e-ISSN: 2320-4230, p-ISSN: 2961-6085

Journal of Drug Discovery and Therapeutics

Available Online at www.jddt.in

CODEN: - JDDTBP (Source: - American Chemical Society)

Volume 12, Issue 03; 2024, 40-58

Simultaneous Enantioseparation and Simulation Studies of Some Drugs using Supercritical Fluid Chromatography

Nidhi Katiyar[1] & Arun Sharma[2]

[1] Research scholar, Department of Pharmacy, Sunrise University, Alwar Rajasthan.

[2] Research supervisor, Department of Pharmacy, Sunrise University, Alwar Rajasthan.

Received: 20-03-2024 / Revised: 14-04-2024 / Accepted: 17-05-2024

Corresponding author: Nidhi Katiyar

Conflict of interest: No conflict of interest.

Abstract:

The enantioseparation of three b-blockers, namely atenolol, metoprolol, and propranolol, was investigated using supercritical fluid chromatography (SFC) on a chiral stationary phase immobilised with amylose tris(3-chloro-5-methylphenylcarbamate). An assessment was conducted to determine the impact of organic modifiers (methanol, isopropanol, and their combination), column temperature, and back pressure on the chiral separation of b-blockers. The best chromatographic separation in terms of resolution, retention, and analysis duration was obtained by utilising a combination of CO2 and 0.1% isopropyl amine in isopropanol: methanol (50:50, V/V), in a ratio of 75:25 (V/V).

When the settings were optimised, the resolution factors (Rs) and separation factors (a) were both higher than 3.0 and 1.5, respectively. Moreover, when the temperature (25e45 ◦C) and pressure (100e150 bars) increased, there was a commensurate drop in the retention factors (k), a, and Rs. Conversely, there was an opposite pattern found with atenolol as the temperature increased. The thermodynamic data obtained from van't Hoff plots indicated that the separation of enantiomers was primarily driven by changes in enthalpy for metoprolol and propranolol, while it was driven by changes in entropy for atenolol. Molecular docking experiments were conducted to provide insight into the process of chiral recognition and the elution behaviour of the enantiomers. The binding energies derived from simulation studies shown strong concordance with both the empirically determined elution sequence and the corresponding free energy values. The approach was verified within the concentration range of 0.5e10 mg/mL for all enantiomers. The range for the limit of detection was 0.126 to 0.137 mg/mL, while the range for the limit of quantitation was 0.376 to 0.414 mg/mL. The approach was effectively used to analyse these substances in pharmaceutical formulations.

INTRODUCTION

The process of isolating enantiomers in order to create pure chiral medicines is now a highly researched topic, particularly in the pharmaceutical sector [1e3]. Multiple publications indicate significant disparities in the pharmacodynamics and pharmacokinetics of enantiomers of the medicine. One of the enantiomers exhibits

the intended pharmacologic action, while the other is either ineffective or linked to unpleasant side effects [1,4]. The variation in the pharmacokinetics mostly stems from the selective binding of medicines (often to plasma proteins), absorption, clearance, and excretion. Therefore, it is necessary to continuously develop analytical and preparative techniques with high resolution and efficiency for assessing chiral purity and conducting pharmacokinetic investigations [4,5]. Beta-adrenoceptor antagonists, often known as beta-blockers, are used to treat various cardiovascular conditions such as hypertension, ischemic heart disease, and migraines. These substances are mostly distributed and promoted as racemic mixes. Nevertheless, the pharmacological action is mostly attributed to the S-enantiomer because of its higher stereoselective affinity for b-receptors. The R-enantiomers exhibit either pharmacological inactivity or toxicity [6e8]. Atenolol is a second-generation beta-blocker that is used for the treatment of hypertension, angina pectoris, and acute myocardial infarction [9]. Metoprolol is a medication that selectively blocks the b1 adrenergic receptors. It is used to treat ischemic heart disease, heart failure, and hypertension [8]. Propranolol, a nonselective beta-blocker, is used for the prevention of migraines, as well as for the management of hypertension and anxiety [7].

Various analytical methods have been employed to separate the enantiomers of these drugs, including thin layer chromatography [10], surface enhanced Raman scattering [11], counter current chromatography [12], electrochromatography [13,14], capillary electrophoresis [15e18], high performance liquid chromatography (HPLC) [9,19e23], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [24e29], and supercritical fluid chromatography (SFC) [30e33]. Among many chiral stationary phases (CSPs), the ones based on polysaccharides (such as cellulose or amylose) are the most often used in these procedures [20,21,23,32,33]. Nikolai and colleagues [24] have measured the quantities of atenolol, metoprolol, and propranolol using liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Chirobiotic V vancomycin-based chiral column, completing the analysis within a 20-minute timeframe. Li et al. [23] recently published a study in which they successfully separated six b-blockers into their enantiomers using HPLC on a Chiralpak IB column. Additionally, they used molecular docking techniques to gain insight into the process of chiral recognition. Nevertheless, there is a scarcity of SFC-based techniques that document the enantioseparation of the medicines examined in this study. Svan et al. [32] used a Chiralpak IB column in combination with SFC-MS/MS to separate the chiral forms of atenolol, metoprolol, propranolol, and the zwitterionic metoprolol acid.

Chiral HPLC is the preferred approach for separating b-blockers, as shown by the literature. Nevertheless, HPLC has many inherent drawbacks, including increased solvent consumption and lengthy analysis time. In contrast, SFC offers an alternate method that utilises environmentally friendly mobile phases. The method use CO2, which is less poisonous and non-polar, as the main component of the mobile phase. It also uses a majority of the CSP typically used in HPLC. Additionally, SFC has the benefit of maintaining column efficiency even when the flow rate is increased, unlike HPLC where column efficiency decreases at a faster rate with higher flow rates. Therefore, it is possible to run Supercritical Fluid Chromatography (SFC) at a faster speed compared to High Performance Liquid Chromatography (HPLC), which leads to a reduction in the time required for analysis

[34,35]. According to a publication, SFC was shown to be more effective than HPLC using a Chiral Art Cellulose-SB column for these medicines, resulting in improved enantioresolution and reduced analytical time [33]. Currently, there have been no documented instances of using the Chiralpak® IG column with the amylose tris(3-chloro-5-methylphenylcarbamate) immobilised chiral stationary phase for separating atenolol, metoprolol, and propranolol into their enantiomers.

The aims of this study were to optimise the conditions for simultaneously separating the enantiomers of atenolol, metoprolol, and propranolol on a Chiralpak® IG column in a single analysis, and to investigate the thermodynamic aspects of chiral separation in order to gain insight into the mechanism of chiral recognition. The choice of polar modifier in the mobile phase significantly impacts the interaction between the analyte and the stationary phase, which in turn affects the resolution of chiral compounds. It has the ability to modify the power of the solvent and the density of the mobile phase. It may also compete with the analytes for adsorption sites and perhaps cause alterations in the structure of the stationary phase [36]. In addition, molecular docking experiments were conducted to determine the binding energy necessary for interacting with the CSP and its correlation with the experimentally determined thermodynamic parameters. The validated technique was also used to analyse the medications in their tablet forms.

Experimental

Chemicals and reagents

Rac-atenolol, rac-propranolol hydrochloride, and rac-metoprolol tartrate reference standards with a minimum purity of 98% were obtained from Sigma Aldrich Chemicals Pvt. Ltd. in Bangalore, India. The enantiomers S(—)-atenolol, R(+)-atenolol, S(—)-metoprolol, R(+)-metoprolol, S(—)-propranolol, and R(+)-propranolol with a minimum purity of 98.0% were purchased from Toronto Research Chemicals Inc. in Ontario, Canada. Methanol, isopropanol, and iso-propylamine (99% purity) of HPLC grade were obtained from Sigma Aldrich Chemicals Pvt. Ltd. (Bangalore, India). The liquid form of carbon dioxide (CO2) with a purity of 99.9% was obtained from SICGIL Industrial Gases Limited in Baroda, India.

Instrumental and chromatographic conditions

The b-blockers were analysed using chromatography using a Waters SFC Investigator system (Milford, MA, USA) that included a fluid supply module, an autosampler with a partial loop volume injection system, a backpressure regulator, a column oven, and a photodiode array (PDA) detector. Data handling was performed using the ChromScope v1.2.1 programme. The Chiralpak® IG column, measuring 250 mm × 4.6 mm with a 5 mm packing, was used to separate all six enantiomers. The column was packed with amylose tris(3-chloro-5-methylphenylcarbamate) immobilised with silica gel. The temperature of the column oven was 40 degrees Celsius. The mobile phase consisted of a blend of carbon dioxide (CO2) and 0.1% isopropyl amine in a combination of isopropanol and methanol (50:50, V/V) in a ratio of 75:25 (V/V). The mobile phase was continuously pumped at a constant flow rate of 4.0 mL/min. The amount of the injection was 10 mL, and the wavelength used for detection was set to 220 nm. The system's backpressure measured 100 bars. The temperature of the sample cooler was maintained at 10 ◦C. The optical rotation measurement was conducted using the MCP 5100 Modular Circular Polarimeter, manufactured by Anton Paar India Pvt. Ltd. (located in Haryana, India). The measurement was performed using a sodium source at a wavelength of 589 nm.

Preparation of stock solutions and calibrators

Stock solutions of rac-atenolol, rac-propranolol hydrochloride, rac-metoprolol tartrate, S(—)-atenolol, R(+)-atenolol, S(—)-metoprolol, R(+)-metoprolol, S(—)-propranolol, and R(+)-propranolol were individually produced in methanol at a concentration of 2500 mg/mL. Their operative solutions (500 mg/mL) were formulated in methanol using their corresponding standard stock solutions. To create linear curves for construction purposes, calibration standards (CSs) were made by diluting enantiomers from their working solutions. The resulting solutions had concentrations of 0.50, 1.00, 2.00, 3.00, 4.00, 6.00, 8.00, and 10.0 mg/mL, respectively. Furthermore, QC samples were prepared at concentrations of 1.50 mg/mL (low), 5.00 mg/mL (middle), and 9.00 mg/mL (high), respectively.

Assay of tablet formulation

To assess the composition of pharmaceutical formulations, we weighed and pulverised 20 tablets of Betaloc® 50 mg (metoprolol tartrate from Astra Zeneca Pharma India Ltd., Ahmedabad, India), Betacap® 10 mg (propranolol hydrochloride from Sun Pharmaceutical Industries Ltd., Mumbai, India), and Betacard® 50 mg (atenolol from Torrent Pharmaceuticals Ltd., Ahmedabad, India). Separate 50 mL volumetric flasks were used to hold an equal quantity of 50 mg metoprolol, 10 mg propranolol, and 50 mg atenolol. Each flask was then filled with 25 mL of methanol. Afterwards, the solutions were subjected to sonication for a duration of 30 minutes and then adjusted to the desired volume using methanol. Their operative solutions (10 mg/mL) were concocted by diluting the original solution with methanol. For the purpose of analysis, a volume of 10 mL was administered to the column in six duplicates. The peak area for all the enantiomers was measured at a wavelength of 220 nm. The quantity of each enantiomer in the tablets was calculated using their respective regression models.

Molecular docking study

The enantiomers were subjected to molecular docking using an Intel dual CPU (2.00 GHz) on the Windows 10 operating system. The enantiomers and the amylose derivatized CSP structures were drawn using Marwin Sketch programme [37]. The structures were converted into three-dimensional (3D) format and then stored in a PDB file. The amylose derivatized CSP structure was docked using Auto Dock Tools (ADT) 4.2. This was done by assigning Gasteiger charges, including nonpolar hydrogen atoms, and storing the structure in PDBQT file format. The docking process allowed for the rotation of all the flexible bonds in the ligands while keeping the receptor stiff [38]. The isomers were modified and stored in the PDBQT format using the same programme. The dimensions of the lattice box employed were 70 Å × 80 Å × 70 Å, with a spacing of 0.375 Å. The Auto Dock Vina programme was used to calculate the binding energy/affinity between the receptor, amylose tris(3-chloro-5-methylphenylcarbamate), and the enantiomer. The result file was then accessed in Discovery Studio Visualizer (Dassault systems Biovia Corporation) for the purpose of virtual screening, molecular docking, investigating the binding site, and determining the interaction and bond length between the stationary phase and the enantiomer.

Results and discussion

Effect of organic modifier

The impact of organic modifiers (methanol, isopropanol, and their combination) on the Chiralpak® IG column was first investigated. This column contains an

immobilised stationary phase of amylose tris(3-chloro-5-methylphenylcarbamate).

The studies were conducted using carbon dioxide (CO2) with a 20% organic modifier that included 0.1% isopropylamine. The trials were carried out at a temperature of 40 degrees Celsius and a back pressure of 100 bars. Isopropylamine, a fundamental additive, demonstrated superior resolution, peak shape, and enough responsiveness for the isomers in comparison to frequently used diethylamine or triethylamine in SFC. Each of the three organic modifiers successfully achieved complete separation of individual enantiomer pairs. However, it was not feasible to concurrently separate all six enantiomers in a single run within the optimal analysis time using methanol and isopropanol, respectively. While these medications cannot be obtained as a combination, it is beneficial to have a single approach instead of three distinct procedures or elution conditions for analysing these drugs.

Figure 1 demonstrates that the S(—) isomers of metoprolol and propranolol eluted together in isopropanol and methanol, whereas the R(+) isomers of atenolol and propranolol eluted in isopropanol. However, all of the enantiomers were completely separated in a combination of methanol and isopropanol (50:50, V/V). Furthermore, the sequence in which enantiomers were separated (with the S(—) isomer preceding the R(+) isomer) remained consistent for all the modifiers. This was verified by gathering the fractions and quantifying their optical rotation, as well as by comparing them to their respective reference standards. Moreover, methanol exhibited higher enantiomer retention compared to isopropanol and their combination. In contrast, the retention of iso-propanol was comparatively lower, particularly for the S(—) isomers of metoprolol and propranolol (Table 1). Nevertheless, the whole study focused on a combination of methanol and isopropanol (50:50, V/V) due to its ability to meet the criteria of separation factor (a≥1.5) and resolution factor (Rs≥1.5), as well as its capability to simultaneously separate all six enantiomers.

Table 1: Thermodynamic parameters for enantiomers on Chiralpak® IG column under different back pressures.

Stereoisomer	100 bar			125 bar			150 bar		
	D*G* (kJ/mol)	D*H* (kJ/mol)	D*S* (J/mol K)	D*G* (kJ/mol)	D*H* (kJ/mol)	D*S* (J/mol K)	D*G* (kJ/mol)	D*H* (kJ/mol)	D*S* (J/mol K)
S(—)-metoprolol	−1.290	−4.232	−9.872	−1.221	−4.248	−10.16	−1.171	−3.385	−7.429
R(+)-metoprolol	−3.989	−11.47	−25.12	−3.928	−13.30	−31.46	−3.881	−13.93	−33.73
S(—)-propranolol	−2.464	−6.448	−13.37	−2.449	−8.031	−18.73	−2.409	−8.296	−19.76
R(+)-propranolol	−4.800	−12.09	−24.46	−4.781	−14.57	−32.87	−4.726	−14.88	−34.07
S(—)-atenolol	−2.864	−6.260	−11.40	−2.871	−7.158	−14.38	−2.844	−7.358	−15.15
R(+)-atenolol	−4.120	−4.878	−2.544	−4.085	−4.751	−2.235	−3.960	−5.008	−3.516

Table 2 provides the impact of various ratios (15, 20, 25, 30, and 35) of methanol to isopropanol (50:50) on the retention duration, retention factors (k), a, and Rs of the enantiomers. The reduction in enantiomer retention was seen with a rise in the organic modifier content. This may be attributed to the enhanced solvating ability of the mobile phase containing CO2. Figure 1A illustrates the relationship between the natural logarithm of the k values and the proportion of methanol:isopropanol (50:50, V/V) in the mobile phase. It is clear that there was a higher level of retention for the R(+) isomer compared to the S(—) isomer for all three b-blockers. Fig. 1B demonstrates a comparable decline in the separation factors (a). However, in the case of atenolol, which is more polar than other b-blockers, the reduction in retention time was more significant and was influenced to a greater extent by the rise in organic modifier content in the mobile phase.

Table 2: Thermodynamic parameters from van't Hoff plots of separation factors versus temperature.

Separation factor (a)	Back pressure (bars)	Correlation coefficient (*r*)	DD*H* (J/mol)	DD*S* (J/mol K)	T_{iso} (°C)	DD*H*/RT	DD*S*/RT
a_1 (*S*(—)- & *R*(+)-metoprolol)	100	0.971	−7101 ± 38	−14.81 ± 0.91	206.4	2.73	1.78
	125	0.975	−9129 ± 54	−21.58 ± 1.08	149.8	3.51	2.60
	150	0.980	−10484 ± 77	−26.12 ± 1.13	128.2	4.03	3.14
a_2 (*S*(—)- & *R*(+)-propranolol)	100	0.974	−5624 ± 29	−11.03 ± 0.83	236.6	2.16	1.33
	125	0.968	−6586 ± 41	−14.28 ± 0.76	188.2	2.53	1.72
	150	0.978	−6547 ± 34	−14.20 ± 0.93	187.9	2.51	1.71
a_3 (*S*(—)- & *R*(+)-atenolol)	100	0.990	2317 ± 16	10.85 ± 0.58	−59.6	0.89	1.31
	125	0.993	2456 ± 19	11.20 ± 0.62	−53.9	0.94	1.35
	150	0.987	2678 ± 14	11.88 ± 0.47	−47.7	1.03	1.43

T_{iso}: Isoelution temperature; column: Chiralpak® IG; mobile phase: a mixture of CO_2 and 0.1% isopropyl amine in isopropanol:methanol (50:50, *V/V*), in 75:25 (*V/V*) ratio; wavelength: 220 nm; flow rate: 4.0 mL/min. RT: retention time.

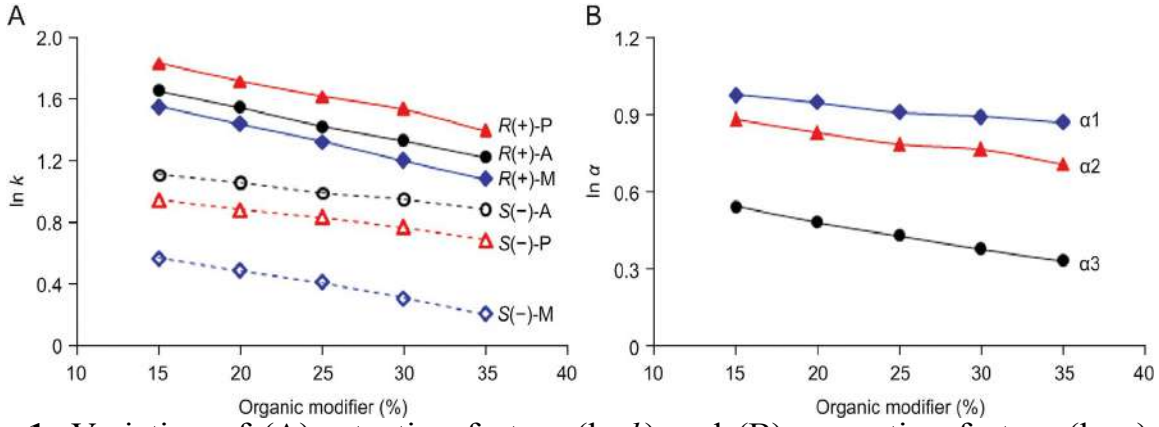

Fig. 1. Variation of (A) retention factors (ln *k*) and (B) separation factors (ln a) for enantiomers of atenolol, metoprolol and propranolol with different percentages of organic modifier. Mobile phase: CO_2: 0.1% isopropyl amine in isopropanol: methanol (50:50, *V/V*). Column: Chiralpak® IG; temperature: 40 °C; back pressure: 100 bars; detection wave- length: 220 nm; flow rate: 4 mL/min. a1, a2, and a3: separation factors between enantiomers of metoprolol (a1), propranolol (a2), and atenolol (a3). A: atenolol; M: metoprolol; P: propranolol.

Effect of back pressure

The solvation capacity of supercritical CO2 is well recognised to increase with higher back pressure, facilitating the fast elution of analytes from the column [39]. The impact of back pressure on enantioseparation was examined at pressures of 100, 125, and 150 bars. The enantiomer retention reduced as the back pressure increased for all the b-blockers (Table 3). An analogous pattern was seen in the resolution factors, with no significant change in separation factors. Moreover, the decline in the preservation of S-isomers was much less compared to the R-isomers as the back pressure increased. At a temperature of 40 ◦C, the Rs values for the enantiomers of metoprolol, propranolol, and atenolol reduced from 5.68 to 5.01, 6.83e6.02, and 3.48e3.26, respectively. This phenomenon may be attributed to the enhanced solvation capacity of supercritical CO2 as the back pressure increases, resulting in accelerated separation of the enantiomers.

Table 3: Molecular docking results for enantiomers of b-blockers.

Enantiomer	Binding energy (kJ/mol)	Ligand efficiency	No. of interactions	Intermolecular bonding interaction		Binding site		Bond length/ distance (Å)	DE_{R-S} (kJ/ mol)
				Category	Type	Enantiomer	CSP		
S(—)-atenolol	−4.37	−0.23	4	H-bond	Conventional H-bond	PheOeC	H of the eCO-NHe	3.00	0.63
				H-bond	Conventional H-bond	C=O of NH_2eCOePh	H of the eCO-NHe	2.92	
				H-bond	Conventional H-bond	H of NH_2eCOe Ph	CO of the eCO-NHe	3.25	
				H-bond	Hydrogen bond	H of CeCHeC	CO of the eCO-NHe	3.03	
R(+)-atenolol	−5.00	−0.26	3	H-bond	Conventional H-bond	H of CeNHeC	CO of the eCO-NHe	2.84	
				H-bond	Conventional H-bond	H of NH_2eCOe Ph	CO of the eCO-NHe	2.92	
				Hydrophobic	Alkyl-alkyl bond	CH_3eCHeCH_3	CH_3 of phenyl ring	3.40	
S(—)-metoprolol	−3.35	−0.18	2	H-bond	p-donor H-bond	p-electrons of the phenyl ring	H of the eCO-NHe	3.26	1.53
				Other	p-lone pair	p-electrons of the phenyl ring	Lone pair on CO of the eCO-NHe	2.98	
R(+)-metoprolol	−4.88	−0.25	5	H-bond	Hydrogen bond	H of -CO-CH_3	CO of the eCO-NHe	3.33	
				H-bond	Hydrogen bond	H of -CO-CH_3	CO of the eCO-NHe	3.35	
				Hydrophobic	p -sigma	CH_3eCHeCH_3	p-electrons of the phenyl ring	3.71	
				Hydrophobic	p -alkyl	p-electrons of the phenyl ring	CH_3 of phenyl ring	4.38	
				Hydrophobic	p -alkyl	p-electrons of the phenyl ring	CH_3 of phenyl ring	5.33	
S(—)-propranolol	−3.95	−0.21	4	Hydrophobic	p -sigma	p-electrons of naphthyl ring	CH_3 of phenyl ring	3.50	1.36
				Hydrophobic	p - p stacked	p-electrons of naphthyl ring	p-electrons of the phenyl ring	4.39	
				Hydrophobic	p -alkyl	p-electrons of naphthyl ring	CH_3 of phenyl ring	5.03	
				Hydrophobic	p -alkyl	p-electrons of naphthyl ring	CH_3 of phenyl ring	4.01	
R(+)-propranolol	−5.31	−0.29	5	H-bond	Hydrogen bond	H of CH_3eCHeCH_3	CO of the eCO-NHe	3.27	
				Hydrophobic	p -sigma	p-electrons of naphthyl ring	CH_3 of phenyl ring	3.41	
				Hydrophobic	p -sigma	p-electrons of naphthyl ring	CH_3 of phenyl ring	3.94	
				Hydrophobic	p - p stacked	p-electrons of naphthyl ring	p-electrons of the phenyl ring	4.32	
				Hydrophobic	p -alkyl	CH_3eCHeCH_3	p-electrons of the	5.27	

CSP: chiral stationary phase (Chiralpak® IG).

Effect of temperature on the enantioseparation

Various investigations [39,40] have indicated that temperature has a significant impact on enantiomeric separations. It has the ability to induce alterations in retention time, selectivity, and resolution. The impact of temperature was investigated in the sub and supercritical range, ranging from 25 ◦C to 45 ◦C with 5 ◦C intervals (Table S3). Like the back pressure phenomenon, the retention of the enantiomers reduced for all three medications at 100 bars as the temperature increased, as seen in Figure 3. However, it is important to mention that there was a change in the order of elution for R(+)-metoprolol and R(+)-atenolol at the subcritical zone at 25 ◦C and 30 ◦C. Under normal supercritical circumstances, the retention time should rise as the temperature increases, as the density of the fluid lowers. This leads to a reduction in the elution strength of the fluid [39]. However, the observed behaviour may be explained by the fact that at higher temperatures, the solubility of the

enantiomers in the mobile phase increases owing to a reduction in the cohesive forces of the fluid. This ultimately results in a decrease in retention. This behaviour is often seen in High Performance Liquid Chromatography (HPLC) separations.

The van't Hoff equation expresses the relationship between the retention factor (k) and temperature.

$$\ln k = -\Delta H/RT + \Delta S/R + \ln (1/b) \quad (1)$$

Here, DH represents the standard molar enthalpy and DS represents the molar entropy for the transfer of the analyte from the mobile phase to the stationary phase. b denotes the phase ratio, whereas R is the ideal gas constant, which has a value of 8.314 J/mol K. Figure 4 displays the graphs of the natural logarithm of retention factors, ln k, plotted against the reciprocal of temperature (1000/T) at various back pressures. The charts exhibited almost linear behaviour for all enantiomers, suggesting that the phase ratio remained relatively constant despite variations in density at various temperatures. Table 1 provides a summary of the standard molar free energy (DG), enthalpy (DH), and entropy (DS) values for the isomers under various back pressures. The enantiomers exhibited negative DG values at various pressures and temperatures, as shown in Table S4. Similarly, the DH and DS values were negative, suggesting that the movement of enantiomers from the mobile phase to the stationary phase was driven by changes in enthalpy.

By examining Figure 5, it is evident that the selectivity of metoprolol and propranolol rises when the temperature decreases under constant pressure. However, a modest reduction in selectivity was noted for atenolol. The correlation between the separation factor and temperature may be mathematically represented as

$$\ln (a) = -\Delta\Delta H/RT + \Delta\Delta S/R \quad (2)$$

$$\ln (a) = -\Delta\Delta G/RT \quad (3)$$

Where DDH and DDS denote differential enthalpy and entropy, respectively. If the enantio-selective separation remains constant across the temperature range under investigation, the relationship between the natural logarithm of a and the reciprocal of T will exhibit a linear pattern. The graphs (Figure 5) exhibited linearity for all enantiomers, suggesting the presence of an isoelution temperature (Tiso) at which the isomers co-elute. Below the isothermal temperature (Tiso), the separation is mostly driven by changes in enthalpy. Additionally, the separation factors may be enhanced by lowering the temperature. At higher temperatures, the chiral separation above Tiso is driven by entropy and is predicted to result in higher separation factors [40]. The values of DDH, DDS, and Tiso were calculated from the regression lines shown in Figure 5 and are reported in Table 2. The DDH and DDS values were negative for metoprolol and propranolol, but the opposite was seen for atenolol at varying pressures. In addition, when the back pressure increased, there was a corresponding rise in the absolute values of DDH and DDS. The magnitudes of DDH/RT were higher than DDS/RT for metoprolol and propranolol, indicating that the separation process was governed by enthalpy. The Tiso values exceeded the operational temperature range for metoprolol and propranolol and may be enhanced by reducing the temperature. However, the Tiso values fell beyond the temperature range investigated for atenolol, resulting in an enantioseparation driven by entropy. The thermodynamic data were analysed using the concept of enthalpy-entropy compensation. The figure comparing DDH and DDS showed a linear relationship, indicating enthalpy-entropy compensation for enantioselectivity (Fig. 2).

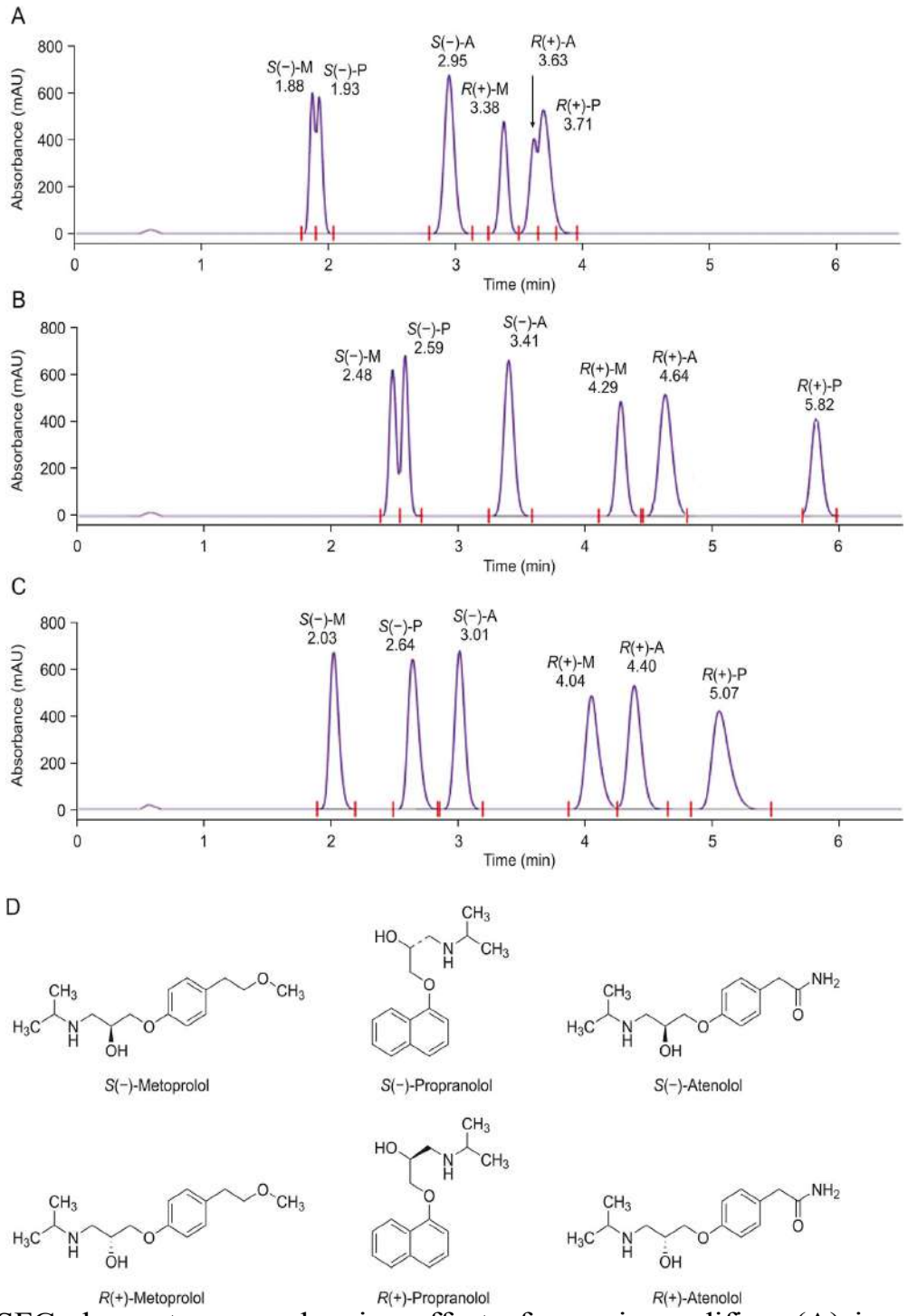

Fig. 2. SFC chromatograms showing effect of organic modifiers (A) isopropanol, (B) methanol, and (C) isopropanol:methanol (50:50, *V/V*) on the separation of enantiomers; (D) chemical structures of enantiomers. Column: Chiralpak® IG; mobile phase: CO_2: 0.1% isopropyl amine in organic modifier (80:20, *V/V*); temperature: 40 °C; back pressure: 100 bars; detection wavelength: 220 nm; flow rate: 4 mL/min.

Molecular docking studies with chiral stationary phases

In order to analyse the elution pattern and gain insight into the process of chiral recognition, we conducted molecular docking using Auto Dock Tools (ADT) 4.2 software. This method enables the prediction of the optimal orientation of tiny molecules for interaction with the stationary phase. Reasonably accurate estimates of binding energy and bond lengths for various interaction modes may be obtained [22,23]. Moreover, it may facilitate the comprehension of the elution patterns of the enantiomers by considering their respective binding energies. The binding energies arise from several intermolecular interactions, including hydrogen bonding, Van der Waals forces, pi-pi interactions, and dipole-dipole interactions. Greater stability of enantiomer-CSP binding is indicated by higher negative values. Figure 6 illustrates the three-dimensional interaction between the enantiomers and the chiral stationary phase. The CSP (chiral stationary phase) has functional groups such as > C]O, –NH–, and a phenyl ring with alkyl and chloro groups. On the other hand, the enantiomers of the CSP have additional features including a carbamoyl group (only in atenolol), eOH, –O–, >C]O, secondary amine, isopropyl groups, and aromatic ring systems (phenyl and naphthyl). Consequently, they are capable of interacting via hydrogen bonding, pi-pi interactions, and hydrophobic interactions. Table 3 displays the binding energy, ligand efficiency, quantity, and type of intermolecular interactions, along with bond lengths.

The enantiomers exhibited different binding energies (measured in kJ/mol) in the following order:

The order of decreasing potency for the listed beta blockers is as follows: S(—)-metoprolol (—3.35), S(—)-propranolol (—3.95), S(—)-atenolol (—4.37), R(+)-metoprolol (—4.88), R(+)-atenolol (—5.00), R(+)-propranolol (The data suggests that R(+)-propranolol had the most potent interaction with the CSP, while S(—)-metoprolol displayed the least potent interaction. Furthermore, the Gibbs free energy (DG) values (in kJ/mol) for the transfer of the enantiomer from the mobile phase to the stationary phase at a pressure of 100 bars and a temperature of 25 ◦C exhibited a similar pattern. The order of the compounds, from least negative to most negative, is as follows: S(—)-metoprolol (—1.290), S(—)-propranolol (—2.464), S(—)-atenolol (—2.864), R(+)-metoprolol (—3.989), R(+)-atenolol (—4.120), R(+)-propranolol (—4.800) (Table S4). These observations align well with the elution pattern found in the experimental data. The elution times of the enantiomers in the given order are as follows: S(—)-metoprolol (1.96 min), S(—)-propranolol (2.59 min), S(—)-atenolol (2.95 min), R(+)-metoprolol (3.79 min), R(+)-atenolol (4.05 min), R(+)-propranolol (5.00 min) (Table S2). In order to establish a connection between binding energy and enantioselectivity, an assessment was conducted to determine the disparity in binding energies between the enantiomers DER-S (in kJ/mol). The metoprolol, propranolol, and atenolol compounds had absolute DER-S values of 1.53, 1.36, and 0.63 kJ/mol, respectively. The significant disparity of 1.53 kJ/mol in metoprolol suggests that the enantiomers may be more readily separated compared to propranolol or atenolol. The values of the medication enantiomers, metoprolol (2.53 kJ/mol), propranolol (2.33 kJ/mol), and atenolol (1.51 kJ/mol), as shown in Table S2, were experimentally determined to have a direct link with the separation factor.

Additionally, the chiral recognition technique may be comprehended by examining the three-dimensional docking

illustrations of enantiomers (Figure 6). The precise alignment of the enantiomers inside the structure of the CSP is crucial for effective separation of chiral compounds. The comprehensive data for each enantiomer is shown in Table 3. Hydrogen bonding and hydrophobic interactions were the primary factors contributing to enantioseparation. The R(+)-propranolol enantiomer, which exhibited strong retention, was shown to interact with the chiral stationary phase (CSP) via four hydrophobic contacts (with bond lengths/distance ranging from 3.41 to 5.27 Å) and one hydrogen bond. The hydrophobic interactions mostly arose from the p-alkyl, p-sigma, and p-p stacking interactions between the p-electrons of the naphthyl ring in the enantiomer and either the alkyl group or the p-electrons of the phenyl ring. Conversely, S(—)-metoprolol, which had the lowest retention, only interacted with the CSP via two points: p-donor, H-bond, and p-lone pair interaction. On the other hand, R(+)-metoprolol exhibited higher retention as a result of two hydrogen bonds formed between the hydrogen of –CO–CH3 and CO of the eCO-NHe group, as well as three hydrophobic interactions involving p-alkyl and p-sigma bonding. The separation of atenolol enantiomers was mainly due to conventional hydrogen bonding. The H-donor/acceptor was either the carbamoyl group of the enantiomer or the carbamate group of the CSP (with bond lengths/distance of 2.84e3.25 Å). While it may be challenging to fully understand the enantioseparation and retention behaviour through molecular docking alone, it is evident that the R(+) enantiomers of the three b-blockers exhibit higher retention due to their increased interactions with the CSP compared to the S(-) enantiomers. Moreover, when comparing this research with previous studies on these medications utilising various chiral stationary phases, several parallels and differences are observed. The study conducted by Li et al. [22,23] used HPLC with vancomycin-bonded and Chiralpak IB columns to analyse some substances. Their findings indicated that for several analytes, the S-enantiomers were eluted first, which aligns with the results obtained in our current study employing Chiralpak IG. However, when a similar column was used in SFC-MS/MS analysis, the elution order was reversed [32]. Furthermore, the simulation studies showed that atenolol had the least change in binding energies across its enantiomers compared to the other two b-blockers [23]. These findings align with the results observed in our current research.

Comparison with reported work

Presently, the techniques used for the simultaneous enantioseparation and measurement of atenolol, metoprolol, and propranolol encompass capillary electrophoresis [15], high-performance liquid chromatography (HPLC) [22,23], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [24], and supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) [32]. The key characteristics of these techniques are outlined in Table 4. Nevertheless, all documented techniques need a separation duration that spans from 10 to 35 minutes. By comparison, the current technique enabled the separation of all six enantiomers in only 6.0 minutes. In addition, most of the documented processes included the use of significant amounts of organic solvents for separation, with the exception of one study that used SFC-MS/MS [32]. In addition, only two approaches have addressed thermodynamic factors and conducted a molecular docking research to gain insights into the interactions between the analytes and the chiral stationary phase [15,22]. Li et al. [23] examined the processes of chiral recognition using the molecular docking

approach. However, this work is the first to discuss the application of SFC approach for chiral separation, specifically focusing on the thermodynamics of drug interaction with the stationary phase and conducting simulation studies to better understand the retention behaviour of enantiomers. In addition, all the enantiomers were separated using the same elution conditions. The present technique resulted in quicker and more effective separations compared to the previous processes, while also minimising the time required for development and validation in the chiral separation of these medications.

Method validation results

The method underwent validation to assess its linearity, limit of detection (LOD = 3.3 s/S, where s represents the standard deviation of the intercept and S represents the slope of the calibration lines), limit of quantitation (LOQ = 10 s/S), specificity, intra-day and inter-day accuracy and precision, as well as recovery, in accordance with the guidelines set by the International Council for Harmonisation (ICH) [41]. The mobile phase used for quantitative investigations consisted of a combination of CO2 and 0.1% isopropyl amine in a mixture of iso-propanol and methanol in a 50:50 volume ratio. Additionally, a 75:25 volume ratio of iso-propanol and methanol was also applied. While it was feasible to achieve satisfactory resolution (Rs>1.5) of the enantiomers using 5% to 20% organic modifier, a concentration of 25% was chosen based on the optimal analysis time, responsiveness, resolution, and selectivity. The calibration curves were created by graphing the peak area in relation to the concentration of the enantiomers. The linearity of each isomer was proven by using least square linear regression on five calibration lines, resulting in a correlation coefficient (r2) of at least 0.9995 and a concentration of 0.5e10 mg/mL.

The method's limit of detection (LOD) and limit of quantification (LOQ) for S(—)-metoprolol, R(+)-metoprolol, S(—)-propranolol, R(+)-propranolol, S(—)-atenolol, and R(+)-atenolol were 0.126/0.381, 0.130/0.394, 0.128/0.389, 0.124/0.376, 0.137/0.414, and 0.132/0.401 mg/mL, respectively. The approach specificity was assessed by comparing the retention duration of the standards and actual samples (pharmaceutical formulations). The findings demonstrated a strong correlation in the assessment of retention time for all the enantiomers, with a coefficient of variation ranging from 0.51 to 1.12 percent. The specific chromatographic properties are reported in Table 5.

The findings for the precision and accuracy of the approach for all enantiomers at three quality control (QC) levels, both within a single day (intra-day) and between different days (inter-day), are shown in Table 5. The accuracy of measurements on a single day varied from 1.2% to 2.9% coefficient of variation (% CV), whereas the precision of measurements across different days ranged from 1.0% to 2.9% (% CV). The method's accuracy was assessed using the standard addition methodology at 80%, 100%, and 120% of the reported value. The findings demonstrated high accuracy within the range of 98.63% to 100.92% (Table 6).

Analysis of pharmaceuticals

The established technique was used to examine these medications in their commercially available formulations. Figure 1 displays the chromatograms illustrating the separation of metoprolol, propranolol, and atenolol enantiomers from respective tablet formulations. The obtained findings demonstrated satisfactory accuracy and precision of the test, as shown in Table 7. Furthermore, the formulations were not affected by any interference from the excipients. In addition, the enantiomeric

purity (or optical purity) of the separated analytes was also assessed. The enantiomeric purity of S(—)-atenolol is 99.5%, with a specific rotation of -24.2° at 25°C in a 1.0 concentration of ethanol. The enantiomeric purity of R(+)-atenolol is 99.6%, with a specific rotation of +24.4° at 25°C in a 1.0 concentration of ethanol. The enantiomeric purity of S(—)-metoprolol is 99.4%, with a specific rotation of -30.2° at 25°C in a 1.0 concentration of ethanol. The enantiomeric purity of R(+)-metoprolol is 99.2%, with a specific rotation of +29.9° at 25°C in a 1.0 concentration of ethanol. The enantiomeric purity of S(—)-propranolol is 99.3%, with a specific rotation of -21.9° at 25°C in a 1.0 concentration of ethanol. The enantiomeric purity of R(+)-propranolol is 99.5%, with a specific rotation of +22.3° at 25°C in a 1.0 concentration of ethanol. In order to demonstrate the importance of the created technique, a statistical analysis was conducted to compare the findings with those obtained from previously described techniques [19,21]. This comparison was carried out using t-test and F-test. The computed t and F values were less than the tabulated values at four degrees of freedom, indicating that there is no substantial difference between the two techniques for any of the medications.

Table 4: Linear range and chromatographic characteristics of enantiomers of metoprolol, propranolol and atenolol.

Enantiomers	Linear range (mg/mL)	Slope (area response/ mg/mL) ± SD	Intercept (area response) ± SD	LOD (mg/mL)	LOQ (mg/mL)	Correlation coefficient (r^2)	Separation factor (a)	Resolution factor (R_s)	Theoretical plates	Tailing factor
S(—)-metoprolol	0.5e10	160.42 ± 13.7	21.9 ± 6.12	0.126	0.381	0.9998	2.53	5.68	5232	1.12
R(+)-metoprolol	0.5e10	163.74 ± 12.6	22.5 ± 6.45	0.130	0.394	0.9995			8823	1.01
S(—)-propranolol	0.5e10	162.34 ± 11.4	22.4 ± 6.32	0.128	0.389	0.9997	2.33	6.83	6989	1.06
R(+)-propranolol	0.5e10	164.57 ± 14.2	22.8 ± 6.18	0.124	0.376	0.9996			9945	1.05
S(—)-atenolol	0.5e10	161.28 ± 12.8	22.2 ± 6.68	0.137	0.414	0.9995	1.51	3.48	7372	1.04
R(+)-atenolol	0.5e10	163.45 ± 15.5	22.6 ± 6.56	0.132	0.401	0.9999			11482	1.06

SD: standard deviation; LOD: limit of detection; LOQ: limit of quantitation.

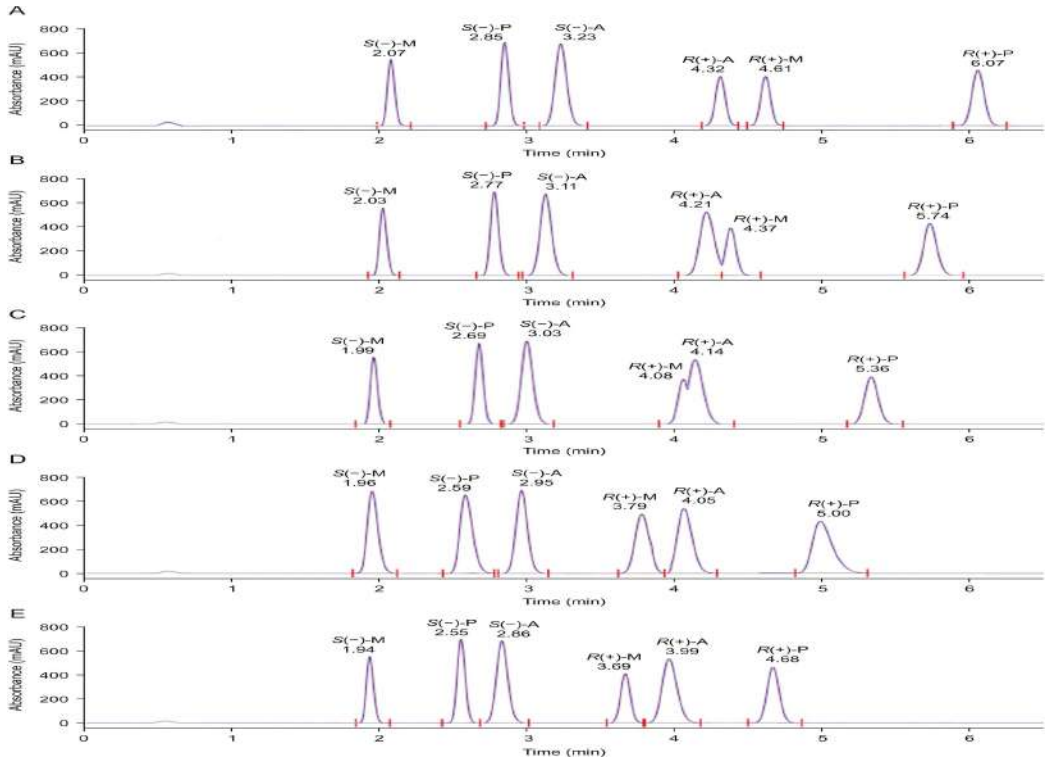

Fig. 3. SFC chromatograms showing effect of temperature (A) 25 °C, (B) 30 °C, (C) 35 °C, (D) 40 °C, and (E) 45 °C on the enantioseparation of the drugs. Column: Chiralpak® IG; mobile phase: a mixture of CO_2 and 0.1% isopropyl amine in isopropanol:methanol (50:50, *V/V*), in 75:25 (*V/V*) ratio; back pressure: 100 bars; detection wavelength: 220 nm; flow rate: 4 mL/min.

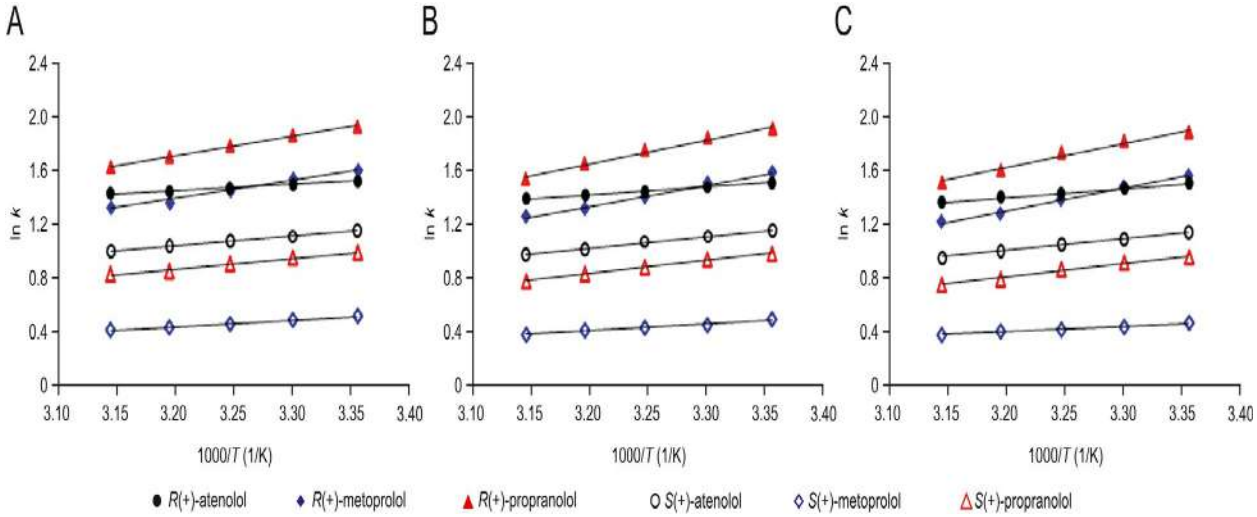

Fig. 4. van't Hoff plots of retention factors (ln k) of atenolol, metoprolol and propranolol enantiomers versus temperature (1000/T) at different back pressures (A) 100 bars, (B) 125 bars, and (C) 150 bars. Column: Chiralpak® IG; mobile phase: a mixture of CO2 and 0.1% isopropyl amine in isopropanol:methanol (50:50, V/V), in 75:25 (V/V) ratio; detection wavelength: 220 nm; flow rate: 4 mL/min.

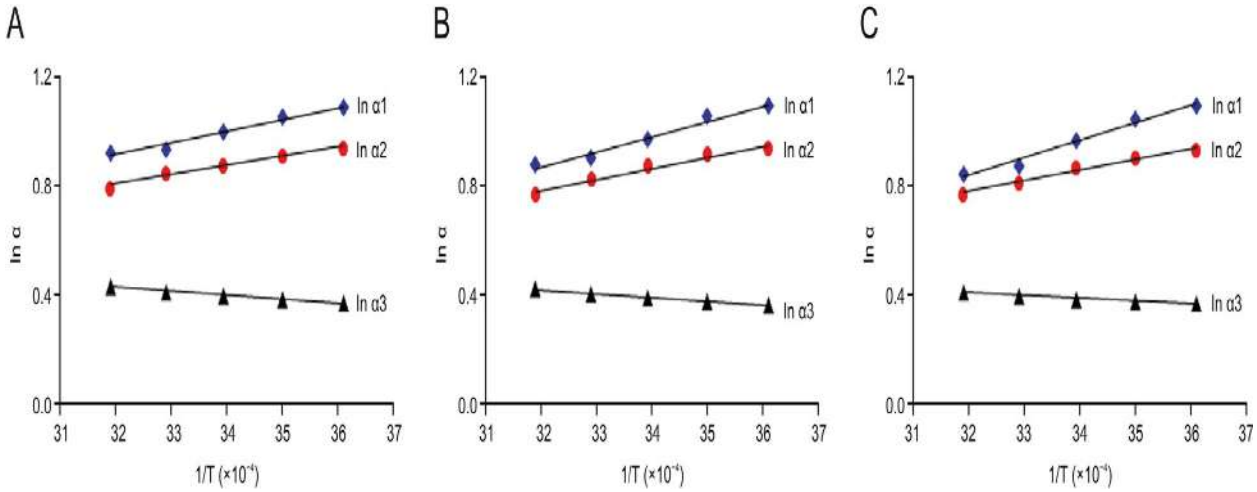

Fig. 5. Changes in the separation factors (ln a) of enantiomers with temperature (1/T) at three different back pressures (A) 100 bars, (B) 125 bars, and (C) 150 bars. Column: Chiralpak® IG; mobile phase: a mixture of CO2 and 0.1% isopropyl amine in isopropanol:methanol (50:50, V/V), in 75:25 (V/V) ratio; detection wavelength: 220 nm; flow rate: 4 mL/ min. ln a1, ln a2, and ln a3 represent separation factors between enantiomers of metoprolol, propranolol, and atenolol, respectively.

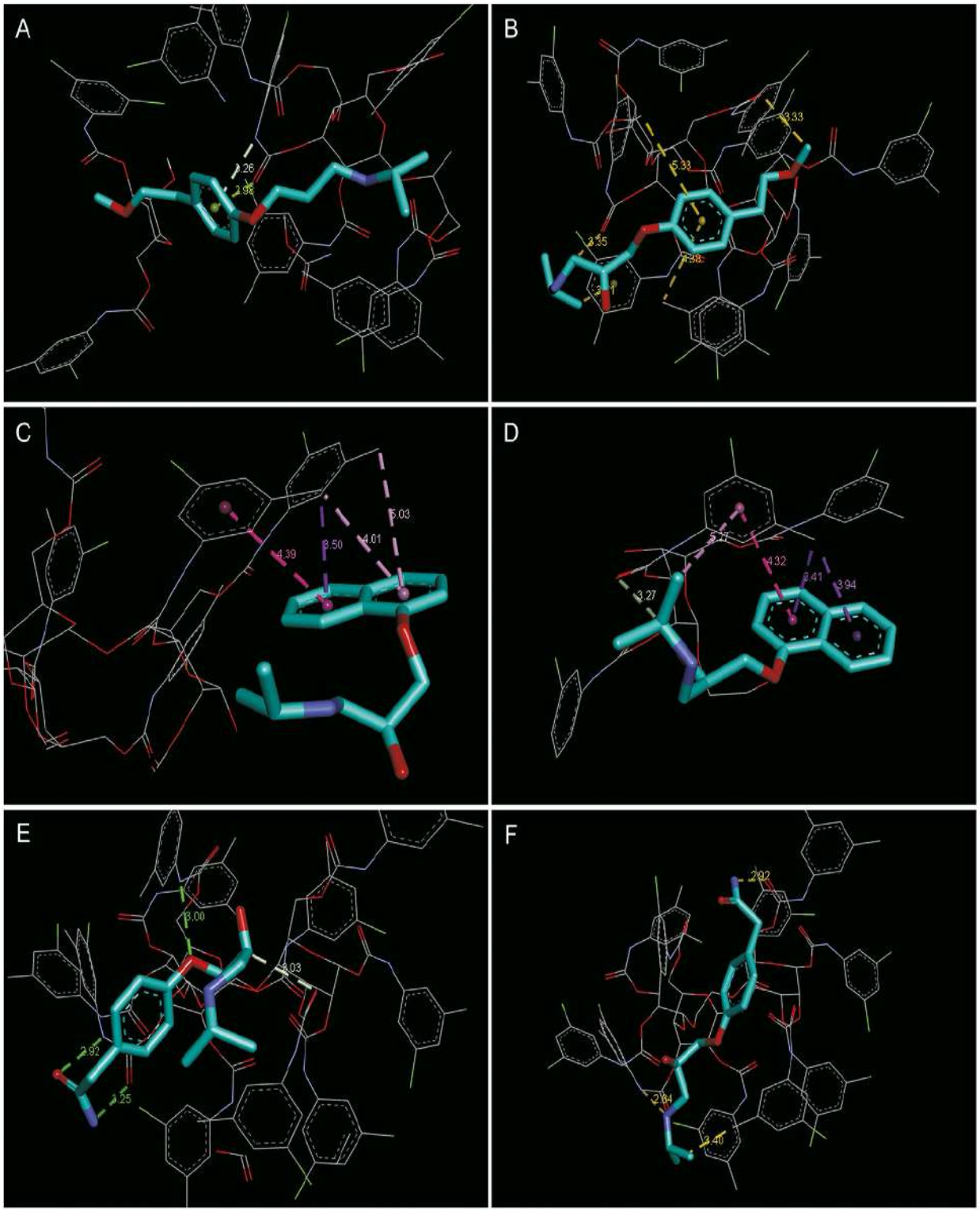

Fig. 6. 3D interaction modes of enantiomers: (A) S(—)-metoprolol, (B) R(+)-metoprolol, (C) S(—)-propranolol, (D) R(+)-propranolol, (E) S(—)-atenolol, and (F) R(+)-atenolol with Chiralpak® IG chiral stationary phase immobilized with amylose tris(3-chloro-5-methylphenylcarbamate.

Conclusions

In this study, we have presented a novel supercritical fluid chromatography (SFC) technique for separating the enantiomers of atenolol, metoprolol, and propranolol on a chiral stationary phase. Our approach utilises a single elution process. The impact of the organic modifier and its percentage had a more significant influence on selectivity compared to the column temperature and back pressure. While methanol and isopropanol could separate the enantiomers individually, a combination of methanol and isopropanol provided the optimal conditions for simultaneously separating them in a single run, with sufficient resolution, selectivity, and chromatographic efficiency. The thermodynamic data indicated that the separation of metoprolol and propranolol was driven by changes in enthalpy, whereas the separation of atenolol was driven by changes in entropy. The elution sequence of the enantiomers observed experimentally and the mechanism for chiral recognition were confirmed by a molecular docking analysis. Moreover, the enantioselectivity of the investigated medicines was significantly influenced by hydrogen bonding and hydrophobic interactions. Ultimately, the SFC approach was successfully used to examine commercially accessible versions of these medications.

References

1. T.J. Ward, K.D. Ward, Chiral separations: a review of current topics and trends, Anal. Chem. 84 (2012) 626e635.
2. C. West, Enantioselective separations with supercritical fluids-review, Curr. Anal. Chem. 10 (2014) 99e120.
3. D. Speybrouck, E. Lipka, Preparative Supercritical fluid chromatography: a powerful tool for chiral separations, J. Chromatogr. A 1467 (2016) 33e55.
4. FDA's policy statement for the development of new stereoisomeric drugs, Chirality 4 (1992) 338e340.
5. I. Ali, Z.A. Al-Othman, A. Al-Warthan, et al., Enantiomeric separation and simulation studies of pheniramine, oxybutynin, cetirizine, and brinzolamide chiral drugs on amylose-based columns, Chirality 26 (2014) 136e143.
6. J. Wu, X. Xiao, Z. Li, et al., Enantioseparation of chiral b-blockers using poly-norepinephrine-coated nanoparticles and chiral capillary electrophoresis, Anal. Bioanal. Chem. 411 (2019) 2121e2129.
7. S. Alwera, R. Bhushan, Liquid chromatographic enantioseparation of three beta-adrenolytics using new derivatizing reagents synthesized from (S)- ketoprofen and confirmation of configuration of diastereomers, Biomed. Chromatogr. 30 (2016) 1772e1781.
8. P.S. Shrivastav, S.M. Buha, M. Sanyal, Detection and quantitation of b-blockers in plasma and urine, Bioanalysis 2 (2010) 263e276.
9. J. Agustian, A.H. Kamaruddin, H.Y. Aboul-Enein, Chromatographic comparison of atenolol separation in reaction media on cellulose tris-(3,5-dimethylphenylcarbamate) chiral stationary phase using ultra fast liquid chromatography, Chirality 24 (2012) 356e367.
10. R. Bhushan, C. Agarwal, Resolution of beta blocker enantiomers by TLC with vancomycin as impregnating agent or as chiral mobile phase Additive, JPC-J. Planar. Chromatogr. e Mod. TLC 23 (2010) 7e13.
11. E. Bodoki, M. Oltean, A. Bodoki, et al., Chiral recognition and quantification of propranolol enantiomers by surface enhanced Raman scattering through su-pramolecular interaction with b-cyclodextrin, Talanta 101 (2012) 53e58.

12. S. Tong, Y. Zheng, J. Yan, et al., Preparative enantioseparation of b-blocker drugs by counter-current chromatography using dialkyl L-tartrate as chiral selector based on borate coordination complex, J. Chromatogr. A 1263 (2012) 74e83.
13. J.M. Park, J.H. Park, Enantiomer separations of basic chiral compounds by capillary electrochromatography on a phosphated b-cyclodextrin-modified zirconia monolith, J. Chromatogr. A 1339 (2014) 229e233.
14. X. Sun, Y. Du, S. Zhao, et al., Enantioseparation of propranolol, amlodipine and metoprolol by electrochromatography using an open tubular capillary modified with b-cyclodextrin and poly (glycidyl methacrylate) nanoparticles, Mikrochim. Acta 186 (2019), 128.
15. W. Li, C. Liu, G. Tan, et al., Molecular modeling study of chiral separation and recognition mechanism of b-adrenergic antagonists by capillary electrophoresis, Int. J. Mol. Sci. 13 (2012) 710e725.
16. S.Q. Hu, W.J. Lü, Y.H. Ma, et al., Chiral separation of b-blockers by MEEKC using neutral microemulsion: analysis of separation mechanism and further elucidation of resolution equation, Electrophoresis 34 (2013) 260e268.
17. S. Dixit, J.H. Park, Application of rifampicin as a chiral selector for enantior- esolution of basic drugs using capillary electrophoresis, J. Chromatogr. A 1453 (2016) 138e142.
18. L. Fang, Y. Du, X. Hu, et al., Carboxymethyl b-cyclodextrin as chiral selector in capillary electrophoresis: enantioseparation of 16 basic chiral drugs and its chiral recognition mechanism associated with drugs' structural features, Biomed. Chromatogr. 31 (2017), e3991.
19. A.K. Singh, E.R.M. Kedor-Hackmann, M.I.R.M. Santoro, Development and validation of a chiral liquid chromatographic method for the determination of atenolol and metoprolol enantiomers in tablet preparations, J. AOAC Int. 84 (2001) 1724e1729.
20. I. Ali, V.D. Gaitonde, H.Y. Aboul-Enein, et al., Chiral separation of b-adrenergic blockers on CelluCoat column by HPLC, Talanta 78 (2009) 458e463.
21. D. Wang, F. Li, Z. Jiang, et al., Chiral recognition mechanisms of four b-blockers by HPLC with amylose chiral stationary phase, Iran, J. Pharm. Res. 13 (2014) 449e457.
22. J. Li, R. Liu, L. Wang, et al., Enantioseparation of chiral pharmaceuticals by vancomycin-bonded stationary phase and analysis of chiral recognition mechanism, Chirality 31 (2019) 236e247.
23. M. Li, Z. Jiang, X. Di, et al., Enantiomeric separation of six beta-adrenergic blockers on Chiralpak IB column and identification of chiral recognition mechanisms by molecular docking technique, Biomed. Chromatogr. 5 (2020), e4803.
24. L.N. Nikolai, E.L. McClure, S.L. MacLeod, et al., Stereoisomer quantification of the b-blocker drugs atenolol, metoprolol, and propranolol in wastewaters by chiral high-performance liquid chromatographyetandem mass spectrometry, J. Chromatogr. A 1131 (2006) 103e109.
25. V.K.H. Barclay, N.L. Tyrefors, I.M. Johansson, et al., Chiral analysis of meto- prolol and two of its metabolites, a-hydroxymetoprolol and deaminated metoprolol, in wastewater using liquid chromatographyetandem mass spectrometry, J. Chromatogr. A 1269 (2012) 208e217.

26. N. De J. Antunes, R.C. Cavalli, M.P. Marques, et al., Stereoselective determination of metoprolol and its metabolite a-hydroxymetoprolol in plasma by LC-MS/MS: application to pharmacokinetics during pregnancy, Chirality 25 (2013) 1e7.
27. P. Sharma, P. Contractor, S. Guttikar, et al., Development of a sensitive and rapid method for quantitation of (S)-(-)- and (R)-(+)-metoprolol in human plasma by chiral LCeESIeMS/MS, J. Pharm. Anal. 4 (2014) 63e79.
28. I. Baranowska, W. Adolf, S. Magiera, Enantioselective determination of metoprolol and its metabolites in human urine high-performance liquid chromatography with fluorescence detection (HPLCeFLD) and tandem mass spectrometry(MS/MS), J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1004 (2015) 79e84.
29. H. Elmongy, H. Ahmed, A.A. Wahbi, et al., Determination of metoprolol enantiomers in human plasma and saliva samples utilizing microextraction by packed sorbent and liquid chromatographyetandem mass spectrometry, Biomed. Chromatogr. 30 (2016) 1309e1317.
30. J. Chen, Y. Hsieh, J. Cook, et al., Supercritical fluid chromatography-tandem mass spectrometry for the enantioselective determination of propranolol and pindolol in mouse blood by serial sampling, Anal. Chem. 78 (2006) 1212e1217.
31. M.K. Parr, B. Wuest, E. Naegele, et al., SFC-MS/MS as an orthogonal technique for improved screening of polar analytes in anti-doping control, Anal. Bioanal. Chem. 408 (2016) 6789e6797.
32. A. Svan, M. Hedeland, T. Arvidsson, et al., Rapid chiral separation of atenolol, metoprolol, propranolol and the zwitterionic metoprolol acid using supercritical fluid chromatographyetandem mass spectrometryeApplication to wetland microcosms, J. Chromatogr. A 1409 (2015) 251e258.
33. K. Kalíkova´, M. Martínkova´, M.G. Schmid, et al., Cellulose tris-(3,5-dimethyl- phenylcarbamate)-based chiral stationary phase for the enantioseparation of drugs in supercritical fluid chromatography: comparison with HPLC, J. Sep. Sci. 41 (2018) 1471e1478.
34. W. Ren-Qi, O. Teng-Teng, N. Siu-Choon, et al., Recent advances in pharma- ceutical separations with supercritical fluid chromatography using chiral stationary phases, TrAC Trends Anal. Chem. 37 (2012) 83e100.
35. C. West, Recent trends in chiral supercritical fluid chromatography, TrAC Trends Anal. Chem. 120 (2019), 115648.
36. L. Nova´kov´a, M. Douˇsa, General screening and optimization strategy for fast chiral separations in modern supercritical fluid chromatography, Anal. Chim. Acta 950 (2017) 199e210.
37. M.F. Sanner, Python: a programming language for software integration and development, J. Mol. Graph. Model 17 (1999) 57e61.
38. G.M. Morris, D.S. Goodsell, R.S. Halliday, et al., Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function, J. Comput. Chem. 19 (1998) 1639-1662.
39. C. West, A. Bouet, S. Routier, et al., Effects of mobile phase composition and temperature on the supercritical fluid chromatography enantioseparation of chiral fluoro-oxoindole-type compounds with chlorinated polysaccharide stationary phases, J. Chromatogr. A 1269 (2012) 325e335.

40. L. Nov´akov´a, M. Douˇsa, J.L. Bernal, M.T. Martín, et al., Effects of organic modifier and temperature on the enantiomeric separation of several azole drugs using supercritical fluid chromatography and the Chiralpak AD column, Biomed. Chromatogr. 28 (2014) 152e158.
41. International Conference on Harmonisation, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline Q2(R1): Validation of Analytical Procedures: Text and Methodology Q2(R1), Geneva, Switzerland, 2005.