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REGULAR ARTICLE

Enantioseparation of chiral pharmaceuticals by vancomycin-bonded stationary phase and analysis of chiral recognition mechanism

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Abstract

The drug chirality is attracting increasing attention because of different biological activities, metabolic pathways, and toxicities of chiral enantiomers. The chiral separation has been a great challenge. Optimized high-performance liquid chromatography (HPLC) methods based on vancomycin chiral stationary phase (CSP) were developed for the enantioseparation of propranolol, atenolol, metoprolol, venlafaxine, fluoxetine, and amlodipine. The retention and enantioseparation properties of these analytes were investigated in the variety of mobile phase additives, flow rate, and column temperature. As a result, the optimal chromatographic condition was achieved using methanol as a main mobile phase with triethylamine (TEA) and glacial acetic acid (HOAc) added as modifiers in a volume ratio of 0.01% at a flow rate of 0.3 mL/minute and at a column temperature of 5°C. The thermodynamic parameters (eg, ΔH, $\Delta\Delta H$, and $\Delta\Delta S$) from linear van 't Hoff plots revealed that the retention of investigated pharmaceuticals on vancomycin CSP was an exothermic process. The nonlinear behavior of $\ln k'$ against $1/T$ for propranolol, atenolol, and metoprolol suggested the presence of multiple binding mechanisms for these analytes on CSP with variation of temperature. The simulated interaction processes between vancomycin and pharmaceutical enantiomers using molecular docking technique and binding energy calculations indicated that the calculated magnitudes of steady combination energy (ΔG) coincided with experimental elution order for most of these enantiomers.

KEYWORDS

chiral pharmaceutical, chiral recognition mechanism, enantioseparation, HPLC-vancomycin CSP, molecular docking, thermodynamic analysis

1 | INTRODUCTION

Pharmaceuticals are a large class of compounds with diverse properties and applications. It has been reported that more than 50% of these pharmaceuticals in current use are chiral compounds.[1,2] Because of the identical physicochemical properties but different biological activities, metabolic pathways, and toxicities of chiral enantiomers, the chiral separation has been a great challenge and still remains a hot issue, which has attracted attention of agricultural, pharmaceutical, and environmental communities.[2-4]

Of the enantioseparation techniques, a high-performance liquid chromatography (HPLC) with chiral

stationary phases (CSPs) has been recognized to be the most predominant method for chiral separation.[5-7] Since the first introduction of macrocyclic antibiotics as chiral selector in 1994 by Armstrong et al,[8] the vancomycin-based CSPs have been widely applied for the separation of various chiral pharmaceuticals, pesticides and industrial materials with liquid chromatography (LC) in different modes. An efficient separation of a number of acidic drugs was obtained using vancomycin immobilized on silica as CSP in reversed phase LC.[9] Two vancomycin-based CSPs with chiral selectors of Chirobiotic V and Chirobiotic V2 were evaluated for the enantioseparation of β-blockers and profens in reversed-phase and polar-organic mode, where it indicated that the better enantioseparation of the selected drugs was achieved on the Chirobiotic V2 column in polar-organic mode.[10] The vancomycin immobilized onto diol hydride-based silica particles as CSP in nano-LC was used to separate the enantiomers of various nonsteroidal anti-inflammatory drugs and β-blockers, showing a better chromatographic separation performance for chiral acidic compounds.[11] Specifically regarding basic pharmaceuticals, R- and S-enantiomers of cinacalcet were effectively separated on a Chirobiotic V column packed with vancomycin as a CSP in polar ionic mode LC, and successively determined by tandem mass spectrometric method.[12] The enantiomeric separation and quantification of metoprolol, propranolol, fluoxetine, and venlafaxine were conducted on commercialized vancomycin CSP in LC, coupled with tandem mass spectrometry.[13] Although some work has been conducted on the enantiomeric separation of several basic drugs based on vancomycin CSP in LC, to the best of our knowledge, the enantiomers amlodipine separation using vancomycin-based CSP has not been previously reported, thus chromatographic conditions of extensive basic drug enantiomers and separation of structurally similar chiral pharmaceuticals should be systematically optimized and evaluated.

In addition, to help improve enantioseparation efficiency of chiral compounds or develop new chiral separation methods, there are critical demands to explore chiral recognition mechanisms. Enantiorecognition mechanisms involve a variety of molecular interactions. With multifunctional groups, cavities, and numerous stereogenic centers, vancomycin was able to provide active sites for hydrogen bond, dipole-dipole interaction, π-π interaction, and electrostatic interactions.[14,15] The interaction between the analyte and the CSP totally depended on chromatographic modes and mobile phase additives.[16,17] It was found that enantiorecognition mechanisms on vancomycin in polar ionic mode was mainly derived from hydrogen bonding, electrostatic forces, and steric hindrance, whereas hydrophobic, electrostatic, and steric interactions were more dominant in reversed-phase mode.[18-20] Unequivocally, the acidic and basic additives in mobile phase impacted chiral recognition mechanism by regulating ion-pairing interaction and altering proton activity of the mobile phase medium, further influencing electrostatic interactions between analyte and the CSP.[21-23] The liophilic ions, as the mobile phase modifiers, have also been used to control retention and selectivity of analytes,[24,25] where enantiorecognition mechanisms of basic pharmaceuticals on a vancomycin-based column in polar ionic mode involved chaotropic interaction between the liophilic ions and the analytes on the CSP.[26] Fortunately, computer simulation methods have been proposed as useful approaches to gain insight into chiral recognition mechanism. For instance, the molecular docking has been employed to simulate the bonding between pharmaceutical enantiomers and CSPs and to predict the order of isomer peaks in chromatographic separation.[11,12,17] Nevertheless, because of the complex interactions between multiple pharmaceuticals and CSPs, there is still a need for comprehensive and extensive understanding on chiral recognition mechanisms and thermodynamics of chiral basic pharmaceuticals separations in vancomycin-based CSPs.

Therefore, the aims of this work, using vancomycin-based CSPs, are to systematically and extensively optimize enantioseparation conditions of amine group-contained basic chiral pharmaceuticals including propranolol, atenolol, metoprolol, venlafaxine, fluoxetine, and amlodipine; to investigate their thermodynamic properties of the chiral separation process so as to provide an extra evidence for the chiral recognition mechanism; and to further explain bonding strength of isomers and possible chiral recognition mechanisms of pharmaceutical racemates based on molecular docking approach.

2 | MATERIALS AND METHODS

2.1 | Instrumentation and chemicals

All the enantiomeric separation experiments were performed on an HPLC system from Agilent Technologies (Palo Alto, CA, USA) with an ultraviolet detector (1260 VWD VL, Agilent Technologies, Germany). S-(−)-propranolol (PHO) and R-(+)-propranolol were purchased from TLC Pharmaceutical Standards Ltd (Canada). Pure R- and S-enantiomers of atenolol (ATL), metoprolol (MTP), venlafaxine (VLX), fluoxetine (FLX), and amlodipine (ADP) were obtained from Toronto Research Chemicals Inc. (Canada). The chemical structures of chiral compounds were shown in Figure 1.

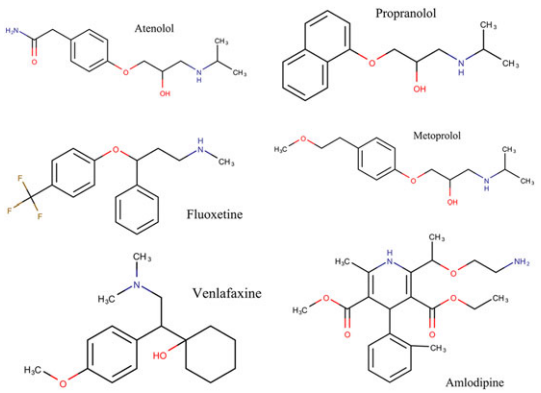


FIGURE 1 Structures of six pharmaceuticals

Methanol (MeOH) of HPLC grade was purchased from Mreda (USA). Triethylamine (TEA) and glacial acetic acid (HOAc) were of analytical grade. The mobile phase was filtered through CNW 0.22-μm filter (CNW, GER) and degassed before use. Milli-Q ultrapure water was used throughout the experiment.

Separate stock solutions of individual enantiomer were prepared by dissolving the substances in methanol at a concentration of 100 mg/L and stored in glass standard bottles (CNW, GER). The working standards of each chiral drug were made up at concentrations of 1 mg/L by mixing appropriate volumes of individual enantiomer solutions and diluting with the same solvent. All solutions were stored at −4°C when not in use. All glassware was deactivated by methanol and dichloromethane to prevent the sorption of analytes to the hydroxyl sites on the glass surface.

2.2 | Chromatographic condition

Enantioselective separations were achieved using a vancomycin-bonded column (5 μm, 150 × 2.1 mm id; Astec Chirobiotic V, Sigma-Aldrich) with an ultraviolet detector (1260 VWD VL, Agilent Technologies, Germany) and an automatic injector (1260 ALS-G1329B, Agilent Technologies, Germany). All the samples of 5 μL at 1 mg/L of concentration were injected into the HPLC for analysis. A computer and an Agilent ChemStation data processing workstation were used to collect and analyze the data. The detection wavelength was set to 226 nm for metoprolol and fluoxetine, 290 nm for propranolol, 237 nm for amlodipine, and 230 nm for atenolol and venlafaxine. Before first use, the column was equilibrated with the prescribed mobile phase until a stable baseline was achieved. Between runs, methanol as a blank sample was injected to avoid interference of the prior sample. The column was operated at temperature range of 5°C to 25°C, controlled with a precise temperature controller (1260TCC-G1316A/Agilent Technologies, USA). The chromatographic system was equilibrated at each temperature for at least 1 hour before each experiment. Methanol, with small amount of TEA and HOAc as modifiers in various volume ratios of 0% to 0.03% (*v*/*v*), was used as mobile phase. The separation of chiral pharmaceuticals was performed at the mobile phase flow rate of 0.2 to 0.4 mL/minute in the reversed-phase mode. Each solution was injected in triplicate with the injection volume of 5 μL.

The resolution (R_s) was main parameter to evaluate the separation efficiency of enantiomers.[27] By measurement of the retention time and peak width, the R_s value for enantiomers could be calculated using the following Equation 1:

$$R_s = 2(t_{R2} - t_{R1})/(w_2 - w_1), \quad (1)$$

where t_{R1} and t_{R2} are the retention time and w_1 and w_2 are base-peak width of the first and second eluted enantiomer, respectively.

To ensure reliable results in the separation process, a strict quality control procedure was followed. The glassware was carefully washed with ultrapure water and methanol to avoid contamination prior to use. To further validate the reproducibility of enantioseparation method, the relative standard deviations (*RSD*) for retention time and R_s of enantiomers were calculated with 6 parallel injections at 1 mg/L of concentration under the optimized chromatographic conditions.

2.3 | Thermodynamic calculations of enantioseparation

The thermodynamic parameters can be accessible by retention factor (k') and the selectivity factor (α), which are related to ΔG, the Gibbs energy, and Δ (ΔG), the difference in the molar Gibbs energy of two enantiomers, respectively[28]:

$$\Delta\,(\Delta G) = -RTln\ \alpha, \quad (2)$$

$$\alpha = k_2^{'}/k_1^{'}, \quad (3)$$

$$\Delta G = -RTlnk^{'}, \quad (4)$$

where R is the gas constant and T is the absolute temperature. From Gibbs free energy equation, Δ (ΔG) can be deduced as follows:

$$\Delta\,(\Delta G) = \Delta\,(\Delta H) - T\Delta\,(\Delta S). \quad (5)$$

In general, the plots of the natural logarithms of chromatographic k' value and α value against the reciprocal of absolute temperature are termed as van 't Hoff plots and are linear.

$$lnk' = -\Delta H/RT + \Delta S/R + ln\varphi, \quad (6)$$

$$ln\alpha = -\Delta(\Delta H)/RT + \Delta(\Delta S)/R, \quad (7)$$

where ΔH and ΔS are the enthalpy and entropy changes, respectively, when the analytes transfer from the mobile phase to the stationary phase. Correspondingly, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ are the difference in enthalpy and entropy changes between two isomers, respectively. The intent of φ is the phase ratio of the column (stationary/mobile).

2.4 | Molecule simulation method

To understand binding mechanism of enantiomers onto CSPs behind the observed results of enantioresolution, the molecular docking was performed for mechanics simulation between molecules using Autodock (version 4.0) software.[29,30] In a molecular mechanics simulation, the primary mechanisms contained H-bonding, van der Waals interaction, steric repulsion, and electrostatic interaction. The Lamarckian genetic algorithm (LGA) was applied to search the conformational and orientational space by iterations of 10. The binding free energy (ΔG) of pharmaceutical onto the vancomycin CSPs was obtained based on the most stable docking conformation by semi-empirical binding-free energy function[31]:

$$\Delta G = \Delta G_{vdw} + \Delta G_{elec} + \Delta G_{H-bond} + \Delta G_{sol} + \Delta G_{confom} + \Delta G_{tor} \quad (8)$$

where ΔG_{vdw}, ΔG_{elec}, and $\Delta G_{H\text{-}bond}$ represent the energies of van der Waals' interaction, electrostatic interaction, and H-bonding, respectively, and ΔG_{sol} is the desolvation upon binding and hydrophobic interaction, ΔG_{confom} models deviation of bond length and bond angle, and ΔG_{tor} represents restriction of internal rotors and global rotation and translation. It should be noticed that there were usually some assumptions in modeling chiral separations of chromatographic techniques using docking method, such as not taking solvation effect, buffer effect and entropy difference into consideration.[30,32]

The molecular three-dimensional structures of chiral pharmaceuticals containing S- and R-isomers were obtained from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/). They were then optimized using the molecular mechanics Powell method.[33] The three-dimensional structure of vancomycin was obtained from the RCSB Protein Data Bank database (http://www.rcsb.org/)[29] and assumed as a macromolecular receptor. The charges of vancomycin in its protonated state were calculated by the Gasteiger-Marsili method.[34] The grid maps were calculated by AutoGrid, and the dimensions of the grid box was set to 40 × 40 × 40 Å, with the grid spacing set to 0.375 Å. The docking conformation possessed the lowest binding free energy because of its being thermodynamically favorable, which was the most stable conformations of the models.

3 | RESULTS AND DISCUSSION

3.1 | Effect of flow rate

The effect of the flow rate of the mobile phase on the enantiomer separation was investigated in the range of 0.2 to 0.4 mL/minute, where a mixture of MeOH, HOAc, and TEA in the volume ratio of 100:0.01:0.02 was used as a mobile phase, and the separation was performed at 25°C. As shown in Figure 2, with the increase of the flow rate, the α values of the six pharmaceuticals were nearly unchanged, whereas their R_s values slightly decreased except for that of VLX, indicating that lower flow rate was preferred for enantioseparation. This observation was likely because of reduced resistance to mass transfer at lower flow rate, which was also obtained for enantioseparation of dansyl-amino acids on norvancomycin-bonded CSP and amino acids or analogues onto cinchona alkaloid-derived zwitterionic CSP.[30] The Van Deemter equation, which described the relationship between height equivalent to a theoretical plate of a chromatographic column (*HETP*) and the flow rate of mobile phase (ν presented as linear velocity), could provide further explanation for this result[31,32]:

$$HETP = A + B/\nu + C\nu \quad (9)$$

where A is the coefficient for eddy diffusion, which relates to particle diameter of stationary phase stuffing; B is the coefficient for longitudinal diffusion, which is proportional to the diffusion coefficient of the mobile phase; and C is the coefficient for resistance to mass transfer. As for liquid chromatography, the longitudinal diffusion coefficient of liquid is significantly smaller than that of gas, which results in the neglection of the second term of Equation 9. Thus, reducing ν values produced the lower *HETP* value and hence the higher resolution of two enantiomers.[33] In addition, a lower flow rate would undoubtedly cause retention time extending from 8 to 17 minutes at a flow rate of 0.4 mL/minute to 20 to 37 minutes at a flow rate of 0.2 mL/minute, and slight

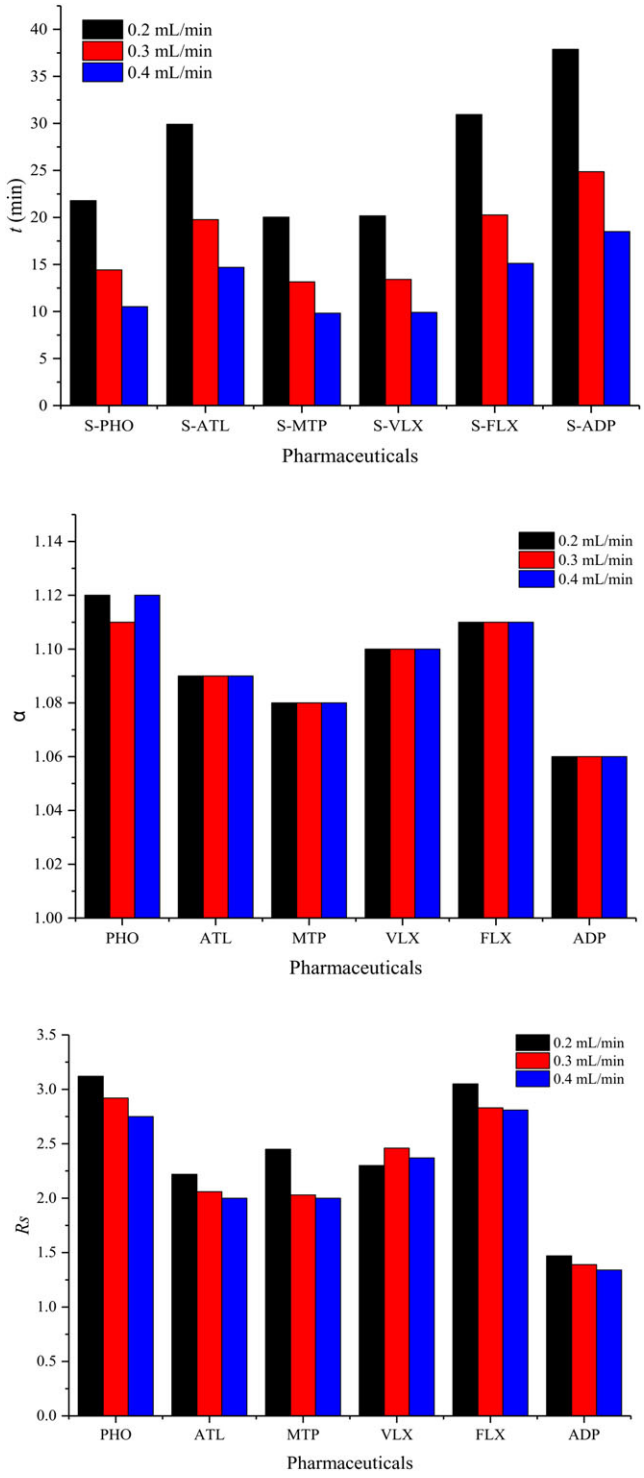


FIGURE 2 Effect of flow rate of mobile phase on the enantioseparation of six pharmaceuticals (chromatographic conditions: Methanol containing 0.01% HOAc and 0.02% TEA, column temperature for 25°C)

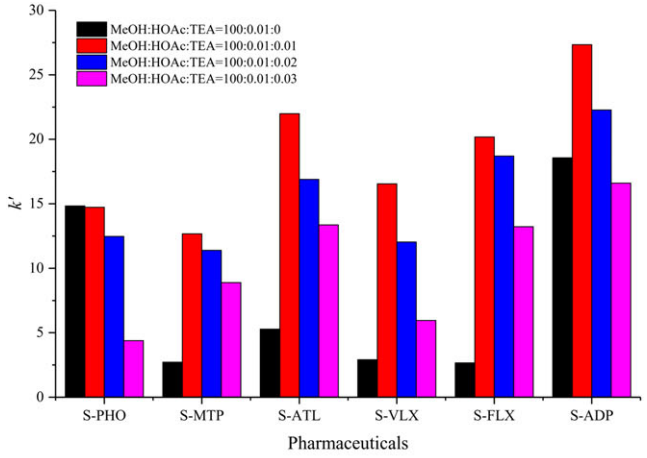
peak broadening was also observed. Considering both of the resolution and retention time, a flow rate of 0.3 mL/minute was selected in this study.

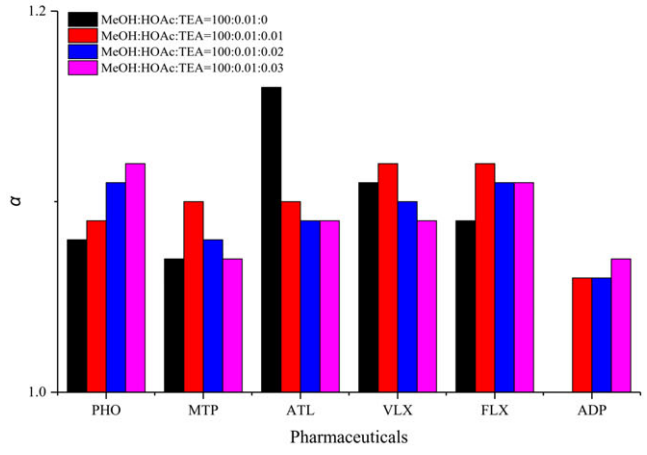
3.2 | Effect of acid and base additives

Our preliminary experiment indicated that all six pharmaceuticals were unable to be eluted and separated within 30 minutes at a flow rate of 0.5 mL/minute using pure methanol as mobile phase (data not presented here). The additives in mobile phase are critical for the enantioseparation, and the effect of acid and base additives has been previously reported by a number of studies.[9,35,36] Various volume ratios of HOAc and TEA added to methanol as mobile phase were selected for assessing effect on enantioseparation at 25°C and a flow rate of 0.3 mL/minute. As shown in Figure 3, at a certain percent of HOAc (0.01%, *v/v*), the addition of TEA had a positive effect on the enantioseperation for most of selected pharmaceuticals, even more for amlodipine that was not separated at all without TEA additives, suggesting that TEA played a crucial role on enantioseparation of these pharmaceuticals. Specifically, increasing TEA percentage from 0.01% to 0.03% led to a decrease in the items of k', R_s, and α for most of the investigated analytes, apart from amlodipine that there was no constant trend in the R_s and α values, and propranolol that its α values slightly raised with increased TEA addition. In the case of certain percent of TEA (0.01%, *v/v*) (as observed in Figure 4), decreased k' values were observed for five of the investigated pharmaceuticals, with increasing HOAc percentage from 0% to 0.03%, while the k' values of venlafaxine achieved the maximum at HOAc percentage of 0.01%. The profiles of selectivity and resolution were found to be highly analyte-dependent. For propranolol, atenolol, and metoprolol, the α and R_s values were decreasing with increasing HOAc percentage, and the overall increased trends were observed in α values for venlafaxine, fluoxetine, and amlodipine, and R_s values for venlafaxine and fluoxetine.

The above observations were associated with the chemical structures of target pharmaceuticals and additives in the mobile phase and stationary phase. The acid basic additives would lead to the alteration of mobile phase pH, further influencing the dissociation and protonation of both analytes and vancomycin that has several ionization constants (*pKa*: 2.9, 7.2, 8.6, 9.6, 10.4, and 11.7) in vancomycin. In the present study, the pH ranges of mobile phase with different ratios of acid basic additives varied from 6.1 to 7.6, which was lower than *pKa* of target analytes ranged from 8.6 to 10.1 and close to isoelectric point of vancomycin (7.2), thus, almost keeping the selected pharmaceuticals and vancomycin in the neutral form.

Additionally, between selected pharmaceuticals, best selectivity and resolution for vancomycin-based CSPs and target analytes were observed for fluoxetine with





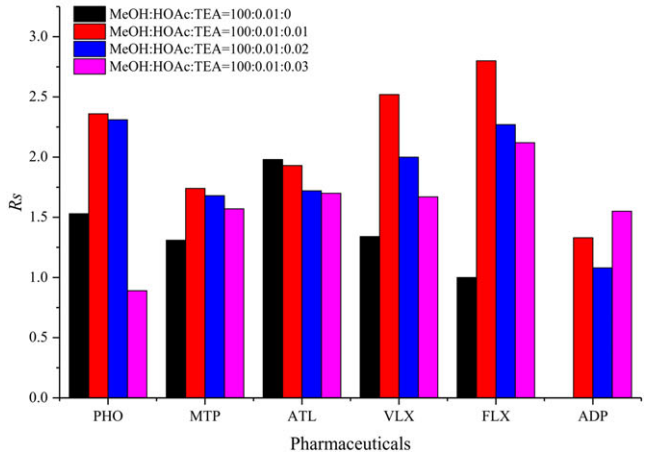
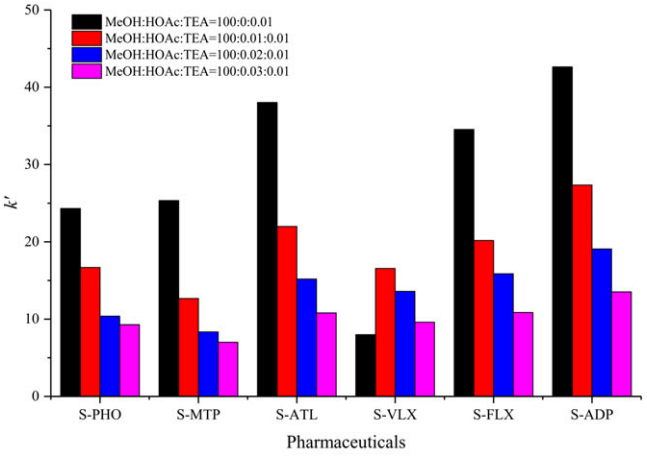
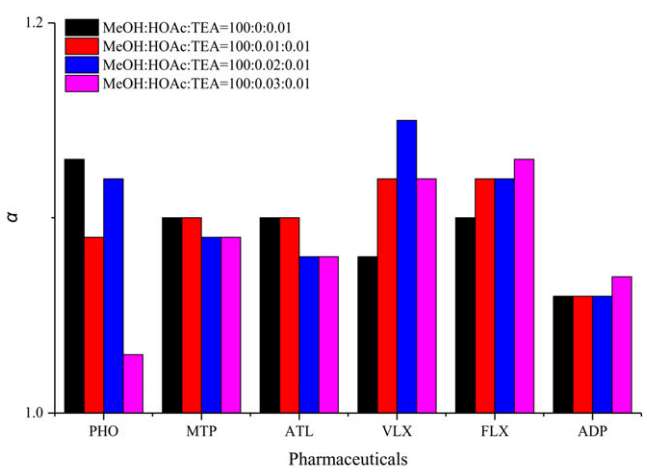


FIGURE 3 Effect of TEA additives on the enantioseparation of six pharmaceuticals under certain percent of HOAc (0.01%, *v/v*) (chromatographic conditions: column temperature 25°C and flow rate of 0.3 mL/min)





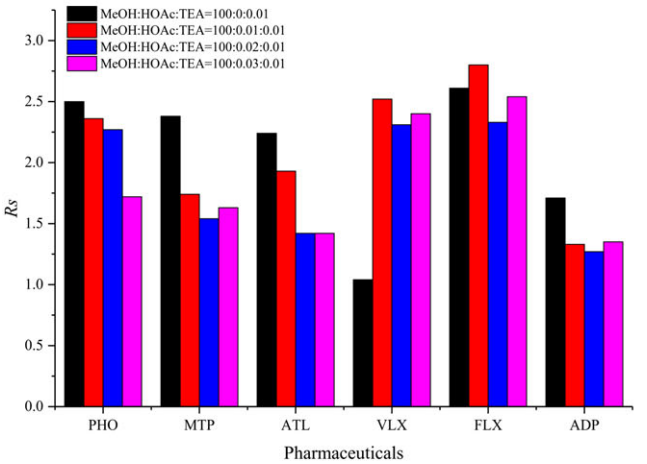


FIGURE 4 Effect of HOAc additives on the enantioseparation of six pharmaceuticals under certain percent of TEA (0.01%, *v/v*) (chromatographic conditions: column temperature 25°C and flow rate of 0.3 mL/min)

highest *pKa* value of 10.1 despite the insignificant linear relationship between *pKa* values of analytes and their enantioseparation efficiency. Those results indicated a vital role of the dipole–dipole interaction between vancomycin-based CSPs and target analytes. In common case, the hydrogen bonding between analytes and CPS should be of less importance in methanol mobile phase system than in nonalcoholic type solvents, eg, acetonitrile-based mobile phase[20] because of the stronger hydrogen bonding between methanol and CSP, hence, dipole–dipole or electrostatic interactions might be a dominated interaction for chiral recognition because of the increased selectivity.[9,35]

It was reported that the acid and base additives could also possibly adjust both polarity and ionic strength of mobile phase.[37] Chemically, both of TEA and basic pharmaceuticals would combine with dissociative silanol groups of the stationary phase.[38] On the one hand, TEA as a masking agent was highly beneficial to improve tailing of the peak shape, but on the other hand, an excess of TEA could compete interaction sites with basic pharmaceuticals on the stationary phase, resulting in negative impact on enantioseparation of pharmaceuticals. The excess of HOAc in mobile phase could cause an increased electrostatic repulsion to target analytes by the protonation of functional groups in vancomycin.[35] Of these pharmaceuticals in the current study, propranolol, atenolol, and metoprolol maintained a good enantioseparation performance at lower HOAc percentage, while venlafaxine, fluoxetine, and amlodipine showed an improved enantioselectivity at higher HOAc percentage, which was not consistent with previous studies where it was confirmed that vancomycin-based CPS gave an increased enantioselectivity with the increase of pH value. The inconsistent result might be somewhat related to diverse ionization constants of investigated pharmaceuticals.[39]

3.3 | Temperature effects and thermomechanical analysis

Because of the instability of vancomycin at the temperature higher than 37°C,[40] the chromatographic column was performed in the temperature range of 5°C to 25°C. The effects of temperature on the enantioseparation of the six chiral pharmaceuticals were shown in Figure 5. As observed for all six pharmaceuticals, the R_s and α values decreased by increasing the column temperature, apart from fluoxetine that had lower R_s value at 10°C. The consistent conclusion was also obtained by Ding et al, who reported that vancomycin CSP performed better enantioselectivity for amino acid derivatives at lower temperature.[41] Additionally, in the tested temperature ranges, the reduced k' values was observed at higher temperature for venlafaxine, fluoxetine, and amlodipine, while there was no significant variation in k' values for propranolol, atenolol, and metoprolol.

The lnk' of the six pharmaceuticals was plotted against $1/T$, and the details of the linear regression equations were given in Table 1. It was clear that higher linearity was obtained for venlafaxine, fluoxetine, and amlodipine with correlation coefficients of 0.85 to 0.96. Correspondingly, the ΔH and ΔS terms were derived from the slope ($-\Delta H/R$) and intercept ($\Delta S/R + ln\varphi$) of the linear plot. The negative values of ΔH were observed

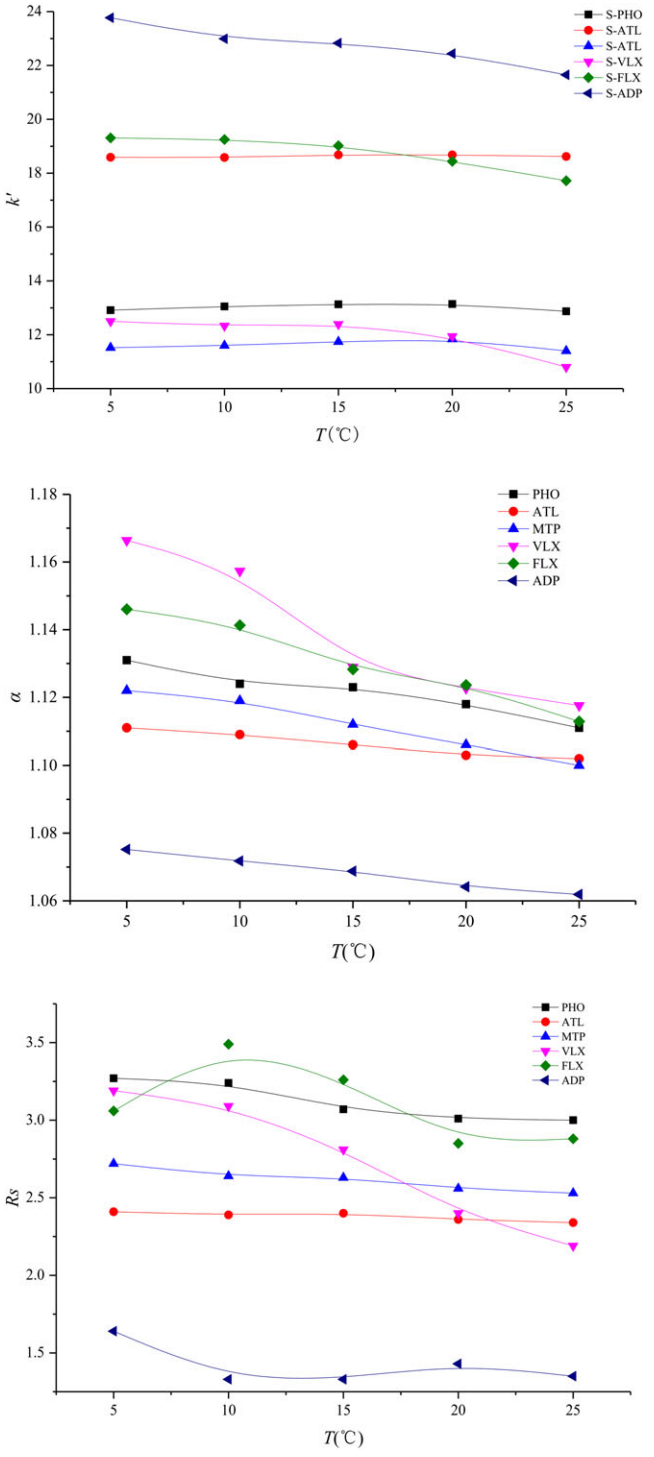


FIGURE 5 Effect of temperature on enantioseparation of six pharmaceuticals (chromatographic conditions: methanol containing 0.01% HOAc and 0.02% TEA, flow rate of 0.3 mL/min)

for venlafaxine, fluoxetine, and amlodipine, indicating that energy was released during transferring of these solutes from mobile phase to stationary phase. For each pharmaceutical, the ΔH or $\Delta S/R + ln\varphi$ terms of *S*-type enantiomer were less negative or higher than those of *R*-type enantiomer, which was in accordance to the

TABLE 1 Thermodynamic parameters for enantiomers of six chiral pharmaceuticals

Compounds	$\ln k'$	r^2	ΔH (kJ/mol)	$\Delta S/R + \ln\varphi$	$\ln\alpha$	r^2	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/mol)	T_{ISO}(°C)
S- propranolol	$\ln k' = 3.065/T + 2.577$	0.001	—	—	$\ln\alpha = 67.94/T\text{-}0.1214$	0.953	−0.565	−1.009	287
R- propranolol	$\ln k' = 14.79/T + 2.633$	0.058	—	—					
S- atenolol	$\ln k' = 14.55/T + 2.975$	0.291	—	—	$\ln\alpha = 36.02/T\text{-}0.0242$	0.982	−0.299	−0.201	1215
R- atenolol	$\ln k' = 23.10/T + 2.946$	0.545	—	—					
S- metoprolol	$\ln k' = 1.879/T + 2.459$	0.0001	—	—	$\ln\alpha = 104.9/T\text{-}0.2708$	0.984	−0.706	−1.571	176
R- metoprolol	$\ln k' = 64.07/T + 2.337$	0.175	—	—					
S- venlafaxine	$\ln k' = 532.0/T + 0.636$	0.908	−4.423	0.636	$\ln\alpha = 192.9/T\text{-}0.5402$	0.923	−1.604	−4.491	84
R- venlafaxine	$\ln k' = 715.8/T + 0.127$	0.848	−5.951	0.127					
S- fluoxetine	$\ln k' = 353.6/T + 1.703$	0.869	−2.940	1.703	$\ln\alpha = 123.2/T\text{-}0.3051$	0.977	−1.024	−2.537	131
R- fluoxetine	$\ln k' = 476.8/T + 1.398$	0.916	−3.964	1.398					
S- amlodipine	$\ln k' = 349.6/T + 1.910$	0.948	−2.906	1.910	$\ln\alpha = 53.11/T\text{-}0.1183$	0.993	−0.442	−0.984	176
R- amlodipine	$\ln k' = 407.0/T + 1.777$	0.962	−3.383	1.777					

elution order of enantiomers (*S*-type first eluted) during the chromatographic separation. Regarding propranolol, atenolol, and metoprolol, the nonlinear relationships of $\ln k'$ versus $1/T$ were presented in Table 1, which could be related to multiple adsorption mechanisms or change in adsorption mechanism within the temperature range of 5°C to 25°C. The similar nonlinear behavior was previously reported for the enantioseparation of metoprolol enantiomers on vancomycin-immobilized CSP[9] and conformationally rigid spirolactam on (R)-N-(3,5-dinitrobenzoyl)phenylglycine-derived CSPs,[42] where this unusual behavior could be ascribed to the temperature-dependent interaction of additives with the stationary phase and/or the target analytes.

The plots of $\ln\alpha$ versus $1/T$ showed a good linearity for all analytes, with correlation coefficients above 0.92. The positive slopes ($-\Delta(\Delta H)/R$) and negative intercepts ($\Delta(\Delta S)/R$) of linear plots were obtained for all pharmaceuticals, giving the negative values of $\Delta(\Delta H)$ from −0.3 to −1.6 kJ/mol and $\Delta(\Delta S)$ from −0.2 to −4.5 J/mol. This result indicated that the chiral separations of these pharmaceuticals were dominantly enthalpically driven. Furthermore, the more negative value of $\Delta(\Delta H)$ and $\Delta(\Delta S)$ represented a good enantioseparation performance.[28] Of these pharmaceuticals, fluoxetine and venlafaxine enantiomers with highest values of $|\Delta(\Delta H)|$ (1.0 and 1.6 kJ/mol) and $|\Delta(\Delta S)|$ (2.5 and 4.5 J/mol) indeed attained excellent resolutions (R_{sFLX} = 2.8, R_{sVLX} = 2.52) and enantioselectivities (α_{FLX} = 1.12, α_{VLX} = 1.12) in the process of seperation.

The iso-enantioselective temperatures (*Tiso*), where the elution order of the enantiomers was reversed, could be calculated as the ratio between $\Delta(\Delta H)$ and $\Delta(\Delta S)$. As presented in Table 1, the *Tiso* values of these pharmaceuticals were pretty high, making the reversion of the enantioelution order impossible under ambient experimental conditions.

3.4 | Method reproducibility and adaptability

Given baseline separation for enantiomers of six pharmaceuticals, the optimal chromatographic condition was obtained with methanol as a main mobile phase containing TEA and HOAc in volume ratio of 0.01%, respectively, a flow rate of 0.3 mL/minute and column temperature of 5°C. Under optimal chiral separation conditions, the chromatographic separation results of each enantiomer were shown in Figure 6, and the *RSD* for retention time and R_s of enantiomers were below 0.92% and 1.80%. The results showed that the method had good reproducibility and separation efficiency.

3.5 | Analysis and prediction of chromatographic retention

The multiple adsorption mechanisms, such as hydrogen-bond, dipole–dipole interaction, π–π interaction, electrostatic interaction, and steric hindrance, have been reported for the enantioseparation of chiral compounds on vancomycin stationary phase.[43,44] The complex structure of macromolecule would provide diverse binding sites, resulting in multiple retention mechanisms. In the view of the three-dimensional structure of vancomycin obtained from the RCSB Protein Data Bank (http://www.rcsb.org/) database, the molecule of vancomycin was assumed to comprise two adsorption active domains for the interaction with analyes,[45] as shown in Figure 7.

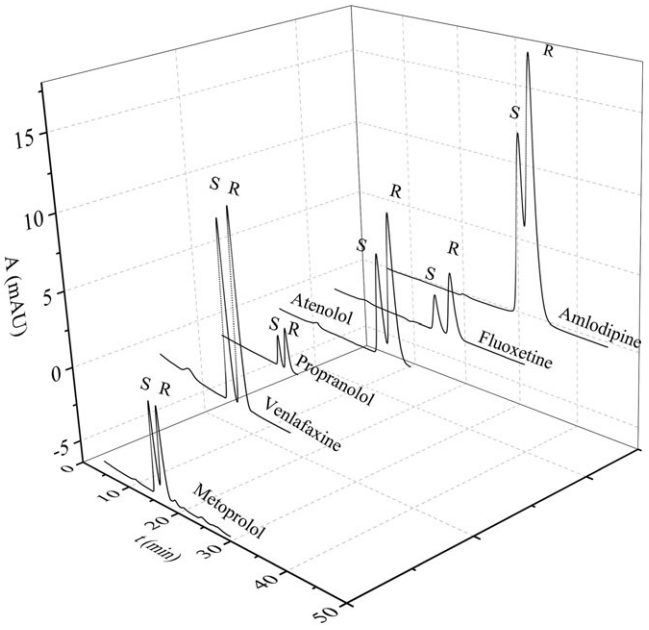


FIGURE 6 Chromatogram for the enantioseparation of six pharmaceuticals under optimized chromatographic conditions (methanol containing 0.01% HOAc and 0.01% TEA, flow rate of 0.3 mL/min, column temperature 5°C)

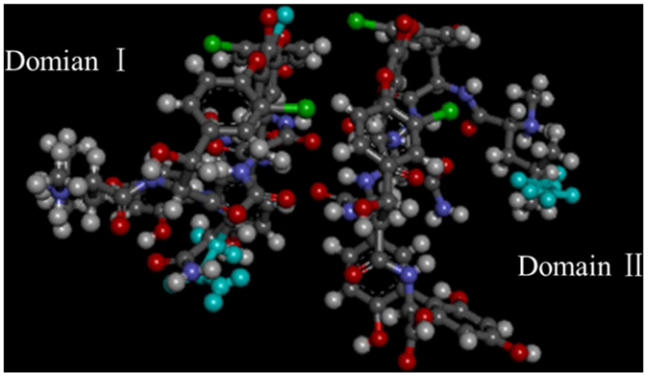


FIGURE 7 Two structural domains of vancomycin

To further understand the micro-mechanisms of pharmaceutical enantiomers binding onto the vancomycin, the molecular docking were performed by Autodock. The calculated, most steady combination energy (ΔG) of pharmaceutical enantiomers with two domains of vancomycin and the differences of binding free energy ($\Delta\Delta G$) for S- and R-type of enantiomers were presented in Table 2. All analyte-vancomycin complexes showed binding free energies in the range of −4.35 to −7.39 kcal/mol, indicating the spontaneity of the binding between analyte molecules and vancomycin. By comparison of the $\Delta\Delta G$ term between two domains of vancomycin, the $\Delta\Delta G$ values of all target analytes on the Domain II were above 0, and their absolute values ($|\Delta\Delta G|$) were greater than those of the Domain I, except for that of venlafaxine, indicating a good corresponding to the experimental elution order of S- and R-type of enantiomers and better enantiomeric separation of analytes on the Domain II. These results suggested that Domain II was the main active site for enantiomeric separation of investigated pharmaceuticals.

Regarding the Domain II, the lowest ΔG value was observed for R-amlodipine (−7.05 kcal/mol), and the highest one was found for S-metoprolol (−4.35 kcal/mol), demonstrating that a stronger affinity between amlodipine and vancomycin, and weaker affinity between metoprolol and vancomycin, respectively. Additionally, the calculated ΔG magnitudes for all enantiomers were in the order of S-metoprolol > R-metoprolol > S-venlafaxine > S-atenolol > R-venlafaxine > S-fluoxetine > R-atenolol > R-fluoxetine > S-propranolol > S-amlodipine > R-propranolol > R-amlodipine. This sequence was somewhat consistent with experimental elution order for the enantiomers of target pharmaceuticals (as shown in Figure 6), except for S- and R- propranolol. Nevertheless, the significant differences were obtained for propranolol between predicted elution order and experimental profile. This deviant result might be because of the limitation of Autodock software, where there was neither the π–π interaction between naphthalene nucleus of propranolol and vancomycin involved nor the solvent effect included in the calculation process of molecular docking.[10,29] Unequivocally, the retention arrangement and mechanism of analyes were strongly controlled by molecular structures. As indicated in Figure 1, the molecules of all analytes comprised benzene rings, which could undoubtedly contribute to binding on the packing of vancomycin by face-to-face π–π interaction. Therefore, the more benzene rings in the structure of propranolol, fluoxetine, and amlodipine, providing stronger π–π interaction, would be responsible for longer retention time of these pharmaceuticals. Similarly, the characteristic side chains of analyes likewise played important roles in retention behavior.

TABLE 2 Most steady combination energy of vancomycin interaction with drug enantiomers (kcal/mol

	Domain I		Domain II	
Compounds	***ΔG***	***ΔΔG***	***ΔG***	***ΔΔG***
S- propranolol	−7.39	−0.29	−6.04	0.98
R- propranolol	−7.10		−7.02	
S- atenolol	−6.09	−0.21	−5.17	0.78
R- atenolol	−5.88		−5.95	
S- metoprolol	−4.79	−0.13	−4.35	0.47
R- metoprolol	−4.66		−4.82	
S- venlafaxine	−4.94	0.80	−5.05	0.46
R- venlafaxine	−5.74		−5.51	
S- fluoxetine	−6.22	−0.27	−5.67	0.33
R- fluoxetine	−5.95		−6.00	
S- amlodipine	−5.75	0.49	−6.13	0.92
R- amlodipine	−6.24		−7.05	

The presence of amino, acylamino, and hydroxyl groups in the molecules were also beneficial to form hydrogen bond and dipolar interaction with vancomycin. For instance, metoprolol with less acylamino group, compared with atenolol, would form weaker hydrogen bond onto vancomycin, resulting in shorter retention time on vancomycin. In addition to hydrogen bond, steric hindrance of alkoxy chain in metoprolol was also a reasonable explanation for the lower retention time of metoprolol enantiomers.

4 | CONCLUSION

Methanol as a main mobile phase with addition of TEA and HOAc as modifiers could be successfully used for the enantiomeric separation of six pharmaceuticals on a vancomycin CPS. An increased retention time, enantioselectivity, and enantioresolution at lower percentage of HOAc and TEA were observed for all the analytes. The reduced flow rate of mobile phase and column temperature were beneficial to efficient enantiomers separation. The thermodynamic parameters revealed that the retention of investigated pharmaceuticals on vancomycin CSP was an exothermic process. The nonlinear van 't Hoff plots of propranolol, atenolol, and metoprolol indicated multiple binding sites and adsorption mechanisms of these anlytes on CSP. In comparing the thermodynamic parameters of six pharmaceuticals, the racemates with higher $|\Delta(\Delta H)|$ and $|\Delta(\Delta S)|$ had excellent resolutions and enantioselectivities of the enantiomer separation. The molecular docking results showed that the calculated differences in binding free energy between S- and R-type enantiomer were above 0, indicating a good agreement with experimentally eluted peak order. The peak order of all the enantiomers somewhat corresponded to binding free energy except for propranolol, which was likely due to the neglect of π–π interaction and solvent effect in the calculation process of molecule docking. These results showed that the molecular modeling technique provided a good perspective to further understand the chiral recognition mechanism, which should be further investigated.

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