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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOTIDE–Mg(II) COMPLEXES

SEPARATION AND MECHANISM OF SEPARATION

ELI GRUSHKA*
Department of Inorganic and Analytical Chemistry, The Hebrew University, Jerusalem (Israel)
and
FRANCIS K. CHOW
Department of Chemistry, State University of New York at Buffalo, Buffalo, NY 14214 (U.S.A.)

SUMMARY

A dithiocarbamate column is used to resolve diphosphate and triphosphate nucleotides with Mg(II) in the mobile phase. A retention mechanism is proposed to account for the retention behavior in the present system. Using this retention model, a linear correlation is found between the concentration of Mg(II) and the k' values of nucleotides. Complex formation constants can also be obtained by this method showing good agreement with those reported in the literature.

INTRODUCTION

The retention orders of solutes and hence the selectivities of chromatographic systems are determined by the thermodynamic processes which control the distribution of the solutes between the stationary and mobile phases. The selectivities, therefore, are a function of such parameters as pH, temperature and additive in the mobile phase. The presence of metal cations in the chromatographic system can significantly alter elution orders and enhance separations.

The use of metal ions in chromatography is a rather old practice which has only recently gained momentum. A review of much of the early work is given in ref. 1. Karger and co-workers[2,3], Vonach and Schomburg[4], Hare and Gil-Av[5] and our group[1,6,7] have all shown the improvement in the selectivities afforded by adding metal cations to either the stationary phase or the eluent.

In a previous paper[6], we have reported the use of a dithiocarbamate–Co(III) column, and Mg(II) in the mobile phase, for the isocratic resolution of a large number of nucleotides. A dithiocarbamate column without cobalt has been used for the separation of nucleotides and nucleosides[8]. This latter approach is particularly interesting because the retention times can easily be controlled with slight changes of pH or Mg(II) concentration in the mobile phase. In this article, a retention mechanism

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is proposed and tested to account for the retention behavior we observed before[8]. Using this mechanism, the capacity ratios (k') of nucleotides can be linearly correlated with the Mg(II) in the mobile phase. From these relationships the formation constants of the nucleotide–Mg(II) complexes can be obtained.

There are several reasons for the use of dithiocarbamate bonded silica in the present study. Our previous experience of using a reversed-phase system to resolve nucleotides and nucleosides in the presence of Mg(II) indicate that changes in the Mg(II) concentration or pH did not have a pronounced effect on the k' values, but rather on the efficiency[6]. With the dithiocarbamate system, on the other hand, a slight change of Mg(II) concentration greatly affected the k' values, which in turn can be correlated to individual complex formation constants with a much better degree of confidence. Detailed discussion on the chromatography of the dithicarbamate system has been made in our previous paper[8]. The complex formation of 8 polyphosphate nucleotides and Mg(II) are studied in this article as a function of Mg(II) concentration and pH.

Horváth *et al.*[9] have recently dealt extensively with measurement of association constants in high-performance liquid chromatography (HPLC). The approach which follows is different from theirs in regard to the derivation of the dependence of k' on the experimental conditions.

THEORETICAL

When a nucleotide is introduced into the column in the presence of Mg(II) in the mobile phase, various equilibria will be established simultaneously. Fig. 1 is a summary of such processes using ATP as model solute.

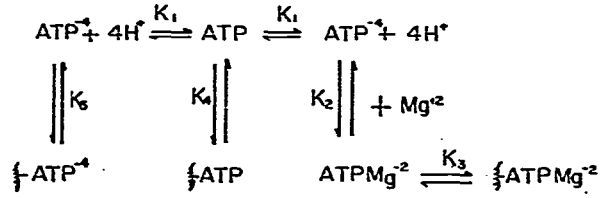

Fig. 1. Schematic representation of the retention mechanism of ATP with Mg(II) in the mobile phase.

Equilibrium constants are given by:

$$K_1 = \frac{[ATP^{-4}]\,[H^+]^4}{[ATP]} \tag{1}$$

$$K_2 = \frac{[ATP\ Mg^{-2}]}{[ATP^{-4}]\,[Mg^{+2}]} \tag{2}$$

$$K_3 = \frac{[S\text{-}ATP\ Mg^{-2}]}{[ATP\ Mg^{-2}]} \tag{3}$$

$$K_4 = \frac{[S\text{-}ATP]}{[ATP]} \tag{4}$$

$$K_5 = \frac{[S\text{-}ATP^{-4}]}{[ATP^{-4}]} \tag{5}$$

where K_1 is the dissociation constant of ATP, K_2 is the complex formation constant between ATP^{-4} and Mg(II), K_3, K_4 and K_5 are the partition constants of ATP Mg^{-2}, ATP and ATP^{-4} between stationary and mobile phases, respectively. The symbol S- indicates species in the stationary phase. The scheme in Fig. 1 is a simplification since individual proton dissociations were not taken into account. However, with the appropriate pH of the mobile phase this simplification is not a bad one.

Obviously, the various species in these equilibria will have individual affinity for the stationary and mobile phases. However, due to the fast equilibrium between ATP^{-4} and ATP Mg^{-2}, the capacity ratio can be written as the weighted average:

$$k' = \alpha k'_0 + \beta k'_\infty \tag{6}$$

Where k'_0 is the value when no Mg(II) is present and k'_∞ is the value when a large excess of Mg(II) is present in the mobile phase, and $\alpha + \beta = 1$. The values of α and β are given by:

$$\alpha = \frac{[\text{ATP}] + [\text{ATP}^{-4}] + [\text{S-ATP}]}{[\text{ATP}] + [\text{ATP}^{-4}] + [\text{S-ATP}] + [\text{ATP Mg}^{-2}] + [\text{S-ATP Mg}^{-2}]} \tag{7}$$

$$\beta = \frac{[\text{ATP Mg}^{-2}] + [\text{S-ATP Mg}^{-2}]}{[\text{ATP}] + [\text{ATP}^{-4}] + [\text{S-ATP}] + [\text{ATP Mg}^{-2}] + [\text{S-ATP Mg}^{-2}]} \tag{8}$$

We assume here that K_5 is negligible due to the formation of the complex between ATP^{-4} and Mg(II). Using eqns. 1–8, α and β can be rewritten as:

$$\alpha = \frac{K_1 + [\text{H}^+]^4 + K_4[\text{H}^+]^4}{K_1 + K_4[\text{H}^+]^4 + [\text{H}^+]^4 + K_1K_2[\text{Mg}^{+2}] + K_1K_2K_3[\text{Mg}^{+2}]} \tag{9}$$

$$\beta = \frac{K_1K_2[\text{Mg}^{+2}] + K_1K_2K_3[\text{Mg}^{+2}]}{K_1 + K_4[\text{H}^+]^4 + [\text{H}^+]^4 + K_1K_2K[\text{Mg}^{+2}] + K_1K_{23}[\text{Mg}^{+2}]} \tag{10}$$

Consequently, k' can be represented by the following expression:

$$k' = \frac{k'_0K_1 + k'_0[\text{H}^+]^4 + k'_0K_4[\text{H}^+]^4 + k'_\infty K_1K_2[\text{Mg}^{+2}] + k'_\infty K_1K_2K_3[\text{Mg}^{+2}]}{K_1 + K_4[\text{H}^+]^4 + [\text{H}^+]^4 + K_1K_2[\text{Mg}^{+2}] + K_1K_2K_3[\text{Mg}^{+2}]} \tag{11}$$

In order to make eqn. 11 useful, several assumptions can be made: (a) The term $[\text{H}^+]^4$ is negligibly small. (b) Since $K_1 \approx 10^{-3}$ and most likely $K_3 \ll K_2$, then we may assume that K_1 and $K_1K_2K_3$ are negligible as compared to K_1K_2. With these assumptions, eqn. 11 reduces to:

$$k' \approx \frac{k'_0}{K_2[\text{Mg}^{+2}]} + k'_\infty \tag{12}$$

Consequently, a plot of k' *vs.* inverse of Mg(II) concentration will give a straight line, the slope of which yields the complex formation constant K_2.

It should be noted that eqn. 12 is similar to the capacity ratio equation proposed by Horváth *et al.*[9]. In fact, when the formation constant is large the two equations reduce to the same expression.

EXPERIMENTAL

Instrumentation

The chromatograph consisted of an Altex (Berkeley, CA, U.S.A.) Model 110 solvent metering pump, an LDC (Riviera Beach, FA, U.S.A.) Spectromonitor I variable-wavelength UV detector monitored at 260 nm, and a Rheodyne (Berkeley, CA, U.S.A.) Model 7120 injection valve. The column and solvent lines were thermostated at 26°C with a home-made water jacket described by Kikta *et al.*[10]. Columns were made of 316 stainless steel 25 × 0.31 cm I.D. using zero dead volume fittings. Chromatograms were recorded with a Heath Model SR 225 strip chart recorder.

Reagent and procedure

Sources of reagents and procedure for preparing the dithiocarbamate bonded phase on Partisil 10 silica gel, has been reported before[6,8]. CHN analysis shows a 3.3 μmole/m^2 surface coverage.

Chromatographic studies

In order to verify the proposed mechanism, eight diphosphate and triphosphate nucleotides that form strong complexes with Mg(II) (refs. 11–13) were used. Mobile phases were prepared from solution containing 0.137 *M* KH_2PO_4 adjusted for various pH and Mg(II) concentrations. The column was equilibrated with at least 100 ml of the phosphate buffer before k' values were measured. Mobile phases were run from low to high pH.

Hold-up times were measured by injecting 10 μl of a solvent having a slightly different composition than that of the mobile phases. Flow-rate of 1.5 ml/min was used during the entire study.

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms illustrating the drastic effect of Mg(II) on the elution of some nucleotides at constant ionic strength. Undoubtedly, such an effect is due mainly to the formation of complexes between Mg(II) and the nucleotides, as we discussed previously[6]. As expected, the addition of Mg(II) to the mobile phase affects the retention of the triphosphate nucleotide more than that of the diphosphate, whereas the nucleoside and monophosphate remain unchanged. Based on these alterations in the retention time we shall now proceed to calculate the complex formation constants.

k' dependence on Mg(II) concentration

Eqn. 12 predicts that k' will be a linear function of the inverse of Mg(II) concentrations. However, it is instructive to discuss the proposed mechanism (Fig. 1) in more detail before making any further use of eqn. 12. Tables I to IV summarize the experimental data. As can be seen, without Mg(II) in mobile phases, the retention times for most of the polyphosphate nucleotides are very long, especially at low pH. Large k' values imply a large magnitude of K_4 and/or K_5 in the retention model given in Fig. 1, *i.e.*, some triphosphate nucleotides are strongly retained. In the presence of Mg(II), k' is greatly reduced. As shown in Fig. 1, the drastic decrease of k' implies

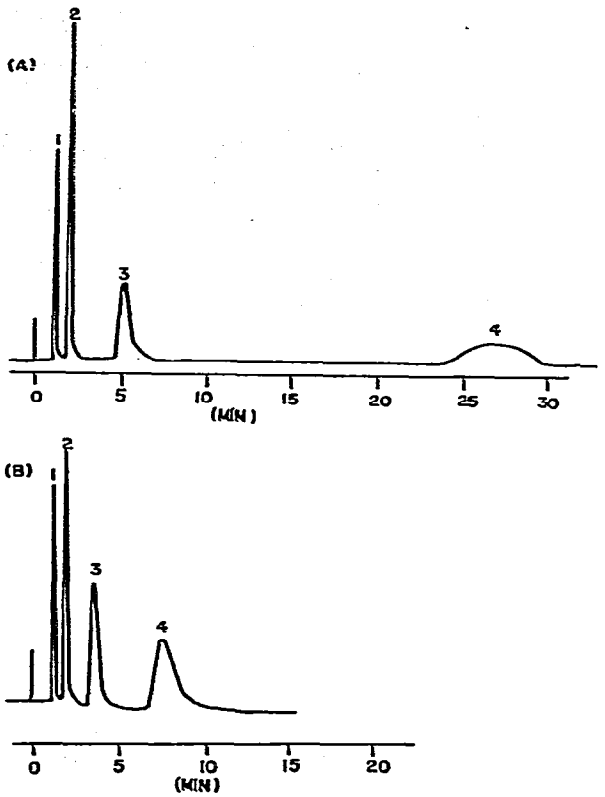

Fig. 2. Separation of some nucleosides and nucleotides. (A) Mobile phase: 0.137 *M* KH_2PO_4 with no Mg(II), pH 6.0, flow-rate 1.5 ml/min. (B) Mobile phase: 0.137 *M* KH_2PO_4 with $8.1 \cdot 10^{-4}$ *M* $MgSO_4 \cdot 7H_2O$, pH 6.0, flow-rate 1.5 ml/min. Peaks: 1 = uridine; 2 = UMP; 3 = UDP; 4 = UTP.

TABLE I

EFFECT OF Mg(II) CONCENTRATION ON k'
pH 7 at 26°C.

Solute	*I/[Mg(II)]**					*Slope***	*log* K_2*****	*Coefficient of determination*
	k_0	*1232.0*	*821.0*	*493.0*	*246.5*			
ADP	4.20	2.30	1.85	1.38	1.09	$1.24 \cdot 10^{-3}$	4.23	0.994
ATP	15.46	3.20	2.07	1.38	0.88	$2.35 \cdot 10^{-3}$	4.57	0.994
GDP	3.80	1.70	1.40	0.94	0.63	$1.10 \cdot 10^{-3}$	4.24	0.979
GTP	12.44	2.37	1.58	1.07	0.57	$1.79 \cdot 10^{-3}$	4.55	0.998
CDP	1.58	0.94	0.77	0.32	0.29	$7.79 \cdot 10^{-4}$	4.02	0.931
CTP	5.40	1.35	0.88	0.51	0.25	$1.12 \cdot 10^{-3}$	4.39	0.998
UDP	1.29	0.70	0.54	0.38	0.23	$4.73 \cdot 10^{-4}$	4.14	0.991
UTP	4.80	1.10	0.65	0.37	0.15	$9.58 \cdot 10^{-4}$	4.40	0.994

* With 0.137 *M* KH_2PO_4 at constant ionic strength.
** Slope is given by eqn. 12 and is determined by least square regression.
*** Corrected for pH and phosphate buffer effects.

TABLE II

EFFECT OF Mg(II) CONCENTRATION ON k'
pH 6.5 at 26°C.

Solute	*1/[Mg(II)]**					*Slope***	*log K_2****	*Coefficient of determination*
	k_0	*1232*	*821.0*	*493.0*	*246.5*			
ADP	7.44	4.02	3.55	3.00	1.78	$2.13 \cdot 10^{-3}$	3.99	0.884
ATP	30.10	5.90	4.73	3.46	1.77	$3.78 \cdot 10^{-3}$	4.34	0.936
GDP	6.36	3.40	2.77	2.38	1.29	$1.99 \cdot 10^{-3}$	3.95	0.915
GTP	28.80	5.30	3.71	2.68	1.37	$3.88 \cdot 10^{-3}$	4.31	0.992
CDP	3.00	2.00	1.54	1.38	0.85	$1.08 \cdot 10^{-3}$	3.89	0.943
CTP	12.04	3.20	2.07	1.54	0.85	$2.31 \cdot 10^{-3}$	4.17	0.990
UDP	2.44	1.40	1.03	0.77	0.54	$8.65 \cdot 10^{-4}$	3.9	0.999
UTP	10.10	2.40	2.07	1.15	0.54	$1.93 \cdot 10^{-3}$	4.16	0.991

* See Table I
** See Table I.
*** See Table I.

TABLE III

EFFECT OF Mg(II) CONCENTRATION ON k'
pH 6.0 at 26°C.

Solute	*1/[Mg(II)]**					*Slope***	*log K_2****	*Coefficient of determination*
	k_0	*1232*	*821.0*	*493.0*	*246.5*			
ADP	12.70	7.40	6.35	5.00	3.21	$4.08 \cdot 10^{-3}$	3.71	0.952
ATP	76.14	18.20	11.77	7.54	4.04	$1.43 \cdot 10^{-2}$	3.95	0.998
GDP	12.26	6.54	4.85	3.92	2.42	$4.01 \cdot 10^{-3}$	3.70	0.984
GTP	54.85	16.50	10.07	6.04	3.00	$1.35 \cdot 10^{-2}$	3.84	0.983
CDP	5.88	3.62	2.85	2.26	1.58	$2.02 \cdot 10^{-3}$	3.68	0.992
CTP	28.93	8.50	5.09	3.35	1.78	$6.72 \cdot 10^{-3}$	3.82	0.990
UDP	4.30	2.60	2.00	1.58	1.02	$1.56 \cdot 10^{-3}$	3.66	0.987
UTP	26.24	6.70	3.78	2.57	1.35	$5.32 \cdot 10^{-3}$	3.91	0.979

* See Table I.
** See Table I.
*** See Table I.

TABLE IV

EFFECT OF Mg(II) CONCENTRATION ON k'
pH 5.5 at 26°C.

Solute	*1/[Mg(II)]**					*Slope***	*log K_2****	*Coefficient of determination*
	k_0	*1230*	*821.0*	*493.0*	*246.5*			
ADP	16.20	10.30	9.22	7.38	5.61	$4.93 \cdot 10^{-3}$	3.60	0.954
ATP	§	38.00	27.57	19.28	10.32	$2.75 \cdot 10^{-2}$	§§	0.993
GDP	15.72	8.84	7.38	6.79	4.38	$4.12 \cdot 10^{-3}$	3.66	0.902
GTP	§	34.22	25.15	16.50	9.15	$2.62 \cdot 10^{-2}$	§§	0.991
CDP	6.96	4.50	3.88	3.31	2.50	$1.96 \cdot 10^{-3}$	3.68	0.968
CTP	49.86	15.60	11.53	7.62	4.42	$1.13 \cdot 10^{-2}$	3.72	0.996
UDP	5.74	3.30	2.88	2.42	1.80	$1.48 \cdot 10^{-3}$	3.66	0.959
UTP	40.40	12.30	9.42	6.00	3.40	$9.08 \cdot 10^{-3}$	3.72	0.989

* See Table I.
** See Table I.
*** See Table I.
§ Not eluted within reasonable time.
§§ Not enough information to determine.

that the various equilibria are shifted by the complex formation between Mg(II) and nucleotides. The effects of K_4 and K_5 on retention becomes less dominating. Furthermore, small k' values imply that the magnitude of K_3 can be rather small. Due to the strong interaction between Mg(II) and nucleotides, the amount of free ionized nucleotides present during the chromatographic process is most likely small and consequently K_5 can be neglected. These are the assumptions, as we discussed in the Theoretical section, which led to the derivation of eqn. 12.

Plots of k' *vs.* $[Mg^{2+}]^{-1}$ are shown in Fig. 3. As expected from eqn. 12, the lines are straight. The slopes of the lines are shown in Tables I–IV, as are log K_2 values extracted from them. In order to obtain the K_2 values from the slopes, the effect of the phosphate buffer in the mobile phase must be taken into account. The species HPO_4^{2-} forms a complex with Mg, and thus the magnesium concentration term in eqn. 12 must be properly corrected. The species $H_2PO_4^-$ forms a very weak complex[14] and its contribution can be neglected. The correction is easily accomplished by simple equilibrium consideration using the literature value for the formation constant of the $MgHPO_4$ complex[14]. The correction becomes significant at pH 6.5 and above.

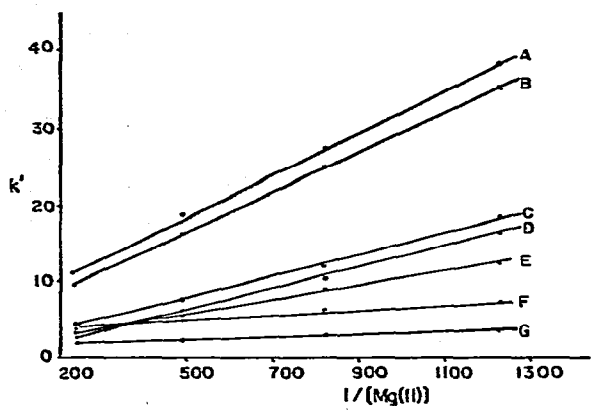

Fig. 3. Correlation of k' of some nucleotides to the inverse of Mg(II) concentrations in the mobile phases. A, ATP with mobile phase pH 5.5; B, GTP with mobile phase pH 5.5; C, ATP with mobile phase pH 6.0; D, GTP with mobile phase pH 6.0; E, UTP with mobile phase pH 5.5; F, ADP with mobile phase pH 6.0; G, CDP with mobile phase pH 6.0.

The results in Tables I–IV show that in all cases the constants for the triphosphates are greater than those for the diphosphates. At pH 7 and 6.5 the K_2 values of the purine triphosphate nucleotide are greater than those of the pyrimidines.

The pH effect on the K_2 values should be noted; K_2 changes, on the average, by about 0.6 log units over the pH range studied here.

Table V compares some values of K_2 obtained in this study with some literature values from two recent references. It should be stressed that the range of values reported in the literature is rather large, as can be seen in ref. 15 (log K values from 3.5 to 4.9). In addition, there are some inherent difficulties in reproducing K_2 values from laboratory to laboratory as discussed by Sigel[12]. Therefore, the agreement shown in Table V is very good.

TABLE V

COMPARISON OF SOME LOG K_2 VALUES WITH SOME LITERATURE VALUES

Solute	*log K_2 (by HPLC)*	*log K_2 (ref. 12)*	*log K_2 (ref. 16)*
ADP	4.23	—	3.17
ATP	4.57	4.24	4.06
GTP	4.55	4.13	4.02
CDP	4.02	—	3.22
CTP	4.39	4.08	4.01
UTP	4.40	4.00	4.02

k' dependence on pH

A change in the pH will cause two related effects. The degree of dissociation of the nucleotides as well as the concentration of HPO_4^{2-} in the mobile phase will change, and consequently the complex formation with Mg(II) will diminish or increase. Both of these effects will alter the elution time. For example, ATP has four ionizable protons, three with low pK values of 2–4 and the fourth of 6.5. At pH 5.5, only 9.09% of the fourth proton is dissociated while at pH 7, 76% will be dissociated; thus ATP will elute much faster at this pH as we reported previously[8]. Tables I–IV show that k' is a strong function of the pH of the mobile phase. Log K_2 values are less sensitive to pH variation in the range studied here. The trend, however, is to decrease log K_2 when increasing the acidity of the mobile phase. This is expected since at low pH, the fully deprotonated nucleotide is no longer the predominating species, and the complex $MgHL^-$ is less stable than MgL^{2-} (L represents the nucleotide). The point that must be made is that K_2 values measured here represent the overall formation constant of all Mg–nucleotide species.

It is interesting to note that the change in k' when changing the pH is more pronounced when Mg(II) is present.

The dependence of the retention times on [Mg(II)] and on pH can be employed advantageously for the separation of nucleosides and nucleotides. Fig. 4 illustrates an efficient use of Mg(II) and pH in gradient elution. The difference between solvent A and solvent B in this chromatogram is the pH. The ionic strength, as well as the amount of Mg(II) present are identified in both A and B. Consequently, the baseline is stable during analysis and column regeneration is easy. Using this step gradient, 18 nucleotides and nucleosides can be resolved in about 17 min.

The temperature dependence of k'

The capacity factors of the nucleotides in the Mg(II)-containing mobile phase should be a strong function of the temperature (T), since all the equilibrium constants in Fig. 1 are temperature dependent. It is of interest, therefore, to examine the change in k' as a function of T.

Table VI shows the capacity ratios of the eight nucleotides at four temperatures: from 26 to 66°C. Fig. 5 shows plots of ln k' *vs.* $1/T$ (in °K) for four solutes. The plots are like the typical Van 't Hoff plots usually observed in chromatography. The slopes of the lines, as obtained from linear regression analysis, are given in Table VI. The correlation coefficients of the regression were very high, with 0.994 being the worst. From the slope, ΔH of solution, can be obtained. However, it is

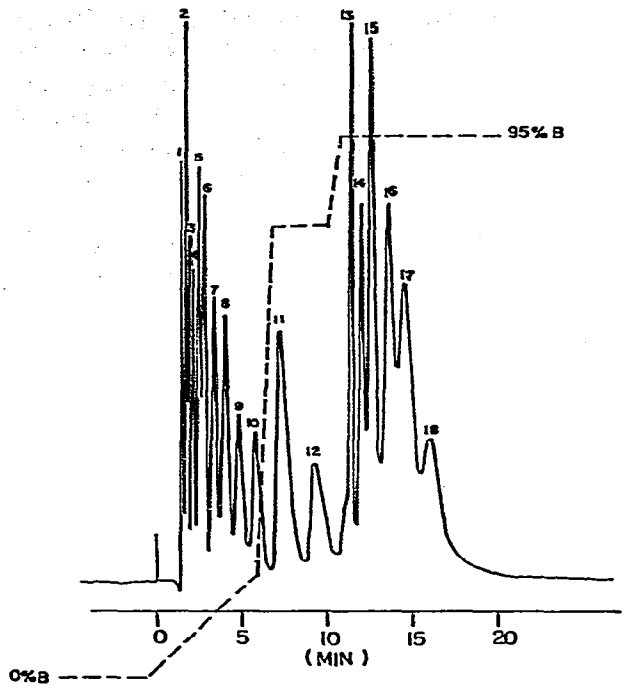

Fig. 4. Separation of some nucleosides and nucleotides by dithiocarbamate column using gradient elution. Mobile phase A: 0.137 *M* KH_2PO_4, $2.0 \cdot 10^{-3}$ Mg(II) at pH 5.5. Mobile phase B; 0.137 *M* KH_2PO_4, $2.0 \cdot 10^{-3}$ Mg(II) at pH 7.0. Gradient profile is shown by the broken line. Peaks: 1 = uridine; 2 = thymidine; 3 = guanosine; 4 = adenosine; 5 = deoxyadenosine; 6 = UMP; 7 = CMP; 8 = dCMP; 9 = GMP; 10 = AMP; 11 = XMP; 12 = UDP; 13 = CDP; 14 = UTP; 15 = CTP; 16 = ADP; 17 = XDP; 18 = XTP.

TABLE VI

EFFECT OF TEMPERATURE ON *k'*

Mobile phase: 0.137 *M* KH_2PO_4 and 0.0024 *M* $MgSO_4 \cdot 7H_2O$ at pH 5.5.

Solute	*k' (ln k') at T°K*				*Slope*
	299	*308*	*317*	*339*	
ADP	6.00 (1.79)	4.46 (1.50)	3.35 (1.21)	2.07 (0.728)	2699
ATP	12.5 (2.52)	9.04 (2.20)	6.05 (1.80)	3.37 (1.22)	3357
GDP	4.34 (1.47)	3.46 (1.24)	2.52 (0.924)	1.55 (0.438)	2661
GTP	9.46 (2.25)	7.08 (1.96)	5.25 (1.66)	2.58 (0.948)	3328
CDP	2.62 (0.963)	2.17 (0.775)	1.86 (0.621)	1.23 (0.207)	1919
CTP	5.23 (1.65)	4.17 (1.43)	3.25 (1.18)	1.85 (0.615)	2667
UDP	1.73 (0.548)	1.54 (0.432)	1.38 (0.322)	1.03 (0.0296)	1326
UTP	3.81 (1.34)	3.21 (1.17)	2.57 (0.944)	1.77 (0.571)	1976

not immediately clear what are the major processes contributing to these ΔH values. To study the effect of Mg(II), experiments should also be done where the temperature dependence of *k'* is obtained with no magnesium ions in the system. Eqn. 12 is not sufficiently rigorous to allow the determination of thermodynamic data concerning the nucleotide–Mg(II) complex formation.

The slopes of the line are interesting, since they can indicate similarities and differences in the chromatographic behavior of the solutes. The slopes of the purine based nucleotides are larger than the pyrimidine ones. Moreover, in the purine family, the slopes of ADP are similar to those of GDP, while ATP slopes are close to those of GTP. The values for the triphosphates are larger than for the related diphosphates, most likely due to the fact that the former are complexed more strongly by the Mg(II).

Plots, such as those in Fig. 5, can help in choosing the chromatographic conditions to optimize the separation, while keeping the retention times reasonable.

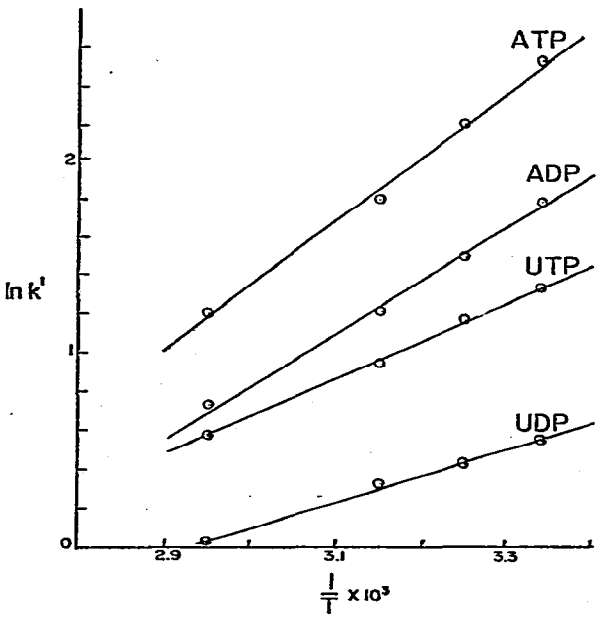

Fig. 5. The dependence of $\ln k'$ on $1/T \times 10^3$. The mobile phase is given in Table VI.

CONCLUSION

A novel approach to the separation of nucleotides using metal–solute complexes is reported. This article provides a semi-theoretical framework to explain some of the retention behaviors, and sheds light on the relationship between the capacity factors and the equilibrium constants in the presence of various concentrations of metal ions in mobile phases. Using this information, prediction of retention and optimization of resolution can be made rather accurately and efficiently. In addition, we believe that the present approach affords a rapid and dependable technique to simultaneously measure stability constants of a large number of complexable species.

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