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(*S*)-naproxen based novel chiral reagent for C-N bond formation: enantioseparation of some β-blockers, determination of absolute configuration and elution order of diastereomers

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Abstract
A new chiral derivatizing reagent has been synthesized from (*S*)-(+)-naproxen and was used for C-N bond formation to prepare diastereomeric amides of (*RS*)-propranolol, (*RS*)-atenolol, (*RS*)-carvedilol and (*RS*)-metoprolol. Derivatization reactions were done at room temperature (30°C for 30 min) under stirring conditions as well as under microwave irradiation (MWI). Separation of diastereomers was achieved by open column chromatography. ^{1}H NMR spectra of the isolated and purified diastereomers were recorded to establish the configurations of the first and second eluting diastereomers (and thus the elution order) and to compare the chromatographic separation characteristics when the diastereomeric mixture was separated using achiral RP-HPLC (using C_{18} column and a binary mixture of MeCN and triethyl ammonium phosphate buffer of pH 3.5 (60:40 *v*/*v*) as mobile phase at a flow rate of 1 mL min^{-1} and UV detection at 230nm). No racemization was observed throughout the study. Test samples of β-blockers were isolated from commercial tablets and then were purified and characterized to be used as racemic standard. The conditions for derivatization and separation were optimized. Lowest energy optimized structures of the two diastereomers were developed using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G basis set which supplemented ^{1}H NMR interpretations and confirmed three dimensional geometry of the diastereomers. Separation method was validated as per ICH guidelines. The limit of detection and limit of quantification for each isomer were 0.4 ng/mL and 1.2 ng/mL, respectively.

Keywords: (*S*)-Naproxen anhydride; β-blockers; RP-HPLC; Diastereomeric separation.

1. Introduction

Efficient reactions for forming carbon-hetero element bonds using a variety of *anhydrides* as condensation reagents are well known. The anhydrides are generally derived from aromatic or aliphatic carboxylic acids. Such condensation reactions proceed

under mild conditions without using harmful substances (e.g., heavy-metal salt or strong base) and form the carbon-hetero element bond at any desired position with high selectivity. These include reactions with alcohols, primary and secondary amines, and aromatic ring.

Presence of undesirable enantiomer in a racemic drug is regarded as impurity. Separation of enantiomers and determination of their purity is still challenging and important. Regulatory agencies in the US, Japan and EC call for registration of single enantiomer and the responsibility lies on the sponsor to demonstrate why registration of racemates and/or fixed amounts of enantiomers is otherwise justifiable[1]. Yet, with "a few" exceptions, chiral synthetic drugs are being approved for marketing and pharmaceutical applications as racemic mixture. Nevertheless, establishing enantiomeric purity of starting materials, reagents and catalysts is equally important because the quality of these compounds limits the enantiomeric purity of the resulting products (e.g., the drug compounds).

If the drug racemates that are relatively poor absorbers in the UV‑vis region are reacted with CDRs having high molar absorptivity (ε) or high fluorescence quantum yield (φ) the diastereomers so formed attain high sensitivity for detection and selectivity for enantioseparation using conventional reversed-phase achiral columns. The chiral columns, if used for direct resolution, are expensive and have limited capacity and stability.

(*S*)-(+)-Naproxen (Npx) is a non-steroidal anti-inflammatory drug and is available as a pure enantiomer. Its systematic chemical name is, (*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid. The literature reveals synthesis of Npx based chiral derivatizing reagents (CDRs) and their application in enantioresolution of certain pharmaceuticals[2-4] the carboxylic group of Npx was activated by introducing different nucleophilic moieties. These served as good leaving groups in a subsequent nucleophilic substitution reaction when the said CDR was reacted with a racemic drug containing amino group. Corresponding diastereomers having amide bond were formed. The large conjugated naphthyl ring having high molar absorptivity (ε >100,000) facilitated detection of diastereomers.

From the literature cited above, and the literature cited therein, on synthesis and application of (*S*)-(+)-Npx based CDRs and considering the application and importance of anhydride type of reagents, it occurred to us that a new CDR could be synthesized from (*S*)-(+)-Npx as its anhydride, due to the presence of carboxylic acid group in Npx, which could be used as a versatile reagent. (*S*)-(+)-Npx based CDRs have not been used for enantioseparation of β-blockers.

β-blockers: Beta blockers are synthetic chiral hydroxyl amine-containing compounds used clinically to treat cardiovascular diseases such as hypertension, coronary heart disease, arrhythmias, sinus tachycardia and myocardial infarction, where they act preferentially upon the β-adrenergic receptors in heart[5]. They cause fewer bronchospastic

reactions and significantly fewer side effects on central nervous system (e.g., depression and nightmares). The sales of antiarrhythmic drugs increased from U.S. $1.8 billion in1999 to U.S. $2.6 billion in 2009; it is anticipated to reach U.S. $3.5 billion in 2015 (*The Worldwide Market for Prescription Cardiovascular Drugs Reports*" from *Kalorama* Information[6]). So far, most of the β-blockers are being used as a racemic mixture despite the fact that pharmacological action is largely confined to (*S*)-(−)-enantiomers showing about 50–500 fold higher activities[7]. The bioavailability of both (*S*)-(−)-propranolol and (*S*)-(−)-metoprolol, in humans, exceeds that of the (*R*)-isomer. (*S*)-(−)-Propranolol is important in therapy against oxidative stress and its beta blocking potency is *ca*. 40 times greater than that of the (*R*)-(+)-enantiomer; metoprolol has been shown to protect red blood cells against phenazine methosulfate (PMS)-induced toxicity. Thus, there is a need to review current prescription of racemic β-adrenergic blockers from the clinical, medical and health point of views.

CDRs for β-blockers: There have been a few reports from this laboratory[8-10] on successful enantioseparation of certain β-blockers as their diastereomers prepared with newly synthesized CDRs based on difluorodinitrobenzene (DFDNB) and cyanuric chloride (CC) moieties using RPHPLC. These moieties added a very strong chromophore for UV absorption and led to sensitive detection of the derivative. The diastereomers prepared with DFDNB based CDRs had a very similar absorption spectrum characterized by a λ_{max} at 340 nm; the spectrum are stable if the solutions are kept in dark, otherwise, a gradual change occurs as a result of a photochemical decomposition of the absorbing chromophore (for DFDNB based derivatives). Many other reagents used for pre-derivatization suffer inherent problems, *e.g*., unstable derivatives, poor detection of certain derivatives, or lack of quantitative yield of the reaction.

Chiral separation of β-adrenergic antagonists by TLC using both direct and indirect modes has been critically reviewed[11]. CDRs, such as, (–)-menthyl chloroformate[12]; (+)-1-(9-fluorenyl) ethyl chloroformate[13]; (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocynate[14]; *N*-trifluoroacetyl-l-prolyl chloride[15]; optically active anhydrides derived from (*R,R*)- and (*S,S*)-tartaric acid such as, *O*,*O*-dibenzoyl derivative[16,17]; *O*,*O*-diacetyl derivative[18] and *O*,*O*-di-*p*-toluoyl derivative[19] have been used for hplc separation of diastereomers of pharmaceutically active compounds containing amino or hydroxyl group, e.g., atenolol and other β-blockers.

Present work: A new CDR was synthesized from (*S*)-(+)-Npx as its anhydride. It was used for synthesis of diastereomers of (*RS*)-propranolol (Prl), (*RS*)-atenolol (Atl), (*RS*)-carvedilol (Cal), and (*RS*)-metoprolol (Mel) (**Fig.1**). The β-blockers were chosen for the present studies based on their high prescription rate and ease of availability and to test the efficiency of the new anhydride reagent for forming carbon-nitrogen bond appearing in the diastereomeric amide products. The diastereomers so synthesized (four pairs) were separated and isolated by open column chromatography. The isolated and purified

diastereomers were used to (i) establish the configurations of the first and second eluting diastereomers (and thus the elution order) by recording their ^{1}H NMR spectra, and (ii) to compare the chromatographic separation characteristics when the diastereomeric mixture was separated using achiral RP-HPLC. HPLC separation method was optimized with respect to chromatographic conditions; it was validated for linearity, limit of detection and limit of quantitation. Structures of the two diastereomers optimized for the lowest energy were developed using the Gaussian 09 Rev. A.02 program and hybrid density functional B3LYP with 6-31G* basis set (based on density functional theory). The configurations established from ^{1}H NMR were correlated with these lowest energy structures. The stability aspect of diastereomers has separately been investigated related to the chemical or composition stability in terms of time of storage and temperature. The above mentioned aspects constitute the novelty of work.

2. Experimental

2.1 Instrumentation

The HPLC system (LC-20AD, Shimadzu, Kyoto, Japan) consisted of a low-pressure gradient unit, low pressure mixing type gradient, DGU-20A5 on-line degasser unit, high pressure mixer, parallel double plunger pump, SPD-M20A diode array detector, SPD-20A/20AV (UV–VIS Detector), CTO-20AC column oven, LC solution and DAO (data access objects) 3.5 operating software; LiChrospher C_{18} column (L×I.D. 25 cm×4.6 mm, 5µm particle size) was from Merck (Darmstadt, Germany). Other instruments used were, Microwave-Multiwave 3000 (800W, Perkin–Elmer, Shelton, CT, USA), Milli-Q system of Millipore (Bedford, MA, USA) to obtain purified water (18.2 MΩcm^3) from double distilled water, pH meter (Cyberscan 510, Singapore), FT-IR spectrometer 1600 (Boardman, OH, USA), spectrophotometer (Shimadzu UV-1601), elemental analyzer (Vario EL III, Hanau, Germany), and Polarimeter (P3001RS Krüss 140, Hamburg, Germany). UV spectra were recorded in methanol and ^{1}H NMR spectra were recorded on 400 MHz (JEOL Inc., Peabody, USA) instrument using $CDCl_3$ as solvent.

2.2 Chemicals

(*S*)-(+)-Npx $[\alpha]_D^{25}$ = (+)67±3.0°, (c = 1, CH_2Cl_2), *ee*: ≥98.0% (GC); (*RS*)-Atl, (*RS*)-Prl, dimethylamino pyridine (DMAP) and N,N'-dicyclohexylcarbodiimide (DCC), were obtained from Sigma–Aldrich (St Louis, MO, USA). Analytical grade reagents such as triethylamine (TEA), trifluoroacetic acid (TFA), phosphoric acid (H_3PO_4), acetic acid (CH_3COOH), concentrated hydrochloric acid (HCl), sodium hydrogen carbonate ($NaHCO_3$), and HPLC grade MeCN and methanol (MeOH) were obtained from E. Merck (Mumbai, India). (*RS*)-Mel marketed as Betaloc (AstraZeneca, Bangalore, India), (*RS*)-Cal marketed as Carca (Intas, Dehradun, India), in racemic form, were purchased from local market.

2.3 Extraction and purification of active pharmaceutical ingredient (API)

Ten tablets of (*RS*)-Mel (each labelled to contain 100 mg) were finely pulverized, and were extracted with 100 mL methanol at 25˚C using sonicator for 15 min. The solution was filtered through Whatman filter paper grade number-1 (particle retention 11 μm) and the residue was further treated thrice with methanol and filtered. The combined filtrate was concentrated in vacuum and left to cool until crystals appeared. The mother liquor was decanted and the crystals were recrystallized from MeOH/H_2O. The crystals were washed with diethyl ether and dried in air. The same procedure was repeated for extraction, isolation and purification of (*RS*)-Cal from its tablets.

2.4 Stock Solutions

The following stock solutions were prepared:

(a) racemic β-blockers (10 mM) in MeCN
(b) Npx anhydride, the CDR, (15mM) in MeCN
(c) triethylammonium phosphate (TEAP) buffer solution by dissolving triethylamine (10mM) in purified water; pH was adjusted to 3.5 by adding phosphoric acid
(d) 0.1% TFA in purified water
(e) Acetate buffer (0.05 M, pH 4.0) in purified water
(f) Borate buffer (0.2 M, pH 9.5) in purified water

2.5 Synthesis and characterization of CDR

Npx anhydride: DCC (206mg, 1 mmol) and DMAP (18.5 mg, 0.15 mmol) were added to a stirred solution of (*S*)-(+)-Npx (460mg, 2 mmol) in 30 mL of dry tetrahydrofuran (THF). The reaction mixture was stirred for 2 h at room temperature under nitrogen atmosphere. It was then filtered to remove the *N,N'*-dicyclohexylurea formed during the reaction and then the filtrate was washed five times with brine solution, five times with water, five times with $NaHCO_3$ and finally with 1N HCl. It was then extracted with 50 mL of ethyl acetate. The ethyl acetate extract was dried over $MgSO_4$. The solvent was evaporated to dryness in vacuum to give symmetrical anhydride of (*S*)-(+)-Npx as white solid. It was recrystallized with methanol. The CDR was stored in a tightly closed container at 4 ^oC. The characterization data are given below. Yield: 95%; mp: 115 ^oC; $[\alpha]_D^{25}$ = +8.33^o (c= 1.05, CH_2Cl_2); UV ($\lambda_{max,}$ 230 nm, CH_2Cl_2); IR(KBr): 3413, 1692, 1633, 1617, 1556, 1331, 1241 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.57-1.59 (d, 6H, CH_3); 3.90 (s, 6H, OCH_3); 7.10-7.14 (m, 4H, Ar); 7.40-7.14 (dd, 2H, Ar); 7.68-7.70 (d, 6H, Ar); Anal. Calcd for $(C_{14}H_{13}O_2)_2O$: C, 76.00%; H, 5.92%. Found: C, 75.88%; H, 5.75%.

It was observed that in the absence of DMAP reaction took 4 h for completion and in presence of DMAP the reaction was completed in 2 h.

2.6 Synthesis of diastereomers via conventional heating and microwave irradiation

To a solution of (*RS*)-Prl (1000 µL, 10 mM), the CDR (Npx anhydride) solution (1000 µL, 15 mM) and borate buffer (500 µL, 0.2 M, pH 9.5) were added. Separate sets of reaction mixture were stirred for 15, 30 and 45 min, each at a temperature of 15, 30 and 45 ^{o}C; acetate buffer (500 µL, 0.05 M, pH 4) was then added to quench the reaction. Effect of ratio of analyte vs CDR (mole ratio of 1:1, 1:1.5, 1:2, and 1:2.5) was investigated for each set of conditions. Stirring for 30 min at 30 ^{o}C with a mole ratio of 1:1.5 was found successful

For microwave irradiation, the reaction mixture (reactants taken in the same molar ratio, as mentioned above) was taken in a 2 mL vial; the reaction mixture was then exposed to MWI for 190 s at 80% of 800 W power. It was then cooled and acetate buffer was added to quench the reaction. Separate sets of reaction mixture were subjected to MWI for 80, 110, 130, 150, 170 and 190 s at 80% power of 800W.

The solvent (from each set of the reaction mixture from the two methods) was evaporated with nitrogen and re-dissolved in 500 µL of MeCN. It was filtered through 0.45 mm filter and 10 µL of this solution was diluted with 100 µL of MeCN; 20 µL of this solution was loaded onto the HPLC column. The reaction was monitored to check the completion of synthesis by injecting the reaction mixture for HPLC analysis for each set of synthesis. The diastereomers synthesized by the two approaches were compared for their separation parameters.

Preparative synthesis of diastereomers: The optimized conditions of synthesis of diastereomers were scaled up to preparative level; the solutions used were, (*RS*)-Prl (155 mg, 60 mM), solution of CDR (397mg, 90mM) and borate buffer (5 mL, 0.2 M, pH 9.5).

Open column chromatography for separation of diastereomers

A glass column (2.5 x 35 cm) was packed with silica gel in *n*-hexane. The mixture of diastereomers (as obtained from preparative synthesis) was loaded. Solvent system consisting of MeOH-DCM (9:1, *v*/*v*) was found successful for adequate separation of the two diastereomers; fractions collected from the column were examined by RP HPLC for verification of purity of each diastereomer. The fractions containing single and identical diastereomer were combined and were concentrated in vacuum. The characterization data of the two separated diastereomers of (*RS*)-Prl is presented below.

First eluting diastereomer (Ds-I): Yield: (85.08%); Color: colourless; ^{1}H NMR ($CDCl_3$, 500 MHz): δ= 1.24 (d, 6H), 1.32 (d, 3H), 2.54 (s, 1H), 3.34 (dd, 2H), 3.67 (m, 1H), 3.98 (s, 3H), 4.19 (m, 1H), 4.61(m,1H), 4.47 (m, 2H), 6.73(dd, 1H, Ar-H),7.23(m, 2H, Ar-H)

7.46(m, 3H, Ar-H), 7.59(t, 2H, Ar-H), 7.79(d, 1H, Ar-H), 7.87(m, 2H, Ar-H), 8.43(m, 2H, Ar-H); Anal. calcd for $C_{30}H_{33}NO_4$: C, 76.41; H, 7.05; O, 13.57; N, 2.97; found: C, 76.29; H, 6.93; O, 13.36; N, 2.75.

Second eluting diastereomer (Ds-II): Yield: (83.14%); Color: colourless; ^{1}H NMR ($CDCl_3$, 500 MHz): δ= 1.24 (d, 6H), 1.33 (d, 3H), 2.26 (s, 1H), 3.34 (dd, 2H), 3.67 (m, 1H), 4.01 (s, 3H), 4.19 (m, 1H), 4.64(m, 1H), 4.36 (m, 2H), 6.74(dd,1H, Ar-H),7.21(m, 2H, Ar-H), 7.45(m, 3H, Ar-H), 7.59(t,2H, Ar-H),7.78(d, 1H, Ar-H), 7.88(m,2H, Ar-H),8.44(m, 2H, Ar-H); Anal. calcd for $C_{30}H_{33}NO_4$: C, 76.41; H, 7.05; O, 13.57; N, 2.97; found: C, 76.19; H, 6.81; O, 13.14; N, 2.56.

2.7 HPLC conditions for separation of diastereomers

The following mobile phases were used for separation experiments:
(I) MeCN-10mM TEAP buffer, (II) MeOH-10mM TEAP buffer,
(III) MeCN-0.1% aq TFA, (IV) MeOH-0.1% aq TFA
These mobile phases were used in an isocratic mode (with the ratio of 90:10, 80:20, 70:30 and 60:40) in 30 min run and by varying flow rate from 0.5 mL min^{-1} to 2 mL min^{-1} (with a difference of 0.5 mL) for each mobile phase. Chromatographic conditions were also optimized by changing the pH (in the range 2.0–6.0) and TEAP buffer concentration (5, 10, 15 and 20mM) and TFA (0.05, 0.1, 0.15 and 0.2%). The mobile phases were filtered through a 0.45mm filter.

2.8 Method Validation

Validation of HPLC separation method was performed according to International Conference on Harmonization (ICH) guidelines[20] for the proposed method, using diastereomers of (*RS*)-Prl prepared with Npx anhydride as the CDR. Peak areas (as provided by the system software) were used for quantitation referring to the calibration curve for the studies related to stabilities and recoveries.

3. Results and Discussion

APIs: Two of the β-blockers, namely, (*RS*)-Cal, and (*RS*)-Mel, were isolated from commercially available tablets. These were purified and characterized. Melting point, yield, λ_{max}, specific rotation, and IR spectra (in KBr) of the purified API were recorded. Each of these was used as a racemic standard. The recoveries were of the order of 96–98% of the quantities reported on the commercial labels.

CDR: The anhydride of (*S*)-(+)-Npx is a symmetrical molecule. It was obtained when two molecules of (*S*)-(+)-Npx reacted in presence of DCC/DMAP in THF (**Fig.2**). In organic synthesis one of the simplest and most efficient methods of acylation is the treatment of amines (or other nucleophile) with anhydrides of carboxylic acid. The

introduction of DCC as coupling reagent had a novel feature that it could be added with carboxyl component and amine component; thus, activation and coupling proceed concurrently. However, the rate of reaction of amine with DCC is much lower in comparison to the rapid rate observed in the addition of carboxylic acid to one of the double bonds of carbodiimide[21].

In an attempt to achieve formation of diastereomers of (*RS*)-Prl (for example) directly with (*S*)-(+)-Npx in presence of DCC there were obtained certain co-eluting and unidentified peaks in HPLC. These may be due to formation of a symmetrical coupling product of (*S*)-(+)-Npx since the N=C group of the intermediate formed by the addition of carboxylic acid, i.e., (*S*)-(+)-Npx, to carbodiimide provided powerful activation leading to coupling. This condition is met with in the present work.

In view of the above, experiments were planned to prepare a symmetrical anhydride of (*S*)-(+)-Npx using DCC. The application of such an anhydride was helpful in preventing the formation of unreactive byproduct such as N-acyl urea if the amine component (i.e., (*RS*)-Prl, the amine group containing analyte) was allowed to react directly with (*S*)-(+)-Npx in presence of DCC because the nucleophilic centre on O-acyl isourea (the intermediate formed by addition of (*S*)-(+)-Npx, being the carboxylic acid) competes with the amine component for acyl residue. Thus, it can be concluded that the application of DCC is justified in activating more for the formation of symmetrical anhydride which in turn was applied for the desired N-acylation.

Enantiomeric purity of CDR: The enantiomeric purity is a parameter different than the chemical purity of the sample. Since (*S*)-(+)-Npx had *ee* of 98%; it was considered that the CDR corresponding to 1% of the other isomer of Npx (if any) was eliminated during synthesis and purification process (of crystallization and recrystallization) of the desired CDR. When (*RS*)-Prl was reacted with the CDR and the product so obtained was investigated on an RP-column using different mobile phases there appeared only two diastereomeric peaks. Had there been the CDR arising from the (*R*)-isomer of Npx there would have resulted corresponding diastereomeric peaks in the chromatogram. Since the PDA detector showed only diastereomeric peaks and confirmed the absence of impurities in the same retention time of the peaks of interest the absence of racemization as well as enantiomeric purity of the recrystallized CDR was confirmed.

Diastereomers: Diastereomers of all the four title compounds [(*RS*)-Prl, (*RS*)-Atl, (*RS*)-Cal, and (*RS*)-Mel] were prepared by their reaction with the symmetrical anhydride of (*S*)-(+)-Npx in borate buffer (0.2 M, pH 9.5), as the catalyzing agent. Reaction scheme for synthesis of the CDR and synthesis of diastereomers of (*RS*)-Prl (as a representative β-blocker) is shown in **Fig.2.** Since the derivatization reaction with the CDR did not involve a direct attack on stereogenic center there was no racemization detected throughout the study. The derivatives are designated as the (*S,R*)- and (*S,S*)-

diastereomers, the first letter refers to the configuration of the CDR and the second to that of the Prl.

The optimized experimental conditions for synthesis of diastereomeric pair of (*RS*)-Prl were: '30 min at 30 ^{o}C by stirring' and '190 s at 80% of 800 W power under MWI', with a 1.5 fold molar excess of CDR. Effect of MWI time on completion of derivatization reaction of (*RS*)-Prl is shown in **Fig.3**. Synthesis of diastereomers of (*RS*)-Atl, (*RS*)-Cal, and (*RS*)-Mel was carried out under the optimized reaction conditions.

Generally, the rate of reaction of enantiomers with chiral molecules is different. The CDR is a chiral molecule and, therefore, the two enantiomers in the (*RS*)-mixture may react at different rates. The derivatization reaction was performed using (*RS*)-β-blocker(s) and the CDR in different mole ratio; slight kinetic resolution was observed when lower ratio of (*RS*)-Prl:CDR (1:1) was applied. The recovery studies of the four eluted diastereomers (as described in "Accuracy and Precision") served as a measure of their yields. Since the recovery of the eluted diastereomers of Prl, Atl and Mpl was found to be more than 94% it was contended that the steric effects due to *tert*-butyl group in these molecules did not adversely affect the derivatization reaction.

The reaction conditions for synthesis of diastereomers were investigated and established keeping in view that β-blockers possess a secondary hydroxyl group besides the secondary amino group. Basic medium (borate buffer, 0.2 M, pH 9.5) made the reaction to occur at amino group. Since the medium has been basic excess of the reagent would not react with hydroxyl function; even after quenching the reaction by adding acetate buffer the solution had pH 8.5.

In the indirect approach, a suitable reactive group (preferably only one function in close proximity to the stereogenic center) should be present in the sample to be analyzed which is prone to a quantitative transformation with the chiral reagent; this condition is met with in the present work.

Stability of Diastereomers: Stability of diastereomers was investigated after short-term (room temperature) and long term (refrigerated at a temperature of 3–5 °C) storage as a function of storage conditions including the container system and their chemical properties. The evaluation also included the stability of the analyte in stock solution and situations encountered during actual sample handling and analysis. HPLC experiments were carried out at an interval of 10 days up to 130 days from the day of synthesis of the diastereomers and it was found that the diastereomers were stable for 120 days under refrigeration conditions (3–5 °C). The chemical stability remains unaffected in the solvents used as mobile phase in HPLC.

Configuration of the diastereomers

Because of the reaction of the carbonyl group of CDR with the amino group of Prl there occurred formation of diastereomers having amide bond. ^{1}H NMR spectra of the purified diastereomers were recorded (**Fig. 4**). Difference in chemical shifts (Δδ) formed the basis for establishing the absolute configuration and discrimination among the chiral diastereomers. In the two diastereomers, the chemical shift values for the peaks of –OH signal are δ 2.54 and δ 2.26. The difference in chemical shift (*i.e.*, $\Delta\delta^{RS}$ value of -0.28) is large enough for a diagnostic –OH signal which can be represented as, [$\Delta\delta^{RS}$(OH) = δ^{R}(OH)– δ^{S}(OH)], where (*R*) and (*S*) descriptors refer to the configuration at the stereogenic centre of the two enantiomeric Prl molecules. The difference in the chemical shift of the –OH signal may be attributed to the formation of H-bonding in one of the diastereomers whereas in the other diastereomer H-bonding is not possible (chemical structures are shown in **Fig.2**). The δ values (in ppm) of –OH proton corresponding to the asymmetric centre in Prl in the first eluting (DsI) and second eluting (DsII) diastereomers are shown in Fig.4 and these correspond to (*S,S*)- and (*S,R*)-diastereomers, respectively.

Further evidence for the formation of H-bond was provided by the DFT based lowest energy structures of the two diastereomers (**Fig.5(i)** and **5(ii)** corresponding to DsII and DsI, respectively) developed (by using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set). **Fig.5(ii)** showed that the naphthyl ring of CDR and the naphthyl ring of Prl are in the same plane; this steric situation favours the formation of H-bond which in turn causes a downfield shift and there is observed a difference in chemical shift of –OH protons in the two diastereomers.

The chemical shift for the –OH proton in the ^{1}H NMR spectrum of the diastereomers is greatly affected by the presence of the H-bond which arises due to specific spatial arrangement. The $\Delta\delta^{RS}$ value obtained for –OH proton has a negative sign. The $\Delta\delta^{RS}$ value between the diastereomeric pair would not be negative for the –OH proton if the structure of the diastereomer is not as represented in **Fig. 5**. The first eluted diastereomer showed a downfield shift for –OH proton while the second eluted diastereomer showed an upfield shift in ^{1}H NMR spectrum; therefore, the first eluted diastereomer has (*S,S*) configuration. The ^{1}H NMR spectrum (**Fig. 4**) and **Fig**. **5(ii)** confirmed that hydrogen bond between –OH proton (in Prl moiety) and the carbonyl oxygen (of Npx moiety) is in one of the diastereomers only and thus it would be the (*S,S*) diastereomer. Such a hydrogen bond formation does not occur in (*S,R*) diastereomer due to the specific spatial arrangement.

DFT based 'lowest energy structures': The present studies are neither intended nor focused on theoretical calculation of the energies of the different configurations of the isomers for which use of different parameters in terms of two dihedral angles, as variables of the potential energy surface, would be required. The default convergence criteria are implemented in Gaussian software itself; the optimization continues simply on submitting the data. Moreover, the diastereomeric molecules are not large and have no metal atom and there was no situation of running out of cycles. Software program

routinely provides results for molecular geometries and energies at a very good level of accuracy. Therefore, the phrase "lowest energy structures" has been used for the structures of the two diastereomers (**Fig.5**) developed (by using the above said Gaussian program).

The diastereomers have been obtained by a synthesis process using a new chiral anhydride type reagent for facile C–N bond formation. The diastereomers were isolated by open column chromatography and the absolute configuration of the diastereomers so isolated was determined by ^{1}H NMR. The stability aspect is related to the chemical or composition stability in terms of time of storage and temperature. The chemical stability remains unaffected in the solvents used as mobile phase in HPLC. The structures shown in **Fig.5** belong to the 'stable diastereomers'. As a result, there is no consideration of torsional energy (or rotational energy barrier) to decide the relative stability of conformations (the diastereomers are not a kind of atropisomers). The diastereomers under question are quite stable species. And the two structures belong to the lowest energy structures which are supplementing the information on configuration (derived from ^{1}H NMR) and the configuration is not changing by rotation at any stage.

The **Fig. 5(ii)** showing the structure for the (*S*,*S*)-diastereomer, for example, corresponds to the "lowest energy". There may be different conformations because of rotations at the bond (i) between carbonyl C and the stereogenic centre bearing methyl group on one side and (ii) the bond between N (of the amide bond) and the C of the $-CH_2$ group in the close proximity of the stereogenic centre bearing –OH group (the two dihedral angles to be considered) on the other side. But, these rotations are not affecting the (absolute) configuration at the two stereogenic centres. The configurations at the two stereogenic centres have been established by ^{1}H NMR separately, as discussed above. Efforts have been made to correlate the establishment of configuration.

HPLC

Retention factor (k), separation factor (α) and resolution (R_s) for the resolved diastereomers of each of the four β-blockers are given in **Table 1**. Selectivity or separation factor (α) is a measure of relative retention of the two adjacent sample components, $\alpha = k_2 / k_1$. The retention factor or capacity factor (k) was calculated as $k_1 = t_1\text{-}t_0/\ t_0$; t_0 is the retention time of a non-retained component (or the first baseline disturbance peak) which is the solvent as it is essentially unretained by the column. In present case void volume peak was observed at around 2.5 min. R_s values (as provided by the system software with the chromatograms) were found to be the same (**Table 1**) for replicate HPLC analysis (n=4). The diastereomers synthesized by MWI and by conventional heating were found to be in agreement in terms of R_s and elution order. Further, the integration data for % peak area for the separation of each pair of diastereomers, as recorded by the system software, verified that the two diastereomers were in the ratio of 1:1. **Table 1** shows that the highest R_s was for the diastereomeric pair

of Prl, and in terms of decreasing order of R_s the four β-blockers can be arranged as Prl>Atl>Mel>Cal. The diastereomers of Mel were found to have greater retention time in comparison with the others.

HPLC conditions were optimized for separation of all the three pairs of diastereomers by varying the concentration of TEAP buffer, pH and flow rate as described above. The mobile phase consisting of MeCN and triethyl ammonium phosphate buffer of 10 mM pH 3.5 (60:40 *v*/*v*) in isocratic mode at a flow rate of 1.0 mL min^{-1} (and detection at 230 nm) was successful in providing baseline separation of the diastereomeric amide derivatives on the C_{18} column. Sections of chromatograms showing baseline resolution of diastereomeric pairs (of racemic β-blockers) are shown in **Fig.6**.

MeCN was found to be better organic modifier in comparison to methanol as larger retention time and broader peaks were observed with MeOH. The reason for broader peak shapes and higher retention times with methanol is because of its lower dielectric constant (33 D) and higher viscosity (0.59 cP at 25 ^{o}C) in comparison to MeCN which has a higher dielectric constant (37.5 D) and lower viscosity (0.343 cP at 25 ^{o}C).

Fig.6 shows that the retention time for the two diastereomers of each of the four analytes was between 3.91 and 7.72 min. There was observed a peak at 6.7 min corresponding to (*S*)-Npx as the sample injected was expected to contain (*S*)-Npx (the leaving group from the anhydride CDR) because the CDR, being an acid anhydride, was a source of reactive acyl group and the reaction afforded equal amounts of the acylated product (the diastereomer) and the carboxylic acid (Npx); there was no interference in the diastereomeric separation as it appeared much later from the diastereomeric mixtures of (*RS*)-Prl, (*RS*)-Atl, and (*RS*)-Cal while it eluted much earlier than the diastereomers of (*RS*)-Mel. Retention time for (*S*)-Npx was found to be 6.4 min when the sample of pure isomer was subjected to hplc under identical conditions.

The values of retention factors for the first eluting and second eluting diastereomers (Table 1) were in agreement with the values of retention factors obtained for DsI and DsII which were run under optimized hplc conditions. Therefore, it can be contended that DsI has (*S,S*)- configuration (and elutes at first) while DsII has (*S,R*)- configuration (and elutes later, with the mobile phase).

Justification for separation and elution order of diastereomers

Separation of diastereomers is considered to be influenced by the distance between the two asymmetric centers in the substrate and the reagent and the distance should be minimized for the best separation. The conformational rigidity around the chiral centers is another important factor for separation. The structures of the diastereomers in the present case satisfy this condition as there is no free rotation near the asymmetric center of the substrate due to partial double bond character of the amide bond.

It remains difficult to distinguish the diastereomers at the time of elution unless the elution order is compared with the standard samples or the configuration of the diastereomers is confirmed and correlated with structures developed by another method. The diastereomers, in the present case, were isolated by open column chromatography and the absolute configuration of the diastereomers so isolated was determined by ^{1}H NMR. The results have been correlated with DFT based lowest energy structures to explain the elution order.

Since there is no ionic group in the diastereomers and the mobile phase causing separation has pH around 3.5 it was inferred that ionic interactions were not playing a role. Thus, the factors contributing to the hydrophobicity of diastereomers are affecting separation process and elution order. The overall hydrophobicity due to (i) the presence of naphthyl group in propranolol and in the CDR, (ii) the partial double bond character of the amide bond in the diastereomers, and (iii) the rheological properties of the mobile phase are responsible for different retention times and different partition coefficients of the (*S,R*)- and (*S,S*)-diastereomers. Therefore, for these different physical properties the diastereomers elute one after another. (*S,R*)-diastereomer is slightly less polar than (*S,S*)-diastereomer. The presence of H-bonding in the (*S,S*)-diastereomer makes it more polar and causes a greater affinity with the mobile phase and thus the same is eluted first. The same explanation is applicable for the diastereomers of all the other β-blockers under study.

Method Validation

To evaluate the validity of method, samples of drug of different concentration in the range of 40 to 200 ng/mL were analyzed. Inter-day and intra-day assay studies were carried out to determine accuracy and precision, and the results are represented as relative standard deviation (RSD). The limit of detection (LOD) and limit of quantification (LOQ) were also evaluated

Linearity, limit of detection and limit of quantitation

Concentration of diastereomer (x) versus peak area (y) were plotted for both diastereomers of Prl prepared with CDR in the range of 40 to 200 ng/mL and linear regression equations were used to determine the slopes and correlation coefficients. A good linear relationship was obtained over this range.

LOD and LOQ represent the concentration of the analyte that would yield a signal to noise ratio (S/N) of 3 and 10, respectively. They were determined by injecting a series of diluted solutions of mixture of diastereomers. LOD was found to be 0.4 ng/mL and LOQ was found to be 1.2 ng/mL for each of the diastereomers prepared with CDR.

Accuracy and Precision

The intra-day precision was carried out by the replicate analysis of five different concentrations (40, 80, 120, 160, 200 ng/mL) of the diastereomers of (*RS*)-Prl on three successive times. The inter-day precision was evaluated through a replicate analysis of five concentrations for a period of three successive days. This was done for all the β-blockers while the data corresponding to diastereomers of Prl prepared with CDR is given as representative. The recovery and RSD for each of the diastereomers were calculated. Small values of RSD and high percentage recoveries indicate the accuracy of the proposed method. The calculated values of RSD varied from 0.92–1.98% and recovery was >94.3%. The results of intraday and inter-day precision are summarized in **Table 2**.

4. Conclusion

Commercial availability of Npx as the pure (*S*)-enantiomer allowed easy synthesis of a symmetrical anhydride of (*S*)-(+)-Npx using DCC under MWI; the acid anhydride is highly reactive and stable at room temperature. Under basic conditions it enabled convenient preparation of diastereomeric amide of certain β-blockers. The method provides an efficient novel carbon-N bond forming reaction by using "aromatic carboxylic anhydride" (i.e., the (*S*)-(+)-Npx anhydride). Besides, presence of hydrophobic naphthyl ring helped RP-HPLC separation of the diastereomers along with facilitation of their on-line detection due to high molar absorptivity related to conjugated system.

In the present case structures of the diastereomers have been confirmed by isolating the diastereomers using open column chromatography and recording their ^{1}H NMR spectrum while literature reports on enantioresolution by indirect approach are limited to synthesis of diastereomers and their HPLC separation only. DFT based lowest energy optimized structures of the diastereomers supplemented confirmation of absolute configuration of the diastereomers. LOD and LOQ were found to be 0.4 ng/mL and 1.2 ng/mL, respectively, for each of the diastereomers under simple reversed-phase HPLC conditions. Optimised and validated RP-HPLC separation conditions can be successfully applied for determination and control of enantiomeric purity routinely in laboratories associated with regulatory agencies and industries, even without resorting to DFT calculations, each time.

5. Acknowledgement

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Table 1. Chromatographic separation data for diastereomers of β-blockers prepared with the CDR

β- blockers for which diastereomers were prepared with the CDR	**Characteristics of separated diastereomers**			
	k_1	k_2	α	R_s
Propranolol	1.77	2.57	1.45	8.26
Atenolol	1.64	2.24	1.36	6.35
Carvedilol	1.57	1.65	1.05	5.98
Metoprolol	2.98	3.21	1.07	6.14

Mobile phase, binary mixture of MeCN and triethyl ammonium phosphate buffer of pH 3.5 (60:40, *v*/*v*) in isocratic mode at a flow rate of 1.0 mL min^{-1};
k_1= retention factor of first eluted diastereomer
k_2= retention factor of second eluted diastereomer
α = separation factor
R_s = resolution.

Table 2. Validation data for separation of diastereomers of (*RS*)-propranolol prepared with the CDR

	First eluting diastereomer			Second eluting diastereomer		
Linearity:						
Range	40-200 ng/mL			40-200 ng/mL		
Slope	5.15			5.43		
Intercept	422.9			349.8		
Correlation coefficient (r^2)	0.993			0.997		
Accuracy and precision:						
Concentration (actual) (ng/mL)	Mean±S.D (observed) (ng/mL)	Recovery (%)	RSD (%)	Mean±S.D (observed) (ng/mL)	Recovery (%)	RSD (%)
Intra-day precision						
40	19.92±0.374	99.6	1.88	19.8±0.251	99.3	1.27
80	39.40±0.362	98.5	0.92	39.1±0.738	97.9	1.89
120	60.36±1.092	100.6	1.81	58.10±0.85	96.8	1.46
160	77.76±0.995	97.2	1.28	77.42±1.153	96.7	1.49
200	99.2±1.944	99.2	1.96	100.2±1.352	100.2	1.35
Inter-day precision						
40	19.44±0.258	97.2	1.33	19.72±0.303	98.6	1.54
80	38.68±0.765	96.7	1.98	38.24±0.412	95.6	1.08
120	59.64±0.697	99.4	1.17	57.69±1.03	96.2	1.79
160	76.10±0.981	95.1	1.29	76.72±1.104	95.9	1.44
200	96.20±1.69	96.1	1.77	94.3±1.593	94.3	1.69
Sensitivity :						
LOD(ng/mL)	0.4					
LOQ(ng/mL)	1.2					

(n = 5), SD = standard deviation, RSD = relative standard deviation

Figure captions:

Figure 1. Structures of (a): (*RS)*-Propranolol, (b): (*RS)*-Atenolol, (c): (*RS)*-Carvedilol, (d): (*RS)*-Metoprolol.

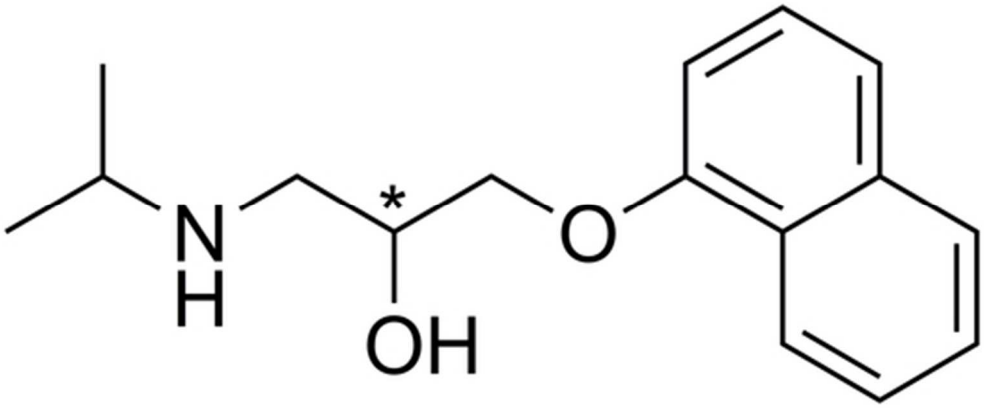
Figure 2. The general scheme for synthesis of naproxen anhydride (the CDR) and synthesis of diastereomers of propranolol (as a representative β-blocker); the diastereomers are designated as the (*S,R*)- and (*S,S*)-diastereomers, the first letter refers to the configuration of the CDR and the second to that of the Prl.

Figure 3. Effect of MWI time on completion of derivatization reaction of propranolol with the CDR

Figure 4. Sections of ^{1}H NMR spectra of diastereomers illustrating chemical shift values for the peaks of –OH (at the stereogenic center) signal at d 2.26 and d 2.54, respectively, in (i) the (*S,R*)-diastereomer, and in (ii) the (*S,S*)-diastereomer. This difference in chemical shift of –OH signal is attributed to the formation of H-bonding in (*S,S*)-diastereomer only.

Figure 5. Lowest energy structures of diastereomers of (*RS*)-propranolol drawn using the program Gaussian 09 Rev. A.02 and hybrid density functional B3LYP with 6-31G basis set; (**i)** and (**ii)** correspond to (*S,R*)-diastereomer (DsII) and (*S,S*)-diastereomer (DsI), respectively.

Figure 6. A section of the chromatogram showing separation of diastereomers of (a) (*RS*)-propranolol, (b) (*RS*)-atenolol, (c) (*RS*)-carvedilol, (d) (*RS*)-metoprolol; Mobile phase: MeCN-TEAP buffer (60:40, *v*/*v*); flow-rate 1.0 mL/ min; injection volume 20μL; the first eluting peak corresponds to (*S,S*)-diastereomer in all the four cases.



Prl Fig 1(A)
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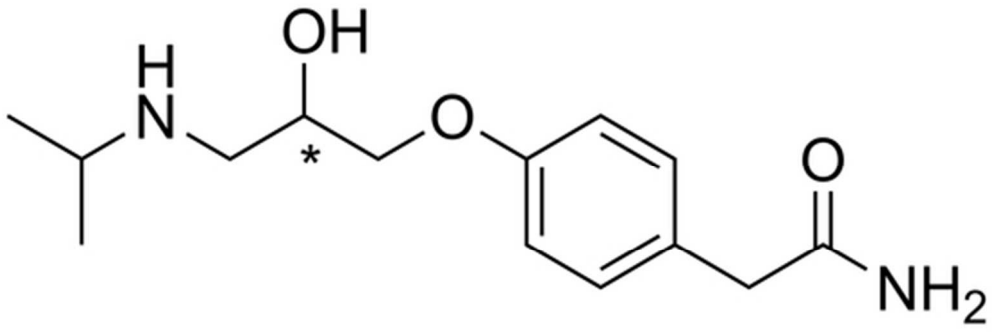

Atl Fig 1(B)
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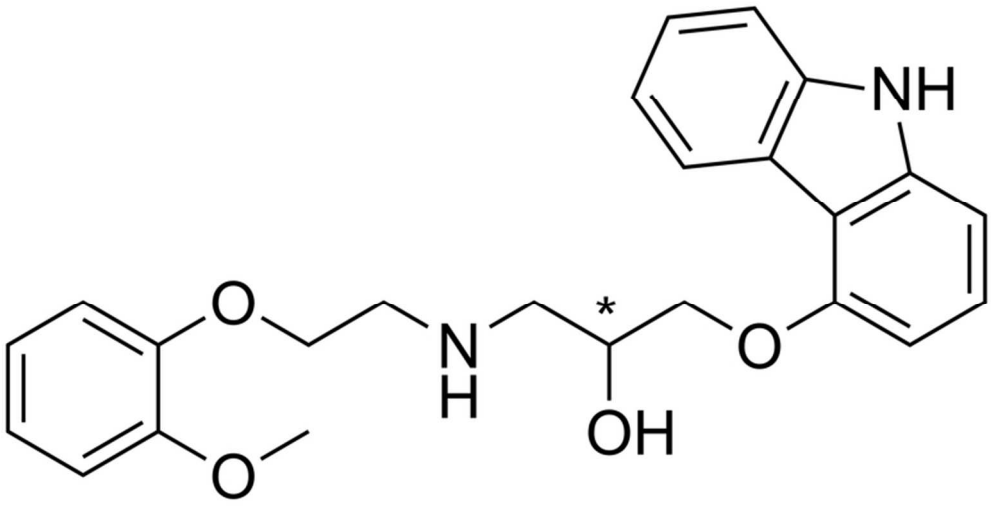

Cal Fig 1(C)
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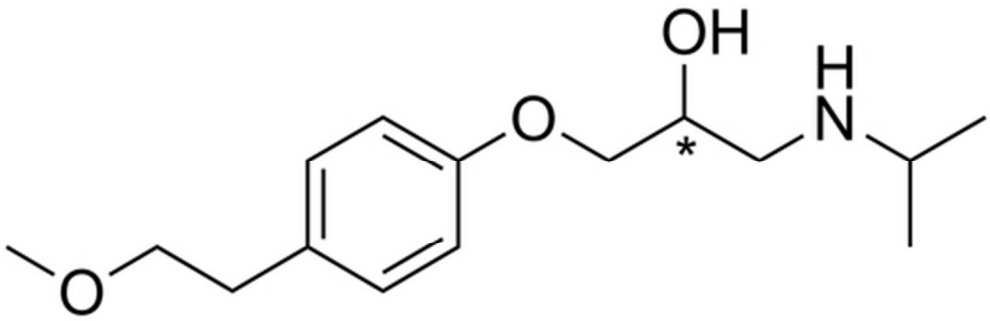

Mel fig 1(d)
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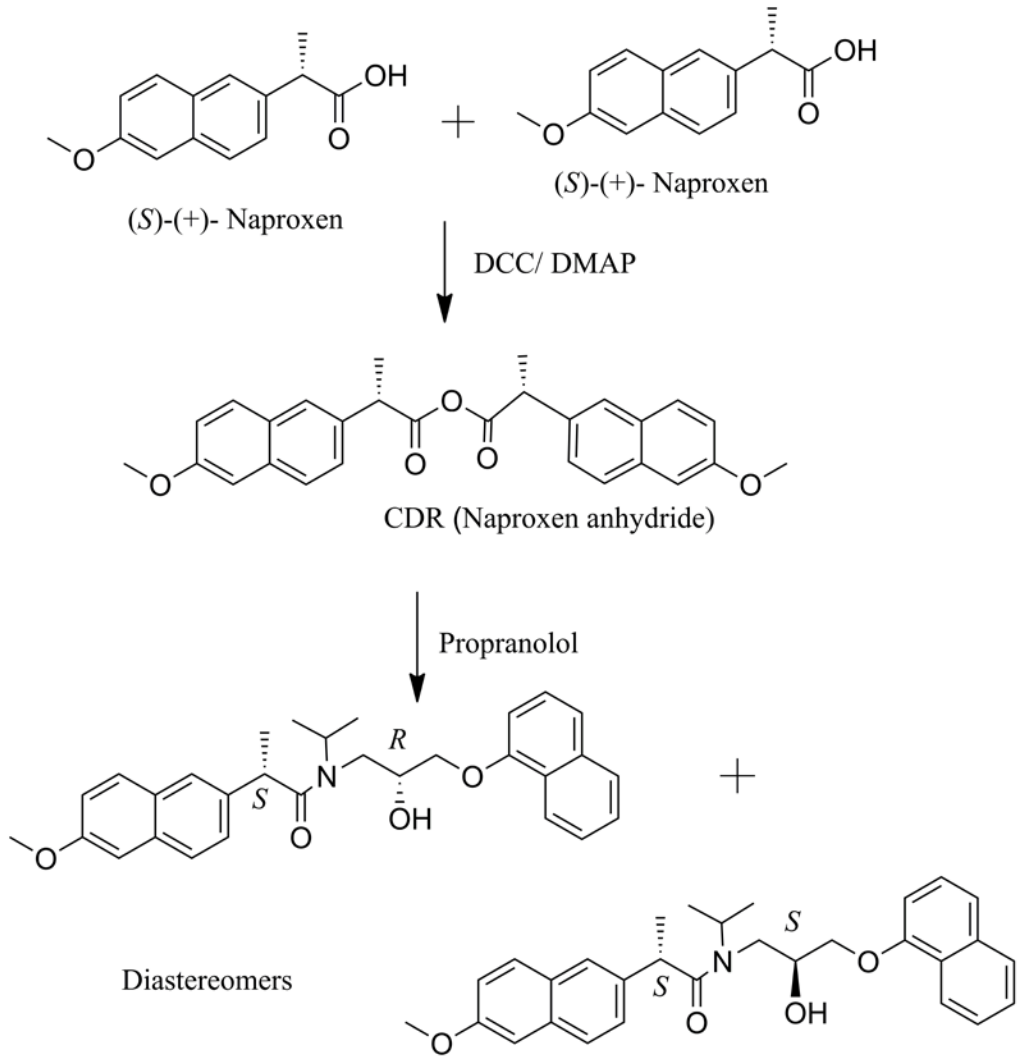

Figure 2. The general scheme for synthesis of naproxen anhydride (the CDR) and synthesis of diastereomers of (*RS*)-propranolol (as a representative β-blocker); the diastereomers are designated as the (*S*,*R*)- and (*S*,*S*)-diastereomers, the first letter refers to the configuration of the CDR and the second to that of the Prl.

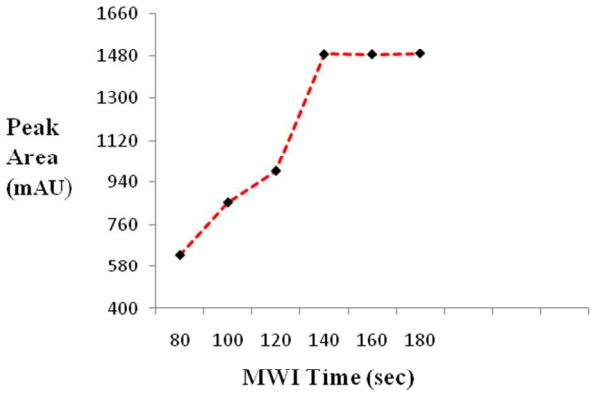

fig 3 effect of MWI
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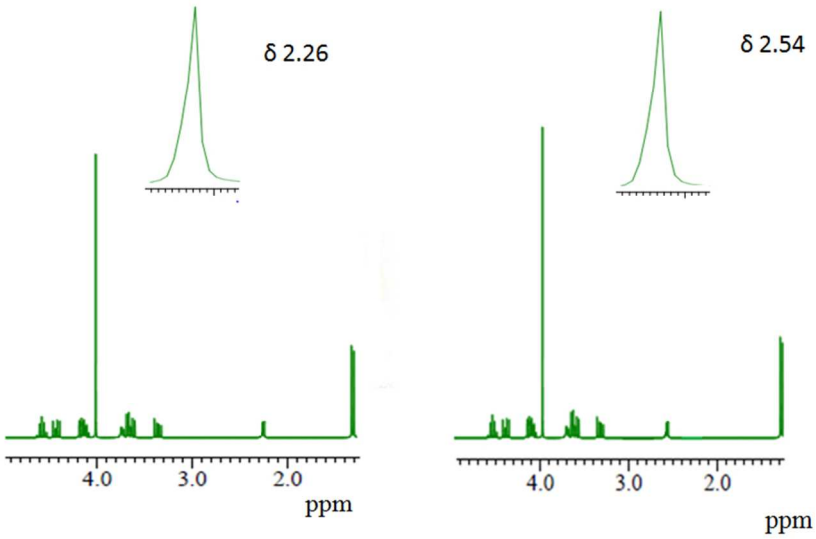

(i) (*S*,*R*) diastereomer (ii) (*S*,*S*) diastereomer

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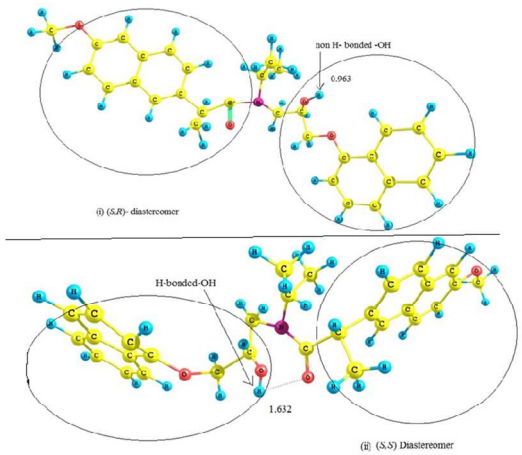

Lowest energy structures of diastereomers of (RS)-propranolol
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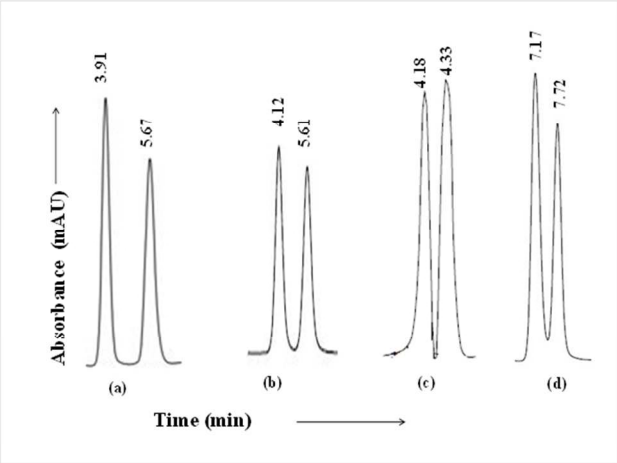

Chromatogram showing diastereomers' separation
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