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Separation of nucleotides using micellar electrokinetic capillary chromatography

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Abstract

The effect of the pH of the background electrolyte and its ionic strength, via Good's buffers, in capillary zone electrophoresis (CZE) on the separation of mono-, di-, triphosphate, adenosine cyclic- monophosphate nucleosides and adenine was investigated. Micellar electrokinetic capillary chromatography (MEKCC) using a cationic surfactant such as dodecyltrimethylammonium bromide offers better separation and shorter analysis than the sodium dodecyl sulfate micellar system. Extension of the elution range could be performed by decreasing the pH and efficiency was improved by adding ethylenediaminetetraacetic acid to the mixture sample reaching 130 000 and 60 000 theoretical plates for c-AMP and GDP, respectively.

Keywords: Nucleotides

1. Introduction

Capillary electrophoresis (CE) has been significantly improved by using small diameter columns [1,2], since it was first described as free solution electrophoresis in capillaries [3]. Separation by capillary zone electrophoresis (CZE) based on differences in the electrophoretic mobility of solutes could be performed with high speed, high efficiency, great mass sensitivity, low sample loading and high resolution [4–7]. Micellar electrokinetic capillary chromatography (MEKCC) is a subtechnique of CE which employs micellar solubilization and electrophoretic migration for separation of solutes [8,9].

MEKCC successfully allows separations where CZE failed, especially for neutral substances of similar structure [10,11]. The common micellar system used in most of the reports was with the anionic surfactant sodium dodecyl sulfate (SDS) which forms micelles with a negative surface charge resulting in an electrophoretic mobility which opposes to the electroosmotic flow (EOF) [9]. Reports on separation using a cationic micellar system have demonstrated that above certain concentrations of surfactants such as cetyltrimethylammoium bromide (CTAB) [12,13] and dodecyltrimethylammonium bromide (DTAB) [10,13], reversal of EOF occurs resulting in a change of polarity of the system. Capillary isotachophoresis (ITP) has long been used for nucleotides because it contributes to efficient separations by concentrating each substance into its ITP zone [14–17]. Recently, Tsuda [18] used high voltage and a phosphate buffer containing 0.5% ethylene glycol for separation of nucleotides in rat

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blood, liver and kidney. Cohen et al. [19] succeeded in retention manipulation of bases, nucleosides and oligonucleotides using a SDS micellar system and adding divalent metals such as Cu(II), Zn(II) and Mg(II) into the buffer. Row et al. [20] also used the SDS micellar system for the separation of normal and modified deoxyribonucleosides and deoxyribomononucleosides. Khur and Yeung [21] could improve the detection limit of nucleotides to the range of 50–100 a*M* of sample injected using indirect fluorescence detection. Gross and Yeung [22] controlled the EOF rate via the buffer concentration to separate nucleoside phosphates. Dolnik et al. [23] and Liu et al. [24] examined the effect of the pH, the ionic strength of the background electrolyte, the addition of anionic and cationic surfactants such as SDS, CTAB, DTAB or amines such as spermine into the buffer on the separation of oligonucleotides, polycitidines and phosphate nucleosides. Huang et al. [25] demonstrated the quantification of ribonucleotides from base-hydrolyzed RNA using CZE and the CTAB micellar system.

Because of their vital role in cells bioenergetics and hormonal regulation systems, metabolites and related nucleotides of the common mono-, di-, and triphosphate nucleosides have often been of particular interest. We report here the effect of the pH and ionic strength of the buffer via Good's buffers on separation of the common nucleoside phosphates, cyclic adenosine monophosphate and adenine in CZE. In this report, we were able to increase the elution range and improve the efficiency of solutes using anionic (SDS), cationic (DTAB) micellar systems by adding ethylenediaminetetraacetic acid (EDTA).

2. Experimental

2.1. Apparatus

A CE system (Model CE-800) by Jasco (Tokyo, Japan) was used throughout in this study. The system was composed of a high-voltage power supply (Model 890-CE), an intelligent UV–Visible detection system (Model 875-CE, operated at the wavelength of 254 nm throughout the experiments), and an integrator (Model 807-IT) for data processing. The effective capillary length was 50 cm. The on-column optical detection was performed by removing the polyimide coating from a short segment (5 mm) of the fused-silica capillary and placing it in the optical path of the detector.

2.2. Materials and reagents

Fused-silica capillary of 50 μm I.D. and 370 μm O.D. was purchased from GL Sciences (Tokyo, Japan). All the nucleotide standards were obtained from Sigma (St. Louis, MO, USA) and adenine, from Wako (Tokyo, Japan). SDS, CTAB, DTAB, disodium hydrogenphosphate, lithium acetate, sodium formate, high-grade sodium and lithium hydroxide and Sudan III were also from Wako. Tris(hydroxymethyl) aminomethane (Tris) was from Nacalai Tesque (Kyoto, Japan). Good's buffers: 2-(N-morpholino) ethanesulfonic acid (MES), Tris(hydroxymethyl) methylglycine (Tricine), 2-(cyclohexylamino) ethanesulfonic acid (CHES), 3-(cyclohexylamino) propanesulfofiic acid (CAPS) and EDTA were from Dojindo Laboratories (Kumamoto, Japan). The pH of Good's buffer was adjusted with high-grade sodium or lithium hydroxide. Standard 1 mg/ml solutions of nucleotides were prepared and stored at −20°C until used. Water was purified using a Milli-Q system and buffers were filtered through 0.45 mm filter units (Millex-HV) from Millipore (Bedford, MA, USA).

2.3. Procedures

Sample injection was carried out manually by elevating the inlet end of the capillary above 15 cm for 30 s. Approximately, 1 nl of sample was injected by using this method. Migration times of an unretained solute, t_0, and that of a micelle, t_{mc}, were determined by using phenol and Sudan III according to Terabe et al. [8,9], where pK_a values of both compounds are 10.0 so that the migration times at pH higher than 8.0 were apparent. The capillary was washed between each analysis with water for 3 min, 0.2 *M* sodium hydroxide for 5 min, water again for 20 min, buffer for 3 min and run for 15 min in order to stabilize the EOF and ensure reasonable reproducibility. The number of theoretical plates for each solute was calculated according to the equation:

N.T.P=$6.28(60 \cdot t_{Ri} \cdot h_i)/A_i$, where t_{Ri} is the migration time of the solute i, h_i, the height of the peak and A_i, the area.

3. Results and discussion

3.1. Effect of pH on nucleoside phosphates and adenine separation by CZE

In order to investigate the influence of the pH of the background electrolyte, Good's buffer such as Tricine–Na (pH 8.1), CHES–Na (pH 9.3), CAPS–Li (pH 10.4) and Tris–Na_2HPO_4 (pH 7.0) were used. Even though the electrophoretic mobility of anionic substances, such as nucleotides, was opposed to the EOF, the migration was from the anode to the cathode. Separation velocities of nucleotides (mono-, di-, triphosphate) did not change significantly in the pH range from 7.0 to 8.1 as shown in Fig. 1. At pH higher than 9.3, resolution among monophosphates decreased as well as di- and triphosphates. Monophosphates migrated faster than diphosphates, which migrated faster than triphosphates. Furthermore, in all three groups, the order of migration was: guanidyl-, adenosyl-, cytidyl- and uridylphosphates for pH 7.0 to 8.1: adenosyl-, guanosyl-, cytidyl- and uridylphosphates for pH 9.3 and cytidyl-, adenosyl-, guanosyl- and uridylphosphates for pH 10.4. Uridyldiphosphate was the exception in the elution order at pH 9.3, probably because of its higher pK_a values than the other members of the structural groups.

3.2. Effect of ionic strength on nucleoside phosphates and adenine separation by CZE

CAPS–Li was chosen to illustrate the influence of the ionic strength of the buffer on the nucleoside phosphates separation. The selectivity of the separation increased with its ionic strength up to 100 m*M*; above this concentration a stabilization occurs as shown in Fig. 2. Increasing the concentration of disodium hydrogenphosphate in the background electrolyte of Tris–Na_2HPO_4 did not improve the separation (data not shown), but, conversely, did lead to a broadening of peaks probably due to temperature elevation in the capillary provoked by higher current intensities. Both for CHES–Na and Tricine–Na background electrolytes, selectivity was improved with longer migration times up to 100 m*M* but not enough to achieve a complete separation. At higher concentrations, resolution rather decreased.

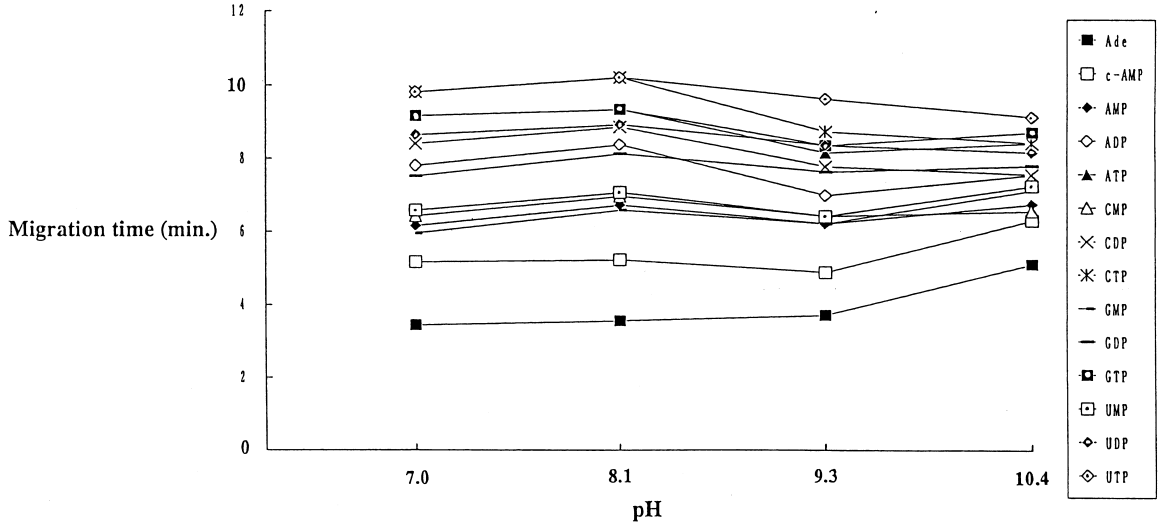

Fig. 1. Separation of adenine and nucleoside phosphates by CZE: influence of background electrolyte pH on migration time. Capillary: 70 cm (separation length 50 cm)×50 μm I.D. Voltage: 25 kV. Background electrolytes: 20 m*M* Tris, 10 m*M* Na_2HPO_4 (pH 7.0), 100 m*M* Tricine–Na (pH 8.15), 100 m*M* CHES–Na (pH 9.3), 150 m*M* CAPS–Li (pH 10.4). Concentrations of the samples were all 1 mg/ml.

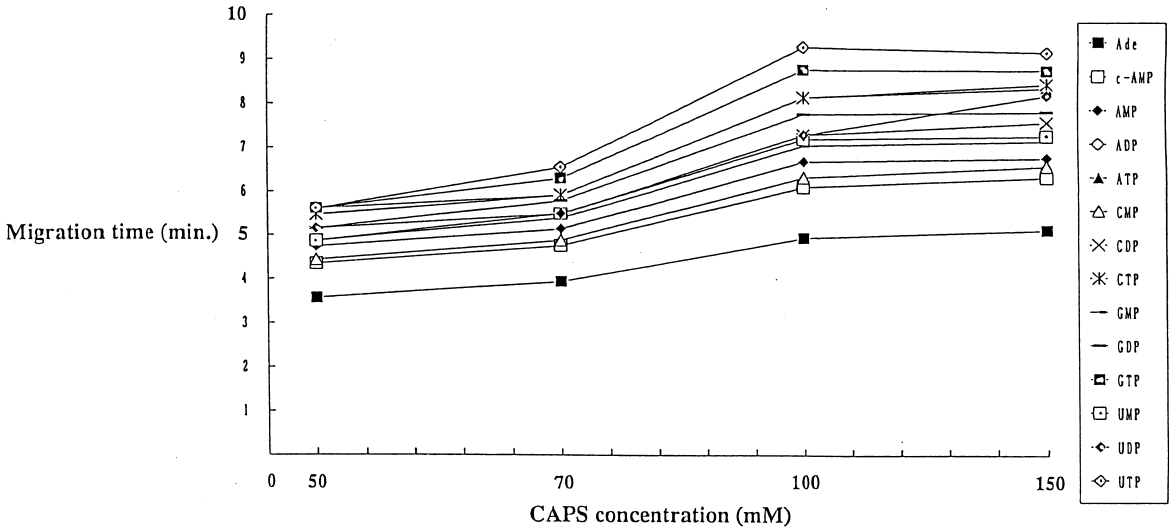

Fig. 2. Influence of ionic strength of background electrolyte on migration times of adenine and nucleoside phosphates: background electrolyte: CAPS–Li (pH 10.4). Other conditions as in Fig. 1.

3.3. MEKCC of nucleoside phosphates and adenine

3.3.1. Separation at neutral pH

Separation of nucleoside phosphates and adenine in the presence of surfactants was investigated next. Including an anionic surfactant such as SDS in the background electrolyte did not change the direction of the migration velocity but decreased its intensity. This was most likely due to a higher electrophoretic mobility of the SDS micelle–solute complex. No substantial changes occurred on the separation by

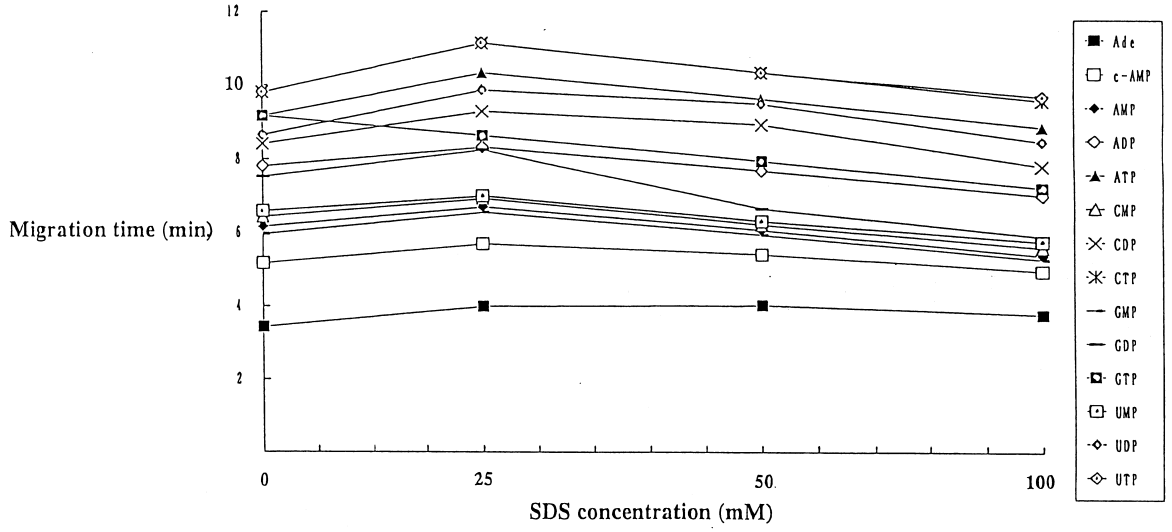

Fig. 3. Separation of adenine and nucleoside phosphates by MEKCC: influence of SDS concentration on migration times. Background electrolyte: 20 m*M* Tris, 10 m*M* Na_2HPO_4 (pH 7.0). Other conditions were as in Fig. 1.

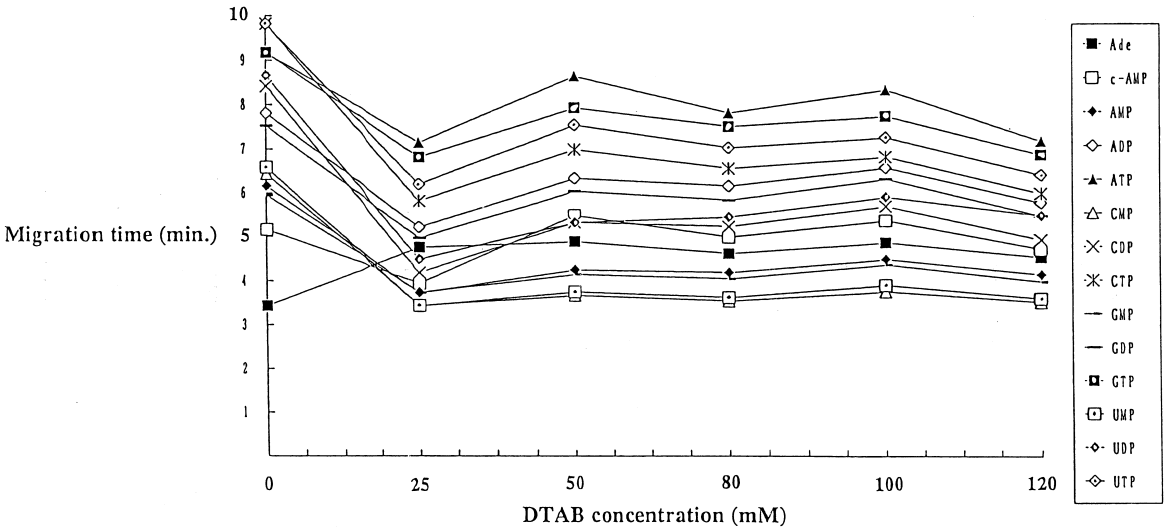

Fig. 4. Influence of DTAB concentration on migration times. Voltage: −20 kV. Other conditions as in Fig. 3.

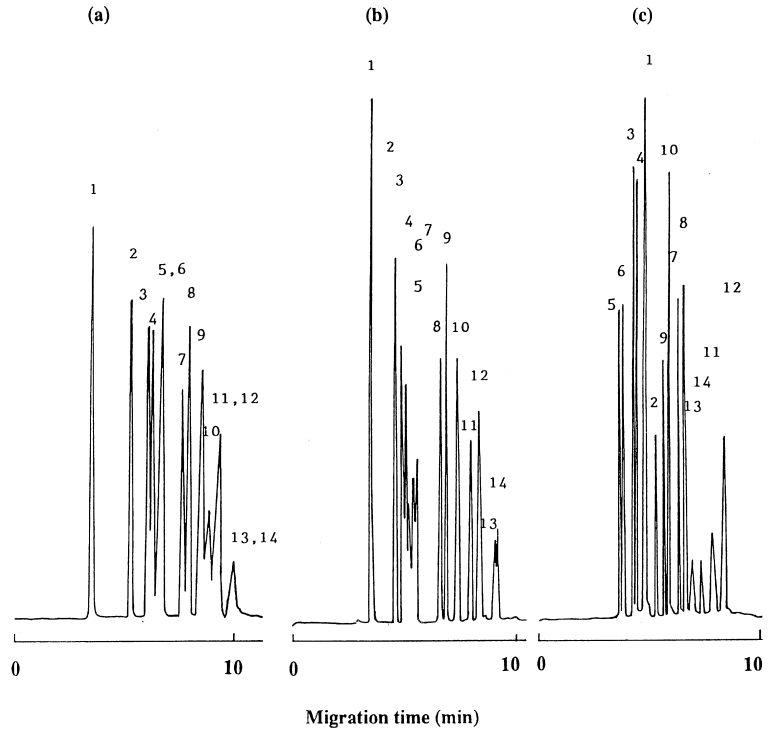

Fig. 5. Electropherograms of adenine and nucleoside phosphates by CZE and MEKCC: background electrolyte: (a) 20 m*M* Tris, 10 m*M* Na_2HP0_4 (pH 7.0); (b) same as in (a) with the addition of 100 m*M* SDS; (c) same as in (a) with the addition of 100 m*M* DTAB. Voltage: (a) and (b), 25 kV; (c), −20 kV. Samples: 1 Adenine; 2 c-AMP; 3 GMP; 4 AMP: 5 CMP; 6 UMP; 7 GDP; 8 ADP; 9 CDP; 10 UDP; 11 GTP; 12 ATP; 13 CTP; 14 UTP. Other conditions were as in Fig. 1.

adding SDS in the range of 0 to 100 m*M* to the background electrolyte (Tris–Na_2HPO_4, pH 7.0) as shown in Fig. 3. Increasing the concentration of SDS, much higher than its critical micelle concentration (CMC, 8 m*M*) did not significantly improve the separation of monophosphates (UTP and CTP, ADP and GTP) from each other. Therefore, we adopted a cationic micellar system by adding DTAB in the same background electrolyte. EOF reversal occurs at a DTAB concentration of about 0.063 m*M* according to [16] which is much lower than its CMC (14 m*M*) [26]. For this reason the polarity of the system was changed and the migration velocity was from the cathode to the anode (negative voltage means that the polarity of the system is reversed). To allow an effective action of DTAB micelles, its concentration must be above its CMC, which would result in a change of polarity of the system by reversing the electrode. Fig. 4 shows that the addition of DTAB at a concentration of 25 m*M* (close to its CMC) to the background electrolyte did not improve the separation. Effective separation appeared at a concentration of 50 m*M*, and at 100 m*M*, the best separation was achieved. At all DTAB concentrations tested here, the separation between CMP and UMP and that between AMP and GMP were not satisfactory. Some representative electropherograms are shown in Fig. 5.

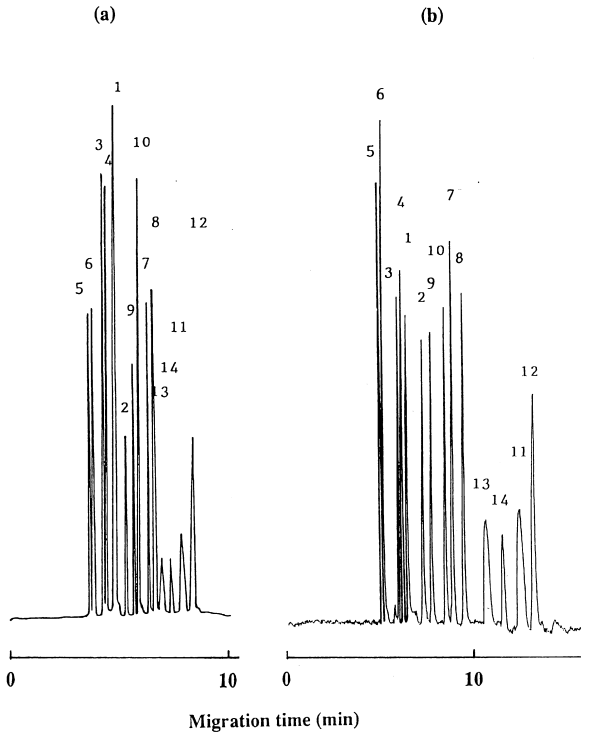

Fig. 6. Electropherogram of adenine and nucleoside phosphates by MEKCC with 100 m*M* DTAB. Background electrolyte: (a) 20 m*M* MES–Na (pH 6.1); (b) 20 m*M* MES–Na (pH 7.0). Voltage: −20 kV. Other conditions as in Fig. 1.

3.3.2. Separation at acidic pH

Otsuka and Terabe [27] have stated that, although the separation depends much on the differential partitioning of solutes, the pH still has an importance in MEKCC. We first tested CZE with various background electrolytes, including 50 m*M* sodium formate, pH 3.8, lithium acetate, pH 4.7, 20 m*M* MES–Na, pH 6.1, without surfactant. These systems gave much longer migration times and could not even elute some of the nucleotides (data not shown). We found that, although the separation between monophosphates and adenine was not complete, a better selectivity between the di- and triphosphate nucleosides was attained by decreasing the pH nearly one unit from 7.0 to 6.1 (Fig. 6a and b).

We noticed that, particularly at lower pH, washing the capillary with alkaline solution between each analysis was nearly essential to achieve good reproducibility of migration times, as previously stated by Otsuka and Terabe [27] and Lambert and Middleton [28].

3.3.3. Elution range

In MEKCC, the peak capacity t_0/t_{mc} is an important parameter which gives the effective elution range of the buffer. 0.148 and 0.296 were the mean values of the peak capacity t_0/t_{mc} for 100 m*M* SDS and DTAB, respectively, added to the background electrolyte 20 m*M* Tris, 10 m*M* Na_2HPO_4 at pH 7.0, which predict that SDS should give the best separation. However, using DTAB as a micellar phase gave a better separation and shorter analysis time as shown in Fig. 5. A higher voltage improved neither separation nor migration times of the SDS system. With 100 m*M* DTAB, the mean values of the peak capacity for the background electrolytes of 20 m*M* Tris, 10 m*M* Na_2HPO_4 at pH 7.0 and 20 m*M* MES–Na at pH 6.1, were 0.296 and 0.217, respec-

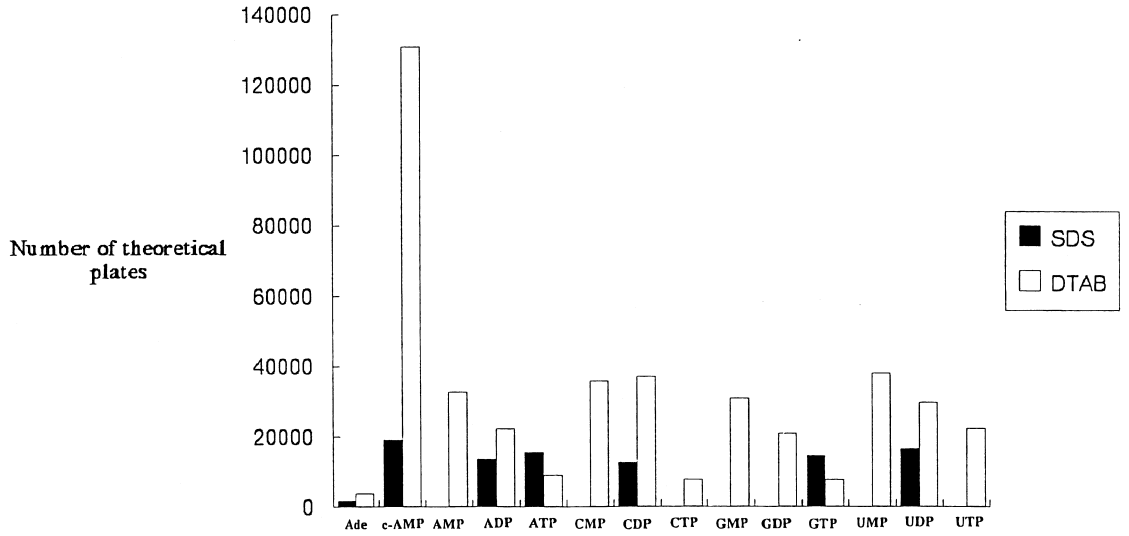

Fig. 7. Efficiency of adenine and nucleoside phosphates in SDS and DTAB micellar system at pH 7.0. Other conditions as in Fig. 5.

tively. These results show that the extension of the elution range could be performed by decreasing the pH of nearly one unit from 7.0 to 6.1 and a coated capillary [23,29] may not be needed for this purpose.

3.3.4. Efficiency

Fig. 7 shows efficiency of adenine and nucleotides which could be separated completely in both SDS and DTAB (100 m*M* each) micellar system with the background electrolyte 20 m*M* Tris, 10 m*M* Na_2HPO_4 at pH 7.0. Using the DTAB micellar system, the number of theoretical plates for adenine, ADP and UDP was almost twice of that obtained with the SDS system and three times for CDP.

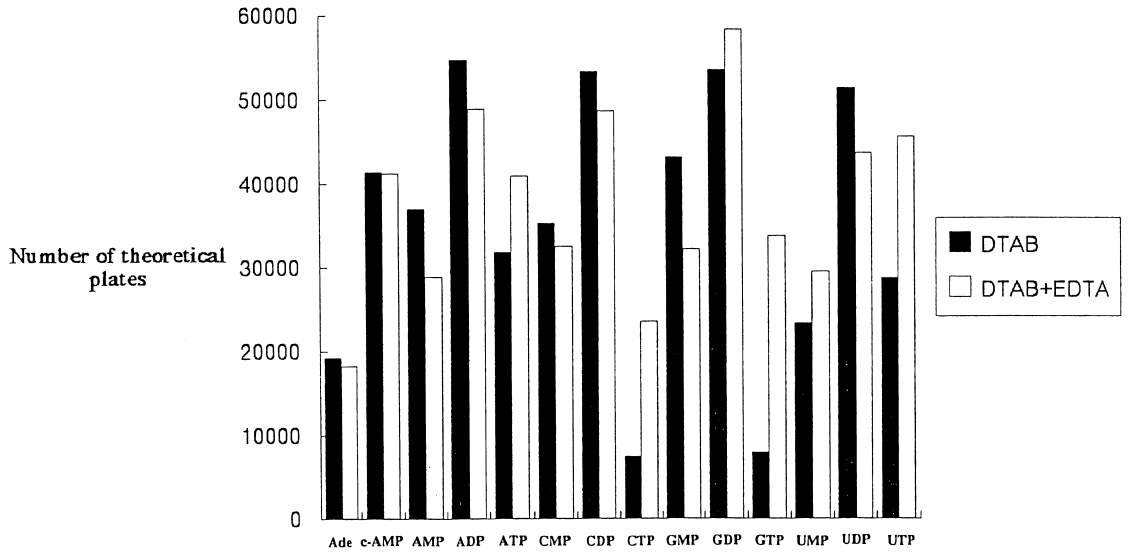

Fig. 8. Effect of 0.1 m*M* EDTA added to the adenine and nucleoside phosphates mixture on efficiency at pH 6.1. Conditions are as in Fig. 6a.

Efficiency of 130 000 theoretical plates was attained for c-AMP which is quite a improvement: nearly seven times compared to the SDS system and close to that found by Tsuda et al. [18] in biological samples. On the other hand, for the nucleoside triphosphates (ATP and GTP), the SDS micellar system gave higher numbers of theoretical plates than the DTAB system.

In Fig. 8, an acidic electrolyte was used. Then, the number of theoretical plates for c-AMP decreased by one third. However, for other adenine and nucleotides, the acidic background electrolyte for the DTAB micellar system increased the efficiency. Especially, nucleoside diphosphates had the highest efficiency reaching nearly 55 000 theoretical plates.

Certain metals, particularly magnesium, are known to have high affinity to triphosphates. To remove any contaminant metals in buffer systems, a chelating agent, EDTA was tried. Adding 0.1 m*M* EDTA in the sample with the acidic DTAB micellar system, as shown in Fig. 8, increased the efficiency of nucleoside triphosphates of 48 000 theoretical plates for UTP and nearly 60 000 for the diphosphate GDP. A great improvement in efficiency occurred specially for CTP and GTP of which the number of theoretical plates was three times higher. Some decrease, though small, was noticed for diphosphates such as ADP, CDP and UDP and monophosphates such as AMP, CMP and GMP.

Fig. 9 shows the electropherogram of the adenine–nucleotides mixture at pH 6.1 with the addition of 0.1 m*M* EDTA in the sample. Although some loss in selectivity occurred by reduction of the elution range ($t_0/t_{mc}=0.267$), it did not compromise the separation. The addition of EDTA would become particularly important for biological samples in which interfering metal ions are expected to be abundant.

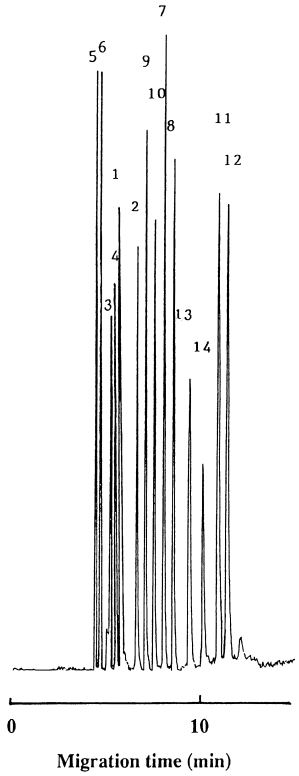

Fig. 9. Electropherogram of adenine and nucleoside phosphates: 0.1 m*M* EDTA was added to the adenine–nucleotides mixture. Conditions are as in Fig. 6.

4. Conclusions

The use of a cationic (DTAB) micellar system gave a shorter analysis time and better separation than the SDS micellar system even though the elution was narrower. Efficiencies of 130 000 and 60 000 theoretical plates were reached for c-AMP and GDP, respectively. Decreasing the pH to 6.1 allowed to increase the elution range useful for complex mixture without coating the capillary. Adding EDTA in the sample increased 3-fold the efficiency for triphosphates such as CTP and GTP. Usually, the complexity of the compounds contained in biological samples prevent to obtain a complete separation. Preliminary tests with biological samples indicated that, although the separations were good, the assignments of peaks would be problems, unless other UV-absorbing substances could either be identified or removed by some pretreatment procedure. Another problem would be that, although intracellular concentrations of nucleotides are usually in the millimolar range as used in the present report, actual samples could be inevitably diluted by cell disruption. Nevertheless, because less than a few nanolitre of a sample is enough for CZE, there is a possibility to use only a single cell injected to the capillary. As surfactants like DTAB and SDS are usually good cell

lytic agents, cell disruption would occur inside the capillary and contents from a disrupted cell would effectively separated without dilution. Such possibility is presently being explored. The DTAB micellar system described above has a potential to offer a possibility of identification and quantification of the nucleoside phosphates and adenine present in many biological fluids.

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