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Determination of 2-chloro-2′-deoxyadenosine nucleotides in leukemic cells by ion-pair high-performance liquid chromatography

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Abstract

A specific isocratic ion-pair HPLC method for the quantitation of mono-, di- and triphosphates of 2-chloro-2′-deoxyadenosine (CdA) in leukemic cells from patients is described. The method is based on an extraction of nucleotides from cells with a solution of perchloric acid containing triethylammonium phosphate followed by an isocratic separation on an Ultrasphere ODS column (250×4.6 mm, 5 μm) with a mixture of 89% triethylammonium phosphate buffer (0.08 *M*, pH 6.1) and 11% methanol as the eluent. UV absorbance at 265 nm was used. The limit of detection was 65 n*M*. Standard curves for the CdA triphosphate (CdATP) were linear within the concentration range of 200 n*M* to 12 μM. The mean overall recovery of CdATP was 90% within a concentration range of standard curves. The within-day and day-to-day coefficients of variation at concentrations of 1.44 μM and 6.25 μM CdATP were <10%. The applicability of the method was demonstrated by in vitro studies of the accumulation of CdA mono-, di- and triphosphates in CCRF-CEM cells and by determination of the cellular pharmacokinetics of CdA nucleotides in leukemic cells from a patient treated with CdA.

Keywords: 2-Chloro-2′-deoxyadenosine 5′-triphosphate; Nucleotides

1. Introduction

2-Chloro-2′-deoxyadenosine (CdA, Cladribine, Leustatin) (Fig. 1) is an adenosine deaminase-resistant purine analogue which has shown very promising activity in the treatment of various lymphoproliferative disorders [1]. CdA used as a single agent has an outstanding activity in hairy cell leukemia with about 90% complete response rate. Results in chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphomas and refractory acute myeloid leukemias, despite lower response rates (44, 63 and 47%), are also very encouraging and make CdA one of the most interesting drugs developed in recent years.

The metabolic pathways of CdA (Fig. 1) include the degradation to 2-chloroadenine (CAde) due to acidic or enzymatic hydrolysis of the glycosidic bond. In order to be effective, CdA, as a prodrug, requires intracellular phosphorylation by deoxycytidine kinase (dCK) to its 5′-monophosphate (CdAMP) and further phosphorylation to the triphosphate (CdATP), which is mainly regarded as being responsible for the cytotoxic effects of CdA. In proliferating cells, CdATP appears to affect DNA synthesis by incorporation into the DNA rather than by inhibition of DNA polymerase [2]. The inhibition of ribonucleotide reductase (RNR) by 0.11–0.28 μM

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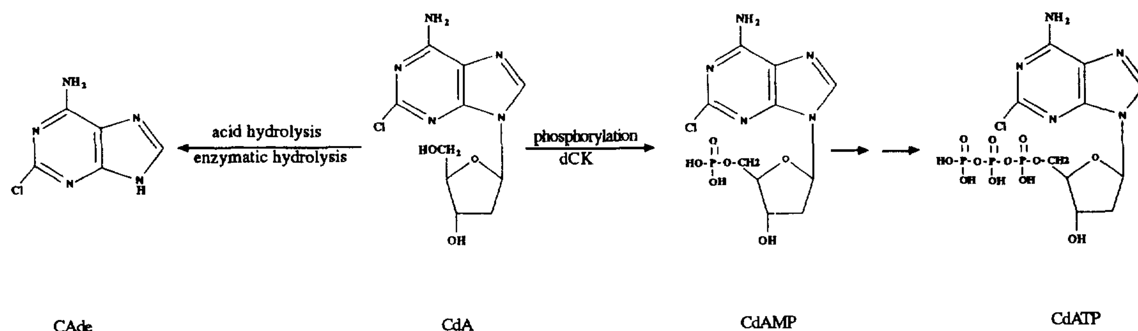

Fig. 1. Structural formulae of the parent compound CdA and its main catabolic (CAde) and anabolic (CdAMP, CdADP, CdATP) products.

CdATP (IC_{50}) also suggests effects on DNA synthesis. In resting cells, CdATP causes the accumulation of DNA strand breaks, induction of poly(ADP) ribosylation, NAD and ATP depletion, and subsequent cell death [2–4].

In order to improve treatment results in patients, individualization of dosing is warranted. Such individualization must be based on correlations between the clinical response and plasma, or intracellular drug concentrations. For antimetabolites, which mostly are prodrugs, it is important to consider the kinetics of the intracellular concentrations of the active metabolites. A weak relationship between clinical response and plasma CdA concentrations was reported in one study [5]. However, in another study, a lack of correlation between plasma CdA and cellular concentrations of CdA nucleotides was observed [6]. In that study, the concentrations of CdA nucleotides were determined indirectly as the concentrations of a parent nucleoside, CdA, resulting from the treatment of cell extracts with alkaline phosphatase. No simple method for the separation of CdA mono-, di- and triphosphates in clinical samples has been published yet. Studies of the biochemical pharmacology of CdA in cell lines in vitro have utilized a radiolabelled drug, and CdA nucleotides were quantitated from the radioactivity of fractions collected after the anion-exchange HPLC analysis of cell extracts [7–9]. The need to monitor intracellular levels of CdA mono-, di- and triphosphates in samples from patients urged us to develop a more specific method.

The present study describes an isocratic ion-pair reversed-phase HPLC assay for the quantitation of mono-, di- and triphosphates of CdA in patients' leukemic cells and in cell lines in vitro.

2. Experimental

2.1. Chemicals and reagents

CdATP was obtained from Sierra Bioresearch (Tucson, AZ, USA). Triethylamine, p.a., was purchased from Fluka (Buchs, Switzerland) and Lymphoprep from Nycomed (Oslo, Norway). RPMI 1640 medium, heat-inactivated fetal calf serum, L-glutamine and penicillin–streptomycin were all from Gibco (Life Technologies, Paisley, UK). Potassium hydroxide, p.a., ammonium dihydrogenphosphate, p.a., sodium chloride, p.a., and phosphoric acid were obtained from Merck (Darmstadt, Germany).

2.2. Cell specimens

Mononuclear cells from patient (CLL) heparinized peripheral blood samples were isolated by standard Ficoll–Hypaque density centrifugation. After separation on Lymphoprep and washing with phosphate-buffered saline (PBS), the cell pellet was dissolved in 2 ml of ice-cold distilled water. A 1-ml volume of 2.7% NaCl was added after 30 s and the volume was adjusted to 10 ml with PBS. This procedure was used to lyze erythrocytes and reticulocytes in order to avoid interferences.

Human T-lymphoblastic leukemic cells (CCRF-CEM), maintained in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin

(100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) at 37°C in a humidified air atmosphere containing 5% CO_2, were used for in vitro experiments.

The cell number of the samples and median cell volume of the samples analysed were determined by a Coulter Multisizer (Coulter Electronics, Luton, UK).

2.3. Extraction procedure

All steps of the extraction were performed on ice. A 200-μl volume of ice-cold 0.4 M perchloric acid containing 0.08 M triethylammonium phosphate (PCA–TEAP) was added to the cell pellet (10^7–$5\cdot10^8$ cells), mixed on a vortex-mixer and brought to pH 6.2 by addition of 100 μl of ice-cold 1.2 M potassium hydroxide–0.4 M ammonium dihydrogenphosphate. Following vortex-mixing and centrifugation in an Eppendorf centrifuge at 14 500 g for 5 min at 4°C, the supernatant was collected. The final volume of the supernatant was measured. A 90-μl aliquot was injected onto the HPLC column directly or the sample was stored at −20°C until analysis.

2.4. Apparatus and chromatographic conditions

The HPLC system consisted of a Milton Roy CM 4000 pump and a variable-wavelength detector (LDC Division, Riviera Beach, FL, USA) and a CMA-240 autosampler (Carnegie Medicine, Stockholm, Sweden). A Macintosh Classic computer (Apple, Chicago, IL, USA), equipped with Chromac 3.1 software (Drew, London, UK) was used for peak integration and data collection.

The column was an Ultrasphere ODS (250×4.6 mm I.D., 5 μm) purchased from Beckman Instruments (Fullerton, CA, USA), equipped with a Guard-Pak precolumn (μBondapak C_{18}; Millipore, Milford, MA, USA). The mobile phase consisted of triethylammonium phosphate buffer (TEAP) (0.08 M, pH 6.1) and methanol (89:11, v/v). The eluent was prepared according to the method of Willis et al. [10] by titrating an appropriate amount of orthophosphoric acid to the required pH with triethylamine and then diluting it to 1 l including methanol. The mobile phase was filtered through a Millipore filter (0.22 μm) and degassed prior to use. The elution was carried out at a flow-rate of 1.5 ml/min (or 1.8 ml/min, respectively) at 265 nm and at ambient temperature (22°C). The temperature of the autosampler was kept at 8°C.

The capacity factors, k', were calculated as $(t_R - t_0)/t_0$, where t_R is the retention time of an individual compound and t_0 is the retention time of an unretained compound, which was determined as the time from injection to the first distortion of the baseline.

2.5. Quantitation

Peak areas and the external standard method were used for quantitation. The molar extinction coefficient was 15 000 mol/l for CdA [11] and it was assumed to be similar for all CdA nucleotides. The intracellular concentration of CdA nucleotides was expressed as μM and was calculated by dividing the total amount of each nucleotide in the sample by the number of cells and the mean cell volume of the sample. Such a calculation assumes that the nucleotides are distributed uniformly in total cell water. Plasma CdA concentrations were determined by an HPLC method previously described [12].

3. Results

The isocratic separation of the standard solution of CdA mono- (CdAMP), di- (CdADP) and triphosphates (CdATP) is shown in Fig. 2. The retention times and capacity factors (in parenthesis) were 15 min (11) for CdAMP, 16.8 min (12.4) for CdADP and 20 min (15) for CdATP.

The ion-pair system is superior to ion-exchange chromatography since it allows a simultaneous separation of the base CAde and the parent nucleoside CdA as well. The elution order of nucleotide mono- < di- < triphosphate was similar to that observed in ion-exchange chromatography [8]. However, the possibility of separating the base and the nucleoside in the same run indicated a mixed retention mechanism.

The validation of the method was performed with a patient's mononuclear cells spiked with known amounts of CdATP and/or with CCRF-CEM cells incubated with CdA in vitro.

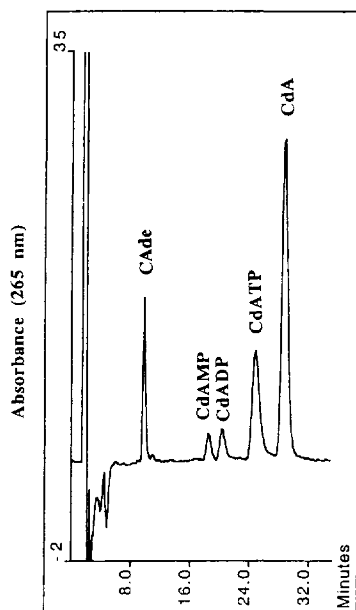

Fig. 2. Isocratic separation of a standard mixture of the base (CAde), the parent nucleoside (CdA) and its mono- (CdAMP), di- (CdADP) and triphosphate (CdATP). Column, Ultrasphere ODS (250×4.6 mm I.D., 5 μm); mobile phase, methanol–triethylammonium phosphate (0.08 *M*, pH 6.1) (11:89, v/v). Detection, 265 nm; 0.001 AUFS; flow-rate; 1.5 ml/min.

3.1. Specificity

No interference with the compounds of interest was observed in the chromatographic profile of the extracts from CCRF-CEM cells incubated with 200 n*M* CdA in vitro (Fig. 3A,B). However, when the extracts from a patient's mononuclear cells were analyzed, a peak of an unknown endogenous compound with the capacity factor of 13.0 interfered with that of CdADP (Fig. 4A,B). Changing the pH of the eluent, the concentration of the ion-pairing agent or the percentage of methanol did not avoid this interference. About 30 min were required for the analysis.

3.2. Recovery

Several extracting agents were used while optimizing the extraction procedure including, among others, 60% methanol, 1 *M* formic acid and 0.4 *M* perchloric acid with or without 0.08 *M* triethylammonium phosphate buffer (PCA, PCA–TEAP). The latter one (PCA–TEAP) was found to be the optimal extractant.

In order to assess the recovery, two calibration

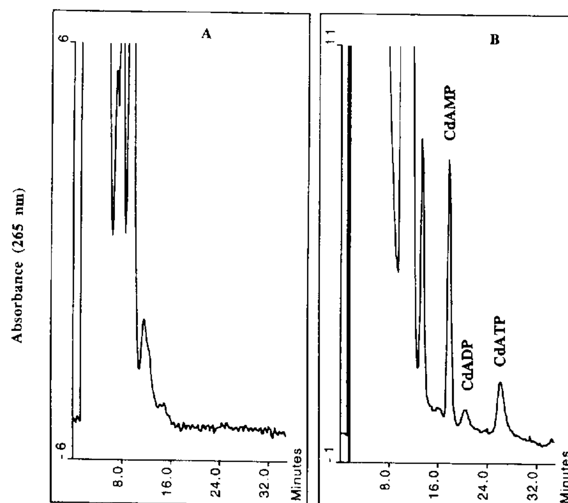

Fig. 3. Separation of CdA nucleotides in PCA–TEAP extracts from CCRF-CEM cells incubated without (A) or with 200 n*M* CdA in vitro for 40 min at 37°C (B). Injection, 23·10^6 cell equivalents; injection volume, 90 μl; detection, 265 nm; 0.001 AUFS; flow-rate, 1.5 ml/min. Other conditions as in Section 2.

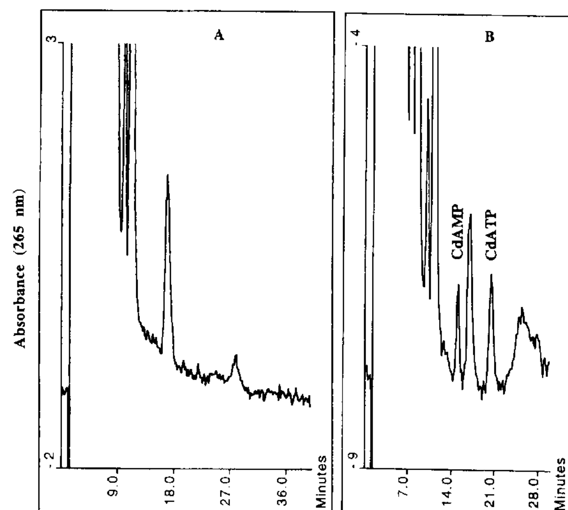

Fig. 4. HPLC profile of PCA–TEAP extracts of a patient's mononuclear cells before (A) and 2 h after treatment with a 2-h i.v. infusion of CdA (5 mg/m^2) (B). Injection, 60·10^6 cell equivalents; injection volume, 90 μl; detection, 265 nm; 0.001 AUFS; flow-rate, 1.8 ml/min. Other conditions as in Section 2.

curves based on the external standard method were set up. One calibration curve was based on PCA–TEAP extracts from mononuclear cells of a previously untreated patient ($50 \cdot 10^6$ cells) spiked with CdATP to seven concentrations in the range of 200 nM to 12 μM. The other one was based on water solutions of a reference standard in the same concentration range. The overall recovery was calculated by comparing the slopes of the regression lines generated for the two sets.

The results obtained are shown in Table 1. The mean recovery calculated by direct comparison of the peak areas was 90.3% and the overall recovery determined by comparing the slopes was 89.3%. Thus, the mean overall recovery determined by two methods was 89.8%, in the concentration range of calibration curves. Additionally, the recovery determined separately and repeatedly at the spiked concentration of 1.44 μM CdATP was 83.4±3.6% (n=3) and that at 6.25 μM CdATP was 86.4±10.2% (n=4). Thus, recovery was independent of concentration.

3.3. Linearity

The calibration curves were linear over the concentration range of 200 nM to 12 μM CdATP and the correlation coefficients obtained were >0.997. The regression equations are presented in Table 1.

The linearity of the correlation between the number of extracted cells and the resulting concentration of CdA nucleotides was checked both for the patient and CCRF-CEM cells. The correlation was found to be linear in the range of 10–180 million cells from a patient's samples (r=0.999) and for 10–50 million CCRF-CEM cells (r=0.994) (data not shown).

In order to optimize conditions for sample handling before the PCA–TEAP extraction, the mononuclear cells from blood of a previously untreated patient with CLL were freshly isolated on Lymphoprep, with or without a subsequent lysis of erythrocytes and reticulocytes. The chromatographic profiles of extracts of non-lyzed or lyzed cells are shown in Fig. 5A,B. An unknown endogenous compound interfering with CdADP diminished after lysis. Also, extracts from freshly separated cells were compared with those isolated from the blood stored on ice for 24 h. It was observed that the concentration of the interfering compound increased enormously both in non-lyzed and lyzed cells (Fig. 5C,D).

3.4. Repeatability

The within-day repeatability of the method was evaluated by analyses (n=4–6) of extracts from a

Table 1
Recovery of CdATP in PCA–TEAP extracts of spiked mononuclear cells (50×16) from a patient

Concentration spiked CdATP (μM)	Recovery (%)
0.2	84.9
0.4	95.5
0.7	96.6
1.5	85.6
3.0	87.7
6.0	92.9
12.0	89.1
Mean±S.D.	90.3±4.7
C.V. (%)	5.2
Regression line for water of CdATP:	$y=2.8119x-32.622$; $r=0.9988$.
Regression line for CdATP recovered from spiked cells from a patient:	$y=2.5118x-17.326$; $r=0.9977$.

$$\text{Overall recovery} = \frac{2.5118}{2.8119} \times 100 = 89.3\%$$

Mean recovery determined by two methods = 89.8%

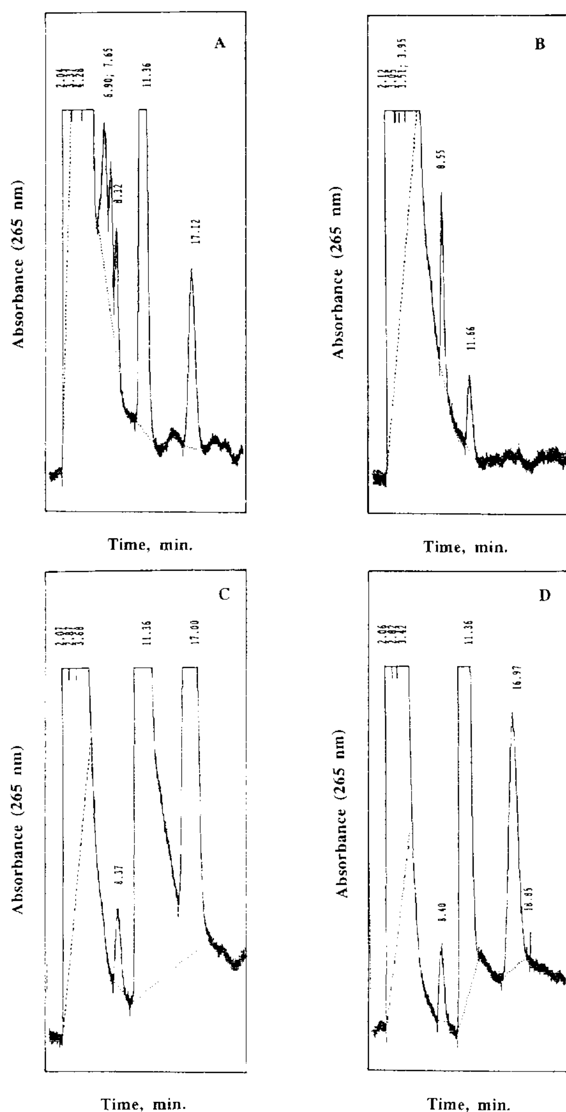

Fig. 5. Chromatograms of PCA–TEAP extracts of blank patient's mononuclear cells isolated freshly without (A) or with lysis of erythrocytes and reticulocytes (B), and extracts of non-lyzed (C) or lyzed cells (D) isolated from blood kept on ice for 24 h. Injection, $40 \cdot 10^6$ cell equivalents; injection volume, 80 μl; detection, 265 nm; 0.001 AUFS; flow-rate, 1.5 ml/min. Other conditions as in Section 2.

patient's mononuclear cells spiked to two concentrations of CdATP. The data are presented in Table 2. The coefficient of variation for extracts of cells spiked to 1.44 μM CdATP was 1.4% and that for cells spiked to 6.25 μM CdATP was 6.2%. To determine the repeatability of the assay for in vitro experiments, CCRF-CEM cells were incubated with 1 μM CdA for 2 h at 37°C, extracted and injected four times. The coefficient of variation determined was 6.2%.The day-to-day repeatability was assessed by analysis of extracts from a patient's cells spiked with CdATP to concentrations of 1.44 μM and 6.25 μM and those of CCRF-CEM cells incubated with CdA in vitro. Sample extracts were stored at −20°C and run on four to six consecutive days. The coefficients of variation were <11%, as shown in Table 2.

3.5. Limit of detection and quantitation

The limit of detection was determined as the concentration giving a signal-to-noise ratio (S/N) of 3:1. The limit of detection of CdATP was 65 nM, corresponding to 5.8 pmol injected onto the column (90 μl). The limit of quantitation was determined as the concentration giving a signal-to-noise ratio (S/N) of 6:1, with within-assay C.V. <15%. The limit of quantitation was 183 nM, corresponding to 16.5 pmol injected (90 μl).

3.6. Stability of the reference compound

The stability of CdATP under various working conditions was studied in order to optimize its extraction from cells. The apparent first-order rate constants and half-lives of CdATP stability under various conditions are shown in Table 3. The half-life of 6.25 μM CdATP in the reference water solution at 8°C was 129 h, indicating that CdATP was undergoing hydrolysis to respective mono- and diphosphates (data not shown). To confirm the importance of the working conditions, the influence of temperature on the degradation of CdATP during the PCA–TEAP extraction was studied. The degradation of CdATP in the extracting agent (PCA–TEAP) kept at ambient temperature (22°C) was about fourteen times faster than that of the one kept on ice (0–4°C).

The stability of CdATP in the PCA solution containing the ion-pairing agent (PCA–TEAP) was much higher than that in PCA alone ($t_{1/2}$ in PCA–TEAP was 4.8 h versus 1.6 h for the one in PCA alone).

Table 2
Within-day and day-to-day repeatability of CdATP quantitation by an HPLC method

PCA extracts	Coefficient of variation (%)			
	Within-day	n	Day-to-day	n
CdATP, 1.44 μM[a]	1.0	4	2.7	6
Patient's cells spiked with 1.44 μM CdATP	1.4	4	4.1	5
CdATP, 6.25 μM[a]	4.1	6	3.4	5
Patient's cells spiked with 6.25 μM CdATP	6.2	4	8.8	4
CEM cells incubated with 1 μM CdA in vitro	6.2	54	10.9	4

[a] Water solution injected directly.

3.7. Stability of CdA nucleotides in whole blood

Stability of CdA nucleotides was studied in blood samples from a patient 1.5 h after receiving 10 mg/m^2 of CdA. Samples of fresh blood were kept at ambient temperature (22°C) and/or on ice water. A considerable degradation of CdAMP and CdATP during storage was observed as shown in Fig. 6.

Samples left at ambient temperature for 10 h showed a 27% decrease in CdAMP and a 25% decrease in CdATP concentrations (compared with that after 1 h storage). Corresponding CdAMP and CdATP concentrations in samples stored on ice water decreased by 15% and 16%, respectively. After 24 h storage at ambient temperature only 24% of the CdAMP concentration was left and no CdATP was observed. While 50% of CdAMP and 75% of CdATP concentrations were recovered in samples kept on ice.

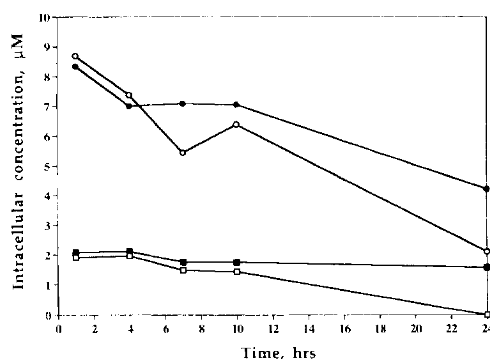

Fig. 6. Degradation of CdA nucleotides in whole blood from a patient treated with CdA orally. Intracellular concentrations of CdAMP after storage of blood at ambient temperature (○) and on ice water (●), and corresponding CdATP concentrations at ambient temperature (□) and on ice water (■) were measured as described in Section 2.

3.8. Stability of CdA nucleotides in cell extracts during storage

Stability of CdA nucleotides in cell extracts during storage was investigated in order to determine the time during which samples could be stored before the HPLC analysis, without significant degradation occurring. The PCA–TEAP extracts from CCRF-CEM cells incubated with 1 μM CdA at 37°C for different times were stored in the freezer for 27 days and then reanalyzed by HPLC. The results are shown in Fig. 7. On average, 95.7±1.6% of CdAMP,

Table 3
Apparent first-order rate constants and half-lives of CdATP stability under various working conditions

CdATP solution in	Concentration (μM)	T (°C)	k (s^{-1})	$t_{1/2}$ (h)	r
Water	6.25	8	$1.5\cdot10^{-6}$	129	0.992
PCA+TEAP[a]	6.25	0–4	$4.4\cdot10^{-6}$	43.8	0.996
PCA+TEAP[a]	6.25	22	$6.1\cdot10^{-5}$	3.2	0.996
PCA+TEAP[a]	25	22	$4.0\cdot10^{-5}$	4.8	0.999
PCA[b]	25	22	$1.2\cdot10^{-4}$	1.6	0.997

[a] 0.4 M perchloric acid + 0.08 M triethylammonium phosphate buffer.
[b] 0.4 M perchloric acid.

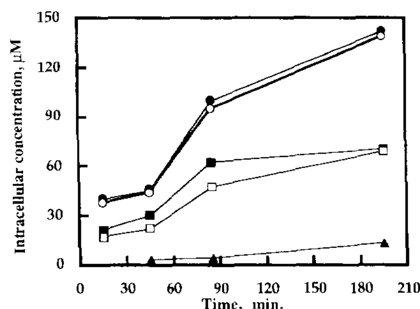

Fig. 7. In vitro accumulation of CdA nucleotides in CCRF-CEM cells incubated with 1 μM CdA at 37°C. The PCA–TEAP extracts were injected either directly onto an HPLC column [CdAMP (●), CdADP (▲), CdATP (■)] or reanalyzed after storage in a freezer for 27 days [CdAMP (○) and CdATP (□)].

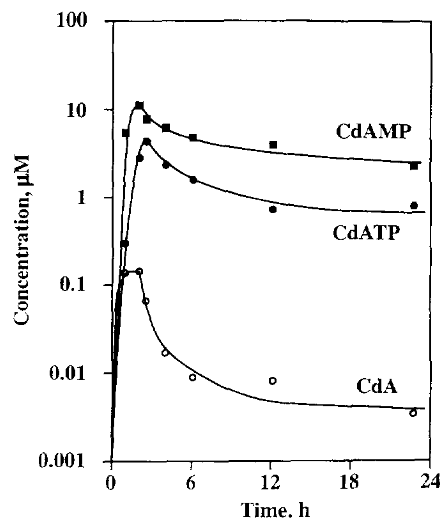

Fig. 8. Pharmacokinetics of intracellular CdAMP (■) and CdATP (●) in a CLL patient given CdA orally (10 mg/m^2). The plasma CdA pharmacokinetics (○) are also shown.

accumulated at the concentrations in the range of 40 to 140 μM, was recovered, and 81.7±11% of CdATP, accumulated in the concentration range of 20 to 70 μM was recovered. The high recovery of CdAMP may partly involve CdAMP resulting from the degradation of CdATP. The accumulation of CdADP represented only about 5–10% of the total nucleotides. Since only small amounts of samples were available for the HPLC reanalysis after 27 days, CdADP could not be adequately quantitated.

3.9. Applicability of the method

With the methodology at hand, the intracellular monitoring of CdAMP and CdATP in leukemic cells from a patient with CLL and treated with CdA (5 mg/m^2, 2-h intravenous infusion) could be studied. Much lower concentrations of CdA mono- and triphosphates were found in the cell extracts from samples from patients treated with CdA (the range of 2 to 10 μM for CdAMP and that of 0.3 to 4.3 μM for CdATP) as compared with those in CCRF-CEM cell extracts.

The pharmacokinetic profiles of the intracellular concentrations of CdA nucleotides compared with that of plasma CdA levels are presented in Fig. 8. The concentrations of CdAMP and CdATP in cells were much higher than that of CdA in plasma. The elimination half-life of CdA, as determined from four data points of the terminal part of the curve, was 8.0 h and the area under concentration versus time curve was 0.479 μM h. The corresponding values for CdAMP were 13.6 h and 97.3 μM h, and those for CdATP were 7.4 h and 27.5 μM h, respectively.

4. Discussion

The objective of our work was to develop a specific, rapid and reliable HPLC method that allowed a separation of mono-, di- and triphosphates of CdA in extracts of leukemic cells.

The gradient ion-exchange HPLC on a Partisil SAX column, widely used for the separation of nucleotides of other anticancer drugs, such as cytosine arabinoside [13–15] and fludarabine [16], did not allow an adequate separation of CdA nucleotides from endogenous compounds (data not shown).

Higher hydrophobicity of CdA nucleotides compared with other endogenous nucleotides suggested using an ion-pair reversed-phase HPLC method instead.

The superiority of the ion-pair HPLC system using triethylammonium phosphate buffers with C_{18} columns over other HPLC systems for the separation of nucleotides was demonstrated previously by Willis et al. [10], Lim and Peters [17] and Molema et al. [18]. The mixed retention mechanism allows the separation of base, nucleoside and respective nucleotides

in the same analysis. High retention of CdA nucleotides in this ion-pair system, based on higher hydrophobicity of CdA compared with adenosine (Ado) and deoxyadenosine (dAdo) [19], allows the separation of the compounds of interest from other endogenous compounds in cell extracts.

The results of the stability study of CdATP underline the importance of the working conditions. Since the external standard method is used for quantitation, the working solutions of CdATP in the required concentration range should be prepared from the concentrated stock solution, stored frozen, immediately prior to HPLC analysis. All steps of the assay, including the cell isolation and extraction, should be performed on ice (0–4°C).

A three-fold difference in half-lives of CdATP in the PCA solutions with and without TEAP was observed. Thus, in order to avoid degradation of CdATP, the addition of the ion-pairing agent, TEAP, to the PCA extracting solution is essential. This extraction procedure provides a good overall recovery.

Another crucial step of the assay is the lysis of erythrocytes and reticulocytes during the separation of mononuclear cells, in order to eliminate chromatographic interference. This problem increases when leukemic cells are not isolated within a few hours after sampling. It is therefore recommended that leukemic cells are isolated fresh as soon as possible after collection. These conclusions are also supported by the results of the stability test of CdAMP and CdATP during storage of whole blood at various temperatures.

Since the previous report [7] and the data from our studies with CCRF-CEM cells showed that the CdADP accumulation represented only about 5–10% of the total nucleotides in vitro, only CdAMP and CdATP are quantitated in the patient's samples. However, high recovery of CdATP was seen in extracts of CCRF-CEM cells incubated with CdA in vitro and stored frozen (−20°C) for nearly a month.

The presented HPLC assay is sufficiently simple, sensitive and reproducible for intracellular drug monitoring, both in vivo and in vitro.

Acknowledgments

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