Journal of Chromatography, 345 (1985) 157—161
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2762

Note

Reversed-phase ion-paired high-performance liquid chromatographic determination of 6-azacytidine in blood

S.V. GALUSHKO* and I.P. SHISHKINA

Institute of Organic Chemistry of the Ukrainian SSR Academy of Sciences, Bioorganic Chemistry Department, 252660 Kiev 94 (U.S.S.R.)

(First received December 6th, 1984; revised manuscript received July 12th, 1985)

6-Azacytidine (6-AzCyt) is an abnormal nucleoside having antitumoural activity [1] (Fig. 1, structure I). In contrast to 5-azacytidine and other antitumour pyrimidine derivatives [2, 3], it has a much lower toxicity. The therapeutic dose of the preparation is 0.5—3 g/kg, and the LD_{50} exceeds 10 g/kg [4].

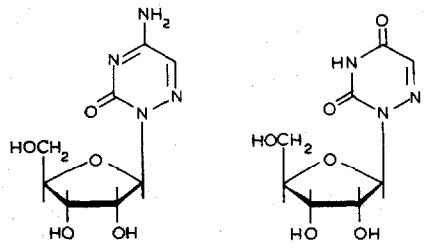


Fig. 1. Structures of 6-azacytidine (I) and 6-azauridine (II).

In order to study the pharmacokinetics of the preparation, a rapid and sensitive method for 6-AzCyt determination in biological substances is required. High-performance liquid chromatography (HPLC) has been used successfully for the analysis of various nucleosides [5—7], and data are available on the chromatographic behaviour of several aza derivatives of pyrimidine nucleosides. Thus, for a study of the stability of 5-aza-2′-deoxycytidine in solution, reversed-phase HPLC on ODS was used [8]. In order to study the kinetics of

0378-4347/85/$03.30

5-azacytidine hydrolysis, ion-exchange chromatography on the cation exchanger Aminex was used [9], but this method lacks stability at high pressures. A method for determining 5-aza-2′-deoxycytidine in plasma by reversed-phase HPLC on a μBondapak phenyl column has been described in the literature but, in this case, full separation of the sample peak from the other components has not been attained [10].

The above data concern only the results of the chromatographic analysis of 5-aza derivatives of pyrimidine nucleosides. Any data concerning the HPLC of asymmetric 6-aza derivatives have not been elucidated in the literature.

This paper is aimed at establishing the optimum conditions for a rapid, selective determination of 6-AzCyt in blood.

EXPERIMENTAL

Materials

6-AzCyt was obtained from the Institute of Molecular Biology and Genetics of the Ukrainian SSR Academy of Sciences. Sodium dodecylsulphate (SDS) (Serva, Heidelberg, F.R.G.), orthophosphoric acid and trichloroacetic acid were obtained commercially (reagent grade) and used without further purification. Heparin, 5000 I.U./ml was from Gedeon Richter (Budapest, Hungary). Water was deionized, double-distilled and filtered for HPLC use.

Chromatographic conditions

The experiments were accomplished on an HG-1305 liquid chromatograph (Nauchpribor Assoc., Orel, U.S.S.R.) equipped with a variable-wavelength detector set at 265 nm (sensitivity 0.005 a.u.f.s.), and on a Waters liquid chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisting of a Model U6K universal injector, a Model 6000A pumping system, a Model 481 variable UV/VIS detector (sensitivity range 2.0—0.001 a.u.f.s.), at a wavelength of 265 nm, and a Model 730 data module. Chromatographic separation was performed at ambient temperature.

The sample components were separated on a Silasorb C_{18} 10-μm (Lachema, Brno, Czechoslovakia) glass column (15.0 × 0.1 cm), connected to an HG-1305 chromatograph, and on a Bondapak C_{18} 10-μm column (30 × 0.4 cm), connected to a Waters liquid chromatographic system. The mobile phase consisted of a 0.2% solution of SDS in 0.3 *M* orthophosphoric acid at a flow-rate of 0.03 ml/min (on the HG-1305 chromatograph) and 0.5 ml/min (on the Waters chromatographic system).

Preparation of samples

A 0.1-ml volume of 50% trichloroacetic acid was added to 0.2 ml of blood for deproteinization. This solution was further mixed with care. Clean supernatant was then isolated by centrifugation at 4000 *g* for 10 min, then injected further into the HPLC system. The concentration of 6-AzCyt could then be determined using a calibration curve.

Calibration curve

Different amounts of 6-AzCyt were added to 0.2-ml samples of blood. The

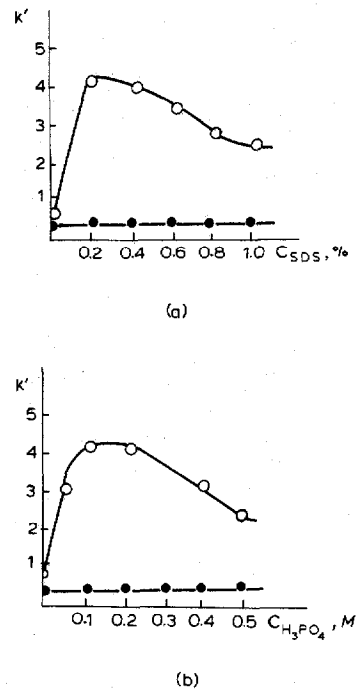
concentration of 6-AzCyt was thus varied from 40 to 1500 μg/ml. Then, deproteinization and centrifugation under the conditions described above were carried out. By injecting a pure supernatant into the HPLC system, the magnitude of the 6-AzCyt peak could be measured. The calibration curve was represented by the relation $y = 0.14x - 2.69$ ($r = 0.998$), where y is the height of the peak and x is the concentration of 6-AzCyt.

Animal studies

To study the pharmacokinetics of 6-AzCyt, 6-AzCyt was given to rats intravenously and intraperitonially in doses of 1 and 2 g/kg, respectively. Samples of blood were drawn from the tail vein and prepared in a manner similar to that discussed above.

RESULTS AND DISCUSSION

6-AzCyt is a highly polar compound and in a neutral mobile phase on C_{18} columns it has a low retention time and so is not retained. But in acid media, the protonated 6-AzCyt molecule is able to form an ion pair with the dodecylsulphate ion, and its retention time rises considerably. The system has a high resolution, which allows the peak of 6-AzCyt (peak 2 in Fig. 3) to be isolated distinctly from the peaks of the other components.



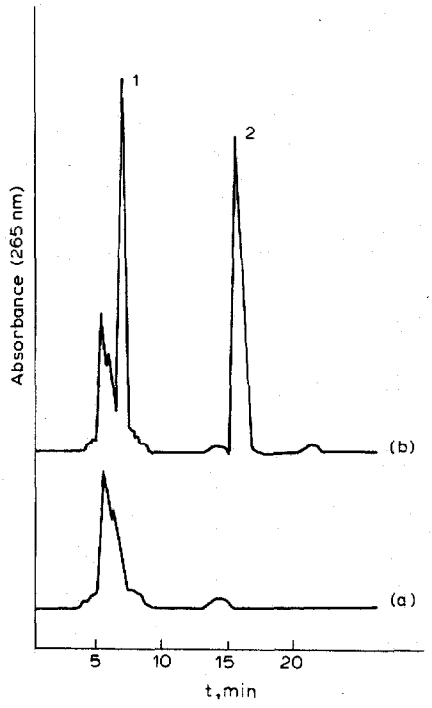


Fig. 2. A plot of capacity factor against concentration of SDS (a) and orthophosphoric acid (b). (○) 6-AzCyt, (●) 6-azauridine. Eluents: 0.1 *M* orthophosphoric acid (a) and 0.2% SDS (b).

Fig. 3. Chromatograms of rat blood before (a) and after (b) administration of 6-AzCyt. Peak 2 = 6-azacytidine.

To optimize the separation of 6-AzCyt from 6-azauridine (a possible metabolite), the effect of SDS and orthophosphoric acid concentration on the capacity factor was studied in detail (see Fig. 2). As follows from Fig. 2, the conditions for distinct separation were provided by the wide range of SDS and orthophosphoric acid concentrations. Optimum selectivity was attained at 0.3% and 0.2 *M* concentrations of SDS and orthophosphoric acid, respectively. A choice of real conditions for the chromatograms was determined by the column resolution. A mobile phase consisting of 0.2% SDS—0.3 *M* phosphoric acid was used in the present study. This composition provided a shorter analysis time (ca. 16 min) and a distinct isolation of the 6-AzCyt peak from the other components.

The chromatograms of blood are depicted in Fig. 3 where the first one (a) was obtained before and the second one (b) after injection of 6-AzCyt. The retention time of peak 1 is close to that for 6-azauridine. One can conclude that the 6-AzCyt metabolism is possibly similiar to that of 5-azacytidine.

As is already known [10], 5-azacytidine is subjected to desamination in vivo. But under the present experimental conditions it is impossible to isolate satisfactorily peak 1 from the concomitant components. Apparently, the negatively charged nucleotide can have possible metabolites, however, they are not retained under these HPLC conditions. 6-AzCyt metabolism is the side problem, and the results of this study will be presented in a forthcoming paper. The dependence of the 6-AzCyt peak height on 6-AzCyt concentration appears to be linear in the range 0—10 mg/ml. Addition of 6-AzCyt before and after the blood deproteinization does not affect the magnitude of the peak. This fact indicates that 6-AzCyt does not bind with blood proteins under the deproteinization conditions presented. The results of determining the 6-AzCyt added to the samples before deproteinization are given in Table I. The lower sensitivity is ca. 12 ng/ml (on the Waters chromatography system) and the signal-to-noise ratio is 5.

The results of the pharmacokinetic studies are presented in Fig. 4. Curve a

TABLE I

RECOVERY DATA

$n = 5$.

Added (μg/ml)	Found (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
10000	9600 ± 190	1.97
5000	5200 ± 75	1.4
2500	2500 ± 100	4.0
1250	1250 ± 25	2.0
620	620 ± 14	2.6
400	350 ± 27	7.7
320	310 ± 21	6.5
200	280 ± 13	4.6
160	165 ± 7.2	4.4
120	120 ± 7.8	6.5
80	80 ± 0	0

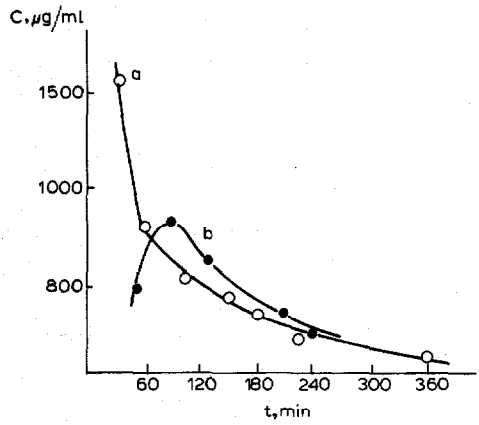


Fig. 4. Blood concentration of 6-AzCyt in two rats. (a) After intravenous injection of 1 g/kg 6-AzCyt; (b) after intraperitoneal injection of 2 g/kg 6-AzCyt.

is obtained after intravenous injection of 6-AzCyt. It is described by the relation 1 g $C = 0.67t$. Its pharmacokinetic parameters are also evaluated. Apparent volume of distribution: 1.33 l kg^{-1}; elimination rate constant: 0.67 h^{-1}; clearance: 0.891 l h^{-1} kg^{-1}; biological half-life: 1.03 h. Curve b is obtained after intraperitonial injection.

Therefore, a sensitive and selective method for the HPLC determination of 6-AzCyt in blood is proposed. This method can be used successfully in pharmacokinetic studies.

REFERENCES

1 A.S. Petrusha, I.V. Alekseeva and V.P. Cherneckii, Conference on Actual Problems of Antitumour Chemotherapy, Vol. 2, Chernogolovka, 1980, Inst. Chim. Phys. Acad. Sci. U.S.S.R., Chernogolovka, 1980, p. 82.
2 E. Velez-Garcia, W.R. Vogler, A.A. Bartolucci and A.N. Arkun, Cancer Treat. Rep., 61 (1977) 1675.
3 N.N. Blokhin and N.I. Perevodcikova, Chemotherapy of Neoplastic Diseases, Meditsina, Moscow, 1984.
4 A.S. Petrusha, I.V. Alekseeva and V.P. Cherneckii, Conference on Actual Problems of Antitumour Chemotherapy, Sverdlovsk, 1982, Inst. Chim. Phys. Acad. Sci. U.S.S.R., Chernogolovka, 1982, p. 115.
5 M. Zakaria and P.R. Brown, J. Chromatogr., 226 (1981) 267.
6 P.R. Brown, Cancer Invest., 5 (1983) 439.
7 P.A. Perrone and P.R. Brown, J. Chromatogr., 307 (1984) 53.
8 K.T. Lin, R.L. Momparler and G.E. Rivard, J. Pharm. Sci., 70 (1981) 1228.
9 K.K. Chan, O.D. Clandini, J.A. Staroscik and W. Sadee, J. Pharm. Sci., 68 (1979) 807.
10 G.G. Chabot, G.E. Rivard, R.L. Momparler, Cancer Res., 43 (1983) 592.