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Ion-pair reversed-phase high-performance liquid chromatography of adenine nucleotides and nucleoside using triethylamine as a counterion

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Abstract

An isocratic HPLC method for the simple and selective determination of adenine nucleoside and nucleotides has been developed. The separation is achieved at room temperature by reversed-phase chromatography (Shiseido, Capcell Pak C18). A mixture of 0.1 *M* triethylamine (TEA) phosphate buffer and methanol (95:5, v/v) is used as a standard eluent. Influence of pH and concentrations of organic modifiers and TEA ion on capacity factors of adenine compounds has been investigated. It has been also found that the TEA ion in the eluent is adsorbed onto the reversed-phase surface. The results clearly demonstrate that ion-pair formation with TEA ion occurs probably both in the mobile phase and on the stationary phase and governs the retention of adenine and nucleotides in the present system. The HPLC system is applied to the analysis of adenine nucleotides formed as intermediates in the synthesis of 3′-phosphoadenosine 5′-phosphosulphate (PAPS) and to the assays of ATPases and 5′-nucleotidase activities in rat liver plasma membrane. This method is a new type of ion-pair reversed-phase HPLC system and is suitable for the separation of highly polar organic anions, especially for adenine nucleotides.

Keywords: Adenine nucleoside; Adenine nucleotides; Nucleosides; Nucleotides

1. Introduction

Recently, reversed-phase columns have been mainly used in high-performance liquid chromatography (HPLC) for the separation of adenine nucleoside and nucleotides [1–3]. Moreover, the introduction of quaternary ammonium alkylates such as tetrabutylammonium (TBA) and cetrimide as counterions to the reversed-phase system has enabled us to perform a rapid and selective separation of these compounds under simple isocratic conditions [4–9]. This excellent technique, however, has a few shortcomings. Since the quaternary ammonium alkylates used as a counterion belong in the group of detergents and are strongly adsorbed on the stationary phase, the passage of a somewhat large volume (about 50–75 column volumes) of the mobile phase is needed for equilibration of the column. This is a serious draw-back in cases where a rapid re-equilibration of the column with a new mobile phase is required. The ion-pair formation between TEA and nucleotides has been utilized as an useful tool for the isolation and purification of many nucleotides. Uematsu and Suhadolnik reported that adenine

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nucleotides such as 5′-AMP, ADP and ATP were strongly retained on XAD-4 column equilibrated with TEA bicarbonate buffer [10]. Based on this finding, we have previously developed a reversed-phase HPLC system for the separation of sulfobromophthalein mercaptides using TEA phosphate buffer as a component of the mobile phase and found that TEA ion behaved as an ion-pairing reagent [11].

The purpose of this work was to establish a new type of ion-pair reversed-phase HPLC system suitable for the separation of adenine nucleotides and nucleoside. The method was applied to the analysis of nucleotides formed in the synthesis of PAPS from adenosine (Ado) and to the assays of ATPases and 5′-nucleotidase activities in rat liver plasma membrane. In the present HPLC system, the equilibration of columns was obtained after the passage of only about 10 column volumes of the mobile phase.

2. Experimental

2.1. Chemicals

Reagent-grade 2′-AMP, 3′-AMP, 2′5′-ADP and 3′,5′-ADP were purchased from Sigma (St. Louis, MO, USA). Adenosine (Ado), 5′-AMP, ADP and ATP were obtained from Wako (Japan). Adenosine 2′,3′-cyclic phosphate 5′-phosphate (ACPP) was prepared by the phosphorylation of Ado with pyrophosphorylchloride [12]. Adenosine 2′,3′-cyclic phosphate 5′-phosphosulfate (ACPPS) and adenosine 5′-phosphosulfate (APS) were prepared by the sulfation of ACPP and 5′-AMP with TEA *N*-sulfonate [13]. PAPS was prepared by treatment of ACPPS with ribonuclease T2 [14]. TEA and phosphoric acid (85 w/w%) were used without further purification. HPLC-grade methanol and acetonitrile were used. All other chemicals were of reagent grade.

2.2. HPLC column

All analyses were performed on a stainless-steel column (15×0.6 cm I.D.) packed with Capcell Pak C18 SG 120 (5 μm, Shiseido, Japan). A stainless-steel guard column (1.0×0.46 cm I.D.) packed with the same resin was used. Columns were packed as described previously [11].

2.3. HPLC apparatus and chromatographic conditions

A Nippon Bunko (Tokyo, Japan) high-performance liquid chromatograph consisting of a Model 880-PU pump and a UV-970 variable-wavelength detector set at 259 nm, and a Shimazu CR-4A electronic integrator (Kyoto, Japan) were used. The column was equilibrated with an eluent at a flow-rate of 1.0 ml/min for 25 min at ambient temperature (22–23°C) prior to sample injection. Unless otherwise indicated, the sample was eluted at the same flow-rate.

A solution consisting of 0.1 *M* TEA-phosphate buffer and methanol (95:5, v/v) was used as a standard eluent.

Retention times (t_R) were measured from the distance between the injection point and the peak maximum on the chromatogram. The mobile phase hold-up time (t_0) was measured by injection of potassium nitrate and/or water. The former was more retained than water below pH 7. Capacity factors (k') was calculated by the formula: $k'=(t_R-t_0)/t_0$.

2.4. Preparation of TEA phosphate buffer

Stock solutions of 0.1 *M* TEA and a mixture of 0.1 *M* TEA and 0.1 *M* phosphoric acid were filtered through a 0.45 μm millipore filter and kept at 4°C. The pH of TEA phosphate buffer was adjusted by admixing of both stock solutions at 23°C.

2.5. Adsorption of TEA on the packing material

The pH (4–9) of the standard eluent consisting of 0.1 *M* TEA-methanol (95:5, v/v) was adjusted prior to the addition of methanol. The reversed-phase column was equilibrated by the passage of 60 ml of the eluent at a flow-rate of 1 ml/min. The equilibrated column was then eluted with 60 ml of a mixture of methanol and acetonitrile (1:1, v/v) at the same flow-rate. The TEA concentration in the eluate was determined by a HPLC method developed newly in the present study as follows: an analytical column (15×0.46 cm I.D.) and a guard column (1.0×0.46 cm I.D.) packed with Capcell Pak CN (5 μm, Shiseido, Tokyo) were equilibrated with an eluent

consisting of 0.05 *M* ammonium hydroxide–acetonitrile–methanol (1.5:9:11, v/v) at a flow-rate of 1 ml/min. An aliquot of the sample (10 μl) containing TEA was injected into the column. TEA was detected at 220 nm.

As shown in Fig. 1, the measurement was completed within about 8 min after injection. A linear correlation between the peak area and the injected amount (1–20 nmol) was observed. The detection limit of TEA was ca. 0.02 nmol/ml.

2.6. Assays of adenine nucleotidase activities of rat liver plasma membrane vesicles

Liver plasma membrane vesicles were prepared from female Sprague–Dawley rats (220–250 g) according to the method Kobayashi et al. [15]. The incubation medium for the assay of Na^+, K^+-ATPase activity contained plasma membrane vesicles (ca. 50 μg protein), 5 m*M* ATP, 140 m*M* NaCl, 14 m*M* KCl, 5 m*M* $MgCl_2$, 2 m*M* ouabain and 40 m*M* Hepes-Tris (pH 7.4) in a final volume of 0.5 ml. The incubation medium for the assay of 5′-nucleotidase activity contained plasma membrane vesicles (ca. 50 μg protein), 5 m*M* 5′-AMP, 10 m*M* $MgCl_2$ and 100 m*M* glycine-NaOH (pH 9.1) in a final volume of 0.5 ml. The reaction was initiated by adding 50 μl of ATP or 5′-AMP stock solution (50 m*M*) to the incubation mixture preincubated for 5 min at 37°C. At appropriate times, aliquots of 50 μl of the reaction mixture were withdrawn and mixed with 50 μl of methanol to stop the reaction. After centrifugation at 15 000 *g* for 5 min at 20°C, 10 μl of supernatant was injected into the chromatograph.

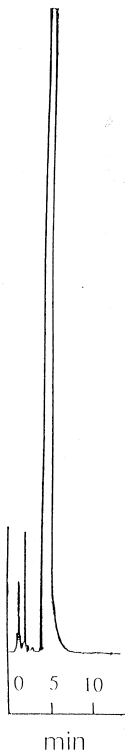

Fig. 1. HPLC elution pattern of triethylamine. The chromatographic conditions are given in Section 2. Injection of 10 μl of a standard solution containing 10 m*M* TEA in acetonitrile.

2.7. Reproducibility of the analytical method

The examination was performed only for 5′-AMP, ADP and Ado generated in the assays of Na^+, K^+-ATPase and 5′-nucleotidase activities. The nucleotides and nucleoside were quantitated from the peak area in the chromatogram. The limit of detection was defined as the amounts of the compounds resulting in a signal-to-noise ratio of 5. Within-day reproducibility of the analytical procedure was tested for the coefficient variation (C.V.) of peak areas for each compound. Three concentrations (5, 10 and 20 nmol/ml) of each compound were included in this study. Five aliquots of each sample were analyzed on the same day.

3. Results and discussion

3.1. TEA ion behaves as a counterion of adenine nucleotides in reversed-phase HPLC system

Previously, we have reported an ion-pair reversed-phase HPLC method for the separation of a number of water soluble mercaptides of sulfobromophthaleine (BSP) in which a simple tertiary amine, TEA, was used as a counterion of these anions [11]. In the present study, we further intend to demonstrate the usefulness of TEA as an ion-pairing reagent for reversed-phase HPLC separation of adenine nucleotides.

Fig. 2a shows the elution pattern of 5′-AMP, ADP and ATP injected into ‘normal’ reversed-phase col-

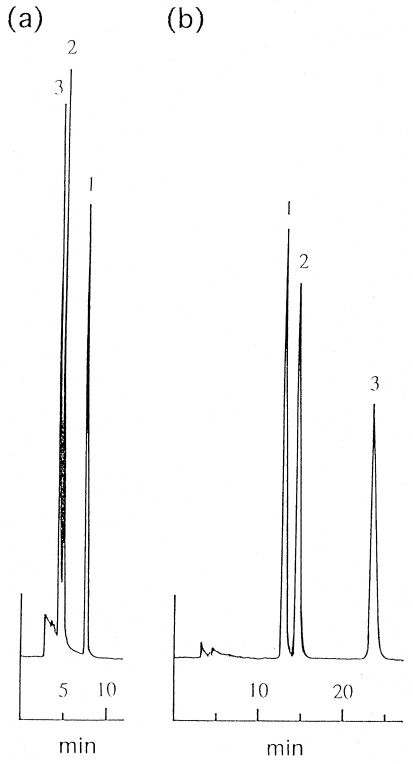

Fig. 2. Evidence that adenine nucleotides are separated as ion-pairs with TEA ion in the reversed-phase HPLC system. (a) Elution pattern of a standard mixture of nucleotides separated by a 'normal' reversed-phase system consisting of 0.1 *M* ammonium phosphate buffer (pH 5) and 5% methanol. (b) Elution pattern of a standard mixture of nucleotides separated by an 'ion-pair' reversed-phase system consisting of 0.1 *M* TEA phosphate buffer (pH 5) and 5% methanol. Injection of 10 μl of a solution containing 0.05 m*M* each of nucleotides. Peaks: 1=5′-AMP, 2=ADP, 3=ATP.

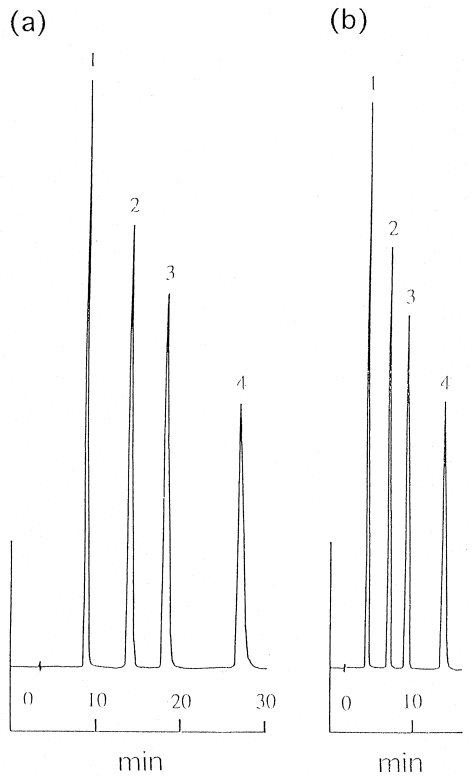

Fig. 3. HPLC elution pattern of a standard mixture of adenine compounds separated by a mobile phase consisting of 0.1 *M* TEA phosphate buffer (pH 8) and 5% methanol. (a) Flow-rate setting was 1 ml/min. (b) Flow-rate setting was 2 ml/min. Peaks: 1=5′-AMP, 2=ADP, 3=ATP, 4=Ado.

umn equilibrated with a mobile phase consisting of 0.1 *M* ammonium phosphate buffer (pH 5) and 5% (v/v) methanol. ATP was eluted first, followed by ADP and 5′-AMP. In contrast, the elution order was reversed in the same column equilibrated with 0.1 *M* TEA phosphate buffer (pH 5) containing 5% (v/v) methanol as shown in Fig. 2b. The drastic increase in the retention times of these nucleotides strongly supports the idea that TEA ion functions as a counterion for the nucleotides.

Fig. 3a shows that the HPLC separation of 5′-AMP, ADP, ATP and Ado in the 'ion-pair' reversed-phase system at pH 8 and a flow-rate of 1 ml/min, and Fig. 3b shows the separation at a flow-rate of 2 ml/min. The resolution of these nucleotides was more excellent at pH 8 than at pH 5 and accelerated as the flow-rate increased. The results shown in Figs. 2 and 3a indicate that the retention times of the compounds are differently affected by the pH of the mobile phase. These results prompted us to study a possible effect of the eluent pH on capacity factors of adenine compounds in the 'ion-pair' reversed-phase system.

It was found that the retention time of Ado decreased dramatically in the 'ion-pair' solvent system in comparison with a 'normal' solvent system consisting of 0.1 *M* ammonium phosphate buffer (pH 8)–5% (v/v)–methanol (ca. 27 vs. 41 min, data not shown). This strongly suggests that TEA ion of the mobile phase is adsorbed by the stationary phase and causes a change in the surface properties.

3.2. Influence of pH of mobile phase on capacity factor

Since Ado is a starting material for the chemical synthesis of PAPS and is also a product generated in the assay of 5′-nucleotidase activity, we included the nucleoside in the systematic study. Moreover, Ado can not form an ion-pair with TEA ion, and thus provides a standard for the solvent effects on the capacity factors of the nucleotides.

Fig. 4 shows the influence of pH on the k' values of 5′-AMP, ADP, ATP and Ado. The pH values shown in Fig. 4 were of 0.1 *M* TEA phosphate buffer before the mixing with 5% of methanol. The results indicate that the resolution of these compounds can be regulated by adjusting the pH of eluent.

The capacity factors of these compounds increased gradually when the pH increased from 4.0 to 5.5. Since the apparent pK_a value of the adenine moiety is 3.65 for Ado, 3.74 for 5′-AMP, 3.95 for ADP and 4.00 for ATP, respectively [16], all of the compounds are partly ionized within the pH range examined. Assuming that the acidic groups in each nucleotide form a neutral complex with TEA ion, the results can be explained by the fraction of un-ionized adenine groups at each pH. Fig. 5 illustrates the relationship between the k' and the apparent fraction (%) of the un-ionized adenine moiety in the mobile phase. The percentage of un-ionized form was calculated from the apparent pK_a of each compound at intervals of 0.5 pH unit over a range of pH 4.0–5.5. A good linear relationship between the k' and the % was observed within the range of pH 4.0–5.5 for Ado and ADP and pH 4.0–5.0 for 5′-AMP and ATP, respectively. These findings provide strong evidence that the primary phosphate groups (pK_a=ca. 1.0) on the adenine nucleotides [16] are neutralized by the complex formation with TEA ion in the mobile phase and that the dissociation of adenine moiety is one of the important factors controlling the retention of these compounds at this acidic pH range.

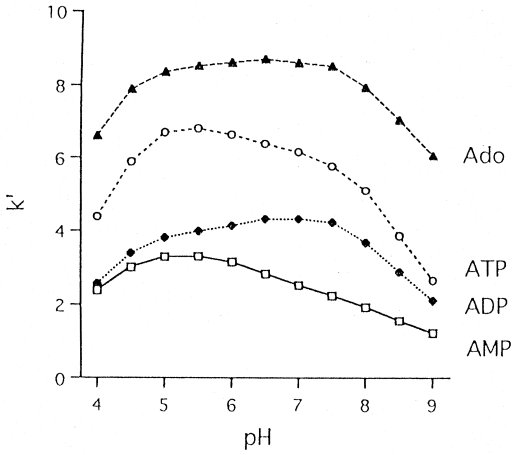

Fig. 4. Influence of pH on the capacity factors of adenine compounds. Other conditions are identical as shown in Fig. 3a. All data points represent the average of three injections.

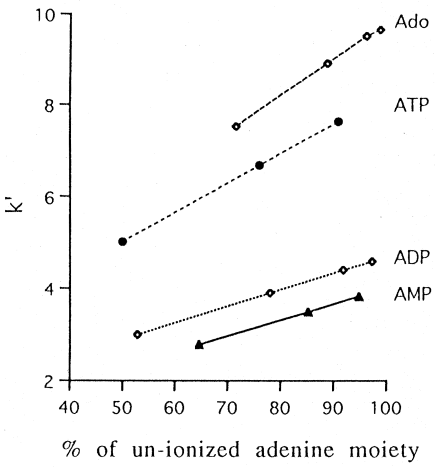

Fig. 5. Correlation between k' values and percentage of un-ionized adenine moiety. The k' values shown in Fig. 4 were used in this plot. The calculation of the unionized fraction of adenine moiety is detailed in the text.

The k' values of 5′-AMP and ATP decreased gradually at pH higher than 6. In contrast, the retention of Ado and ADP increased consecutively until pH 7.5. This interesting phenomenon remains to be investigated. The k' values of all the compounds drastically decreased at a pH higher than 8. This may be explained by the extensive adsorption of TEA ion at alkaline pH on the stationary phase. Thus, it is probable that TEA ion will compete with the TEA complex of nucleotide for the available adsorption sites, if these species are assumed to take up the same area of the stationary phase.

Fig. 6 shows the influence of the eluent pH on the k' values of ACPP, 2′,5′-ADP and 3′,5′-ADP which are important intermediates occurring in the chemical synthesis of PAPS from Ado. The results indicate

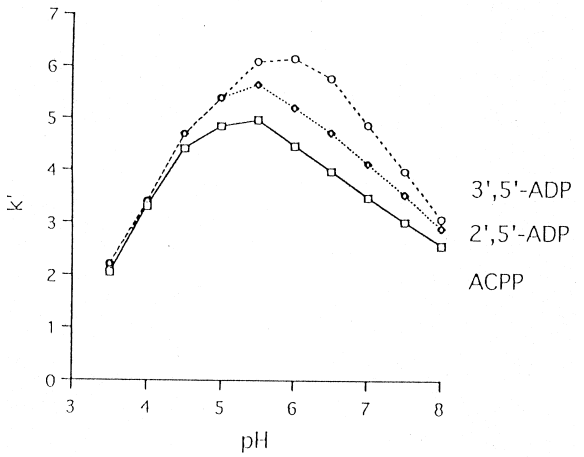

Fig. 6. Influence of pH on the capacity factors of ACPP, 2′5′-ADP and 3′,5′-ADP. The conditions are identical as shown in Fig. 4. All date points represent the average of three injections.

that excellent resolution of these nucleotides can be obtained at around pH 6.5.

Fig. 7 shows a typical HPLC separation of a standard mixture containing PAPS and several nucleotides closely associated with each other in the biological synthesis of PAPS and its decomposition [26]. The separation was carried out at pH 7.5 by the use of the standard eluent.

Table 1 shows the retention data for eleven adenine compounds in the eluent systems consisting of 0.1 *M* TEA phosphate buffer (pH 6.5 or 8.0) and 5% methanol. The results suggest that the polarity of the nucleotides is one of the important factors affecting their retention order [3,9]. APS, ACPPS and PAPS are the sulfated derivatives of 5′-AMP, ACPP and 3′,5′-ADP, respectively. The retention times of these sulfated nucleotides were longer than those of their respective parent nucleotides. Since the pK_a values of sulfated compounds may be less than those of phosphates, the sulfated nucleotides probably more readily and extensively combine with TEA ion as compared to their parent nucleotides.

3.3. Adsorption of TEA ion on the packing material

We examined the adsorption of TEA ion on the stationary phase using the standard eluent containing 5% (v/v) of methanol.

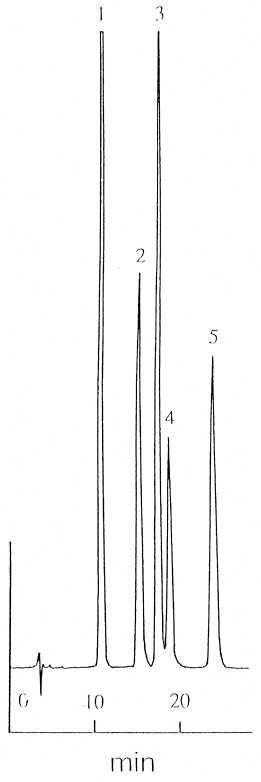

Fig. 7. HPLC elution pattern of a standard mixture of PAPS and related nucleotides. Eluent: 0.1 *M* TEA phosphate buffer (pH 7.5) and 5% methanol. Peaks: 1=5′-AMP (1 nmol), 2=3′,5′-ADP (0.65 nmol), 3=APS (1.1 nmol), 4=PAPS (0.55 nmol), 5=ATP (0.7 nmol).

Fig. 8a shows a plot of the amount of TEA adsorbed on the stationary phase as a function of pH of the mobile phase. As expected from the apparent pK_a (10.7) of TEA, the adsorption of TEA species increased dramatically when the pH of the buffer increased from 8.0 to 9.0. The result well supports the idea that the extensive adsorption of TEA species brings about the decrease of the capacity factors of adenine compounds at alkaline pH as shown in Fig. 4.

Fig. 8b shows the reciprocal plot of the amount of TEA species adsorbed as a function of TEA concentration in the mobile phase. Fairly good linearity was observed over the concentration range of TEA examined here. The monolayer capacity of the stationary phase for TEA species can be calculated from the intercept of the curves in Fig. 8b and the

Table 1
Retention data of adenine nucleoside and nucleotides

Compounds	pH 6.5 (t_0 = 3.00 min)		pH 8.0 (t_0 = 3.02 min)	
	t_R (min)	k'	t_R (min)	k'
5′-AMP	11.9	2.97	9.0	1.98
3′-AMP	29.0	8.65	25.9	7.58
2′-AMP	57.0	18.00	43.1	13.27
5′-ADP	18.1	5.03	14.4	3.77
5′-ATP	25.7	7.57	18.7	5.19
2′,5′-ADP	14.6	3.88	11.4	2.77
3′,5′-ADP	17.2	4.73	12.0	2.97
ACPP	15.6	4.20	10.4	2.44
APS	17.3	4.78	11.6	2.87
PAPS	19.9	5.63	14.3	3.74
ACPPS	21.7	6.23	15.7	4.20
Ado	32.3	9.77	27.2	8.01

Eluent: 0.1 *M* TEA phosphate buffer–methanol (95:5, v/v); flow-rate: 1 ml/min at 22–23°C. Data represent means of three injections.

apparent binding constant of the counterion to the stationary phase is obtained from the slope. The results are shown in Table 2.

3.4. Regulation of capacity factor with organic modifier

It is well known that in reversed-phase HPLC, a linear relationship between the logarithm of capacity factor and the percent of organic modifier in the mobile phase exists over a limited range of the concentration [17,18]. Since the TEA-nucleotide complex can be regarded as a kind of hydrophobic compound, we speculated that such a logarithmic relationship might exist also between the capacity factors of adenine nucleotides in the present HPLC system and the concentration of methanol or other modifier in the eluent.

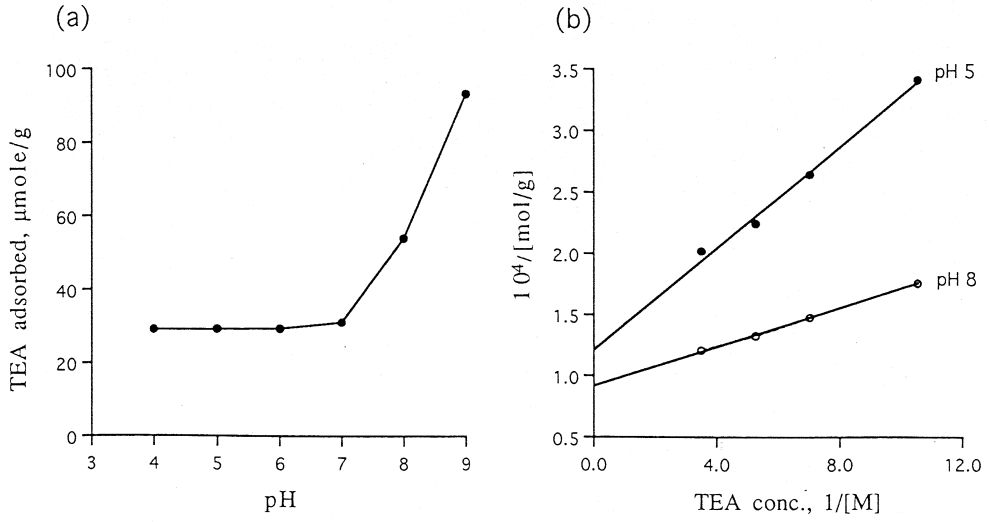

Fig. 8. Adsorption of TEA ion on the solid phase. (a) Influence of pH on the adsorption of TEA ion. (b) Reciprocal plot of the amount of TEA ion adsorbed as a function of TEA concentration in the eluent. The conditions are detailed in Section 2.

Table 2
Adsorption of triethylamine ion on the stationary phase

pH of buffer	Methanol % v/v	Adsorption capacity[a] mmole/g	Binding constant[a] $[M]^{-1}$
5.0	5	82.5	5.8
8.0	5	108.7	11.5

[a] Values were obtained from the data shown in Fig. 8b.

Fig. 9 shows a plot of the data for six adenine compounds of interest over a range of 3–10% (v/v) of methanol in the standard eluent. Since the retention times of 2′-AMP and 3′-AMP were very long (ca. 60 min for 2′-AMP) in the standard eluent of pH 6.5, we carried out the experiments for these AMP isomers only at pH 8.0. As shown in Fig. 9a,b, excellent linearity was observed over this range at both pHs, with the correlation coefficients for all six compounds being better than 0.995.

Contrary to the previous results [3], the elution order of the 2′ and 3′ isomers were reversed in the present system, as shown in Fig. 9b. Assuming that the isomers also behave as neutral complexes with TEA under the present conditions, it seems possible that the observed eluent order might be regulated by a factor other than the polarity such as stacking interaction [3,20].

Fig. 10 shows the effect of acetonitrile added to the standard eluent consisting of 0.1 *M* TEA phosphate buffer (pH 6.5 or 8.0) and 5% methanol on the $\ln k'$ of Ado and three adenine nucleotides. As expected, the capacity factors of all the compounds were dramatically reduced by the addition of very small amounts of acetonitrile. Over a range of 0.249–1.48% (v/v) (corresponding to 0.25–1.5 ml of acetonitrile added to 100 ml of the standard eluent), excellent linearity between the $\ln k'$ and the percent

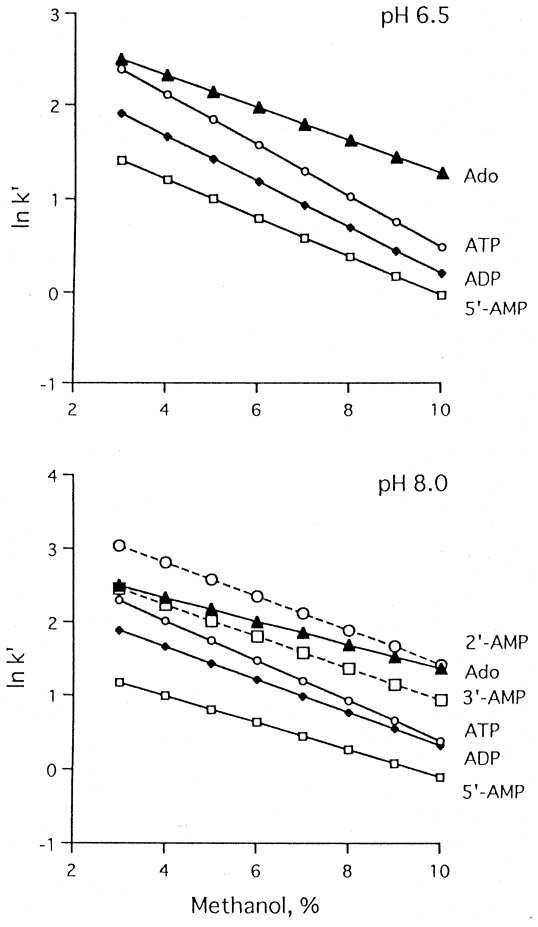

Fig. 9. Dependence of $\ln k'$ values on the concentration of methanol. All data points represent the average of three injections.

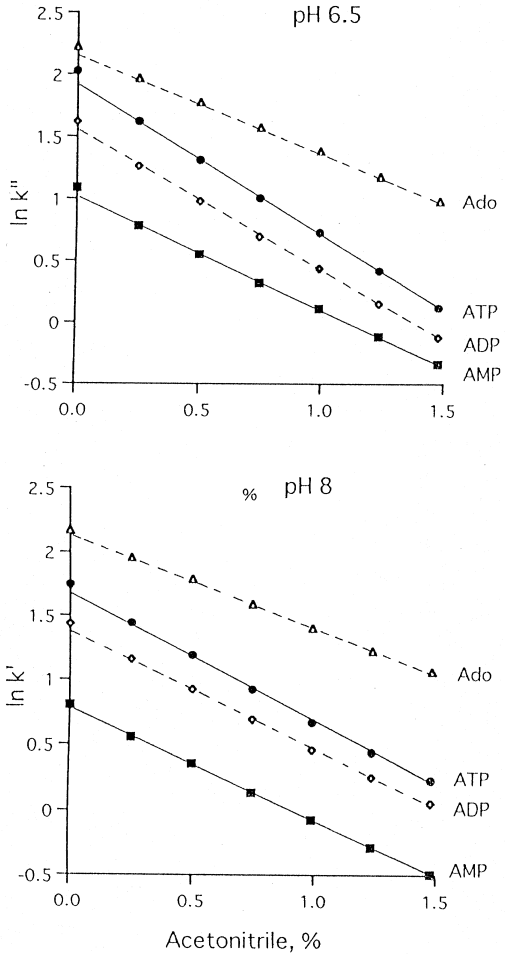

Fig. 10. Dependence of $\ln k'$ values on the concentration of acetonitrile. All data points represent the average of three injections.

of the modifier was observed for four compounds examined.

Although the logarithmic relationship between the k' values and the concentration of organic modifier would not be theoretically reasonable [19], the relationship has been employed as a useful parameter in the optimization of solvent composition to provide maximum resolution [18]. We applied the logarithmic relationship for the selection of eluate composition in the assays of ATPases and 5′-nucleotidase activities in rat liver plasma membrane.

As illustrated in Fig. 11a,b, the separation system allowed complete resolution of 5′-AMP, ADP and ATP included in the assay mixture. Fig. 11c shows the HPLC pattern of the standard mixture of Ado and three adenine nucleotides separated with the same eluent adopted for the assay of 5′-nucleotidase. Fig. 11d illustrates the separation of the substrate, 5′-AMP, and the product, Ado, in the assay mixture of 5′-nucleotidase.

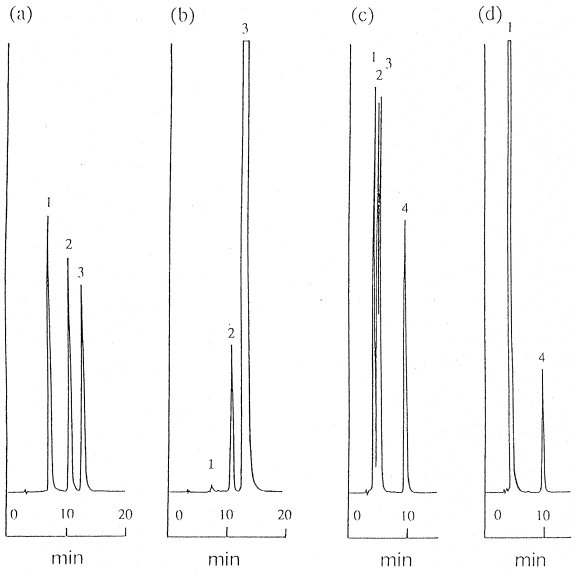

Fig. 11. HPLC separation of adenine compounds by the solvent systems adopted for the assays of ATPases and 5′-nucleotidase activities. Eluent for the assay of ATPases used in (a) and (b): 0.1 *M* TEA phosphate buffer (pH 8)–methanol–acetonitrile (95:5:0.5, v/v); eluent for the assay of 5′-nucleotidase used in (c) and (d): 0.1 *M* TEA phosphate buffer (pH 8)–methanol–acetonitrile (95:5:2, v/v). (a) and (c) are the elution patterns of standard mixtures. (b) and (d) are the separation of incubation mixtures. Peaks; 1=5′-AMP, 2=ADP, 3=ATP, 4=Ado.

A linear correlation between peak area and injected amount (10–500 pmol) was observed for all the compounds. The lowest 5′-AMP, ADP or Ado amount detectable was ca. 5 pmol. The reproducibility of peak areas was directly related to the precision of the injection. The average variation in peak areas was about 2% for all compounds.

3.5. Influence of TEA concentration on capacity factor

The effect of TEA concentration in the mobile phase containing 5 v/v % of methanol on the

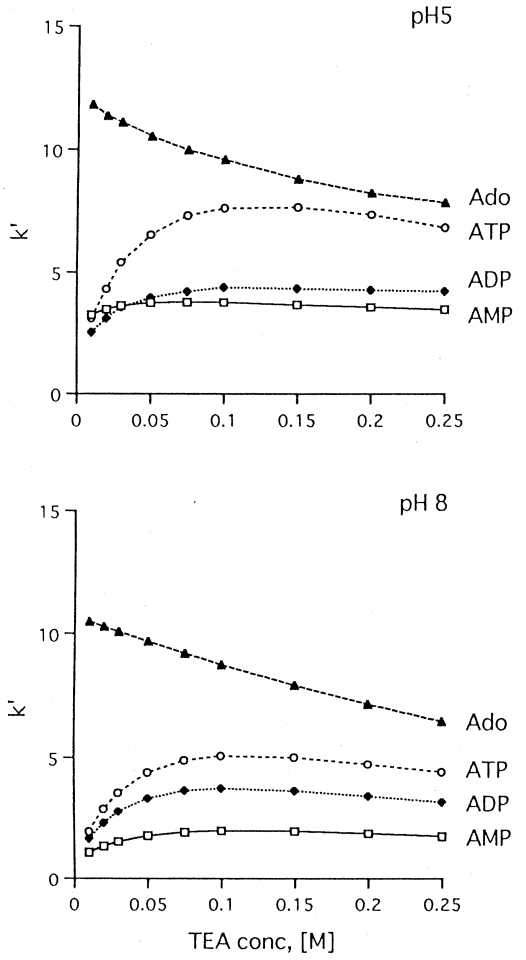

Fig. 12. Dependence of k' values on the TEA concentration. All data points represent the average of three injections.

retention of adenine nucleotides and Ado was investigated at pH 5.0 and pH 8.0.

Fig. 12 shows the capacity factors of four adenine compounds as a function of TEA concentration. The results show that the retention of these compounds can be also regulated by adjusting the TEA concentration in the mobile phase.

The retention of Ado gradually decreased with increasing TEA concentration at both pHs. A linear relationship ($r=0.995$) existed between the $\ln k'$ values for Ado and the TEA concentration over a range of 0.01–0.25 *M*, especially at pH 8 (data not shown). The results may be explained by solvophobic effect [21] of the TEA ion in the buffer as an organic modifier. That is, an increasing adsorption of THA ion onto the hydrophobic ligands may cause a decrease in the capacity of the stationary phase to take up Ado.

On the other hand, the k' values of the nucleotides initially rose to a maximum from which it gradually declined. The fall in the k' of the nucleotide at higher concentrations of TEA may be also explained by the solvophobic effect of TEA ion in the eluent.

3.6. Retention mechanism of adenine nucleotides

The parabolic dependence of the k' values of adenine nucleotides on TEA concentration found in this study is qualitatively similar to the results found by other workers [5,22–24]. They used tetrabutylammonium [5], cetyl trimethylammonium [22], *n*-decylsulfate [23] and tetrapentylammonium [24] as a counterion for the separation of nucleotides, aryl sulphonic acids, catecholamines and aryl carboxylic acids, respectively. Both the groups of Horvath et al. [23] and Melin et al. [24] proposed their respective

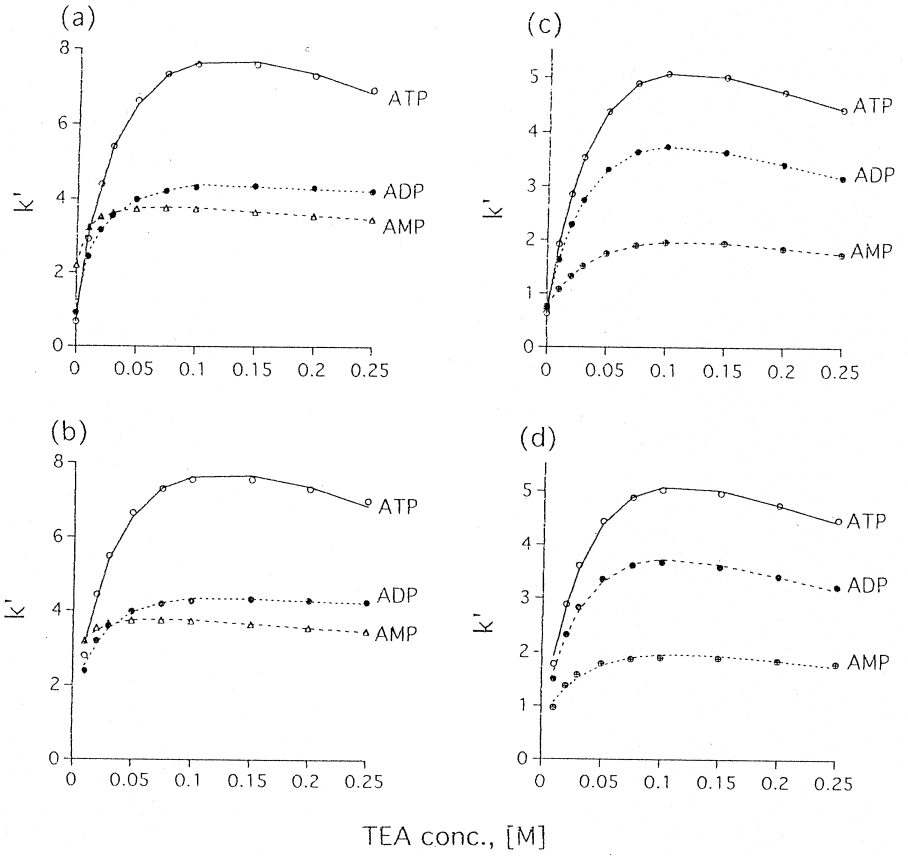

Fig. 13. Illustration of the fitting of the data shown in Fig. 12 to the models of Eqs. (1) and (2). (a) and (b): the fitting of the data obtained at pH 5 to Eqs. (1) and (2), respectively. (c) and (d): the fitting of the data obtained at pH 8 to Eqs. (1) and (2), respectively. Lines represent the observed values. Circles show the values obtained from least-squares fit of the data to Eqs. (1) and (2).

retention models and explained their observations by a combination of following three mechanisms: (1) ion-pair formation between the sample ion and the counterion bound to the stationary phase, (2) ion-pair formation in the mobile phase, and (3) binding of the complex to the stationary phase.

Horvath et al. [23] proposed the following equation to describe the dependence of the k' on the counterion concentration:

$$k' = (k_0 + B[\mathrm{H}])/(1 + K_2[\mathrm{H}])(1 + K_3[\mathrm{H}]) \quad (1)$$

where k_0 is the capacity factor of the sample in the absence of counterion in the mobile phase, K_2 is the association constant for the sample and the counterion, K_3 is the binding constant of the counterion to the stationary phase, [H] is the counterion concentration in the mobile phase, and B is the product of the two constants related to the character of column and the two equilibrium constants, which is given by $\phi[\mathrm{L}]K_2K_4$, $\phi[\mathrm{L}]K_3K_5$ or $\phi[\mathrm{L}]K_1K_6$. ϕ is the ratio of the volume of stationary phase to the volume of the mobile phase, [L] is the total monolayer capacity of the stationary phase, K_1 and K_4 are the binding constants of the sample and the complex to the stationary phase, respectively, K_5 is the association constant for the sample and the counterion already bound to the stationary phase, and K_6 is the association constant for the counterion and the sample ion already bound to the stationary phase.

A plot of k' vs. [H] according to Eq. (1) (corresponds to Eq. 13 in [22]) yields a parabolic retention curve.

As the second approach, if we assume that all of the sample ions introduced into the chromatographic system are distributed to the stationary phase as the complex with the counterion, then we can neglect the k_0 in Eq. (1) and simplify the equation to:

$$k' = B[\mathrm{H}]/(1 + K_2[\mathrm{H}])(1 + K_3[\mathrm{H}]) \quad (2)$$

Assuming that the inorganic counterion species, phosphate ions, can bind to the stationary phase only as the ion-pair with the organic counterion, Eq. (2) is identical with Eq. (24) proposed by Melin et al. [24].

In order to test the validity of Eqs. (1) and (2) and to estimate the apparent equilibrium constants, the data shown in Fig. 12 were analyzed by a nonlinear least-square fit. The computer program used for the fitting procedure was a MULTI written by BASIC language [25]. The algorithm used for the fitting was the Damping Gauss Newton Method [25].

The k_0 values used in the fitting of the data to Eq.

Table 3
List of the parameters of Eqs. (1) and (2), which are evaluated from the data shown in Fig. 12

	K_2 $[\mathrm{M}]^{-1}$	K_3 $[\mathrm{M}]^{-1}$	B $[\mathrm{M}]^{-1}$	k_0
(a) The parameters of Eq. (1)				
pH 5.0				
5′-AMP	109±10[a]	1.3±0.1[a]	445±47[a]	2.20
ADP	55±5	1.3±2.1	287±15	0.90
ATP	21±1	3.3±0.2	295±13	0.66
pH 8.0				
5′-AMP	6.9±7.4	6.7±7.2	48±0.4	0.76
ADP	8.5±12	8.4±12	120±0.7	0.72
ATP	9.1±0.4	7.4±0.3	162±0.3	0.63
(b) The parameters of Eq. (2)				
pH 5.0				
5′-AMP	376±46	0.6±0.1	1531±170	
ADP	94±11	0.5±0.2	463±38	
ATP	31±4	2.2±0.5	373±24	
pH 8.0				
5′-AMP	67±12	1.2±0.4	164±19	
ADP	34±5	2.8±0.5	206±13	
ATP	25±0.5	3.2±0.5	229±10	

[a] The standard deviations of parameters calculated by the program.

(1) were obtained from the experiments in which the normal reversed-phase systems consisting of 0.01 *M* ammonium phosphate buffer (pH 5.0 or 8.0) and methanol (95:5, v/v) were used. The goodness of fit was judged by the plot of the observed and calculated values as shown in Fig. 13. Table 3 shows the apparent equilibrium constants calculated by the computer. Since Eqs. (1) and (2) have a weakness that the values obtained for K_2 and K_3 can not be readily distinguished from each other, we judged them by the measured apparent values for K_3 shown in Table 2. We have assumed that the values of K_3 for the binding of TEA ion to the stationary phase are smaller than those of K_2 for the association of nucleotides and TEA ion.

As shown Fig. 13, both Eqs. (1) and (2) showed a good fitness to the data for pH 5 and pH 8. Judging from visual inspection, however, the retention data were found to conform better to Eq. (1) than Eq. (2). This implicated that we can not neglect the k_0, i.e., the equilibrium between the sample ion and the stationary phase under the present HPLC system, especially at the low concentration ranges of TEA ion.

Although these phenomenological approaches cannot specify each item in parameter *B*, the present observations strongly support the idea that the retention of adenine nucleotides in this system can occur by a combination of 'dynamic ion-exchange' mechanism, i.e., the ion-pair formation taking place between the sample ion and the counterion bound to the stationary phase, and the ion-pair formation in the mobile phase and binding of the complex to the stationary phase.

References

[1] R.A. Hartwick, S.P. Assenza, P.R. Brown, J. Chromatogr. 186 (1979) 647.
[2] A. Stocchi, L. Cucchiarini, M. Magnani, L. Chiarantin, P. Palma, G. Crescentini, Anal. Biochem. 145 (1985) 118.
[3] D.L. Ramos, A.M. Schoffstall, J. Chromatogr. 261 (1983) 83.
[4] M. Hill, A. Dupaix, P. Volfin, A. Kurkdjian, B. Arrio, Methods in Enzymology, 148 (1987) 132–141, Academic Press, New York.
[5] D.R. Webster, G.D. Boston, D.M. Patno, J. Liq. Chromatogr. 8 (1985) 603.
[6] O.C. Ingebreten, A.M. Bakken, J. Chromatogr. 242 (1982) 119.
[7] G.H.R. Rao, J.D. Peller, K.L. Richards, J. McCullough, J.G. White, J. Chromatogr. 229 (1982) 205.
[8] E. Juengling, H. Kammermeier, Anal. Biochem. 102 (1980) 358.
[9] J.D. Schwenn, H.G. Jender, J. Chromatogr. 193 (1980) 285.
[10] T. Uematsu, R.J. Suhadolnik, J. Chromatogr. 123 (1976) 347.
[11] K. Sano, I. Kinoshita, R. Mihara, Y. Ikegami, T. Uesugi, J. Chromatogr. 579 (1992) 63.
[12] J. Tomasz, A. Simoncsits, J. Carbohydr. Nucleosides Nucleotides 2 (1975) 315.
[13] R. Cherniak, E.A. Davidson, J. Biol. Chem. 230 (1964) 2986.
[14] J.P. Horwitz, J.P. Neenana, R.S. Misra, J. Zozhin, A. Huo, K.D. Phlips, Biochim. Biophys. Acta 480 (1977) 379.
[15] K. Kobayashi, Y. Sogame, H. Hara, K. Hayashi, J. Biol. Chem. 265 (1990) 7737.
[16] J.D. Smith, Methods in Enzymology, 112 (1967) 350, Academic Press, New York.
[17] H. Colin, N. Ward, G. Guiochou, J. Chromatogr. 149 (1978) 169.
[18] R.A. Hartwick, C.M. Grill, P.R. Brown, Anal. Chem. 51 (1979) 34.
[19] R.P.W. Scott, Chem. Anal. 98 (1980) 117.
[20] P.R. Brown, E. Grushka, Anal. Chem. 52 (1980) 1210.
[21] C. Horvath, W. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[22] J.H. Knox, G.R. Laird, J. Chromatogr. 122 (1976) 17.
[23] C. Horvath, W. Melander, I. Molnar, P. Molnar, Anal. Chem. 49 (1977) 2295.
[24] A. Tilly-Melin, Y. Askemark, K.-G. Wahlund, G. Schill, Anal. Chem. 51 (1979) 976.
[25] K. Yamaoka, Y. Tanigawa, Y. Nakagawa, T. Uno, J. Pharmacobio-dyn. 4 (1981) 879.
[26] M.L. Tsang, J. Lemieux, J.A. Schiff, T.B. Bojarski, Anal. Biochem. 74 (1976) 623.