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Stability and analysis of 2-chloro-2′-deoxyadenosine, 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine and 2-chloroadenine in human blood plasma

Synnöve Lindemalm,[1] Freidoun Albertioni[1] and Jan Liliemark[1,2]
Departments of [1]Clinical Pharmacology and [2]Oncology, Karolinska Hospital, 171 76 Stockholm, Sweden,
Tel: (+46) 8 729 58 32; Fax (+46) 8 33 13 43.

Cladribine (2-chloro-2′-deoxyadenosine, CdA) is a purine nucleoside analog with activity against lymphoproliferative and autoimmune disorders. 2-Chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA), a derivative of CdA with better acid stability, shows a similar *in vitro* spectrum of activity as CdA. 2-Chloroadenine (CAde) is the major catabolite of both CdA and CAFdA. We have developed a high performance liquid chromatography method to measure CdA, CAFdA and their metabolite CAde in plasma. This method employees an internal standard, chloroadenosine (CAdo), and a C_8 solid-phase extraction to isolate and concentrate the substances. Chromatographic separation was achieved using a C_8 reverse-phase column, with UV detection at 265 nm, which gives a limit of detection of 1 nmol/l for all substances. The method was reproducible with intra- and inter-assay coefficients of variations below 6% at 50 nmol/l and at 5 nmol/l below 23%. The average recoveries of CdA, CAde, CAFdA and the internal standard were higher than 70%. Stability studies of authentic patient samples show that samples containing CdA should be kept in a refrigerator or on ice to prevent degradation. Plasma containing CAde should not be kept at −20°C for longer than 10 weeks before analysis. CdA and CAFdA remain almost stable during storage at −20°C for 12 weeks.

Key words: 2-Chloro-2′-arabino-fluoro-2′-deoxy-adenosine, 2-chloro-2′-deoxyadenosine, chloroadenosine, reverse-phase HPLC, stability, storage.

Introduction

The anticancer drug cladribine, 2-chloro-2′-deoxyadenosine (CdA; Figure 1), was first synthesized more than 25 years ago.[1] CdA is a deoxyadenosine analog resistant to deamination by adenosine deaminase. The drug has a broad range of *in vitro* activity against both lymphoid and myeloid neoplasm but possesses no activity against multiple myeloma specimens and many solid tumor cell lines.[2] CdA demonstrates activity against both dividing and non-dividing cells.[3]

CdA is unstable at low pH and is also deglycosylated by bacterial nucleoside phosphorylases to 2-chloroadenine[4] (CAde; Figure 1). CdA crosses the cell membrane mainly by facilitated diffusion and is phosphorylated intracellularly by deoxycytidine kinase (dCK) and to some extent by deoxyguanosine kinase (dGK).[5] Since CdA is resistant to adenosine deaminase, the mono- and triphosphorylated forms of the drug accumulate rapidly in the cell. The triphosphate is incorporated into DNA by proliferating cells. The precise mechanism by which CdA acts has not been established, but in resting cells CdA causes DNA strand breaks, activation of poly(ADP)ribosylation, depletion of NAD and apoptosis.[6]

In the treatment of hairy cell leukemia, CdA has become the drug of choice,[7,8] with a response rate of more than 90% (80% complete remission). Preliminary results obtained in other lymphoproliferative disorders, acute myeloid leukemia in children and autoimmune diseases have also been encouraging.[2,9,10]

2-Chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA; Figure 1) is an analog of deoxyadenosine which is more acid stable than CdA. CAFdA is currently under preclinical evaluation.[4]

Previously published methods used to measure CdA are radioimmunoassay,[3] fluorescence detection[11] and high performance liquid chromatography (HPLC) with UV.[12,13] Generally the immunological assay may suffer from cross-reactivity with endogenous substances. In contrast to immunological assays, HPLC can identify and measure metabolites. Our previous HPLC method for determination of plasma CdA used guaneran as an internal standard.[12,13] With that method we have so far not been able to separate CAFdA from guaneran.

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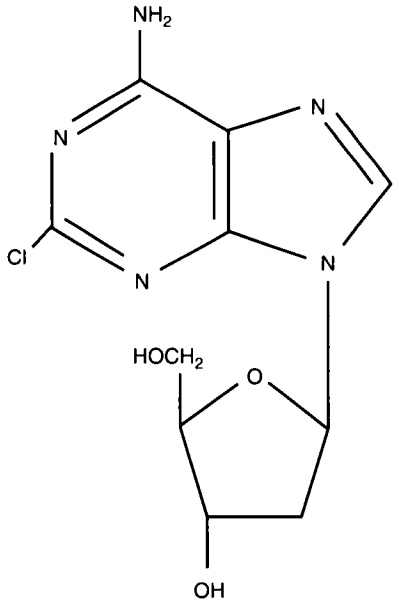
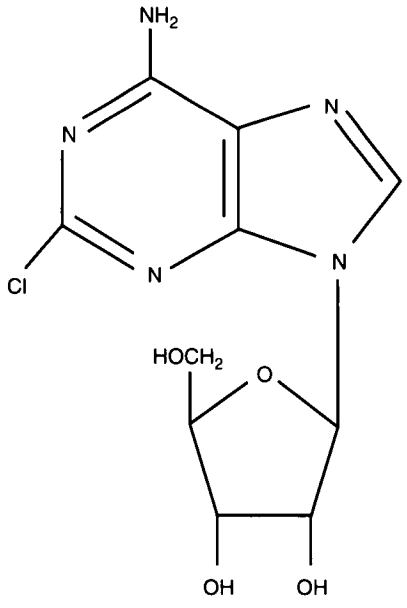
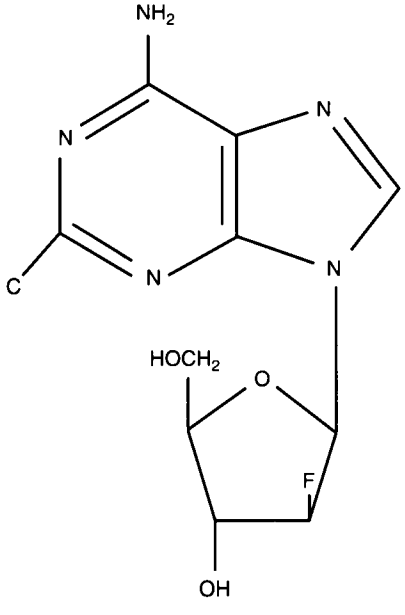
Correspondence to F Albertioni



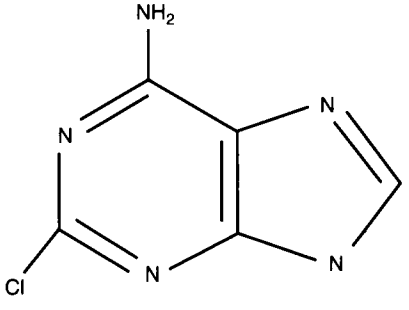
2′-Chloro-2′-deoxyadenosine (CdA)
MW 285.7



Chloroadenosine (CAdo)
MW 301.7



2-Chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA)
MW 303.7



2-Chloroadenine (CAde)
MW 169.5

Figure 1. Structural formulae of CdA, CAde, CAFdA and the IS used (CAdo).

After oral administration of CdA the bioavailability is about 50%, probably due to degradation to CAde in the acid environment of the stomach.[14,15] Previous analytical methods have not been able to measure simultaneous CAFdA and the catabolic product CAde. Furthermore, when the pharmacokinetic properties of CAFdA will be described in early clinical trials, it is important to also study the pharmakokinetics of CAde.

The aim of the study was to develop one method

which can be used for specific measurement of the concentration of either of these new and promising anticancer drugs (CdA and CAFdA) and their plasma catabolic product (CAde) in plasma. We have also investigated the stability of the drugs.

Materials and methods

Chemicals

CdA was provided by Dr Zygmunt Kazimierczuk (The Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland). CAFdA was a gift from Dr Howard Cottam (University of California, San Diego, CA). CAdo and other chemicals for the experiments were puchased from Sigma (St Louis, MO). CAde was prepared by acid hydrolysis of CdA (0.1 mol/l HCl) at 37°C. The reaction was stopped when no CdA could be detected spectrophotometrically. The recovery of CAde was 98% as determined spectrophotometrically. The CAde prepared this way had an identical absorption spectrum and capacity factor as CAde previously supplied by Dr Zygmunt Kazimierczuk. The UV absorption spectrum of the chromatographic peak of CAde from the hydrolyzate was also examined with a photodiode array detector with a similar result.

The concentration of CdA, CAFdA and CAde was determined spectrophotometrically with a Hitachi U-2000 spectrophotometer, a molar absorptivity of 15000 AU/mol was used for CdA[1] and CAFdA and 12600 AU/mol for CAde at 264 nm, pH 7.0.

Plasma sample preparation

For the solid-phase extraction, a VacMaster ((International Sorbent Technology, Mid-Glamorgan, UK) was used. C_8 (isolute 100 mg, part 290-0010-B) solid-phase extraction cartridges (International Sorbent Technology) were conditioned before use with 2 ml of methanol and 2 ml of 10 mmol/l phosphate buffer, pH 7.0. Then, 200 μl (1 μmol/l) of CAdo was added as internal standard (IS) to 1 ml plasma and mixed with 1 ml 10 mmol/l phosphate buffer, pH 7. Standard samples were prepared by spiking blank plasma with 2, 5, 10, 20, 50, 100, 200 and 500 nmol/l of CAde, CdA and CAFdA used to construct calibration curves. The mixture was applied on the cartridge with a flow rate of about 2.5 ml/min. The cartridge was then washed sequentially with 3 ml of 1% acetonitrile in phosphate buffer, pH 7.0, and dried by pulling air for 15 s. The compounds were eluted with 2 ml of 5% methanol in ethyl acetate. The elute was collected in glass test tubes and evaporated to dryness under a stream of nitrogen in a water bath (37°C). The residue was finally dissolved in 50 μl of the mobile phase and 20 μl was injected by a CMA-240 Carnegie autosampler (Carnegie Medicine, Stockholm, Sweden). CAde, CdA, CAFdA and CAdo were separated from endogenous compounds on a high-speed C_8 column (HSpecosphere 3CR C8, 80 × 4.6 mm, 3 μm; Perkin Elmer, Norwalk, CT) at room temperature. A mobile phase consisting of 10 mmol/l phosphate buffer, pH 3.0, with 6.5% acetonitrile was delivered isocratically by a Milton Roy CM 4000 multiple solvent delivery system pump (Milton Roy, Rochester, NY), at a flow rate of 1.5 ml/min. Drug concentration was determined at 265 nm with a Milton Roy variable wavelength UV detector SM 3100.

Table 1 gives a summary of the chromatographic conditions used.

Assay validation

The intra- and inter-assay variabilities of CAde, CdA and CAFdA were examined at three concentration levels, i.e. 5, 50 and 200 nmol/l, in pooled plasma from healthy donors. The recovery of each compound was assessed by comparing peak area after extraction with the peak area ratios obtained from direct injection of equivalent quantities of pure standard. The capacity factor, k', of each compound was calculated as $(t_r - t_0)/t_0$, where t_r is the retention time of the compound and t_0 is the retention time of the first distortion of the baseline.

Table 1. Summary of conditions for HPLC analyses of CdA, CAde and CAFdA

Parameter	Assay conditions
Column	HSpecosphere 3CR C8 (80 × 4.6 mm, 3 μm) Perkin Elmer
Extraction	solid-phase extraction, C_8 100 mg (part 290-0010-B)
Mobile phase	sodium phosphate buffer (10 mM, pH 3.0), 6.5% acetonitrile
Temperature	ambient
Flow rate	1.5 ml/min
Detection	Milton Roy variable wavelength UV detector at 265 nm
Internal standard	chloroadenosine (CAdo)
Capacity factor, k'	1.0 for CAde 6.7 for CAdo 9.1 for CdA 14.3 for CAFdA

Analysis of data

Chromatographic data were collected and processed on a IBM compatible computer equipped with Chromatography Station for Windows (CSW; Watrex, Praha, Czech Republic). CAde, CdA and CAFdA concentrations in samples were determined by comparing the ratios of their respective peak areas to that of the IS with those of a standard curve determined from at least eight data points in the range 2–500 nmol/l. The slopes of the standard curves were determined with linear regression.

Drug stability

CdA, CAFdA and CAde was added separately to pooled plasma from three healthy donors at a concentration of 100 nmol/l. Each group were then devided to five subgroups. The samples were stored at −20°C. The samples were analyzed after 0, 4, 9, 12 and 24 weeks, and CAdo was used as IS.

Stability test

To stimulate common procedures for handling blood samples, peripheral blood samples were collected in heparinized glass tubes before and immediately after a 2 h infusion of CdA (5 mg/m^2), in a patient with chronic lymphocytic leukemia (CLL) given CdA i.v. The samples were kept at room temperature (21°C), in a refrigerator (5°C) or in ice water (0°C) for 0, 2, 6, 18, 24, 48 and 72 h. In order to increase the recovery of CdA and CAde from whole blood and to facilitate hemolysis of blood cells, 9 volumes of distillated water were immediately added to each tube as soon as they were removed. The mixture was vortexed in glass tubes for 30 s and the hemolyzed samples were frozen immediately at −20°C prior to analysis. The rest of the sample was centrifuged (1500 r.p.m., 5 min). The plasma was collected and then stored at −20°C. The experiment was conducted in duplicate.

The plasma samples were analyzed with HPLC as described above.

To analyze the whole blood samples, 5 ml of 100% ethyl acetate was added, well mixed for 5 min in a IKA-Wibrax-VXR shaker (Janke & Kunkel, Staufen, Germany). The mixture was centrifuged (2000 r.p.m., 10 min). The supernatant was collected in glass test tubes and evaporated to dryness under a stream of nitrogen in a water bath (37°C). The residue was finally dissolved in 50 μl of the same mobile phase as plasma samples and analyzed as earlier described.[12]

For the *in vitro* stability test, blood samples from three healthy donors were spiked with 1 μmol/l CdA. They were kept at 37, 21, 5 and 0°C, and analyzed after 0, 1, 2, 4 and 24 h. Plasma and blood samples were prepared and analyzed with HPLC as earlier described, but guaneran was used as IS.

Patients

Six patients with B cell CLL, three male and three female (49–71 years old), with normal liver and kidney functions received CdA orally (10 mg/m^2) for three consecutive days according to an ongoing protocol. During day 3, blood samples were collected into heparinized tubes before and 1, 2, 4, 6, 12, 24, 36, 48 and 72 h after drug administration. Samples were immediately put on ice and the plasma was collected after centrifugation.

Results

Chromatography

CdA, CAde and CAFdA was well separated from each other, endogenous compounds and the IS (CAdo) (Figure 2). The capacity factor, k', for CAde was 1.0 and for CAdo was 6.7, the corresponding value for CdA was 9.1 and for CAFdA was 14.3.

Recovery

Drug recovery was determined by comparing spiked plasma samples with aqueous solutions of CAde, CdA and CAFdA. Mean overall recovery of CAde was 85 ± 13% (n = 27), of CdA was 76 ± 10% (n = 27) and of CAFdA was 75 ± 11% (n = 27). The IS, CAdo, had a recovery of 84 ± 13% (n = 27).

Precision of the method

Table 2 shows the results of a precision study, of both intra- and inter-assay variability for three levels of plasma CAde, CdA and CAFdA. The variability was markedly concentration dependent with a CV of below 6% for both inter- and intra-assays at a concentration of 50 nmol/l. At a concentration of 5 nmol/l, intra-assays variability was below 19% and inter-assay variability less than 23%.

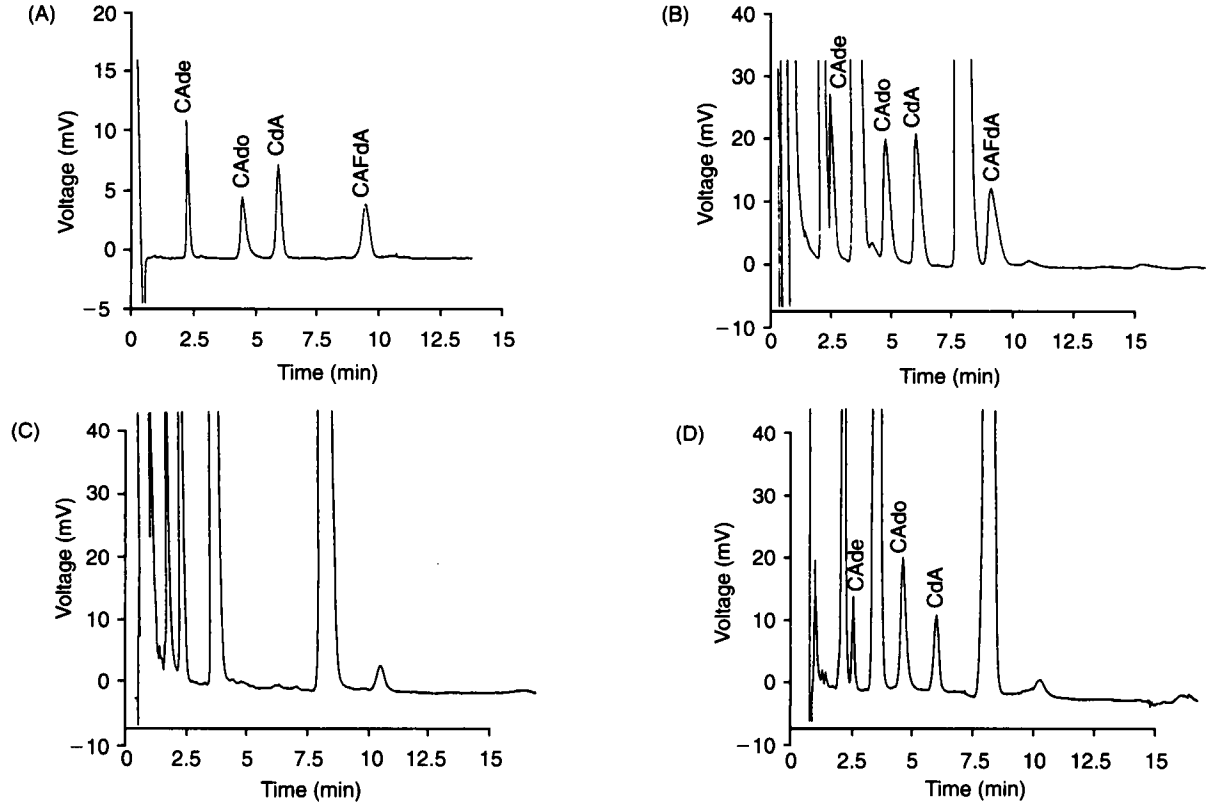


Figure 2. Representative chromatograms of (A) a water solution containing CAde, CdA, CAFdA and the IS used (CAde), (B) a blank plasma supplemented with 100 nmol/l CAde, CdA, CAFdA and the IS, (C) a blank plasma from a patient and (D) a patient's plasma sample collected 1 h after a single oral dose of CdA.

Table 2. Intra- and inter-assay variabilities of CAde, CdA and CAFdA methods in blank human plasma

	Spiked level (nmol/l)	Measured mean (nmol/l)	CV (%)	*n*
Intra-assay				
CAde	5	5.6	9.3	7
	50	46.0	1.8	7
	200	199.9	6.8	5
CdA	5	5.4	18.4	7
	50	50.6	5.6	7
	200	202.3	5.0	5
CAFdA	5	6.2	16.4	7
	50	48.5	1.3	7
	200	194.4	5.1	5
Inter-assay				
CAde	5	6.0	13.0	10
	50	54.3	4.5	10
	200	204.7	4.1	10
CdA	5	6.0	22.7	10
	50	52.1	4.7	10
	200	198.7	6.4	10
CAFdA	5	6.5	11.2	10
	50	50.5	4.0	10
	200	200.7	11.2	0

Limit of detection and quantitation

The limit of detection of CAde, CdA, CAFdA and IS defined as at least three times the baseline noise signal corresponds to a concentration of less than 1 nmol/l using 1 ml plasma for all substances injected into the column. Analogous values for the limit of quantitation defined as at least five times the baseline noise signal correspond to less than 2 nmol/l. Lower concentrations could, however, be quantitated by injecting larger sample volumes.

Linearity

The linearity of these methods was studied in spiked plasma solutions in the concentration range of 2.0–500 nmol/l. The results from linear regression analysis are presented in Figure 3.

Drug stability

The stability of plasma stored at −20°C was studied

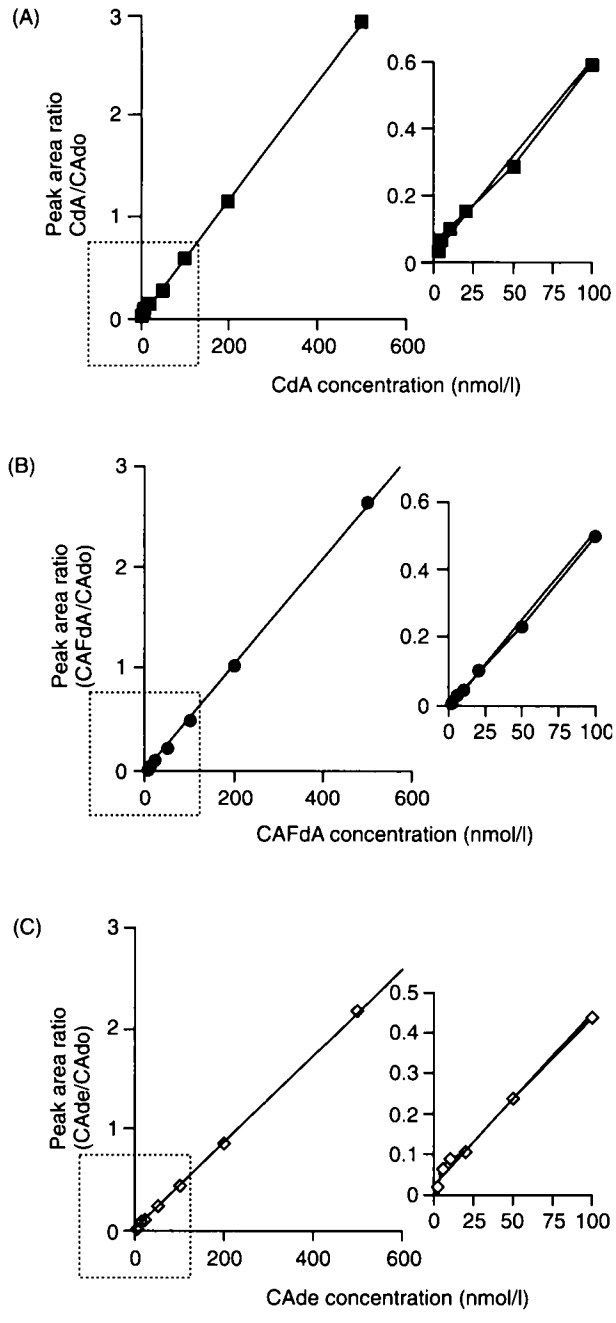


Figure 3. Calibration curve for the assays of CdA (A), CAFdA (B) and Cade (C). The results from linear regression analysis were: CAde, slope = 0.004, *y*-intercept = 0.002 and $r = 1.000$; CdA, slope = 0.006, *y*-intercept = 0.004 and $r = 1.000$; CAFdA, slope = 0.005, *y*-intercept = 0.0056 and $r = 0.999$.

after 0, 4, 9, 12 and 24 weeks (Figure 4). After 24 weeks CAde is degraded to less than 50%. CAFdA is almost intact (87%) and CdA is degraded to less than 75%.

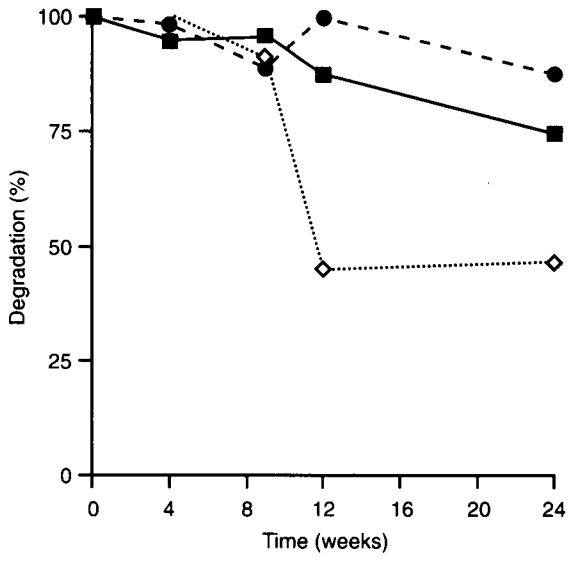


Figure 4. Plasma containing CdA (⊞), CAde (◊) and CAFdA (●) stored at −20°C, and analyzed after 0, 4, 9, 12 and 24 weeks.

Stability test

CdA and CAde in a blood sample from a CdA-treated patient were reasonably stable (below 25% degraded) throughout the experiment at 0 and 5°C, while they were rapidly degraded at 21°C (Figure 5a). A slight redistribution of drug from plasma to erythrocytes was apparent during the first hours (Figure 5b). *In vitro* this redistribution was not so marked (Figure 6).

Clinical application of the method

Figure 7 shows the kinetics of CdA and CAde in plasma over a period of 72 h following the last of three oral administration of 10 mg/m^2 of CdA in six patients.

Discussion

This method for simultaneous determination of CdA, CAFdA and CAde is rapid and simple. In contrast to our previously published method,[12] the IS is CAdo which has a similar chemical structure as CdA, CAFdA and CAde (Figure 1). Furthermore, the mobile phase has been simplified and contains only phosphate buffer and acetonitrile.

The bioavailability of CdA is 37–50%.[14,16] After oral administration a large proportion of CdA is degraded to CAde (Figure 7). The role of CAde for

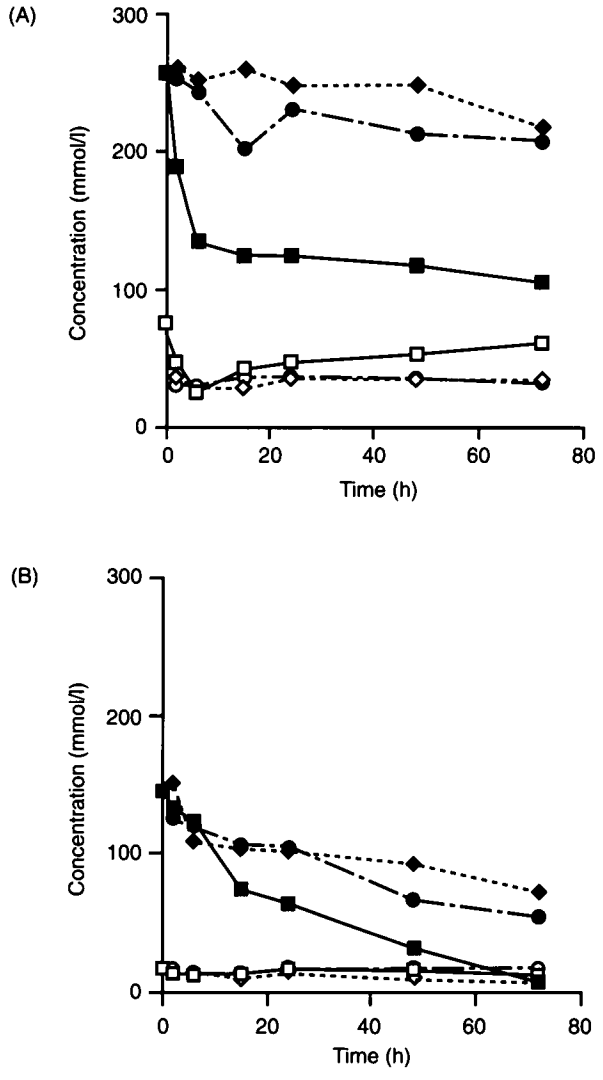


Figure 5. Semilogaritmic plot of blood (A) and plasma samples (B) from a patient treated with CdA infusion, stored at 21°C (□), 5°C (◊) and 0°C (○). The samples were analyzed after 2, 6, 15, 24, 48 and 72 h. CdA (gray) and CAde (white).

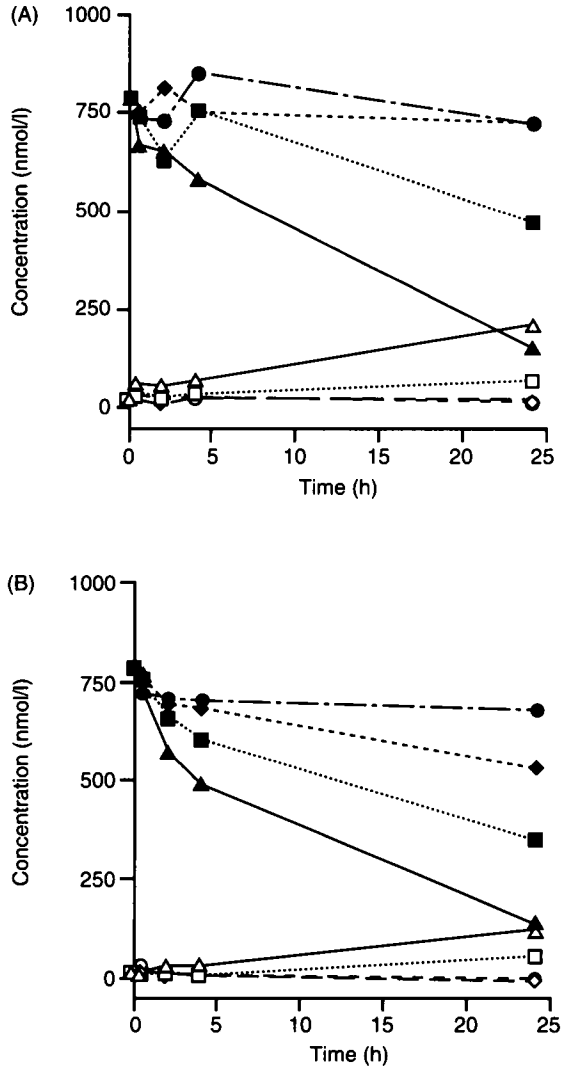


Figure 6. Blood from three healthy donors spiked with 1 μmol/l CdA stored at 37°C (△), 21°C (□), 5°C (◊) and at 0°C (○). CdA (gray) and CAde (white). Semilogaritmic mean plot of blood (A) and plasma samples (B).

the toxicity and efficacy of CdA treatment needs to be elucidated further. The first step is to delineate its pharmacokinetics. The method presented is a useful tool for this purpose.

CAFdA is a new promising drug candidate with activity orally in SCID mice with human CLL.[4] CAFdA is more stable at low pH than CdA and might therefore be a better drug for oral application. CAFdA has not yet entered clinical trials. This method provide a possibility to delineate the pharmacokinetics of CdA, CAFdA and their catabolite CAde in plasma simultaneously. The plasma extraction procedures used in the present study are simple and reproducible, with acceptable intra- and inter-assay variabilities at high and intermediate plasma concentrations. Near the limit of detection the variability increases considerably. CAdo was chosen as IS instead of guaneran, which was used previously, because guaneran interferes with CAFdA in the assay. Furthermore, CAdo is commercially available while guaneran is not. The limit of quantitation (2 nmol/l) is sufficient to determine the pharmacokinetics of CdA and CAde in plasma during a 72 h period following oral administration of CdA.

The extent of the formation of CAde, a catabolic product of CdA detected in the plasma of patients treated with orally administrated CdA, indicates that

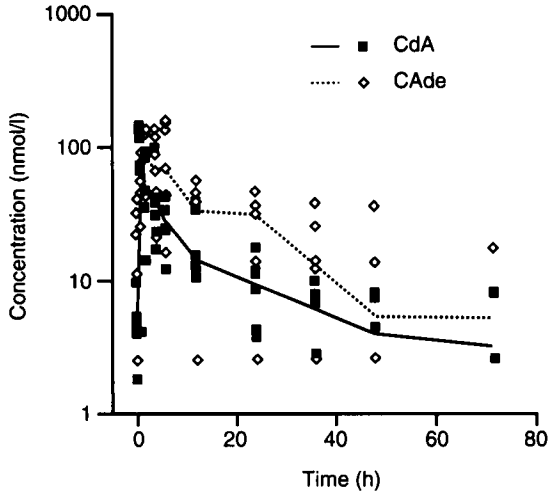


Figure 7. Semilogaritmic plot of mean plasma CdA and CAde concentration–time curve, and individual values, obtained in six patients treated for CLL with CdA.

a substantial part of the oral dose may be deglycosylated before absorption or as a first-pass effect. Further data on this subject will be presented elsewhere.

The stability test showed that the temperature at which samples are stored is of the utmost importance for the quality of data obtained. In contrast to some other drugs,[17] the distribution of CdA or CAde between plasma and erythrocytes did not alter the results of the determination of plasma CdA or CAde. Likewise, the duration of storage before separation and freezing should be minimized. During the first hours, redistribution of drug from plasma to erythrocytes probably plays a role, while deglycosylation to CAde is apparent when samples are stored at room temperature. However, the majority of CdA lost from plasma during prolonged storage at room temperature cannot be recovered as CAde. These results were very similar in spiked samples and the authentic patient samples. It is unlikely that these problems are exceptional for CdA. However, data on the effect of storage on plasma concentration data obtained are scarce in general.

Acknowledgments

The study was performed in accordance with the principles stated in the Declaration of Helsinki. The study was approved by the Swedish drug agency (#151:1603/94) and the local ethics committee (#94/325).

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