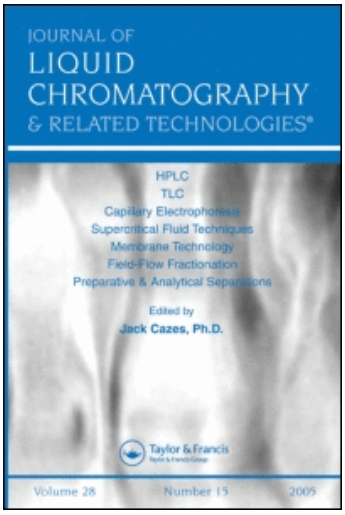

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Chromatographic Behavior of Deoxyribonucleosides with Respect to Organic Modifier Content in the Mobile Phase

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To cite this Article Kim, Jae-Duck , Row, Kyung Ho , So, Myoung Seup , Polunina, I. A. and Larin, A. V.(1995) 'Chromatographic Behavior of Deoxyribonucleosides with Respect to Organic Modifier Content in the Mobile Phase', Journal of Liquid Chromatography & Related Technologies, 18: 15, 3091 — 3104
To link to this Article: DOI: 10.1080/10826079508010435
URL: http://dx.doi.org/10.1080/10826079508010435

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(15), 3091-3104 (1995)

CHROMATOGRAPHIC BEHAVIOR OF DEOXYRIBONUCLEOSIDES WITH RESPECT TO ORGANIC MODIFIER CONTENT IN THE MOBILE PHASE

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ABSTRACT

A chromatographic behaviour of five deoxyribonucleosides (dCyd, dUrd, dGuo, dThd, and dAdo) has been studied under isocratic conditions of reversed-phase HPLC. The Waters column packed by 10 μm μ-Bondapak C18 was used. The volume fraction (F) of organic modifier was changed from 0.05 to 0.30, and to 0.12 for methanol and acetonitrile, respectively. Futhermore, the capacity factors with a mobile phase of 100 % water (k'_w) have been measured. Linear regression has been carried out for the log k′ vs. F data and the intercepts and the slopes of these plots were calculated.

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The slope magnitudes of different solutes were almost the same for each organic modifier. In all cases, the experimental values of k'_w were higher than extrapolated ones. For each solute, extrapolation of log k' vs. F gives practically the same intercept values for both organic modifiers. It was concluded that the intercepts calculated in the region of small content of organic modifier are independent on the nature of organic modifier and correlate with deoxyribonucleosides nature only.

INTRODUCTION

Deoxyribonucleosides are the important components of DNA, and they play an essential role in life-functions. Many of their derivatives have powerful physiological, antiviral and antitumor effects. In recent years the use of HPLC technique to analyse DNA-fragments has increased significantly. A number of publications describe the HPLC protocols to separate the mixtures of deoxynucleosides and other solutes of industrial, medical, and other interest [1-4].

Earlier the retention behaviour of deoxyuridine and its derivatives, and the dependence of the logarithm of capacity factor (log k') on the methanol concentration of the mobile phase were investigated under HPLC conditions elsewhere [5]. Correlations were studied between the retention data and physico-chemical parameters. The obtained multiple regression equations revealed that the hydrophobic (reversed-phase chromatographic retention) properties of the solutes have great influence on the DNA incorporation reaction. Previously the chromatographic selectivity of nucleosides and deoxynucleosides as a function of methanol concentration was reported by Gehrke et al. [6].

A review [1] underlines that the capacity factor with a mobile phase of 100 % water (k'_w) is the best measure of chromatographic retention and simultaneously a good predictor and descriptor of the solute hydrophobicity in biological systems. More often the

deoxynucleoside elutions were performed by using methanol and phosphate buffers as mobile phase components under gradient conditions [1-3]. This mode of chromatography can not give any sufficient information to estimate the values of k'_w. The rare data obtained under the isocratic mode of HPLC [2] do not allow us to extrapolate the value of log k'_w from the experimental results. So, the value of k'_w, which serves as a measure of the solute hydrophobic properties, is not defined accuracy for the deoxynucleosides.

The purpose of this work was to investigate the chromatographic behaviour of five deoxynucleosides on the mobile phase content of organic modifiers and to estimate more precisely the hydrophobic parameters k'_w of these substances.

THEORETICAL

The exact mechanism of solute retention in reversed-phase HPLC has been the subject of numerous studies [1, 5-8]. Ideally, knowledge of this mechanism would allow a priori prediction of solute retention and solute hydrophobic character in the case of reversed-phase HPLC. The difficulty in elucidating the mechanism of solute retention behaviour lies in the many interactions that a solute may undergo in both the stationary and mobile phases. For a given reversed-phase adsorbing material, the change in the mobile phase composition plays a primary role in the retention of compounds. The general approach assumes that retention under reversed-phase HPLC conditions obeys the linear relationship [7]:

$$\log k' = \log k'_w - S \times F \qquad (1)$$

where k′ refers to the solute capacity factor, k'_w is the value of k′ for water as mobile phase, F is the volume-fraction of organic modifier in the mobile phase, and S is a constant for a given solute and mobile-phase organic solvent.

The k'_w parameter is independent of any specific organic modifier effects, but it is dependent on the solute's structure and polar functionalities [8]. The k'_w value is difficult to measure directly. It is most often estimated as an intercept of the dependence (1) by the extrapolation of plot of log k′ vs. volume percent organic modifier.

Numerous papers have documented the validity of eqn.1, as well as modest deviations from this relationship [1, 5, 7-9]. While plots of log k′ vs. percent organic modifier often appear to be linear, they will always exhibit some curvature and are best fit by a quadratic equation [10]. Deviations from linearity were characterized with water-rich eluents, and good linear correlations were usually observed with mobile phases containing 30-50% of organic modifier. It was found in [5, 7-10] that the measured log k′ values of different solutes in the methanol/water and acetonitrile/water mixtures varied in a highly nonlinear manner vs. either percent or mole fraction of organic component. Johnson et al. showed in [9] that these curves can be linearized by plotting of log k′ values vs. a spectroscopic function of the mobile phase: the so-called ET(30) index. This approach and other ones were reviewed by Dorsey and Khaledi in [1].

Reversed phase slope and intercept values of eqn. (1) can be regarded as a measure of the hydrophobic character of the solutes. The intercept values are in the correlation to the partition coefficients of the compounds in the chromatographic stationary and mobile phase. The slope values can be regarded as a measure of the contact hydrophobic surface area of the solutes [5]. In all cases, the correct

measurements of the eqn. (1) parameters should be helpful to characterize the chromatographic and physico-chemical properties of the solutes. In this connection, it will be interesting to testify the validity of eqn. (1) in the region of the small content of organic modifier, and to compare the extrapolated values of k'_w of deoxyribonucleosides to the directly measured ones.

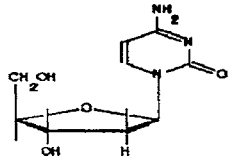
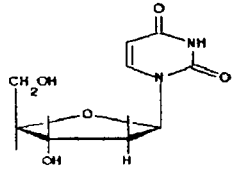
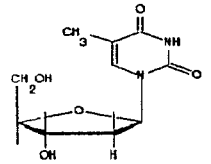
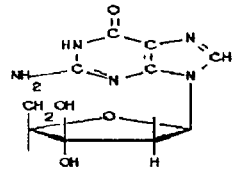
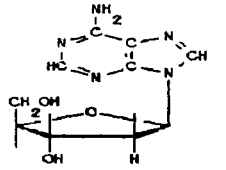
EXPERIMENTAL

Chemicals. The chromatographic behaviour of 5 deoxyribonucleosides with respect to composition of mobile phase was studied in this work. Table 1 gives the IUPAC names and the chemical structures of the solutes investigated. All deoxyribonucleosides were chromatographically pure and were purchased from Sigma (St. Louis, MO, U.S.A.). Standard solutions of solutes were prepared in the HPLC-grade water in 50 ppm concentration. HPLC-grade water, methanol and acetonitrile were obtained from Baker (Phillipsburg NJ, U.S.A.).

HPLC equipment. Throughout the study we used the following equipment: Waters Model 600 liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 600E Multisolvent Delivery System, a UV-visible tunable wavelength adsorbance detector (Waters 486), U6K injector (2 mℓ sample loop). The data aquisition system was CHROMATE (V.2.1, Interface Eng., Korea) installed in PC. A Waters column (30×0.39 cm) packed by μ-Bondapak C_{18} reversed phase material of 10 μm particle size was used.

Methods. The modifier concentration was ranged from 0 to 30% (v/v). Aliquots of 5 μℓ were injected directly for HPLC analysis. The elutions were performed by using an isocratic protocol at a flow rate

TABLE 1
Structure of Deoxyribonucleosides Studied

Name	Structure	Abbreviation
2′-Deoxycytidine		dCyd
2′-Deoxyuridine		dUrd
2′-Deoxythymidine		dThd
2′-Deoxyguanosine		dGuo
2′-Deoxyadenosine		dAdo

of 1 ml/min. Absorbance was monitored at 254 nm with a sensitivity of 2 and 0,001 a.u.f.c. All separations were done at ambient laboratory temperature. The number of theoretical plates N was calculated for all both solutes and mobile phases. The average value of N was 5270, that corresponds to the N value reported in [2]. The dead volume was measured by introducing of 20 $\mu\ell$ of methanol, and the value of dead volume was equal to 2.95 ml.

RESULTS AND DISCUSSION

The experimental data of reversed-phase HPLC retention of five investigated deoxynucleosides in water-methanol and water-acetonitile mobile phases with respect to the content of organic modifier are presented in the Figures 1 and 2. In both cases, the retention of deoxynucleosides decreases with an increase in concentration of modifier in a semi-logarithmic relationship. Compared to methanol, acetonitrile offers approximately twice the elution power for nucleosides, but there is not any significant difference in their selectivity for separation of the nucleosides.

The slopes and intercepts of experimental dependences of log k′ of each solute vs. organic modifiers content were calculated according to eqn. (1). These coefficients and the values of k'_w experimentally measured by using a pure water as mobile phase are presented in the Tables 2 and 3. The empirical coefficients (log k'_w and S) of eqn.(1) were calculated with and without the experimental values of k'_w. The correlation coefficients (r^2) were always higher than 0.98, with the exception of dAdo data, calculated for both organic modifers by using the experimental value of k'_w.

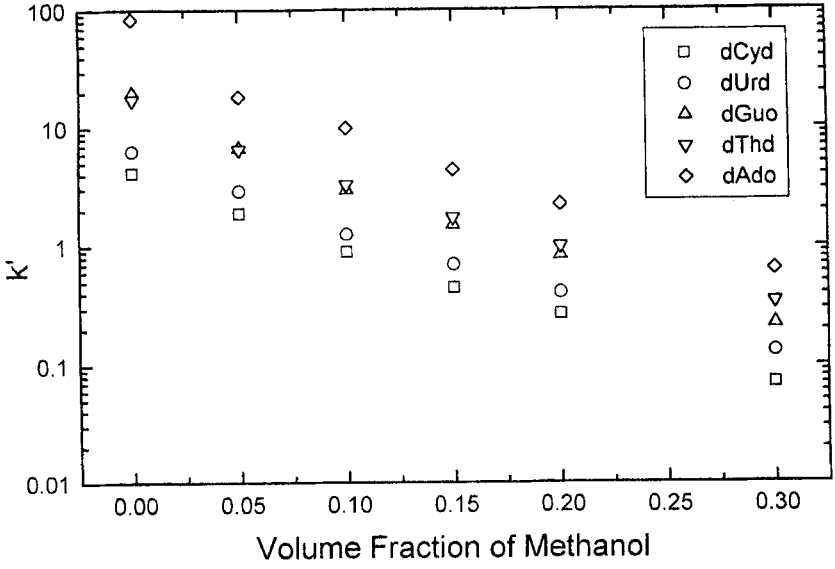


Figure 1. The plot of capacity factors against the methanol volume fraction for deoxyribonucleosides.

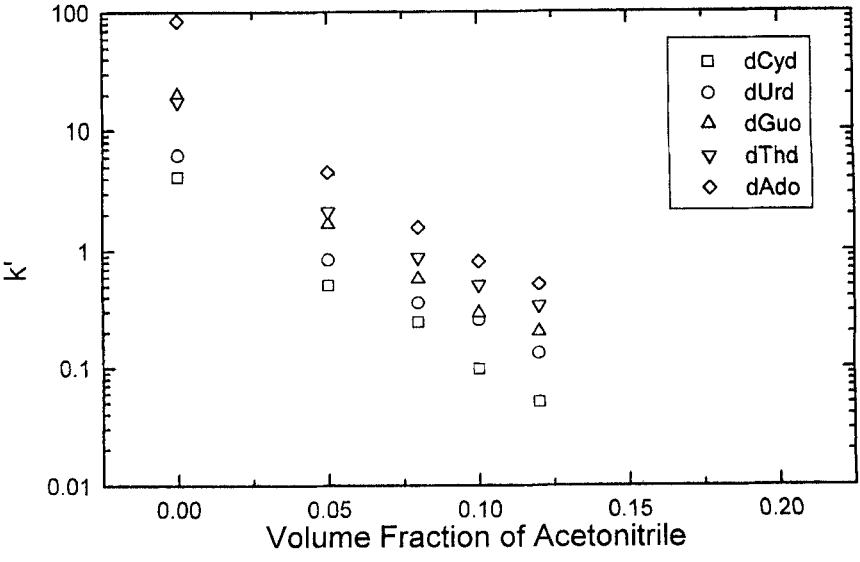


Figure 2. The plot of capacity factors against the acetonitrile volume fraction for deoxyribonucleosides.

TABLE 2
Intercepts (log k'_w) and Slopes (S) of Relationship (1) for Methanol as Organic Modifier

	Solute	log k'_w	S	r^2	k'_w(ext.)[1]	k'_w[2]
	dCyd	0.53	-0.06	0.998	3.39	4.13
	dUrd	0.67	-0.05	0.993	4.68	6.30
3)	dGuo	1.11	-0.06	0.995	12.88	19.31
	dThd	1.08	-0.05	0.990	12.02	17.59
	dAdo	1.51	-0.06	0.996	32.36	84.55
			$\overline{S_{ave}}$[5]=-0.06			
	dCyd	0.57	-0.06	0.999	3.71	
	dUrd	0.73	-0.06	0.991	5.37	
4)	dGuo	1.20	-0.06	0.990	15.85	
	dThd	1.16	-0.06	0.987	15.14	
	dAdo	1.71	-0.07	0.964	51.29	
			$\overline{S_{ave}}$[5]=-0.06			

1) extrapolated values
2) experimental values
3) calculations without the experimental value of k'_w
4) calculations with the experimental value of k'_w
5) averages

The slopes S of different solutes calculated for each organic modifier are approximately coincident. The average values of this slopes (see Tables 1 and 2) calculated with and without experimental values of k'_w are not significantly different, and changed from -0.13 to -0.16 for acetonitrile only. Thus, the slopes S characterize the properties of organic modifier in the case of deoxynucleoside elution under reversed-phase HPLC conditions.

TABLE 3
Intercepts ($\log k'_w$) and Slopes (S) of Relationship (1) for Acetonitrile as Organic Modifier

	Solute	$\log k'_w$	S	r^2	k'_w(ext.)[1]	k'_w[2]
	dCyd	0.48	-0.15	0.984	3.02	4.13
	dUrd	0.48	-0.11	0.990	3.02	6.30
3)	dGuo	0.86	-0.13	0.985	7.24	19.31
	dThd	0.88	-0.11	0.994	7.59	17.59
	dAdo	1.32	-0.14	0.992	20.89	84.55
			$\overline{S_{ave}}$[5]=-0.13			
	dCyd	0.58	-0.16	0.994	3.80	
	dUrd	0.72	-0.14	0.983	5.25	
4)	dGuo	1.19	-0.17	0.980	15.49	
	dThd	1.16	-0.14	0.982	14.45	
	dAdo	1.78	-0.19	0.969	60.26	
			$\overline{S_{ave}}$[5]=-0.16			

(For notations, see TABLE 2)

The difference in the slopes of each organic modifier can be explained by the competitive equilibrium between solute and solvent molecules for the sites on the stationary phase surface. Since the stationary phase possesses a non-polar surface, the less polar molecules will have a better affinity for this surface, and they will be adsorbed, therefore, more strongly. Acetonitrile is less polar solvent than methanol, it can be confirmed by the comparison of their polarity indexes and Hildebrand's solubility parameters. Moreover, unlike methanol, acetonitrile is a very weak hydrogen bond donor.

Due to these properties, it could be expected the preferential adsorption of acetonitrile molecules than methanol ones from aqueous mixtures on reversed-phase modified silica. This assumption was proofed experimentally. Even at 10% organic modifier concentration, acetonitrile was found to solvate the stationary phase to a much higher degree than methanol [11]. Thus, the two-fold higher slope of the curve plots that was found in the acetonitrile/water system could be explained by more preferable adsorption of acetonitrile molecules than methanol ones on the adsorbent surface.

The elution order of deoxynucleosides is the same in the different mobile phases (Figures 1,2). When the modifier concentration is less than 5%, the retention value increases as following: dCyd - dUrd - dThd -dGuo - dAdo. Here dCyd, dUrd, and dThd contain the pyrimidine bases, and dGuo and dAdo contain the purine bases. This result can be connected with an increasing of molecule size and, therefore, with an increasing of the surface area of solute molecule [9]. The retention order on Bondapak column in pure water corresponds probably to the hydrophobic properties of investigated deoxynucleosides, i.e. their affinity for this surface.

As seen in Figures 1 and 2, the elution order of dThd and dGuo is inversed when the methanol concentration in the eluent is increased from 5 to 10 % and more. The same phenomenon was reported in [2], where the elution order of dG and dT was changed by simply varying of the elution conditions. It must be noted that dG and dT represent a pair that is very difficult to separate. Perhaps, this is due to their specific interaction with the modifier molecules and free hydroxyl groups on silica surface.

The k'_w values extrapolated from the experimental dependences of log k' vs. organic modifier content do not coincide with the k'_w values measured directly. In fact, the experimental k'_w values are higher than

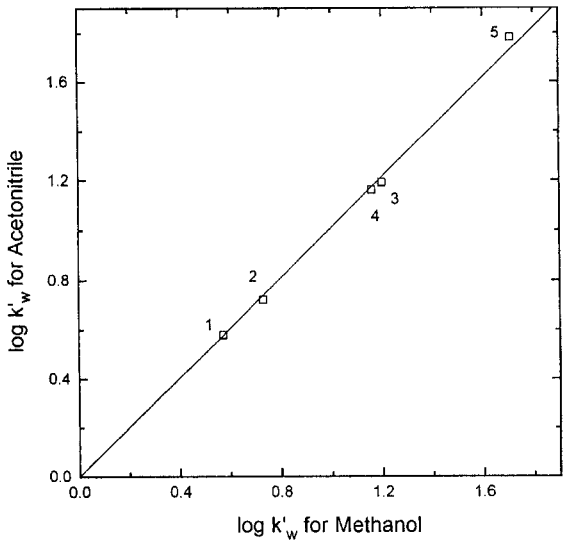


Figure 3. Comparison of intercepts calculated by using the experimental values of k'_w.
(deoxyribonucleosides: 1 = dCyd, 2 = dUrd, 3 = dGuo, 4 = dThd, and 5 = dAdo)

extrapolated ones. The disparities of experimental and extrapolated k'_w values are increased in acetonitrile as organic modifier. For example, the ratio of experimental and extrapolated k'_w values for dAdo is more than 4. The errors in the predicting the k'_w values are reduced when the experimental k'_w values are used for calculations. But in all cases, the difference between the experimental and extrapolated values of k'_w is sufficiently high.

Unexpected result has been obtained in this study. As seen in Figure 3, the dependence of the log k'_w intercepts calculated for acetonitrile vs. the log k'_w intercepts calculated for methanol as organic modifier is fitted by straight line arising from origin. That is, the intercepts of eqn.(1) calculated by using the experimental

values of k'_w for different organic modifiers are coincident. This fact is in contradiction with some data mentioned in review [1]. So, the intercepts calculated in the region of the small content of organic modifier are independent on the nature of organic modifier. Probably, the high level of a coincidence has an accidental character.

Thus, for each solute, the extrapolated and the experimental values of k'_w are not equal, but for different organic modifiers, the extrapolated k'_w values of the solute are the same. This important conclusion is confirmed indirectly by numerous regression correlations between the extrapolated values of k'_w and other hydrophobic and biological properties of solutes. The extrapolated values of k'_w measured in the regions of different content of organic modifier appear to be in the functional dependence on hydrophobic parameters of solutes. For example, the authors in [12] have obtained some good linear correlations for various groups of heteroaromatic compounds with eluents containing 50-70 % of methanol.

ACKNOWLEDGEMENTS

This work was performed in the high-purity separation laboratory of INHA University (Inchon, Korea) under support of Korea Science and Engineering Foundation (Agreement No. 2451-3662 of 1994.11.8) and Korea Institute of Science and Technology.

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Received: March 19, 1995
Accepted: May 25, 1995