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Note

Sample preparation for the determination of 5-aza-2′-deoxycytidine in plasma by high-performance liquid chromatography

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5-Aza-2′-deoxycytidine (5-AZA-CdR) has been demonstrated to be a potent antileukemic agent in animal models [1—3]. This nucleoside analogue is currently undergoing clinical trials in patients with acute leukemia [4]. 5-AZA-CdR is a chemically unstable compound [5] and is also rapidly deaminated by human cytidine deaminase [6]. Due to its instability it is not feasible to develop a radioimmunoassay for this agent. A bioassay [4] has been used to estimate the concentration of 5-AZA-CdR in plasma, but this assay lacks specificity and is time-consuming.

The attempt to assay 5-AZA-CdR in patients using high-performance liquid chromatography (HPLC) has been hampered by the presence of interfering ultraviolet-absorbing substances and the low concentrations of this agent in plasma. In this paper we describe a simple and very effective method for the preparation of plasma samples for HPLC analysis. This method has been used to monitor plasma levels of 5-AZA-CdR in patients with acute leukemia in order to perform preliminary pharmacokinetic analysis on this agent.

EXPERIMENTAL

Materials

5-AZA-CdR was synthesized by Dr. A. Piskala of the Czechoslovak Academy of Science (Prague, Czechoslovakia). 5-Azacytidine, the internal standard, was obtained from Sigma (St. Louis, MO, U.S.A.). Normal human control serum (Quality I) was obtained from American Monitoring Canada (Montreal, Canada). Sulfonic acid type cation-exchange resin (Aminex A-6) was obtained from Bio-Rad Labs. Canada (Mississauga, Canada). Silver perchlorate was

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obtained from BDH Canada (Montreal, Canada). Small plastic columns (65 × 6 mm) and the aspiration apparatus (Vac-Elut) were obtained from Analytichem International (Harbor City, CA, U.S.A.). Blood was obtained by intravenous aspiration into heparinized tubes (5 U/ml). After centrifugation at 15 600 *g* for 15 min, the plasma was stored at −70°C.

Preparation of cation-exchange column

The resin was suspended in 20% methanol and allowed to settle by gravity overnight at room temperature. To 1 vol. of the settled resin 9 vols. of 20% methanol were added and after mixing 0.5 ml of the suspension was placed in small plastic columns which were attached to the aspiration apparatus. The column was washed with 1 ml of methanol, 2 ml of 2 *M* hydrochloric acid and 2 ml of distilled water using gentle suction. The column was then ready for injection of the sample.

Preparation of methanolic silver perchlorate

Silver perchlorate (1.04 g) was dissolved in 100 ml of methanol, and 43 μl of 70% perchloric acid were added to give a final concentration of 0.05 *M* silver perchlorate and 0.005 *M* perchloric acid. This solution could be stored in a tightly sealed bottle for at least one month at room temperature without detectable loss of activity.

Sample preparation

To 500 μl of plasma in a 1.5-ml Eppendorf polypropylene centrifuge tube 10 μl of 5-azacytidine, the internal standard (I.S.) (50 μg/ml), and 1.0 ml of the methanolic silver perchlorate were added. After mixing, the tube was placed at room temperature for 10 min, on ice for 20 min, and then centrifuged at 15 600 *g* for 15 min. The supernatant (1.2 ml) was placed into the cation-exchange column, allowed to drain and then centrifuged at 800 *g* for 5 min. The column was then washed by centrifugation with 1.3 ml of 66.7% methanol, 0.5 ml of 5% methanol and 1.0 ml of distilled water (twice). The column was eluted with 0.1 ml of 1.5 *M* ammonium phosphate (pH 7.4). The final eluent represents a four-fold concentration of the original plasma sample. Reference samples containing 0.1, 0.2, 0.4 and 1.0 μg/ml 5-AZA-CdR in control serum were assayed routinely. All samples were assayed immediately or stored at −70°C prior to HPLC analysis.

HPLC assay

The apparatus used for the analysis consisted of an LKB Model 2150 pump, an LKB Uvicord SII (Model 2238) UV detector, set at 254 nm, and an Ultrasphere-ODS column (150 × 4.6 mm; 5 μm particle size). The mobile phase was 0.05 *M* ammonium phosphate (pH 6.8) with a flow-rate of 2 ml/min.

Sensitivity, linearity, recovery and precision

The minimum quantifiable concentration of 5-AZA-CdR was 0.1 μg/ml at a signal-to-noise ratio of 5. Since the sample preparation produced a four-fold concentration of the sample, the minimum detectable concentration in plasma was about 0.03 μg/ml.

In order to determine the linear detection range 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 μg/ml 5-AZA-CdR and 1.5 μg/ml 5-azacytidine (I.S.) were placed in control human serum. The peak height ratio of 5-AZA-CdR over 5-azacytidine versus the 5-AZA-CdR concentration was found to be $y = 0.012 + 0.544x$ with a coefficient of correlation of 0.999.

The percentage recovery of the samples was calculated by comparing the peak heights obtained from the normal control serum spiked with the compounds with the peak height obtained by direct injection of the pure compound in 1 *M* phosphate buffer (pH 7.0). The mean recoveries of five replicate samples containing 4 μg/ml each of 5-AZA-CdR and 5-azacytidine were 80.5% (coefficient of variation, C.V. = 3.6%) and 78.8% (C.V. = 2.3%), respectively. The mean recoveries carried out under similar conditions for samples containing 0.2 μg/ml each of 5-AZA-CdR and 5-azacytidine were 84.0% (C.V. = 5.0%) and 83.5% (C.V. = 8.3%), respectively.

The precision of the assay was determined using control human serum containing 1 μg/ml each of 5-AZA-CdR and 5-azacytidine. For the within-batch ($n = 10$) precision, the mean peak height ratio of 5-AZA-CdR over 5-azacytidine was 0.742 ± 0.025 (C.V. = 3.4%). Between-day precision using duplicate assays of the same standard, performed by sample preparation in four batches over two consecutive days ($n = 8$) and analyzed at the same time by HPLC, was 0.800 ± 0.036 (C.V. = 4.5%).

RESULTS

In Fig. 1 are shown the HPLC patterns of plasma from a leukemic patient that has been extracted with 0.4 *M* perchloric acid (Fig. 1A) or with methanolic silver perchlorate and eluted through a cation-exchange column (Fig. 1B

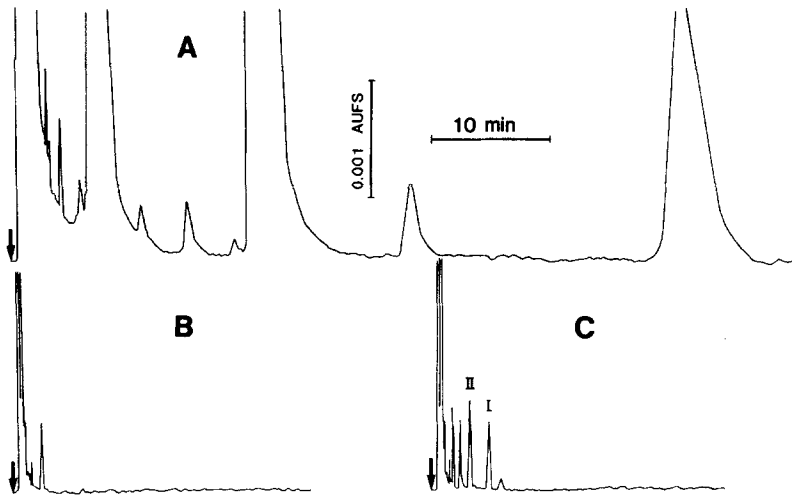

Fig. 1. Typical chromatograms of plasma from a leukemic patient that had been extracted with 0.4 *M* perchloric acid (A) or methanolic silver perchlorate, and passed through a cation-exchange column (B and C) as described in Experimental. 5-Azacytidine (II) and 5-AZA-CdR (I), each at a concentration of 1 μg/ml, were added to plasma in C. The samples were analyzed by HPLC as described in Experimental.

and C). The plasma sample extracted with perchloric acid shows many interfering UV peaks throughout the chromatogram, and some peaks had a very long retention time (60 min). The same plasma sample extracted with methanolic silver perchlorate and eluted through a small cation-exchange column showed the absence of all the major UV peaks (Fig. 1B). When 5-azacytidine and 5-AZA-CdR were added to the plasma sample and treated by the same procedure, two sharp peaks with a retention time of 3.3 and 4.9 min ap-

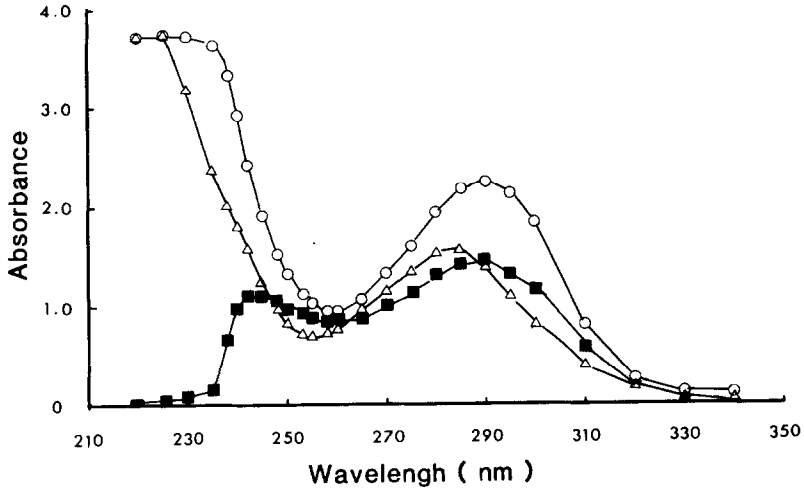

Fig. 2. Spectrum of deproteinized control human serum obtained by 0.4 *M* perchloric acid (△), methanol (○) or methanolic silver perchlorate (■). Each extract represents a 1:3 dilution from the original serum and read against its own reagent blank with water substituting the serum.

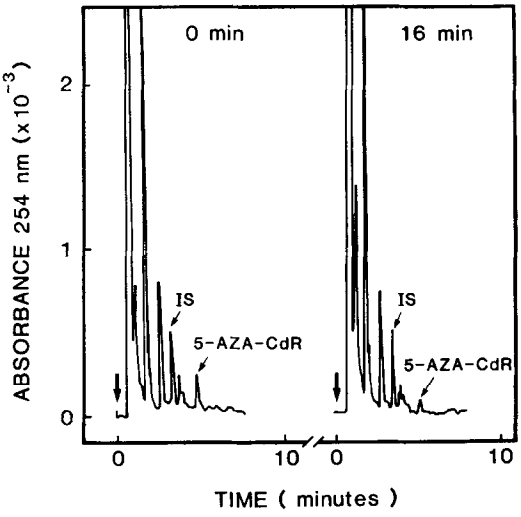

Fig. 3. Representative chromatograms of a leukemic patient (patient 4 in Table I) receiving a continuous intravenous infusion of 5-AZA-CdR at a rate of 1.0 mg/kg/h. The steady-state plasma (0 min) was taken after 24 h of infusion and the infusion was then interrupted to obtain blood samples at 2, 4, 8, 12 and 16 min. Only 0 and 16 min are shown in the figure. IS is the internal standard, 5-azacytidine.

peared on the chromatogram; two minor peaks that also appeared on the chromatogram probably represent the decomposed formyl derivatives of these 5-azacytosine compounds (Fig. 1C).

The UV spectra of the supernatant of normal human serum extracted with perchloric acid, methanol and methanolic silver perchlorate are shown in Fig. 2. Methanol alone was the least effective process for the removal of UV-absorbing substances, whereas the other two methods were about equally effective in removing UV peaks in the region above 240 nm. However, below 240 nm the methanolic silver perchlorate was much more effective than perchloric acid in removing UV peaks.

The present assay method was used to monitor the plasma concentration of leukemic patients undergoing therapy with 5-AZA-CdR. Fig. 3 shows a typical chromatogram of plasma from a patient and Table I summarizes the analytical results for four patients. The average steady-state plasma concentration was found to be 0.56 μg/ml (range 0.36—0.76 μg/ml) and the mean elimination half-life was 12 min (range 10—15 min).

TABLE I

HPLC ANALYSIS OF PLASMA FROM PATIENTS WITH ACUTE LEUKEMIA ADMINISTERED CONTINUOUS INTRAVENOUS INFUSION OF 5-AZA-CdR

5-AZA-CdR was administered at a rate of 1.0 mg/kg/h for 40—60 h.

Patient	Age (years)	Weight (kg)	Diagnosis*	Steady-state plasma concentration (μg/ml)	Elimination half-life** (min)
1	14	50	AML	0.43	15
2	17	62	ALL	0.36	12
3	47	72	ALL	0.67	10
4	21	62	ALL	0.76	11

*AML = Acute myelocytic leukemia; ALL = acute lymphocytic leukemia.
**To determine the elimination half-life, the blood specimens were obtained as described in Fig. 3.

DISCUSSION

The sample preparation method described in this communication can effectively remove all UV-interfering peaks (Fig. 1) and at the same time preserve and concentrate 5-AZA-CdR from plasma samples. The first step of this method, which consists of mixing the plasma with methanolic silver perchlorate, has two major effects: first, the reagent acts as a protein precipitant and also removes chloride and some other anions; second, the silver ion can complex and remove many UV-absorbing materials (Fig. 2). The second step in the sample preparation involves the use of a small cation-exchange column. The pK of the amino group on the triazine moiety of 5-AZA-CdR was determined to be 2.6 [7] which allows the binding of this analogue to the sulfonic group of the resin, and permits the column to be washed with methanol and water to remove non-cationic impurities. The 5-AZA-CdR was then eluted out in a con-

centrated form with ammonium phosphate at neutral pH, whereas the strongly cationic materials remained bound to the column. This two-step process facilitated the identification and quantification of 5-AZA-CdR on the chromatogram.

Since the plasma concentration of chloride is around 0.1 equiv./l, the silver ions contained in the precipitating reagent are largely removed from the solution by precipitation with chloride ions. When the concentration of silver perchlorate was lower than 0.05 *M* it was much less effective in removing UV peaks (data not shown), suggesting that trace amounts of silver ion must be present to complex the UV-absorbing materials. Since the column was designed to receive up to 25% in excess volume of the extract used in the present method, it could well tolerate some variations in silver ion concentration in the extract resulting from the different plasma anion content in plasma from patients.

5-Azacytidine was an excellent internal standard in the present method. 5-Azacytidine differs from 5-AZA-CdR only by the presence of a hydroxyl group at the 2′-position of the ribose moiety and thus, while it is slightly more hydrophilic, it has similar chemical properties of 5-AZA-CdR to survive all the purification processes. Since 5-azacytidine itself is an effective antineoplastic agent, the present method could also be applied to the assay of 5-azacytidine in plasma by alternating the role of 5-azacytidine and 5-AZA-CdR.

The sample preparation method was applied successfully for monitoring plasma concentrations of 5-AZA-CdR in leukemic patients by HPLC (Table I and Fig. 3). In patients that were administered 5-AZA-CdR at a rate of 1.0 mg/kg/h the steady-state plasma concentration of this agent ranged from 0.36 to 0.76 μg/ml and the estimated elimination half-life was in the range 10—15 min. These results illustrate that the assay may be used to assist clinical evaluation of 5-AZA-CdR as well as to adjust the dose for administration.

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REFERENCES

1 F. Šorm and J. Veselý, Neoplasma, 15 (1968) 339.
2 J. Veselý and A. Čihák, Cancer Res., 37 (1977) 3684.
3 R.L. Momparler and F.A. Gonzales, Cancer Res., 38 (1978) 2673.
4 G.E. Rivard, R.L. Momparler, J. Demers, P. Benoit, R. Raymond, K.T. Lin and L.F. Momparler, Leuk. Res., 5 (1981) 453.
5 K.T. Lin, R.L. Momparler and G.E. Rivard, J. Pharm. Sci., 64 (1981) 1228.
6 G.G. Chabot, J. Bouchard and R.L. Momparler, Biochem. Pharmacol., 32 (1983) 1327.
7 G.G. Chabot, G.E. Rivard and R.L. Momparler, Cancer Res., 43 (1983) 592.