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Hydrophobicity parameters of 2-chloro-2′-deoxyadenosine and some related analogues and retention in reversed-phase liquid chromatography

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Abstract

Hydrophobicity parameters expressed as the logarithm of the partition coefficient (log P) and ionization constants (pK_a) for 2-chloro-2′-deoxyadenosine and some related nucleoside analogues (2-fluoroarabinosyladenine, 2-chloroadenosine and 5′-chloro-5′-deoxyadenosine) are reported. The effects of methanol concentration and the pH of the mobile phase on the retention of the studied compounds in a reversed-phase system were examined. The relationship between hydrophobicity parameters and the retention in the HPLC system was investigated. A fairly good linear correlation was obtained when log k' values ($r = 0.960$) and/or log k'_w values (extrapolated from the relationship between log k' and the percentage of methanol to 100% water) ($r = 0.959$ and 0.967, respectively) were correlated with log P values.

1. Introduction

2-Chloro-2′-deoxyadeonosine (CdA; Cladribine) (Fig. 1) is a purine analogue that has shown great therapeutic efficacy in phase I and II clinical trials in lymphoid malignancies [1–3]. Although well tolerated by patients after oral administration, the bioavailability of CdA is only approximately 50% [4]. Several factors are of primary importance for oral absorption, including drug aqueous solubility, resistance to loss from the absorption site due to enzymatic or chemical degradation and the partition coefficient of the drug.

The pH partition hypothesis for gastrointestinal drug absorption assumes that only the non-ionized form of the drug passes through a biological membrane which is regarded as lipoidal in nature [5]. The more lipophilic character the drug possesses, the more easily it passes through the membranes and enters the blood circulation. Knowledge of the partition coefficient (P) of CdA is therefore important when predicting the drug absorption through the wall of gastrointestinal mucosa. Moreover, the determination of lipophilicity may provide useful information about the potential of the drug for penetration of the blood–brain barrier and thus drug delivery to the CNS.

The n-octanol–water system was chosen by Hansch and Dunn [6] as the standard for the determination of P, as several properties of n-

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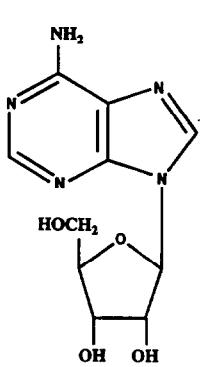
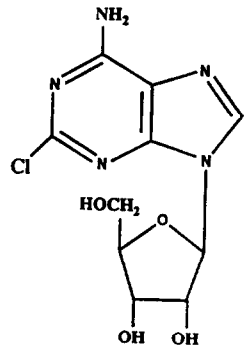
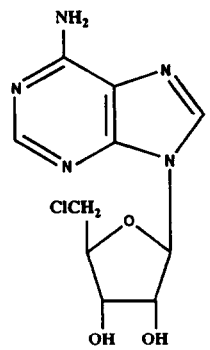


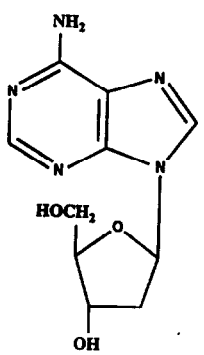
Ado



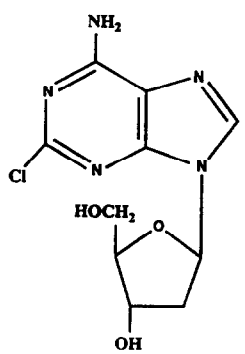
2-Cl-Ado



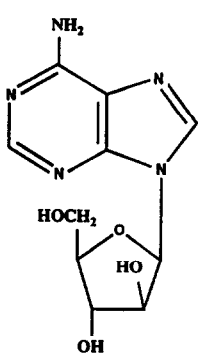
5′-Cl-5′-dAdo



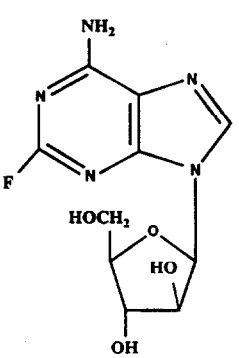
dAdo



CdA



ara A



F-ara A

Fig. 1. Structural formulae of the compounds studied.

octanol allowed it to serve as a model for the hydrophobic and hydrogen-bonding effects that might be encountered in biological membranes. The shake-flask method for the determination of P is conventional, but laborious and time consuming.

The isotropic nature of octanol [7] is in contrast to the strongly anisotropic typical biomembrane (with phospholipids and cholesterol molecules forming a bilayer in which proteins and other lipids are incorporated). Therefore, there have been numerous proposals to use HPLC to determine the hydrophobicity of the drug [7–10]. A chemically bonded phase does not behave as a liquid and hence the retention in a reversed-phase LC system seems to resemble the dynamic partitioning in biological membranes more than the static liquid–liquid distribution in the *n*-octanol–water system.

The purpose of this paper is to report the apparent partition coefficients (*P* values determined by the shake-flask method) and ionization constants (pK_a values determined by UV spectrophotometry) of CdA and some structurally related nucleoside analogues including 2-fluoroarabinosyladenine (F-araA), 2-chloroadenosine (2 Cl-Ado) and 5′-chloro-5′-deoxyadenosine (5′-Cl-5′-dAdo) (Fig. 1) and to investigate the relationship between hydrophobicity parameters and retention in a reversed-phase LC system.

2. Experimental

2.1. Materials

The nucleosides Ado, dAdo, 2-Cl-Ado, araA and 5′-Cl-5′-dAdo and 1-octanol were obtained from Sigma (St. Louis, MO, USA). F-araA was a generous gift from Dr Ze've Shaked (Berlex, Alameda, CA, USA). CdA was synthesized by Dr. Zygmunt Kazimierczuk (Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland). Methanol was of HPLC grade (J.T. Baker, Deventer, Netherlands). Analytical-reagent grade potassium dihydrogenphosphate (KH_2PO_4), sodium dihydrogenphosphate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogenphosphate ($Na_2HPO_4 \cdot 2H_2O$), citric acid ($C_6H_8O_7 \cdot H_2O$), formic acid (HCOOH), acetic acid (CH_3COOH), orthophosphoric acid (H_3PO_4) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany) and potassium hydroxide (KOH) from Kebo (Spånga, Sweden).

2.2. Determination of partition coefficients (P)

The shake-flask method according to Cheung and Keney [11] was utilized for the determination of *P* values for CdA, 2-Cl-Ado, F-araA and 5′-Cl-5′-dAdo. Individual solutions for nucleosides with concentrations in the range 10^{-6}–10^{-5} *M* in 0.05 *M* KH_2PO_4 (pH 7) presaturated with 1-octanol were used for recording the UV spectra from 300 to 200 nm with a Hitachi U-2000 spectrophotometer. The absorbances at 260 nm were plotted against their concentrations and these linear plots were used for the calculation of the molar absorptivity (ε_{max}).

Volumes of 5 ml of freshly prepared buffer solutions were transferred into individual centrifuge tubes and 5 ml of 1-octanol presaturated with the buffer were added. The mixtures were shaken 100 times followed by centrifugation at 1000 *g* for 1 h. The partitioning between buffer and 1-octanol was performed at 22°C. The aqueous and the organic phases were separated and the UV spectra in the aqueous phases were recorded. The apparent *P* value of each nucleoside was determined according to the equation

$$P = \frac{(c)_{aq,0} - (c)_{aq,1}}{(c)_{aq,1}} \quad (1)$$

where $(c)_{aq,0}$ and $(c)_{aq,1}$ are the concentrations in the aqueous phases before and after partitioning, respectively. This calculation of the *P* values was used because only limited amounts of the compounds studied were available and the method using the approximation of *P* values from the decrease in the concentrations in the aqueous phase has been reported previously [12].

2.3. Determination of capacity factors (k′)

The capacity factors (k') were determined isocratically on a high-speed C_{18} (3-μm) column

(80 × 4.6 mm I.D.) (Perkin-Elmer, Norwalk, CT, USA) at 22°C using a Shimadzu (Kyoto, Japan) LC-9A pump, a CMA-240 autosampler (Carnegie Medicine, Stockholm, Sweden) and a Milton Roy (LDC Division, Riviera Beach, FL, USA) variable-wavelength detector at 265 nm. A Macintosh Classic computer (Apple, Chicago, IL, USA) equipped with Chromac 3.1. software (Drew, London, UK) was used for collecting the HPLC data. Aqueous mobile phases of 0.01 *M* KH_2PO_4 with various percentages of methanol and pH values at a flow-rate of 1 ml/min were used. The pH was adjusted with a few drops of either KOH or H_3PO_4 before methanol was added using a PHM 62 standard pH meter (Radiometer, Copenhagen, Denmark). A 20-pmol amount of each compound was injected.

The k' values were calculated as $(t_r - t_0)/t_0$, where t_r is the retention time of an individual compound and t_0 is the retention time of an unretained compound, which was determined as the time from injection to the first distortion of the baseline.

Linear regression analyses for the relationship between log k' or log k'_w and log P were performed on an IBM AT personal computer.

2.4. Determination of ionization constants (pK_a)

The ionization constants of all the compounds studied were determined by UV spectrophotometry according to Albert and Serjeant [13]. The method depends on the direct determination of three variables: the absorbances of the molecular species (neutral molecule), the totally ionized species and the partly ionized species.

Aqueous stock solutions of the compounds with concentrations of $2 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ *M* were prepared. Glass-distilled water free from carbon dioxide was used for the preparation of all solutions and buffers.

The stock solutions were diluted tenfold with appropriate non-absorbing buffers whose pH values were chosen so that the compounds to be measured were present either as molecular species (phosphate buffer, pH 7.22) or totally ionized species (0.1–2 *M* HCl). Spectra in the range 200–300 nm of both species were compared for each compound and a wavelength was chosen at which the greatest difference between the absorbances of the two species was observed. The analytical wavelength chosen was 280 nm for Ado, dAdo, araA, CdA, 2-ClAdo and 5'-Cl-5'-dAdo and 250 nm for F-araA. A_i (absorbance of the ionized species) and A_m (absorbance of the molecular species) were then measured.

To determine the absorbance of the partly ionized species, the stock solutions were diluted as before but with 0.01 *M* buffers of citrate–phosphate (pH 3–3.6), formate (pH 3.7) and acetate (pH 4.3) and with 0.1–0.5 *M* HCl. The pH of all buffers was measured at 22°C. The absorbance of these solutions, A, was determined and the pK_a value for each compound was calculated from the appropriate equation:

$$pK_a = pH + \log\left(\frac{A - A_m}{A_i - A}\right) \quad \text{when } A_i > A_m \quad (2)$$

$$pK_a = pH + \log\left(\frac{A_m - A}{A - A_i}\right) \quad \text{when } A_i < A_m \quad (3)$$

where A is the absorbance of a partly ionized substance measured in a buffer of a known pH.

3. Results and discussion

The ionization constants and the partition coefficients of CdA and related nucleoside analogues are given in Table 1.

When the pK_a values of Ado, dAdo and araA are compared with those of their analogues with a halogen atom (chlorine or fluorine) at the C-2 position of the purine ring, a tendency towards significantly lower values (by 2.5 pH units on average) is observed. In the case of CdA, substitution with a chlorine atom at the C-2 position of the purine ring decreases the basicity of the N-1 atom [14], which was confirmed by the finding of differences in protonation sites (the N-1 atom of dAdo and the N-7 atom of CdA) and differences in the pK_a values (1.4 for CdA compared with 3.8 for dAdo) [15]. This change renders the drug resistant to adenosine deaminase (ADA).

The pK_a value of CdA determined spectro-

Table 1
Partition coefficients and capacity factors of CdA and related nucleoside analogues

Compound	pK_a	$P \pm$ S.D.	Log P	k'	Log k'
Ado	3.47 (3.5)[a]	0.105 ± 0.003[a]	−0.979	25.52	1.41
araA	3.40	0.111 ± 0.004[a]	−0.955	19.01	1.28
F-araA	0.90	0.158 ± 0.015	−0.801	31.99	1.51
dAdo	3.79 (3.8)[b]	0.245 ± 0.002[a]	−0.611	35.24	1.55
2-ClAdo	0.79	0.429 ± 0.046	−0.368	87.90	1.94
5′-Cl-5′-dAdo	3.90	0.881 ± 0.051	−0.055	105.93	2.03
CdA	1.28 (1.4)[b]	1.059 ± 0.053	0.025	101.16	2.01

pK_a values were determined by UV spectrophotometry, P values by the shake-flask method (at four different concentrations of the compound), capacity factors by HPLC on a high-speed C_{18} (3 μm) column (80 × 4.6 mm I.D.) with a mobile phase of 0.01 M KH_2PO_4 with 3% MeOH (pH 7.0), flow-rate 1 ml/min and detection at 265 nm.
[a] Data from ref. 11.
[b] Data from ref. 15.

photometrically seems to be slightly lower than that in the literature (1.28 *vs.* 1.4). Although variations in pK_a values determined with different methods (such as UV spectrophotometry and potentiometry) have been reported [16], there may be other possible explanations. As the totally ionized species of the bases must be determined at not less than 2 pH units below pK_a, stronger acids, such as 1 M HCl, should be used for diluting the compounds with expected $pK_a < 2$. However, the acid-catalysed hydrolysis of the glycosidic bond of CdA [15], 2-Cl-Ado and to a lower extent also F-araA (data not shown) has been observed. In this way the accuracy of spectrophotometric determination of pK_a of such compounds may differ from that when other methods are used. Thus, the pK_a of 2-Cl-Ado and F-araA may be slightly higher than those we determined.

The position of the chlorine substituent is apparently of importance for the pK_a of the compound, as the pK_a of 5′-Cl-5′-dAdo, an adenosine analogue with a chlorine atom in the C-5 position of the sugar moiety, was found to differ from that of 2-Cl-Ado with a chlorine atom in the purine ring instead (3.90 for 5′-Cl-5′-dAdo *vs.* 0.79 for 2-Cl-Ado).

As regards the lipophilicity, these data clearly confirm that the introduction of a halogen atom into the molecule increases the lipophilic character of the compound [17,18]. The replacement of the hydrogen atom at the C-2 position of the purine ring in the molecules of Ado and dAdo with a chlorine atom increased the P values *ca.* fourfold (0.429 for 2-Cl-Ado *vs.* 0.105 for Ado, and 1.059 for CdA *vs.* 0.245 for dAdo). When the chlorine atom replaced the hydroxy group at the C-5 position of the sugar moiety, an eight-fold difference in partition coefficients of the halogenated and the parent compounds was found ($P = 0.881$ for 5′-Cl-5′-dAdo *vs.* 0.105 for Ado). In agreement with the literature [19], higher P values were observed for purine nucleosides and their halogenated analogues than their pyrimidine counterparts (Table 2).

As the traditional shake-flask method has a number of practical disadvantage [20], there has been considerable interest in the development and utilization of relationships between *n*-octanol–water partition coefficients (log P) and

Table 2
Partition coefficients of some purine and pyrimidine nucleosides and their halogenated analogues

Compound	P	Log P
Ado	0.105	−0.979
5′-Cl-5′-dAdo	0.881	−0.055
araC[a]	0.009	−2.050
5′-Cl-araC[a]	0.195	−0.710

[a] Data from ref. 25.

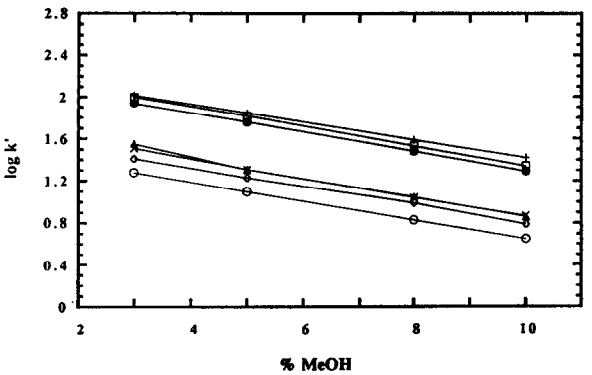


Fig. 2. Effect of the concentration of methanol in the mobile phase (pH 7) on the logarithm of the capacity factors ($\log k'$) of (○) araA, (◇) Ado, (▲) dAdo, (×) F-araA, (●) 2-ClAdo, (□) CdA and (+) 5'-Cl-5'-dAdo. Other conditions as in Experimental.

capacity factors ($\log k'$) in reversed-phase LC systems.

3.1. Retention in the reversed-phase LC system

We investigated the influence of methanol concentration and pH of the mobile phase on the retention of the compounds studied in reversed-phase LC. Good linearity between $\log k'$ for the compounds studied and the percentage of methanol in the mobile phase was observed at pH 7.0 (Fig. 2). However, the elution order of araA, dAdo and 5'-Cl-5'-dAdo did not follow the retention order predicted by P values (Table 1).

When mobile phases with various contents of methanol and pH were used, araA was always

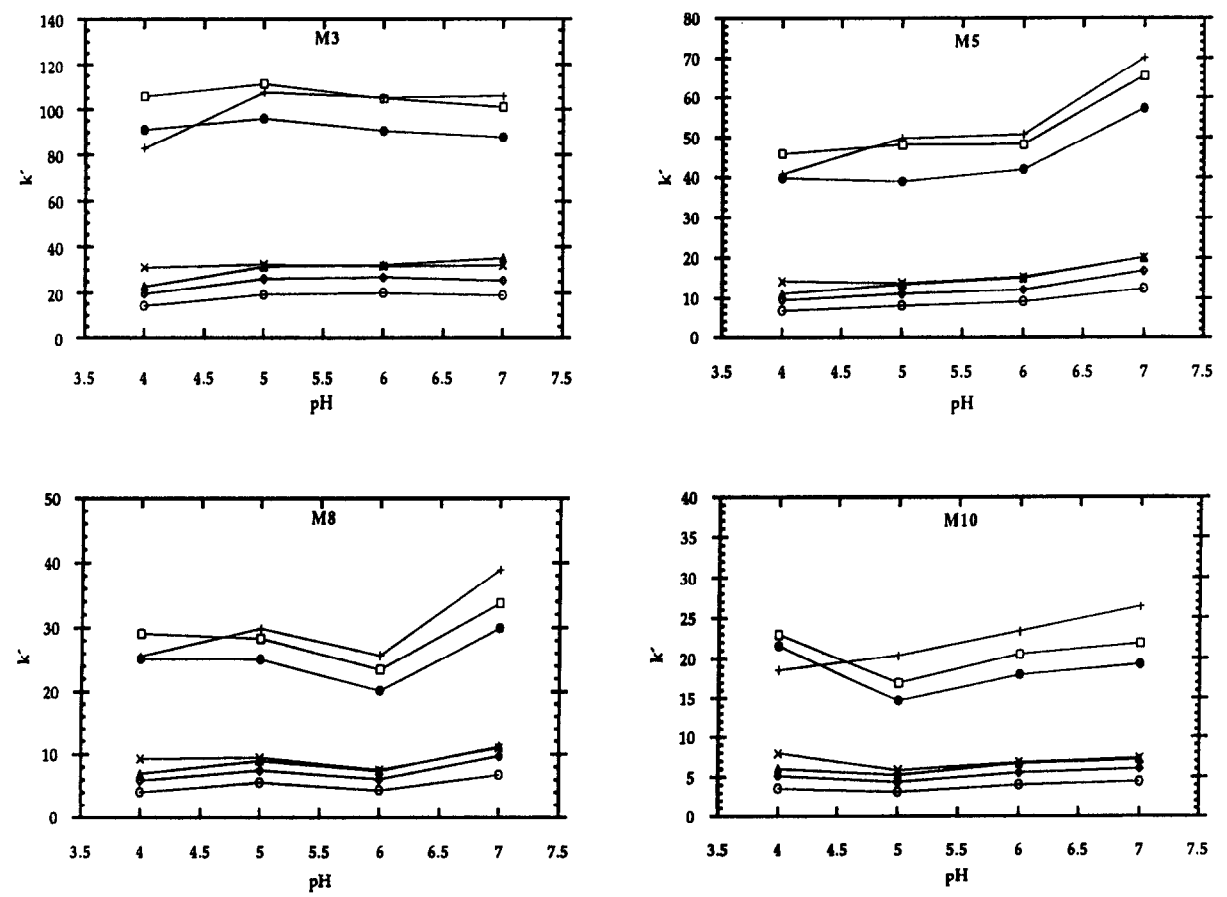


Fig. 3. Effect of pH of the mobile phase on capacity factors (k') of (○) araA, (◇) Ado, (▲) dAdo, (×) F-araA, (●) 2-ClAdo, (□) CdA and (+) 5'-Cl-5'-dAdo in the mobile phase with 3% (M3), 5% (M5), 8% (M8) and 10% (M10) of methanol.

eluted before Ado (Fig. 3). The same elution order in the reversed-phase system was observed by Cheung and Keney [11], despite the fact that the P value determined by the shake-flask method for araA was slightly higher than that of Ado (0.110 for araA *vs.* 0.105 for Ado). The calculated P value was lower (0.09) [11] but it was the same for both araA and Ado. In general, fairly large differences have been observed among partition coefficients determined by the shake-flask method by various workers [21,22]. Thus, a knowledge of the retention characteristics in a reversed-phase system is of great importance when the hydrophobicity is evaluated.

Despite its higher hydrophobicity compared with F-araA, dAdo ($P = 0.245$) was eluted before F-araA ($P = 0.158$) when the pH of the mobile phase was in the range of 4–5. This can be explained by the ionization of dAdo (pK_a = 3.8) with pH values in the range of $pK_a \pm 1$ or $pK_a \pm 2$ as dAdo was eluted after F-araA (pK_a = 0.90) when the pH of the mobile phases was 7.0.

In the mobile phases with pH > 5 and with more than 3% of methanol, 5′-Cl-5′-dAdo was retained longer than expected from its partition coefficient. Its elution order changed only when the pH of the mobile phase was changed from 4 to 5, seemingly owing to the ionization as the pK_a determined spectrophotometrically was 3.90.

Optimum separation of all compounds was accomplished with a mobile phase containing 8% of methanol at pH 7.0 (Fig. 4).

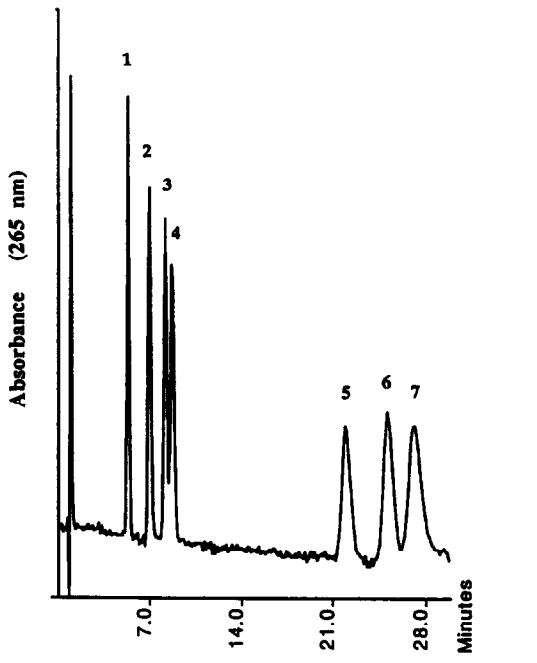


Fig. 4. Reversed-phase chromatographic separation of (1) araA, (2) Ado, (3) F-araA, (4) dAdo, (5) 2-ClAdo, (6) CdA and (7) 5′-Cl-5′-dAdo. Column, high-speed C_{18} (3 μm) (80 × 4.6 mm I.D.); mobile phase, 0.01 M KH_2PO_4 (pH 7.0) with 8% of methanol; flow-rate, 1 ml/min; detection 265 nm, 0.01 aufs; 20 pmol of each nucleoside injected.

3.2. *Correlation between partition coefficients and capacity factors*

In order to investigate whether the hydrophobicity of the compounds studied could be predicted by HPLC, the log k' values determined with a mobile phase containing 3% of methanol were plotted against log P values determined by the shake-flask method (Table 1, Fig. 5).

Using this monocratic approach, with a single mobile phase, a fair linear correlation was obtained as shown by the equation

$$\log k' = 2.06(\pm 0.062) + 0.72(\pm 0.094) \log P \quad (4)$$

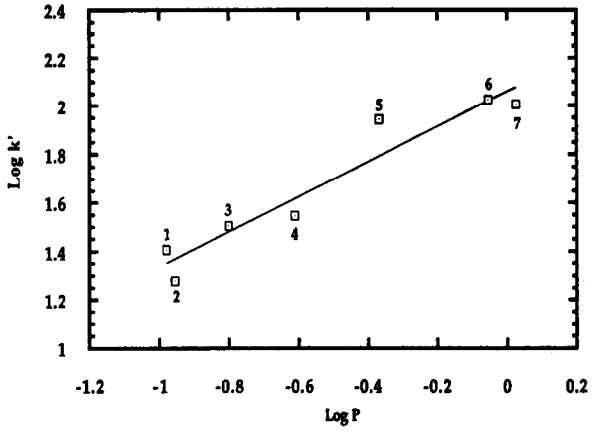


Fig. 5. Linear plot of log k' *vs.* log P of (1) Ado, (2) araA, (3) F-araA, (4) dAdo, (5) 2-ClAdo, (6) 5′-Cl-5′-dAdo and (7) CdA in a mobile phase of 0.01 M KH_2PO_4 (pH 7.0) with 3% of methanol. Other conditions as in Fig. 4.

$n = 7$, $r = 0.960$, S.D. = 0.095

where n is the number of compounds studied, r is the correlation coefficient and S.D. is the standard deviation of the equation.

However, strong evidence has been provided by several studies that extrapolated $\log k'_w$ values are more closely related to $\log P$ than the isocratic capacity factors [7,23,24], so $\log k'_w$ was proposed to describe the hydrophobicity of the solute directly.

When the polycratic approach was applied (using several mobile phases containing different percentages of methanol), both quadratic

$$\log k' = \log k'_w(\text{quadr}) + A\varphi^2 - S(\text{quadr})\varphi \quad (5)$$

and linear

$$\log k' = \log k'_w(\text{lin}) - S(\text{lin})\varphi \quad (6)$$

equations, where A and S(quadr, lin) are constants for a given solute–solvent combination and φ is the volume fraction of the organic modifier, were used to describe the relationship between solute retention and the composition of the binary mobile phase. The resulting data from regression analyses given in Table 3 clearly demonstrate a very good linear correlation with $r > 0.997$, despite the fact that the methanol content, φ, was in the range 0.03–0.10.

When $\log k'_w$ and $\log P$ were correlated, as good a linear correlation as that obtained using the monocratic approach was obtained, as represented by the equations

$$\log k'_w(\text{quadr}) = 2.35(\pm 0.057) + 0.74(\pm 0.087)\log P \quad (7)$$

$n = 7$, $r = 0.967$, S.D. = 0.088

$$\log k'_w(\text{linear}) = 2.33(\pm 0.063) + 0.73(\pm 0.095)\log P \quad (8)$$

$n = 7$, $r = 0.959$, S.D. = 0.096

Recently, several investigators have studied the relationship between lipophilicity and retention in a reversed-phase system for different classes of nucleosides and nucleoside analogues. Good linear correlations between log P values in n-octanol–water and retention times [17], $\log k'$ using the monocratic approach [11,25] or extrapolated $\log k'_w$ determined by the polycratic approach [26] were reported.

This work shows that hydrophobicity parameters of nucleoside analogues can be calculated from their $\log k'$ or $\log k'_w$ values. As many nucleoside analogues are used as drugs in the treatment of cancer and AIDS, the importance of physico-chemical properties including log P and pK_a should be emphasized, not only for retention in reversed-phase systems, but mostly for drug absorption and biological activity in general.

Based on the similarity of CdA lipophilicity

Table 3
Calculation of $\log k'_w$ values from the relationship between the percentage of methanol, φ, and $\log k'$ for nucleoside analogues

Compound	Regression[a]			
	Quadratic $\log k'_w$	r	Linear $\log k'_w$	r
Ado	1.63	0.999	1.67	0.9989
araA	1.55	1.000	1.55	1.000
F-araA	1.82	1.000	1.77	0.999
dAdo	1.89	0.998	1.82	0.997
2-ClAdo	2.22	1.000	2.23	1.000
5′-Cl-5′-dAdo	2.29	1.000	2.28	1.000
CDA	2.29	1.000	2.29	1.000

[a] Regression analyses were performed using the quadratic Eq. 5 and the linear Eq. 6 for the extrapolation of retention to $\varphi = 0$ (100% water); r = regression correlation coefficient.

(expressed as log P) with that of another clinically used drug, azidothymidine (AZT) (0.025 for CdA *vs.* 0.038 [11] or 0.05 [19] for AZT), which is known to enter the CNS, one would expect CdA to be able to penetrate the blood–brain barrier. This is indeed the case, as has recently been shown [3,27].

4. Acknowledgement

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