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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

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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 $\mu g\,mL^{-1}$ for araCTP and of 0.01 $\mu g\,mL^{-1}$ 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-β-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.

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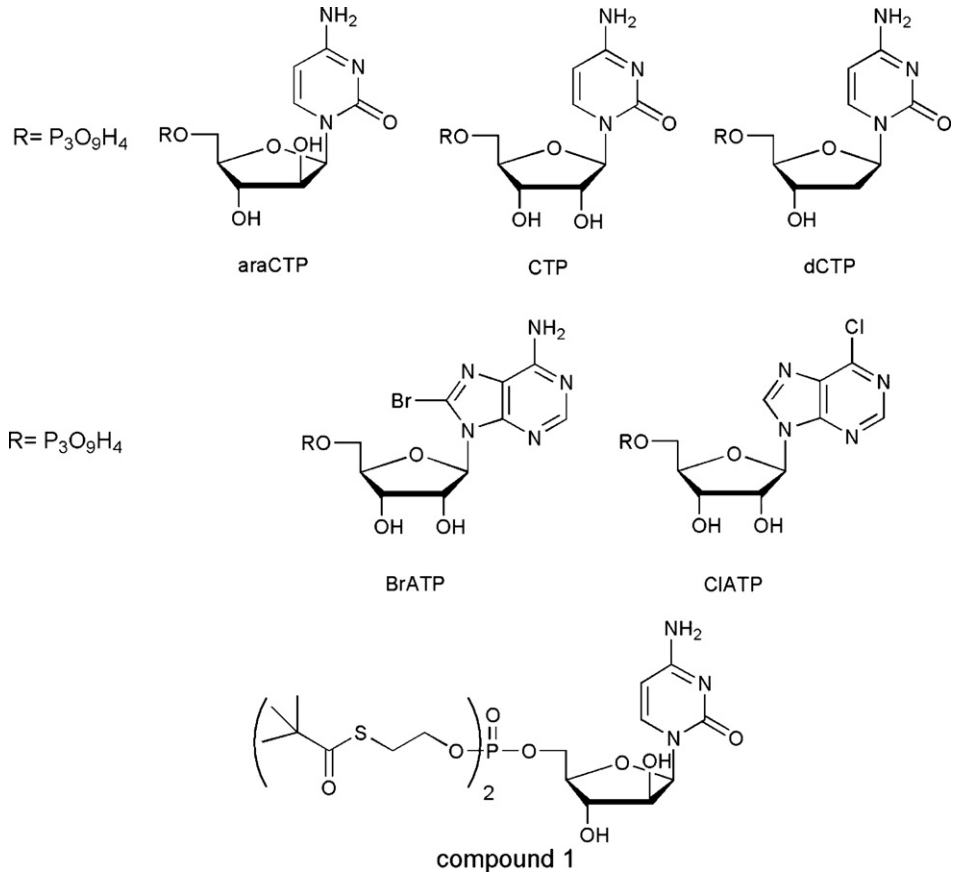

Fig. 1. Chemical structures of araCTP, CTP, dCTP, BrATP (internal standard) ClATP (internal standard) and bis(SATE) araCMP.

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 (ClATP) 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 (Valde-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 springe filters (0.45 μm) 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