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Comparative investigation of the retention behaviour of nucleoside derivatives on alumina stationary phases in thin-layer chromatography and high-performance liquid chromatography

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ABSTRACT

The retention of 21 natural and synthetic deoxyuridine derivatives was determined by high-performance liquid chromatography (HPLC) using an alumina column with water–2-propanol or dichloroethane–methanol mobile phases in various volume ratios. The log k' (capacity factor) values from HPLC were compared with the R_M [log $(1/R_E - 1)$] values determined by thin-layer chromatography (TLC) on alumina layers using water–2-propanol or dichloroethane–methanol mobile phases. The highest correlation coefficient (0.93) between the log k' values and the R_M values was obtained when dichloroethane–methanol–acetic acid (50:50:0.01) and 100% water were used as mobile phases both in TLC and HPLC, respectively. It was found that the retention of the nucleoside derivatives did not depend linearly on the concentration of the stronger component in the mobile phase. The length of the alkyl chain of the substituent at position 5 of the deoxyuridine has a negligible impact on the retention. The presence of the double and triple bounds in the substituent, however, significantly influenced the retention properties of the nucleosides on alumina layers. Principal component analysis proved that the reversed phase (water–2-propanol) and adsorption eluents (dichloroethane–methanol) show different selectivity.

INTRODUCTION

Alumina seems to be a promising substitute for silica in high-performance liquid chromatography (HPLC) due to its higher stability at extreme pH values [1]. Successful separations of some aromatic compounds [2], heroin derivatives [3], proteins [4] and various drugs [5] were achieved on alumina columns. When alumina was combined with anion-exchange columns, it was found to be a highly selective stationary phase for the preconcentration of sulphate from complex matrices [6]. Experiments were carried out to evaluate the synthetic procedures for the chemical modification of alumina for HPLC [7] and to compare the octadecyl-bonded alumina and silica for reversed-phase HPLC [8].

Due to its high versatility and simplicity thin-layer chromatography (TLC) can be used as a rapid pilot method in the search for HPLC separation conditions [9].

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However, the correlation between the retention in a TLC and HPLC system is not always good enough for the adequate prediction of HPLC parameters [10].

Synthetic nucleotides have many biological effects [11]; they can incorporate into DNA [12], resulting in the modification of some enzymatic processes [13]. Their incorporation rate into DNA depended on their hydrophobic and steric properties determined by chromatographic methods and the inductive effect of the substituents at position 5 of deoxyuridines also played an important role [14].

As the triphosphate group of nucleotides highly dominates their chromatographic properties, the retention behavior of the corresponding nucleoside derivatives were investigated on alumina stationary phase by TLC and HPLC in order to reveal the most important physico-chemical properties of the compounds governing their retention and the predictive power of TLC technique for HPLC.

EXPERIMENTAL

Alumina support of particle size 5 μm was produced by the research group of Dr. L. Zsembery at the Research Institute of the Hungarian Alumina Trust (Budapest, Hungary). A 15 cm × 4 mm I.D. column was filled in our laboratory with a Shandon analytical HPLC packing pump (Shandon Southern Products, Runcorn, UK). The HPLC equipment consisted of a Liquopump Model 312 (Labor MIM, Budapest, Hungary), a Cecil CE-212 spectrophotometer (Cambridge, UK) used as a detector, a Valco injector (Houston, TX, USA) with 20-μl sample loop, a Waters 740 integrator (Milford, MA, USA) or a Radelkisz Type OH-814/1 recorder (Radelkisz, Budapest, Hungary). The determination of the theoretical plate number was carried out as previously described [15]. The dead volume of the column was determined by injecting a 10% $NaNO_3$ solution. The nucleoside derivatives, deoxyuridine (**1**), 5-methyl-(**2**), 5-ethyl-(**3**), 5-*n*-propyl- (**4**), 5-butyl-(**5**), 5-hexyl- (**6**), 5-heptyl- (**7**), 5-octyl- (**8**), 5-vinyl- (**9**), 5-*E*-pentenyl- (**10**), 5-*E*-hexenyl- (**11**), 5-*E*-heptenyl- (**12**), 5-*E*-octenyl- (**13**), 5-propynyl- (**14**), 5-butynyl- (**15**), 5-pentynyl- (**16**), 5-hexynyl- (**17**), 5-heptynyl- (**18**), 5-octynyl- (**19**), 5-isopropyl- (**20**) and 5-bromovinyldeoxyuridine (**21**) were synthesized by the research group of Dr. J. Sági and described elsewhere [16–19]. The other chemicals were of HPLC purity. The nucleoside derivatives were dissolved separately in the eluents to give a concentration of 50 μg/ml. The retention of the nucleoside derivatives was determined in various eluent mixtures as water–2-propanol (0, 40, 50, 60, 70, 80 and 90%, v/v of 2- propanol) and dichloroethane–methanol (50, 60, 70, 80 and 90%, v/v of methanol). The effect of trifluoroacetic acid (TFA) concentration on the retention of nucleoside derivatives was investigated at 0.050, 0.025 and 0.01% (v/v) concentrations. The flow-rate was 0.8 ml/min. The detection wavelength was 260 nm. Each determination was carried out in triplicate. The calculation of the theoretical plate number and the log k' (capacity factor) values of the nucleoside derivatives were carried out as previously described [20].

DC-Fertigplatten Aluminiumoxid 60 F_{254} (Merck, Darmstadt, Germany) were used for TLC without any pretreatment. The nucleoside derivatives listed above were dissolved separately in methanol to give a concentration of 5 mg/ml, and 2 μl of each solution were spotted onto the plates. Dichloroethane–methanol–acetic acid (50:50:1) and water–2-propanol (1:9 and 85:15, v/v) were applied as eluents. After development the plates were dried at 105°C, and the nucleoside spots were detected via ultraviolet.

Each determination was run in quadruplicate. The R_M [log $(1/R_F\text{-}1)$] values were separately calculated for each eluent and nucleoside.

It was supposed that the main distinguishing mark between adsorption and reversed-phase chromatography for both TLC and HPLC is the relative polarity of the mobile and stationary phases, *i.e.* the stationary phase has to be more polar than the mobile phase in adsorption chromatography. This suggests that the chemical bonding of apolar substituents to the polar support is not a prerequisite for the reversed-phase separation mode. The validity of the hypothesis mentioned above was proved to be true for unimpregnated cellulose [21]. The application of the aqueous eluent systems may help in the elucidation of the relative polar or non-polar character of alumina compared to water–2-propanol mixtures.

To find the similarities and dissimilarities among various chromatographic systems and to assess the predictive power of TLC for HPLC, principal component analysis (PCA) was applied on the retention data [22]. The results of the PCA can give us information about the clustering of the variables according to their relationship and reveals possible backgrond variables which may have concrete physicochemical meaning, and in this way the number of variables can be decreased. The HPLC [water, water–2-propanol (1:9), dichloroethane–methanol–TFA (1:9:0.05 and 1:9:0.01)] and TLC [dichloroethane–methanol–acetic acid (50:50:0.01), water–2-propanol (1:9 and 85:15)] systems were taken as the variables during PCA. The log k' and R_M values of the nucleosides in the corresponding eluent system were taken as observations. As deoxyuridine was retained on the column in water it was excluded from the calculations. The PCA was carried out on the correlation matrix. The sum of the variance explained was set to 99.9%. The two-dimensional non-linear map of the obtained principal component loadings and variables was also calculated [23]. The iteration was carried out to the point where the difference between the maximum errors of the last two iterations was lower than 10^{-8}. The eluent systems or nucleosides showing similar retention characteristics are near to each other on the maps, whereas the eluent or compounds having opposite or very different retention behavior are widely separated.

The linear regression analysis between the chromatographic retention data was carried out using the Drugidea (Chemichro, Budapest, Hungary) software system.

RESULTS AND DISCUSSION

The log k' and R_M values obtained for the deoxyuridine and its derivatives in two HPLC and three TLC systems are listed in Table I. The correlation coefficients between the retention data obtained in various chromatographic systems are listed in Table II in a correlation matrix. It was surprising that the highest correlation coefficient (0.93) was obtained when log k' values with 100% water and R_M values with dichloroethane–methanol–acetic acic (50:50:0.01) were compared. In order to reveal some important physico-chemical characteristics governing the retention of the compounds on alumina, the hydrophobicity, electronic and steric parameters of the substituents at 5 position were taken from the compilation of Hansch and Leo [24]. The actual values for deoxyuridine derivatives have already been published [12]. The correlation study revealed that neither the hydrophobicity nor the molar refractivity (bulkiness of the substituent) play on important role in the retention. The Swain–

TABLE I

LOG k' VALUES AND THE MEAN $100R_M$ VALUES AND INDUCTIVE ELECTRONIC PARAMETER ($\mathcal{F}$) [12] OF THE INVESTIGATED DERIVATIVES

(I) log k' values obtained with 100% water as mobile phase; (II) log k' values obtained with methanol–dichloroethane–trifluoroacetic acid (90:10:0.05); (III) $100R_M$ values obtained with dichloroethane–methanol–acetic acid (50:50:0.01); (IV) $100R_M$ values obtained with water–2-propanol (1:9); (V) $100R_M$ values obtained with water–2-propanol (85:15); ($\mathcal{F}$) inductive electronic parameter of the 5 substituent.

Substituent	I	I	III	IV	V	$\mathcal{F}$
H	Inf.	Inf.	30	−1	start	0.00
Methyl	0.244	0.847	108	45	−29	−0.04
Ethyl	0.129	0.524	81	18	−33	−0.05
Propyl	0.222	0.284	80	11	−36	−0.06
Butyl	0.148	0.225	62	2	−40	−0.06
Hexyl	0.157	0.195	53	−5	−37	−0.06
Heptyl	0.140	0.109	47	−9	−31	−0.06
Octyl	0.177	0.085	39	−11	−9	−0.06
Vinyl	0.272	0.530	89	31	−30	0.07
E-Pentenyl	0.238	0.028	64	3	−34	0.03
E-Hexenyl	0.195	0.303	56	3	−29	0.03
E-Heptenyl	0.230	0.100	59	3	−38	0.03
E-Octenyl	0.219	0.254	43	−2	−4	0.03
Propynyl	0.746	0.234	167	114	−15	0.15
Butynyl	0.722	0.029	149	94	−15	0.15
Pentynyl	0.676	−0.253	154	85	−16	0.15
Hexynyl	0.678	0.000	164	91	−17	0.15
Heptynyl	0.685	0.885	146	77	−17	0.15
Octynyl	0.681	1.038	144	78	−20	0.15
Iso-propyl	0.023	0.364	64	−5	−39	−0.05
Bromovinyl	0.0254	0.480	112	25	−27	–

Loupton type [24] electronic parameter ($\mathcal{F}$) describing the strength of the inductive effect of the substituents at the 5 position showed significant correlation to the retention data. The correlation coefficients are shown also in Table II. It means that only the electronic properties are involved in the retention, and no partition phenomena take place even when hydro–organic mobile phases are used.

Each derivative showed unusual retention behavior on the alumina column. Their log k' value decreased between 0 and 40% of 2-propanol concentration in

TABLE II

MATRIX OF THE CORRELATION COEFFICIENTS BETWEEN THE DATA LISTED IN TABLE I

	I	II	III	IV	V	$\mathcal{F}$
I	1.00	0.72	0.93	0.84	0.64	0.93
II	0.72	1.00	0.74	0.57	0.60	0.64
III	0.93	0.74	1.00	0.84	0.44	0.84
IV	0.84	0.57	0.84	1.00	0.46	0.75
V	0.64	0.60	0.44	0.46	1.00	0.64
$\mathcal{F}$	0.93	0.64	0.84	0.75	0.64	1.00

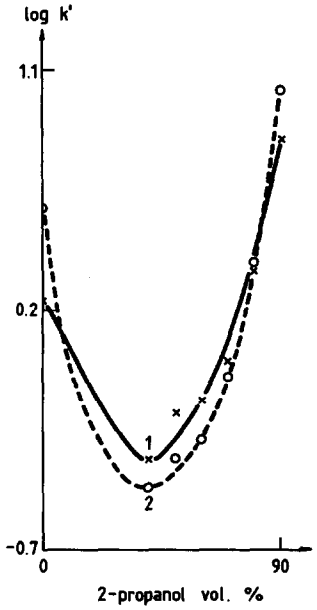


Fig. 1. Effect of 2-propanol concentration on the log k' value of 5-methyl- (1) and 5-octynyldeoxyuridine (2) in aqueous eluent systems.

aqueous systems according to the general rule of reversed-phase separation mode, reached a minimum and then increased again at higher 2-propanol concentrations (Fig. 1).

Opposite to our expectations, the nucleosides also showed anomalous retention behaviour in organic eluent systems (Fig. 2). The retention increased at higher methanol concentration, although methanol is a stronger eluent than dichloroethane. The

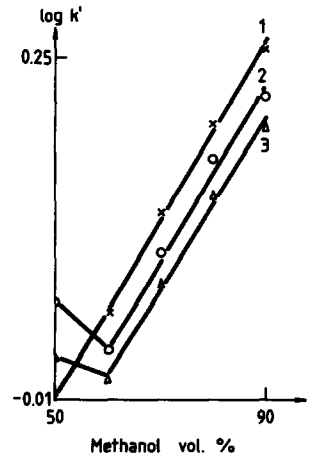
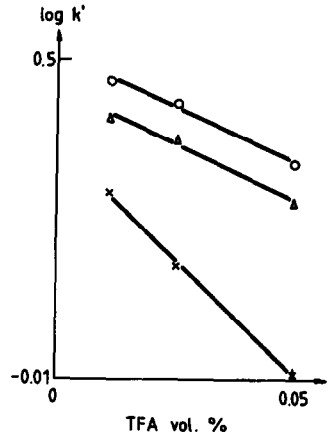


Fig. 2. Dependence of the log k' value of 5-ethyl- (1), 5-propyl- (2) and 5-butyldeoxyuridine (3) on the methanol concentration in the eluent (dichloroethane–methanol mixtures, containing 0.05%, v/v, TFA).



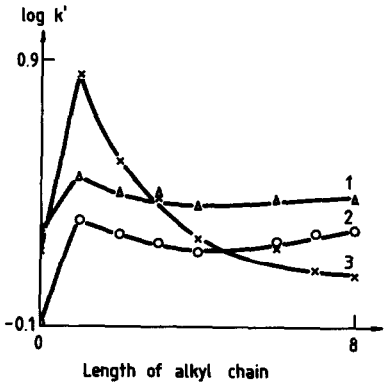


Fig. 3. Dependence of the log k' value of deoxyuridine (○), 5-methyl- (△) and 5-hexyldeoxyuridine (×) on the TFA concentration in the eluent (dichloroethane–methanol, 1:9, v/v).

Fig. 4. Dependence of the log k' values of some nucleosides on the length of saturated alkyl substituent. (1) Dichloroethane–methanol–TFA (1:9:0.01); (2) dichloroethane–methanol–TFA (1:9:0.05); (3) water–2-propanol (1:9).

retention decreased linearly with increasing concentration of TFA (Fig. 3) which proves the predominance of polar interactions between solute and support as outlined above. A strongly non-linear dependence of the retention on the length of alkyl substituents was observed (Fig. 4). The chromatographic behavior of deoxyuridine considerably deviates from that of the substituted ones. This is probably due to the fact that is has a higher capacity to form hydrogen bond with the alumina surface resulting in increased retention. The low dependency of retention on the length of

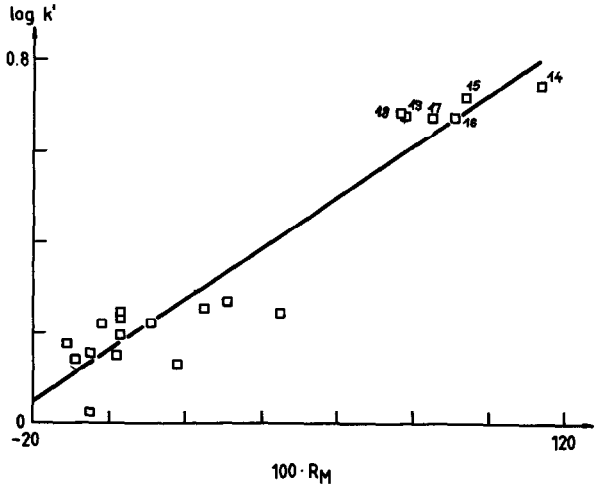


Fig. 5. Relationship between the log k' (eluent, water) and R_M (eluent, dichloroethane–methanol–acetic acid, 50:50:0.01) values of some nucleoside derivatives.

TABLE III

RELATIONSHIPS BETWEEN THE HPLC AND TLC RETENTION BEHAVIOR OF SOME NUCLEOSIDE DERIVATIVES AND RESULTS OF PRINCIPAL COMPONENT ANALYSIS

No. of principal component	Eigenvalue	Sum of variance explained (%)
1	3.69	56.69
2	1.03	71.43
3	1.01	85.89
4	0.61	94.64

Eluent	Principal component loading			
	No. of principal component			
	1	2	3	4
Water (HPLC)	0.96	0.03	−0.09	0.09
Water–2-propanol (1:9) (HPLC)	0.12	−0.31	0.93	−0.15
Dichloroethane–methanol–TFA (1:9:0.05) (HPLC)	0.83	0.22	0.09	−0.07
Dichloroethane–methanol–TFA (1:9:0.01) (HPLC)	−0.24	0.86	0.35	0.27
Dichloroethane–methanol–acetic acid (50:50:0.01) (TLC)	0.94	−0.18	0.02	0.27
Water–2-propanol (1:9) (TLC)	0.96	−0.03	0.01	0.23
Water–2-propanol (85:15) (TLC)	0.68	0.34	−0.08	−0.61

alkyl chain was a result of the fact that the alumina column under our experimental conditions is not suitable for the separation of these derivatives.

The correlations between HPLC and TLC systems were significant in some cases, as shown in Fig. 5, although the eluent systems were different. These relationships did not have any predictive value for the HPLC separation of the nucleosides.

The results of PCA are compiled in Table III. The first two components account for about 70% of the total variance. This means that two background variables include the majority of the information content of the seven eluent systems. It must be emphasized that the two hypothetical variables need not have any concrete physical meaning. The calculation only proves their mathematical possibility.

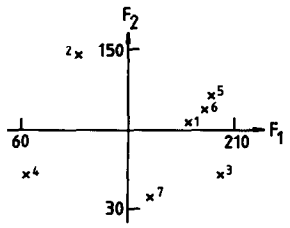


Fig. 6. Two-dimensional non-linear map of principal component loadings. Number of iterations: 97. Maximum error: $2.01 \cdot 10^{-2}$. (1) HPLC; eluent, water. (2) HPLC; eluent, water–2-propanol (1:9). (3) HPLC; eluent, dichloroethane–methanol–TFA (1:9:0.05). (4) HPLC; eluent, dichloroethane–methanol–TFA (1:9:0.01). (5) TLC; eluent, dichloroethane–methanol–acetic acid (50:50:0.01). (6) TLC; water–2-propanol (1:9). (7) TLC; water–2-propanol (85:15).

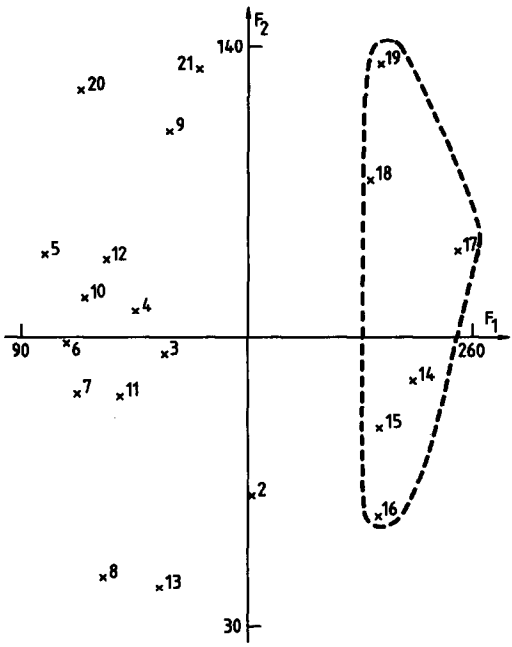


Fig. 7. Two-dimensional non-linear map of principal component variables. Number of iterations: 59 Maximum error: $3.16 \cdot 10^{-2}$. Number refer to nucleoside derivatives in Experimental. The compounds in the circle contained triple bond.

Neither the HPLC (points 1–4) nor the TLC systems (points 5–7) form separate clusters on the two-dimensional non-linear map of principal component loadings (Fig. 6). This results proves that the eluent composition has a higher impact on the retention characteristics then the type of chromatographic system. Each HPLC system is widely separated from the others, that is the separation capacity of the eluents is different. Points 1, 5 and 6 form a distinct cluster. However, this cluster contains very different chromatographic systems. The nucleoside derivatives having a triple bond in the alkyl chain form a loose cluster on the two-dimensional non-linear map of principal component variables (Fig. 7). It also proves that alumina stationary phase is sensitive to the π–π interactions, *i.e.* the inductive electronic properties with all of the investigated mobile phases. The length of the alkyl chain does not separate the compounds, *i.e.* the hydrophobic interactions are not important in the retention.

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