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Synthesis of a Series of Quinoline-Based New Chiral Reagent and its Application in Separation of Racemic Mexiletine followed by Liquid Chromatography and Confirmation of Results Using Molecular Modelling; A Complete Study

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In present work, *L*-valine-based new chiral derivatives were prepared by introducing hydrophobic groups. The prepared derivatives were then used to prepare quinoline-based chiral reagents under mild amidation reaction and esterification reaction. Spectroscopic techniques, such as HRMS, FT-IR, ^{1}H NMR and CHNS analysis, were used to characterize the synthesized chiral reagents. The synthesized series of reagents was then used to derivatize diastereomers of racemic mexiletine and these diastereomers were separated using the RP-HPLC (a derivatization approach of enantioseparation). The mobile phase for the analysis consisted of acetonitrile and buffer solution. The impact of modifying mobile phase pH and concentrations was optimized to separate diastereomers. The lowest energy-minimize optimized diastereomer structures, as well as the design of separation processes and elution orders, were also developed through the use of density functional theory (DFT) calculations. Following ICH guidelines, the limits of detection (0.161 ng/mL) and quantification (0.483 ng/mL) were determined, together with the retention-factor (k), selectivity-factor (α), resolution-factor (*RS*) and technique validation.

Keywords: 8-Quinolinecarboxylic acid, (*RS*)-Mexiletine, Covelant derivatization, Enantioseparation, Chiral reagents, RP-HPLC.

INTRODUCTION

Quinoline is a fused ring (benzene and pyridine) aromatic molecule [1-3]. Thus, it shows excellent UV-visible and fluorophoric activities and offers remarkable detection sensitivity in different spectroscopic methods [4-9]. In the presence of a polar group (nitrogen atom) in the molecular structure, quinoline acts as a suitable ligand with various polar analytes. Due to this, quinoline and its derivatives have been used to prepare chemosensors and such chemosensors have been used to detect metal ions, organic small molecules, pharmaceuticals and chromatographic applications [6-9]. Quinolines are naturally occurring aromatic molecules available at low cost and facile, easy modification in their chemical structure; here in this report, we prepared a series of new chiral reagents. In order to introduce chirality in the molecule, *L*-valine and its chiral derivatives are used. The derivatives of *L*-valine were synthesized by adding hydrophobic molecules such as methyl, ethyl, cyclohexyl and benzyl groups. These molecules were then converted into the activated chiral reagents by modifying its carboxylic group into reactive pentafluorophenol ester [9]. These chiral reagents were then used to derivatize racemic mexiletine (target analyte) and an enantioseparation method was developed.

(*RS*)-Mexiletine [(*RS*)-Max; l-(2,6-dimethylphenoxy)-2-aminopropane)] is a racemic drug which is sold and administered as a racemate. It is a non-selective voltage-gated sodium channel blocker belonging to the IB-antiarrhythmic group.

Compared to the (*S*)-enantiomer, the (*R*)-enantiomer of Mex exhibits greater affinity with human serum proteins. Also, both enantiomers of Mex have different therapeutic activities [8]. Various methods for enantioseparation have been developed for racemic mexiletine, including direct separation and covalent derivatization methods. The application of covalent derivatization in establishing chiral purity has the advantage over other reported methods [8]. The covalent derivatization methods for enantioseparation show high sensitivity towards chromatographic UV-visible detection and provide fast elution with high resolution compared to other reported methods [10]. Thus, these methods are considered important for trace analysis [7-11]. The literature indicates that the numerous chiral derivatizing reagents have been produced for covalent derivatization, *e.g.*, chiral reagents based on Marfey's reagent, (*S*,*S*)-O, O2-di-*p*-toluoyl tartaric acid anhydride, cyanuric chloride, chloroformates, Sanger reagent, (*S*)-(–)-(N)-trifluoroacetyl-prolyl chloride, (1*S*)-(–)-camphanic chloride, divinyl dicarboxylates, levofloxacin esters, 2-anthroyl chloride, (*S*)-naproxen ester, *etc*. [7-13] provided easy derivatization and sensitive detection (LOD and LOQ).

In this study, novel chiral reagents based on quinoline were synthesized and employed to derivatize (*RS*)-Mex into diastereomers under microwave irradiation (MWI) heating conditions. Racemic mexiletine was transformed into a highly sensitive molecule for UV-visible detection by introducing the newly prepared quinoline-based chiral reagents moiety and the outcome is a susceptible detection using an HPLC PDA-detector. The synthesized diastereomeric pair was introduced into the HPLC's C_{18} column and the eluting phase consisted of acetonitrile (ACN) and triethylamine phosphate (TEAP) buffer. In addition, the elution order and molecular configuration were also established using the energy-minimized developed structures of all the synthesized diastereomers. The current method's preciseness, linearity, limit of quantitation (LOQ) and limit of detection (LOD) have all been verified.

EXPERIMENTAL

Racemic mexiletine and 8-quinolinecarboxylic acid (8-QC), *L*-valine, 3-{[(ethylimino)methylidene]amino}-*N,N*-dimethylpropan-1-amine (EDC), pentafluorophenol (PFP), oxalyl chloride, methyl bromide, ethyl bromide, bromocyclohexane, bromomethyl benzene and bromomethyl pyridine were purchased from Sigma-Aldrich, USA. The solvent and other reagents used in current study were purchased from Avra Chemicals (India).

Chromatographic system and equipments: Shimadzu HPLC with manual injector (20 µL), non-polar column (C_{18}), PDA detector and LC solution software for analysis was used for the present studies. Apart this, microwave reactor, FT-IR instrument, pH meter, NMR spectrometer (500 MHz), UV-visible spectrometer and elemental analyzer were also used to characterize the synthesis products.

Stock solutions: The stock solutions of QCRs (1 mmol/L) were prepared in acetonitrile and the stock solution of (*RS*)-Mex (1 mmol/L) was prepared in 1 M $NaHCO_3$. TEAP-buffer (triethylammonium phosphate buffer; 3.5 pH; 10 mM) was prepared as reported in the literature [8,9].

Synthesis of derivatives of *L*-valine (V1-V6): *L*-Valine (5 mmol) and K_2CO_3 (20 mmol) was dissolved in 30 mL dry THF and then under heating condition, a dropwise solution of methyl bromide (5 mmol) in 20 mL dry THF was added into the above solution. The reaction was set to stir overnight under refluxing conditions. The reaction progress was tracked with thin-layer chromatography [14]. After the reaction, the solid residue was removed by passing the solution from the filter paper. The filtrate was then dried at reduced pressure and the final single substituted purified product (**V2**) was obtained by column chromatography [15].

Similarly, the other derivatives (**V3-V6**) of *L*-valine were synthesized under the substitution reaction. The structures of the *L-valine* (**V1**) and prepared derivatives of L-valines (**V2-V6**) are given in Fig. 1.

V2: Colour: off-white solid; yield: 61%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 5-91-6.02 (m, 1H), 2.99-3.03 (m, 1H), 2.46-2.48 (dd, 3H), 2.21-2.29 (m, 1H) and 0.92-0.98 (dd, 6H).

V3: Colour: off-white solid; yield: 72%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 4.58-4.79 (m, 1H), 3.40-3.44 (m, 1H), 2.68-2.85 (dm, 2H), 2.18-2.26 (m, 1H), 1.18-1.21 (t, 3H) and 0.92-0.97 (dd, 6H).

V4: Colour: off-white solid; yield: 52%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 4.08-4.14 (m, 1H), 3.06-3.11 (m, 1H), 2.68-2.77 (m, 1H), 2.06-2.11 (m, 1H), 1.74-1.82 (m, 2H), 1.52-1.69 (m, 3H), 1.34-1.46 (m, 5H) and 0.92-0.99 (dd, 6H).

V5: Colour: off-yellow solid; yield: 56%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 7.20-7.34 (Ar, m, 5H), 4.30-4.35 (m, 1H), 3.92-3.96 (dd, 1H), 3.79-3.84 (dd, 1H), 2.08-2.16 (m, 1H) and 0.91-0.97 (dd, 6H).

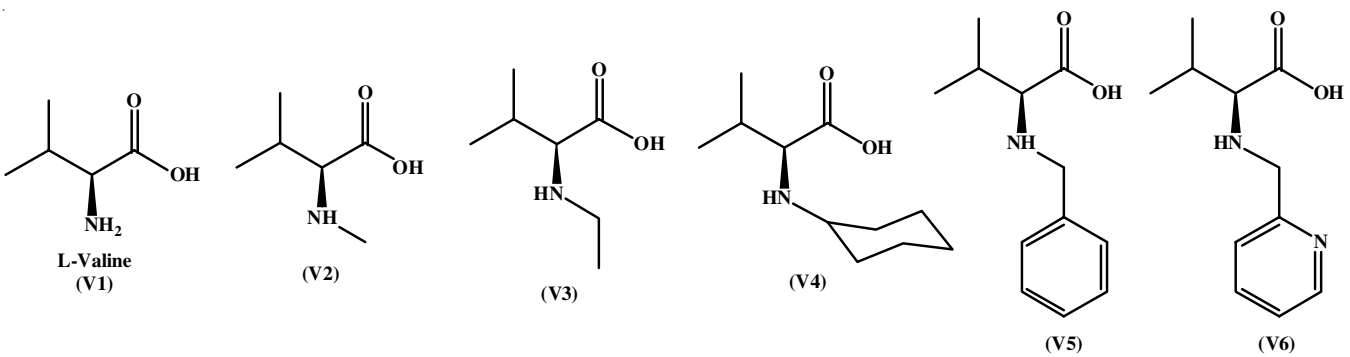

Fig. 1. Structures of derivatives of *L*-valine and its prepared derivatives

V6: Colour: pale-yellow solid; yield: 64%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.51-8.52 (dd, 1H), 7.61-7.64 (t, 1H), 7.46-7.49 (m, 1H), 7.26-7.28 (d, 1H), 4.72-4.76 (m, 1H), 4.13-4.18 (dd, 1H), 4.02-4.06 (dd, 1H), 3.38-3.42 (m, 1H), 2.07-2.14 (m, 1H) and 0.92-0.98 (dd, 6H).

Synthesis of chiral derivatives of 8-quinolinecarboxylic acid (QV1-QV6): The carboxylic group of 8-quinolinecarboxylic acid (8-QC) was activated by oxalyl chloride and pyridine [9,14-19]. The acid carbonyl group was then converted to amide of prepared derivatives of L-valine (**V1-V6**), under substitution reaction as shown in Fig. 2 (Synthesis of **QV-4**) [9]. The characterization data of all the synthesized derivatives (**QV1-QV6**) are given below.

QV-1: Colour: yellowish-white solid; yield: 99%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.86-8.88 (dd, 1H), 8.25-8.28 (dt, 1H), 8.08-8.10 (dt, 1H), 7.57-7.59 (m, 1H), 7.47-7.49 (t, 1H), 6.02-6.31 (br, 2H), 3.51-3.55 (m, 1H), 2.04-2.11 (m, 1H) and 0.98-1.04 (dd, 6H).

QV-2: Colour: yellowish-white solid; yield: 98%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.87-8.88 (dd, 1H), 8.24-8.27 (dt, 1H), 8.08-8.10 (dt, 1H), 7.58-7.60 (m, 1H), 7.46-7.49 (t, 1H), 5-92-6.04 (m, 1H), 3.01-3.05 (m, 1H), 2.46-2.47 (dd, 3H), 2.20-2.28 (m, 1H) and 0.92-0.99 (dd, 6H).

QV-3: Colour: pale yellow solid; yield: 99%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.85-8.87 (dd, 1H), 8.22-8.25 (dt, 1H), 8.07-8.10 (dt, 1H), 7.56-7.59 (m, 1H), 7.47-7.50 (t, 1H), 4.57-4.78 (m, 1H), 3.38-3.41 (m, 1H), 2.67-2.83 (dm, 2H), 2.16-2.25 (m, 1H), 1.19-1.22 (t, 3H) and 0.92-0.97 (dd, 6H).

QV-4: Colour: pale yellow soild; yield: 97%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.86-8.88 (dd, 1H), 8.25-8.27 (dt, 1H), 8.08-8.11 (dt, 1H), 7.57-7.59 (m, 1H), 7.46-7.49 (t, 1H), 4.08-4.14 (m, 1H), 3.06-3.11 (m, 1H), 2.68-2.77 (m, 1H), 2.06-2.11 (m, 1H), 1.74-1.82 (m, 2H), 1.52-1.69 (m, 3H), 1.34-1.46 (m, 5H) and 0.92-0.99 (dd, 6H).

QV-5: Colour: yellow solid; yield: 98%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.86-8.87 (dd, 1H), 8.24-8.26 (dt, 1H), 8.10-8.11 (dt, 1H), 7.56-7.59 (m, 1H), 7.44-7.48 (t, 1H), 7.21-7.34 (Ar, m, 5H), 4.29-4.33 (m, 1H), 3.91-3.94 (dd, 1H), 3.78-3.85 (dd, 1H), 2.09-2.17 (m, 1H) and 0.92-0.97 (dd, 6H).

QV-6: Colour: brownish-yellow solid; yield: 96%; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ ppm: 8.87-8.89 (dd, 1H), 8.24-8.27 (dt, 1H), 8.07-8.09 (dt, 1H), 7.57-7.60 (m, 1H), 7.46-7.48 (t, 1H), 8.51-8.52 (dd, 1H), 7.61-7.65 (t, 1H), 7.47-7.49 (m, 1H), 7.25-7.27 (d, 1H), 4.73-4.78 (m, 1H), 4.12-4.17 (dd, 1H), 4.02-4.07 (dd, 1H), 3.38-3.41 (m, 1H), 2.07-2.14 (m, 1H) and 0.92-0.98 (dd, 6H).

Synthesis of quinoline based chiral reagents (QCR1-QCR6): Under the dry conditions, a solution of 561 mg (3 mmol) pentafluorophenol and 400 mg obtained purified compounds **V2** (3 mmol) was prepared in 35 mL dry THF. Under the stirring condition, a dropwise addition of 630 mg (4 mmol) EDC and 365 mg (3 mmol) 4-DMAP in 30 mL dry THF was prepared in this solution [20,21]. The final solution was left to stir at room temperature for 2.5 h. The reaction mixture was treated with 1N HCl solution and extracted with ethyl acetate. The synthesized compound was then characterized. Similarly, other QCRs (**QCR-1, 2, 3, 5** and **6**) were also synthesized. The structures of QCRs are shown in Fig. 3.

The ^{1}H NMR spectrum of all the synthesized **QCRs (1-6)** and **QVs (1-6)** are the same because pentafluorobenezene doesn't have any H-proton, so the number of protons remains the same. Thus, the synthesis of QCRs were confirmed by HRMS and the purity was confirmed by TLC and HPLC.

QCR-1: Colour: yellowish-white solid, yield: 96%; HRMS ($C_{15}H_{15}FN_2O_3P_2$): 353.06 ($M+H^+$); Anal. calcd. (found) % for $C_{15}H_{15}FN_2O_3P_2$: C, 51.02 (51.15); H, 4.32 (4.29); N, 7.91 (7.95).

QCR-2: Colour: pale yellow solid, yield: 98%; HRMS ($C_{16}H_{17}FN_2O_3P_2$): 367.06 ($M+H^+$); Anal. calcd. (found) % for $C_{16}H_{17}FN_2O_3P_2$: C, 52.24 (52.47); H, 4.53 (4.68); N, 7.81 (7.65).

QCR-3: Colour: yellow solid, yield: 95%; HRMS ($C_{17}H_{19}FN_2O_3P_2$): 381.08 ($M+H^+$); Anal. calcd. (found) % for $C_{17}H_{19}FN_2O_3P_2$: C, 53.45 (53.96); H, 4.98 (5.04); N, 7.46 (7.37).

QCR-4: Colour: orange yellow, yield: 94%; HRMS ($C_{21}H_{25}FN_2O_3P_2$): 435.13 ($M+H^+$); Anal. calcd. (found) % for $C_{21}H_{25}FN_2O_3P_2$: C, 57.68 (58.07); H, 5.78 (5.80); N, 6.51 (6.45).

QCR-5: Colour: yellow-brown solid, yield: 97%; HRMS ($C_{22}H_{21}FN_2O_3P_2$): 443.10 ($M+H^+$); Anal. calcd. (found) % for $C_{22}H_{21}FN_2O_3P_2$: C, 59.62 (59.73); H, 4.73 (4.79); N, 6.57 (6.33).

QCR-6: Colour: brownish yellow solid, yield: 92%; HRMS ($C_{21}H_{20}FN_3O_3P_2$): 444.10 ($M+H^+$); Anal. calcd. (found) % for $C_{21}H_{20}FN_3O_3P_2$: C, 56.78 (56.89); H, 4.61 (4.55); N, 9.26 (9.48).

Synthesis of diasteromers of (*RS*)-Mex with QCRs: Synthesis of diastereomers was performed under a microwave reactor. In a vial, 40 µL (50 nmol) solution of (*RS*)-Mex, 45 µL (45 nmol) solution of **QCR-6** and 4 µL of triethylamine (base) was transferred and heated for 60 s in a microwave reactor at 70% (700 W) [8,22]. The molar ratio used for Mex and QCR was 1:1.25. After the reaction, aliquots (15 µL) were

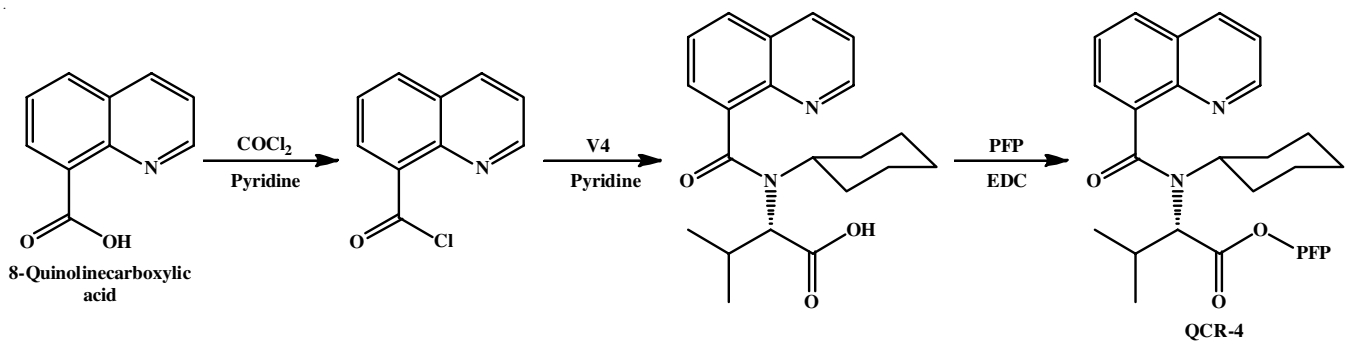

Fig. 2. Synthesis of chiral reagent (**QCR-4**)

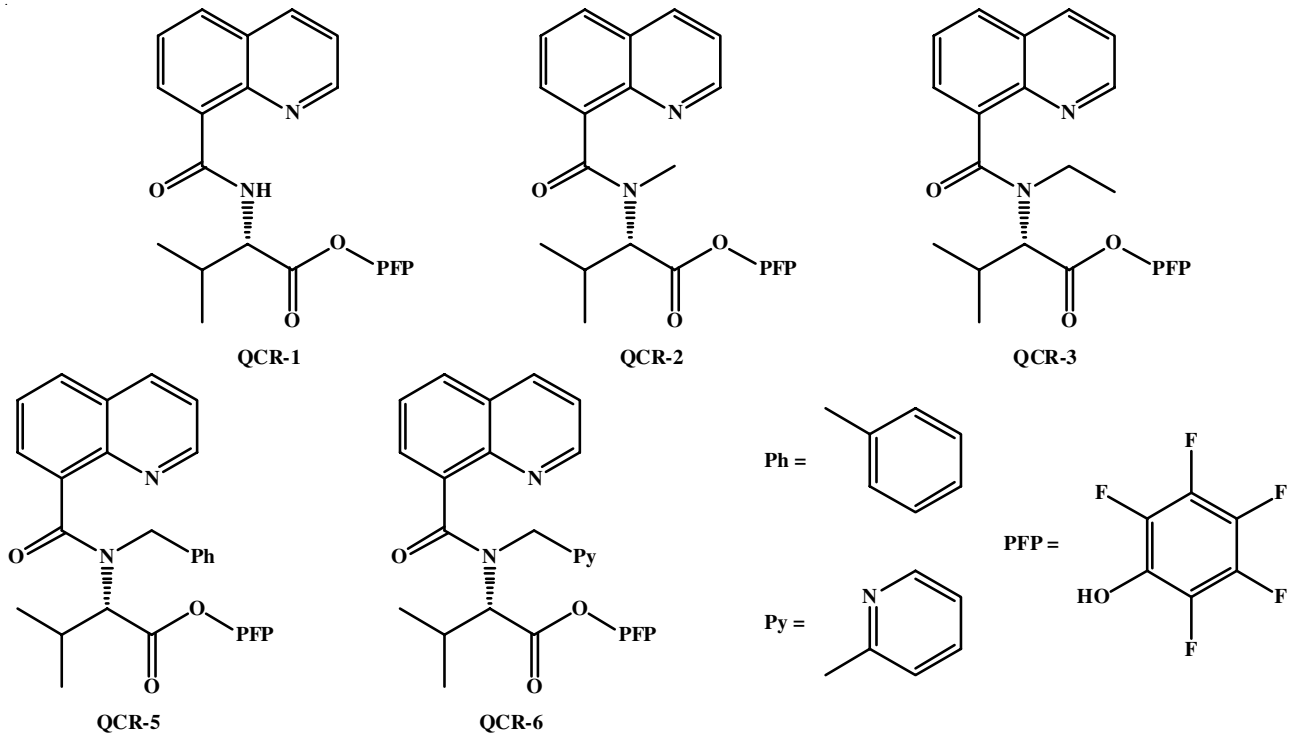

Fig. 3. Structures of remaining chiral reagent

taken from the vial, diluted with acetonitrile (10 times) and passed through a syringe filter. A 20 µL of the prepared solution was applied to HPLC for analysis. Likewise, the remaining QCRs were used to prepare the (*RS*)-Mex diastereomers. The synthesis of diastereomers with **QCR-6** is shown in Fig. 4. The effect of the excess reagent, reaction time, pH and microwave heating were investigated during this research.

HPLC and conditions: The gradient mode was used for RP-HPLC analysis of prepared diastereomer of (*RS*)-Mex with QCRs. Here, acetonitrile and triethylamine-phosphate buffer (TEAP) was used in 80-20%, 70-30%, 60-40% and 55-45% (in a linear gradient). Before the analysis, the mobile phases were pre-treated (filtered, sonicated and degassed). The UV detection was carried out on 294 nm wavelength and the flow rate of the RP-HPLC system was maintained at 1.0 mL/min.

Method validation: The diastereomers prepared with **QCR-6** were used for validation studies. The validation was carried out according to the ICH guidelines. The least square method (MS Excel software) was used to calibrate graphs, slope and correlation coefficient. The concentration range used was 30-3000 ng/mL.

RESULTS AND DISCUSSION

Synthesis of derivatives of L-valine, QVs, QDR and diastereomers: The derivatives of L-valine were prepared by substituting (S_N^1 or S_N^2) the halogen group (leaving group) from methyl bromide/ethyl-bromide/bromocyclohexane/ bromomethyl benzene/bromomethyl-pyridine (Fig. 1). The reaction was performed under reflux conditions for overnight and yielded a good amount of the product [15].

Acylation of 8-qunilolinecarboxylic acid (8-QC): The carboxylic group of 8-QC was converted to an acyl group in the presence of oxalyl group (chlorinating reagent) (Fig. 2). The oxalyl chloride, in the presence of a catalyst (pyridine), is considered an excellent reagent for acylation for the carboxylic groups [9,14,15] and yields nearly 100% of acyl chloride under a fast S_N^2 reaction.

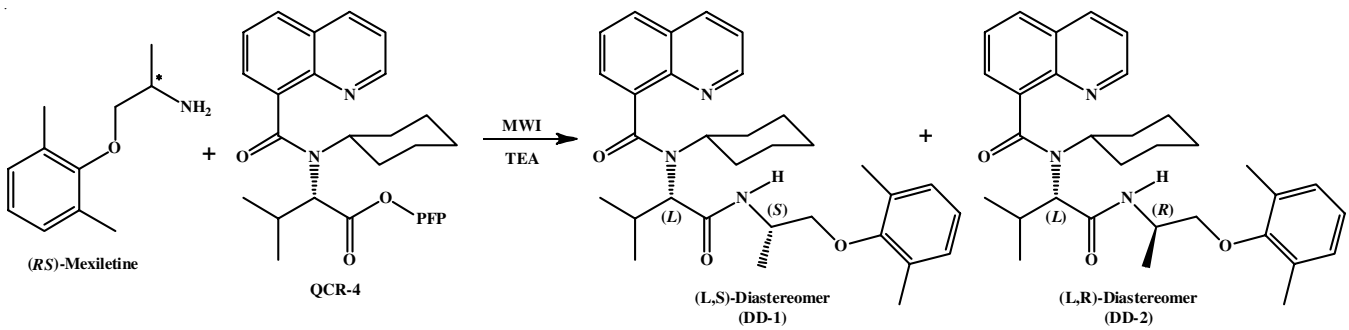

Fig. 4. Synthesis of diastereomers of (*RS*)-Mex with **QCR-4**

Synthesis of QVs: Acyl chlorides are very sensitive towards the nucleophilic-substitution reaction [23]; thus, acyl chloride reacts quickly and efficiently with the molecules that possess amino groups in their structure and yields the desired amide bond under substitution reaction [9,23]. Followed by same mechanism, here, the amides of qunilines (QVs) with derivatives of L-valine (**V1-V6**) are synthesized. The amino group of derivatives of L-valines (**V1-V6**) reacted quickly with the acyl group of quinoline-acyl chloride and produced nearly 100% yield of the desired amides (**QV1-QV6**) (Fig. 2).

Synthesis of QCRs: In order to synthesize **QCRs (1-6)**, the carboxylic groups of the QVs were activated by pentaflourophenol (PFP) under an esterification reaction (Figs. 2 and 3). Pentafluorophenol (PFP) is an excellent leaving group and its esters give a fast and efficient synthesis of relative amides or ester [9]; thus, it is vastly used as a reagent in peptide synthesis [9]. The carboxylic group of QVs were treated with PFP in the presence of EDC and yielded desire PFP esters (QCRs; yield 95-98%). EDC removes the hydroxyl group from the carboxylic function group and the hydrogen group from amine or alcohol, producing amide or esters [24]. During the synthesis of QCRs, the reaction doesn't take place on the asymmetric centre of the molecule; thus, no racemisation was observed during synthesis. Further, the enantiopurtiy was confirmed by chiral HPLC analysis (on cellulose-based chiral column).

Synthesis of diastereomers: The esters of PFPs are very reactive towards the nucleophilic substitution (similar to acyl chlorides) [23]; thus, prepared QCRs react very quickly with the amino group of (*RS*)-Mex and yield the desired diastereomers [25,26], for instance, as a representative shown synthesis of diastereomers of (*RS*)-Mex with **QCR-4** (DD1 and DD2). The synthesis was carried out under microwave heating conditions [9]. Due to the high reactivity of the QCRs (PFP esters), the reaction was finished quickly (in just 60 s at 70% power of MWI, 700 W). During synthesis, racemization wasn't observed because the reaction wasn't performed on an asymmetric center [27]. The chromatographic peaks of equal intensity support the statement that no racemization occurs during the synthesis. Various conditions were applied to investigate the stability of the QCRs, such as pH, storage temp, moisture, light radiations, *etc*. The QCRs were found stable over five months in dry and low-temperature conditions.

The diastereomers prepared with **QCR-1**, **QCR-2**, **QCR-3**, **QCR-4**, **QCR-5** and **QCR-6** are named as DA1 and DA2, DB1 and DB2, DC1 and DC2, DD1 and DD2, DE1 and DE2 and DF1 and DF2, respectively. Here, "1" represents (*L*,*S*)-diastereomer of Mex and "2" represents (*L*,*R*)-diastereomer of Mex.

RP-HPLC analysis: The prepared diastereomers of (*RS*)-Mex with QCRs were separated on the RP-HPLC. Fig. 5 shows the separation chromatogram of resolution of the diastereomers (DD-1 and DD-2) prepared with **QCR-4** as representative. The separation was achieved on the C_{18} Column (RP-HPLC). The chromatographic separation data (retention time, retention factor, separation factor and resolution) of the prepared diastereomers were calculated and reported in Table-1.

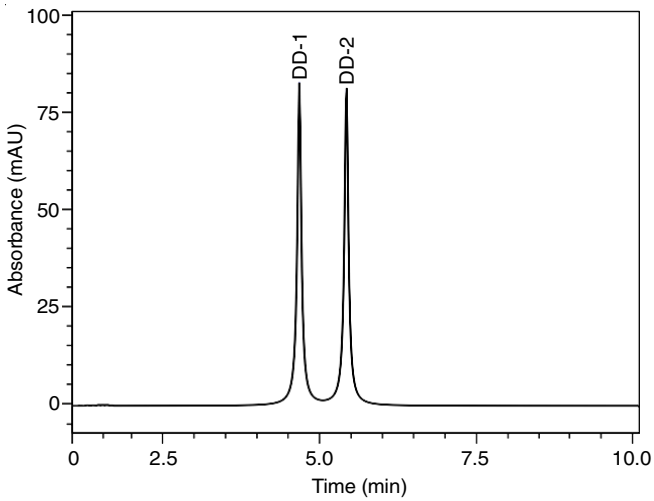

Fig. 5. RP-HPLC chromatogram of enantioseparation of diastereomers of (*RS*)-Mex prepared with **QCR-6**

It was observed, in most of the cases, the (*S*,*S*)-diastereomer (DA-2, DB-1, DC-1, DD-1, DE-1, DF-2) elute before then (*S*,*R*)-diastereomer (DA-1, DB-2, DC-2, DD-2, DE-2, DF-1). The elution orders of the separated diastereomers are given in Table-1. The linear gradient mode of the combination of TEAP buffer (pH 3.5; 30%) with acetonitrile (70%) was a successful mobile phase for the current experiment. The efficiency of the mobile phase was investigated by modifying the pH range (2 to 6) concentration range of the buffer solution (4 to 24 mM). Apart from that, an organic solvent such as methanol was used for the experiment. Still, the results with acetonitrile were better due to its low viscosity and low UV cut-off and thus doesn't interfere in the detection of diastereomers. Acetonitrile provides sharp peaks and lower elution time for diastereomers compared to other organic solvents [28,29]. For the current study, the different flow-rates (0.25 to 1.5 mL/min) of the eluting phase for the RP-HPLC system were investigated. 1 mL/min flow rate

TABLE-1
CHROMATOGRAPHIC SEPARATION DATA OF DIASTEREOMERS OF (*RS*)-Mex PREPARED WITH QCRs

Chiral reagent	Chromatographic separation data of diastereomers of (*RS*)-Mex			Calculated separation data for diastereomers of (*RS*)-Mex			
	First peak time (min)	Second peak time (min)	First eluted diastereomer	k_1	k_2	α	Rs
QCR-1	4.96	6.21	DA-2	2.81	3.77	1.34	6.26
QCR-2	5.14	6.44	DB-1	2.95	3.96	1.32	6.51
QCR-3	5.27	6.74	DC-1	3.05	4.18	1.37	7.35
QCR-4	6.87	8.58	DD-1	4.21	5.60	1.32	8.55
QCR-5	4.69	5.71	DE-1	2.61	3.39	1.64	5.14
QCR-6	4.72	5.48	DF-2	2.63	3.21	1.22	6.08

of eluting phase (70% acetonitrile and 30% TEAP) was found successful for the current study (separation of diastereomers).

Elution order and DFT optimized 3D structures: The DFT calculations (on Gaussian Software) were performed to establish the elution order of the prepared diastereomers. The optimized 3D structures were used to study the molecular geometry, separation behaviour and elution order during separation on the C_{18} column of HPLC.

The optimized structures of the diastereomers prepared with **QCR-4** (DD-1 and DD-2) and **QCR-6** (DF-1 and DF-2) are shown in Fig. 6 as representative. The DD-1 (*L*,*S*-diastereomer) was organized in such a way that the aromatic rings of Mex and 8-QC stabilize on maximum distance (due to hindered structure); thus, molecule size became bigger compared to DD-2 (*L,R*-diastereomer). Literature shows the bigger diastereomer in a diastereomeric pair has more surface to expose during the reverse phase chromatographic separation, thus eluting first compared to a small diastereomer. Meanwhile, the small diastereomer shows more hydrophobic properties (due to its small size) then the big one; thus, it interact more with non-polar column material, thus eluting in the last [7-10]. In present study, due to bigger size, DD-1 has more polarity, more surface and interact higher with polar mobile phase; thus, DD-1 elute faster (elution time 6.87 min) compared to DD-2 (elution time 8.58 min).

A similar hypothesis was applied to diastereomers DF-1 and DF-2. Among them, DF-2 has bigger 3D structure and the aromatic rings of Mex, pyridine and 8-QC are arranged to form a bigger structure compare to DF-1. The DF-2 elute first (elution time 4.72 min) and DF-1 elute in last (elution time 5.48 min). In the diastereomers, the hydrophobic substituents attached in L-valine (methyl, ethyl, cyclohexyl, methylbenzene, methylpyridine) play an important role during the chromatographic interactions. The substituent with higher hydrophobic carbons interacts more with column material (non-polar interaction); thus, they took more time to elute than less carbons containing substituent. The aromatic ring having substituent elutes fast due to polar dipole interaction; therefore, methylbenzene and methylpyridin-containing diastereomers elute faster than non-aromatic substituent. Similarly, the DFT calculations for all the synthesized diastereomers were performed and elution orders were also established. The elution time and elution sequence of the separated diastereomers are given in Table-1.

Validation

The validation of the current analytical method was performed as per ICH regulations [30]. The validation study was conducted on diastereomers prepared with **QCR-6** (DF-1 and

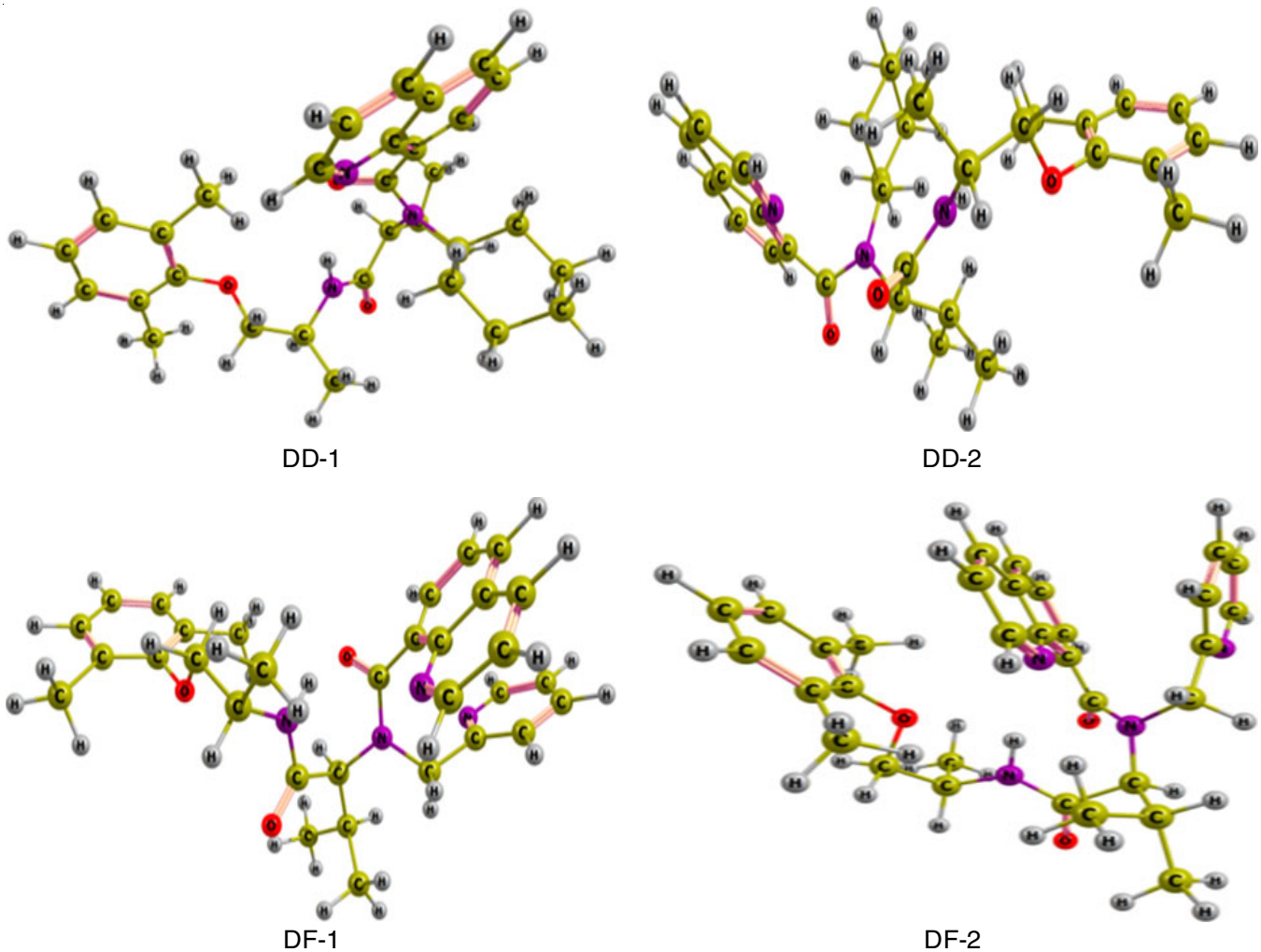

Fig. 6. FT optimized 3D structures of diastereomers of (*RS*)-Mex (DD-1, DD-2, DF-1 and DF-2) prepared by **QCR-4** and **QCR-6**, respectively

DF-2) as a representative, as described in literature [8,9,31]. The concentration range of 30-3000 ng/mL was used for the validation and the detection limit (LOD), the quantification limit (LOQ), linearity, accuracy, precision and relative standard deviation (RSD) were determined. Stabilities and recoveries were quantified and examined using the RP-HPLC system-generated peak region. The estimated recovery values for the first and second eluting diastereomers are 98.64 and 99.20% for the inter-day assay and 99.32 and 99.78% for the intra-day assay. It was found that the LOD and LOQ were, respectively, 0.161 ng/mL and 0.483 ng/mL.

The current work shows better results compared to the previous reports [7-10,18,23,27,28] and also better separation of the diastereomers of (*RS*)-Mex in terms of the separation factor (1.22), resolution (6.08), elution time (4.72-5.48 min) and retention time (2.36-3.21). The results obtained in this work are also better for the chiral separation of (*RS*)-Mex.

Conclusion

The current work shows an excellent analytical method to detect enantiomeric purity of (*RS*)-Mexiletine, even in very low concentrations. This method report an efficient synthesis of 8-qunilolinecarboxylic acid (8-QC) based activated chiral reagents (QCRs) and their application in easy microwave synthesis of diastereomers of the (*RS*)-Mex. A clean RP-HPLC separation was achieved for the prepared diastereomers with very high sensitivity detection (LOD = 0.161 ng/mL and LOQ = 0.483 ng/mL). The gradient mobile phase (acetonitrile and TEAP buffer) was found sufficient to separate all the prepared diastereomers cleanly and efficiently (low elution time). Elution time and elution mechanism were explained with DFT-optimized 3D structures. This is an excellent analytical method to identify the enantiomeric impurities and estimate trace amounts of the racemic organic compounds that contain amino or alcoholic functional groups in their structure.

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CONFLICT OF INTEREST

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

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