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Polyacrylamides as hydrophilic selectors in non-aqueous capillary electrophoresis

Yukihiro Esaka[a,*], Shinsuke Inagaki[a], Daisuke Uchida[a], Masashi Goto[a], Kenji Kano[b]

[a]*Department of Pharmaceutical Analytical Chemistry, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan*
[b]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

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Abstract

Polyacrylamides (PAAms) were investigated as hydrophilic selectors in non-aqueous capillary electrophoresis (CE). Separation of 10 substituted benzoates and unsubstituted benzoate as model samples was greatly improved by the addition of PAAms in acetonitrile–CE. The migration behavior indicates that the carbonyl moiety of PAAms works as a good hydrogen-accepting site toward hydrogen-donating analytes such as 4-hydroxybenzoate anion (4OH-BA) in acetonitrile. PAAms also serve as electron-accepting agents with its amide proton interacting with the dissociated carboxyl groups of the benzoates. The ion–dipole interaction is useful to control the migration behavior of benzoates without hydrogen-donating substituents. The overall mode of the interaction is similar to that of polyethylene glycol (PEG) 20 000 reported previously, but the complex formation constant of poly(*N-tert*.-butyl)acrylamide (PBAAm) with 4OH-BA estimated here was 130-fold larger than that of PEG 20 000. This would be ascribed to the strong basicity of the carbonyl oxygen atoms of PBAAm as compared with the ether oxygen atoms of PEG. Furthermore, a copolymer of (*N-tert*.-butyl)acrylamide–acrylamide [70:30 (in feed)] exhibited a complex formation constant of about fourfold larger toward 4OH-BA than PBAAm, most probably due to decrease in steric hindrance from the *tert*.-butyl groups. Adrenaline and its six precursors have been separated successfully using the PAAms.

Keywords: Nonaqueous capillary electrophoresis; Hydrophilic selectors; Polyacrylamides; Poly(*N-tert*.-butyl)acrylamide; Adrenaline; Benzoic acids

1. Introduction

Non-aqueous capillary electrophoresis (NACE) is a relatively new, developing field in capillary electrophoresis (CE) with apparnet potential. Of significant interest in NACE is the use of interactions as separation modes, quite different from those applied in aqueous systems. Availability of non-aqueous media in CE means that the migration behavior of analytes can be controlled by changing not only the selectors but also the environment in which the interactions occur. This feature of the methodology will be very advantageous in designing separation systems. Especially, hydrophilic interactions such as hydrogen-bonding, dipole-related, and/or ionic interactions are expected to be valuable in NACE possessing hydrophobic surroundings, since those hydrophilic interactions are, in principle, thermodynamically strengthened in non-aqueous systems compared with those in aqueous ones [1–11]. On the

*Corresponding author. Tel.: +81-582-373-931; fax: +81-582-375-979.

E-mail address: esaka@gifu-pu.ac.jp (Y. Esaka).

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other hand, the style of selectors is also an important factor to effectively control separations. If we use selectors with highly effective forms to reflect interactions between the selectors and analytes on the migration behavior of analytes such as ionic selectors and polymeric ones in non-aqueous media, we will get separation systems with novel selectivity remarkably different from those we have employed in aqueous media.

In our previous papers, CE separations of organic compounds using polyethers as hydrophilic selectors have been proposed [9,12–15]. Polyethylene glycol (PEG) added to aqueous running solutions in capillary zone electrophoretic (CZE) separation works as a hydrogen-bonding base with the ether oxygen atoms toward hydrogen-donating substituents of benzoate anion analytes to improve separation remarkably [12,13]. Polyether chains of Tween 20 and Brij 35 in the mixed micelles with SDS interact with hydrogen-donating analytes to accelerate their transfer to the micelle phases [14,15]. The mixed surfactant systems were successfully applied to the micellar electrokinetic chromatographic (MEKC) separation of substituted benzenes. In these aqueous systems, the hydrogen-bonding interaction would work effectively with the assistance of the hydrophobicity of the polyethers and/or micelles. On the other hand, in CZE separation of substituted benzoate in an acetonitrile system, PEG works as both hydrogen-acceptor (with the ether oxygen atoms) and electron-acceptor (with the terminal hydroxyl groups) toward hydrogen-donating substituents and dissociated carboxyl groups of the analytes, respectively [9]. Especially, the dipole–ion interaction between the terminal hydroxyl groups and the dissociated carboxyl groups plays a significant role in the acetonitrile system, while it works minimally in aqueous systems because of strong solvation. As a result, quite different migration behavior of analytes was observed in non-aqueous systems, which realized improved separation as compared with those in aqueous systems containing PEG.

In the present paper, we will propose use of polyacrylamides (PAAms) as hydrophilic selectors for CE in acetonitrile, using benzoates as model analytes. The amide groups of PAAms have both electron-donating (hydrogen-accepting) activity with the carbonyl oxygen atoms and electron-accepting (hydrogen-donating) activity with the amide protons. The former activity is much stronger than the ether oxygen atoms of PEG. The migration behavior of the analytes in the present separation systems will be compared with those in the acetonitrile systems containing PEG 20 000 reported previously. Quantitative analysis of the interaction between analytes and the matrix agents will be discussed. On the other hand, separation modes based on such hydrophilic interactions should be useful for analysis of biological substances which often have various kinds and numbers of hydrophilic function groups in relation to their functions in organisms. From this point of view, we have also studied the separation of a group of biologically important analytes, adrenaline and its precursors, briefly.

2. Materials and methods

2.1. Apparatus

All separations were performed on a laboratory-made system consisting of a Matsusada HCZE-30 PNO high-voltage power supply (Siga, Japan), a Jasco CE-970 detector (Tokyo, Japan) and a Shimadzu C-R6A Chromatopac integrator. Polydimethylsiloxane-coated capillaries (DB-1) were obtained from J&W Science (CA, USA). The capillaries have internal diameters of 100 μm and an outer diameter of 365 μm. The total length of the capillaries was 1000 mm for separation of benzoates and 875 mm for separation of adrenaline and its precursor, while their effective lengths were 750 mm and 190 mm, respectively.

2.2. Reagents and chemicals

Spectrum-grade acetonitrile was obtained from Nacalai Tesque (Kyoto, Japan). Substituted polyacrylamides were polymerized in our laboratory from (*N*-*tert*.-butyl)acrylamide (BAAm) and acrylamide (AAm) (see below) obtained from Aldrich (Milwaukee, WI, USA) and Nacalai Tesque, respectively. *N*,*N*,*N*,*N*-Tetramethylethylenediamine (TEMED) and ammonium peroxodisulfate for the polymerization were purchased from Nacalai Tesque. Benzoic acid and 10 substituted benzoic acids (4-acet-

amidobenzoic acid, 4-acetoxybenzoic acid, 4-dimetylaminobenzoic acid, 4-hydroxybenzoic acid, 4-aminobenzoic acid, terephthalaldehydic acid, 4-nitrobenzoic acid, 4-toluic acid, *ortho*-phthalaldehydic acid and salycilic acid) as model analytes were purchased from Nacalai Tesque. L-Noradrenaline, L-dopa, dopamine and L-phenylalanine were obtained from Nacalai Tesque. L-3-Methoxytyrosine and adrenaline were obtained from Sigma (St. Louis, MO, USA). L-Tyrosine was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Tetra(*n*-butyl)ammonium perchlorate [$(n\text{-butyl})_4NClO_4$] was prepared from tetra(*n*-butyl)ammonium bromide as reported in our previous report [9]. Tetra(*n*-butyl)ammonium hydroxide [$(n\text{-butyl})_4NOH$] and tetra(*n*-butyl)ammonium bromide were purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were of analytical grade.

2.3. Procedure

2.3.1. Electrophoresis

The concentrations of the analytes in sample solutions were usually 0.02 m*M* each for benzoates and 0.05 m*M* each for adrenaline and its precursors. The coated capillaries were rinsed with pure acetonitrile for acetonitrile systems at the beginning of daily experiments. These capillaries were further rinsed for 30–60 s with running electrolyte solutions to be used before each run. Since electroosmotic flow was not observed or was sufficiently weak in this study, anionic samples (benzoates) and cationic samples (adrenaline and its precursors) were injected on the cathodic side and the anodic side, respectively. The applied voltage was fixed at 20 kV and −20 kV for the separation of the anionic samples and the cationic samples, where the current was 12 μA and 17 μA, respectively. The detection wavelength was set at 220 nm.

2.3.2. Polymerization

Typical procedures were as follows [16]: a total of 3.9 m*M* monomers (BAAm and AAm) were dissolved in a mixture of water and acetonitrile (50 ml and 9 ml, respectively), followed by bubbling N_2 through the solutions for 5 min to displace O_2 which interferes with these polymerizations. Immediately, 4.3 mmol TEMED and 2.2 mmol ammonium peroxodisulfate were added in this order as stimulator and initiator for polymerization, respectively. Polymerization reactions were stopped by spontaneous precipitation of polymers. The precipitations were dried at 80 C° for 5 h in vacuo. Elemental analysis was performed to determine the copolymer composition. Compositions of BAAm and AAm in two copolymers (80:20 and 70:30 in feed) were estimated to be 90:10 and 85:15 in products, respectively.

3. Results and discussion

3.1. CZE separations of benzoates in acetonitrile containing polyacrylamides

Fig. 1 shows CZE separations of unsubstituted benzoate and 10 substituted benzoates: 4-acetamidobenzoate ($4CH_3CONH$-BA), 4-acetoxybenzoate ($4CH_3COO$-BA), 4-dimetylaminobenzoate [$4(CH_3)_2N$-BA], 4-hydroxybenzoate (4OH-BA), 4-aminobenzoate ($4NH_2$-BA), 4-formylbenzoate (4CHO-BA), 4-nitrobenzoate ($4NO_2$-BA), 4-toluate ($4CH_3$-BA), 2-formylbenzoate (2CHO-BA), benzoate (BA), salycilate (2OH-BA), in the absent (A) and present (B) of 0.20% poly(*N-tert.*-butyl)acrylamide (PBAAm) in acetonitrile containing

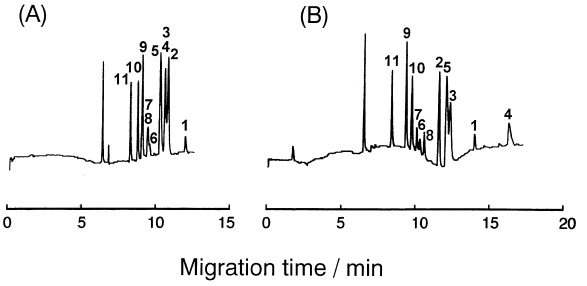

Fig. 1. Electropherograms of benzoates in the absence (A) and presence (B) of 0.20% poly(*N-tert.*-butyl)acrylamide in acetonitrile containing 5.7 m*M* tetra(*n*-butyl)ammonium perchlorate and 0.3 m*M* tetra(*n*-butyl)ammonium hydroxide. Applied voltage [operating current]: 20 kV [12 μA]. Capillary (coated; μ-sil DB-1): 1000 mm (effective length 750 mm)×100 μm I.D. Analytes: 1=4-acetamidobenzoate, 2=4-acetoxybenzoate, 3=4-dimetylaminobenzoate, 4=4-hydroxybenzoate, 5=4-aminobenzoate, 6=4-formylbenzoate, 7=4-nitrobenzoate, 8=4-toluate, 9=2-formylbenzoate, 10=benzoate, 11=salycilate.

5.7 m*M* $(n\text{-butyl})_4NClO_4$ and 0.3 m*M* $(n\text{-butyl})_4NOH$ to dissociate carboxyl groups of analytes. As seen in (B), almost complete separation of the benzoates was achieved by the addition of PBAAm, though the peaks of $4(CH_3)_2N$-BA and $4NH_2$-BA overlapped in part with each other. Compared with (A) and (B), the decrease in the electrophoretic mobility (m_{ep}) of 4OH-BA was much more larger than those of the other benzoates. The decrease in m_{ep} means the interaction of non-ionic PAAms with the analytes. Thus, it can be considered that the phenomenon is just ascribed to the hydrogen-bonding interaction between the phenolic hydroxyl group of 4OH-BA and the carbonyl oxygen atoms of PBAAm and is utilized in this separation system.

We also attempted to use copolymers of (*N-tert.*-butyl)acrylamide (BAAm) and acrylamide (AAm) [BAAm:AAm=80:20 and 70:30 (in feed)] as modifiers. Migration behavior of the analytes in acetonitrile using the copolymers was almost similar to those using PBAAm. However, a copolymer with a larger ratio of AAm interacted with the analytes more strongly. Four-times smaller concentration (%, w/v) of the copolymer (70:30) was enough to cause a given extent of the migration compared with PBAAm. Decreased ratio of BAAm with the ''bulky'' *N-tert.*-butyl group in copolymers of BAAm and AAm would result in stronger hydrophilic interactions between the amide groups of the copolymers and analytes, although the ''hydrophobic'' *N-tert.*-butyl groups in the copolymers would play a role in part to solubilize the polymer in acetonitrile. In addition, the operation at lower concentrations of PAAms provides a practical advantage in the spectrophotometric detection, because of the slight absorption of the amide groups at a conventional detection range of the wavelength. The structures of the polymers are shown in Fig. 2.

3.2. Analysis of the interactions between the benzoates and polyacrylamides

For detailed and quantitative analysis of the interactions working in the separation systems, we estimated complex formation constants (K) between the benzoates and the PAAms (PBAAm and the copolymers). Assuming a stoichiometric interaction

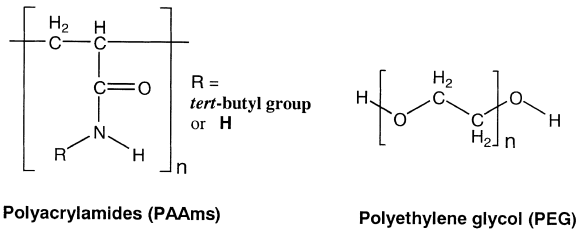

Fig. 2. Structures of the synthesized polyacrylamides and polyethylene glycol.

between the analyte and the polymers (PAAms in this study), the electrophoretic mobility (m_{ep}) is expressed as a function of the concentration of the polymers [12]:

$$m_{ep} = m_{ep,f} + K(m_{ep,c} - m_{ep,f})[\text{polymers}] \quad (1)$$

where the subscripts c and f denote complexed and free analytes, respectively. In order to eliminate the effect on m_{ep} arising from the change in the viscosity, we will define $m_{ep}/m_{ep,o}$ as a relative value of m_{ep} against the electrophoretic mobility of a reference compound with $K \cong 0$ ($m_{ep,o}$). Thus Eq.(1) can be rewritten as:

$$m_{ep}/m_{ep,o} = m_{ep,f}/m_{ep,o} + K\left[(m_{ep,c} - m_{ep,f})/m_{ep,o}\right][\text{polymers}] \quad (2)$$

Here, salicylate (2OH-BA) was chosen as a reference compound because the interaction with PAAms in the acetonitrile system was very weak, probably due to the intramolecular hydrogen-bonding formation. K values were evaluated from the slopes of the linear part of the $m_{ep}/m_{ep,o}$ versus the polymer concentration profiles by assuming that $m_{ep,c} - m_{ep,f} = -m_{ep,f}$.

Fig. 3 shows the profiles of $m_{ep}/m_{ep,o}$ against change in the concentration of PBAAm as a selector. 4OH-BA has a particularly larger absolute value of slope reflecting its specifically stronger interaction with PBAAm compared with those of the other analytes. This profile indicates a remarkable change in the migration order of these analytes, suggesting the usefulness of PAAms as selectors in acetonitrile systems. Judging from Fig. 2, 0.20% seems to be one

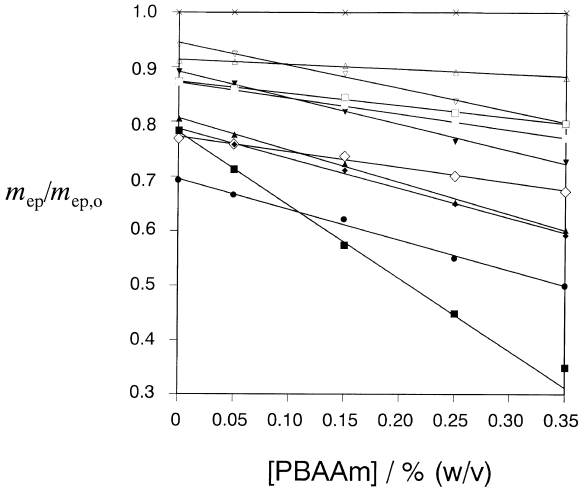

Fig. 3. Relative electrophoretic mobility ($m_{ep}/m_{ep,o}$) of benzoates as a function of the concentration of poly(*N-tert.*-butyl)acrylamide. Symbols: ●=4-acetamidobenzoate, ▲=4-aminobenzoate, ♦=4-dimetylaminobenzoate, ■=4-hydroxybenzoate, ◇=4-acetoxybenzoate, ○=4-formylbenzoate, □=4-nitrobenzoate, ▼=4-toluate, △=2-formylbenzoate, ▽=benzoate, ×=salycilate (as a reference).

of the optimum concentration of PBAAm (see Fig. 1B).

Table 1 summarizes the *K* values of the benzoates with PBAAm and the copolymers (BAAm:AAm= 80:20 and 70:30 in feed) estimated according to Eq. (2) as well as those with PEG 20 000 in acetonitrile [9]. The results for the PBAAm and the copolymers will be interpreted in terms of the hydrogen-bonding interaction of hydrogen-donating substituents of the benzoates with the carbonyl oxygen atoms of the PAAms and the ion–dipole interaction of the dissociated carboxyl groups with the amide protons of the PAAms.

The three upper benzoates possessing hydrogen-donating substituents in Table 1: 4OH-BA, $4CH_3CONH$-BA and $4NH_2$-BA have larger *K* values than the other benzoates. The order of magnitude of *K* values is fundamentally consistent with the magnitude of the hydrogen-donating activities of the substituents of the benzoates. This strongly supports that the hydrogen-bonding interaction with the carbonyl oxygen atoms of the PAAms works significantly as a separation mode in these systems. Here, we will assume that the *K* values of 4OH-BA are predominantly responsible for the hydrogen-bonding interaction. When the *K* values of 4OH-BA with PBAAm were expressed as the per unit concentration of the interaction sites (the carbonyl oxygen atoms) in the PAAms, the value is almost 400-fold larger than the corresponding value for the ether oxygen atoms of PEG 20 000. Moreover, the value for the copolymer (70:30) is almost 1200-fold larger than that for PEG 20 000 due to a decrease in steric hindrance from the *tert.*-butyl groups.

In addition to the strong hydrogen-bonding inter-

Table 1
Estimated values of complex formation constants (*K*) with modifiers

Analyte	$K \cdot 10^3$ (%, w/v)[a]				Acidity of substituents	Basicity of COO^- (Hammet σ)
	PBAA	Copolymer (80:20)	Copolymer (70:30)	PEG 20 000 [9]		
4OH-BA	1720±140	4800±800	6140±1140	13.0	1	3 (−0.37)
$4CH_3CONH$-BA	810±150	1830±80	2510±290	13.0	2	5 (0)
$4NH_2$-BA	730±70	1890±150	2110±220	9.8	3	2 (−0.66)
$4(CH_3)_2N$-BA	690±130	1700±200	2180±250	6.8	–	1 (−0.83)
$4CH_3$-BA	540±130	1100±310	1290±320	6.0	–	4 (−0.17)
BA	440±50	890±40	1040±240	5.9	–	5 (0)
$4CH_3COO$-BA	360±80	830±140	790±290	6.8	–	7 (0.31)
4CHO-BA	330±30	630±110	610±340	6.0	–	8 (0.42)
$4NO_2$-BA	250±40	430±20	410±100	5.1	–	9 (0.778)
2CHO-BA	100±30	200±20	100±100	4.2	–	–
2OH-BA[b]	0[b]	0[b]	0[b]	0[b]	Intramol. H-bond	Very weak

[a] % (w/v) is the unit of the concentration of the polymers. Concentration of polymers was changed in the range of 0–0.35%, 0–0.15% and 0–0.10% for PBAA, copolymer (80:20) and copolymer (70:30), respectively, for estimation of *K* values of the benzoates except 4OH-BA. Only for 4OH-BA, the ranges were 0–0.25%, 0–0.10% and 0–0.075%, respectively.

[b] These values were estimated using 2OH-BA as a reference.

action, the ion–dipole interaction between the dissociated carboxyl groups of the benzoates and the amide protons of PAAms would also work effectively in the acetonitrile system, because the order of magnitude of the *K* values of the lower five *para*-substituted benzoates and unsubstituted benzoate in Table 1 is almost consistent with the magnitude of the electron density on dissociated carboxyl groups of the benzoates, judging from Hammet σ values of the benzoates (see Table 1) [17,18].

3.3. Application to separation of adrenaline and its six precursors

As separation targets, adrenaline and its six precursors (L-noradrenaline, L-dopa, dopamine, L-phenylalanine, L-3-methoxytyrosine, L-tyrosine) were selected, which are biologically important compounds and possess a variety of hydrophilic function groups: undissociated carboxyl groups, ammonium groups, phenolic hydroxyl groups and alcoholic hydroxyl groups.

Fig. 4 shows electropherograms of the seven analytes as cations in the absence (A) and presence (B) of the copolymer (80:20) in acetonitrile containing 5.7 m*M* $(n\text{-butyl})_4NClO_4$ and 0.3 m*M* perchloric acid to make the analytes cations. Addition of

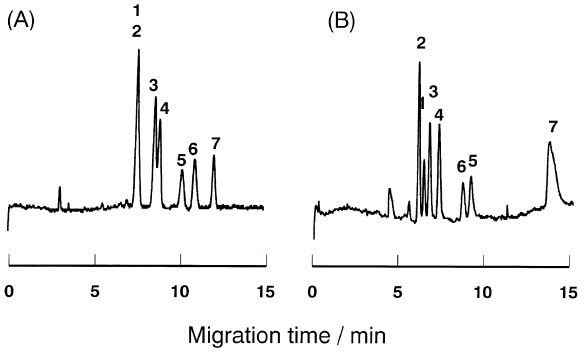

Fig. 4. Electropherograms of adrenaline and its six precursors in the absence (A) and presence (B) of 0.07% of the copolymer of (*N-tert.*-butyl)acrylamide–acrylamide [80:20 (in feed)] in acetonitrile containing 5.7 m*M* tetra(*n*-butyl)ammonium perchlorate and 0.3 m*M* perchloric acid. Applied voltage [operating current]: −20 kV [17 μA]. Capillary (coated; μ-sil DB-1): 875 mm (effective length 190 mm)×100 μm I.D. Analytes: 1=L-phenylalanine, 2=dopamine, 3=adrenaline, 4=L-noradrenaline, 5=L-tyrosine, 6=L-3-methoxytyrosine, 7=L-dopa.

the copolymer caused remarkable change in their migration order and resulted in improvement of separation. A baseline resolution was achieved by addition of 0.07% of the copolymer. Judging from the remarkable delay of migration, L-dopa seems to interact most strongly with the copolymer. L-Dopa has a primary ammonium group, an undissociated carboxyl group, and two phenolic hydroxyl groups and thus, will possess the strongest hydrogen-bonding acidity among all of the analytes. Therefore, these changes in migration behavior of analytes would be responsible for hydrogen-bonding interaction between analytes and the copolymer. It should be mentioned that, in these separations under acidic conditions, electroosmotic flow toward cathodic side was observed and strengthened with increase in the concentration of the copolymers. This was reason that some analytes were detected faster in the presence (B) than in the absence (A) of the copolymer.

Table 2 summarizes the *K* values of the seven analytes, estimated according to Eq. (2) using profiles of [copolymer (80:20)] vs. $m_{ep}/m_{ep,o}$, in which ''o'' denotes adrenaline as a reference, and the kinds and number of hydrogen-donating substituents which the analytes possess. The order of hydrogen-donating activity of substitutes will be as follows: undissociated carboxyl groups>primary ammonium groups>secondary ammonium groups>phenolic hydroxyl groups>alcoholic hydroxy groups. Table 2 will demonstrate that an analyte possessing a larger number of substituents with stronger hydrogen-donating activity interacts with the copolymer more strongly. It strongly supports our hypothesis that the hydrogen-bonding mode works effectively in this separation system.

4. Conclusion

This report demonstrates the significance of the use of PAAms as selectors in non-aqueous separation systems. PAAms appeared to have remarkable ability for highly selective separation of organic compounds possessing hydrophilic moieties as biological compounds. As an application, successful separation of adrenaline and its six precursors using an acetonitrile system containing PAAms was demonstrated. These

Table 2
Estimated values of complex formation constants (K) with the copolymer (80:20)

Analyte	$K \cdot 10^3$ (%, w/v)[a]	Functional groups of analytes				
		–COOH	$-NH_3^+$	$-NH_2^+-$	Phenolic –OH	Aliphatic –OH
L-Dopa	6600	1	1	–	2	–
L-Tyrosine	3600	1	1	–	1	–
L-3-Methoxytyrosine	1800	1	1	–	1	–
L-Noradrenaline	1300	–	1	–	2	1
L-Phenylalanine	1000	1	1	–	–	–
Dopamine	300	–	1	–	2	–
Adrenaline	0[b]	–	–	1	2	1

[a] % (w/v) is the unit of the concentration of the polymer. Concentration of copolymer (80:20) was changed in the range of 0–0.10% for estimation of K values.
[b] These values were estimated using adrenaline as a reference.

acetonitrile systems will be useful for separation of many other biological substances. Related study is in progress.

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