Separation Behavior of Biological Constituents Having *cis*-Diol Groups through Interactions with Phenylboronic Acid Sites Introduced on the Inner Wall of a Fused-Silica Capillary

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Phenylboronic acid sites were introduced onto the inner wall of a fused-silica capillary through the polymerization of 3-acrylamidophenylboronic acid after activating the wall by a silane coupling reagent. The phenylboronic acid-modified capillary was examined from various viewpoints of separation chemistry. Nucleosides having *cis*-diol groups were mainly used as model samples. The phenylboronic acid-modified capillary successfully separated a mixture of nucleosides, for example, an adenosine and deoxyadenosine mixture, in capillary electrophoresis. The separation was based on an interaction between the *cis*-diol groups and the phenylboronic acid sites on the capillary wall. The cooperation of borate migration buffer with the modified capillary could improve the resolution. The separation of a D- and L-glucoses mixture was also indicated with the coexistence of β-cyclodextrin in electrophoresis using a modified capillary. Furthermore, mixtures of nucleosides were separated with satisfactory results by chromatography utilizing a solute-wall interaction in the modified capillary, where the eluate was delivered through the capillary by a gravity method without any electrophoresis. The separation of biological constituents in this study, which took advantage of the interaction between the solutes and the inner wall of the capillary, is interesting as a new separation technique in not only capillary electrophoresis, but also capillary chromatography.

It is known that boronic acid can form cyclic esters with certain compounds including the *cis*-diol group.[1,2] The reaction is reversible; the forward reaction proceeds under an alkaline condition and the backward one under an acidic condition. Ester formation enables the separation and concentration of biological constituents comprising the *cis*-diol group. Glyco- and nucleoproteins, nucleic acids, and saccharides were, in fact, successfully separated.[3—6] Cellulose,[3] polyacrylamide gel,[4] glass bead,[5] and polymer[6] holding arylboronic acids were prepared for the affinity chromatographic separation of *cis*-diols. Moreover, packing agents having phenylboronic acid sites were also reported for high-performance liquid chromatography (HPLC).[7,8]

Recently, capillary electrophoresis (CE) has received much attention as a powerful tool for separating various samples. In CE using unmodified fused silica, the adsorption of solutes on the capillary wall often becomes a serious problem. The surfaces of the capillary are, then, chemically modified so as to reduce or eliminate the interaction ability of the solute to the capillary wall.[9,11] For example, fused-silica capillaries were modified with polyacrylamide,[9] polyethylene glycol,[10,11] polyethylsiloxane chains,[10,11] and so on. Furthermore, capillary electrochromatography (CEC) has also been intensively studied.[12] The force driving the mobile phase in CEC is electrical, as in CE. In many respects, however, CEC is a chromatographic technique much closer to HPLC than CE. Charged analytes in CEC exhibit selectivities resulting from a combination of electrophoretic and chromatographic partitioning. CEC can be performed in open tubular as well as packed capillaries. In open-tubular CEC, the stationary phase is bonded to the capillary wall; for example, the cyclodextrine,[13] C18 phase,[14,15] and fluorinated C8 phase.[16]

We have proposed that solute-wall interactions, which are induced by some specific modification of the capillary wall, is aggressively utilized for improving the separation selectivity. Here, the polymerization of 3-acrylamidophenylboronic acid was carried out in fused-silica capillary in order to introduce phenylboronic acid sites onto the inner wall of the capillary. A mixture of nucleosides having *cis*-diol groups was subjected to CE using a modified capillary. They were successfully separated on the basis of an interaction between the *cis*-diol group and phenylboronic acid sites on the inner wall. The preparation of the modified capillary, the cooperation of the capillary with a borate migration buffer, and the separation of a D- and L-glucoses mixture are also discussed in this study. Furthermore, chromatographic usage of the modified capillary was also examined. The separation mode using the solute-wall interaction in the capillary is attractive in CEC, capillary chromatography, as well as CE. We briefly described the preliminary results of this investigation in a recent communication.[17]

Experimental

Reagents. Adenosine, deoxyadenosine, inosine, deoxyinosine, guanosine, deoxyguanosine, uridine, and deoxyuridine were purchased from Nacalai Tesque. Acryloyl chloride and 3-aminophenylboronic acid hemisulfate were received from Tokyo Kasei Kogyo and Aldrich, respectively. 5-Aminonaphthalene-2-sulfonic acid (ANA) and β-cyclodextrin (CD) were purchased from Wako Pure Chemical Industries, Ltd. The other reagents used were commercially available and of special grade. Ion-exchanged water was distilled before use.

Labeling of D- and L-Glucoses with ANA. Saccharides are derivatized by a Schiff-base formation between the aromatic amine of a labeling reagent and the aldehyde form of a saccharide, followed by reduction of the Schiff base to a stable product.[18] Here, to 200 μl of a ANA solution (0.5 mol dm^{-3}) was added 200 μl of a glucose solution (D-isomer, 1.0 mol dm^{-3} and L-isomer, 0.25 mol dm^{-3}). The mixture was heated at 90 °C for 10 min with stirring. Next, 80 μl of the reducing agent, sodium cyanoborohydride (0.3 mg μl^{-1}), was added, and the mixture was heated for an additional 60 min at 90 °C. Finally, the samples were diluted 5 times with distilled water and stored at −5 °C. Injected samples were prepared from the stock solution before use.

Preparation of Phenylboronic Acid-Modified Capillary. Phenylboronic acid-modified capillary was made while referring to the preparation of an ordinary polyacrylamide-modified capillary.[19] A new capillary tube of 50 μm i.d. (Polymicro Technologies) was treated with 1.0 mol dm^{-3} sodium hydroxide for 30 min and washed with distilled water and then acetonitrile. A solution containing 4 ml of acetonitrile, 16 μl of [3-(methacryloyloxy)propyl]trimethoxysilane, and 32 μl of acetic acid was passed through the capillary for 30 min; the capillary was then left 90 min, and washed with acetonitrile. 3-Acrylamidophenylboronic acid, which was synthesized from acryloyl chloride and 3-aminophenylboronic acid hemisulfate,[20] was dissolved in 1.5 ml of distilled water to be 5 mg ml^{-1}. To the solution were added 7.5 μl of *N,N,N′,N′*-tetramethylethylenediamine and 15 μl of 10% ammonium peroxodisulfate solution. The solution was passed through the capillary for 10 min by syringe and left for 1 night. Finally, the capillary was washed with distilled water for 1 h.

Apparatus and Procedures. An ordinary CE apparatus with a spectrophotometric detector was used for electrophoresis. The capillary was filled with a migration buffer in advance. A 10 mmol dm^{-3} Tris-HCl (pH 7.0, 8.0, and 9.0), 10 mmol dm^{-3} carbonate (pH 9.0, 9.5, and 10.0), and 10 mmol dm^{-3} borate (pH 9.0 and 10.0) buffers, and 10 mmol dm^{-3} carbonate buffer (pH 9.5) containing 5 mmol dm^{-3} CD were used as migration buffers. Mixture samples of adenosine and deoxyadenosine, inosine and deoxyinosine, guanosine and deoxyguanosine, uridine and deoxyuridine, or ANA-labeled D- and L-glucoses were introduced into a capillary for 30 s from 15 cm height by siphoning. An a applied voltage of 15 kV was used, and these nucleosides and glucoses were migrated and detected at 267 and 220 nm, respectively, with a modified SPD-6AV spectrophotometric detector (Shimadzu Co.). The CE apparatus without applying any voltage was used as a chromatographic system. The introduced sample and the eluate (10 mmol dm^{-3} carbonate buffer; pH 9.5) were fed in the capillary by a gravity method from 15 cm height.

Results and Discussion

Preparation of Phenylboronic Acid-Modified Capillary. The concentration of ammonium peroxodisulfate as an initiator was, first, examined using 5 mg ml^{-1} of a 3-acrylamidophenylboronic acid solution. Electropherograms for a mixture of adenosine and deoxyadenosine, which were obtained by capillaries prepared at 0.05 and 0.1% of the initiator, were compared. The use of a 0.1% initiator concentration gave a better resolution. However, a higher concentration than that led to quick gelation, which prevented passing of the monomer solution through the capillary. Therefore, it was recommended that 0.1% of the initiator concentration be used for preparing the capillary.

Next, 3-acrylamidophenylboronic acid was dissolved in distilled water at 1, 3, and 5 mg ml^{-1} and saturated concentration (about 10 mg ml^{-1}). Electropherograms for the capillaries prepared at these monomer concentrations were compared with each other, where the mixture of adenosine and deoxyadenosine was used as a sample and the initiator concentration was 0.1%. There was little difference in the resolution and peak shape of the electropherograms. Then, 5 mg ml^{-1} of the monomer concentration was used in this study. In the case of a polyacrylamide-modified capillary, however, a much higher concentration of the monomer (acrylamide; about 30 mg ml^{-1}) was used for polymerization. When taking this into consideration, the amount of 3-acrylamidophenylboronic acid might not be sufficient to coat the total surface of the capillary wall. However, the lifetime and reproducibility of the capillary, as mentionted below, were improved through these examinations, and the capillary was confirmed to be a practical value.

Electroosmotic Flow and Lifetime of a Phenylboronic Acid-Modified Capillary. In order to confirm the introduction of the phenylboronic acid sites onto the capillary, the migration velocities of acetone as a neutral marker were examined. Figure 1 shows the migration velocities obtained by using unmodified and phenylboronic acid-modified capillaries. The migration velocities obtained with the modified capillary were found to be much smaller than those of an unmodified one. This result means that the inner wall of the capillary was coated or modified through the polymerization of 3-acrylamidophenylboronic acid. However, there still existed enough electroosmotic flow in the modified capillary, in contrast to the negligible one in the case of the ordinary polyacrylamide-modified capillary. The difference must be due to the dissociation of phenylboronic acid sites on the wall; furthermore, there may be some contribution by the silanol group residues, which can act effectively as negative charge sites.

Standing the modified capillary about 2 weeks without any use after the preparation showed no lowering of the separation ability. The capillary provided good reproducibility within 30 measurements in electrophoresis. However, when a pH higher than 10 was used for a migration buffer, the lifetime tended to become shorter. The relatively short lifetime might be attributed to a low coating percentage of phenylboronic acid sites on the inner wall of the capillary.

Electropherogram of Nucleosides Obtained by Using a Phenylboronic Acid-Modified Capillary. Adenosine,

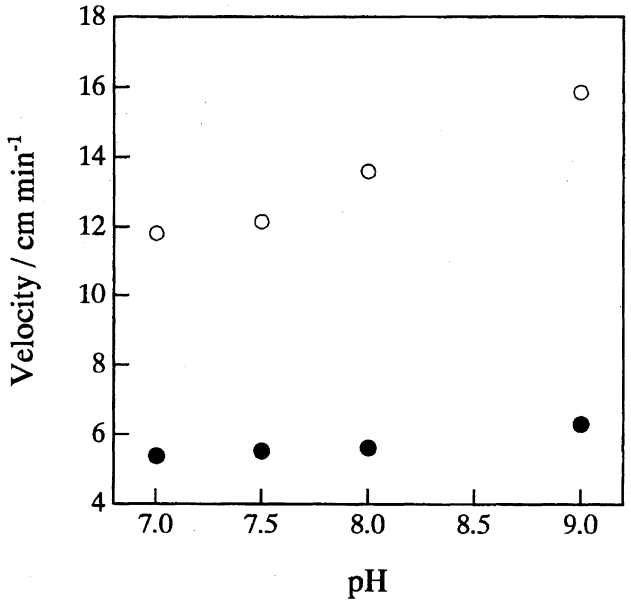

Fig. 1. Migration velocities of acetone as a neutral marker when using (○) unmodified and (●) phenylboronic acid-modified capillaries. Conditions: Capillary, 60 cm of 50 μm i.d. fused silica (effective length 40 cm); applied voltage, 15 kV; migration buffer, 10 mmol dm^{-3} Tris-HCl buffer(pH 7.0, 7.5, 8.0, 9.0); injection, siphoning (15 cm×30 s); and detection, 267 nm.

inosine, guanosine, and uridine have a *cis*-diol group in a molecule, while deoxyadenosine, deoxyinosine, deoxyguanosine, and deoxyuridine have none. First, a mixture of adenosine and deoxyadenosine was subjected to CE using the phenylboronic acid-modified capillary. Figure 2 shows electropherograms produced by using various pH migration buffers: pH 7.0 and 8.0 (10 mmol dm^{-3}Tris-HCl), pH 9.0 (10 mmol dm^{-3} Tris-HCl and 10 mmol dm^{-3} carbonate) and 10.0 (10 mmol dm^{-3} carbonate). The resolutions increased with increasing pH. The pK_a value of phenylboronic acid is reported to be 8.7,[21] although the value of phenylboronic acid sites on the capillary wall has not been examined. It was also confirmed that the first peak was due to deoxyadenosine, and the second one due to adenosine. When using unmodified and polyacrylamide-modified capillaries, no separation was observed at all under the same conditions as described in Fig. 2. Therefore, it would be concluded that separation of the mixture was achieved through interactions between the phenylboronic acid sites on the capillary wall and the *cis*-diol groups.

As shown in Fig. 2, at pH 9.0, carbonate buffer indicated a better separation than did the Tris-HCl one. The result may have been caused because 2-amino-2-hydroxymethyl-1,3-propanediol in the buffer interacts with phenylboronic acid sites on the capillary, although very weakly, in competition with adenosine as an analyte. Other mixture samples, inosine and deoxyinosine, guanosine and deoxyguanosine, and uridine and deoxyuridine, were also similarly separated under the conditions of pH 9.0 and 10.0 (10 mmol dm^{-3} carbonate buffer); for example, the electropherograms of a mixture of

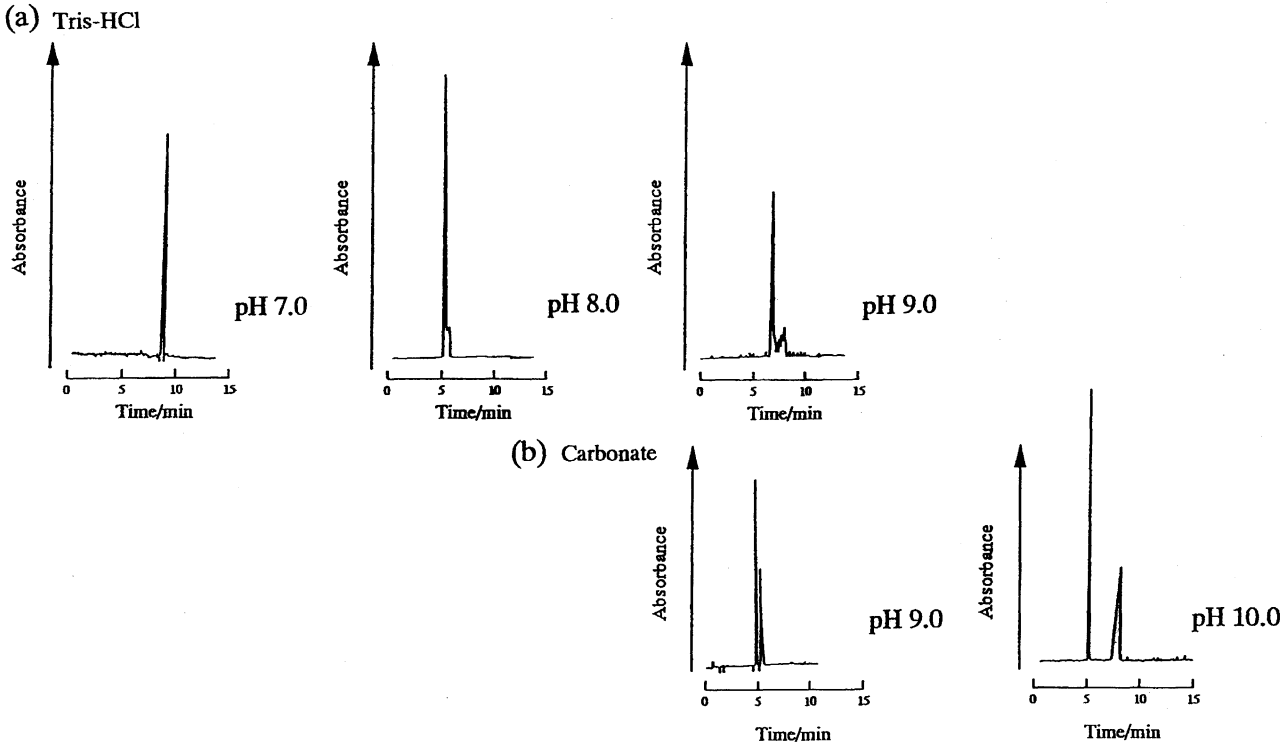

Fig. 2. Effect of pH on electropherograms of the mixture of adenosine and deoxyadenosine when using phenylboronic acid-modified capillary. (a) 10 mmol dm^{-3} Tris-HCl buffer (pH 7.0, 8.0, and 9.0) and (b) 10 mmol dm^{-3} carbonate buffer (pH 9.0 and 10.0). The experiments were carried out under the same conditions as described in Fig. 1 except for migration buffers and sample of each 0.5 g dm^{-3} adenosine and deoxyadenosine.

uridine and deoxyuridine are shown in Fig. 3. The nucleosides were separated by taking advantage of the solute-wall interaction in this study. The separation mode will be studied from the viewpoint of not only CE, but also CEC.

Cooperation of a Phenylboronic Acid-Modified Capillary with Borate Buffer. An unmodified capillary was used to separate a nucleoside mixture (adenosine and deoxyadenosine). Electropherograms obtained at pH 3.0 and 5.0 (25 mmol dm^{-3} Tris-phosphate buffer) and pH 9.0 (10 mmol dm^{-3} Tris-HCl buffer) are shown in Fig. 4. Only pH 3.0 indicated a slightly top separation on the electropherogram. No separation was observed at pH 5.0 and 9.0. In a series of experiments, however, a borate migration buffer was found to indicate the ability for separating nucleosides, even in an unmodified capillary (Fig. 5). A mixture of adenosine and deoxyadenosine was detected with a base-line separation using the unmodified capillary and 10 mmol dm^{-3} borate buffer (pH 9.0 and 10.0). There was no change between the resolutions under the two pH conditions. Boronic acid in the buffer would interact with the *cis*-diol group of adenosine to alter the charge- to-mass ratio. Although the borate buffer showed good separations in electrophoresis, it could not separate any samples at all in chromatography mentioned below.

Interestingly, cooperation of the present modified capillary with the borate buffer (pH 9.0) could improve the resolution to a large extent. The time distance between the two peaks of nucleosides indicated more than 5 min. The resolution, consequently, came to 6.56 (the resolution (R_s) was here calculated using the equation $R_s = 2(t_2 - t_1)/1.70(W_1 + W_2)$; t_n is the migration time and W_n is their half-width). It seems to be impossible to achieve a remarkable improvement by any additive effect in the migration buffer. The results obtained here support the possibility that chemically modifying the capillary wall could be a promising way to provide a new separation selectivity.

Possibility of the Optical Resolution of Monosaccharides. Recently, optical resolution has been achieved by means of CE. For example, Terabe et al.[22,23] reported that micellar electrokinetic chromatography, which is a mode of CE, gave a good result for the enantiomeric separation of dansyl-D- and L-amino acids using cyclodextrins together with sodium dodecyl sulfate. When cyclodextrin is added to a micellar solution, some solutes, such as dansyl-D- and L-amino acids, must be partitioned into the hydrophobic cavity of cyclodextrin, which has a chiral structure. The interaction also plays an important role in the process of optical resolution.

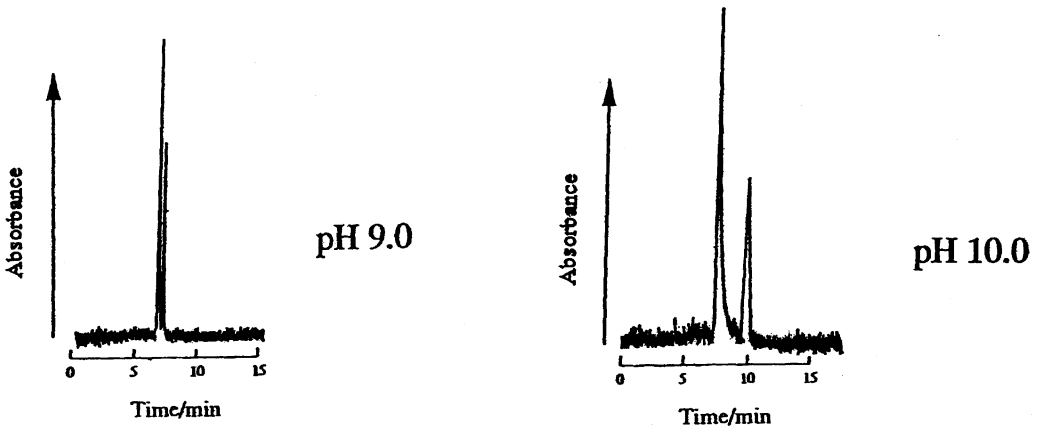

Fig. 3. Electropherograms of the mixture of uridine and deoxyuridine when using phenylboronic acid-modified capillary. The experiments were carried out under the same conditions as described in Fig. 1 except for migration buffers of 10 mmol dm^{-3} carbonate (pH 9.0 and 10) and sample of each 0.5 g dm^{-3} uridine and deoxyuridine.

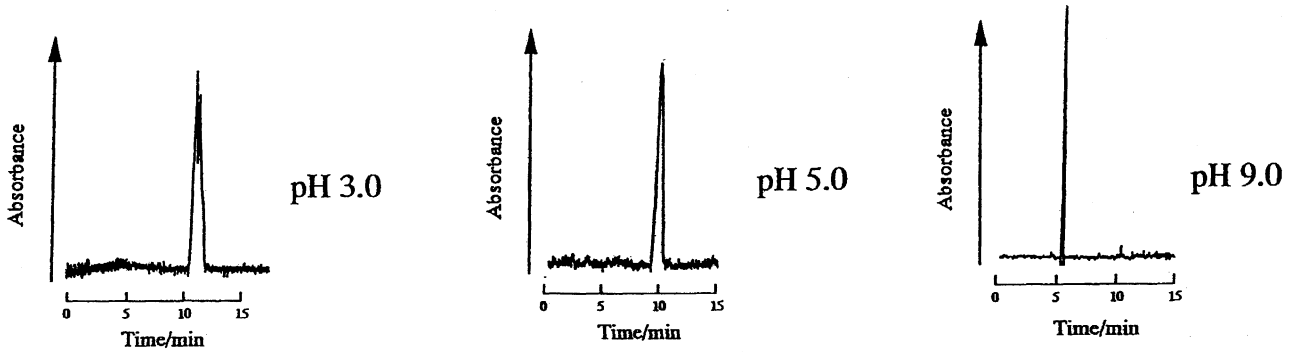

Fig. 4. Electropherograms of the mixture of adenosine and deoxyadenosine when using unmodified capillary. The experiments were carried out under the same conditions as described in Fig. 1 except for migration buffers of 25 mmol dm^{-3} Trisphosphate buffer (pH 3.0 and 5.0) and 10 mmol dm^{-3} Tris-HCl buffer (pH 9.0) and sample of each 0.5 g dm^{-3} adenosine and deoxyadenosine.

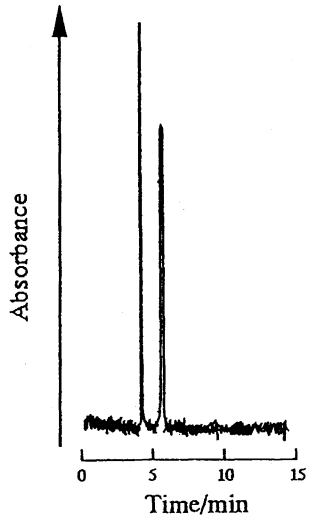

Fig. 5. Electropherograms of the mixture of adenosine and deoxyadenosine when using unmodified capillary and borate buffer. The experiments were carried out under the same conditions as described in Fig. 1 except for migration buffer of 10 mmol dm^{-3} borate buffer (pH 9.0) and sample of each 0.5 g dm^{-3} adenosine and deoxyadenosine.

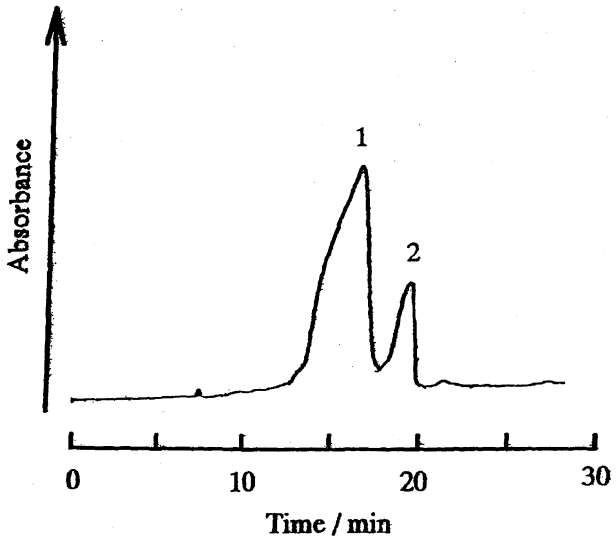

Fig. 6. Electropherogram of the mixture of ANA-labeled D- and L-glucoses when using phenylboronic acid-modified capillary together with CD. 1, ANA-labeled D-glucose and 2, ANA-labeled L-glucose. Conditions: Capillary, 60 cm of 50 μm i.d. fused silica (effective length 40 cm); applied voltage, 15 kV; migration buffer, 10 mmol dm^{-3} carbonate buffer (pH 9.5); injection, siphoning (15 cm×30 s); detection, 220 nm; and sample of 0.1 mmol dm^{-3} D-glucose and 0.025 mmol dm^{-3} L-glucose.

The possibility of the optical resolution of monosaccharides was examined in electrophoresis using a phenylboronic acid-modified capillary. The experiment was carried out for a mixture of ANA-labeled D- and L-glucoses (molar concentration ratio 4 : 1) in 10 mmol dm^{-3} carbonate migration buffer (pH 9.5) containing 5 mmol dm^{-3} CD. The mixture was stereospecifically separated (Fig. 6). The D-isomer moved faster than did the corresponding L-isomer; they were identified by their retention times and peak areas. The separation could not be observed using other capillaries, such as unmodified and polyacrylamido-modified ones under the same conditions. Therefore, the separation mechanism must be attributed to an interaction of the complex between ANA-labeled glucose and CD with the phenylboronic acid sites on the inner wall of the capillary. The specific separation surroundings could cause peak-broadening. In any case, the result seems to be interesting for optical resolution as initial data by the modified capillary.

Chromatography Using a Phenylboronic Acid-Modified Capillary. Chromatographic separation was examined, in which the eluate was fed by a gravity method, not by electrophoresis. Mixture samples of adenosine and deoxyadenosine, inosine and deoxyinosine, guanosine and deoxyguanosine, and uridine and deoxyuridine, as well as each individual sample, were subjected to a chromatographic system using unmodified, polyacrylamide-modified, and phenylboronic acid-modified capillaries. All of the nucleosides

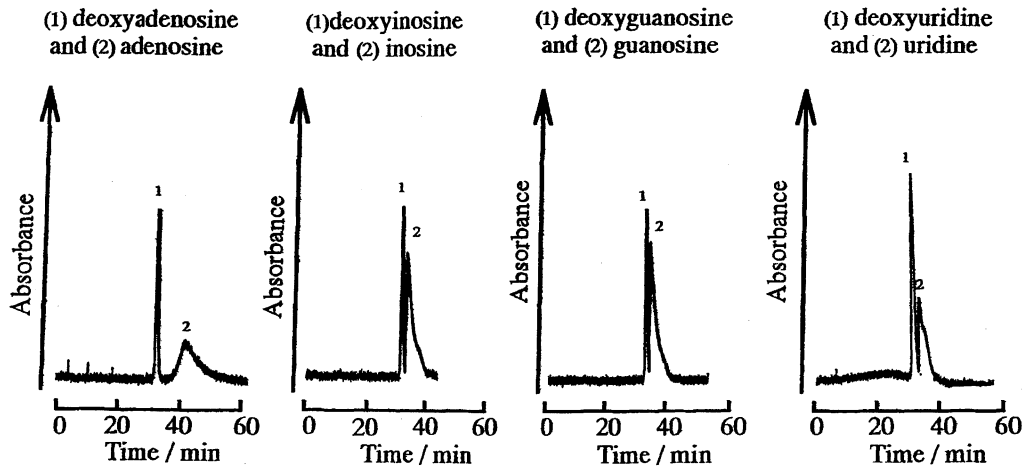

Fig. 7. Chromatograms of the mixture of nucleosides when using phenylboronic acid-modified capillary. Conditions: Capillary, 60 cm of 50 μm i.d. fused silica (effective length 40 cm); migration buffer, 10 mmol dm^{-3} carbonate buffer (pH 9.5); injection, siphoning (15 cm×30 s); and detection, 267 nm.

were detected at the same time after around 25 min using the unmodified and polyacrylamide-modified capillaries, whereas they were separated within 50 min using the phenylboronic acid-modified capillary. Chromatograms are shown in Fig. 7; the first peaks were due to compounds having no *cis*-diol group and the second ones due to those having the group. This means that chromatographic separation is based on an interaction between the *cis*-diol group and the phenylboronic acid site on the capillary wall.

Chemiluminescence (CL) has been shown to be a highly sensitive detection method in both flow-injection analysis and HPLC. Recently, several studies have utilized tris(2,2′-bipyridine) ruthenium(II) ion ($Ru(bpy)_3^{2+}$) as a CL reagent. The oxidant, $Ru(bpy)_3^{3+}$, generally reacts best with tertiary, next secondary, and primary alkyl amines.[24,25] It was found here that nucleotides responded to the chemiluminescence system of $Ru(bpy)_3^{3+}$. Therefore, the combination of capillary chromatography using the modified capillary with the CL detection of $Ru(bpy)_3^{3+}$ might lead to a useful separation and detection method for extremely small amounts of samples. Chromatography with CL detection is now being investigated.

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