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Short Communication

Simultaneous determination of 6-azacytidine, 6-azauridine, arabinoside cytosine and arabinoside uracil in blood by liquid chromatography*

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Introduction

Cytosine arabinoside (Ara-C) and 6-azacytidine (6-azaCyd) (Fig. 1) are abnormal nucleosides having antitumour activity. 6-azaCyd has a much lower toxicity than Ara-C and can be used in high doses [1]. Ara-C is subjected to deamination and *in vivo* yields inactive uracil arabinosie (Ara-U). A simultaneous administration of these nucleosides is seen to be promising for two reasons: (1) 6-azaCyd is a selective inhibitor for RNA synthesis, whereas Ara-C inhibits DNA synthesis [2]; and (2) 6-azaCyd is a substrate for cytidine deaminase [3], so that the presence in blood of this compound in concentration much greater than Ara-C content can retard Ara-C deamination. In order to study the pharmacokinetics and metabolism under conditions of combined administration a rapid method for their simultaneous determination in blood was required.

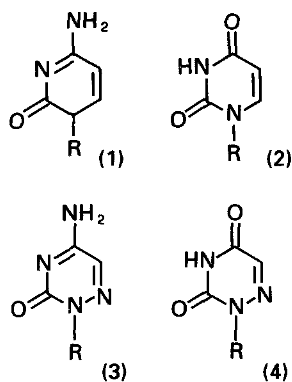

Figure 1
Structures of Ara-C (1) and Ara-U (2) (R = arabinose); 6-azaCyd (3) and 6-azaUrd (4) (R = ribose).

HPLC is widely used to determine the synthetic analogues of pyrimidine nucleosides in different media and results obtained have been summarized and reviewed [4]. The behaviour of pyrimidine and 6-aza triazine nucleosides in reversed-phase LC has been studied [5] and an approach that enables the choice of optimal conditions for the separation of these compounds proposed [6]. Several HPLC assays for Ara-C and Ara-U in various biological fluids have been described. Ion-exchange [7], dual reversed-phase and ion-exchange [8] and reversed-phase columns [9–11] have been used. A method for determining 6-azaCyd in blood by ion-pair LC also has been described [12]. The above data concern only the results of chromatographic analysis of Ara-C and 6-azaCyd in blood after individual administration or chromatographic separation of artificial mixtures. Any data concerning the simultaneous determination of Ara-C, 6-azaCyd and products of their deamination in blood have not been found in the literature.

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This paper describes optimum conditions for the rapid simultaneous determination of these compounds in blood.

Experimental

Chromatographic conditions

The experiments were performed using a LKB (Bromma, Sweden) liquid chromatographic system consisting of a Model 2140 rapid spectral detector set at 265 nm, a Model 2150 dual HPLC pumps, a model 2152 LC controller, a Model 2155 column oven, a Model 2154 injector and Model 2220 recording integrator. The column used was an Octadecyl Polyol Si 100 (stainless-steel column 250 × 4.6 mm i.d., particle size 5 μm) (Serva, Heidelberg, Germany). The mobile phase was phosphate buffer (pH 5.1; 0.1 M H_3PO_4 adjusted with 0.1 M sodium hydroxide) at a flow rate of 0.5 ml min^{-1}. Separation was performed at 35°C.

Materials

6-azaCyd and 6-azaUrd were obtained from the Institute of Molecular Biology and Genetics of the Ukrainian Academy of Sciences. Ara-C, Ara-U and uridine was obtained from Chemical Dynamic Corp. (South Plainfield, NY, USA). Acids (H_3PO_4, $HClO_4$), and sodium hydroxide were obtained commercially (analytical-reagent grade) and were used without further purification. Water was doubly distilled and filtered for HPLC use.

Preparation of samples

A 50 μl volume of 5 M $HClO_4$ was added to 0.2 ml of rat blood in a heparinized vial. Clean supernatant was isolated by centrifugation at 10,000 rpm for 2 min, then injected into LC system. The injected volume was 50 μl.

Results and Discussion

All the nucleosides studied are ampholites but within the pH range 2–8 they exhibit either basic (Ara-C, 6-azaCyd), or acidic (Ara-U, 6-azaUrd) characteristics. The pK value of Ara-C and Ara-U does not differ considerably from that for natural analogues in contrast to 6-azaCyd and 6-azaUrd [7]. The replacement of the carbon atom in the pyrimidine ring at position 6 by the more electronegative nitrogen atom appreciably increases the acidic and decreases the basic properties of the compounds. The changes in the acid–base properties of the compounds significantly affects their chromatographic behaviour. Figure 2 shows the dependence of the capacity factors of the nucleosides studied upon the eluent pH at constant ionic strength. The sharp decrease in the capacity factors with transition of the compounds from the molecular into the ionized form is observed for 6-aza-derivatives at much lower pH than that for Ara-C, Ara-U and uridine. As can be seen from Fig. 2 the optimum pH lies within the range 3.5–5.5. As long as Ara-C is partially protonated, it is possible, under these conditions, to separate to a maximum extent uridine and Ara-C. It should be noted that uridine, one of the endogenous interferences, co-eluted with Ara-C when an eluent with a pH value of 6.5 was employed [11]. The experiment shows that the 0.1 M phosphate buffer at pH 5.1 enables the separation of 6-azaCyd, 6-azaUrd, Ara-C and Ara-U from the other components of the plasma (Fig. 3).

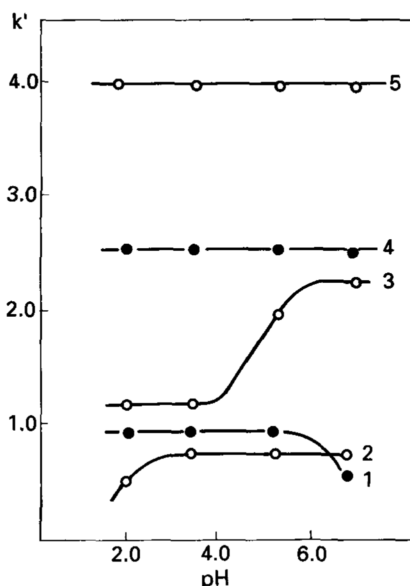

Figure 2
Effect of pH on the retention of nucleosides. Eluent: 0.01 M phosphate buffer in 0.1 M $(NH_4)_2SO_4$. Urd 1,6-aza; 2,6-azaCyd; 3, Ara-C; 4, Urd; 5, Ara-U.

Standard curves (based on peak area) are found to be linear in the range 0.2–200 μg ml^{-1}. Linear regression of these curves gave correlation coefficients of 0.995, 0.996, 0.993, 0.997 and straight-line equations of area (Y) against amount (X): $X = (0.00163Y - 16.3)$ μg l^{-1}; $X = (0.00159Y - 31.8)$ μg l^{-1}; $X =$

Table 1
Validation studies performed by adding known amounts of Ara-C, Ara-U, 6-azaCyd and 6-azaUrd to blank blood

Conc. (μg ml^{-1})	Compound	Assay conc. mean ± SD (n = 5, μg ml^{-1})	Recovery (%)	RSD Within-day (n = 5)	RSD Between-day (n = 5)
20.00	Ara-C	19.68 ± 0.30	98.42	5.2	6.8
	Ara-U	19.97 ± 0.42	99.83	4.0	7.2
	6-AzaCyd	20.25 ± 0.36	101.26	5.6	4.8
	6-AzaUrd	20.01 ± 0.62	100.04	3.0	4.3
50.00	Ara-C	49.28 ± 0.49	98.55	2.3	3.2
	Ara-U	49.82 ± 1.02	99.64	4.9	5.4
	6-AzaCyd	50.02 ± 0.84	100.03	3.8	4.4
	6-AzaUrd	48.92 ± 1.12	98.98	1.6	1.7

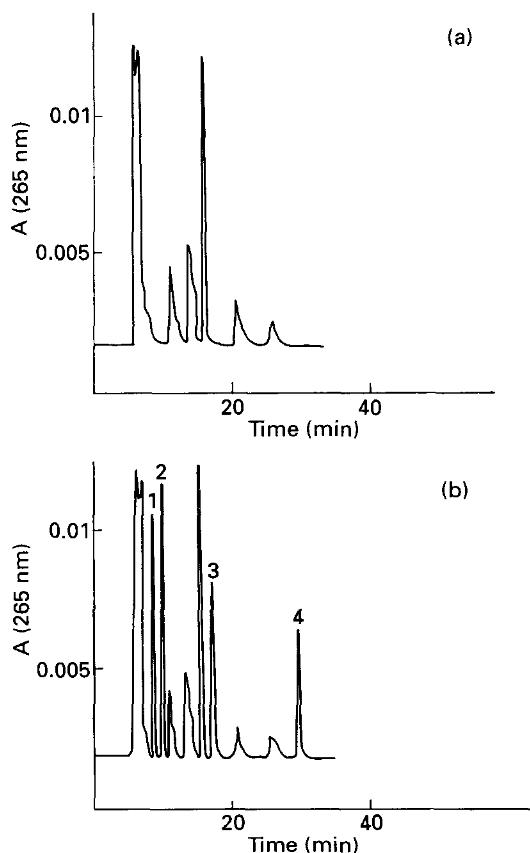

Figure 3
Chromatograms of rat blood samples. (a) Blank blood, (b) 1, AzaCyd (6.0 μg ml^{-1}); 2, AzaUrd (4.0 μg ml^{-1}); 3, Ara-C (10.0 μ ml^{-1}); 4, Ara-U (5.0 μg ml^{-1}). Eluent: 0.1 M phosphate buffer, pH 5.1.

$(0.0159Y - 31.7)$ μg l^{-1}; and $X = (0.00156Y - 46.9)$ μg l^{-1} for: Ara-C, 6-azaCyd, 6-azaUrd and Ara-U, respectively. The standard deviations about the regression lines were: 0.0410, 0.0395, 0.0450 and 0.0455, respectively. The limit of detection was found to be 0.24 μg ml^{-1} (6-azaCyd, 6-azaUrd), 0.3 μg ml^{-1} (Ara-C) and 0.6 μg ml^{-1} (Ara-U) at a signal-to-noise ratio of 2. Validation and recovery studies were performed by adding known amounts of nucleosides to blank rat blood at two different concentrations (Table 1).

The method proposed is being used for the pharmacokinetics studies of 6-azaCyd and Ara-C under combined administration in animals and the results obtained will appear in a future publication.

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