Establishment and Evaluation of the Novel Tetramethylammonium-L-Hydroxyproline Chiral Ionic Liquid Synergistic System Based on Clindamycin Phosphate for Enantioseparation by Capillary Electrophoresis

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ABSTRACT Much attention has been paid to chiral ionic liquids (ILs) in analytical chemistry, especially its application in capillary electrophoresis (CE) enantioseparation. However, the investigation of chiral ionic liquids synergistic systems based on antibiotic chiral selectors has been reported in only one article. In this work, a novel chiral ionic liquid, tetramethylammonium-L-hydroxyproline (TMA-L-Hyp), was applied for the first time in CE chiral separation to evaluate its potential synergistic effect with clindamycin phosphate (CP) as the chiral selector. As observed, significantly improved separation was obtained in this TMA-L-Hyp/CP synergistic system compared to TMA-L-Hyp or a CP single system. Several primary factors that might influence the separation were investigated, including CP concentration, TMA-L-Hyp concentration, buffer pH, types and concentrations of organic modifier, applied voltage, and capillary temperature. The best results were obtained with a 40 mM borax buffer (pH 7.6) containing 30 mM TMA-L-Hyp, 80 mM CP, and 20% (v/v) methanol, while the applied voltage and temperature were set at 20 kV and 20°C, respectively. *Chirality 27:598–604, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: enantioseparation; synergistic effect; ionic liquids; capillary electrophoresis; antibiotics

Nowadays, enantiomeric separation has gained more and more attention since different enantiomeric forms of chiral compounds have different physiological, toxicological, or pharmacodynamic properties.[1] Recognizing the importance of chiral effects, the US Food and Drug Administration and the European Medicines Agency have issued guidelines requiring information on the enantiomeric purity of the optically active compounds prior to their marketing.[2] For this reason, many effective chiral separation techniques have been explored such as high-performance liquid chromatography (HPLC), gas chromatography (GC), etc. Capillary electrophoresis (CE) has been proven to be an excellent method in this field for its high separation efficiency, fast separation, low sample consumptions, and feasibility.[3] In CE, most enantiomeric separations are carried out by the use of a buffer solution containing chiral selectors, mainly cyclodextrins (CDs) and their derivatives,[4] polysaccharides,[5] macrocyclic antibiotics,[6,7] etc. Unfortunately, sometimes the aforementioned chiral selectors used alone may not provide adequate selectivity and resolution. Hence, more than one chiral selector is required to improve the enantioseparation.

Ionic liquids (ILs) are defined as materials consisting of only ionic components with low melting points.[8] They have a number of unique chemical and physical properties including negligible vapor pressure, low melting point, high solubility power, and being air- and moisture-stable.[9,10] These properties enabled ILs to be used in many fields such as organic synthesis, electrochemistry, and so on.[11] To date, the application of ILs in separation analysis has received considerable attention. Since ILs were first introduced by Armstrong et al.[12] in 1999, they have been used as a suitable stationary phase in GC[13] and additive/stationary phase in HPLC[14]. ILs can be used as background electrolyte (BGE) or additives in capillary zone electrophoresis (CZE)[15,16] and nonaqueous capillary electrophoresis (NACE),[17] as well as supported coatings of the capillary wall in capillary electrochromatography (CEC).[18] Recently, several studies have reported the application of chiral ILs (CILs) as chiral selectors alone or as additives to cyclodextrins (CDs) and their derivatives for CE enantioseparation,[19–26] and researchers have noted that the combination of CDs and CILs exhibited a significant synergistic effect during the enantioseparation process.[21–25] As far as we know, however, the investigation of chiral ionic liquid synergistic systems based on antibiotics chiral selectors has been reported in only one article.[24]

Tetramethylammonium-L-hydroxyproline (TMA-L-Hyp) IL (Fig. 1a), belonging to the short-chain tetraalkylammonium-based Ils, has never been previously investigated. Compared with long-chain ammonium surfactants, short-chain tetraalkylammonium-based ILs are relatively more hydrophilic and, thus, can be used in high concentrations for low

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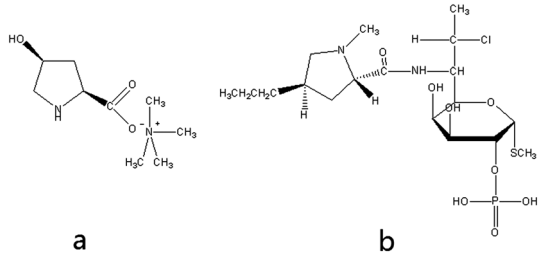

Fig. 1. Structures of the TMA-L-Hyp IL (**a**) and CP (**b**).

conductivity. Recently, we reported the use of a new antibiotic, clindamycin phosphate (CP), which belongs to the lincosamides family as a novel chiral selector in CE.[7] CP consists of an amide portion, an amino portion (tertiaryamine), a phosphate ester portion, and an aglycon portion in each molecule (Fig. 1b). It possesses not only high solubility but also low viscosity in both water and alcohols. Furthermore, due to the lack of aromatic rings in the structure, it exhibits very weak UV absorption. In this work, a novel TMA-L-Hyp IL synergistic system based on CP was established for the first time. Under the same condition, the enantioseparation capability of the single system (TMA-L-Hyp IL or CP) and the dual system (TMA-L-Hyp IL and CP) were investigated and compared to search for possible synergistic effects. Several separation conditions that might influence this dual system were examined and optimized, including CP concentration, TMA-L-Hyp concentration, buffer pH, types and concentrations of organic modifier, applied voltage, and capillary temperature.

EXPERIMENTAL

Chemicals and Reagents

CP (purity > 99%) was supplied by Jiangsu Institute for Food and Drug Control (Nanjing, China). Tetramethylammonium-L-hydroxyproline ionic liquid (purity >99%) was purchased from Shanghai Cheng Jie Chemical (Shanghai, China). Propranolol hydrochloride (PRO) was purchased from Sigma (St. Louis, MO). Nefopam hydrochloride (NEF), citalopram hydrobromide (CIT), and chlorphenamine maleate (CHL) were supplied by Jiangsu Institute for Food and Drug Control. All these drug samples were racemic mixtures.

Methanol, isopropanol, and acetonitrile, all of HPLC grade, were purchased from Jiangsu Hanbon Sci. & Tech. (Nanjing, China). Sodium hydroxide, hydrochloric acid, and sodium tetraborate decahydrate, all of analytical grade, were purchased from Nanjing Chemical Reagent (Nanjing, China). Double-distilled water was used throughout all the experiments.

Apparatus

Electrophoretic experiments were performed with an Agilent 3D capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), which consisted of a sampling device, a power supply, a photodiode array UV detector (wavelength range from 190 nm to 600 nm), and a data processor. The whole system was driven by Agilent ChemStation software (Revision B.02.01) for system control, data collection, and analysis. It was equipped with a 50 cm (41.5 cm effective length) × 50 μm i.d. uncoated fused-silica capillary (Hebei Yongnian County Reafine Chromatography, Hebei, China). A new capillary was flushed for 20 min with 1 M NaOH, 20 min with 0.1 M NaOH, and 30 min with water. At the end of each day, it was flushed successively with 0.1 M NaOH (10 min) and water (10 min). Between consecutive injections, the capillary was rinsed with 0.1 M NaOH (2 min), water (2 min), and running buffer (5 min). Sample injections were performed by pressure (50 mbar, 5 s). Enantioseparations were performed at a constant applied voltage in a range of 12–28 kV, and the temperature of capillary was controlled at 14–28°C using an air-cooling system.

Experimental Procedures

Buffer solution was a 40 mM borax buffer containing methanol (20% v/v), if not stated otherwise. The running buffer was freshly prepared by dissolving CP and tetramethylammonium-L-hydroxyproline IL in the buffer solution with a specified pH, and then pH was adjusted exactly to a desired value by adding a small volume of 1 M hydrochloric acid or sodium hydroxide solution using a microsyringe. Each buffer solution was filtered through a 0.45 μm pore membrane filter prior to use. The racemic drugs were dissolved in water. Final sample solutions for injection were all at a concentration of 0.4 mg/ml.

Calculations

Separation factor (α) and chiral resolution (R_s) were calculated by the equations [1] and [2], respectively:

$$a = t_2/t_1 \quad (1)$$

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \quad (2)$$

Where t_1, t_2 are the migration time of the first and second eluted enantiomer, respectively, and w_1, w_2 are the corresponding widths at the peak base. The electroosmotic flow (EOF) was expressed by equation [3]:

$$\mu_{eof} = lL/t_0V \quad (3)$$

Where l is the effective capillary length, L is the total capillary length, t_0 is the migration time of neutral maker (thiourea), and V is the applied voltage.

RESULTS AND DISCUSSION

Figure 2 shows the comparative electropherograms of the dual system and the single CP system. It can be observed that the R_s of the tested drugs in the dual system had a significant increase compared with the single system: the R_s of the PRO, NEF, CHL, and CIT increased from 2.21, 3.01, 2.53, 0.95 to 3.63, 4.21, 3.55, 1.35, respectively. This indicates the existence of synergistic effect with the combination of TMA-L-Hyp and CP in the separation system. It is worth noting that all the studied racemic drugs could not be enantioseparated when the TMA-L-Hyp IL was used alone.

In order to evaluate the stereoselectivity of the dual system toward the tested drugs, the effects of a series of separation conditions on enantioseparation were investigated.

Effect of CP and TMA-L-Hyp IL Concentrations on Enantioseparation

The concentration of chiral selector is critical to the enantioseparation for the migration time and resolutions of enantiomers. To obtain the optimum concentrations of CP and TMA-L-Hyp IL, their effects on the resolution of tested drugs were investigated.

The effect of CP concentration was investigated through fixing the concentration of TMA-L-Hyp IL at 30 mM while changing the CP concentration (20, 40, 60, 80, 100 mM) with 40 mM borax buffer solutions (pH 7.6) containing 20% v/v methanol. As can be observed in Table 1, it was evident that the R_s of all the tested drugs increased generally as the CP concentration ranged from 20 to 80 mM, then decreased with the CP concentration ascending from 80 to 100 mM. Moreover, Table 1 shows that the migration time of all the analytes increased in parallel with the increasing CP concentration due to the strengthened interaction between chiral selectors and the analytes as well as the increased viscosity of the running buffer. Thus, 80 mM CP was chosen to separate the tested drugs.

Furthermore, the CP concentration was kept at 80 mM while the TMA-L-Hyp IL concentration was changed over

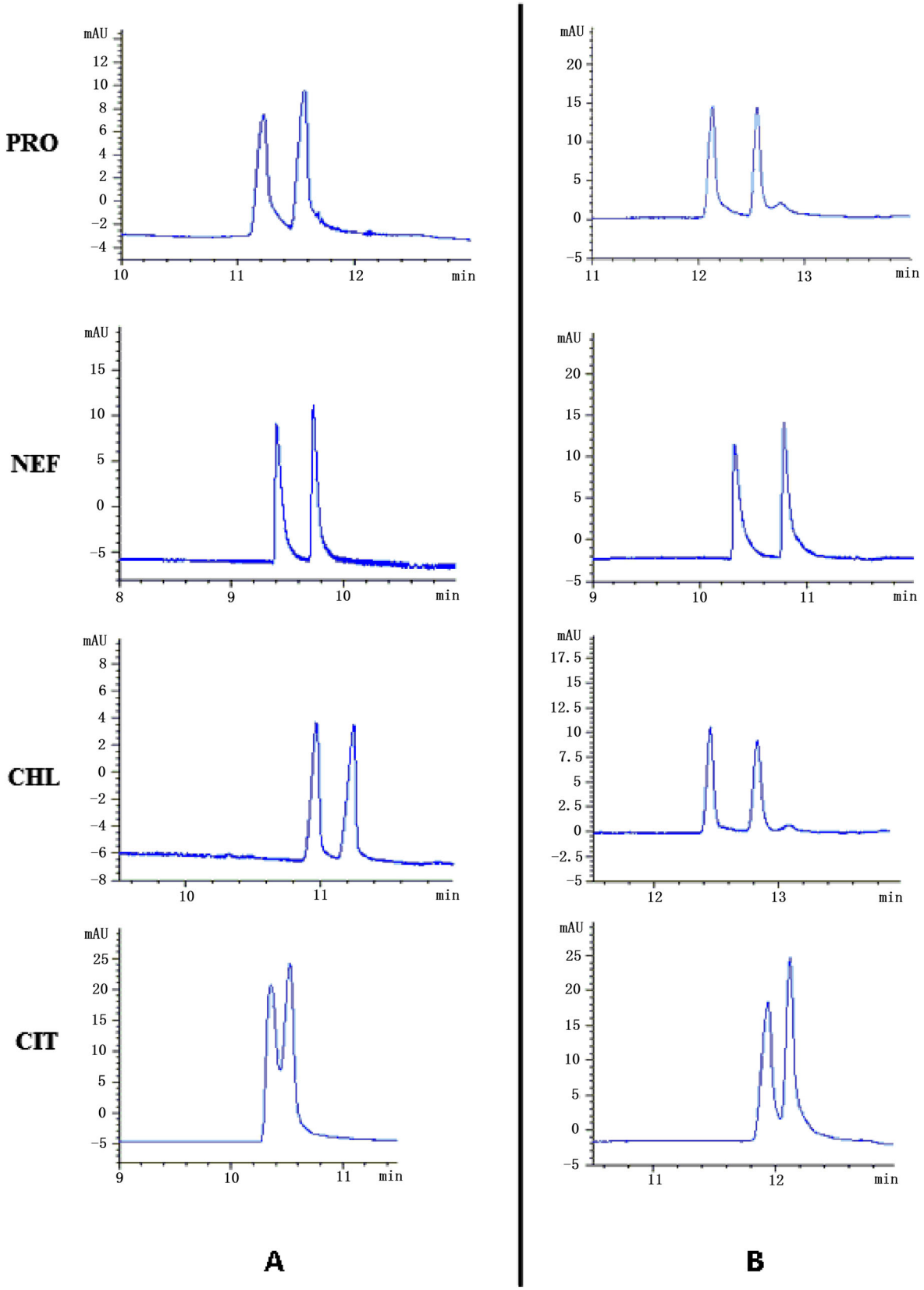

Fig. 2. Electropherograms of PRO,NEF, CHL,CIT in the circumstances (**A**) 80 mM CP (**B**) 80 mM CP and 30 mM TMA-L-Hyp IL. Other Conditions: 40 mM borax buffer solution (pH 7.6)with methanol (20% v/v); applied voltage, 20 kV; capillary temperature, 20°C; fused-silica capillary, 50 cm (41.5 cm effective length) × 50 m id; UV absorption at 289 nm (PRO), 230 nm (NEF), 265 nm (CHL) and 237 nm (CIT).

the range of 10 mM to 50 mM. Table 2 shows the effect of TMA-L-Hyp IL concentration on R_s and α of the tested analytes. The migration time of all the analytes increased with the ascending IL concentration. This effect might be partly attributed to the increase of the buffer viscosity and the adsorption of IL cation on the capillary wall, which led to the

TABLE 1. Effect of CP concentration on the enantioseparation of the studied drugs

	CP concentration (mM)									
	20		40		60		80		100	
Chiral Compound	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α
PRO	7.964/8.038	0.53/1.01	7.991/8.112	1.51/1.02	9.470/9.713	2.69/1.03	12.130/12.550	3.63/1.03	15.065/15.709	1.22/1.04
NEF	7.466/7.535	0.59/1.01	7.609/7.727	0.94/1.02	8.604/8.834	1.81/1.03	10.371/10.777	4.21/1.04	14.032/15.090	2.79/1.08
CHL	7.876/7.993	0.64/1.01	8.129/8.223	1.16/1.01	9.702/9.908	2.30/1.02	12.446/12.825	3.55/1.03	15.598/16.190	0.88/1.04
CIT	7.952	—	8.058/8.112	0.59/1.01	9.373/9.493	1.02/1.01	11.933/12.118	1.35/1.02	16.804/17.156	0.72/1.02

Conditions: 40 mM borax buffer (pH 7.6) containing 30 mM TMA-L-Hyp IL and 20% (v/v) methanol; CP concentration, as shown in Table 1; "—" : single peak. All other conditions as in Figure 2.

TABLE 2. Effect of ILs concentration on the enantioseparation of the studied drugs

	ILs concentration (mM)									
	10		20		30		40		50	
Chiral Compound	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α	t_1 / t_2 (min)	R_s / α
PRO	11.557/11.949	3.05/1.03	11.610/12.007	3.54/1.03	12.130/12.550	3.63/1.03	12.982/13.395	3.58/1.03	14.324/14.955	3.00/1.04
NEF	9.808/10.215	3.59/1.04	9.964/10.398	3.88/1.04	10.371/10.777	4.21/1.04	10.927/11.493	3.69/1.05	16.701/18.065	2.38/1.08
CHL	11.681/12.023	3.17/1.03	12.003/12.370	3.53/1.03	12.446/12.825	3.55/1.03	13.984/14.376	2.14/1.03	22.388/23.870	1.77/1.07
CIT	10.939/11.111	1.19/1.02	11.401/11.593	1.26/1.02	11.933/12.118	1.35/1.02	13.165/13.396	1.03/1.02	20.223/20.919	0.85/1.08

Conditions: 40 mM borax buffer (pH 7.6) containing 80 mM CP and 20% (v/v) methanol; TMA-L-Hyp concentration, as shown in Table 2. All other conditions as in Figure 2.

decrease of EOF from 1.17×10^{-4} $cm^2V^{-1}s^{-1}$ to 5.41×10^{-5} $cm^2V^{-1}s^{-1}$. The R_s of the analytes increased as the concentration added up to 30 mM, and subsequently tended to drop with the concentration ascending from 30 mM to 50 mM. The decrease may be explained by more Joule heat caused by the increased ionic strength of the running buffer, which led to a broad peak. It is worth noting that sometimes the reduced EOF would also, to some extent, influence the R_s of drug enantiomers because the prolonged migration time would provide more chances for the enantiorecognition during the separation process. Thus, we investigated the influence of EOF on enantioseparation by decreasing the applied voltage from 20 kV to 12 kV in the single CP system. As expected, the migration times of the four tested analytes increased by about 6 to 8 min. However, the decreased applied voltage did not result in significantly increased R_s and α for the analytes compared to the ILs synergistic system (Supporting Information, Fig. S1). This observation indicated, from another aspect, that the improved enantioseparation in the CP/chiral ILs system was mainly attributed to the synergistic effect between chiral selector and the CILs.

Taking the R_s and proper migration time into consideration, 80 mM CP and 30 mM TMA-L-Hyp IL were chosen as the optimum values for the four analytes.

Effect of pH on Enantioseparation

In the CE enantioseparation, buffer pH is one of the most important parameters because it determines the EOF of the capillary, the extent of ionization and mobility of analytes, CILs, and the chiral selector. In addition, it influences the interactions among chiral selector, chiral ILs, and analytes and results in the changing of resolution. In this study, the effect of pH for the tested drugs on enantioseparation was studied by varying the pH (7.2–7.8) of the 40 mM borax buffer (containing 20% v/v methanol) with 80 mM CP and 30 mM TMA-L-Hyp IL. It can be observed from Figure 3 that as the buffer pH ascended from 7.2–7.6, the R_s of all studied enantiomers tended to increase; however, when the pH was over 7.6, the R_s of analytes decreased. This trend gave important information on the interactions among the CP, TMA-L-Hyp IL, and the analytes. Since the degrees of protonation in drugs, CP, and TMA-L-Hyp IL were dependent on pH, the optimum pH would point to the most compatible state of the amino function in the drugs for binding or reacting to the groups in TMA-L-Hyp IL (hydroxyl or amino) and the groups in CP (amino, hydroxyl, or phosphate). In the present system of the TMA-L-Hyp-drugs-CP combination, it can be supposed that two kinds of main bindings exist among the chiral selectors, CIL, and analytes according to the structures of CP, TMA-L-Hyp IL, and the analytes. One might be the hydrogen-bonding among the amino, hydroxyl groups in the drug, the amino, hydroxyl groups in CP, and the hydroxyl group in TMA-L-Hyp IL; the other one might be the electrostatic interaction among the amino group in the drug, the phosphate group in CP, and the carboxyl group in TMA-L-Hyp IL. Thus, the optimum pH value was 7.6 for the analytes.

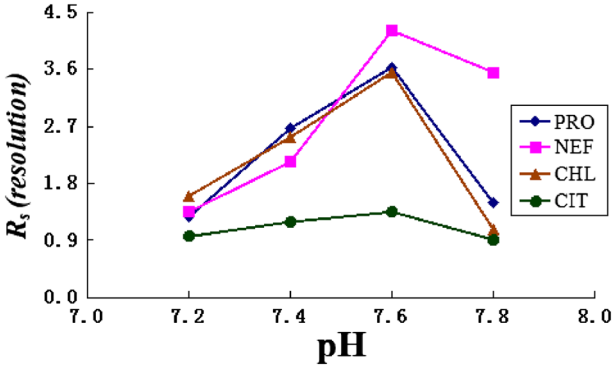

Fig. 3. Effect of pH on the R_s of the studied drugs. Conditions: 40 mM borax buffer containing 80 mM CP, 30 mM TMA-L-Hyp ionic liquid and 20% (v/v) methanol; pH, 7.2 to 7.8. All other conditions as in Figure 2.

Effect of the Types and Concentrations of Organic Modifier on Enantioseparation

The use of an organic modifier has a great effect on migration time and R_s. It not only affects the effective charge of the enantiomers and chiral selectors, the viscosity of the BGE, and the EOF, but also the complexation interaction involved in the enantioseparation mechanism when antibiotics are used as the chiral selectors.[27]

In this work, methanol, isopropanol, and acetonitrile were added separately to the running buffer with a concentration of 20% (v/v). From the results of the experiment, when the isopropanol was used, the peaks of the analytes and the solvent were found to be mutually interfered. It can be observed from Supporting Information, Figure S2. that methanol had better results, with satisfactory resolutions and proper migration time compared to acetonitrile. Thus, methanol is the best choice for the tested enantiomers.

Furthermore, the effect of the concentration of methanol (0%–30% v/v) on the separation was studied. According to the results shown in Table 3, the migration time was prolonged with the increase of the methanol concentration because of the changing of the buffer viscosity, the EOF, and the interactions among the complexing antibiotic, analytes, and the TMA-L-Hyp IL. Additionally, the R_s of the four compounds increased generally when the methanol concentration increased from 0% to 20%; however, the R_s decreased with further increasing of the methanol concentration. As a result, the optimum methanol concentration for the studied analytes was 20% (v/v).

Effect of Applied Voltage and Capillary Temperature on Enantioseparation

The applied voltage affects the enantioseparation through three main aspects: column efficiency, migration time, and R_s. Generally, an optimum voltage value is obtained during the separation. In this study, the influence of applied voltage was investigated from 12 to 28 kV. As Supporting Information, Figure S3a describes, an increase of applied voltage resulted in a general decrease of the migration time of all studied analytes. As to R_s, when the voltage value was lower than 20 kV, the R_s of the analytes increased with the increase of the voltage value. It can be illustrated by the improving of the column efficiency. However, with the voltage value increasing continually, the R_s of the tested substances decreased due to the generation of Joule heating, which can result in the broadening of the peaks. Thus, the applied voltage was set at 20 kV.

Buffer viscosity, pKa, and complexation interaction among the chiral selector, CILs, and the analytes are affected by the capillary temperature. It can be observed from Supporting Information, Figure S3b that when the capillary temperature changed from 14 to 28°C, the enantiomeric migration time

TABLE 3. Effect of the concentration of methanol on the Rs of the studied drugs

Chiral Compound	Methanol concentration (v/v)							
	0%		10%		20%		30%	
	t_1 / t_2 (min)	R_s.	t_1 / t_2 (min)	R_s.	t_1 / t_2 (min)	R_s.	t_1 / t_2 (min)	R_s.
PRO	6.568/6.690	0.63	9.205/9.464	1.93	12.130/12.550	3.63	16.256/17.074	2.71
NEF	5.799/5.901	1.23	7.850/8.076	2.04	10.371/10.777	4.21	13.384/14.176	3.51
CHL	6.561/6.683	1.24	9.120/9.354	2.07	12.446/12.825	3.55	16.782/17.344	1.51
CIT	6.445	—	8.904/9.034	0.58	11.933/12.118	1.35	15.244/15.579	0.99

Conditions: 40 mM borax buffer (pH 7.6) containing CP 80 mM and 30 mM TMA-L-Hyp ionic liquid; Methanol concentration, 0% to 30% (v/v); "—" : single peak. All other conditions as in Figure 2.

decreased. As to R_s., it increased with the capillary temperature ranging from 14 to 20°C, then decreased when the capillary temperature ascended from 20 to 28°C. Therefore, 20°C was chosen for enantioseparation of the studied drugs.

The run-to-run reproducibility was evaluated by five replicate testings. All separations were performed under the optimum separation conditions as illustrated in Figure 2. The variations of migration times (the RSD values of the migration times) were less than 2.8%. It can therefore be concluded that the TMA-L-Hyp synergistic system based on CP can provide satisfactory reproducibility.

CONCLUSIONS

A novel chiral ionic liquid (TMA-L-Hyp) synergistic system based on the antibiotic chiral selector (CP) was established in this study for the first time. In the dual system, four pairs of enantiomers were successfully enantioseparated and the separations were significantly improved compared to the single CP system. The R_s. of the PRO, NEF, CHL, and CIT increased from 2.21, 3.01, 2.53, 0.95 to 3.63, 4.21, 3.55, 1.35, respectively. For the single system where TMA-L-Hyp IL was used alone in the studied conditions, all the studied drugs could not be separated. Additionally, several separation conditions that might influence this dual system were optimized, including CP concentration, TMA-L-Hyp concentration, buffer pH, types and concentrations of organic modifier, applied voltage, and capillary temperature.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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