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Sensitive Detection and Enantioseparation of β-Blockers with Quinoline Based Active Chiral Reagents using HPLC: Separation Mechanism and DFT Modelling

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A sensitive and effective method for the detection and enantioseparation of β-blockers was developed. Three quinoline-based active chiral reagents were prepared followed by an amide and ester formation reaction. The synthesized active chiral reagents were characterized with spectroscopic techniques such as NMR, FT-IR, UV, HRMS and elemental analyzer. The active reagents were used in the diastereomerization of the racemic β-blockers (propranolol, metoprolol and carvedilol). Synthesized diastereomeric pairs were then subjected to RP-HPLC for chromatographic separation. The eluting phase, which consists of a buffer solution with acetonitrile, was used for analysis. The separation analysis results were optimized in the presence of variables such as eluting phase ratio, concentration and pH. Additionally, the separation process, elution order and stable conformer of diastereomers were studied by developing minimized energy structures of prepared diastereomeric pairs using DFT.

Keywords: Quinoline-8-carboxylic acid, β-Blockers, Diastereomerization, Enantioseparation, Active reagents, RP-HPLC.

INTRODUCTION

β-Blockers are the aromatic derivatives of β-amino alcohols, also known as β-agonists or amino alcohols. These are used as drugs commonly in the treatment of cardiovascular and respiratory diseases [1]. The β-blockers are commonly obtained as a racemic mixture; thus, the racemic mixture of the β-blockers is prescribed for the treatment. The studies show that the (*S*)-enantiomer of the β-blockers is responsible for all desired actions, while the (*R*)-enantiomer is considered as an impurity due to side effects or possesses less desired action. The unwanted enantiomer causes side effects on the human body, such as dizziness, paraesthesia, gastrointestinal irritation, aches in muscles and asthmatic wheezing [2,3].

Enantiomers behave differently in chiral environments, thus showing different metabolisms inside the animal body and producing different pharmaceutical and toxicological effects [3,4]. Due to this, the demand for single enantiomers as the drug has increased. Also, before permitting a racemic drug to the market, the regulatory agencies ask for complete information on racemic drugs in pharmacodynamics and pharmacokinetics [4]. Thus, separation and detection of enantiomers have gained colossal attention these days. The chromatographic separation methods are the most promising methods among other techniques due to their high sensitivity, quickness, cost-effective and ease of performance [5,6]. The separation of enantiomers *via* derivatization (indirect approach of enantioseparation) enhances separation by introducing a desired organic molecule into the enantiomer. It also enhances the UV-visible detection sensitivity with chromatographic interactions [7-9]. Due to this, an indirect method of enantioseparation of few important β-blockers is developed and for this, we synthesized three quinoline based chiral reagents.

Quinolines are naturally occurring inexpensive aromatic molecules that show outstanding UV-visible activities with high sensitivity during spectroscopic detection due to the fused

pyridine and benzene rings system [10,11]. The polar groups in the molecule make quinoline an excellent ligand to interact with other polar molecules and functional groups [12]. Due to this, quinoline derivatives have been used to develop various chemosensors for different spectroscopic methods, such as the fluorescence detection of small organic molecules, metal ions and pharmaceuticals [13-15]. Quinolones with desired functional groups are facile to easy modification in the chemical structure to a new quinoline derivative [13]. In this report, three quinoline-based activated chiral reagents were synthesized. Quinolines are achiral molecules; thus, to introduce the asymmetry into the reagents, the L-valine derivatives were used. Three L-valine derivatives (**LV1-LV3**) were synthesized by modifying the L-valine structure with the addition of the hydrophobic groups. Then, under the substitution reaction, the L-derivatives were introduced to the quinoline molecule (**QLV1-QLV3**). The synthesized molecule then converted activated chiral reagents (**QCR1-QCR3**) by inserting reactive pentafluorophenol into the carboxylic group of **QLVs**. The synthesized activated chiral reagents were then used to diastereomerization racemic β-blockers (propranolol, metoprolol and carvedilol) and a new enantioseparation method was developed.

In the current report, three quinoline-based activated chiral reagents have been synthesized and used for derivatizing β-blockers (propranolol, metoprolol and carvedilol) in a microwave reactor. The derivatization with QCRs transforms racemic β-blockers into highly sensitive organic molecules toward the PDA detector commonly used in chromatography (RP-HPLC). The synthesized pair of diastereomers of β-blockers was applied to the C_{18} column of RP-HPLC. The mobile phase consisting of triethylaminephosphate buffer and acetonitrile was used for separation. The energy-minimized structure of the prepared diastereomeric pair was developed using DFT and the elution order, conformer and 3D configuration of diastereomeric pairs were established. The accuracy, linearity and preciseness of the developed method were established and the limit of quantitation (LOQ) and limit of detection (LOD) have also been validated.

EXPERIMENTAL

The chemicals *viz*. racemic propranolol, metoprolol and carvedilol, quinoline-8-carboxylic acid (8-QC), L-valine and bromo-subsitutents were purchased from Sigma-Aldrich, USA. Analytical grade solvent and other reagents were purchased from Avra chemicals (India).

HPLC system and other instruments: HPLC system (Shimadzu) equipped with 20 µL injector (Manual), C_{18} achiral column, PDA-detector was used for analysis. The obtained results were proceed with LC solution software. Other than this, UV-visible-spectrometer, pH-meter, FT-IT instrument, 500 MHz NMR-spectrometer and elemental analyzer were used to characterize the products.

Synthesis of L-valine derivatives (LV1-LV3): A 30 mL solution of L-valine (5 mmol) was prepared in dry THF and 20 mmol K_2CO_3 was added under heating. In this solution, a solution of cyclohexylbromide (5 mmol) in 15 mL dry THF was added slowly. The reaction solution was set to stir for 20 h, under refluxing and also the reaction was continuously monitored with TLC [16]. Upon completion, the reaction solution was filtered and the solid residue was discarded. The filtrate was then concentrated and dried under reduced pressure. The column chromatography was performed to obtain final purified product (**LV1**) (**Scheme-I**) [17].

Similarly, under the substitution reaction another derivatives (**LV2** and **LV3**) of L-valine were also synthesized. The molecular structures of **LV2** and **LV3** (derivatives of L-valine) are given in Fig. 1.

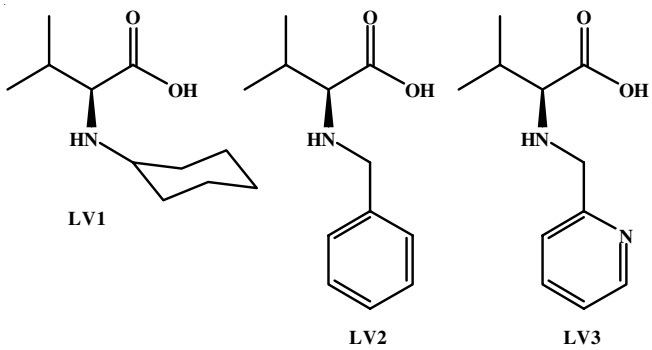


Fig. 1. Derivatives of L-valine (LV1-LV3)

LV1: Colour: Off-white solid; yield: 54%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.92-0.99 (6H, dd), 1.34-1.46 (5H, m), 1.52-1.69 (3H, m), 1.74-1.82 (2H, m), 2.06-2.11 (1H, m), 2.68-2.77 (1H, m), 3.06-3.11 (1H, m) and 4.08-4.14 (1H, m).

LV2: Colour: Off-yellow solid; yield: 57%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.91-0.97 (6H, dd), 2.08-2.16 (1H, m), 3.79-3.84 (1H, dd), 3.79-3.84 (1H, dd), 4.30-4.35 (1H, m) and 7.20-7.34 (5H, m, Ar).

LV3: Colour: pale-yellow solid; yield: 65%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.92-0.98 (6H, dd), 2.07-2.14 (1H, m), 3.38-3.42 (1H, m), 4.02-4.06 (1H, dd), 4.13-4.18 (1H, dd), 4.72-4.76 (1H, m), 7.26-7.28 (1H, d), 7.46-7.49 (1H, m), 7.61-7.64 (1H, t) and 8.51-8.52 (1H, dd).

Synthesis of quinoline-8-carboxylic acid based chiral derivatives (QLV1-QLV3): The oxalyl chloride was used to activate the quinoline-8-carboxylic acid's (8-QC's) carboxylic group in the presence of pyridine catalyst (base) [16,18-20]. The acid chloride group than converted to amide, in a substitution reaction, in presence of suitable nucleophile (amino group of L-valine) and results the chiral derivatives of 8-QCs (**QLV1** and **QLV2**) were formed [21] (**Scheme-I**). Similarly, the other derivatives were also synthesized and the molecular structures of **QLV1**, **QLV2** and **QLV3** are given in Fig. 2.

QLV-1: Colour: pale-yellow soild; yield: 98%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.92-0.99 (6H, dd), 1.34-1.46 (5H, m), 1.52-1.69 (3H, m), 1.74-1.82 (2H, m), 2.06-2.11 (1H, m), 2.68-2.77 (1H, m), 3.06-3.11 (1H, m), 4.08-4.14 (1H, m), 7.46-7.49 (1H, t), 7.57-7.59 (1H, m), 8.08-8.11 (1H, m), 8.25-8.27 (1H, dt) and 8.86-8.88 (1H, dd).

QLV-2: Colour: yellow solid; yield:97%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.92-0.97 (6H, dd), 2.09-2.17 (1H, m), 3.78-3.85 (1H, dd), 3.91-3.94 (1H, dd), 4.29-4.33 (1H, m), 7.21-7.34 (5H, m,Ar), 7.44-7.48 (1H, t), 7.56-7.59 (1H,

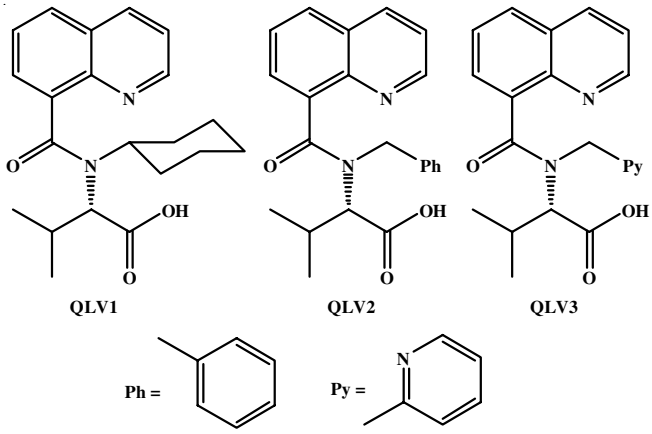


Fig. 2. Structure of the prepared QLVs

m), 8.10-8.11 (1H, dt), 8.24-8.26 (1H, dt) and 8.86-8.87 (1H, dd).

QLV-3: Colour: brownish-yellow solid; yield: 98%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 0.92-0.98 (6H, dd), 2.07-2.14 (1H, m), 3.38-3.41 (1H, m), 4.02-4.07 (1H, dd), 4.12-4.17 (1H, dd), 4.73-4.78 (1H, m), 7.25-7.27 (1H, d), 7.47-7.49 (1H, m), 7.61-7.65 (1H, t), 8.51-8.52 (1H, dd), 7.46-7.48 (1H, t), 7.57-7.60 (1H, m), 8.07-8.09 (1H, m), 8.24-8.27 (1H, dt), 8.87-8.89 (1H, dd).

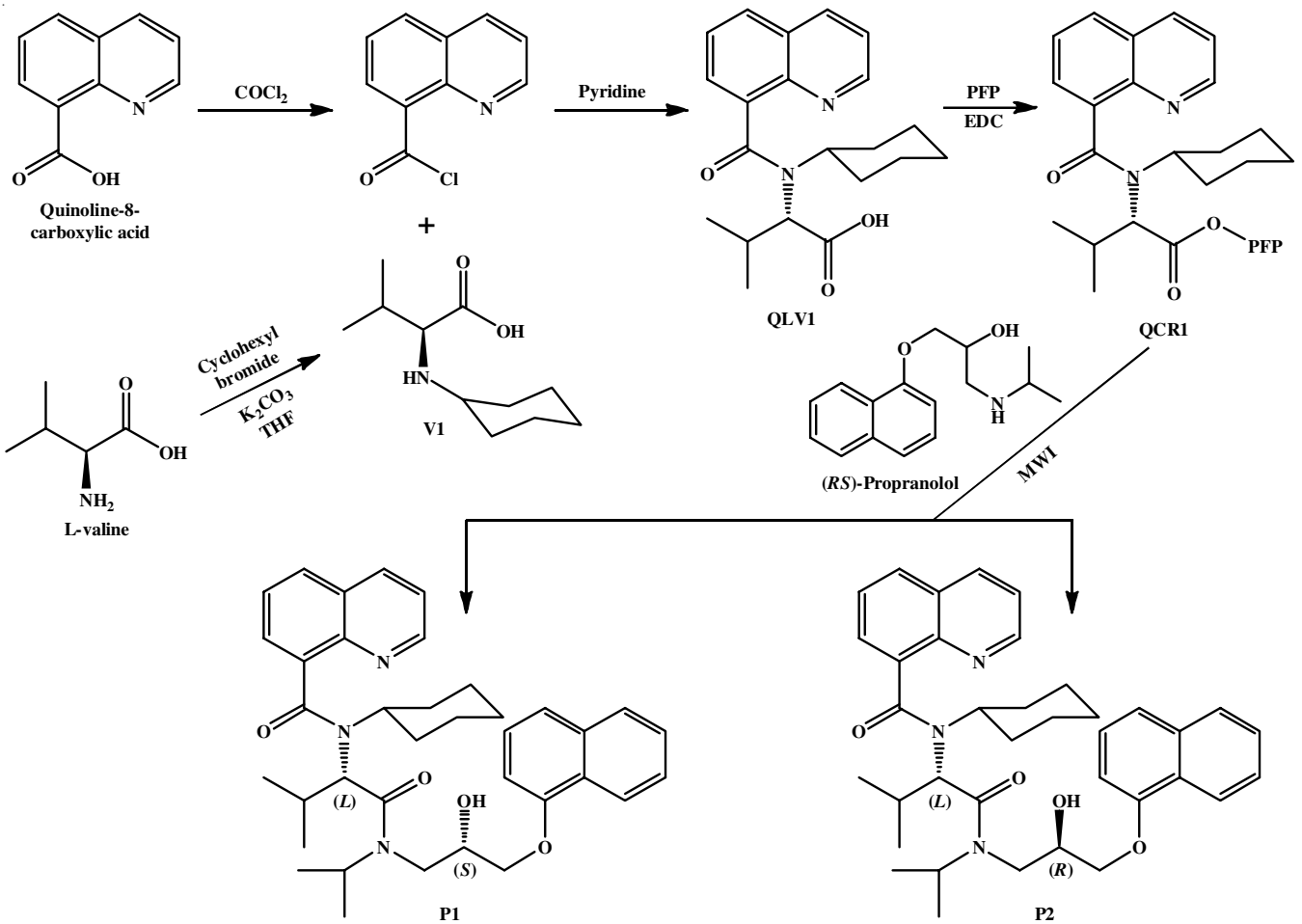
Synthesis of quinoline-8-carboxylic acid based active chiral reagents (QCR1-QCR3): Pentafluorophenol (434 mg, 2 mmol) was mixed with obtained product (**QLV1**; 267 mg, 2 mmol) and dissolved in 25 mL dry THF under dry N_2 condition (**Scheme-I**). The solution of EDC (420 mg, 2.7 mmol) and 4-DMAP (245 mg, 2 mmol) was added dropwise in this solution [22,23] and left to stir for 150 min at room temperature. After this, 1 N HCl solution was poured into the reaction and extracted three-times with ethyl acetate and the molecular structures of **QCR2** and **QCR3** are given in Fig. 3.

The spectrum obtained from ^{1}H NMR for QCRs (**1-3**) and QLVs (**1-3**) are same due to pentafluorobenzene (in the absence of the proton) not appearing in proton NMR. Thus, the numbers of the protons that appear in the spectrum of the QCR and QLVs are the same. The synthesis and purity of the QCRs were confirmed with HRMS and TLC.

QCR-1: Colour: Orange-yellow solid, yield: 92%; Anal. calcd. (found) % for $C_{21}H_{25}N_2O_3P_2F$: C, 57.68 (58.07); H, 5.78 (5.80); N, 6.51 (6.45). HRMS: 435.12 ($M+H^+$).

QCR-2: Colour: Yellow-brown solid, yield: 96%; Anal. (found) calcd. % for $C_{22}H_{21}N_2O_3P_2F$: C, (59.73) 59.62; H, (4.79) 4.73; N, (6.33) 6.57. HRMS: 443.11 ($M+H^+$).

QCR-3: Colour: Brownish-yellow solid, yield: 93%; Anal. calcd. (found) % for $C_{21}H_{20}N_3O_3P_2F$: C, 56.78 (56.89); H, 4.61 (4.55); N, 9.26 (9.48). HRMS: 444.12 ($M+H^+$).



Scheme-I: Synthesis of the V1, QLV1, QCR1 and diastereomers of the propranolol (P1 and P2) as representative

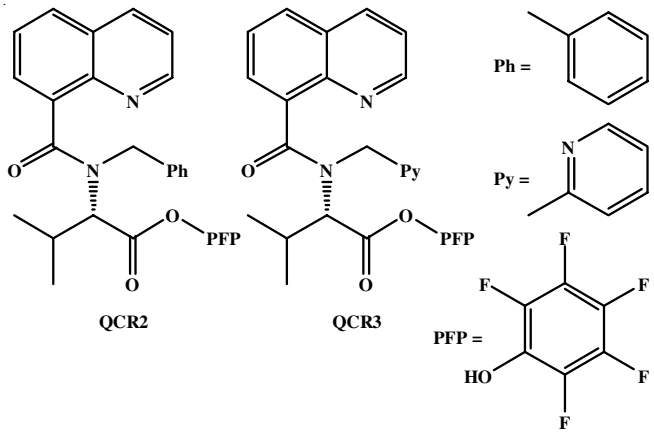


Fig. 3. Structures of remaining activated chiral reagents (QCR2 and QCR3)

Diastereomerization of β-blockers with QCRs: Diastereomerization of β-blockers was done under microwave heating conditions [15,24]. The dilute solution of (*RS*)-propranolol (45 µL, 60 nmol) and **QCR1** (50 µL, 65 nmol) were mixed in a microwave vial and 5 µL triethylamine was added to make the solution alkaline. The resulting solution was then microwave heated for 1 min to prepare diastereomers. The propranolol and QCR were used for the reaction in 1:1.25 molar ratio. A 10 µL aliquot was taken from the reaction solution and diluted 20 times with acetonitrile. The filtered dilute solution of diastereomers was then conducted HPLC analysis. Similarly, other diastereomers of β-blockers were also prepared with **QCRs** (**2** and **3**).

The conditions for synthesis were optimized in the presence of variables such as reaction time, excess reagent, pH and microwave-heating timings.

HPLC and conditions: The HPLC analysis of prepared diastereomers of β-blockers was performed in gradient mode; in this, triethylaminephosphate buffer and acetonitrile were used as an eluting phase in ratios of 45-55%, 40-60%, 30-70% and 20-80% (in a linear-gradient). The solvents and eluting phase were pretreated (filtered and degassed on a sonicator) before applying to the HPLC system. The detection of the diastereomers was set on 294 nm wavelength and the eluting phase's flow rate was set at 1 mL/min.

Method's validation: The validation was performed as per ICH guidelines, as a representative, on diastereomers of propranolol prepared with **QCR3**. The validation parameters (graph, correlation coefficient and slope calibration) were calculated using the least square method on MS Excel software. The concentration range used for the validation was 60-6000 ng/mL.

RESULTS AND DISCUSSION

Synthesis of derivatives of L-valine, QLVs, QCR and diastereomers: The L-valine was converted to its substituted derivatives under the substitution reaction, where the bromo-group (leaving group) of the cyclohexyl bromide or bromomethyl benzene or bromomethyl pyridine was substituted with an amino group of L-valine [17] and yields around 70-75% yield. The carboxylic group of the 8-QC, in the presence of chlorinating reagent (oxalyl chloride and pyridine), was converted to acyl chloride under S_N^2 substitution. The chlorinating reagent, oxalyl chloride and catalyst (pyridine), is considered an excellent reagent for the acylation of carboxylic groups [17,18,23] and yields nearly 100% of the desired product. Acylchloride plays an important part towards the nucleophile attack and helps in the easy conversion of an ester of amide bond in the presence of suitable nucleophiles under the substitution reaction and yields a remarkable amount of products [25-27]. Following this, acyl chloride of 8-QC and the amino group of L-valines (**LV1-LV3**) were allowed to react in the presence of the catalytic amount of pyridine. The reactants were quickly reacted and yielded nearly 100% of the desired molecule (**QLV 1-3**). All the synthesized products were then characterized by the spectroscopic methods.

Synthesis of QCRs: Active chiral reagents (**QCRs**) were prepared by converting the carboxylic group of the **QLVs** to activated ester of pentaflourophenol (PFP). The PFP in esters is considered an excellent leaving group; thus, these esters react quickly with suitable nucleophiles (amino group of β-blockers) and produce more stable desired amides or esters [21]. The carboxylic group of the **QLVs** were converted to PFP esters in the presence of coupling reagent EDC and base DMAP. Thus, it yielded the desired PFP ester (activated chiral reagent) with an excellent yield (nearly 98-99% yield) [15,28]. The synthesis of **QCRs** was quick and mild and the substitution reaction didn't take place on asymmetric carbon; thus, no racemization was observed during synthesis. The purity of **QCRs** was further confirmed by chiral HPLC analysis.

Diastereomerization*:* PFP esters are very unstable in the presence of nucleophiles; thus, QCRs quickly reacted with an amino group of the β-blockers (nucleophile) and yielded desired diastereomeric pairs of β-blockers with high yield (**Scheme-I**) [27,29,30]. The reaction was performed in a microwave reactor for fast synthesis [15,21]. Also, no racemization was observed because the reaction was performed on a non-chiral carbon centre. The further purity of the prepared diastereomeric pair was confirmed by HPLC separation analysis (Fig. 4); the two equal intensity peaks in the chromatogram give indirect evidence that racemization or kinetic resolution didn't occur during the microwave synthesis. The diastereomers of β-blockers prepared with QCRs were abbreviated as described in Table-1.

RP-HPLC analysis: The obtained diastereomerization products were analyzed on the RP-HPLC for the separation. The chromatogram of diastereomeric pairs (P5 and P6) of propranolol prepared with **QCR3** is shown as a representative in Fig. 4. The clean separation of all diastereomeric pairs was achieved on the C_{18} column (achiral and non-polar). The separation result is used to calculate the chromatographic data values and is provided in Table-2. The separation results show that in most of the cases, the L-(*S*)-diastereomers elute first compared to L-(*R*)-diastereomers (elution order of diastereomers is given in Table-2).

The eluting phase consisted of triethylaminephosphate buffer (20%) and acetonitrile (80%) in a linear gradient mode found sufficient to separate all the prepared diastereomeric pairs. The pH 3.5 of the solution was maintained acidic for

TABLE-1
DIASTEREOMERIC PAIRS OF β-BLOCKERS PREPARED WITH QCRs AND ELUTION ORDER

β-Blocker	QCR1		QCR2		QCR3	
	L-(*S*)-diastereomer	L-(*R*)-diastereomer	L-(*S*)-diastereomer	L-(*R*)-diastereomer	L-(*S*)-diastereomer	L-(*R*)-diastereomer
Propranolol	P1	P2	P3	P4	P5	P6
Metoprolol	M1	M2	M3	M4	M5	M6
Carvedilol	C1	C2	C3	C4	C5	C6

TABLE-2
CHROMATOGRAPHIC SEPARATION DATA OF DIASTEREOMERS OF RACEMIC β-BLOCKER PREPARED WITH QCRs

Chiral reagent	First peak time (min)	Second peak time (min)	First eluted diastereomer	k_1	k_2	α	*Rs*
	Chromatographic separation data of diastereomers of propranolol			Calculated separation data for diastereomers of propranolol			
QCR1	5.78	7.19	P1	3.12	4.35	1.39	4.70
QCR2	5.41	7.08	P3	2.86	4.05	1.41	5.66
QCR3	5.27	6.98	P5	2.76	3.98	1.44	5.71
	Chromatographic separation data of diastereomers of metoprolol			Calculated separation data for diastereomers of metoprolol			
QCR1	5.52	6.88	M1	2.94	3.91	1.32	5.44
QCR2	5.18	6.74	M4	2.70	3.81	1.41	6.24
QCR3	5.11	6.71	M6	2.65	3.79	1.43	6.42
	Chromatographic separation data of diastereomers of carvedilol			Calculated separation data for diastereomers of carvedilol			
QCR1	6.38	7.81	C1	3.55	4.57	1.28	5.72
QCR2	5.81	7.42	C3	3.15	4.30	1.36	6.44
QCR3	6.28	7.51	C5	3.48	4.36	1.25	4.92

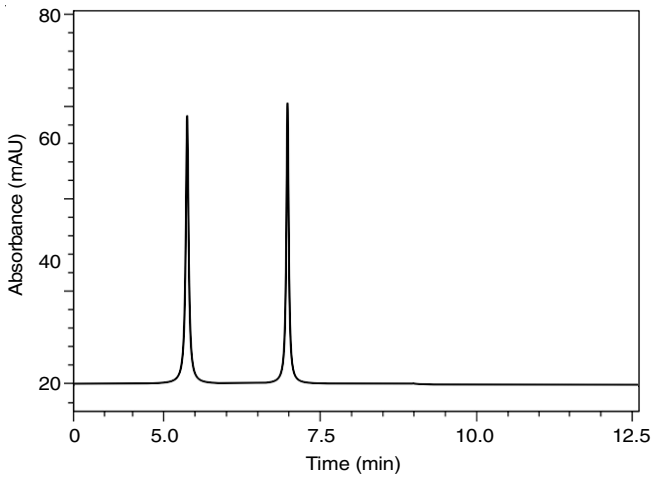


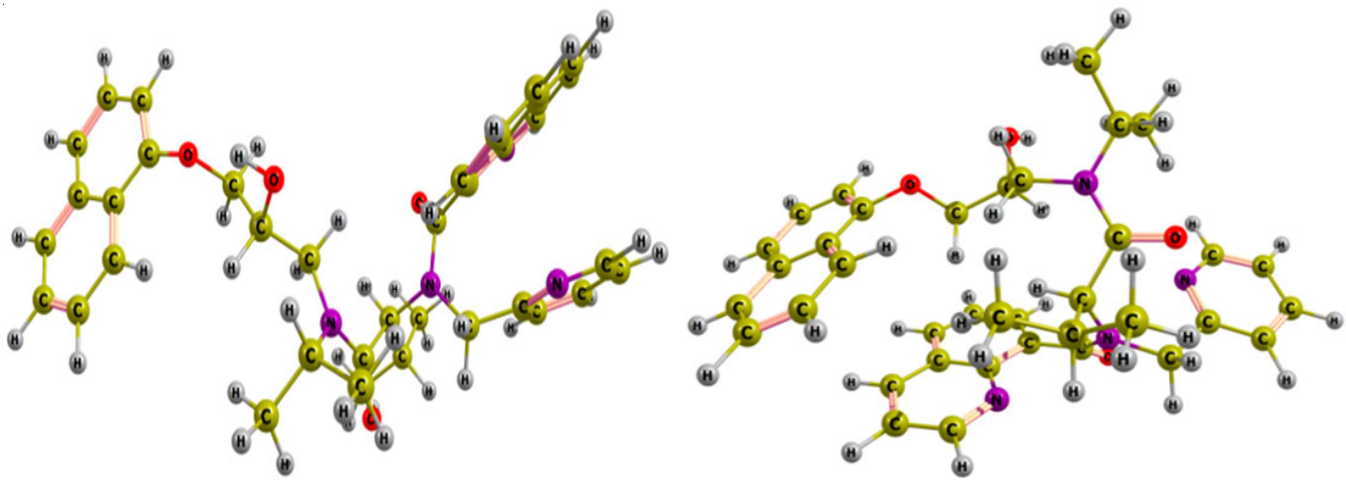
Fig. 4. RP-HPLC chromatogram of enantioseparation of diastereomers of (*RS*)-propranolol prepared with QCR3

good separation. The different eluting phase variables, such as organic modifier concentration, pH of eluting phase, flow rate and concentration of buffer, were changed to optimize the separation of efficiency of the developed method. Methanol as an alternative organic modifier was also tested for separation. Acetonitrile was a better organic modifier for the eluting phase due to higher polarity, low density and low UV cut-off. Also, in the presence of acetonitrile, the peaks appear in chromatograms were sharper [26,31]. The eluting phase at 3.5 pH gives better separation rather than the lower or higher acidic eluting phase. A 1mL/min flow rate of eluting phase for HPLC was found suitable for separation. The lower flow rate causes low resolution and the higher flow rate causes high system pressure; thus, the eluting phase was run on the HPLC system with a 1 mL/min flow rate.

Elution order and DFT optimized 3D structures: The DFT calculations using Gaussian Software were performed to confirm the elution order and separation mechanism results. The 3D structures of the diastereomeric β-blocker derivatives were developed and used to study molecular geometry and chromatographic interaction with a non-polar C_{18} column.

The 3D optimized structure of diastereomeric pair P5 and P6 are shown in Fig. 5 as representative. The P5 molecule's spatial structure is arranged so that it has a bigger structure than the molecule P6. Thus, molecule P5 has more surface to expose to the eluting phase and shows more hydrophilic properties. Due to higher polar-polar interaction with the eluting phase, the P5 diastereomer elutes first. The P6 diastereomer has less surface to expose to the eluting phase and shows more hydrophobic properties; due to this, P5 interacts more with non-polar column material and elutes in the last [15,23,32]. Apart from this, the substituent groups (cyclohexane, methylbenzene and methylpyridine) attached to the L-valine molecule showed an important role in elution time. A polar group containing aromatic rings (methylpyridine substituent) increases the polar interaction between the diastereomer and eluting phase. Thus, methylpyridine substituent in diastereomeric pairs decreases the elution time. While hydrophobic aliphatic substituent in diastereomer increases the hydrophobic interactions with non-polar column material. Thus, aliphatic substituent containing diastereomeric pairs elute in the end.

A similar hypothesis was applied to each prepared diastereomeric derivative and the elution order of diastereomeric pairs was established. In each case, the bigger diastereomer in the diastereomer shows more polarity and interacts more with the eluting solvents and elutes first compared to the smaller diastereomer in a diastereomeric pair. The elution order of prepared diastereomeric derivatives is given in Table-2.



P5 (L,*S*-diastereomer) **P6** (L,*R*-diastereomer)

Fig. 5. Optimized 3D structures of diastereomers of (*RS*)-propranolol (P5 and P6) prepared by QCR3

Validation: The current method was validated for accuracy, robustness, linearity and precision as described in ICH regulations [35]. The diastereomeric pair (P5 and P6) of propranolol prepared with QCR3 was used as a representative to validate the developed method as described in the literature [15,21,34,35]. The concentration of the samples was used in the range of 60-6000 ng/mL and the detection limit (LOD), quantification limit (LOQ), linearity, accuracy, precision and relative standard deviation (RSD) were calculated. The recoveries and stabilities of the method were examined and quantified by peak region obtained in the RP-HPLC system. The calculated recovery of the diastereomeric pair was more than 98% for inter and intra-day assay (98.56 and 98.92% for inter-day assay and 98.21 and 99.08% for intraday assay). The developed method showed a remarkable sensitivity for the detection of diastereomeric pairs due to lower LOD and LOQ values (0.178 ng/mL and 0.534 ng/mL, respectively).

The results [separation factor (1.25-1.44), resolution (4.70-6.44), elution time (5.11-7.81 min) and retention factor (2.65-4.57)] obtained by the developed method for separation of diastereomers of β-blockers were found better compared to the other reported work on enantioseparation. Also, the results obtained in the current report are better when compared with another report on direct chiral separation of β-blockers.

Conclusion

The developed method describes an efficient synthesis of quinoline-8-carboxylic acid based chiral reagents and their use in the diastereomerization of β-blockers. The synthesized chiral reagents (QCRs) react quickly with amino groups of β-blockers and play an important role in the separation of synthesized diastereomeric pairs. The diastereomers were prepared quickly and efficiently under microwave conditions. A clean separation of the prepared diastereomeric pairs was achieved on RP-HPLC with low elution time in an efficient polar mobile phase consisting of acetonitrile and triethylaminephosphate buffer. The detection sensitivity of the developed method was found to be very low (LOD = 0.178 ng/mL and LOQ = 0.534 ng/mL) in the presence of quinoline fluorophore in diastereomers. Elution time, separation and molecular interaction were explained with energy-minimized 3D structures. This method is excellent for the enantioseparation of the β-blockers due to high sensitivity, low elution time, high resolution and accessible synthesis of diastereomeric derivatives. This method can be used to identify the enantiomeric impurities or in trace analysis of racemic compounds that contain active nucleophiles in the structure.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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