1-Butyl-3-methylimidazolium-based ionic liquids and an anionic surfactant: Excellent background electrolyte modifiers for the analysis of benzodiazepines through capillary electrophoresis

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ABSTRACT

In this study, we found that adding 1-butyl-3-methylimidazolium-based ionic liquids (ILs) and sodium dodecyl sulfate (SDS) as modifiers in the background electrolyte (BGE) for capillary electrophoresis enhanced the separation of benzodiazepines. In particular, 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([BMIM][NTf$_2$]) was the best IL additive for the separation system because its anionic moiety interacted favorably with the benzodiazepines. We added SDS because of its known effect on the separation of hydrophobic analytes. We optimized the separation conditions in terms of the concentrations of the IL, SDS, and organic solvent, the pH, and the BGE's ionic strength. The optimal BGE, containing 170 mM [BMIM][NTf$_2$] and 10 mM SDS, provided baseline separation, high efficiency, and satisfactory peak shapes for the benzodiazepines. The separation mechanism was based on heteroassociation between the anionic moiety of the IL and the benzodiazepines, with SDS improving the resolution of the separation. The limits of detection for the seven analytes ranged from 2.74 to 4.42 µg/mL. We subjected a urine sample to off-line solid phase extraction (SPE) prior to the analysis of its benzodiazepine content. Our experimental results reveal that the combination of [BMIM][NTf$_2$] and SDS provides adequate separation efficiency for its application to CE analyses of benzodiazepines after SPE concentration.

1. Introduction

Ionic liquids (ILs) are compounds comprising organic cations and inorganic or organic anions that have melting points below 100 °C [1]. Because the choice of the anionic and cationic components affects the properties of the ILs, they can be regarded as designer solvents [2]. ILs exhibit many interesting properties, including excellent solvation over a wide range of viscosities and temperatures [3], negligible vapor pressure, and non-volatility. ILs have been used in analytical separations [1,4] as (i) the stationary phases of gas chromatography (GC) columns [5,6], on account of their low volatility and thermal stability, and (ii) as stationary phases for liquid chromatography (LC) columns [7,8], because their non-molecular characteristics result in unusual selectivities. Therefore, the use of ILs provides GC and LC methods with additional variables for successful separation and analysis.

Much effort has been exerted recently in the application of ILs to capillary electrophoresis (CE). For example, ILs have been used as support coatings covalently bonded to the inner walls of capillaries [4]; when cationic imidazolium-based ILs were applied, the electroosmotic flow (EOF) of the capillary was reversed relative to that of the uncoated capillary. Qin and Li [9] used this technique to weaken the interactions between the analytes and the capillary wall to determine metal or ammonium ions. ILs have also been used as dynamic wall coatings in aqueous CE [1]. Qi et al. [10] used ILs in the BGE to analyze the anthraquinones present in Chinese herbs; in such cases, the ILs play dual roles: as EOF modifiers and as species that associate with the analytes. ILs have also been applied to non-aqueous CE, where dynamic coating of the capillary wall is greatly decreased and/or inexistent [1,11]. The resulting improved resolution has been attributed to heteroassociation between the ILs and the analytes. Vaher et al. [12] separated polyphenolic compounds by applying 1-alkyl-3-methylimidazolium-based ILs possessing various counter anions. ILs have also been used as modifiers to improve separations performed through micellar electrokinetic chromatography (MEKC) [1]. Mwongela et al. [3] employed imidazolium-based ILs in polymeric surfactant systems to analyze both achiral and chiral compounds. The addition of the ILs appears to increase the ionic strength and decrease the EOF, thereby influencing the analytes' migration times. Tian et al. added

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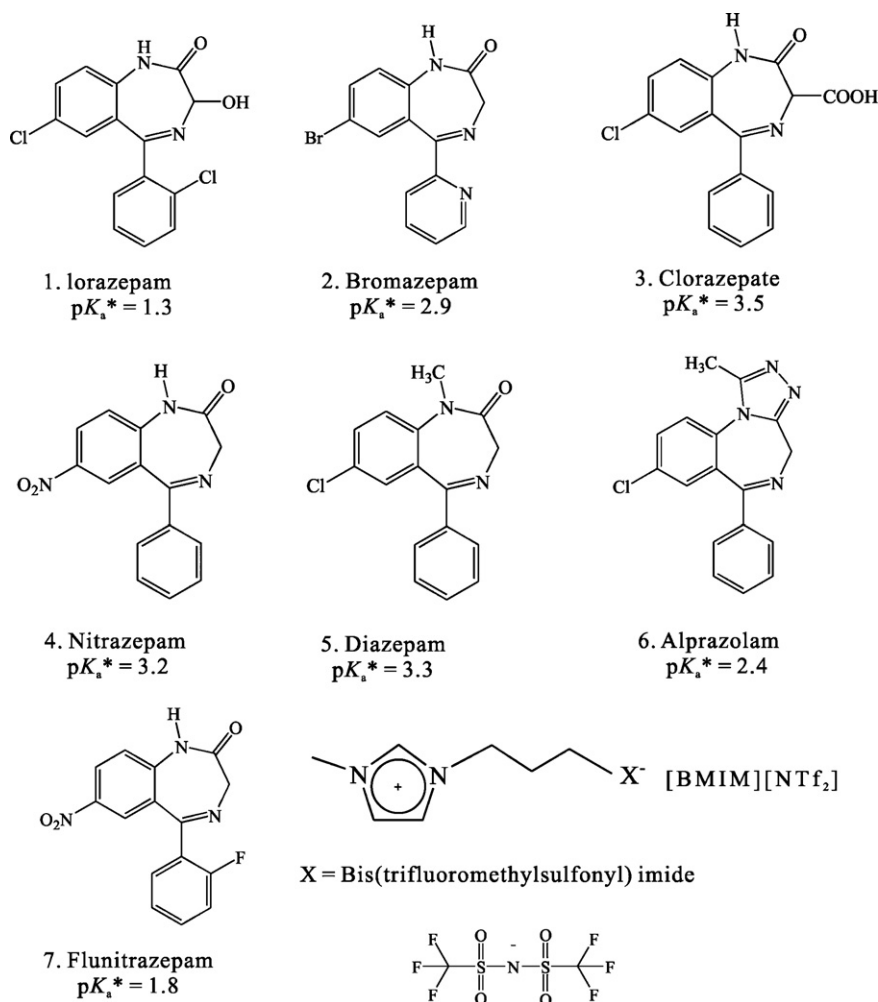E-mail address: yzhsieh@mail.nctu.edu.tw (Y.-Z. Hsieh).

Fig. 1. Structures of benzodiazepines and [BMIM][NTf$_2$]. *Values of pK_a are those of the conjugate acids of the benzodiazepines.

1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF$_4$]) as a modifier for the MEKC separation of lignans. The BMIM$^+$ cation appears to modify the sodium dodecyl sulfate (SDS) micelles and, thereby, change the degree of solute partitioning [13].

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 desirable to monitor their concentrations in the body [14]. CE is a suitable method for separating benzodiazepines because of its rapidity, efficiency, and selectivity. Although SDS micelles have been employed previously to determine benzodiazepines [15–17], the resolution of this approach is poor when simultaneously analyzing several benzodiazepines. To obtain the highest degree of resolution of benzodiazepines, we investigated the effects of adding ILs and SDS as modifiers in CE separations. Among the known ILs, imidazolium-based ILs have been used most commonly in CE [18–21] because their cheaper prices or simpler syntheses have made them as the first choice for analytical scientists. In particular, 1-butyl-3-methylimidazolium–based ILs have been used most often in both aqueous and non-aqueous CE. Hence, in this study, we selected 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([BMIM][NTf$_2$]) as a modifier to analyze a mixture of benzodiazepines, including diazepam, clorazepate, bromazepam, nitrazepam, alprazolam, flunitrazepam, and lorazepam (Fig. 1). We optimized the separation conditions in

terms of the concentrations of the IL, SDS, and organic solvent, the pH, and the ionic strength. In addition, we determined the effects that the interactions of the ILs and SDS with the benzodiazepines had on their CE separation. We then used the developed method to analyze benzodizepines in spiked urine samples after solid phase extraction (SPE).

2. Materials and methods

2.1. Chemicals and reagents

All reagents and chemicals were of analytical grade. Lorazepam, bromazepam, clorazepate, nitrazepam, diazepam, alprazolam, and flunitrazepam standards were obtained from Sigma–Aldrich (St. Louis, MO, USA). 1-Methylimidazole (99%), 1-bromohexadecane (C$_{16}$H$_{33}$Br, 99%), and 1-methylpyrrolidine (98%) were purchased from Acros Organics (Geel, Belgium). Lithium bis(trifluoromethanesulfonyl)imide (LiNTf$_2$, 97%) was purchased from Alfa Aesar (Ward Hill, MA, USA). Disodium hydrogenphosphate (Na$_2$HPO$_4$), sodium dihydrogenphosphate (NaH$_2$PO$_4$), and sodium hydroxide (NaOH) were purchased from Fluka (Buchs, Switzerland). SDS was purchased from J.T. Baker (Phillipsburg, NJ). Methanol was purchased from Echo Chemical (Miaoli, Taiwan). Ethyl acetate was purchased from Grand Chemical (Bangkok, Thailand). Citric acid was obtained from Merck (Darmstadt, Germany). Dichloromethane was obtained from Tedia (Fairfield, OH, USA).

Deionized water was purified through a Millipore Milli-Q water system (Milford, MA, USA).

2.2. Apparatus

A Beckman P/ACE MDQ CE system (Fullerton, CA, USA) was used to effect the separations. A diode array detector was employed for detection. Separations were performed in a 50-cm (effective length: 40 cm) × 50-μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), which was assembled in cartridge format. A personal computer running 32 Karat software controlled the P/ACE instrument and allowed data analysis. Samples (80 μg/mL) were pressure-injected at 3.45 kPa for 3 s. The detection wavelength was set at 230 nm. The separations were performed under a positive applied potential (20 kV). Prior to use, the separation capillary was preconditioned (at 138 kPa) sequentially with MeOH (10 min), 1 M HCl (10 min), deionized water (2 min), 1 M NaOH (10 min), and then deionized water again (2 min).

2.3. Synthesis of ILs

[BMIM][Cl] was prepared using a slight modification of a literature procedure [22]. 1-Methylimidazole (0.15 mol) was reacted with 1-chlorobutane (0.15 mol) for 48 h at 80 °C. The resulting salt was washed twice with ethyl acetate to yield a yellow liquid. After drying under vacuum for 2 days, [BMIM][Cl] was obtained as a highly viscous liquid.

[BMIM][NTf$_2$] was synthesized through the reaction of [BMIM][Cl] (0.035 mol) and LiNTf$_2$ (0.035 mol) in acetone (50 mL) at room temperature for 6 h. After removing the solvent under rotary evaporation, the residue was dissolved in CH$_2$Cl$_2$ (100 mL) and washed with water (3× 25 mL) and then the solvent was removed under rotary evaporation [23]. [BMIM][Cl] and [BMIM][NTf$_2$] were analyzed qualitatively using ^{1}H NMR spectroscopy.

2.4. Preparing standards and urine samples

Stock standard solutions (1 mg/mL) of the seven benzodiazepines were prepared in MeOH and refrigerated at 4 °C. Prior to analysis, each stock solution was diluted to 80 μg/mL with MeOH and water (1:1, v/v) to form the working solution. The [BMIM][NTf$_2$] BGEs were prepared at concentrations ranging from 100 to 240 mM in 10–30 mM phosphate buffer containing 50–60% MeOH and 5–20 mM SDS. Between runs, the capillary was flushed (at 138 kPa) sequentially with MeOH (1.5 min), water (1.5 min), 0.1 M NaOH (1 min), water (1.5 min), and the BGE (5 min). Calibration curves were obtained after preparing solutions of the standards individually at 10, 20, 50, 100, and 150 μg/mL. Experiments were performed four times at each concentration. Samples of human urine were collected and frozen at −20 °C; when required for an assay, they were thawed and warmed to room temperature.

2.5. Solid phase extraction

Oasis® MCX column-type cartridges were obtained from Waters (Milford, MA, USA). The cartridges (3 mL/60 mg) were first conditioned with methanol (2 mL) and H$_2$O (2 mL). The loading sample was a drug-free urine sample (2 mL) spiked with seven benzodiazepines (1000 μg/mL, 25 μL) and citric acid/disodium hydrogenphosphate buffer (1 M, 100 μL); the pH was 5.0. The concentration of benzodiazepines in the final solution was 11 μg/mL. The column was washed sequentially with 0.1 M HCl (0.5 mL) and methanol (0.5 mL). The elution solution was a mixture of dichloromethane, isopropyl alcohol, and sat. ammonium hydroxide (78:20:2, v/v/v); the mixture was dried under a stream of nitrogen

gas [24]. For assaying, the residue was dissolved in a mixture of methanol (125 μL) and water (125 μL).

3. Results and discussion

In preliminary experiments, we observed poor separation efficiency of the seven benzodiazepines when using SDS surfactant as the sole modifier. For example, when using 5 mM phosphate buffer (pH 8.5) containing 65 mM SDS and 55% MeOH, the analysis required a long time (ca. 40 min) to achieve separation. In corresponding experiments using the ILs, we first investigated the effect of the cationic moiety of the ILs by comparing the performance of [BMIM][Cl] and [EMIM][Cl] as modifiers. Our results revealed that [BMIM][Cl] separated the benzodiazepines with better resolution. Next, to study the effect of the anionic moiety of the ILs on the separation of the benzodiazepines, we compared the performance of [BMIM][Cl], [BMIM][PF$_6$], and [BMIM][NTf$_2$] as additives. We found that [BMIM][NTf$_2$] had a dramatic effect on the separation of these analytes. To achieve satisfactory separation using [BMIM][NTf$_2$] in the BGE with SDS as a modifier, we investigated the combined effects of the concentrations of [BMIM][NTf$_2$], SDS, and organic solvent, the pH of the phosphate buffer, and the ionic strength on the migration time and selectivity of the benzodiazepines.
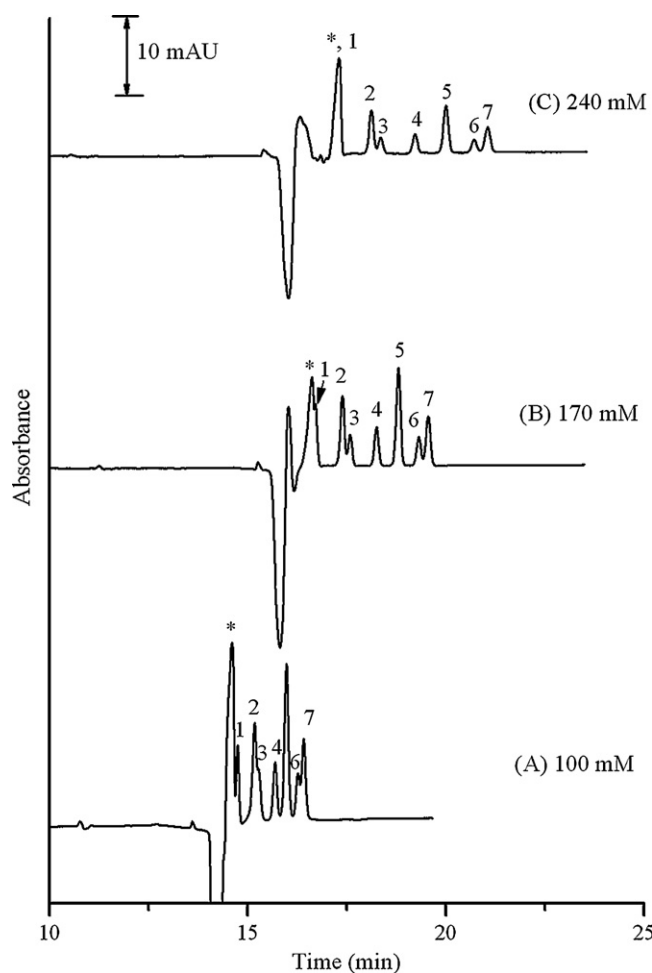

Fig. 2. Electropherograms of the seven benzodiazepines recorded at [BMIM][NTf$_2$] concentrations of (A) 100, (B) 170, and (C) 240 mM. Conditions: 20 mM phosphate buffer (pH 7.0) containing 55% MeOH; sample concentration, 80 μg/mL in MeOH/water (1:1, v/v); separation voltage, 20 kV; detection at 230 nm. Peak identification: (*) system peak; (1) lorazepam; (2) bromazepam; (3) clorazepate; (4) nitrazepam; (5) diazepam; (6) alprazolam; (7) flunitrazepam.
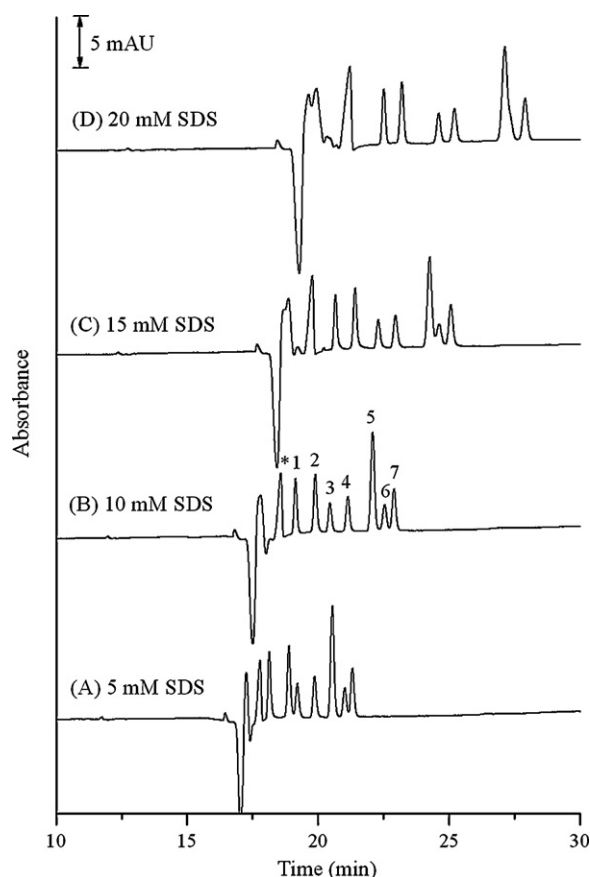
Fig. 3. Electropherograms of the seven benzodiazepines recorded at SDS concentrations of (A) 5, (B) 10, (C) 15, and (D) 20 mM. Conditions: 20 mM phosphate buffer (pH 7.0) containing 170 mM [BMIM][NTf$_2$] and 55% MeOH; sample concentration, 80 μg/mL in MeOH/water (1:1, v/v).

3.1. Effect of [BMIM][NTf$_2$] concentration

In aqueous CE, the capillary wall is coated and the direction of the EOF is reversed after adding imidazolium ions to the BGE. In contrast, the degree of dynamic coating by imidazolium ions is decreased in non-aqueous CE [1,7]. Because we used a high content of organic solvent in this study, we first investigated the influence of the IL on the direction of the EOF, applying reverse polarity voltage and a BGE containing 170 mM [BMIM][NTf$_2$] and 55% MeOH. Neither the EOF marker nor the analyte signal was observed when the voltage was set at −20 kV. After increasing the concentration of [BMIM][NTf$_2$] to 240 mM, we still did not observe any signals for the analytes within 60 min. When we applied a separation voltage of +20 kV, however, the signals for the analytes emerged. These results indicate that adding [BMIM][NTf$_2$] to the BGE did not lead to a significant degree of coating of the capillary wall. We suspect that the direction of the EOF was not influenced by the added imidazolium ions in the BGE because the BGE contained an organic solvent content of greater than 50%. Thus, the main role of [BMIM][NTf$_2$] was that of an association agent, not as an EOF modifier.

Fig. 2 displays the electropherograms obtained when using [BMIM][NTf$_2$] in the BGE at concentrations ranging from 100 to 240 mM—in the absence of SDS—at a constant buffer pH (pH 7.0) and a constant concentration of organic solvent. The resolution of the signals for bromazepam, clorazepate, nitrazepam, diazepam, alprazolam, and flunitrazepam increased upon increasing the [BMIM][NTf$_2$] concentration, but the signal for lorazepam remained overlapped with the system peak. The mobility and

selectivity of the analyte changed significantly upon changing the concentration of [BMIM][NTf$_2$]. At 170 mM [BMIM][NTf$_2$], we observed the best separation of the seven analytes; therefore, we employed this concentration in our subsequent experiments.

3.2. Effects of the concentrations of SDS and organic solvent

Fig. 3 reveals the effect of the SDS concentration (5–20 mM) on the separation of the analytes in the BGE containing 170 mM [BMIM][NTf$_2$]. An increase in the SDS concentration improved the resolution of the signals for lorazepam, bromazepam, clorazepate, and nitrazepam. For diazepam, alprazolam, and flunitrazepam, 10 mM SDS provided the optimal resolution; when the SDS concentration exceeded 15 mM, the signals for diazepam and alprazolam migrated together. The improved separation efficiencies for the BGEs containing 5–10 mM SDS were probably due to synergistic effects between the ILs and SDS. Overall, the best results were obtained when using 10 mM SDS in the BGE.

We added organic solvent in this system to increase the solubility of [BMIM][NTf$_2$] in the BGE ([BMIM][NTf$_2$] is only slightly soluble in water). In a preliminary experiment, we attempted to separate the benzodiazepines through non-aqueous CE using MeOH/ACN mixtures [50–100% (v/v)] containing 170 mM [BMIM][NTf$_2$] (pH 5.0), but we could not obtain satisfactory separation. Because [BMIM][NTf$_2$] is quite soluble in MeOH, we employed MeOH at concentrations greater than 50% to improve the solubility of the IL in the BGE for CE. When we increased the concentration of MeOH in the BGE from 50 to 60% (v/v), the separation time increased (Fig. 4). We suspect that the decrease in the EOF was due
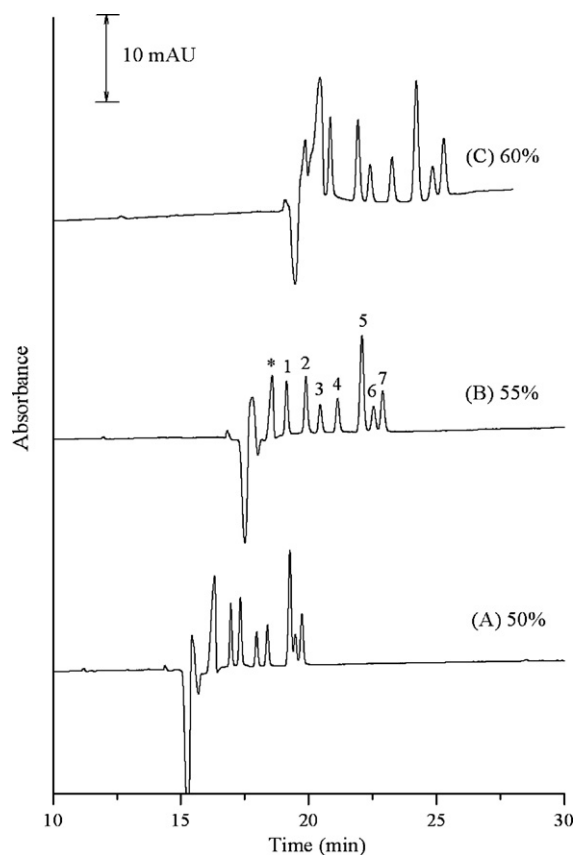

Fig. 4. Electropherograms of the seven benzodiazepines recorded at MeOH concentrations of (A) 50, (B) 55, and (C) 60%. Conditions: 20 mM phosphate buffer (pH 7.0) containing 170 mM [BMIM][NTf$_2$] and 10 mM SDS.

to the decreased dielectric constant, zeta potential, and viscosity of the BGE. Because micelles are difficult to form in BGEs containing more than 50% MeOH, it appears that the analytes' enhanced separation was a result of solvophobic interactions with individual surfactant molecules. Because adding 55% MeOH to the BGE provided the optimal resolution and a stable baseline, we employed this concentration in our subsequent experiments.

3.3. Effects of buffer pH and electrolyte concentration

Changing the BGE's pH affects both the EOF and the charge of the analyte. Fig. 5 reveals the effect of the pH (pH 6.0–8.0) of the phosphate buffer on the analytes' migrations, while maintaining the other separation conditions constant. The EOF increased upon increasing the pH from 6.0 to 8.0. Because the analytes possessed no net charge in the BGE within this pH range, the main effect on their migration times was caused by a variation of the EOF. At pH 8.0, the resolution of diazepam, alprazolam, and flunitrazepam was poor; at pH 6.0, the analysis time was ca. 28 min. Therefore, the optimal conditions for the separation of these analytes involved a pH of 7.0.

Fig. 6 displays the effect of the phosphate concentration in the BGE on the analytes' separation. Although the addition of the IL also contributed to the electrolyte's ionic strength [23], we kept its concentration constant in this study. The EOF decreased upon increasing the phosphate concentration, presumably due to narrowing of the double-layer thickness at the capillary wall. At 10 mM phosphate, the EOF was too fast to completely resolve the peaks for diazepam, alprazolam, and flunitrazepam. At 30 mM phosphate, the analysis time was extended to 27 min. Hence, the best resolution for all of the analytes within a reasonable analysis time occurred when we added 20 mM phosphate to the BGE.
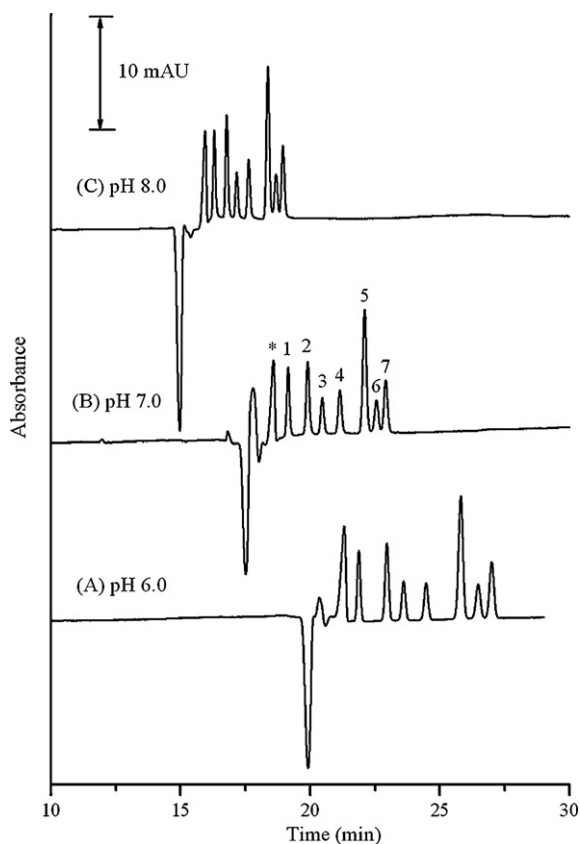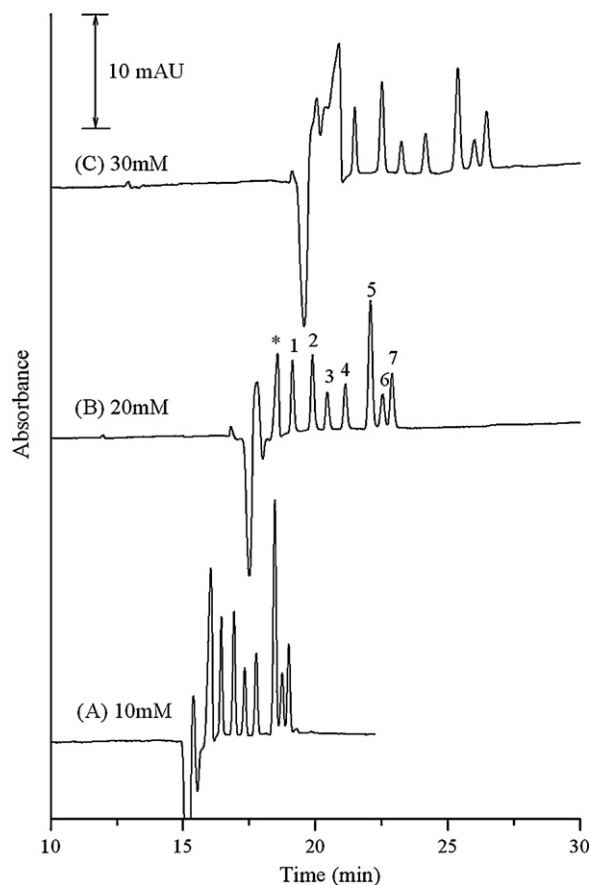

Fig. 6. Effect of phosphate concentration on CE analysis. Conditions: phosphate buffer (pH 7.0) containing 170 mM [BMIM][NTf$_2$], 55% MeOH, and 10 mM SDS. Inset: effect of phosphate concentration on the EOF.

3.4. Mechanism of separation using [BMIM][NTf$_2$] and SDS

Although the analytes possessed no net charge in the BGE at pH 7.0, their signals appeared after that of the EOF marker. This phenomenon suggested that the association of the benzodiazepines with the free anions was the cause of their separation. In addition, the migration order of the analytes was the same for the BGEs containing either [BMIM][NTf$_2$] or [BMIM][NTf$_2$]/SDS, but it differed from that obtained when the BGE containing SDS only. These experimental results suggest that [BMIM][NTf$_2$] was the dominant agent in the separation mechanism, with the anionic moiety of [BMIM][NTf$_2$] involved in heteroassociation interactions with the analytes and SDS improving the resolution through hydrophobic, electrostatic, and/or hydrogen bonding interactions. Thus, the different degrees of association of the individual benzodiazepines allowed their mixture to be separated adequately.

3.5. Calibration curve and detection limits

Table 1 lists the ranges of linearity, limits of detection (LODs), relative standard deviations (RSDs) of the migration times and peak areas, and peak efficiencies for the analytes under the optimized separation conditions for a BGE containing 170 mM [BMIM][NTf$_2$] and 10 mM SDS. The benzodiazepines were completely resolved within 23 min. The calibration curve was linear from 10 to 150 μg/mL and the coefficients of determination (r^2) were all greater than 0.9924. The LODs [signal-to-noise (S/N) ratio = 3] of the seven analytes ranged from 2.74 to 4.42 μg/mL. The theoretical plate numbers (per meter) ranged from 2.64×10^5 to 3.52×10^5 [unnormalized (absolute) plate numbers ranged from

Fig. 5. Effect of pH on CE analysis. Conditions: 20 mM phosphate buffer containing 170 mM [BMIM][NTf$_2$], 55% MeOH, and 10 mM SDS. Inset: effect of pH on the EOF.

Table 1
Calibration curves, coefficients of determination (r^2), limits of detection (LODs), relative standard deviations (RSDs), and theoretical plate numbers for benzodiazepines separated through CE.

	Lorazepam	Bromazepam	Clorazepate	Nitrazepam	Diazepam	Alprazolam	Flunitrazepam
Calibration curve[a]	$y = 163x - 759$	$y = 177x - 1295$	$y = 104x - 400$	$y = 157x - 1294$	$y = 211x - 1851$	$y = 107x - 613$	$y = 128x - 1110$
Coefficient of determination	0.9982	0.9973	0.9976	0.9924	0.9960	0.9975	0.9969
LOD (S/N = 3; μg/mL)	4.23	4.42	4.23	3.63	2.74	3.17	3.63
LOQ (S/N = 10; μg/mL)	14.1	14.7	14.1	12.1	9.14	10.6	12.1
RSD (%; $n = 5$)							
(a) Migration time (min)	0.18	0.23	0.18	0.16	0.15	0.15	0.16
(b) Peak area	5.30	2.43	4.68	6.39	4.85	7.07	9.59
Theoretical plates (N, per meter)[b]	3.52×10^5	2.79×10^5	2.95×10^5	2.75×10^5	2.64×10^5	2.75×10^5	2.83×10^5

[a] Calibration line (10–150 μg/mL): peak area (arbitrary units) = slope × concentration (μg/mL) + y-intercept.
[b] $N = 5.54(t_R/W_{1/2})^2$; t_R, migration time; $W_{1/2}$, width at half peak height; in 20 mM phosphate buffer (pH 7.0) containing 170 mM [BMIM][NTf$_2$], 10 mM SDS, and 55% MeOH.

1.06×10^5 to 1.41×10^5]. The RSDs of the migration times for the seven analytes were all less than 0.23% under the optimized separation conditions. These results imply that combining ILs with SDS for the analysis of benzodiazepines provides satisfactory repeatability.

3.6. Analyzing spiked urine samples

We employed SPE prior to performing CE separation of the benzodiazepines in a urine sample to eliminate interference from the urine sample and to concentrate the analytes. Fig. 7 reveals that the presence of the seven analytes in the spiked urine sample was discernable without interference from any unknown compounds. The
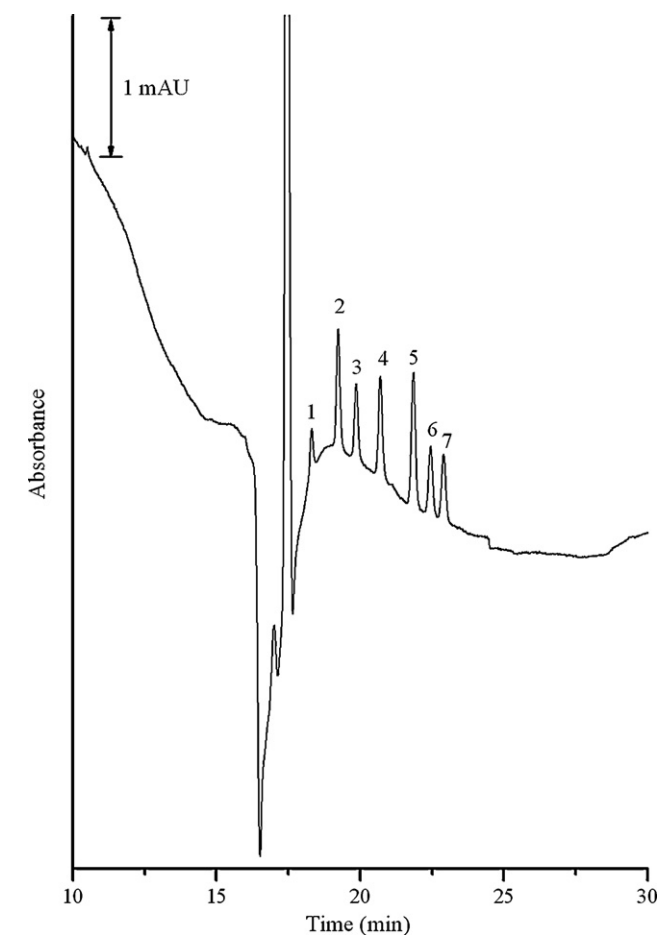

Fig. 7. CE electropherogram, obtained using [BMIM][NTf$_2$] and SDS, of a urine sample spiked with the seven benzodiazepines. Analysis was performed under the optimized conditions. Other conditions were the same as those used to obtain Fig. 2.

analytes were confirmed by their migration times and absorbance spectra. Under the optimized SPE conditions, the recoveries were 50.1% for lorazepam, 88.2% for bromazepam, 81.8% for clorazepate, 74.7% for nitrazepam, 78.0% for diazepam, 68.5% for alprazolam, and 72.6% for flunitrazepam; the repeatabilities (RSD; $n = 4$) of the extractions were 2.3, 5.4, 5.4, 9.8, 9.7, 2.8 and 5.1%, respectively.

4. Concluding remarks

In this study, we used CE to separate a mixture of seven benzodiazepines within 23 min after adding an IL to the BGE and using SDS as a modifier. The addition of [BMIM][NTf$_2$] and SDS provided high selectivity and improved the resolution of the seven analytes of interest, presumably because the different degrees of association of the individual benzodiazepines allowed their mixture to be separated satisfactorily, relative to the results obtained using the IL or SDS alone. [BMIM][NTf$_2$] appeared to play a dominant role during the separation process. The anionic moiety of [BMIM][NTf$_2$] behaved as a heteroassociation site for the benzodiazepines; added SDS improved the resolution of the separation. The optimal BGE for separation of the benzodiazepines comprised 170 mM [BMIM][NTf$_2$] at pH 7.0, 20 mM phosphate buffer, 10 mM SDS, and 55% MeOH. We also applied this method, in conjunction with SPE, to the successful analysis of the seven benzodiazepines in a spiked sample of human urine. Our results suggest that ILs can contribute significantly to CE-based separations.

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