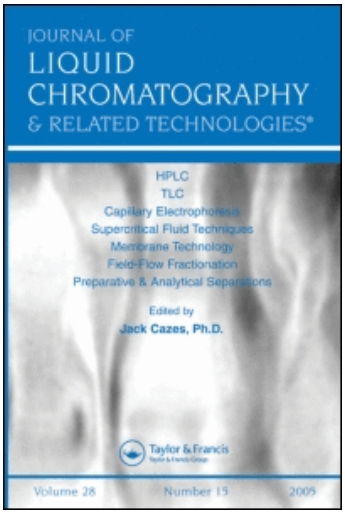

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STUDY OF REVERSED-PHASE HPLC RETENTION AND DETECTION OF ADENOSINE AND RELATED COMPOUNDS AS A FUNCTION OF pH AND SOLVENT COMPOSITION

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ABSTRACT

The retention, UV absorbance, and electrochemical response of adenosine and some of its precursors and metabolites under varying mobile phase composition was investigated using a UV detector on line with an electrochemical detector. The primary interest was focused on adenosine. Experimental retention data sets of this compound obtained on two columns in buffered mobile phases of different pH values modified with methanol and/or acetonitrile were fitted through adequate equations. The physical meaning of the fitted parameters was discussed, besides the dependence of these equation coefficients on the type of organic modifier and composition of mobile phase. The possibility of predicting the retention behaviour of adenosine on the basis of a limited number of data was also explored.

INTRODUCTION

The ribonucleoside Adenosine (Ado) has been suggested to be involved in a variety of physiological processes such as the regulation of tissue blood flows,[1-3] the neurotransmission,[4-6] or the pathophysiology of migraine.[7] However, a thorough study of the physiological roles of Ado necessitates the

optimisation of a reliable and sensitive method, such as the reversed phase HPLC (RP-LC) procedure, for the simultaneous determination of Ado and related compounds in biological tissues and fluids.

In general, we believe that an in-depth understanding of the retention behaviour and responsiveness of these compounds to different modes of detection is particularly essential for the optimisation of a simultaneous determination of these biomedically important compounds by HPLC. At present, several investigations on separation of nucleotides, nucleosides, and bases by RP-LC have been described[8-11] but only a little work has been done on detection activity of these compounds under different chromatographic conditions.[12] In any case, it has been reported that although these compounds are most often detected by UV absorbance when analysis is carried out by HPLC,[1,4,8-11,13-16] some of them can also be detected by electrochemical (EC) detection[12,17] or by combination of these two detection modes.[18]

In this investigation, we studied the retention behaviour of the purine bases Xanthine (Xan) and Hypoxanthine (Hyp), the nucleoside Ado and the nucleotides ATP, ADP, AMP, and IMP under varying mobile phase composition. In addition, we examined the manner in which UV absorbance and EC activity of these compounds varies in response to changes in chromatographic buffer pH and organic modifier content. These particular compounds were selected for analysis for two reasons. First, they are included between the precursors and the degradation products of Ado, which was the compound of main interest in this study, and second, they represent typical polar organic analytes that can be used to elucidate retention mechanisms in RP-LC, since, until now, the theory of chromatographic retention of solutes under conditions of RP-LC has not been fully understood.[19,20]

EXPERIMENTAL

Chromatographic System and Conditions

The liquid chromatography system consisted of a Shimadzu LC-9A pump, a model 7125 syringe loading sample injector fitted with a 20 μL loop (Rheodyne, Cotati, CA), a chromatographic column, a Shimadzu UV- Visible spectrophotometric detector (Model SPD-10A), and a Gilson EC detector (Model 141) equipped with a glassy carbon electrode. The UV detector was placed prior to, and in series, with the EC detector so that one or both could be used in any chromatographic experiment. The detection of the analytes was performed at 260 nm and/or 1.4 V vs the Ag/AgCl reference electrode. The two detectors were interfaced to a Pentium PC (at 200 MHz) via a 14-bit AD-DA card. The same computer was also used to carry out all calculations reported in this paper with programs written by the authors and/or with the microsoft excel solver.

Two chromatographic columns were used: a 250 × 4.1 mm Hamilton (PRP-1) polymeric reversed phase column (7 μm spherical; poly(styrene-divinylbenzene)) and a 100 × 4.6 mm Alltech reversed phase column (3 μm Adsorbosphere Catecholamine). Hold-up times for both columns, t_0, were estimated using replicate injections of pure water under all different chromatographic conditions we used, and found to be 2.14 min for the Hamilton column and 1.12 min for the Alltech one. Since the mobile phase composition was systematically varied in this work (see below), the columns were equilibrated with the new eluent for at least 15 min in order for the baseline of the system to be stabilized. The volume flow rate of all mobile phases used was 1.0 mL/min. All separations were carried out isocratically at ambient temperature.

Chemicals, Standard Solutions, and Mobile Phases

Nucleotide, nucleoside, and base standards from Sigma Chemicals Co. (St. Louis, MO) were dissolved to a concentration of 100 μg/mL in 0.05 M phosphate buffer (pH 6.9) except, Xan, which was initially dissolved into a buffer of pH 10.0 because of its insolubility in pH 6.9. Working solutions of standards (4-0.32 μg/mL) were made by an appropriate dilution of the stock solutions so that each analyte gave approximately the same response in the UV and/or EC detector. Solutes were injected individually or together depending upon their retention times and resolution. The solute retention times and peak areas were obtained from the average value of at least three runs at each pH and percentage of organic modifier we used in this investigation. All solutions were kept refrigerated at 4°C when not in use.

Different mobile phases were prepared by combining an aqueous phosphate buffer with an organic modifier of HPLC-grade to obtain solutions in the range of 0 to 15% (v/v) organic. The organic modifier was MeOH or ACN. Analytical-reagent grade components were used for the preparation of aqueous phosphate buffers with constant ionic strength (0.05 M) in the pH range 2.1 to 6.9. The compositions of the mobile phases used for individual experiments in this investigation are specified in Table 1. All the eluents were filtered through a mixed esters membrane filter (0.45 μm, Schleicher & Schuell, Germany), sonicated and degassed under vacuum for 5 min before use.

RESULTS AND DISCUSSION

Detection

EC Detection

The EC detection of Ado and related compounds was initially studied under different conditions of organic modifier content and pH. For MeOH as

Table 1

The UV Absorbance (mAU.s) and EC Response (nC) of Ado[a] as a Function of pH and Organic Modifier Content

Org. Modif.	% Org. Modif.	pH 2.1 UV	2.1 EC	2.6 UV	2.6 EC	3.0 UV	3.0 EC	4.4 UV	4.4 EC	5.7 UV	5.7 EC	6.9 UV	6.9 EC
None	0	1.05	160	1.01	273	0.96	ND[c]	0.87	ND	0.85	ND	0.70	ND
MeOH	1	---[b]		1.04	344	---		0.95	ND	---		---	
	2	---		1.05	308	---		0.93	ND	---		---	
	3	1.11	108	1.04	281	1.04	246	0.90	ND	0.89	ND	0.91	ND
	5	1.13	156	1.04	235	1.04	306	0.98	291	0.89	335	0.89	ND
	10	1.11	157	1.04	240	1.01	233	0.91	322	0.90	371	0.89	230
	15	---		1.06	143	---		---		1.02	328	0.92	284
ACN	1	1.14	241	1.10	297	1.08	ND	0.97	ND	0.96	ND	0.97	ND
	2	---		1.08	369	---		1.03	ND	---		---	
	3	1.18	113	1.12	271	1.12	ND	1.04	ND	1.05	ND	1.13	ND
	5	1.10	138	1.08	205	1.09	279	1.08	ND	1.15	ND	1.03	ND
	10	---		---		---		---		1.20	ND	1.12	ND

[a] Peak area per 16 ng of Ado injected. For other chromatographic conditions see experimental section. [b] Dashes indicate not tested mobile phase compositions. [c] Not determined EC peak areas due to an excessive background current.

an organic modifier concentrations of 1, 2, 3, 5, 10, and 15% were used, and for ACN 1, 2, 3, and 5%. For each composition and for aqueous eluent three pH values were studied, 2.6, 5.7, and 6.9. The oxidation potential of the EC detector was set at 1.4 V, since it was known that Ado yields a detector response only at high electrode potentials.[12] The high operating voltage used in this study gave a high background current of the mobile phase in some chromatographic systems making it impossible to obtain any EC response, see Table 1.

This preliminary work also indicated that electrode responsiveness varied markedly among the compounds examined. Generally, the order of electroactivity of purine derivatives tested were bases > nucleosides > nucleotides. More precisely, only AMP among all adenine nucleotides studied exhibited a relatively strong EC signal, the peak area of AMP was ~0.6 times lower than that of Ado. The response of ADP was greater than that of ATP but both gave considerably lower response than AMP. As concerns the other nucleotide studied, IMP, it was barely detectable giving either no or only a weak response. On the other hand, we found that, contrary to the nucleotides which are poorly detected by EC detection, the bases Hyp and Xan responded strongly to the EC detec-

tor. The peak areas of Hyp and Xan were ~2.3 and ~1.6 times higher than that of Ado, respectively.

At pH 2.6 the effect of MeOH or ACN on the EC responsiveness of Ado and related compounds is rather complex, since EC detection was not so reproducible. However, despite the relative instability of the EC response, there was a significant decrease in the detector response at 15% MeOH or 5% ACN respectively. In addition, the peak areas determined when MeOH was used as an organic modifier were close to those when ACN was used instead of MeOH.

As concerns the effect of the chromatographic buffer pH on the EC detector response, it was not examined extensively because of excessive background current in some eluents pH. However, practical peak measurements were possible, for example, in eluents containing 15% MeOH at three different pH values, 2.6, 5.7, and 6.9. Chromatograms performed in those particular chromatographic systems showed that for Ado and Hyp there was an increase in the detector response as the pH was increased from 2.6 to 5.7 and then the detector response decreased with increasing pH from 5.7 to 6.9.

All above findings suggest that a determination of AMP and of its degradation products Ado, Hyp, and Xan is possible by an EC method only at particular pH values and organic modifier content, since the requirement of high applied potential for this determination gave a background current out of range in many chromatographic eluents. As a consequence of these limitations chromatographic data obtained with an EC detector can not be used in an extensive investigation of the retention behavior of Ado and related compounds. It was decided, therefore, to solve this problem by the combined use of an EC and a UV detector.

Combined UV and EC Detection

Ado, Hyp, and Xan were chosen to be studied using combined UV and EC detection, since they gave relatively higher response than other compounds tested. Chromatograms were obtained under different conditions of organic modifier concentration and pH. The compositions of the mobile phases examined are specified in Table 1, where it is also indicated which of them are compatible with the EC detection at high electrode potentials such as that used in the present work.

For UV detection the wavelength was set at 260 nm. At that wavelength UV detection is feasible for all compounds tested. However, Ado gave higher UV absorance than the other two compounds. The peak areas of Hyp and Xan were 0.64 and 0.56 times lower than that of Ado respectively. In addition, UV detection was very reproducible and the UV absorbance for Hyp and Xan remained rather constant under different conditions of organic concentration

and pH, whereas for Ado there was a slight decrease in the UV absorbance with increasing pH over the pH range 2.1-6.9, see Table 1.

In accordance with our earlier consideration, Ado, Hyp, and Xan could be detected either with UV or electrochemistry or both, at least under particular chromatographic conditions. We prefer, in general, EC detection as the most sensitive and specific technique;[21] unfortunately at high electrode potentials such as that utilized in the present work EC detector selectivity is limited. However, EC detection could be useful in the identification of Ado, for example, by the disappearance of the corresponding chromatographic peak after applying an electrical potential lower than 1.4 V, instead of treating the sample with adenosine deaminase.[12,13,15] On the other hand, another important criterion in the choice of the detection mode is the stability of response. As concerns this criterion, we concluded that the UV detector was more reliable and took less time to be stabilized under experimental conditions used in this communication. In any case, combination of two detection modes offers optimum results, especially for the analysis of complex biological samples, since it can solve the problem of the interference by comparison peak ratios between detector channels recorded by standards and those produced by samples.

Retention

Elution Behavior and Resolution of all Purine Derivatives Tested

The chromatographic retention times of all solutes examined in the preliminary work by EC detection are limited, see EC detection section. However, on the basis of these data some general observations were made. Nucleotides as highly charged compounds eluted first, followed by the less charged bases and nucleosides, as expected in a RV-LC system. In particular, at pH 2.6 without any organic modifier, the elution order was: ATP, ADP, AMP, IMP, Hyp, Xan, and Ado. However, only Ado exhibited a relatively long retention time, 11.51 min, on the Alltech Adsorbosphere column used under the above chromatographic conditions, while all other compounds eluted in the first 4.5 min. Furthermore, it was found that with all solutes tested except Ado there was a little change in their retention with increasing pH, while the retention of Ado increased strongly as the pH was raised.

As concerns the effect of organic modifier content on the elution behavior of compounds tested, we found that for each compound, retention time decreased with increasing MeOH concentration, in the small range of MeOH examined, from 0 to 15%, although the relative elution position of solutes did not change.

In addition, in all experiments where ACN was used instead of MeOH as an organic modifier, the effect was similar to that of MeOH except for faster elution. This effect is expected in reversed phase systems, since ACN is less polar than MeOH.

Moreover, it is worth noting that the extensive chromatographic retention data of Ado, Hyp, and Xan obtained by the combined use of an EC and a UV detector (see Table 2 for the retention times of Ado, the retention times of two other compounds are not given) were in agreement with the above pattern of purine compounds elution.

In conclusion, all these findings suggest that a simultaneous change of pH and organic modifier content seems to be not enough to get efficient separation of these particular analytes with isocratic mode, primarily as a result of the fact that the retention of these compounds, except Ado, were relatively insensitive

Table 2

Retention Times (min) for Ado[a] as a Function of pH and Organic Modifier Content

		pH					
Org Modif.	**% Org. Modif.**	**2.1**	**2.6**	**3.0**	**4.4**	**5.7**	**6.9**
None	0	6.49	9,94	14.76	47.47	50.05	49.44
MeOH	1	---[b]	8.84	---	34.47	---	---
	2	---	8.06	---	28.19	---	---
	3	4.63	6.97	9.34	23.57	25.05	25.37
	5	4.07	5.95	7.76	17.77	20.51	20.87
	10	3.35	4.62	5.85	11.18	12.64	14.68
	15	---	3.89	---	---	8.60	8.31
ACN	1	4.58	7.18	8.67	22.15	24.64	25.34
	2	---	5.15	---	14.55	---	---
	3	3.29	4.56	5.09	9.96	11.33	11.58
	5	2.79	3.73	3.79	5.91	6.64	6.26
	10	---	---	---	---	3.70	3.37

[a] Measured by UV absorbance on the PRP-1 column. For other chromatographic conditions see experimental section. [b] See footnotes in Table 1.

to the pH changes of the mobile phase. In contrast, a successful resolution of these compounds could be obtained only by using an adequate ion-pairing agent and/or a gradient elution.

However, the retention behavior of Ado is worth being investigated further, since Ado constitutes a compound that can be used as a model for investigating the separation mechanism in reversed phase systems. In addition, the results of this investigation should have a considerable clinical and research utility in light of the growing interest in this biomedically important substance.

Regression Analysis of Ado Data

Ado represents a typical polar organic compound that can be used to unravel the solvophobic elution rules. For the retention of such compounds, when RP-LC methods were used, the pH and the solvent composition of the mobile phase proved to be critical.[11,22-25] In order to determine if the RP-LC behavior of Ado could be predicted we systematically determined the effects of each mobile phase variable on the retention behavior of Ado.

pH Effects

The variation of k with pH at constant organic modifier content is described in the case of a weak monoprotic base such as Ado by the following equation:[26]

$$k=\frac{k_0 + k_1 K_a^{-1} 10^{-pH}}{1+ K_a^{-1} 10^{-pH}} \qquad (1)$$

k_0 and k_1 are the capacity factors of the neutral and protonated (positively charged) species, respectively, in that buffered aqueous-organic eluent and K_a is the dissociation constant of the protonated basic compound in the same eluent. It should be noted here that aqueous-organic K_a and/or pK_a values can not be derived reliably from Eq. (1) since the pH values inserted into Eq. (1) are measured before the addition of organic modifier to the aqueous buffer (a much more convenient procedure).[22,23,27] However, in our study only a small content of organic modifier was used (1-15% MeOH or 1-10% ACN), since Ado at acidic pH shows rather low retention, necessitating low concentrations of organic modifier for analysis. Therefore, it is expected that the addition of an organic modifier to the mobile phase at low concentrations may have little effect on the dissociation of both the basic solute and the buffer and not produce errors in the calculation of aqueous-organic pK_a values.

The experimentally obtained capacity factors for Ado in all the tested compositions of the mobile phase were fitted to Eq. (1) with good accuracy, using

pH values measurements in the aqueous buffer prior to mixing with organic modifier. A non linear least square procedure (regression analysis) was used for fitting Eq.(1) to the experimental data of Ado represented in Table 2. The regression analysis was carried out with a statistical program written by the authors and/or using the microsoft excel solver.

No remarkable differences were found in the parameters evaluated with the different regression fitting procedures. The good agreement between fitted equation and the experimental data can be deduced from the standard deviation of residuals, s, in Table 3 and from Figure 1, where the curves shown were calculated using the parameters in Table 3.

On the basis of the results represented in Table 3, some observations can be made: 1) The calculated aqueous pK_a of Ado, 3.47, compares favorably with the literature value, 3.52;[28] 2) The aqueous-organic pK_a values increase with increasing the organic modifier percentage in the mobile phase. This effect, already observed,[29] is expected, since an increase in the organic modifier concentration leads to a decrease in the dielectric constant of the mixture and con-

Table 3

Results of Regression Analysis of Eq. (1) for the Full Experimental Retention Data Set of Ado at Each Different Organic Modifier Content Shown in Table 2 and in Figure 1

Org. Modif.	% Org Modif.	k_0	k_1	pK_a	s[a]
None	0	22.63	0.95	3.47	0.663
MeOH	3	10.86	0.78	3.43	0.168
	5	8.60	0.90	3.59	0.260
	10	5.36	0.81	3.82	0.472
ACN	1	10.68	1.08	3.57	0.279
	3	4.32	0.63	3.63	0.184
	5	1.98	0.34	3.38	0.154

[a] Computed as the root of the sum of squares of the deviations between measured and estimated k values (residuals) divided by the degrees of freedom for the full data set (in all Tables.)

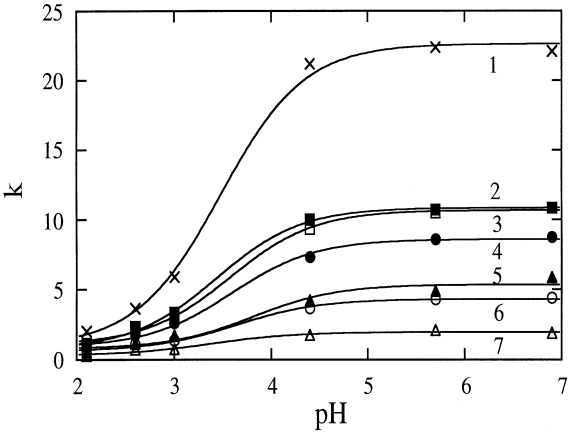

Figure 1. Plots of k vs. pH for Ado at different organic modifier percentages in the mobile phase: (1) 0%, (2) 3%, (4) 5%, (5) 10% MeOH and (3) 1%, (6) 3%, (7) 5% ACN. Drawn lines constructed using Eq. (1) and coefficients in Table 3.

sequently to a decrease in the dissociation of protonated Ado in the same mixture. The decrease of the pK_a value at 5% ACN should be inaccurate owing to the difficulty in computing accurately the inflection point in a sigmoidal curve constructed by very low capacity factors; 3) In general, Ado shows a slightly weaker basicity in the mobile phase modified with MeOH than that modified with ACN; 4) In the case of Ado the neutral form is the most strongly retained species ($k_0 > k_1$ in all cases) and the higher the content of organic modifier in the aqueous buffer the more pronounced the decrease in k_0 and k_1 values.

On the other hand, the ideal sigmoidal curve dependence of the capacity factor on pH for Ado in all the tested compositions of the mobile phase, shown in Figure 1, give evidence of the presence of exclusively hydrophobic interactions between Ado and the tested reversed phase sorbent.[22] From this Figure some other important points are evident as well: 1) There is a number of pH-organic percentage combinations that give similar retention. For example, the retention time of Ado was the same at pH 3.0 and 0% organic modifier as at pH 6.9 and 10% MeOH; 2) Ado, which is largely neutral in the pH range from 4.4 to 6.9, does not undergo a significant change in retention in this pH range; 3) 3% MeOH and 1% ACN are isoeluotropic mobile phases, i.e. they have the same strength, since the retention behavior of Ado is roughly equivalent over the pH range 2.1-6.9 in these organic modifier compositions. All the above observations provide some assurance that the best mobile phase can be found for the separation of Ado in case it is found in a complicated sample.

Summarizing all the above mentioned results, it can be concluded that in general, a good agreement of the theoretically predicted and the measured data was found for Ado in all the tested compositions of the mobile phase over the pH range 2.1-6.9. However, the aim of the present report is to describe the retention of Ado as a function of pH on the basis of a rather limited number of experiments. Typically, three HPLC runs are required for the description of the variation of k with pH at each mobile phase concentration via Eq. (1), but a less sensitive to experimental errors description should be obtained from at least four initial chromatograms. For checking this hypothesis, we selected capacity factors of Ado in all the tested mobile phase compositions for three or four different pH values taking into account that: 1) changes in retention as a function of pH result from changes in the ionization of solute and; 2) dissociation of an ionogenic solute extend one pH unit above and below pK_a. Thus, retention factors of Ado at three or four different mobile phase pH values including the pK_a of solute for each organic modifier content should enable us to predict the retention behavior of Ado over a broad range of pH via Eq. (1) accurately.

Table 4 represents a summary of the results of regression analysis for Ado derived from a limited number of experiments (the footnotes of the same Table indicate the actual mobile pH values selected). By comparing Table 3 and 4 no

Table 4

As Table 3 Except for Reduced Data Sets

Org. Modif.	% Org. Modif.	(4)[a]	(3)[b]	(4)	(3)	(4)	(3)	(4)	(3)
None	0	22.71	22.11	0.88	1.32	3.48	3.55	0.670	0.986
MeOH	3	10.93	10.86	0.69	0.73	3.44	3.45	0.203	0.203
	5	8.57	8.75	0.77	0.57	3.57	3.47	0.276	0.372
	10	5.79	5.86	0.86	0.34	4.00	3.47	0.570	0.815
ACN	1	10.76	10.84	0.88	0.78	3.57	3.54	0.338	0.377
	3	4.31	4.41	0.50	0.38	3.60	3.48	0.209	0.255
	5	1.91	1.93	0.21	0.20	3.31	3.31	0.189	0.184

[a] The number of limited points used in this regression fitting procedure (input k data for pH of 2.1, 3.0, 4.4, and 6.9). [b] The number of limited points used in this regression fitting procedure (input k data for pH 2.1, 3.0, and 6.9).

remarkable differences were found in parameters estimated by the whole data set or by the reduced ones. The maximum standard deviation of residuals between the k values of the regression lines and the experimental k values varies from 0.663 (for the case of all experimental retention data used in the regression analysis) to 0.986 (for the case of the most limited ones).

Organic Modifier Content Effects

Another single-parameter relationship studied in this report is the variation of retention of Ado with organic modifier content at particular mobile phase pH values over the pH range 2.1-6.9. The effect of solvent strength (as controlled by the percentage or the volume fraction, φ, of an organic modifier in a mobile phase of a given pH) on the retention of a solute at a constant pH value is usually expressed as:[11,23,24]

$$\ln k = \ln k^0 + A\varphi + B\varphi^2 \qquad (2)$$

k^0 is the value of k for aqueous mobile phase and A, B are constants for a given chromatographic system (column, mobile phase organic solvent and pH) and solute. A simple regression analysis of retention data of Ado, shown in Table 2, has been carried out according to Eq.(2). The regression coefficients and the standard deviations of residuals, s, calculated are summarized in Table 5.

Table 5

Results of Regression Analysis of Eq. (2) for the Full Experimental Retention Data Set of Ado at each Different pH Value of the Mobile Phase Modified with MeOH or with ACN, Shown in Table 2

Org Modif.	Para-meter	2.1	2.6	3.0	4.4	5.7	6.9
MeOH	$\ln k^0$	0.70	1.29 (2.28)[a]	1.77	3.01	3.05	3.00
	A	-20.1	-15.6 (-27.0)	-20.5	-26.7	-20.6	-16.7
	B	74	39 (66)	84	110	52	28
	s	0.021	0.034 (0.044)	0.021	0.042	0.105	0.176
ACN	$\ln k^0$	0.69	1.30 (2.28)	1.74	3.01	3.03	3.04
	A	-55.2	-53.5 (-65.9)	-61.9	-73.4	-60.0	-60.0
	B	355	431 (375)	440	496	266	240
	s	0.054	0.062 (0.027)	0.073	0.078	0.099	0.072

[a]The values in parentheses represent regression coefficients derived from retention data of Ado obtained in the Alltech Adsorbosphere column, shown in Figure 2.

The results in Table 5 indicate that: 1) Eq.(2) satisfactorily describes the retention of Ado as a function of solvent composition; 2) The intercepts, $\ln k^0$, for MeOH and for ACN as organic modifiers are closely coincident at each pH value in the region of the small content of organic modifier used; 3) There is a weak correlation between values of A and B constants and mobile phase pH, since no significant change is caused by changing mobile phase pH, by itself.

Another view of experimental and predicted results is demonstrated in Figure 2. The plots of ln k vs volume fraction of MeOH or ACN in this Figure depict that: 1) In general the effect of a particular organic modifier content was to decrease the retention times. However, this decrease is more pronounced in the mobile phase modified with ACN than that modified with MeOH; behavior consistent with that predicted in reversed phase systems, since ACN has a slightly higher solvent strength than MeOH[30]; 2) The dependence of the retention of Ado on the organic modifier content is also closely related to the stationary phase used. Particularly, when an organic modifier increases, we observe a more rapid decrease in the retention of Ado for the Alltech column than that for the PRP-1 one, although this comparison is attempted at the same pH value, 2.6, where the tested compound is ionized to the same extent. The same conclusion arises also from the values, in Table 5, of regression coefficients A and B derived for the two columns at mobile phase pH 2.6; 3) For a

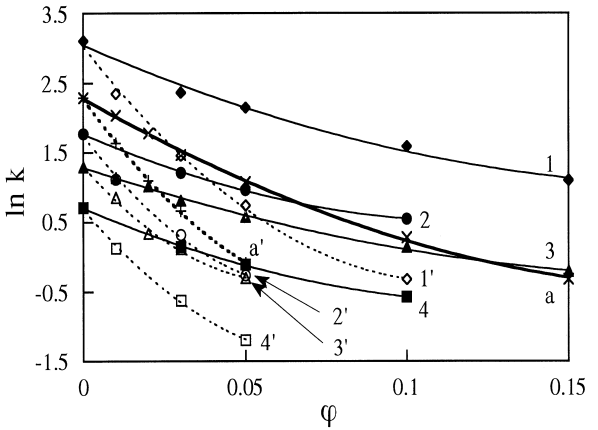

Figure 2. Plots of ln k vs. MeOH (solid lines) or ACN volume fraction (dotted lines) for Ado at different pH values: (1, 1') 5.7, (2, 2') 3.0, (3, 3', a, a') 2.6, and (4, 4') 2.1. Drawn lines (thin for the PRP-1 column and thick for the Alltech Adsorbosphere one) constructed using Eq. (2) and coefficients in Table 5. Lines (a) and (a') represent data obtained on the Alltech Adsorbosphere column.

given chromatographic column the curvature of plots obtained at different pH values is, to a first approximation, independent of the pH, since it remains fairly constant for each organic modifier, but the corresponding values of intercepts are changed strongly for pH < 4.4. For higher pH values, however, the values of ln k^0 are independent of pH, as is normally the case for neutral solutes. See also the results of regression analysis in Table 5, since plots of ln k vs φ at pH values of 4.4 and 6.9 are not present in Figure 2, because they are approximately coincided with that at pH 5.7. So the parameters A and B of Eq. (2) characterize fairly the properties of the organic modifier in the case of Ado, whereas the values of ln k^0 are dependent on sample ionization at each pH value. The values of ln k^0 decrease markedly when the extent of protonation of Ado increases at pH values < 4.4 and consequently the hydrophobicity of the mixture of protonated and non-protonated forms of Ado, which exists at each of these pH values, decreases.

Thus, it can be concluded that the values of ln k^0 obtained at each pH value are not dependent on the kind of organic solvent employed, but, exclusively on the ionization fraction of Ado. However, we should point out that the intercepts, ln k^0, for ACN and for MeOH as organic modifier, obtained by a regression analysis of ln k data dropping those correspond to φ=0 are not coincided (Table 6) and they are different from ln k^0 values determined experimentally. This observation is of great importance in the physical meaning of the ln k^0 value especially in case high concentrations of organic modifier are used for

Table 6

As Table 5 Except for Reduced Data Sets

Org. Modif.	Para-meter	pH 2.1		2.6		3.0		4.4		5.7		6.9	
MeOH	lnk^0	0.61[a]	0.71[b]	1.28	1.29	1.67	1.77	2.93	3.05	2.74	3.11	2.50	3.10
	A	-16.6	-19.7	-15.4	-17.2	-17.0	-20.1	-23.2	-26.5	-12.6	-23.2	-3.73	-23.8
	B	49	69	38	57	58	79	82	103	11	80	-39	105
	s	0.103	0.029	0.034	0.106	0.102	0.029	0.070	0.067	0.259	0.244	0.422	0.593
ACN	lnk^0	0.57	0.71	1.36	1.29	1.59	1.77	2.78	3.05	2.86	3.11	2.93	3.10
	A	-46.7	-53.8	-57.5	-49.8	-50.4	-60.1	-56.6	-71.8	-53.0	-66.6	-55.2	-61.1
	B	227	316	491	360	267	388	246	442	213	387	204	245
	s	0.134	0.071	0.075	0.073	0.181	0.097	0.193	0.105	0.176	0.453	0.123	0.089

[a] Regression coefficients derived from all experimental k data of Ado at each mobile phase pH value except that corresponding to φ = 0. [b] Regression coefficients derived from experimental k data of Ado in only three different organic modifier concentrations, φ, (0, 0.05, and 0.10 for MeOH or 0, 0.03, and 0.05 for ACN) at each mobile phase pH value.

analysis (for a discussion see ref. [31]). On the contrary, only three data points, which, however, include k values obtained at φ=0, give us an accurate description of the retention behavior of Ado under varying organic modifier composition, as is evident from a comparison of the results obtained from the whole and reduced data sets (Tables 5 and 6).

CONCLUSIONS

In summary, the following conclusions can be drawn from the results obtained in the present work: 1) The knowledge of experimental details and conditions is essential to interpret data fully. For this reason the performance of an extensive experimental study is much more preferable than the use of experimental data taken from literature; 2) Only AMP, Ado, Hyp, and Xan among purine derivatives tested responded to the EC detector; 3) The manner in which the EC detector response of these compounds varies under different mobile phase composition can hardly be described extensively because of excessive background current of the mobile phase in some chromatographic systems; 4) The background current of the mobile phase increased with increasing pH and with decreasing organic modifier content; 5) An EC detector used on-line with a UV detector can be valuable in characterizing Ado; 6) Elution using a solvent gradient and/or an adequate ion-pairing agent is necessary for the complete separation of purine compounds tested; 7) The pH and the solvent composition of the mobile phase proved to be critical only for the retention of Ado.

As a result it is possible to draw some general conclusions from the systematic study of the influence of pH and percentage of the organic modifier on the retention of this compound: a) Retention times of Ado can be predicted accurately by Eqs. (1) and (2) on the basis of a limited number of experimental retention times; b) The values of ln k^0 obtained at each pH value by regression analysis using Eq.(2) have a physical meaning, serve as a good description and predictor of Ado hydrophobicity in biological systems, and do not depend on the organic modifier employed only in case that experimental data which correspond to φ = 0 are included in fitting procedures; c) The dissociation constant of Ado depends on the kind and amount of the organic modifier; d) This systematic study has potential value for optimizing the separation of Ado and provides researchers with adequate initial estimates of parameters in case the retention behavior of Ado, as a function of simultaneous change in pH and solvent composition, is modeled.

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