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Highly sensitive high-performance liquid chromatographic assay for 1-β-D-arabinofuranosylcytosine-5′-stearyl phosphate (cytarabine-ocfosfate)

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Abstract

An ion-pair HPLC method for the determination of 1-β-D-arabinofuranosylcytosine-5′-stearyl phosphate (cytarabine-ocfosfate **I**) was developed, using a phenyl-bonded column under reversed-phase conditions with a mobile phase of acetonitrile–buffered water (pH 6.8) (50:50) for isocratic elution. A reproducible sample clean-up was achieved by solid-phase extraction. In order to reach the low limit of detection of 2 ng/ml, an enrichment switching system was used. The present validation leads to a limit of quantification of 5 ng/ml with a coefficient of variation (C.V.) of 10%. The total time of measurement was shortened by a back-flush procedure to restore the conditions after each run. UV detection at 275 nm was applied. The recoveries for plasma samples ranged from 56.4 to 64.1%, regardless of drug concentrations. The intra-assay C.V. was about 4% (40 measurements at four different concentrations). The inter-assay recovery (ten measurements over ten days) at a plasma concentration of 50 ng/ml was 57% with a C.V. of 8.25%. Based on this HPLC method, the pharmacokinetics of **I** were measured during a clinical phase I/II study.

1. Introduction

1-β-D-Arabinofuranosylcytosine-5′-stearyl phosphate (cytarabine-ocfosfate, **I**) is a recently developed derivative of cytosine-arabinoside (ARA-C) that differs from the latter by the presence of a stearyl phosphate side-chain (Fig. 1). By this modification **I** has gained lipophilic properties and may therefore be absorbed by the gut mucosa after oral administration. This change offers the potential for a broad clinical application, especially for disorders in which ARA-C is currently used in low to conventional doses [1–3]. The assumed hepatic metabolism of **I** is characterized by stepwise degradation through the microsomal P-450 system, ω-oxidation and β-oxidation followed by the last step of disconnecting the phosphate moiety by phosphodiesterase [4–7]. Thus **I** is prodrug of ARA-C. To evaluate the relationship between the two drugs and the clinical importance of the distinct pharmacokinetics and pharmacodynamics of **I**, we developed a highly sensitive HPLC method

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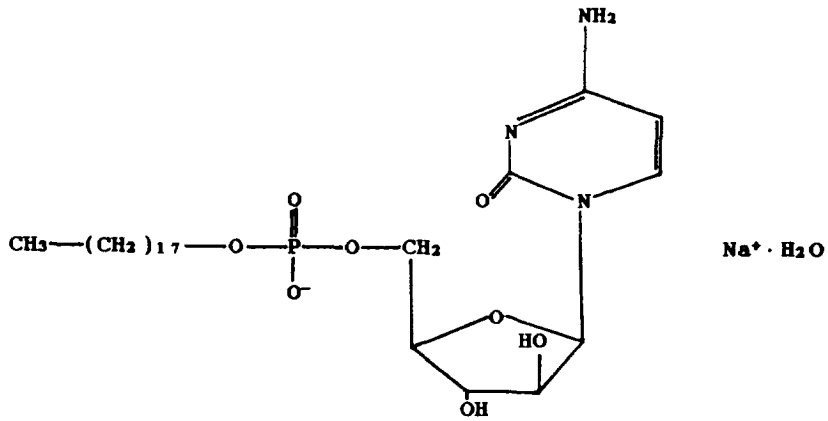

Fig. 1. Structure of 1-β-D-Arabinofuranosylcytosine-5'-stearyl phosphate (cytarabine-ocfosfate, I).

that allows the sensitive determination of I in plasma and urine.

2. Experimental

2.1. Chemicals and solvents

The substances used and solutions were all of analytical quality. Compound I was supplied by Nippon Kayaku (Tokyo, Japan)/Asta Medica (Frankfurt, Germany). Water, methanol and acetonitrile and the C_{18} material with a particle size of 40 μm for cartridge filling were obtained from Baker (Gross-Gerau, Germany). Na_2HPO_4 and NaH_2PO_4 was purchased from Merck (Darmstadt, Germany). Tetrabutylammonium sulfate (Pic A low UV) was furnished by Waters/Millipore (Eschborn, Germany). A Visiprep solid-phase extraction vacuum manifold system, manufactured by Supelco (Bad Homburg, Germany) was used in combination with 1-ml filled tubes and 3-ml unfilled tubes, which were sealable by cover platelets.

2.2. HPLC apparatus

The HPLC apparatus (Fig. 2) consisted of two Waters M 510 pumps, a membrane pulse damper from Chemdata (Frankfurt, Germany), a Waters Model 490 UV multi-wavelength absorbance detector, two Model 7000 switching valves (Rheodyne, Cotati, CA, USA) and one Rheodyne Model 7125 injection value. A 10-ml loop for sample loading was combined with the injection valve. In order to improve the resolving power and to shorten the retention time, the analytical column was cooled to 8°C. The analytical column (250 × 4 mm I.D. Nucleosil 120-7 C_6H_5), manufactured by Macherey–Nagel (Düren, Germany) proved most appropriate for the respective measurements. Merck provided the enrichment cartridges (4 × 4 mm I.D., C_{18}, 5 μm). An empty column (250 × 4 mm I.D.) (Macherey–Nagel) was hand-filled with C_{18} material (Baker, 40 μm) and inserted between the pulse damper and the enrichment valve to prevent contamination of the enrichment cartridge and the analytical column.

2.3. Quantification and data accumulation

The external standard method was used for quantification. The peak areas were plotted against the amounts of I in aqueous standards and spiked urine and plasma samples. The detector signal was changed to digital format and registered on an IBM-compatible 386 processor computer system. The data accumulation was performed by the NINA program from Nuclear-Interface (Münster, Germany). The Topfit pharmacokinetic program [8–10] was used for the calculation of pharmacokinetic parameters.

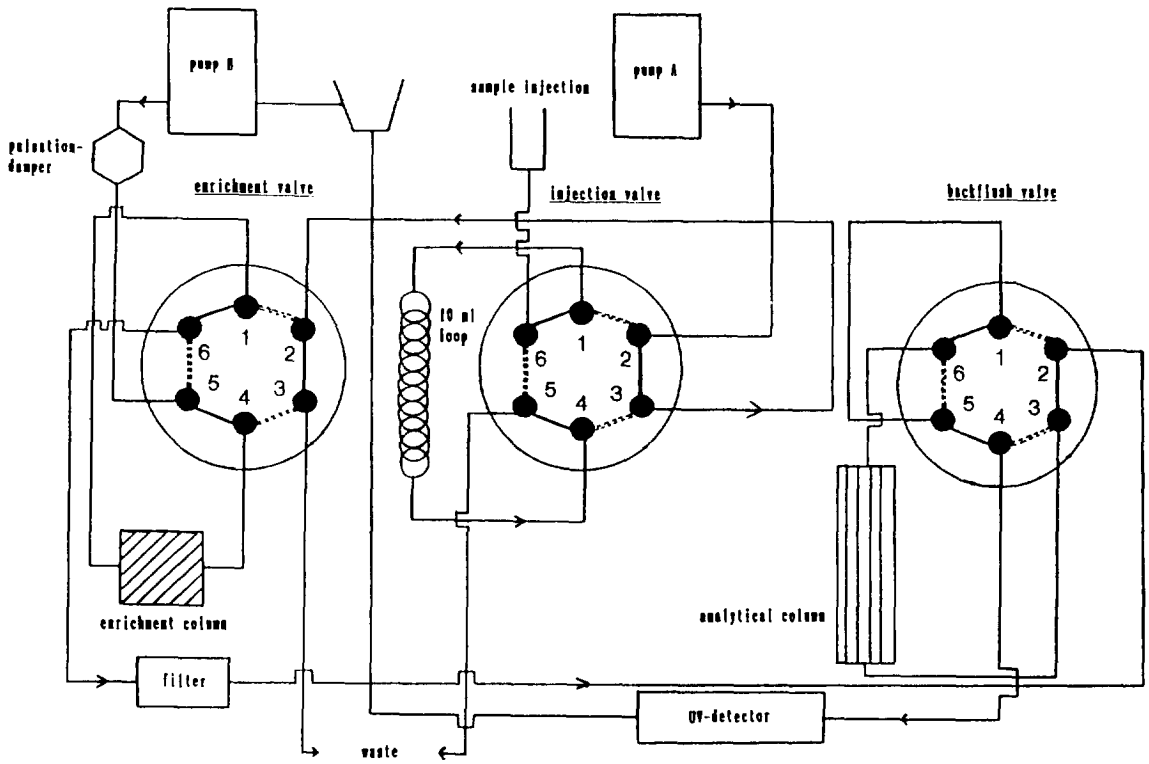

Fig. 2. Schematic representation of the HPLC apparatus.

2.4. Chromatographic conditions

Buffer water was composed of 0.015 mol of Na_2HPO_4, 0.025 mol of NaH_2PO_4 and 0.005 mol of tetrabutylammonium sulfate per litre of water (HPLC quality). A 50:50 (v/v) mixture of buffered water and acetonitrile was used as the analytical mobile phase. The mobile phase for the enrichment system consisted of buffered water–acetonitrile (70:30, v/v). Buffered water–acetonitrile (20:80, v/v) was necessary for mixing the sample additive. Additionally, a cleaning solution composed of methanol–buffered water (60:40, v/v) was used.

2.5. Flow directions and valve operations

Fig. 2 shows the valve-switching system. After complete injection of the sample volume the injection valve was switched, thus directing the flow of the mobile enrichment phase through the 10-ml sample loop to the enrichment valve and through the enrichment cartridge at 2 ml/min. The flow was maintained for 12 min. Whereas **I** interacts with the C_{18} material and is thus retained, all other substances with lower affinity for the C_{18} material are eluted to waste. After switching the enrichment valve, pump B directs the analytical mobile phase together with the retained **I** in a reversed flow through the enrichment cartridge to the analytical column and finally to the detector at a flow-rate of 0.6 ml/min. This step marks the start of analysis ($t = 0$). After a 10-min analysis time, the back-flush procedure is started by switching the back-flush valve and subsequently the enrichment valve, thus separating the enrichment cartridge from the circulation in the analytical part. The reverse flow through the analytical column is maintained for 11 min, during which the next enrichment run can be performed. The total duration of analysis is about 22 min. The analytical column is

cooled to 8°C and the enrichment column is equilibrated to room temperature.

2.6. Sample preparation

Tube preparation procedure

Tubes (3 ml) were filled with an average of 200 mg of 40-μm C_{18} material in methanol suspension. Cover platelets were placed at top and bottom to hold the C_{18} material in between. This was followed by activation of the solid-phase material with different solutions, which were sucked by the vacuum of the Visiprep cartridge system through the tubes. The order of solutions was 2 ml of methanol, 4 ml of acetonitrile, 4 ml of HPLC-quality water and 2 ml of enrichment solution. In general, the clean-up procedures were the same for urine and plasma. For urine samples it was more suitable to use already filled 1-ml tubes from Supelco and consequently to apply smaller volumes of solvents for the activation procedure. The order of solutions was 1 ml of methanol, 2 ml of acetonitrile, 2 ml of HPLC-quality water and 2 ml of enrichment solution.

Solid-phase extraction

Solid-phase extraction was carried out with a Visiprep solid-phase extraction vacuum manifold system to clean plasma and urine samples. In order to reduce the aggregation of **I** particles, 1 ml of the sample additive was mixed with 2 ml of the plasma sample to enhance the lipophilic character. This solution was added immediately to prepared tubes while the vacuum pump was running at high power. Subsequently, 4 ml of enrichment solution, 4 ml of cleaning solution, 4 ml of HPLC-quality water and 0.5 ml of analytical solution for pre-elution were added. As urine usually contains only small amounts of proteins, a similar cleaning procedure was not required for urine samples. Thus, the volume of enrichment solution, cleaning solution and HPLC-quality water could be reduced by half. Pre-elution with 0.5 ml of analytical solution was not necessary. Regardless of whether urine or plasma samples were analysed, 5 ml of the analytical mobile phase were added to the tubes for slow terminal elution of **I**. As a low flow-rate was essential for achieving a reproducible recovery, extractions were performed at reduced vacuum. The eluate consisted of a purified aliquot of **I** dissolved in analytical solution. It was collected in glass test-tubes and subsequently diluted with 3.33 ml of HPL-quality water before measurement.

3. Results

3.1. Separation parameters

Fig. 3b and c show chromatograms for urine and plasma samples spiked with 25 ng/ml of **I**. These measurements revealed that the capacity factor was about 2, the tailing factor was nearly 1.42 and the number of theoretical plates was about 3800 m^{-1}.

3.2. Selectivity and detection and quantification limits

Native plasma and urine samples from ten healthy volunteers were measured under the conditions described above. No co-eluting peak was detectable in ten zero control samples at the characteristic retention time of **I** (e.g., Figs. 4b and 5b). Sixteen plasma and urine samples were spiked with 2 ng/ml (limit of detection) to ensure a signal-to-noise ratio of 3:1 at this concentration (Figs. and 4a and 5a). Then ten plasma and ten urine samples were spiked with 5 ng/ml of **I**. The standard deviation (S.D.) in plasma and urine was about 6% and 4%, respectively; the coefficient of variation (C.V.) was about 10% and 7%, respectively. The present validation measurements revealed a limit of detection (LOD) of 2 ng/ml and a limit of quantification (LOQ) of 5 ng/ml, although even lower concentrations of the drug were still detectable.

Without cooling the analytical column to 8°C, a lipophilic substance is retained at the same retention time as **I**. At lower temperature, micelles of **I** were less fluid, hence the contact intensity with the column material was reduced

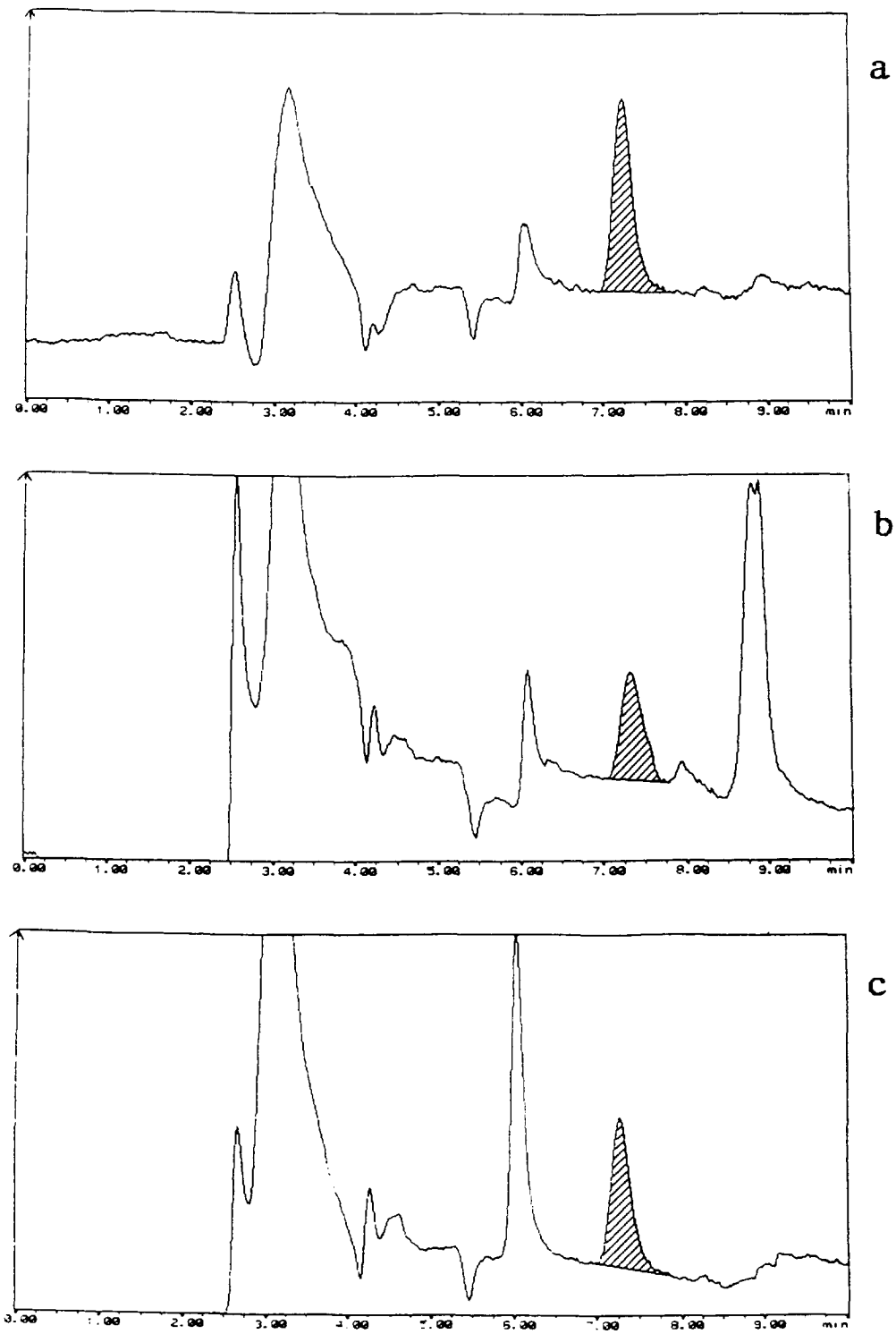

Fig. 3. Chromatograms for 25 ng/ml of I (marked area) in (a) aqueous standard, (b) spiked plasma and (c), spiked urine.

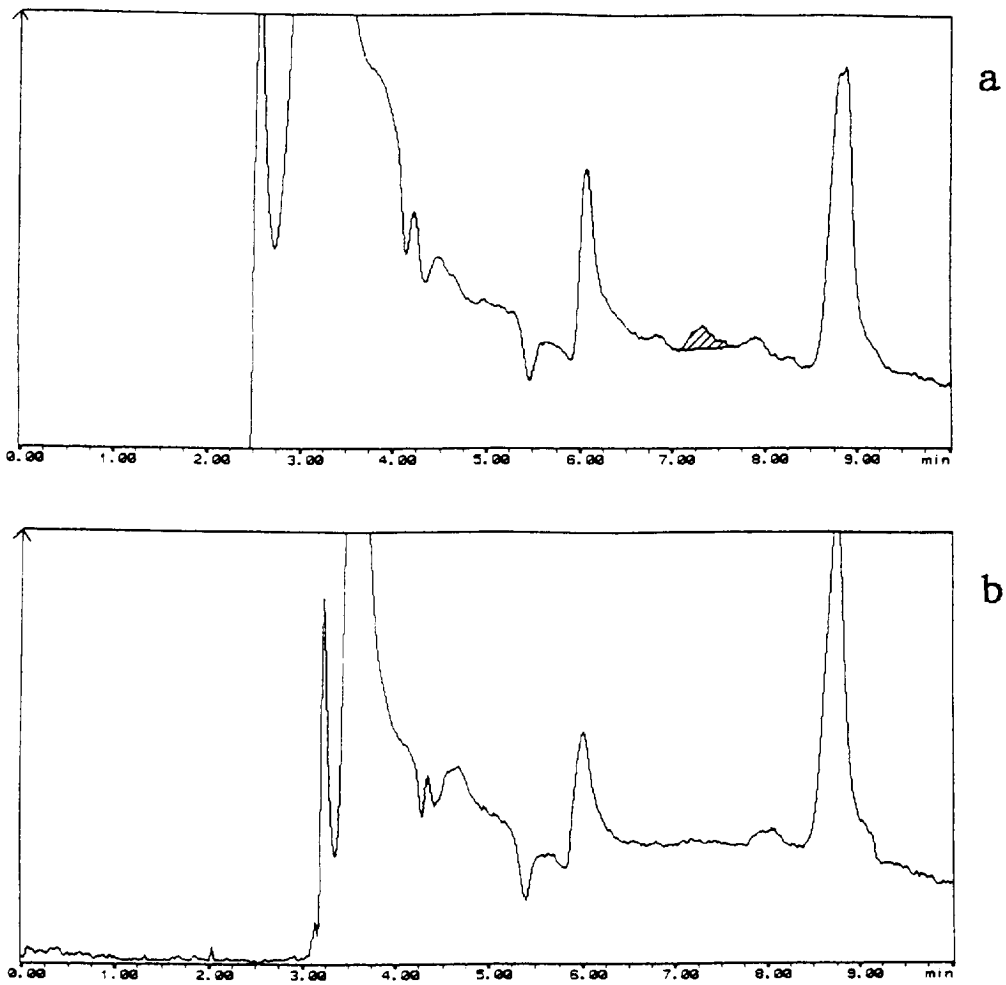

Fig. 4. Chromatograms for (a) 2 ng/ml of I (marked area) in spiked plasma and (b) control plasma.

and subsequently the retention time was shorter. The other so far unidentified substances did not show this temperature-dependent behaviour of I.

3.3. Calibration and linearity

Aqueous standards were measured at ten different concentrations (5, 10, 25, 50, 75, 100, 175, 250, 400 and 500 ng/ml) on five consecutive days. The concentration of I in the aqueous standards was linear with respect to the measured concentrations with $y = 2.250x + 3.39$ and a correlation coefficient $r = 0.9994$. Subsequently, 100 spiked plasma samples were measured on ten days to produce ten different calibration graphs, each with the same concentration as in aqueous standards. The overall slope for the plasma samples was 1.322 with a $r = 0.99839$ and C.V. = 7.8%. The equation was $y = 1.322x + 3.11$.

3.4. Recovery and intra- and inter-assay C.V.

The overall recovery was calculated from the ratio of the slopes of the regression curves for the plasma samples and the aqueous standards. On this basis the recovery was 58.76% for plasma samples with S.D. = 4.58%. The re-

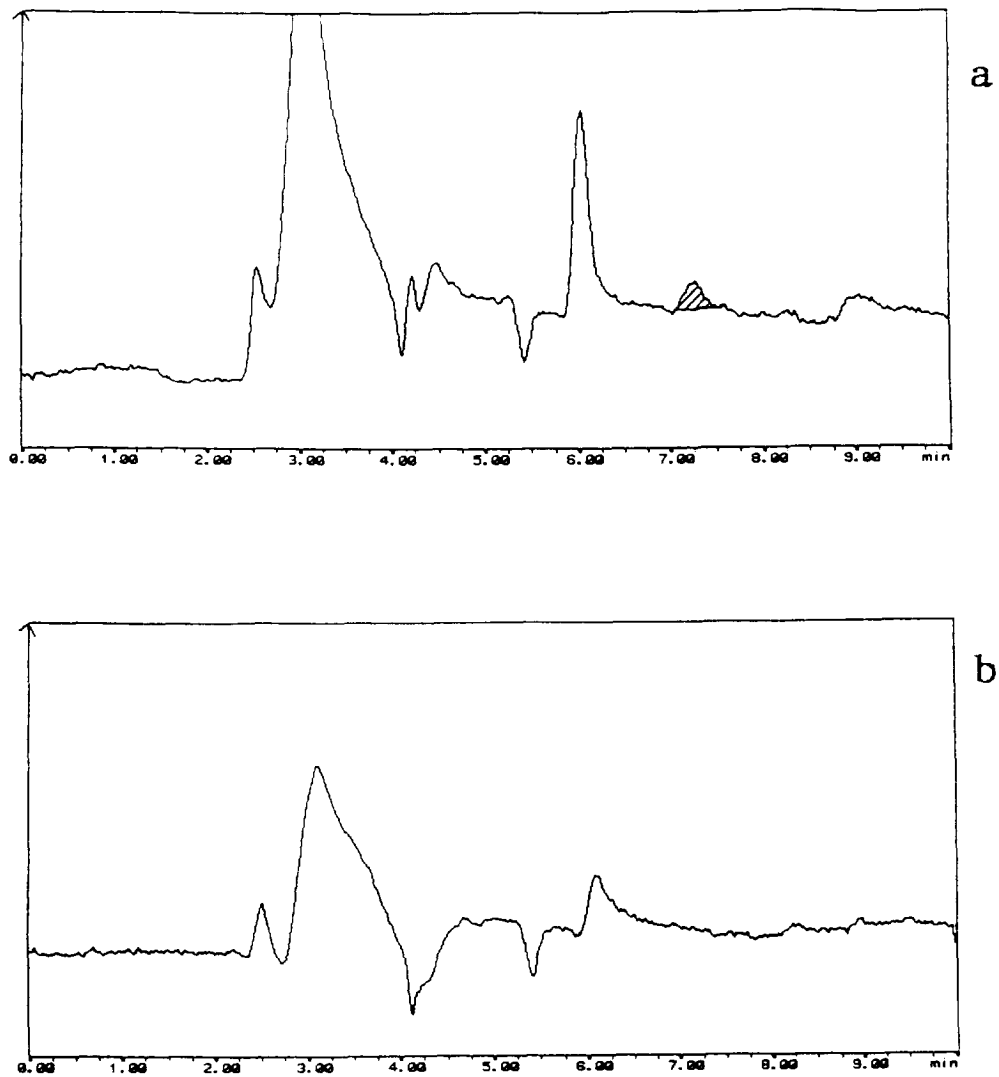

Fig. 5. Chromatograms for (a) 2 ng/ml of **I** (marked area) in spiked urine and (b) control urine.

covery of urine samples was 60.11% with S.D. = 5.03%. The intra-assay C.V. for plasma samples (4%) was calculated on the basis of ten measurements each performed at four different concentrations (Table 1). These measurements revealed an intra-assay recovery of 56.4–64.1% for plasma samples, regardless of the drug concentrations. The intra-assay recovery of urine samples was in the same range (Table 2).

The inter-assay C.V. was determined by

Table 1
Intra-assay recovery and concentration-dependent standard deviation for plasma samples ($n = 10$)

I in plasma (ng/ml)	Standard deviation (ng/ml)	Average recovery (%)
5	0.26	64.07
25	1.41	56.46
100	6.65	57.95
500	29.50	56.36

Table 2
Intra-assay recovery and concentration-dependent standard deviation for urine samples ($n = 10$)

I in urine (ng/ml)	Standard deviation (ng/ml)	Average recovery (%)
5	0.34	53.02
25	1.03	55.52
100	7.02	53.49
500	26.60	60.19

measuring plasma samples with a spiked concentration of 50 ng/ml on ten consecutive days stored at room temperature. The mean recovery value of **I** was about 57.11% with C.V. = 8.25%. If the sample–sample additive mixture was added to the tubes during too long a time, the recovery was decreased owing to obstruction of the cover platelets.

3.5. Pharmacokinetic data

Pharmakokinetic analysis for **I** was carried out during three subsequent cycles of therapy in a 68-year-old male patient suffering from end-stage chronic lymphocytic leukaemia. During the first cycle of therapy the patient received 100 mg/body (body surface area = 2 m^2) of **I** per day, which was increased to 300 and 600 mg/body in subsequent cycles. The plasma concentration curve for the second cycle is shown in Fig. 6. Each 14-day treatment cycle was preceded by a single application of the respective doses on day −2 for the determination of

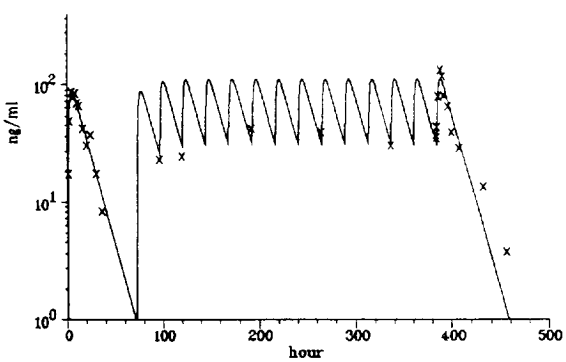

Fig. 6. Plasma concentration curve for a 14-day treatment cycle with 300 mg/body per day, which was preceded by a single application of **I** 72 h earlier.

Table 3
Pharmacokinetic parameters from one patient receiving 100, 300 and 600 mg of **I** daily over a period of 14 days

Pharmacokinetic parameter	100 mg	300 mg	600 mg
AUC (ng h/ml)	646	1690	4635
Half-life (h)	8.9	10.0	8.5
Lag time (h)	1.98	0.82	1.55
Maximum concentration (ng/ml)	50.1	89.0	262.0

baseline pharmacokinetic parameters, and indicated linear pharmacokinetics and a one-compartment model. The curve was well fitted to the measured concentrations by the Topfit pharmacokinetic program with a regression coefficient of 0.98 [8–10].

The dose-dependent and dose-independent parameters are given in Table 3. The area under the curve (AUC) and the peak concentration increase with increase in the applied dose. The lag time (120–48 min) varies depending on the resorption conditions. The elimination half-life is 9 h. No **I** was found in the urine (LOD = 2 ng/ml).

3.6. Stability of cytarabine-ocfosfate

Compound **I** was dissolved in acetonitrile–HPLC-quality water (5:95, v/v) and kept at room temperature for 1 month. Repeated measurements revealed no degradation of **I** over the whole observation period.

4. Discussion

Cytosine-arbinoside (ARA-C) represents one of the most active single agents in the treatment of acute leukaemias and malignant lymphomas and is an essential compound in frequently applied combination regimens [11–13]. Its chemical nature, however, makes a parenteral application mandatory, which results in some limitations and inconvenience to patients. Hence the development of **I** as an orally applicable derivative holds substantial benefit and may even

replace ARA-C in certain therapeutic situations [14–16].

An essential prerequisite for this perspective is the proof of a comparable clinical efficacy and the achievement of adequate plasma and even intracellular ARA-C concentrations after the administration of **I**. The development of a reliable and sensitive method for the evaluation of its pharmacokinetic and pharmacodynamic properties supports this approach.

The only published HPLC method for the determination of **I**, by Nakanuma et al. [15], is based on a complicated sample preparation procedure with a time-consuming evaporation step and is therefore not suitable for broad clinical studies. The procedure described here appears more advantageous and easily applicable. It uses solid-phase extraction for sample clean-up considering the high protein binding of **I** (nearly 98%; data not shown) [17]. The structure of **I** (Fig. 1) shows a hydrophilic and a highly lipophilic part. Therefore, **I** tends to form micelles and aggregates in a mainly hydrophilic solvent, causing inadequate retention. Sufficient recovery is achieved however, by adding a lipophilic component to the sample. A similar effect is also observed at lower temperatures of the analytical column: the micelles are less fluid, so the contact intensity with the column material is reduced. This results in an inverse reaction between a short retention time and a low column temperature.

The tendency to create micelles leads to an inhomogeneous solution and probably to higher affinity to the wall of glass or plastic tubes. Hence it is very important to shake the test-tubes containing **I**, if only an aliquot is removed. The use of buffered tetrabutylammonium sulfate results in suppression of the acid function by ion pairing. Thereby the hydrophilic character and the ability to create micelles of **I** are reduced by ion pairing. The weakly basic function is neutralized by the phosphate buffer system. Hence it was suitable to use a phenyl-bonded column under reversed-phase conditions, as the crucial feature of dissolved **I** was its lipophilic character.

The back-flush valve was switched immediately after receiving the detector signal of **I** to ensure a reverse flow for about 11 min through the analytical column. Thus the following run is not influenced by substances with a higher retention time than that of **I** retained from previous analyses. This valve-switching method is well established for the HPLC detection of low-concentration substances [18,19]. An internal standard was not necessary, owing to constant recoveries. We finally recycle the analytical eluent in routine analysis, for ecological and economic reasons.

The pharmacokinetic data show that the intestinal uptake of **I** rises with increase in the given dose (Table 3). The long lag time indicates resorption in the more distal part of the small intestine, similar to the resorption conditions of fatty acids. Compound **I** did not appear in the urine of our study patient. It is likely that the drug will be metabolized to cytosine-arabinoside and subsequently to uracil-arabinoside, substances which are eliminated renally [14,16].

The proposed HPLC method allows the rapid routine monitoring of patient plasma levels during antileukaemic therapy with **I** (Fig. 6) [14,16]. The method is quicker, cheaper, more appropriate and shows a much higher efficiency at an equivalent low LOD than the previously unique method described by Nakanuma et al. [15].

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