and dCTP in a human follicular lymphoma cell line. This method involves solid phase extraction (SPE) using a weak anion-exchanger (WAX) cartridge, a porous graphitic carbon high-performance liquid chromatography (HPLC) column separation, and tandem mass spectrometry (MS/MS) detection. By using a triple quadrupole mass spectrometer operating in negative ion multiple reaction monitoring (MRM) mode, the method was able to achieve a lower limit of quantification (LLOQ) of 0.1  $\mu\text{g mL}^{-1}$  for araCTP and of 0.01  $\mu\text{g mL}^{-1}$  for both CTP and dCTP. The method was validated and used to determine the amount of araCTP, CTP and dCTP formed after incubation of araC and an araCMP prodrug in the human follicular lymphoma cell line RL.

## 1. Introduction

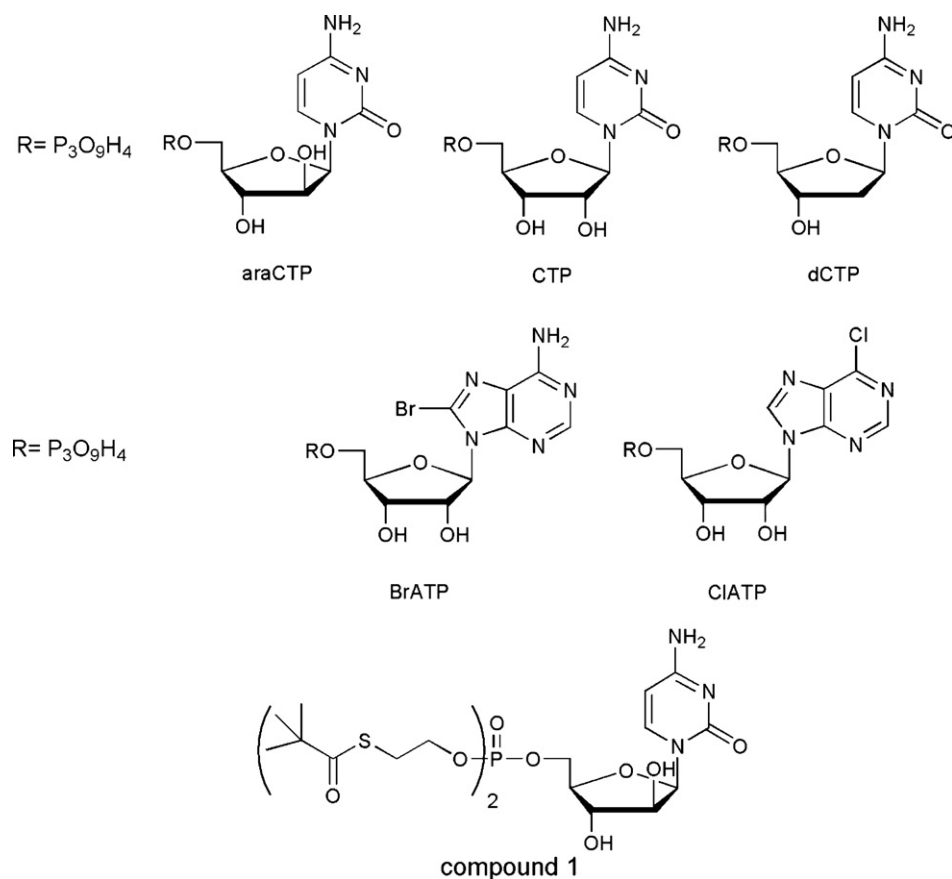
Nucleoside analogs represent a group of cytotoxic antimetabolites used for the treatment of haematological malignancies and solid tumors [1]. AraC (1- $\beta$ -D-arabinofuranosylcytosine, cytarabine) a deoxycytidine analog, is one of the most important antileukemic drug currently available for the treatment of acute myeloid leukaemia [2,3]. The cellular metabolism of araC is similar to that of its physiological deoxyribonucleoside counterpart (dC). Once inside the cell, araC is successively phosphorylated by cellular kinases to its 5'-monophosphate (araCMP) (Fig. 1), 5'-diphosphate (araCDP), and then cytotoxic 5'-triphosphate derivative (araCTP) which interferes with the biosynthesis of DNA [4–6]. The enzyme deoxycytidine kinase (dCK), is known to catalyze the first step of the sequential phosphorylation of araC to araCTP. As the therapeutic efficiency of araC strongly depends on its intracellular conversion to the corresponding 5'-triphosphate form, there is a correlation

between the cellular accumulation of araCTP and the cytotoxicity effect of araC *in vitro* and in clinical samples [3]. Moreover, *in vitro* and *in vivo* prolonged treatment with araC has resulted in the emergence of drug resistant cells with diminished sensitivity to the drug, thus contributing to treatment failures [3,7,8]. Several mechanisms of resistance to araC have been reported [9] and among them, dCK deficiency has been associated with reduced araCTP concentrations in various cells and animal models [10]. Consequently, the intracellular quantification of araCTP is critical in the clinical pharmacokinetic data for determination of an appropriate dosing regimen and to detect resistance mechanisms.

However, biological activity of araC depends not only on the intracellular concentrations of araCTP but also on the endogenous nucleotide pools (CTP and dCTP) since araCTP will compete with dCTP for incorporation into DNA, and to a lesser extent with CTP for incorporation into RNA. In this respect, the concentration ratios of araCTP versus dCTP (araCTP/dCTP) and of araCTP versus CTP (araCTP/CTP) could be considered as a predictive marker of therapeutic efficacy [11]. Consequently, direct access to such ratios in individual patient samples could prove to be helpful in understanding and/or predicting which patients will respond to araC-containing regimens.

\* Corresponding author. Tel.: +33 467143210; fax: +33 467042029.

E-mail addresses: isabelle.tournier@univ-montp2.fr, tournier@univ-montp2.fr (I. Lefebvre).



**Fig. 1.** Chemical structures of araCTP, CTP, dCTP, BrATP (internal standard) CIATP (internal standard) and bis(SATE) araCMP.

The measurement of intracellular concentration of therapeutic nucleosides triphosphates (NuTPs) by LC/MS/MS is especially challenging because of the presence of very high levels of endogenous compounds. Mass spectrometry provides a detection method with sensitivity and specificity suitable for intracellular measurements but it is not typically compatible with LC methods for resolving nucleotides. Furthermore, nucleotides are difficult to analyse by traditional LC/MS/MS methodologies because of their polar nature that hampers their retention by reversed phase (RP) chromatography using typical aqueous-organic mobile phases. Some chromatographic separations have been successfully developed and validated using anion exchange chromatography or reversed phase with ion-pairing agents. As an example, the use of weak anion exchange (WAX) column coupled to MS/MS detection has been described in the literature [12]. However, high salt concentrations and non-volatile ion-pairing agents preclude the use of mass spectrometry (MS) for detection. Therefore, the type of ion-pair agent and its subsequent concentration should be optimized. Thus, the ion-pair agent would act as a counter ion for the acidic nucleotides, thereby facilitating their elution from the column. For instance, dimethylhexyl amine (DEA) [13–19] or tetrabutylammonium (TBA) [20,21] have been reported in the literature. As endogenous CTP and araCTP exhibit the same molecular mass and the same MS/MS transitions, a chromatographic baseline separation between these compounds was required prior to MS detection. Several assays for the detection and determination of araCTP have been published using tritium-labelled araC [22]. Separation of metabolites was performed using ionic chromatography with UV detection [23] or scintillation spectrometry [24], but never by LC/MS/MS. This paper describes the development and validation of an ion-pair LC/MS/MS method for the simultaneous analysis

of araCTP, CTP and dCTP in human follicular lymphoma cell line (RL) and in a dCK-deficient human follicular lymphoma cell line (RL-G) using a porous graphitic carbon (PGC) stationary phase coupled with electrospray ionization (ESI) mass spectrometry. One application of this method is illustrated by an experiment in cell lines.

## 2. Materials and methods

### 2.1. Chemicals

2-Chloroadenosine 5'-triphosphate (CIATP) and araCTP were synthesized following a published procedure [25].  $^{13}C_9,^{15}N_3$ -Cytidine 5'-triphosphate (CTP\*),  $^{13}C_9,^{15}N_3$ -2'-deoxycytidine 5'-triphosphate (dCTP\*), 2'-deoxycytidine 5'-triphosphate (dCTP), cytidine 5'-triphosphate (CTP), 8-bromoadenosine 5'-triphosphate (BrATP) used as internal standard (IS) for NuTP were obtained from Sigma-Aldrich (Saint Quentin, France). The bis(SATE) prodrug of araCMP (compound 1, Fig. 1) was synthesized following a previously published procedure [26].

High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Carlo Erba (Val-de-Reuil, France). Other chemicals, 99.5% (v/v) diethylamine and 99% (v/v) ammonium acetate and glacial acetic acid from Fluka, 99% (v/v) hexylamine from Acros Organics, 28% (v/v), rectapur concentrated  $NH_4OH$  from Fisher Scientific. Ultrapure water was from a Milli-Q purifier (Millipore, France). Nitrogen was from Air Liquide (Paris, France). GV spring filters (0.45  $\mu m$ ) were from Millipore (Yonezawa, Japan). Phosphate buffered saline (PBS) was from Biowhittaker (Emerainville, France). RPMI 1640 (order number 21875) medium-containing L-glutamine, penicillin-streptomycin

**Table 1**  
Method gradient profile, flow-rate 0.3 mL min<sup>-1</sup>.

Time (min)	Percentage of mobile phase in the eluent	
	A	B
0	100	0
15	70	30
25	70	30
35	0	100
36	100	0
51	100	0

(A) HA (5 mM) with DEA (0.4%, v/v), the pH was adjusted to 10 with glacial acetic acid. (B) Acetonitrile and water (1/1, v/v).

and fetal bovine serum heat-inactivated were obtained from Gibco (Cergy Pontoise, France).

## 2.2. Instrumentation

LC instrumentation included an ACQUITY™ Ultra Performance Liquid Chromatography integrated system from Waters (Milford, MA, USA), set at 272 nm. MS experiments were performed using a triple quadrupole mass spectrometer TSQ Quantum Ultra™ (Thermo Fisher Scientific Inc., Waltham, MA, USA).

**LC conditions.** Separation was performed on a hypercarb column, 5 μm, 50 mm × 2.1 mm (ThermoFisher). The mobile phase consisted of two eluents. Eluent A was hexylamine (HA, 5 mM) with DEA (0.4%, v/v), the pH was adjusted to 10 with glacial acetic acid. Eluent B was acetonitrile/water (50/50, v/v). The flow-rate was 0.3 mL min<sup>-1</sup> and the temperature of the analytical column was maintained at 30 °C. The autosampler temperature was kept at 5 °C. The method gradient profile was presented in Table 1. 10 μL of sample was injected with full loop mode.

**Mass spectrometric conditions.** Ionization was performed using an electrospray ionization source operating in the negative ion mode. The sheath and auxiliary gas were set at 45 and 35 arbitrary units (au), respectively (all N<sub>2</sub>). The collision gas (argon) pressure was set at 1.7 mTorr. The spray voltage was 3000 V. The capillary was heated to 380 °C. A 0.7 full width half mass (FWHM) resolution (unit resolution) of the hyperquads was used for all transitions. The source collision-induced dissociation (CID) was not used. The instrument was optimized for araCTP, CTP\*, dCTP\*, CTP, dCTP, CIATP and BrATP by infusing a 2.5 μg mL<sup>-1</sup> solution of each compound in acetonitrile/water (50/50, v/v) at 10 μL min<sup>-1</sup>. Data was acquired using Xcalibur 2.0 software (Thermo Fisher Scientific Inc.). The multiple reaction monitoring (MRM) transitions of *m/z* 585–159, *m/z* 525–159, *m/z* 482–159, *m/z* 494–159, *m/z* 482–159, *m/z* 478–159 and *m/z* 466–159 were chosen for BrATP, CIATP, araCTP, CTP\*, CTP, dCTP\* and dCTP, respectively.

## 2.3. Cell culture

The RL-7 cells are derived from a human follicular lymphoma [27]. The resistant variant RL-G which is dCK-deficient was obtained by exposure to progressively increased concentrations of gemcitabine over a 12-month period as previously described [28]. Both lines were grown at 37 °C in the presence of 5% CO<sub>2</sub>, in RPMI 1640 containing L-glutamine and supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units mL<sup>-1</sup>) and streptomycin (0.1 mg mL<sup>-1</sup>). The medium was renewed every 3 days.

## 2.4. Stock and working standard solutions

Stock standard solutions of araCTP, BrATP and CIATP (500 μg mL<sup>-1</sup>) were prepared precisely by weighing out 0.5 mg of powder which was dissolved in 1 mL of ultrapure water. Stock

solutions of CTP\* and dCTP\* (50 μg mL<sup>-1</sup>) were prepared by diluting commercial solutions (1 mM) in MilliQ water. A series of working standard solutions of araCTP, CTP\*, dCTP\* were prepared by subsequent dilution of the above-mentioned stock standard solutions in MilliQ water to reach concentration ranges of 0.5–50 μg mL<sup>-1</sup> for araCTP, 0.05–50 μg mL<sup>-1</sup> for CTP\* and 0.05–50 μg mL<sup>-1</sup> for dCTP\*. Working standard solutions of the internal standards, BrATP and CIATP (50 μg mL<sup>-1</sup>) were also prepared by subsequent dilution of the above-mentioned stock standard in MilliQ water. Stock and working standard solutions were stored at –80 °C.

## 2.5. Cell extract preparation

Cell extracts were prepared from the human follicular lymphoma cell line (5 × 10<sup>6</sup> cells/sample). Cell culture medium was removed by centrifugation (290 × g, 10 min, 5 °C) and cells were washed three times with cold phosphate-buffered saline (PBS, 3 × 500 μL/5 × 10<sup>6</sup> cells). After centrifugation (290 × g, 10 min, 5 °C), the cell pellet was resuspended in a solution containing 60% methanol (250 μL/5 × 10<sup>6</sup> cells), vigorously vortex mixed for 15 s and kept overnight at –20 °C to precipitate proteins. The following day, cell debris were removed by centrifugation at 13,200 × g for 3 min at room temperature, and the supernatant was extracted using WAX SPE procedure or directly stored at –80 °C for samples used to study matrix effect).

## 2.6. WAX SPE procedure

The cartridges were preconditioned by passage of 2 mL of methanol and 2 mL of milliQ water. Supernatant obtained during cell extract preparation was diluted with 1.75 mL of solution MeOH/H<sub>2</sub>O (60/40, v/v). This biological sample was passed through the preconditioned cartridges at a rate of 1 drop s<sup>-1</sup>. The cartridges were then washed (2 mL of 50 mM ammonium acetate buffer, pH 4.5) and the target analytes (NuTP) were eluted with 2 mL of MeOH/H<sub>2</sub>O/NH<sub>4</sub>OH (24/5/1, v/v/v). Finally, sample was evaporated to dryness under a nitrogen stream at room temperature. Samples were stored at –80 °C until LC/MS/MS analysis.

## 2.7. Validation procedure

Validation of the analytical method was performed according to the FDA guidance [29]. The validation parameters included specificity, linearity, intra- and inter-assay precision and accuracy, extraction recovery and stability.

The specificity of the proposed method toward endogenous cell extract compounds was tested in five different batches of drug-free cell extract by analyzing blanks, samples containing no drug but the internal standard and cell extract samples spiked with araCTP, dCTP\* and CTP\* at their corresponding 3 × LOQ levels and internal standard at 4 μg mL<sup>-1</sup> (Fig. 2A and B).

Three different calibration curves were performed in cell extracts and analyzed on three separate days. Each calibration curve consisted of one “blank” cell extract and seven calibration standards. Calibration spiked cell extract standards were prepared fresh daily by dilution of working standard solutions of unlabelled araCTP and labelled compounds (CTP\* and dCTP\*) as well as the IS (BrATP) with 250 μL of cell pellets (5 × 10<sup>6</sup> cells) obtained before proteins precipitation step. Spiked cell extract calibration standards at seven different concentration levels 0.10, 0.30, 0.50, 1.00, 2.00, 4.00 and 8.00 μg mL<sup>-1</sup> for araCTP, 0.01, 0.03, 0.10, 1.00, 2.00, 10.00 and 30 μg mL<sup>-1</sup> for CTP\*, 0.01, 0.03, 0.30, 0.50, 1.00, 4.00 and 8.00 μg mL<sup>-1</sup> for dCTP\* were prepared (Table 2). Each calibration sample contained 4 μg mL<sup>-1</sup> of the internal standard. Then, all samples (calibration standard and QC) were loaded on phase extraction

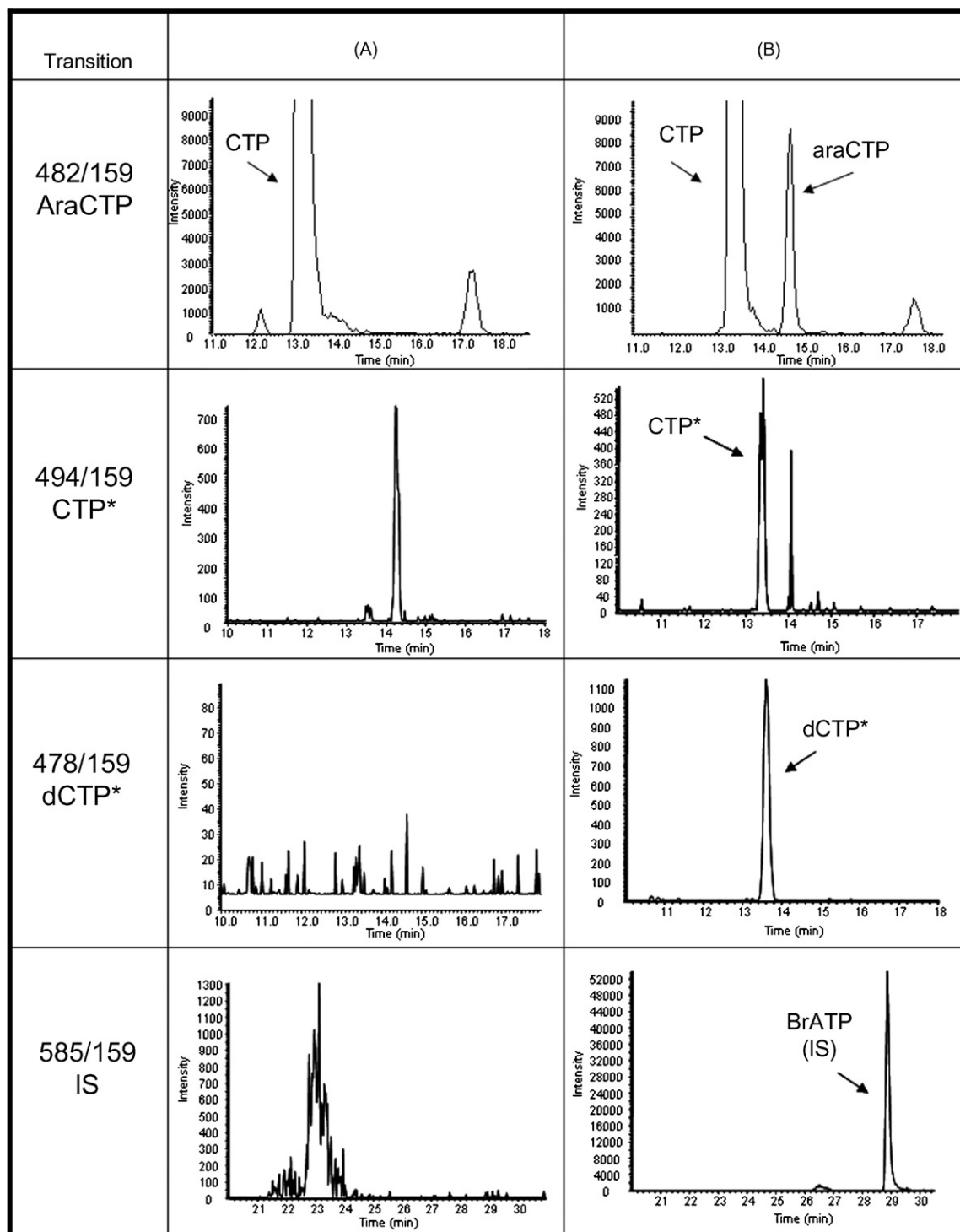
support (solid phase extraction (SPE)) Oasis® WAX cartridges and analyzed by LC/MS/MS. The calibration curves were drawn by plotting the peak area ratio of each compound (araCTP, CTP\*, dCTP\*) to the signal area of the internal standard (BrATP) versus the nominal concentration ( $\mu\text{g mL}^{-1}$ ) of each analytes in cell extract.

Six independent replicates ( $n=6$ ) of the quality control (QC) samples were also prepared at four concentration levels 0.1, 0.3, 4 and  $8 \mu\text{g mL}^{-1}$  for araCTP, 0.01, 0.03, 10 and  $30 \mu\text{g mL}^{-1}$  for CTP\* and 0.01, 0.03, 4 and  $8 \mu\text{g mL}^{-1}$  for dCTP\* and tested the same day in order to assess intra-day variability. All QC samples were freshly

prepared for each run. Inter-assay performance was monitored in four replicates at three levels during three different days.

### 2.7.1. Accuracy and precision

The accuracy was determined by comparing the means of the concentrations found in the quality control samples with the theoretical values. Precision was represented as coefficient of variation (CV) in percentage (%CV). The acceptance criteria for within- and between-batch precision were 20% or less of the coefficient of variation for lower limit of detection (LLOQ) and 15% or less



**Fig. 2.** Ion chromatograms of (A) blank cell extract, (B) cell extract spiked with araCTP ( $0.3 \mu\text{g mL}^{-1}$ ), CTP\* ( $0.03 \mu\text{g mL}^{-1}$ ), dCTP\* ( $0.03 \mu\text{g mL}^{-1}$ ) and BrATP ( $4 \mu\text{g mL}^{-1}$ ) and (C) samples containing no drug but the internal standard.

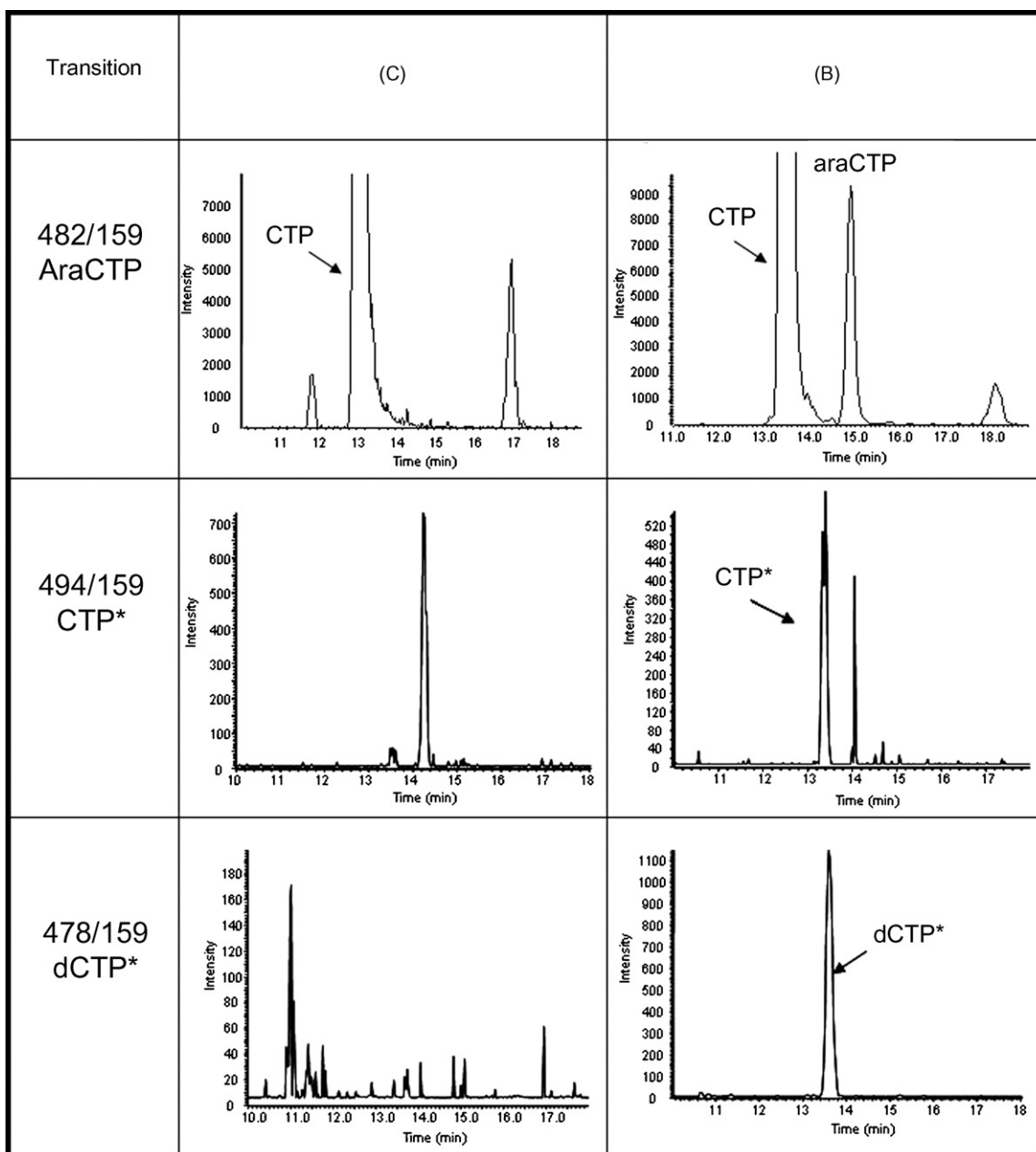


Fig. 2. (Continued)

of the CV for the other concentrations, and for accuracy were 80–120% of the nominal value at the LLOQ and 85–115% of the nominal value at the other concentrations. The lower limit of detection (LOD) was determined at a signal-to-noise (S/N) ratio

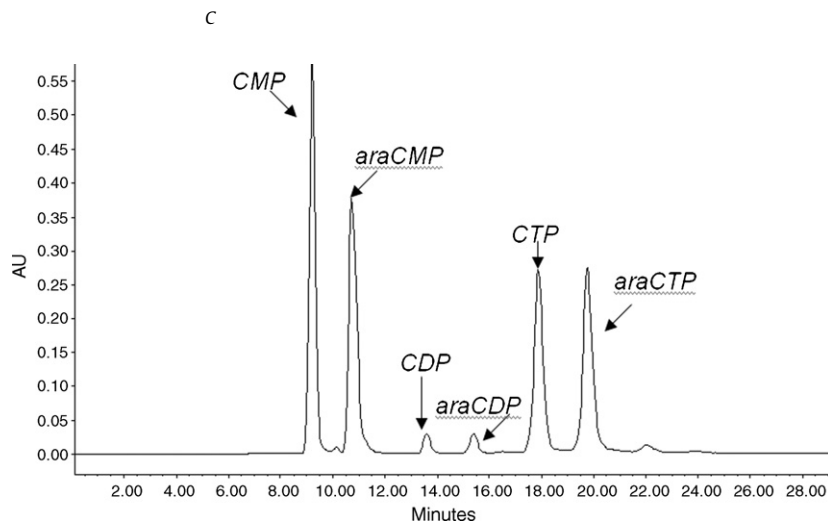
of 3. The lower limit of quantification, the lowest concentration in the calibration curve that can be determined with acceptable accuracy and precision, was measured at a minimum S/N ratio of 10. The between-run precision (BRP), and accuracy of

Table 2

Linearity of araCTP, CTP\* and dCTP\* in cell extract from RL-7 cells was determined by analysing three separate analytical batches. Each batch contained standards consisting of seven points in three replicates for each concentration. Data were represented as mean of three samples.

Curves ranges ( $\mu\text{g mL}^{-1}$ )			araCTP		CTP*		dCTP*	
araCTP	CTP*	dCTP*	CV (%)	RE (%) <sup>a</sup> mean (n=3)	CV (%)	RE (%) <sup>a</sup> mean (n=3)	CV (%)	RE (%) <sup>a</sup> mean (n=3)
0.1	0.01	0.01	8.9	0	6.7	0	5.7	0
0.3	0.03	0.03	2.4	5.7	7.5	6.7	7.6	13.3
0.5	0.1	0.3	8.5	0.4	6.1	8	2.4	7.7
1	1	0.5	0.3	-10.1	3.7	-12	3.5	-12.8
2	2	1	6.9	-1.1	8.9	-1.6	6.7	0.4
4	10	4	4.1	-8.5	3.1	-9.5	5.6	-7.1
8	30	8	2.9	5.5	1.3	3.7	13.2	-5.1

<sup>a</sup> [(Mean measured concentration – spiked concentration)/spiked concentration] × 100.



**Fig. 3.** Chromatogram obtained at  $\lambda = 272$  nm for a mixture of araCTP, CTP, araCDP, CDP, CMP and araCMP in water. Separation was performed on a hypercarb column, 5  $\mu\text{m}$ , 50 mm  $\times$  2.1 mm (ThermoFisher). The mobile phase consisted of two eluents. Eluent A was water with hexylamine (HA, 5 mM) and DEA (0.4%, v/v), the pH was adjusted to 10 with glacial acetic acid. Eluent B was acetonitrile/water (50/50, v/v). The flow-rate was 0.3 mL min<sup>-1</sup> and the temperature of the analytical column was maintained at 30 °C.

the method were evaluated on three separate days by four replicates analysis of quality control containing araCTP, dCTP\* and CTP\* at concentration of 0.1, 4, 8 ng mL<sup>-1</sup>, 0.01, 4, 8 ng mL<sup>-1</sup> and 0.01, 10, 30 ng mL<sup>-1</sup>, respectively in cell extract from RL-7 cells. An estimate of the BRP was obtained by one-way analysis of variance (ANOVA) for each test concentration using “run day” as the classification variable. The BRP was estimated as  $\text{BRP} = 100 \times (\sqrt{(\text{MS}_{\text{bet}} - \text{MS}_{\text{wit}})/n/\text{GM}})$ .  $\text{MS}_{\text{wit}}$ ,  $\text{MS}_{\text{bet}}$ ,  $n$ , GM, represented the within-groups mean square, the between-groups mean square, the number of replicate observations within each run and the grand mean, respectively. These parameters were calculated using the software Statview for Windows Version 5.0 (SAS Institute, Cary, USA).

Stability experiments were performed to evaluate the stability of each analyte and IS in cell extract sample or in free cell extract sample under different temperature and timing conditions and under conditions used during the extraction procedure. Three aliquots of QC free cell extract of mid concentration were subjected to the following conditions: three freeze–thaw cycles (–80 °C to room temperature), storage (3 weeks at –80 °C), storage at room temperature for 1 h under acidic conditions or 3 h under basic conditions used for SPE WAX extraction.

The stability of QC prepared in cell extract of mid-concentration was also assessed after addition of MeOH/H<sub>2</sub>O (60/40, v/v) and kept for 24 h at –20 °C. Post-preparation stability was assessed by analyzing the extracted QC samples kept either in the autosampler at 4 °C for 24 h or at room temperature for 7 h that exceeded the routine preparation time of samples.

## 2.8. Extraction recoveries

Extraction recoveries were carried out at concentrations of 0.1, 4 and 8  $\mu\text{g mL}^{-1}$  for araCTP, 0.01, 10, 30  $\mu\text{g mL}^{-1}$  for CTP and 0.01, 4 and 8  $\mu\text{g mL}^{-1}$  for dCTP, and 4  $\mu\text{g mL}^{-1}$  for BrATP with five samples of each concentration. For the determination of the absolute recovery of various analytes and the IS from the extraction procedure, the method described by Matuszewski et al. [30] was applied. The absolute recovery was determined by a comparison of the peak areas derived from cell extract samples (QC samples at three concentrations, with five samples for each concentration) spiked before proteins precipitation step with the peak areas derived from cell extract samples spiked after the extraction procedure with the same amount of analyte. To determine the extraction recovery of BrATP we used CIATP as IS. This IS was used to determine the extraction recovery of other compounds.

## 2.9. Matrix effect

We studied the matrix effect on ionisation only for araCTP because this compound was suspected to be present in cell extract at a lower concentration and it was the compound of interest. The matrix effect before and after SPE extraction was evaluated by comparing the results of analysis of three sets of samples in according to Matuszewski et al. [30]. In the first set (A) two levels of standards, dissolved in HPLC mobile phase, were analyzed in triplicate (six samples). In the second set (B), samples were spiked with two levels of araCTP after proteins precipitation step. Samples were not

**Table 3**  
Intra-assay performance data for araCTP, CTP\* and dCTP\* in cell extract from the human follicular lymphoma cell line RL-7. Six separately spiked samples at each concentration were analyzed in the same run ( $n = 6$ ).

Compound	Nominal concentration ( $\mu\text{g mL}^{-1}$ )	Measured concentration ( $\mu\text{g mL}^{-1}$ ) mean $\pm$ S.D. ( $n = 6$ )	Accuracy (%)	Precision (%)
araCTP	0.100	0.102 $\pm$ 0.007	102.0	6.9
	0.300	0.315 $\pm$ 0.021	105.0	6.9
	4.00	3.85 $\pm$ 0.34	96.3	8.1
	8.00	7.80 $\pm$ 0.82	97.5	10.5
CTP*	0.010	0.010 $\pm$ 0.001	100	12.6
	0.030	0.031 $\pm$ 0.003	103.3	11.7
	10.00	10.07 $\pm$ 0.91	100.7	9.0
	30.00	28.89 $\pm$ 3.36	96.3	11.6
dCTP*	0.010	0.010 $\pm$ 0.001	100.0	7.9
	0.030	0.032 $\pm$ 0.003	93.4	10.8
	4.00	4.16 $\pm$ 0.47	96.0	11.4
	8.00	8.44 $\pm$ 0.63	94.5	7.5



**Table 4**

Assessment of accuracy and precision in cell extract from RL-7 cells. Inter-assay was carried out at three concentrations on three different days with four replicates ( $n = 12$ ).

Concentration ( $\mu\text{g mL}^{-1}$ )		Accuracy (%)	Precision (%), between-run
Spiked	Found (mean $\pm$ S.D.)		
<b>araCTP</b>			
0.1	0.103 $\pm$ 0.008	103.1	4.7
4	4.16 $\pm$ 0.38	103.9	7.1
8	7.95 $\pm$ 0.90	99.3	4.1
<b>CTP*</b>			
0.01	0.0102 $\pm$ 0.0012	102.0	1.6
10	10.24 $\pm$ 0.88	102.4	1.3
30	30.68 $\pm$ 2.89	102.5	7.6
<b>dCTP*</b>			
0.01	0.010 $\pm$ 0.001	100.7	2.5
4	4.22 $\pm$ 0.41	105.6	7.9
8	8.29 $\pm$ 0.72	103.6	6.7

subjected to SPE extraction. In the third set (C), samples from cell extract were extracted using SPE and then spiked with two levels of araCTP. For sets, A–C, the peak area ratios were calculated between analyte and BrATP (IS). The matrix effect (ME) with or without used SPE was calculated as follows:  $ME (\%) = C/A \times 100$  and  $ME (\%) = B/A \times 100$ , respectively.

### 2.10. Application: incubation of cells with araC or bis(SATE)araCMP prodrug

For each experiment, 5 million cells were incubated in presence of araC (10  $\mu\text{M}$ ) or bis(SATE)araCMP prodrug (10  $\mu\text{M}$ ) at 37 °C under a 5% CO<sub>2</sub> atmosphere for 6 h. After incubation, cells were harvested by centrifugation (1200 rpm, 10 min, 5 °C), washed three times with 500  $\mu\text{L}$  of cold PBS then internal standard (BrATP) was added. The metabolites present in the cell pellet were extracted by addition of 250  $\mu\text{L}$  of solution MeOH/H<sub>2</sub>O (60/40, v/v). This solution was vigorously vortex mixed for 15 s and stored overnight at –20 °C. The day after, cell debris were removed by centrifugation at 13,000 rpm for 3 min at room temperature and the supernatant, which contained the intracellular metabolites, was diluted with 1.75 ml of a methanol/H<sub>2</sub>O (60/40, v/v) solution. Then, the samples were loaded on phase extraction support (SPE) using a procedure previously described.

## 3. Results and discussion

### 3.1. Method development

Because araCTP has the same molecular mass as CTP the endogenous nucleotide, naturally present in cells and also the same major daughter ion ( $m/z$  159), chromatographic separation between these two compounds was required prior to MS detection and quantification. During the last decade, porous graphitic carbon has emerged as a new packing material for polar analytes [31] including nucleotides [32,33] and this material was used to resolve structurally related compounds. In addition, due to its chemical

nature and physical structure, the PGC stationary phase tolerates a wide pH range (0–14). Therefore, we decided to test whether the separation of araCTP and CTP could be performed using PGC as the stationary phase. In preliminary PGC experiments, we have shown that this stationary phase provides adequate selectivity for the separation of araCMP from CMP [33]. However, the mobile phase (0.1% formic acid with ACN) used in this experiment eluted only nucleosides and nucleoside 5'-monophosphates while the nucleoside 5'-triphosphates were trapped on the column. As previously demonstrated [32], even a high concentration of formic acid (50 mM) did not elute nucleoside 5'-triphosphates, presumably due to insufficient competition with electronic interactions between triphosphates and the PGC surface [34].

Due to the high polarity of the analytes and in order to improve their separation, the addition of different ion-pairing agents in the aqueous mobile phase was investigated. The ion-pairing agent acts as a counter ion for the acidic nucleotides, thereby facilitating their elution from the column. Alkylamines were shown to be promising ion-pairing agents for acidic analytes [35] and we tested several alkylammonium salts. By using 20 mM TEAAc in the aqueous mobile phase, CTP and araCTP could not be separated. The effect of TEAAc concentration on chromatographic behaviour was checked in the 5–50 mM range but no improvement in selectivities was observed. As previously described by Coulier et al. [36], hexylamine may be used as ion-pairing agent to analyse nucleotides on a reversed phase column. Thus, we have shown that the amount of hexylamine could be minimized to 5 mM and led to good retention of analytes and minimal contamination in the MS source.

Under chromatographic conditions described above, we observed a peak tailing probably due to strong interactions between the PGC phase and the phosphate groups. To improve the peak shape, the effect of diethylamine (DEA) as a modifier was evaluated at various concentrations. The ion pairing between DEA and the phosphate group was expected to reduce interactions between analytes and PGC, and thus may improve peak shape [32]. With the addition of 0.4% DEA, reasonable peak shapes were

**Table 5**

Recovery percentages obtained for araCTP, dCTP\*, CTP\* and BrATP in cell extract from the human follicular lymphoma cell line RL-7.

	Concentration ( $\mu\text{g mL}^{-1}$ )									
	Low			Middle				High		
	0.1 araCTP	0.01 dCTP*	0.01 CTP*	4 BrATP	4 araCTP	4 dCTP*	10 CTP*	8 araCTP	8 dCTP*	30 CTP*
Yield (%)	81.9	60	73.8	81	81.9	73	84.6	75.2	72.5	63
CV (%) ( $n = 5$ )	5.1	12.9	19.6	1.31	2.2	4.4	7.7	2.6	2.4	15.9

**Table 6**  
Matrix effect (ME).

	Concentration araCTP 4 $\mu\text{g mL}^{-1}$	8 $\mu\text{g mL}^{-1}$
Standard solutions (A) Ratio (drug/IS) (mean $\pm$ S.D.) (n = 3)	2.87 $\pm$ 0.19	6.17 $\pm$ 0.23
Cell extract spiked after protein extraction and before SPE extraction (B) Ratio (drug/IS) (mean $\pm$ S.D.) (n = 3)	2.06 $\pm$ 0.09	4.44 $\pm$ 0.29
Cell extract spiked after protein extraction and with SPE extraction (C) Ratio (drug/IS) (mean $\pm$ S.D.) (n = 3)	3.01 $\pm$ 0.09	6.21 $\pm$ 0.14
ME % without SPE	71.8	72.0
ME % with SPE	106.0	100.6

(A) Two concentration points of standards were dissolved in HPLC mobile phase and analysed in triplicate. (B) Three cell extracts were spiked with two levels of araCTP, samples were not extracted by SPE. (C) Three cell extracts were extracted by SPE and spiked with two levels of araCTP. ME values were calculated as follows: without SPE, ME (%) =  $B/A \times 100$  and with SPE, ME (%) =  $C/A \times 100$ .

obtained (Fig. 3). All these assays were initially performed by UV detection.

It was essential to optimize the pH of the mobile phase in order to obtain significant retention of the analytes, peak shapes and correct mass spectrometry response. A pH value of 10 for the mobile phase A was found to be the optimum for the nucleotides retention. A pH of 7 was required for the mobile phase B, especially to allow the formation of ion-pair between hexylamine and nucleotides, thus reducing electronic interactions between triphosphates and the PGC surface. As illustrated in Fig. 3, this method allowed chromatographic separation between each couple of nucleotides: araCMP/CMP, araCDP/CDP and araCTP/CTP. The optimal MS response was obtained with ESI in negative mode.

### 3.2. Validation study

#### 3.2.1. Specificity

Blank matrix from five batches was tested for endogenous interference. Under optimized HPLC and MS conditions, blank cell extracts showed no significant interfering peaks at the retention times of each analytes or the IS (Fig. 2).

#### 3.2.2. Linearity

For araCTP and CTP\*, calibration curves were constructed using a linear regression ( $Y = aX + b$ ) and a  $1/X$  weighted regression was used. For dCTP\*, the best fit was obtained by using a quadratic regression model with  $1/X^2$  weighting.

The mean coefficients of determination ( $r^2$ ) for the validation runs were 0.9937 for araCTP, 0.9960 for CTP\* and 0.9865 for dCTP\*. The calibration curves (Table 2) for both analytes were acceptable according to the validation criteria as the back-calculated values were within  $\pm 15\%$  of the nominal concentrations ( $\pm 20\%$  at the LLOQ) in the 3-day validation. The LLOD and the LLOQ in cell extracts were respectively 0.05 and 0.1  $\mu\text{g mL}^{-1}$  for araCTP, 0.005 and 0.01  $\mu\text{g mL}^{-1}$  for both CTP\* and dCTP\*.

**Table 7**  
Concentrations of araCTP, CTP and dCTP ( $\mu\text{g mL}^{-1}$ ) observed in parental RL-7 and resistant RL-G cell lines after 6 h of exposure to 10  $\mu\text{M}$  of araC or of prodrug of araCMP. Ratio of araCTP concentration in RL-7 and RL-G cell lines.

Compound incubated	RL-7 cell line			RL-G cell line			[araCTP] <sub>RL-7</sub> /[araCTP] <sub>RL-G</sub>
	[araCTP] <sup>a</sup>	[CTP] <sup>a</sup>	[dCTP] <sup>a</sup>	[araCTP] <sup>a</sup>	[CTP] <sup>a</sup>	[dCTP] <sup>a</sup>	
araC (10 $\mu\text{M}$ )	>8 CV = 6.6%	8.37 CV = 13.4%	0.53 CV = 23.6%	<0.1 CV = 12%	7.99 CV = 21.0%	0.06 CV = 12.9%	>177
Incubation prodrug of araCMP (10 $\mu\text{M}$ )	1.32 CV = 5.9%	9.87 CV = 5.8%	1.02 CV = 7.8%	0.18 CV = 10.9%	10.04 CV = 10.6%	0.10 CV = 16%	7
Blank cell culture	ND <sup>b</sup> –	6.71 CV = 3.5%	0.91 CV = 1.7%	ND –	9.52 CV = 6.4%	0.09 CV = 6.1%	–

<sup>a</sup> Data was represented as mean of three separate experiments (n = 3).

<sup>b</sup> Not detected.

#### 3.2.3. Intra- and inter-day accuracy and precision

Tables 3 and 4 show the validation results for intra- and inter-day assay accuracy and precision. For the QC samples, the intra-day and inter-day accuracy of araCTP were from 96.3% to 105% and the inter-day and intra-day precision was less than 10.5%. The inter-day and intra-day accuracy of dCTP\* were from 93.4% to 105.6% and the inter-day and intra-day precision was less than 11.4%. For CTP\*, the inter-day and intra-day accuracy were from 96.3% to 103.3% and the inter-day and intra-day precision was less than 12.6%. These results satisfied the acceptance criteria and consequently the analytical method was judged to be reproducible in terms of intra- and inter-day accuracy and precision.

#### 3.2.4. Stability

Stability during sample handling (repeated freeze–thaw and ambient thaw) and for processed samples (extracts) was tested. All analytes (araCTP, dCTP\* and CTP\*) and IS (BrATP and ClATP) demonstrated to be stable through three freeze–thaw cycles ( $-80^\circ\text{C}$  to room temperature), storage 3 weeks at  $-80^\circ\text{C}$ , storage at room temperature for 1 h under acidic conditions and 3 h under basic conditions (used for SPE WAX extraction).

The stability of QC in cell extracts prepared prior to extraction and kept for 24 h at  $-20^\circ\text{C}$  was also assessed. The samples remained stable.

Post-preparation stability was assessed by analyzing the extracted QC samples kept either in the autosampler at  $4^\circ\text{C}$  for 24 h or at room temperature for 7 h, duration that exceeded the routine preparation time of samples.

In all conditions tested, QC samples remained stable.

#### 3.2.5. Recovery and matrix effect

Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of matrix recovery samples prepared by adding compound to post-extraction matrix blanks at the corresponding QC concentrations. The mean recoveries were at least 75.2%, 60%, 63% and 81% for araCTP, dCTP\*

CTP\* and BrATP, respectively, for all three QC concentrations from cell extracts (Table 5). The effects of ion suppression on the peaks before and after SPE extraction were compared (Table 6). These results indicated that SPE extraction allow to reduce matrix effects.

### 3.3. Incubation studies: application

To circumvent cellular resistance due to a reduced intracellular concentration of araCMP, prodrugs of araCMP (compound 1, Fig. 1) have been developed. These compounds are expected to enter cells by passive diffusion and then to convert into araCMP, thus overcoming reduced nucleoside transport and/or deoxycytidine kinase deficiency. We have previously demonstrated that mononucleoside phosphotriester derivatives bearing two SATE moieties as transient phosphate protecting groups were able to deliver the corresponding 5'-mononucleotide inside the cell through an esterase mediated activation process [37]. Using a dCK-expressing and deficient lymphoma cell model, we compared the ability of compound 1, comparatively to araC, to allow the intracellular delivery of araCMP and thus circumvent araC resistance caused by dCK deficiency and/or lack of nucleoside transport.

Human follicular lymphoma cell lines RL-7 and RL-G were incubated for 6 h with araC or the bis(SATE) araCMP prodrug at 10  $\mu$ M. Concentrations of araCTP, CTP and dCTP determined by LC-MS-MS are reported in Table 7. Incubation of araC in RL-7 cells resulted in the accumulation of araCTP. In contrast, a very small amount of araCTP was detected in RL-G cells. Since the first phosphorylation step of araC is catalyzed by deoxycytidine kinase, this data was consistent with the lack of dCK activity in RL-G cell line. Conversely araCTP was formed upon incubation of either RL-7 or RL-G cells with the araCMP prodrug. However, araCTP concentration was found at an 7-fold lower concentration in RL-G cells than in RL-7 cells. This lower accumulation of araCTP in RL-G cells is presumably due to other cellular mechanisms involved in araC resistance such as increased level of 5'-nucleotidase or deficiency in nucleoside transport [38]. The ratio of [araCTP]<sub>RL</sub>/[araCTP]<sub>RL-G</sub> was 25-fold higher in araC-treated cells than in araCMP prodrug-treated cells (Table 7). Thus, demonstrating that the pronucleotide of araCMP was able to deliver araCTP inside cells and to overcome deoxycytidine kinase-mediated resistance. Our results are thus consistent with previously published data [39]. As shown in Table 7, the intrinsic dCTP levels in RL-G cell line were 10-fold lower than in the parental RL cell line, while the CTP levels of the resistant cell lines were not affected. This discrepancy in dCTP pools has been already observed in AG6000 cells line having dCK deficiency [40].

## 4. Conclusions

We have developed and validated a new LC/MS/MS method to quantify araCTP, CTP and dCTP in human cell extracts. As the levels of endogenous compounds (CTP and dCTP) may vary according to cell extracts, stable isotope labelled nucleotides (CTP\* and dCTP\*) were used to build standard curves and for needs of validation. Sample preparation was performed by solid phase extraction. Separation of nucleotides was achieved on a PGC stationary phase and this material has a great potential for nucleotides analysis. Hexylamine and DEA have proved to be excellent ion-pairing reagents for CTP and araCTP separation. Finally, this method was successfully applied to a pharmacokinetic study in a human follicular lymphoma cell line. The ratio of [araCTP]/[dCTP] and [araCTP]/[CTP] were monitored after the incubation of cells with araC and of a bis(SATE) prodrug of araCMP.

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