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Chromatographic properties of cytosine, cytidine and their synthetic analogues

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Abstract

A direct reversed-phase high-performance liquid chromatographic (RP-HPLC) assay was used for the study of the effects of methanol concentration, pH, flow-rate of the mobile phase and column temperature on the retention of the natural nucleic acid components cytosine and cytidine and their synthetic 1-β-D-arabinofuranosyl, 5-aza and 6-aza analogues. The pK_a values were also determined. The greatest changes were observed with changes in pH. The relationship between the capacity factors and the hydrophobicity of the compounds studied was also investigated.

Keywords: Cytosine; Cytidine; Nucleosides; Nucleobases; Nucleic acids; Arabinosylcytosine; 5(6)-Azacytosine; 5(6)-Azacytidine; 5-Azaarabinosylcytosine

1. Introduction

Synthetically prepared analogues of naturally occurring nucleobases, nucleosides and nucleotides play an important role in chemotherapy. Arabinosylcytosine (araC) is a cell cycle (S-phase) specific anti-tumour agent used in the treatment of leukaemias and lymphomas. Its clinical utility is limited by its rapid deamination to a biologically inactive arabinosyluracil [1]. The success with araC encouraged the search for other cytidine analogues, and it was logical to take into consideration structural changes in the pyrimidine ring. 5-Azacytidine (5-azaCyd) differs from deoxycytidine in the presence of a nitrogen at the 5-position on the heterocyclic ring. Its cytostatic action is exerted against acute myeloblastic leukaemias. 5-Aza-araC possesses similar metabolism and therapeutic effects to 5-azaCyd and araC [2]. Base 5-azacytosine (5-azaCyt) has not found wide clinical use but after incorporation into DNA it could be responsible for the inhibiting effect of 5-azaCyd on DNA methylation [3]. 6-Aza analogues of naturally occurring nucleobases and nucleosides possess antitumour activity and they have lower cytotoxicity [2].

In this work, we tried to test their relationships through the study of various factors and their hydrophobicity (log P) by RP-HPLC.

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2. Experimental

2.1. Chemicals

Cytosine and cytidine were purchased from Pharma-Waldhof (Mannheim, Germany). The analogues studied (Fig. 1) were synthesized at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences (Dr. A. Píškala, 5-aza and 6-aza analogues; Dr. J. Beránek, araC). Potassium dihydrogenphosphate (KH_2PO_4) and potassium hydroxide (KOH) were purchased from Merck (Darmstadt, Germany), sodium dihydrogenphosphate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogenphosphate ($Na_2HPO_4 \cdot H_2O$), citric acid ($C_6H_8O_7 \cdot H_2O$),

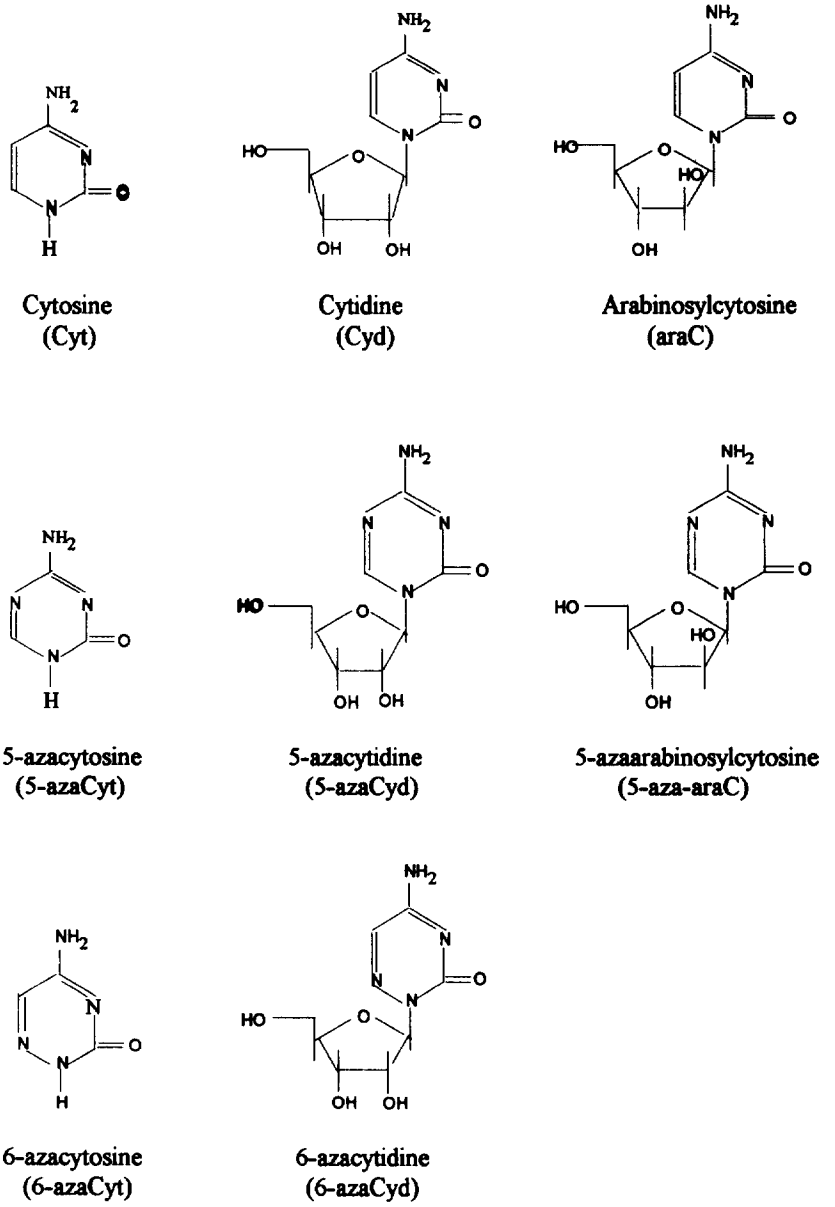

Fig. 1. Structures of the compounds studied.

acetic acid (CH_3COOH) and hydrochloric acid (HCl) from Lachema (Brno, Czech Republic), methanol (CH_3OH) and orthophosphoric acid (H_3PO_4) from Fluka (Buchs, Switzerland) and *n*-octanol from Aldrich (Milwaukee, WI, USA). Doubly distilled, deionized water was obtained with an Elgasta Spectrum SC 20 system (ELGA, High Wycombe, UK). Single-compound stock solutions of the studied compounds were prepared at concentrations of $5 \cdot 10^{-4}$ *M*. A mixture containing 1 ml of each compound was diluted to a final volume of 10 ml with doubly distilled, deionized water.

2.2. Equipment

A Pye Unicam HPLC system (Philips, Cambridge, UK) was used, consisting of a PU 4030 controller, PU 4011 gradient pump, PU 4031 column oven, PU 4020 variable-wavelength UV detector and Rheodyne Model 7125 injector (20-μl sample loop). Peaks were recorded with a Model 4880 data station, version 1.1 (Ati Unicam, Cambridge, UK).

2.3. Determination of ionization constants (pK_a)

Ionization constants (pK_a) were determined by UV spectrophotometry according to Albert and Serjeant [6], using a Perkin-Elmer (Norwalk, CT, USA) Model 557 double-beam UV–Vis spectrophotometer.

2.4. Chromatographic conditions

Reversed-phase separations were performed on a 250 × 5 mm I.D. stainless-steel column, commercially packed (Philips) with octadecyl-bonded polymer gel of 5-μm particle size (Spherisorb 5 ODS). The mobile phase consisted of 0.01 *M* KH_2PO_4 solution containing various concentrations of methanol (0, 3, 5, and 8%, v/v). The pH (3.5–7.0) was adjusted with a few drops of either H_3PO_4 or KOH. The mobile phase was filtered through 0.45-μm nylon 66 membranes (Supelco, Bellefonte, PA, USA) and degassed. The system was operated at ambient temperature with a flow-rate of 1.3 ml/min. The UV detector was set at 275 nm. When studying the influence of temperature and flow-rate, the temperature range was 25–45°C and the flow-rate range was 1.0–1.5 ml/min.

2.5. Determination of apparent partition coefficient (P)

The apparent partition coefficients (*P*) were determined and calculated to the authors [5,7] with minor changes to the procedure. An amount of 2 mg of each compound was dissolved in octanol-saturated 0.01 *M* KH_2PO_4 and then 0.01 *M* KH_2PO_4-saturated octanol was added. The mixture was shaken on a shaker in the tube for 10 min at room temperature. Separation was achieved by centrifugation at 1500 r.p.m. (600 g) for 50 min. The concentration of each compound in both the octanol and phosphate phases was measured by HPLC as described above.

3. Results and discussion

3.1. Ionization constants

The ionization constants and log *P* and log *k'* values of studied compounds are given in Table 1. We compared the ionization constants of Cyt, Cyd, araC and their 5-aza and 6-aza analogues which possess a nitrogen atom at position 5 or 6 on the heterocyclic ring. It was observed that the

Table 1
Ionization constants (pK_a) and log *P* and log *k'* values of the compounds studied

Compound	pK_a	Log *P*	log *k'*
Cyt	4.25 (4.60)[a]	−0.59	0.03
Cyd	4.22 (4.22)[a]	−2.08	0.19
araC	4.15	−2.09	0.28
5-azaCyt	3.52	−1.94	−0.21
5-azaCyd	3.32	−2.41	0.16
5-azaC	3.18	−2.50	0.25
6-azaCyt	2.80	−1.06	−0.09
6-azaCyd	2.57	−1.97	−0.07

[a] Data from Ref. [13].

pK_a values decrease in the order Cyt, 5-azaCyt, 6-azaCyt and in the order Cyd, 5-azaCyd, 6-azaCyd. Unfortunately, we could not compare the pK_a values of araC and its 5-aza and 6-aza analogues because of the unavailability of 6-aza-araC. The results show that the presence of a nitrogen atom in the heterocyclic ring reduces the basicity of bases and nucleosides. A similar problem has been investigated by other workers [8], who compared the values for adenosine, deoxyadenosine, arabinosyladenine and their analogues with a chlorine atom at the C-2 position on the purine ring. 2-Chlordeoxyadenosine decreased the basicity of the N-1 atom, which was confirmed by the finding of differences in protonation sites [9]. This change in the protonation sites leads to resistance of the drug to adenosine deaminase [8]. The same conclusion can be made about the effect of the isosteric change of carbon at the 5- or 6-position by nitrogen in the pyrimidine base, but these compounds are deaminated by cytidine deaminase [2].

3.2. Retention in the chromatographic system

Bases and nucleosides are usually separated by RP-HPLC with 0.01 *M* KH_2PO_4 combined with (0–10%) of methanol as the mobile phase at a pH in the range 4–6. The elution order of bases and nucleosides depends on the pH [10]. The elution of compounds in RP-HPLC decreases with increasing concentration of organic modifier (methanol or acetonitrile) in the mobile phase.

The influence of methanol concentration (0, 3, 5 and 8%, v/v) and pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) was investigated. It was observed that the pairs of analogues Cyd–5-azaCyd and araC–5-aza-araC have the same retention on the column at methanol concentrations of 0–5% at pH 7.0. With 8% of methanol in the mobile phase both pairs elute in one peak. The effect of methanol concentration in the mobile phase on $\log k'$ is depicted in Fig. 2; k' decreases with increasing of methanol concentration. The study of the influence of methanol concentration at various pH values shows that the retention of the compounds decreases with increasing methanol concentration and with decreasing pH of the mobile phase. In some cases, k' increased slightly at higher methanol concentrations and lower pH. The effect of pH on k' of the compounds with 0, 3, 5 and 8% of methanol in the mobile phase is depicted in Fig. 3.

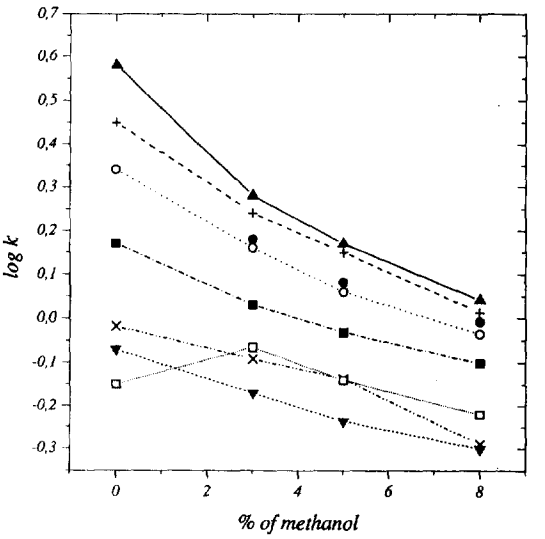

Fig. 2. Effect of the concentration of methanol in the mobile phase on log k' in the RP system for (■) Cyt, (○) Cyd, (▲) araC, (▼) 5-azaCyt, (●) 5-azaCyd, (+) 5-aza-araC, (×) 6-azaCyt and (□) 6-azaCyd. Chromatographic conditions: sample, 20 μl of standard solution; column, Spherisorb 5 ODS, 250 × 5 mm I.D.; mobile phase, 0.01 *M* KH_2PO_4 (pH 7); flow-rate, 1.3 ml/min; UV detection at 275 nm; temperature, 24°C.

When the pH and methanol concentration were changed, some changes in elution order were observed as a consequence of solute–solvent interactions, isosteric substitution and hydrogen bond formation between polar functional groups of the compound and the mobile phase (co-elution of Cyt and 5-aza-Cyt at pH 3.5 and 4.0 or distinguishing the peaks of Cyd, 5-azaCyd, araC and 5-aza-araC at pH 4.0 without methanol). It seems that the effect of methanol in the mobile phase is negligible.

We also investigated the influence of temperature in the range 25–45°C and at various methanol concentrations. Cyt, 5-azaCyt, 5-aza-araC and 6-azaCyd were studied and k' decreased with increase in temperature. The k' values of

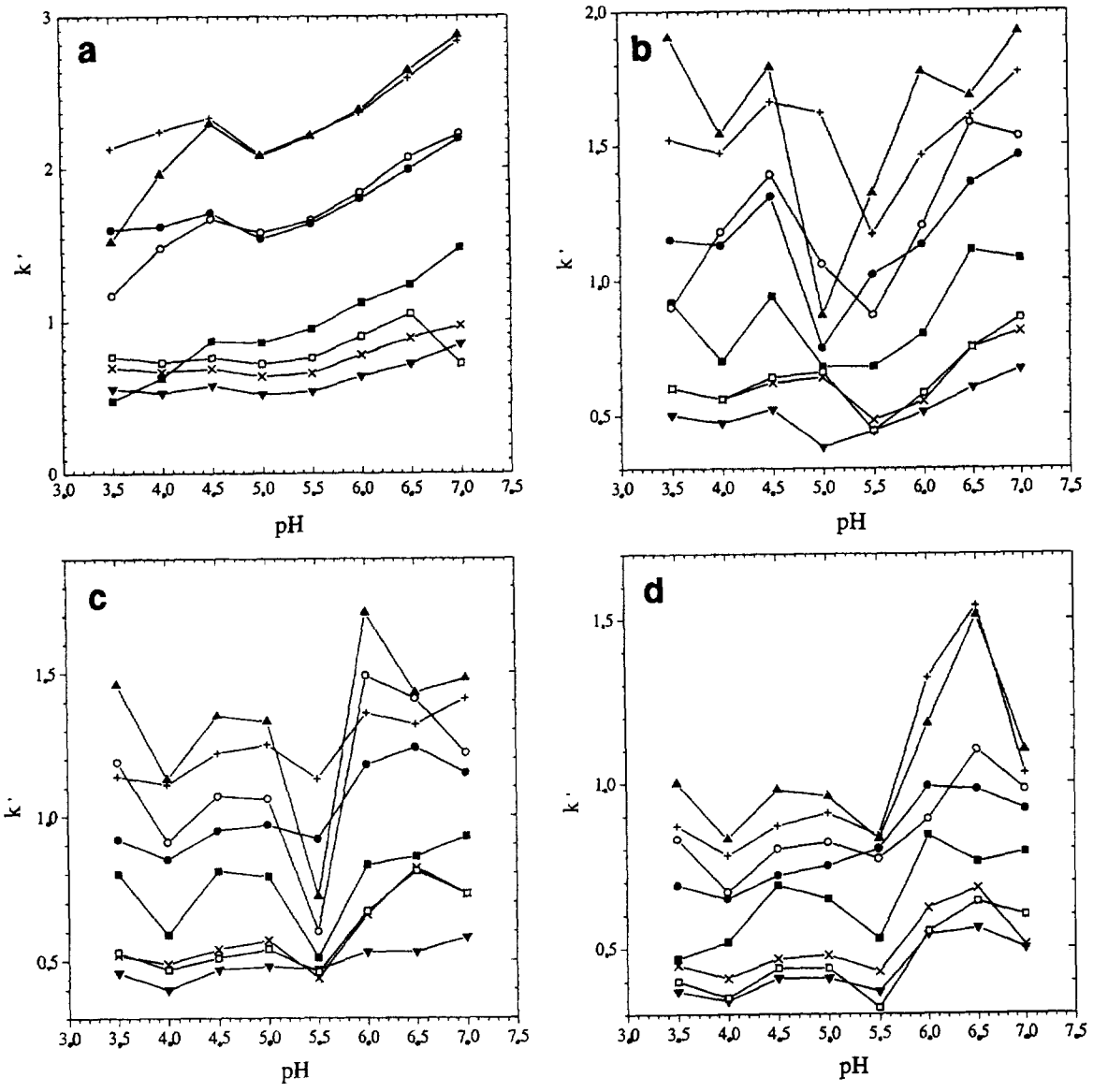

Fig. 3. Effect of pH on capacity factors (k') of (■) Cyt, (○) Cyd, (▲) araC, (▼) 5-azaCyt, (●) 5-azaCyd, (+) 5-aza-araC, (×) 6-azaCyt and (□) 6-azaCyd with a mobile phase containing (a) 0, (b) 3, (c) 5 and (d) 8% (v/v) of methanol. Chromatographic conditions as in Fig. 2.

the compounds studied intermittently decreases and increases at some temperatures (Fig. 4).

The flow-rate (1.0, 1.1, 1.2, 1.3, 1.4, and 1.5 ml/min) has no influence on the k' values of the compounds studied (data not shown). Our results and those of similar studies by other workers [1,8] confirm that an increase in flow-rate is more suitable for rapid separation than an increase in temperature because of the mass transfer term in both the mobile and stationary phases [11]. The optimum separation of the compounds is shown in Fig. 5.

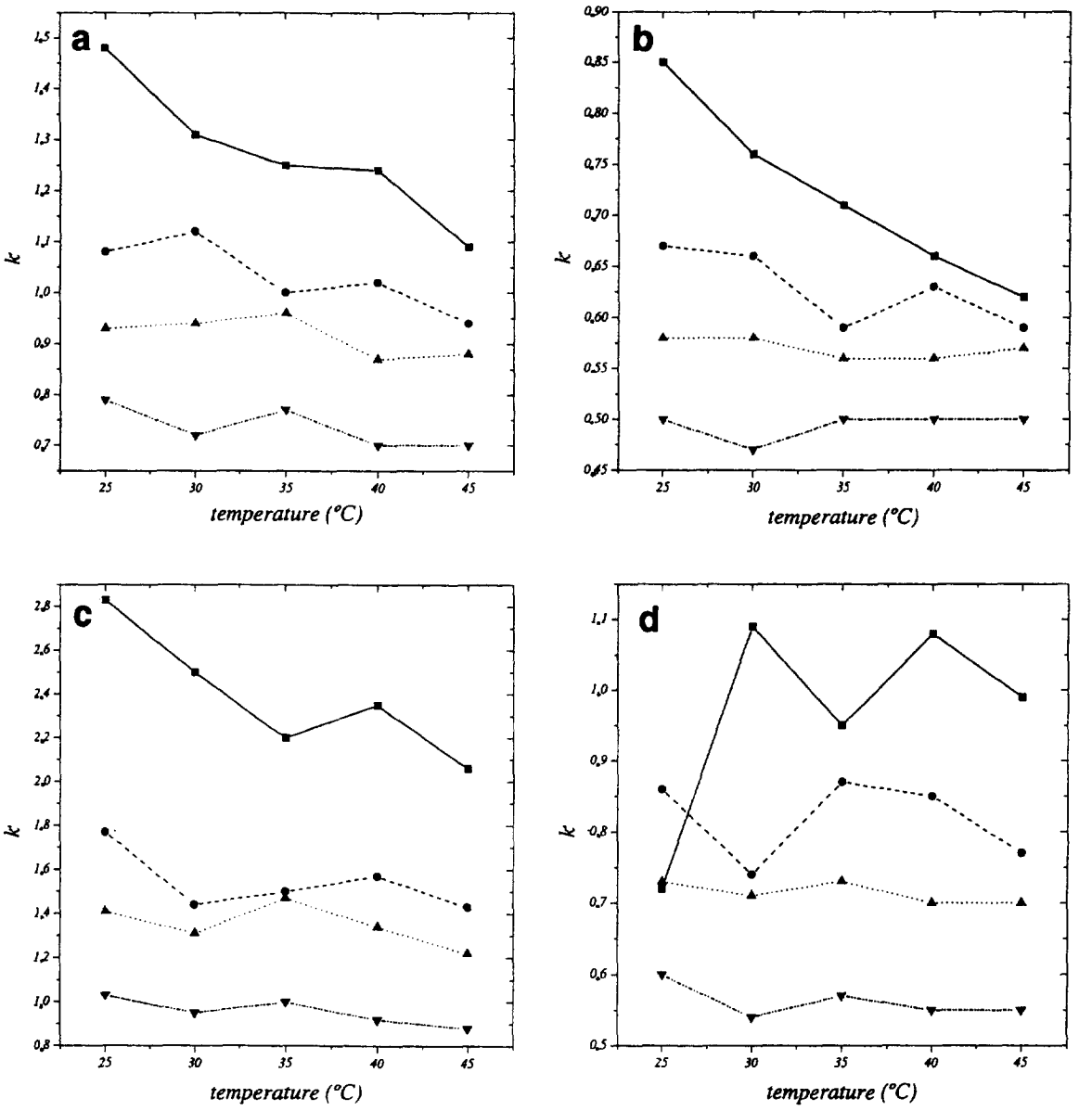

Fig. 4. Effect of temperature on capacity factors (k') of (a) Cyt, (b) 5-azaCyt, (c) 5-aza-araC and (d) 6-azaCyd with a mobile phase containing (▼) 8, (▲) 5, (●) 3 and (■) 0% (v/v) of methanol. Chromatographic conditions as in Fig. 2.

3.3. Correlation between log P and retention in RP-HPLC

Hydrophobicity, one of the most important physico-chemical properties, is expressed as the logarithm of the apparent partition coefficient (log P) of the compound between n-octanol and aqueous phases. Cheung and Kenney [4] reported the use of the Collander equation [12] for model compounds: $\log k' = a\log P + b$. This relationship has been shown to be valid for congeners only. The chromatographic conditions

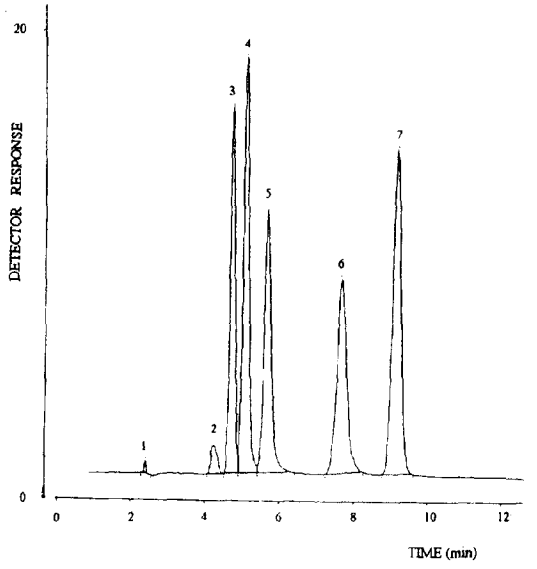

Fig. 5. RP-HPLC separation of the compounds studied. Chromatographic conditions as in Fig. 2. Peaks: 1 = mobile phase; 2 = 5-azaCyt; 3 = 6-azaCyt; 4 = 6-azaCyd; 5 = Cyt; 6 = Cyd; 5-azaCyd; 7 = araC, 5-aza-araC.

as described above were used. We tried to determine whether the hydrophobicity can be predicted from the k' values obtained with LC, to find a correlation between log P and log k', but no correlation was found (log $k' = -0.123$ log $P - 0.158$, $r = -0.457$, $n = 8$). The negative value of correlation coefficient indicates that there is an indirect dependence between log k' and log P. The capacity factors cannot be predicted on the basis of hydrophobicity.

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