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Research Article

Chaotropic salts: Novel modifiers for the capillary electrophoretic analysis of benzodiazepines

This paper describes a CE method for analyzing benzodiazepines using the chaotropic salts lithium trifluoromethanesulfonate (LiOTf), lithium hexafluorophosphate (LiPF$_6$), and lithium bis(trifluoromethanesulfonyl)imide (LiNTf$_2$) as modifiers in the running buffer. Although adequate resolution of seven benzodiazepine analytes occurred under the influence of each of the chaotropic anions, the separation efficiency was highest when bis(trifluoromethanesulfonyl)imide (Tf$_2$N$^-$) was the modifier. We applied affinity CE in conjunction with linear analysis to determine the association constants for the formation of complexes between the Tf$_2$N$^-$ anion and the benzodiazepines. According to the estimated Gibbs free energies, the interactions between this chaotropic anion and the benzodiazepines were either ion–dipole or ion–induced dipole interactions. Adding chaotropic salts as modifiers into CE buffers is a simple and reproducible technique for separating benzodiazepines.

1 Introduction

Chaotropic salts, also called "structure breakers," are electrolytes that disrupt the natural hydrogen bonding of water to increase its disorder [1]. In the Hofmeister series, chaotropic ions are those having large size and low charge, such as the anions SCN$^-$, ClO$_4^-$, and I$^-$; in contrast, small, highly charged ions such as F$^-$, SO$_4^{2-}$, and H$_2$PO^{4-} are considered to be kosmotropic [2]. When a hydrophobic compound is dissolved in an aqueous solution, hydrated molecules gather around it, forming a "clathrate" to isolate the hydrophobic domain; intermolecular hydrogen bonds nearby the clathrate stabilize the hydrophobic compound [3]. Adding chaotropic ions disrupts the solvation shell of the analyte, increasing its apparent hydrophobicity [4]. The phenomenon of decreasing the structure of water and increasing the opportunity for hydrophobic compounds to interact with other ions to form complexes is called the chaotropic effect [5].

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The chaotropic effect is often found in the salting-out of protein precipitates [3, 6]. Ammonium sulfate is the classic example of an agent that causes proteins to separate from solution, because the chaotropic ions expose the hydrophobic domain of the protein and then like-to-like interactions enlarge the protein size, causing precipitation. Hashem and Jira [4] observed the satisfactory separation of protonated beta-blockers when using chaotropic anions as mobile phase additives for HPLC. Adding chaotropic additives provided a significant change in the resolution without the need to vary the column type or add an ion-pairing reagent. The addition of ClO$_4^-$ was more effective at separating the beta-blockers than was the addition of H$_2$PO$_4^-$. LoBrutto *et al.* [7, 8] demonstrated that the concentration of the chaotropic anion notably affects the retention of protonated basic analytes on RP HPLC columns. The improvement in the retention factor is due to the chaotropic anions disrupting the analytes' solvation shells, increasing their hydrophobicity and, hence, enhancing the interactions of the analytes with the stationary phase. The analyte retention factors increase in the order PF$_6^-$>ClO$_4^-$ ∼ BF$_4^-$>H$_2$PO$_4^-$.

Cationic and anionic ionic liquids (ILs) can be sorted as either chaotropic or kosmotropic in terms of their thermodynamic parameters, such as the viscosity *B*-coefficient and hydration entropy [1]. The physicochemical properties of chaotropic salts and their applications in analytical chemistry have been studied widely [9–15]. Based on IL viscosity data, Pandey *et al.* [9] discovered that bis(trifluoromethanesulfonyl)imide (Tf$_2$N$^-$) is a stronger chaotropic ion than hexafluorophosphate

(PF_6^-), and that 1-butyl-3-methylimidazonium (BMIM$^+$) is a stronger kosmotropic cation than 1-ethyl-3-methylimidazolium (EMIM$^+$). Berthod *et al.* [10] used ILs as additives for the RPLC separation of basic cationic compounds, finding that electrostatic interactions between positively charged analytes and anionic additives affect the retention factors enormously. The separation mechanism is influenced by the chaotropic character and involves the formation of ion pairs. Vaher *et al.* [11] applied ILs to NACE [11]; these ILs were used as electrolytes in organic media for the analysis of uncharged phenols and aromatic acids. The anions of the ILs had a much greater effect on the separation resolution than did the cations. The formation of heteroconjugated species between the uncharged analyte and the anions of the ILs altered the mobility of the complex.

CE is a separation method that combines the advantageous features of online detection, high separation efficiency, low sample consumption, and rapid analysis. Affinity CE (ACE) is a CE method used to study the specific interactions between analytes and additive ligands [16–19]. The effective mobility of an analyte is affected by its interactions with ligands present within the electrolyte. Association constants for these interactions can be obtained through linear analysis, *e.g.*, from double reciprocal plots of the mobility data against the ligand concentration.

Benzodiazepines are sedative, hypnotic, and anxiolytic drugs. Because insomnia, anxiety, and depression occur frequently in modern society, the rate of benzodiazepine consumption is increasing. Overdoses of these drugs can cause acute symptoms; therefore, it is very important to be able to monitor the concentrations of these drugs in the body. Benzodiazepines are basic drugs containing amino groups; their values of pK_a range from 1.8 to 4.6, *i.e.*, they exhibit neutral characteristics in buffers having values of pH greater than 6.0. Thus, micellar EKC (MEKC) is a suitable CE method for separating benzodiazepines [20]. Although the addition of organic solvent can improve the resolution of MEKC, increasing its content in the buffer usually results in decreasing reproducibility.

In this present study, we improved the resolution of the CE method of detecting benzodiazepines through the addition of only one modifier (*cf.* the need for both surfactant and organic solvent in MEKC). We separated seven benzodiazepines using CE after adding the chaotropic salts lithium trifluoromethanesulfonate (LiOTf), lithium hexafluorophosphate (LiPF$_6$), and lithium bis(trifluoromethanesulfonyl)imide (LiNTf$_2$) as additives to the running buffer. Herein, we discuss the effects of each chaotropic anion on the separations. Under the optimized separation conditions, we used ACE in conjunction with linear analysis to determine the association constants for complex formation between the chaotropic anions and the benzodiazepines. We discuss the nature of the interactions between the chaotropic anions and the benzodiazepines based on the estimated Gibbs free energies.

2　Materials and methods

2.1　Chemicals and reagents

All reagents and chemicals were of analytical grade. Benzodiazepine standards – diazepam, clorazepate, chlordiazepoxide, bromazepam, nitrazepam, alprazeolam, and flunitrazepam (Fig. 1) – were purchased from Sigma–Aldrich (St. Louis, MO, USA). The three chaotropic salts, LiOTf (97%), LiPF$_6$ (98%), and LiNTf$_2$ (97%), were purchased from Acros
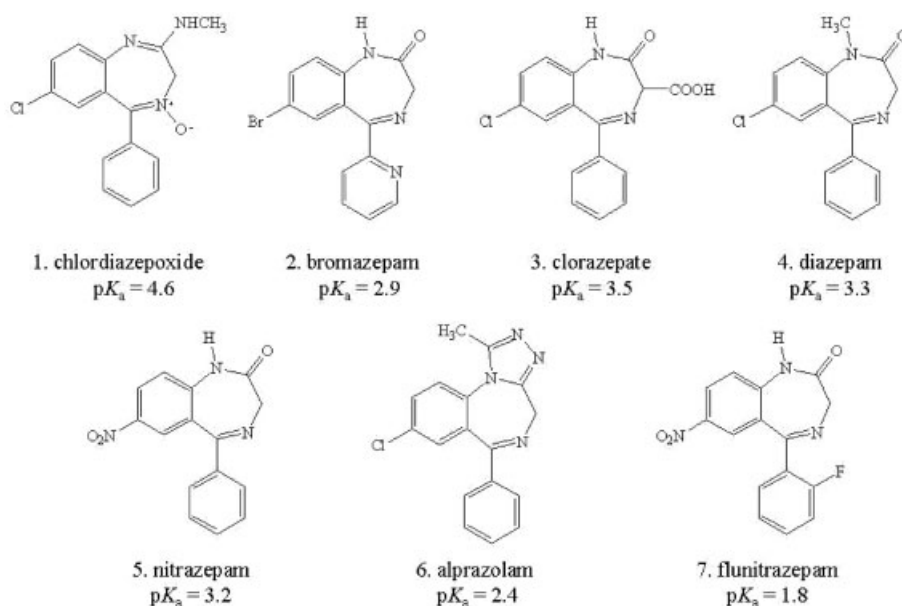

Figure 1. Structures of the seven benzodiazepines.

1. chlordiazepoxide
pK_a = 4.6

2. bromazepam
pK_a = 2.9

3. clorazepate
pK_a = 3.5

4. diazepam
pK_a = 3.3

5. nitrazepam
pK_a = 3.2

6. alprazolam
pK_a = 2.4

7. flunitrazepam
pK_a = 1.8

Organics (Geel, Belgium), Sigma–Aldrich, and Alfa Aesar (Ward Hill, MA, USA), respectively. Sodium dihydrogen phosphate (NaH_2PO_4) and sodium hydroxide (NaOH) were supplied by Fluka (Buchs, Switzerland). Disodium hydrogen phosphate anhydrous (Na_2HPO_4) was purchased from Scharlau (Barcelona, Spain). Methanol (99.9%) was purchased from Echo Chemical (Miaoli, Taiwan). Water was purified through a Milli-Q water system (Millipore, Milford, MA, USA).

2.2 Apparatus

A Beckman P/ACE MDQ CE system (Beckman, Fullerton, CA, USA) was used to effect the separations. A DAD was employed for detection. Separations were performed in a 50 cm (40 cm effective length) × 50 µm id fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA). The capillary tube was assembled in the cartridge format. A personal computer using 32 Karat software controlled the P/ACE instrument and allowed data analysis. Samples (40 µg/mL) were pressure-injected at 3.45 kPa for 3 s. The detection wavelength was set at 230 nm. The separation proceeded with a positive applied potential (15 kV). Prior to use, the capillary was preconditioned sequentially with methanol (10 min), 1 M HCl (10 min), deionized water (2 min), 1 M NaOH (10 min), and deionized water (2 min).

2.3 Standards and buffers

Stock standard solutions (1 mg/mL) of the seven benzodiazepines were prepared in methanol and refrigerated at 4 °C. Prior to analysis, the stock solution was diluted to 40 µg/mL using methanol and water (1:1 v/v) as the working solution. Solutions of the chaotropic salts LiOTf, $LiPF_6$, and $LiNTf_2$ were prepared in 20 mM phosphate buffer (pH 7.0) at concentrations ranging from 200 to 400 mM. Between runs, the capillary was flushed sequentially with methanol (3 min), water (7 min), and running buffer (5 min).

2.4 ACE

To determine the association constants and mechanisms for the interactions of the benzodiazepines, the anion exhibiting the strongest chaotropic effect, Tf_2N^-, was selected for ACE analysis. The concentration of Tf_2N^- ranged from 100 to 500 mM in a 5 mM phosphate running buffer (pH 7.0). The sample (20 µg/mL) was pressure-injected into the capillary at 3.45 kPa for 5 s. The separation proceeded under a positive applied potential (15 kV). Data were processed using OriginPro 7.0 software.

DMSO was employed as a flow marker to calculate the viscosity correction factor (ν) in running buffers containing various concentrations of Tf_2N^-; it was injected into the capillary at 3.45 kPa for 3 s and detected at 220 nm. The measurement proceeded with a pressure separation of 20.7 kPa.

The peak appearance times for DMSO were applied to the following equation to correct the analyte's electrophoretic mobility [17]

$$\nu = \frac{t_1}{t_2} = \frac{\eta_1}{\eta_2}$$

where η_1 and η_2 are the viscosities in the presence and absence of the chaotropic salt, respectively, and t_1 and t_2 are the corresponding migration times for the arrival of DMSO at the detector.

3 Results and discussion

3.1 Effect of the concentration of chaotropic salt

We selected the three chaotropic IL salts LiOTf, $LiPF_6$, and $LiNTf_2$ as additives for the CE analysis of benzodiazepines. Because strongly chaotropic anions significantly affect the retention behavior of amines during HPLC, we used these salts as buffer modifiers to improve the separation efficiency of CE. The anions TfO^-, PF_6^-, and Tf_2N^- (Fig. 2) exhibit strong chaotropic effects because of their low charges and large sizes. The cations of common salts, namely Li^+, Na^+, and K^+, lie in the middle of the Hofmeister series and do not exhibit notable kosmotropicity or chaotropicity. Thus, we suspected that these cations would have little effect on the separation efficiency and, therefore, we selected the Li^+ salts of our chaotropic anions.
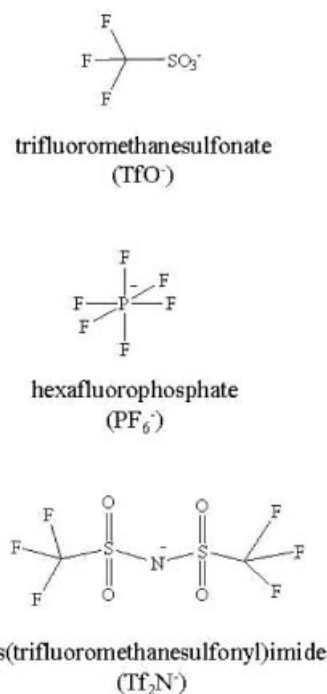

Figure 2. Structures of the three chaotropic anions.

Figure 3 indicates that increasing the TfO^- concentration from 200 to 400 mM increased the resolution of the separation of the seven benzodiazepines. At 400 mM, we observe six peaks for the analytes within 12 min, with only peaks 4 and 5 remaining unresolved. Because the solubility of TfO^- in the buffer was limited to 400 mM, no further improvement in resolution was possible. Figure 4 displays the effect of the PF_6^- concentration on the separation of the analytes. The time required for migration of all of the analytes increased from 8 to 32 min upon increasing the concentration of PF_6^- from 200 to 400 mM. Although signals for all seven of the analytes were resolved in the buffer containing 400 mM PF_6^-, the long separation time required was a drawback of this system. Figure 5 indicates that the analytes' migration times increased upon increasing the Tf_2N^- concentration from 200 to 400 mM. The analytes were all resolved adequately within 16 min in the buffer containing 300 mM Tf_2N^-. Even though the analytes were completely separated in a 400 mM Tf_2N^- buffer, the total separation time was extended to 26 min.

Figure 4. Separation of benzodiazepines using PF_6^- as modifier at (A) 200, (B) 300, and (C) 400 mM. Other conditions were the same as in Fig. 3.

In these electropherograms, chlordiazepoxide, which possesses the highest value of pK_a, was the first to migrate after the EOF marker; flunitrazepam, having the lowest pK_a, migrated last. Thus, it appears that the order of migration of these benzodiazepines followed their values of pK_a from high to low, with the exception of bromazepam. We suspect that the degrees of ionization of these analytes affected their interactions with the chaotropic anions. The analyte having the lowest pK_a was readily deprotonated in the buffer to become a neutral compound; when the chaotropic anion interacted with this compound, the complex possessed the highest negative charge and, therefore, migrated last in the electropherogram. We also studied the effect of the buffer pH on the separation, but observed no significant improvement in the analytes' resolution upon varying the buffer pH from 5.0 to 8.0. Thus, the pH of the buffer does not play a major role during the separation of these analytes.
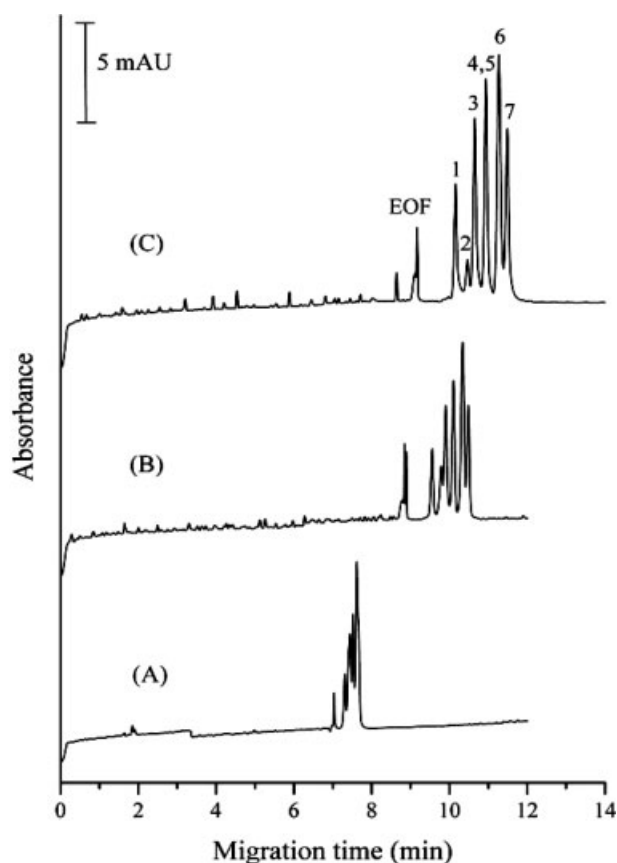

Figure 3. Separation of benzodiazepines using TfO^- as modifier at (A) 200, (B) 300, and (C) 400 mM. Conditions: BGE, 20 mM phosphate buffer (pH 7.0); sample concentration, 40 μg/mL; injection, 3.45 kPa for 3 s; separation voltage, 15 kV; detection at 230 nm; effective capillary length, 40 cm. Peak identification: 1, chlordiazepoxide; 2, bromazepam; 3, clorazepate; 4, diazepam; 5, nitrazepam; 6, alprazolam; 7, flunitrazepam.
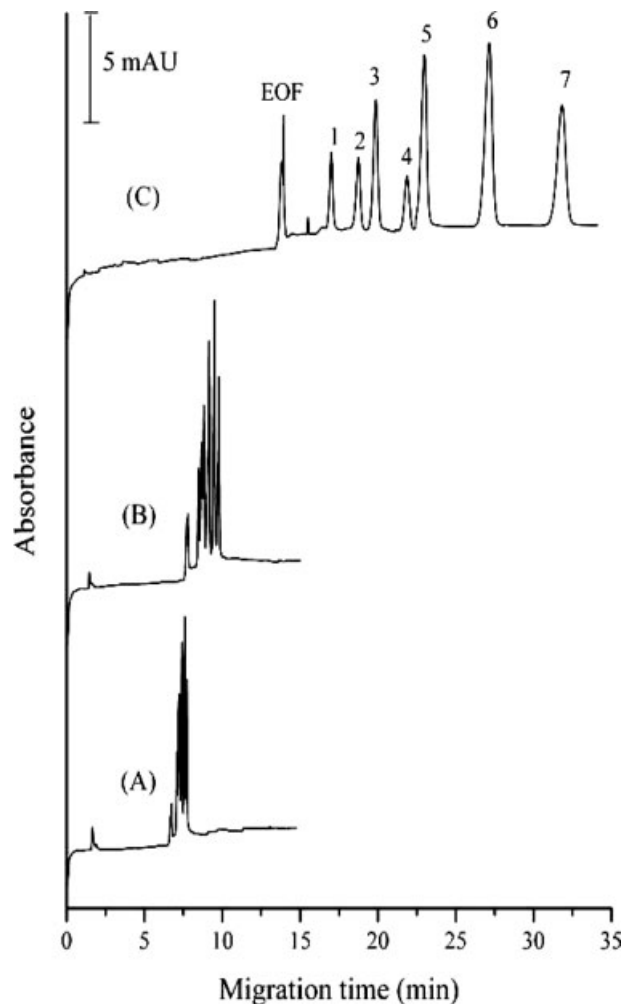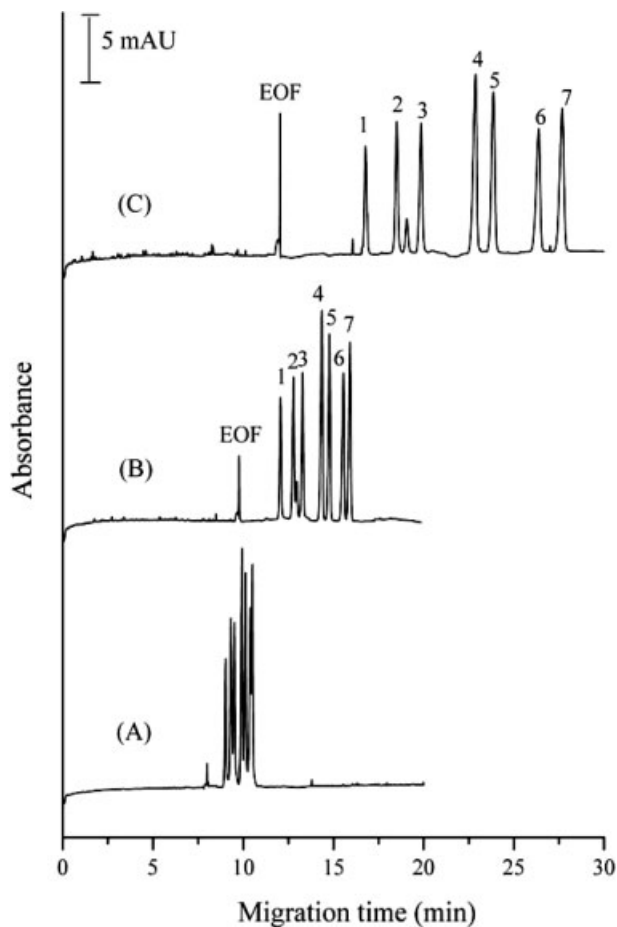
Figure 5. Separation of benzodiazepines using Tf_2N^- as modifier at (A) 200, (B) 300, and (C) 400 mM. Other conditions were the same as in Fig. 3.

3.2 Effect of the nature of the chaotropic salt

From a comparison of the separation efficiencies on the analytes in the presence of 300 mM TfO^-, PF_6^-, or Tf_2N^- in the buffer, we found that the most satisfactory separation of the seven analytes occurred when using Tf_2N^- as the buffer additive. The analytes were not completely separated after adding either 300 mM TfO^- or PF_6^- to the buffer. Pandey *et al.* [9] reported previously that Tf_2N^- is a stronger chaotropic anion than PF_6^-, which is consistent with Tf_2N^- being larger than PF_6^-. Our results indicate that adding an anion of greater chaotropic character, such as Tf_2N^-, improved the resolution of the separation, presumably because the chaotropic anion strongly disrupted the analytes' hydration, increasing their hydrophobicity and strengthening their interactions with the chaotropic anion. Because TfO^- is of similar size to PF_6^-, we expected these two anions to display similar chaotropic behavior. Indeed, the separation efficiency was similar when either TfO^- or PF_6^- was present in the buffer.

3.3 Peak efficiency and reproducibility

Table 1 list the analytes' migration times, the RSDs of these migration times, and the peak efficiencies under the optimized separation conditions when using the buffer containing 300 mM Tf_2N^-. The benzodiazepines were completely resolved within 16 min without the need to add an organic solvent as modifier. Although a high concentration of Tf_2N^- resulted in a high current (*ca.* 110 μA), which led to increased Joule heating, the theoretical plate numbers (*per* meter) reveal that the peak efficiency remained satisfactory, ranging from 1.72×10^5 to 2.15×10^5. The separation of the benzodiazepines was highly reproducible under the optimized separation conditions; the RSDs of the migration times for the seven analytes were all less than 0.38%. These results imply that the only modifier required for CE to be used as a reproducible method for analyzing benzodiazepines is a chaotropic salt.

Table 1. Average migration times, RSDs of migration times, and theoretical plate numbers for benzodiazepines in CE separation using a buffer containing 300 mM Tf_2N^-

Compound	Migration time (min)	RSD (%; *n* = 4) migration time	Theoretical plates (*N*)[a]
Chlordiazepoxide	11.70	0.326	2.14×10^5
Bromazepam	12.39	0.326	1.95×10^5
Clorazepate	12.89	0.341	2.11×10^5
Diazepam	13.92	0.332	1.72×10^5
Nitrazepam	14.32	0.368	2.15×10^5
Alprazolam	15.06	0.363	2.01×10^5
Flunitrazepam	15.42	0.377	2.10×10^5

a) $N = 5.54(t_R/W_{1/2})^2$; t_R, migration time; $W_{1/2}$, width at half peak height; in 20 mM phosphate buffer (pH 7.0) containing 300 mM Tf_2N^-.

3.4 Evaluation of complexation constants by ACE

To determine the mechanism of interaction between the chaotropic anion Tf_2N^- and the benzodiazepines, we used ACE to simultaneously evaluate the association constants for the seven complexes. Figure 5 indicates that the migration time of the EOF shifted as a result of the increase in buffer viscosity associated with increasing the concentration of Tf_2N^-. Thus, we had to allow for viscosity correction when calculating the analyte's effective mobilities; *i.e.*, multiplying the viscosity of the buffer containing the chaotropic salt by the viscosity correction factor (ν) of the EOF maker [21]. Figure 6 presents a plot of the nonlinear regression analysis according to the corrected effective electrophoretic mobilities of the benzodiazepines as a function of the concentration of Tf_2N^-. We observe that the corrected effective mobilities reached a plateau when the separation buffer contained more than 500 mM Tf_2N^-.
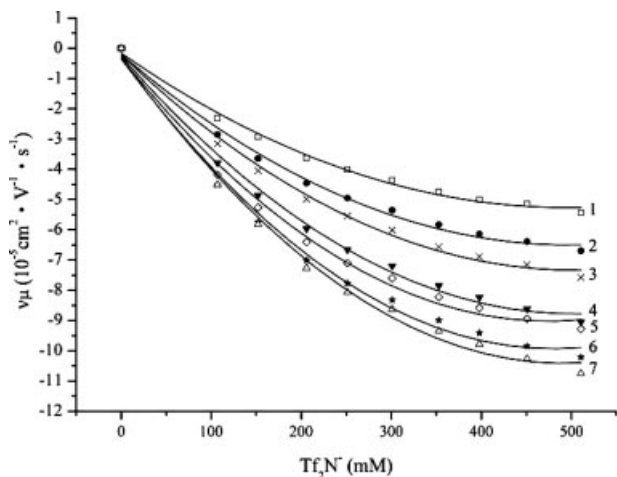
Figure 6. Variation of the corrected effective mobilities of the benzodiazepines plotted as a function of the Tf_2N^- concentration in the running buffer. The solid line represents the results of nonlinear curve fitting. The conditions for ACE are described in Section 2.

Table 2 provides the results of linear analysis using the double-reciprocal equation proposed by Rundlett and Armstrong [22]

$$\frac{1}{\mu - \mu_0} = \frac{1}{K(\mu_c - \mu_0)} \times \frac{1}{[L]} + \frac{1}{\mu_c - \mu_0}$$

where K is the association constant, [L] is the concentration of the chaotropic salt, μ is the corrected benzodiazepine mobility at the chaotropic salt concentration [L], and μ_0 and μ_c are the electrophoretic mobilities of the free and complexed benzodiazepine, respectively. Plotting $1/(\mu - \mu_0)$ *versus* $1/[L]$ allowed us to determine that the association constants ranged from 3.0 to 4.0 M^{-1} ($R^2 > 0.9976$), corresponding to Gibbs free energies ($\Delta G = -RT\ln K$) of *ca.* 3.0 kJ/mol. Hence, the interactions between the chaotropic anion Tf_2N^- and the benzodiazepines are either ion–dipole or ion–induced dipole interactions [21]. Because the ion–dipole interactions between this chaotropic anion and these analytes are relatively weak, we must also consider the existence of other possible effective interactions. The other chaotropic anions, PF_6^- and TfO^-, had less of an effect on the analyte's resolution than did Tf_2N^-. Furthermore, it will be interesting to study the effects of chaotropic anions on analytes possessing various functional groups.

Adding a modifier in the buffer to increase the separation efficiency is a common practice in CE analyses; the top choice is usually a surfactant, as is the case for MEKC. In addition, if several analytes are present simultaneously, organic solvents are often added to the buffer to alter the partition coefficient between the analyte in the bulk and in the micelles to improve the resolution. Nevertheless, the addition of organic solvent sometimes worsens the reproducibility as a result of eva

Table 2. Association constants determined using the double-reciprocal equation

Compound	Equation[a]	R^2	K (M^{-1})[b]
Chlordiazepoxide	$y = -3372x - 11591$	0.9990	3.44
Bromazepam	$y = -2725x - 9468$	0.9995	3.47
Clorazepate	$y = -2493x - 8239$	0.9994	3.30
Diazepam	$y = -2072x - 6910$	0.9995	3.33
Nitrazepam	$y = -1786x - 7146$	0.9992	4.00
Alprazolam	$y = -1671x - 6388$	0.9991	3.82
Flunitrazepam	$y = -1737x - 5717$	0.9976	3.29

a) Equation for the least-squares regression: a plot of $1/(\mu - \mu_0)$ (*y*-axis) against $1/[Tf_2N^-]$ (*x*-axis).
b) K = intercept/slope.

poration. Although other methods have been devised to increase the resolution, most of them are complicated and/or time-consuming. Thus, the addition of a chaotropic salt to the CE buffer appears to be a simple alternative method for improving the separation of analytes. Varying the concentration of the selected chaotropic salt provided satisfactory resolution without the need to add a surfactant or organic solvent. The resolution depended on the nature of the ion–dipole or ion–induced dipole interactions between the chaotropic anions and the benzodiazepines, in much the same way as micelle–analyte interactions affect the resolution of MEKC. The process of optimizing the separation conditions for this CE-based method is, however, much simpler than that required for MEKC. Our developed CE method appears highly suitable for the simple, rapid, and simultaneous determination of several neutral analytes.

4 Concluding remarks

We have examined the use of the chaotropic anions TfO^-, PF_6^-, and Tf_2N^- as modifiers for CE separation. The chaotropic effects of these anions toward benzodiazepines strongly influenced the analytes' migratory behavior. The analytes migrated after the EOF marker toward the cationic detection end as a result of the uncharged benzodiazepines complexing with the negatively charged chaotropic anions. A mixture of seven benzodiazepines was adequately resolved within 16 min after adding $LiNTf_2$ to the separation buffer. Tf_2N^-, which was the strongest chaotropic anion among the three that we tested, exhibited the greatest effect on the analytes' resolution. Using ACE, we determined that the mechanism of complexation between the chaotropic anion Tf_2N^- and the benzodiazepines involved ion–dipole or ion–induced dipole interactions. Merely varying the concentration of the chaotropic anion in the buffer (*i.e.*, without the need to add any surfactant or organic solvent) allowed us to optimize the separation of these benzodiazepines. Presently, we are investigating the influence of chaotropic salts on analytes posses-

sing various functional groups to gain insight into the general effectiveness of these modifiers in CE analyses.

The authors have declared no conflict of interest.

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