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Inosine and 2′-deoxyinosine and their synthetic analogues: lipophilicity in the relation to their retention in reversed-phase liquid chromatography and the stability characteristics

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Abstract

The purines and among them inosine synthetic nucleoside derivatives and analogues belong to a group of compounds to which the attention is being paid because of their biological activities. Relationships of their various parameters are being investigated because of their effect on biological (antineoplastic, virostatic, immunosuppressive) properties. Hydrophobicity parameters expressed as the logarithm of the partition coefficient (log P) and the capacity factor k' for naturally occurring inosine, 2′-deoxyinosine, 2′-deoxyadenosine and 2′-deoxyguanosine and for inosine synthetic analogues 5′-deoxyinosine, 5′-chloro-5′-deoxyinosine and 2′,3′-dideoxyinosine were measured. The effect of methanol percentage in the mobile phase and its pH on the retention of the studied compounds in a reversed-phase system was also examined. There was a good correlation between the lipophilicity expressed as log P and capacity factor k'. It was also determined that dissociation has a marginal effect on capacity factor k' in this group of nucleoside derivatives as the k' values were almost unchanged at various pH of the mobile phase used. The stability of the all investigated compounds was investigated in basic, neutral and acidic conditions. The values of the reaction constant k_1 were calculated and effects of nucleoside structural characteristic on stability are discussed.

Keywords: Inosine; Inosine derivatives; Reversed-phase HPLC; Lipophilicity; Stability

1. Introduction

Nucleic acids as the basis of genetic information represent an important part of the living matter in which, along with other macromolecular components — mainly proteins — enable the run of basic processes vital for life and reproduction.

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One of the components of nucleic acids' building blocks is inosine and its 2′-deoxyanalogue-2′-deoxyinosine [1]. The role of nucleic acids and the study of regulation of their functions on a molecular level stimulated the development of nucleoside chemistry. A large number of synthetic nucleoside analogs have been prepared [1] whose potential antitumor, antiviral, and immunosuppressive activities have been studied. Inosine derivatives drew attention because of their antiviral activity and because nucleoside analogs of inosine play an important role in various biochemical pathways of living cells and organisms. Thus, compounds with potentially different antiviral, antitumor and carcinogenic properties can arise in the organism through its metabolism. Therefore, the study of these compounds is important not only from a theoretical, but also from a practical point of view, since e.g. didanosine (2′,3′-deoxyinosine, ddI) belongs among the few promising compounds in anti-HIV therapy [2,3].

The therapeutic drugs' problem exists in the small bioavailability of potentially useful substances [4]. The drugs' aqueous solubility and the partition coefficient of these compounds seem to belong among factors of primary importance for their biological activity observed.

The pH partition hypothesis for gastrointestinal drug absorption assumes that only the non-ionized form of the drug passes through a biological membrane that is regarded as lipoidal in nature [5]. The more lipophilic character a drug possesses, the more easily it passes through the membranes and enters the blood circulation and intra-cellular compartments [5]. Measurement of the partition coefficient (P) of new synthetic nucleoside analogues is therefore important when predicting their absorption through the wall of gastrointestinal mucosa and various cellular membranes. The revered-phase liquid chromatography seems to be a suitable method for the investigation of new potential drugs as the 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 [6,7]. The purpose of this work is to measure the apparent partition coefficients of inosine, 2′-deoxyinosine and their structurally related synthetic analogues 5′-deoxyinosine, 5′-chloro-5′-deoxyinosine and 2′,3′-dideoxyinosine, to asses an effect of ionization on hydrophobicity/retention in a HPLC system and to investigate the relationship between lipophilicity and retention in a reversed-phase LC system as this nucleoside series consist of substances with gradually increasing lipophilicity. 2′-Deoxyadenosine and 2′-deoxyguanosine are also included among the compounds investigated to obtain a broader understanding of lipophilicity relationships among naturally occurring purine nucleosides. Additionally, The goal of this work consisted of obtaining data regarding the nucleosides' stability at various pH and elucidating the effect of structural modifications on this parameter (reaction constant k_1).

2. Material and methods

2.1. Chemicals and reagents

The nucleosides inosine, 2′-deoxyinosine, 2′-deoxyadenosine, 2′-deoxyguanosine were obtained from Pharma-Waldhof (Manheim, Germany), 2′,3′-dideoxyinosine and 1-octanol were products of Sigma (St. Louis, MO, USA). 5′-Deoxyinosine and 5′-chloro-5′-deoxyinosine were synthesized according to the published procedure [8]. Formulas of the all nucleosides investigated are shown at Fig. 1. Methanol of the HPLC grade was obtained from Fischer Scientific UK Ltd. (Longhborough, UK). Analytical reagent grade potassium dihydrogenphosphate and potassium hydroxide were purchased from Fluka Chemie AG, Buchs, Switzerland. Orthophosphoric acid and hydrochloric acid were purchased from Merck, Darmstadt, Germany.

2.2. Determination of partition coefficients (P)

The shake-flask method according to Cheung and Keney [9] was utilized for determination of P values of the all nucleoside included into this investigation. Individual solutions of nucleosides

(10^{-6} M in 0.05 M KH_2PO_4, pH 7, presaturated with 1-octanol) were prepared and their UV spectra were recorded at 200 to 300 nm with a Hitachi U-2000 spectrophotometer (Hitachi, Japan).

Volumes of 5 ml of freshly prepared buffer solution of the particular nucleoside 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

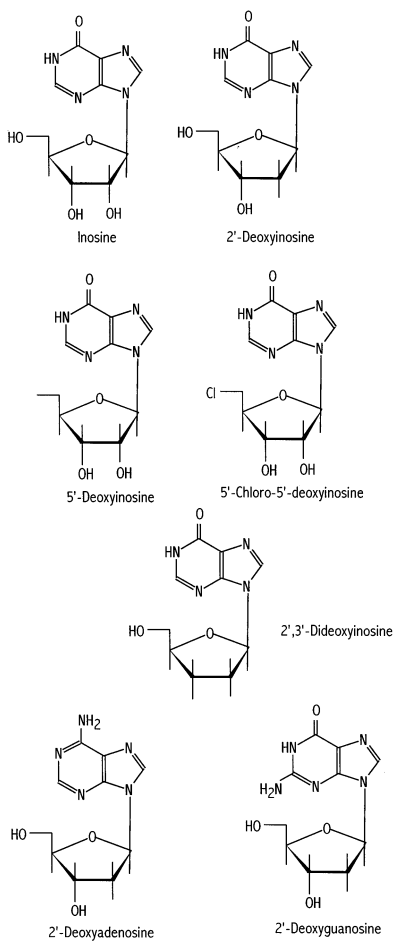

Fig. 1. Chemical structures of the nucleoside investigated.

$$P = (c)_{aq,0} - (c)_{aq,1}/(c)_{aq,1},$$

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. Additionally, this method was used previously for similar compounds [10] and the use of the approximation of P values from the decrease in the concentrations in the aqueous phase has been reported in literature at other occasions [11].

2.3. Determination of capacity factors (k')

The capacity factors (k') were determined isocratically using a base deactivated silica column Hypersil 4.6 × 150 mm (5 μ Hypersil® BDS C18, United Kingdom) at 22°C. The experiments were performed using HPLC system of Waters 2690 Separation Module complemented with Waters 996 Photodiode Array Detector with build-in Millenium software (Waters, Milford, USA). Aqueous mobile phases of 0.01 M KH_2PO_4 with various percentages of methanol and pH values at a flow-rate of 1.0 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 240 pH/ion-meter (Radiometer, Copenhagen, Denmark). 20-pmol amounts of each compound were injected.

The k' values were calculated as $(t_r - t_o)/t_o$, where t_r is the retention time of an individual compound and t_o is the retention time of an unretained compound (methanol), 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 $\log P$ are being performed on a Compaq Deskpro personal computer.

2.4. Stability studies

Separate solutions of the particular nucleoside (1 mg/ml) in 0.1 N HCl, NaCl and NaOH were freshly prepared and kept at the thermostatically controlled temperature 20°C. Samples of these

Table 1
The capacity factors of the nucleosides investigated using the mobile phase with various methanol contents. (The effect of pH was marginal as discussed in the Section 3.1).

Nucleoside	k' in phosphate buffer — MeOH (pH 7)			
	0% MeOH	3% MeOH	5% MeOH	8% MeOH
I	9.37	3.75	2.23	1.12
2′dI	12.17	4.91	2.95	1.46
5′dI	22.16	9.98	6.28	3.43
5′Cl I	24.01	20.42	12.79	6.05
2′,3′ddI	20.07	16.97	9.61	4.79
2′dA	21.99	18.35	10.96	5.73
2′dG	14.25	5.81	3.52	1.83

solutions were analyzed by the reversed-phase HPLC method at appropriate time. The concentration of the remaining nucleoside was determined from a linear regression equation relating the peak area of the nucleoside to concentration.

The first-order rate constants k_1 were calculated according to the following formula

$$k_1 = 2.3\log(([A])/([A_t]))/t,$$

where $[A]$ is the initial nucleoside concentration and $[A_t]$ is the nucleoside concentration at the time t.

The half-life time $t_{1/2}$ of the particular nucleoside first-order reaction was calculated as $t_{1/2} = 0.69/k_1$

3. Results and discussion

The partition coefficients and the capacity factors k' of the all nucleosides investigated are shown in Tables 1 and 2. 2′-Deoxyadenosine served as the reference compound for us as its log P determined (-0.62) is in a very good agreement with the log P value (-0.611) published by other authors [10]. The comparison of the log P values indicates the lower lipophilicity of inosine derivatives compared to adenosine and guanosine nucleosides. Comparison of the log P values of 2′-deoxy nucleosides, 2′dI (-1.33), 2′dG (-1.23) and 2′dA (-0.62) shows similar lipophilicity of inosines and guanosines (Table 2). There is the indication that lipophilicity of adenine nucleosides is lower. This is probably because of the absence of a carbonyl (=O) substitution at the purine system (Fig. 1) that is then less easily hydrated. The removal of hydroxyls (–OH) from inosine nucleoside molecule increased lipophilicity. Thus inosine is less lipophilic than 2′-deoxyinosine which is less lipophilic than 2′,3′-dideoxyinosine (log P values are -1.4, -1.33, -0.98). However, the more profound effect on the nucleoside lipophilicity is obtained by the removal of the

Table 2
The $-\log P$–$\log k'$ (pH 7, 8% methanol) relationships in the group of nucleoside investigated (the linear regressions)

	Nucleoside		log P	log k'		a	b	c	d	e
1	Inosine	I	−1.4	0.56			*	*	*	*
2	2′-Deoxyinosine	2′dI	−1.33	0.59		*	*	*	*	*
3	5′-Deoxyinosine	5′dI	−0.87	0.73			*	*		*
4	5′-Chloro-5′-deoxyinosine	5′Cl I	−0.73	1.1			*		*	*
5	2′,3′-Dideoxyinosine	2′,3′ddI	−0.98	0.92			*		*	*
6	2′-Deoxyadenosine	2′dA	−0.62	1.01		*				*
7	2′-Deoxyguanosine	2′dG	−1.23	0.64		*				*
					n	3	5	3	4	7
					A	1.3793	1.4517	1.3867	1.5208	1.4964
					B	−0.5968	−0.6447	−0.5983	−0.6931	−0.6753
					r	0.9998	0.9136	0.8958	0.9233	0.9171

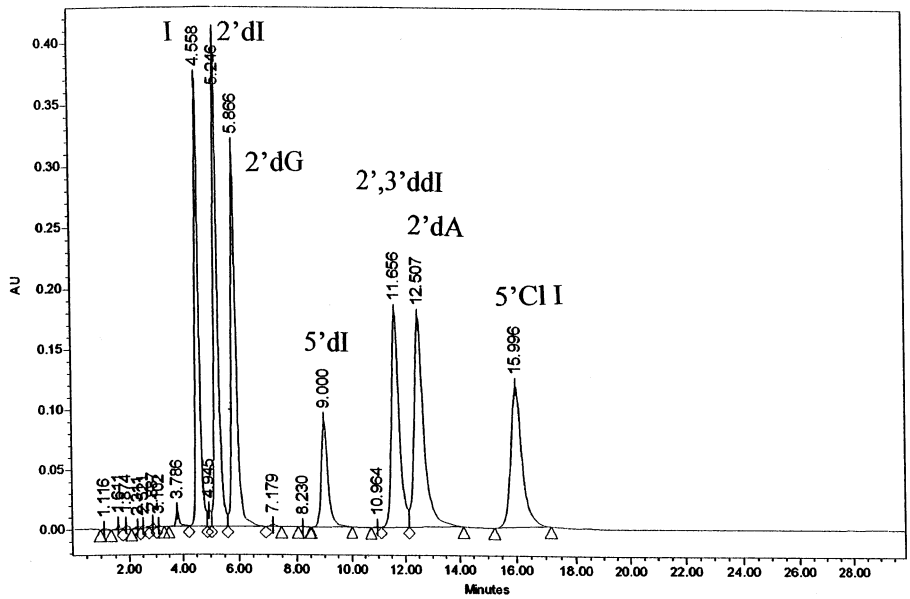

Fig. 2. Reversed-phase chromatographic separation of nucleosides investigated. Column, base deactivated silica column Hypersil 4.6 × 150 mm (5 μ Hypersil® BDS C18; mobile phase, 0.01 M KH_2PO_4 (pH 7.0) containing 8% of methanol; flow-rate 1 ml/min; detection 255 nm, 20 pmol of each nucleoside injected.

hydroxyl at the position 5′, as its interactions with the purine system are stronger than in the case of the hydroxyl at the position 2′. The lipophilicity of 5′dI is almost three times higher than that of 2′dI (P of 5′dI = 0.135; P of 2′dI = 0.0468). The lipophilicity of 5′dI is even higher than the log P value of 2′,3′ddI. 5′-Chloro-5′-deoxyinosine, 5′Cl I was the most lipophilic among the inosine nucleosides included in this series ($P = 0.186$, $\log P = -0.73$). However, this is still lower lipophilicity than the one determined for 2′dA ($P = 0.240$, $\log P = -0.62$). These data are clearly in an agreement with other published results [10,12–15] indicating that the introduction of halogen atom into the molecule increases the lipophilic character of the compound.

As the traditional shake-flask method has a number of practical disadvantages [16], there has been significant interest in the development and utilization of relationships between n-octanol-water partition coefficients (log P) and capacity factors (log k') in reversed-phase liquid chromatography systems [10,17,18].

3.1. Retention in the reversed-phase liquid chromatography system

The elution order of investigated compounds followed the retention order predicted based on log P values for I, 2′dI and 2′dG. However, some changes of order were observed for the more lipophilic compounds as 5′dI switched place with 2′,3′ddI and 5′Cl I switched place with 2′dA (Tables 1 and 2, Fig. 2).

The similar observations were made when mobile phases with various contents of methanol and pH were used. It was noted that the concentration of methanol significantly influenced the elution characteristics. The k' values changed 10 times for the least hydrophobic substance, inosine, when the methanol content increased from 0% to 8%. They changed more than six times for the most hydrophobic substance, 5′Cl I. The change of the k' values was linear in the range of the methanol content from 3% to 8% while it was more substantial when the change from 3% to 0% of methanol in a mobile phase took place (Table 1).

The changes in the capacity factor values at various methanol concentrations in the mobile phase used for all the compounds investigated are shown at Table 1.

The pH of mobile phases had no effect on retention of substances investigated at the column thus reflecting the marginal level of ionization of these nucleosides at different pH. This is not surprising when the pK_a value of inosine (pK_a 1.2) and guanosine and adenosine (pK_a 1.6 and 3.45, respectively) are compared. The data available in scientific literature [10] reporting on the effect of the mobile phase pH values indicate already limited effect of pH in reversed-phase chromatography system for various adenosine nucleosides. The absence of the amino ($-NH_2$) group in the structure of inosine nucleosides when compared to adenosine (and guanosine) nucleosides is obviously responsible for the change in pK_a values and also it affects the behavior of the particular nucleoside at various pH values.

The optimum separation of all compounds was accomplished with a mobile phase containing 8% of methanol at pH 7 (Fig. 2) mainly because the lower values of k' of particular compounds.

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 8% of methanol at pH 7 were plotted against $\log P$ values determined by the shake-flask method (Table 2, Fig. 3).

Using this monocratic approach, with a single mobile phase, a fair linear correlation was obtained. The results of these linear regression analyses are presented in Table 2. The correlation characteristics were calculated for various groups of nucleosides included in this study in order to show that capacity factors of various nucleosides are affected not only by lipophilicity but also by other factors, such as the nucleoside molecule shape and the region of chemical modification. Basically, the correlation coefficient r values were above 0.9 for various groups of the nucleosides investigate, including the group containing all the nucleosides investigates.

Regarding the obtained correlation, it is very interesting to note that in the inosine group, the

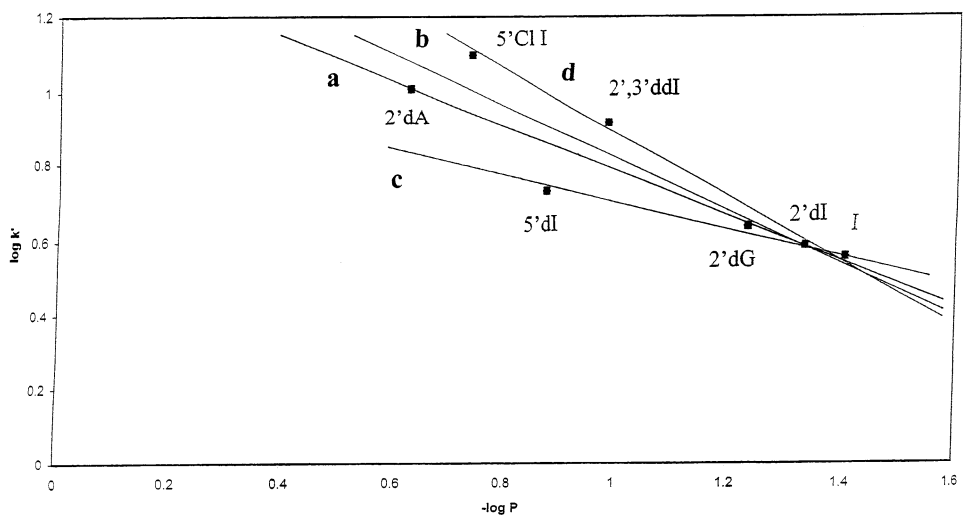

Fig. 3. Linear plot of $\log k'$ vs $\log P$ of the nucleosides investigated in a mobile phase of 0.01 M KH_2PO_4 (pH 7.0) containing 8% of methanol. (The regression analysis groups a–d as in Table 2).

Table 3
Stability of the investigated nucleosides at various conditions

	Nucleoside	k_1 (0.1 N HCl) [1/s]	$t_{1/2}$ (0.1 N HCl/20°C)/ $t_{1/2}$ (0.1 N NaOH/20°C) [hours]	k_1 (0.1 N NaCl)	k_1 (0.1 N NaOH) [1/s]
1	I	Stable	N/A	Stable	Stable
2	2′dI	$(1.85 \pm 0.04)\cdot 10^{-4}$/17	1.04	Stable	Stable
3	5′dI	Stable	N/A	Stable	Stable
4	5′Cl I	Stable	N/A	Stable	Stable
5	2′,3′ddI	$(7.12 \pm 1.82)\cdot 10^{-3}$/2	0.027/28	Stable	$(6.85 \pm 1.60)\cdot 10^{-6}$/4
6	2′dA	$(1.50 \pm 0.11)\cdot 10^{-4}$/8	1.28	Stable	Stable
7	2′dG	$(7.29 \pm 0.83)\cdot 10^{-5}$/6	2.63	Stable	Stable

removal of one or two hydroxyls does not diminish the lipophilicity-capacity factor correlation obtained under used experimental conditions. Also, the substitution of the hydroxyl at the position 5′ of the sugar moiety does not interfere with the above-mentioned correlation. However, the removal of the substituent from the position 5′, resulting in the formation of 5′-deoxyinosine, makes the obtained compound to deviate from the $\log P$–$\log k'$ relationship. This is probably due to the fact that substituent present at the position 5′ of the sugar moiety (–OH or –Cl in our study) with some degree of electronegativity interacts with the purine base. It influences the position of the base in the relation to the sugar moiety. When an electronegative substituent is missing, the purine base is free to occupy an energetically more advantageous position, thus changing the overall form of the nucleoside molecule (see Fig. 1). This was confirmed by the addition of 2′-deoxyguanosine to the series and in some extends also by the addition of 2′-deoxyadenosine. 2′-Deoxyguanosine possesses the amino group in its structure, which is capable of limited interaction with the position 5′ of the sugar moiety. On the other hand, 2′-deoxyadenosine is lacking such group and possesses in its structure only the amino group at the purine system that is not capable of any direct interaction with the sugar moiety. This amino group can interact with a sugar moiety indirectly through aromatic system of a purine ring. This makes 2′-deoxyadenosine to deviate from the established relationship.

To summarize these results, it may be concluded that better correlation is obtained when the nucleoside molecule modifications are more uniform in the set analyzed and that the r value calculated depends on that uniformity.

3.3. Stability of investigated nucleosides at various pH

Stability of the nucleosides investigated was followed in solutions of a particular nucleoside in 0.2 N HCl, NaCl and NaOH. As shown in Table 3, all the nucleosides were stable in 0.1 N NaCl but some of them degraded in 0.1 N HCl. It is interesting to note that only nucleosides lacking a hydroxyl group at position 2′ (or 2′ and 3′) were not stable in 0.1 N HCl solution. Inosine, 5′dI and 5′Cl I were stable under our experimental conditions. The stability of 2′dI was similar to 2′dA while 2′dG was more stable, probably because the interaction of its amino group at position 2 (Fig. 1) interaction with the sugar moiety (as discussed earlier). The removal of both hydroxyl at positions 2′ and 3′ led to much lower stability of 2′,3′ddI. The stability of this compound is so low that this was the only nucleoside for which it was possible to record degradation in 0.1 N NaOH.

This data correspond with the known fact that purine dideoxy nucleosides possess a very low stability that is lower than the stability of pyrimidine dideoxy nucleosides. An acid environment further lowers their nucleoside bond stability [19,20]. This is important as 2′,3′-dideoxy purine

nucleosides have anti-HIV activity and their instability of these compounds in acidic conditions complicates their oral administration [21]. Obviously, the substitution of a purine base by an electronegative atom (i.e. fluorine) has a stabilizing effect on nucleoside bond due to purine base–nucleoside moiety interaction [22]. This also explains the high stability of nucleosides with fully hydroxylated sugar moiety when compared to nucleosides with a lower number of hydroxyls or other substituents at the sugar part.

In conclusion, it may be summarized shortly that this work shows that hydrophobicity parameters of nucleoside analogues (log P) are in relation to their log k' values. Their physico-chemical properties, such as stability, are directly related to the structural changes in nucleoside molecule. As many nucleoside antimetabolites are used as drugs in the treatment of cancer, AIDS and viral diseases, the importance of physico-chemical properties including log P and retention/capacity characteristics, should be emphasized, not only for analytical purposes, but mostly for the assessment of drug absorption and biological activity in general.

Acknowledgements

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