

## Synthesis of Cyanuric Chloride based Chiral Reagent for RP-HPLC Enantioseparation of (*RS*)-Propranolol

H.S. AL-SHEHRI<sup>1</sup>, V. ALWERA<sup>2</sup>, K.C. NILUGAL<sup>3</sup>, K.K. JOSHI<sup>4</sup> and S. ALWERA<sup>2,\*</sup> 

<sup>1</sup>Chemistry Division, King Khaled Military Academy, SANG, 11495, Riyadh, Saudi Arabia

<sup>2</sup>Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee-247667, India

<sup>3</sup>School of Pharmacy, Management and Science University, Shah Alam-40100, Malaysia

<sup>4</sup>Department of Chemistry, Lovely Professional University, Phagwara-144001, India

\*Corresponding author: E-mail: ashiv.kumalwera@gmail.com

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In this work, four cyanuric chloride based chiral reagents were prepared *via* nucleophile substitution of chlorine atom by L-proline derivatives and characterized by UV, FT-IR, HRMS, NMR and elemental analysis. Racemic propranolol was chosen for the chiral recognition study. The prepared chiral reagents were used in the synthesis of diastereomeric derivatives of (*RS*)-propranolol, under microwave heating conditions. RP-HPLC was used to separate the prepared diastereomeric derivatives. The effect of varying eluting phase concentrations and sample concentrations was optimized. The DFT calculations were performed using Gaussian 09 Rev A.02 to create the lowest energy optimised structures of diastereomeric derivatives. LOD (0.324 ng mL<sup>-1</sup>), LOQ (0.972 ng mL<sup>-1</sup>), calibration range (0.02-2.0 mg mL<sup>-1</sup>), correlation-coefficient (0.999), and recovery were the validation parameters for the present method (99.09 and 99.81 % for inter-day assay and 98.47 and 99.72 % for intra-day assay).

**Keywords:** (*RS*)-Propranolol, Cyanuric chloride, Chiral reagent, Derivatization, RP-HPLC.

### INTRODUCTION

Cyanuric chloride, an agrochemical class active ingredient, is an inexpensive and readily accessible chemical [1]. It is also known as trichloro-*s*-triazine or 2,4,6-trichloro-1,3,5-triazine. Cyanuric chloride has been used, as backbone, in synthesis of varieties of herbicides (atrazine, simazine, propazine, cyanazine, *etc.*) [1]. Cyanuric chloride has been used in environmental applications, as a catalyst in varieties of chemical reactions [2,3], organic synthesis [4,5], preparation of photoelectric materials [6-9], nanoparticles and polymer for energy storage [6,10-13], synthesis of the porous and microporous polymer [14], preparation of chiral stationary phase [15,16], chiral derivatizing reagents [17-21] and synthesis of pharmaceuticals [1,22]. The optically pure amino acids or amines based cyanuric chloride derivatives have been explored widely for enantioseparation of the varieties of racemic compounds [17-21]. According to literature [15,16], the cyanuric chloride-based chiral stationary phases (CSPs) are easy to synthesize and show remarkable separation capacity for a huge range of racemic pharmaceuticals.

Cyanuric chloride shows trifunctionality in the presence of three chlorine atoms (leaving group) and undergoes sequential and managed displacement of three Cl atoms in presence of appropriate nucleophiles (alcohols, amines, thiols, *etc.*). It is a useful source for adding chromophore into poor UV absorbing compounds since it is easy to alter the cyanuric chloride moiety and has good UV absorbing characteristics. Indirect separation of enantiomers of racemic amino alcohols, amino acids and racemic amines has been widely utilized using cyanuric chloride based chiral reagents [17-21].

(*RS*)-Propranolol (1-(1-methylethylamino)-3-(1-naphthyl-oxy) propan-2-ol) is a non-selective  $\beta$ -adrenolytics (Fig. 1). (*RS*)-Propranolol is the first developed  $\beta$ -adrenolytics and considered as an essential medicine according to WHO list [23,24]. It is used for the treatment of anxiety, panic attacks, hypertension as well as to avoid migraine headaches and angina. (*RS*)-Propranolol is marketed as a racemic combination (*R* and *S*-enantiomers), but the desirable action is only associated to (*S*)-enantiomer and show multiple times (more the five hundred

times) higher activity compared to (*R*)-enantiomer [25]. The variety of the activated chiral reagents have been used for enantio-separation of (*RS*)-propranolol such as (1*R*,2*R*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [26] and 2,3,4,6-tetra-*O*-acetyl- $\beta$ -D-galactopyranosyl isothiocyanate [27]. Also, the enantiomeric-separation of (*RS*)-propranolol and its derivatives has been achieved on chiral stationary phases such as immobilized acid glycoprotein and ovomucoid [28], cellulose *tris*(3,5-dimethylphenylcarbamate) [29] and polysaccharide and Pirkle-type chiral stationary phases (CSPs) [30,31].

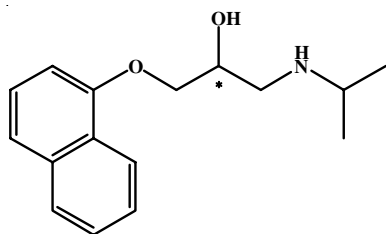


Fig. 1. Structures of (*RS*)-propranolol

In this work, a complete and successful method of the synthesis of new chiral reagents and their use in separation of racemic (*RS*)-propranolol using by RP-HPLC are described. Here, the activated chiral reagent of cyanuric chloride were prepared using a substitution reaction of derivatives of L-proline (CR1-4) and characterized with UV, IR, HRMS, NMR, and elemental analysis. The chiral reagent of high molar absorbing chiral moieties was used to make diastereomeric derivatives of racemic propranolol (*RS*)-propranolol under microwave heating conditions. After the derivatization, chiral reagents convert (*RS*)-propranolol into high molar absorbing molecules, allowing for sensitive detection with a UV-Visible detector. The  $C_{18}$  column of the RP-HPLC was used to separate synthesized diastereomeric derivatives pairs of (*RS*)-propranolol. The organic phase (methanol or acetonitrile) with triethylammonium phosphate (TEAP) buffer in distilled water was used as the eluting solvent for chromatography. Also, the separation mechanism for the diastereomeric derivatives was established. Additionally, the molecular structures of the diastereomeric derivatives were developed for the lowest energy using the Gaussian 09 Rev. A.02 program, the 3D molecular configuration and the elution order of the diastereomeric derivatives were established. The current system's linearity, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) were all validated.

## EXPERIMENTAL

The chemicals *viz.* cyanuric chloride, assay 99%, L-proline, Boc-L-proline, phenol, aniline and racemic propranolol were obtained from Sigma-Aldrich (St Louis, USA). Chloroform, dichloromethane, sodium bicarbonate, ethyl acetate, ethanol, tetrahydrofuran, phosphoric acid and triethylamine (TEA) of analytical reagent grade, acetonitrile and methanol of HPLC grade were obtained from E. Merck (Mumbai, India).

**Chromatographic system:** HPLC system (LC-20AD, Shimadzu, Japan) consisted of a DGU-20A5 on-line degasser

unit, low-pressure gradient unit, low pressure mixing type gradient, parallel double plunger pump, manual injector with 20  $\mu$ L volume loop size, high pressure mixer, SPD-M20A diode array detector, SPD-20A/20AV (UV-VIS detector), CTO-20AC column oven and LC solution and DAO (data access objects) 3.5 operating software. LiChrospher  $C_{18}$  column (L  $\times$  I.D. 25 cm  $\times$  4.6 mm, 5  $\mu$ m particle size) was from Merck (Darmstadt, Germany).

Other instruments used in present investigations were Microwave-Multiwave 3000 (800 W, Perkin-Elmer, Shelton, USA), pH meter Cyberscan 510 (Singapore), Milli-Q system of Millipore (Bedford, USA) to obtain purified water (18.2  $M\Omega$   $cm^3$ ) from double distilled water. FT-IR Spectrometer (Nicolet-6700, Thermo Scientific, USA), elemental analyzer (Vario EL III, Germany), and NMR spectrometer 400 MHz (JEOL Inc., USA). UV spectra were recorded in MeOH (Shimadzu, UV-2450 spectrophotometer).

**Preparation of stock solutions:** A stock solution of  $NaHCO_3$  (0.1 M), (*RS*)-propranolol and chiral reagents (1-4) were prepared respectively in water, MeOH and MeCN. A solution of triethylammonium phosphate (TEAP) buffer (10 mM) was also prepared in double distilled water.

### Synthesis of derivatives of L-proline

**Phenyl ester of L-proline (Ph-O-L-proline):** Under the nitrogen atmosphere, 0.88 g (4 mmol) boc-L-proline and 0.40 g (4 mmol) phenol were dissolved in 15 mL dry DCM and then the solution of EDC (0.72 g; 4.2 mmol) in 15 mL dry DCM was added dropwise. The reaction was allowed to stir for 3 h. After the reaction, the mixture was washed with 1N HCl solution and extracted with DCM. The organic layer was concentrated under reduced pressure [32,33]. The dried product was treated with 1N HCl solution (Boc deprotection) and washed with DCM [34,35]. The organic layer was concentrated under reduced pressure.

Similarly, methyl ester of L-proline ( $CH_3$ -O-L-proline) and phenyl amide of (Ph-N-L-proline) were prepared by using methanol and aniline, respectively, instead of phenol (Fig. 2).

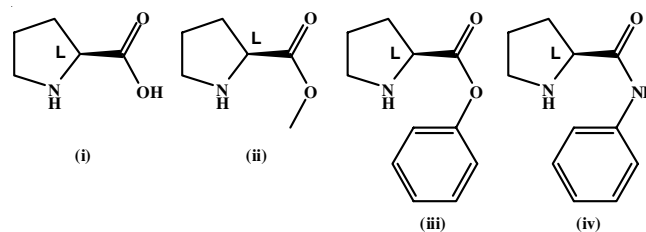


Fig. 2. L-proline and its synthesized derivatives

**$CH_3$ -O-L-proline:** Yield: 98%, colour: white,  $[\alpha]_D^{25} = -80^\circ$  ( $c = 0.5$ , MeOH), UV (nm, in MeCN): 222 ( $\lambda_{max}$ ), IR (KBr,  $cm^{-1}$ ): 2980, 2892, 2850, 1740, 1470, 1330, 1180 and 857.  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  1.72 (m, 2H), 1.85 (m, 1H), 1.94 (m, 1H), 3.2 (m, 2H), 3.56-3.64 (m, 2H) and 3.71 (s, 3H). HRMS: Calcd. for  $C_6H_{11}NO_2$ : 130.0823 ( $M^+ + H$ ), found 130.1012. Anal. calcd. (found) % for  $C_6H_{11}NO_2$ : C, 55.80 (55.68); H, 8.58 (8.26); N, 10.84 (10.92).

**Ph-O-L-proline:** Yield: 92%, colour: white,  $[\alpha]_D^{25} = -72^\circ$  ( $c = 0.5$ , MeOH), UV (nm, in MeCN): 230 ( $\lambda_{max}$ ), IR (KBr,

cm<sup>-1</sup>): 3040, 2985, 2830, 1748, 1608, 1485, 1465, 1320, 1180, 1020, 986 and 864. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.70-1.90 (m, 3H), 2.01-2.06 (m, 1H), 2.95-3.10 (m, 2H), 3.80 (m, 2H), 7.12 (dd, 2H), 7.45 (t, 2H) and 7.65 (t, 1H). HRMS: Calcd for C<sub>11</sub>H<sub>13</sub>NO<sub>2</sub>: 192.0980 (M<sup>+</sup>+H), found 192.1124. Anal. calcd. (found) % for C<sub>11</sub>H<sub>13</sub>NO<sub>2</sub>: C, 69.09 (68.85); H, 6.85 (6.34); N, 7.32 (7.12).

**Ph-N-L-proline:** Yield: 94%, colour: white, [α]<sub>D</sub><sup>25</sup> = -76° (c = 0.5, MeOH), UV (nm, in MeCN): 233 (λ<sub>max</sub>), IR (KBr, cm<sup>-1</sup>): 3391, 3060, 2950, 2868, 2820, 1689, 1620, 1540, 1440, 1350, 1240, 1160, 1070, 878 and 736. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.71-1.94 (m, 3H), 2.04-2.08 (m, 1H), 2.85-3.04 (m, 2H), 3.64-3.68 (m, 1H), 3.78-3.81 (m, 1H), 7.14-7.18 (m, 1H), 7.46 (m, 4H) and 8.8 (s, 1H). HRMS: Calcd for C<sub>11</sub>H<sub>14</sub>NO<sub>2</sub>: 191.1140 (M<sup>+</sup>+H), found 191.1020. Anal. calcd. (found) % for C<sub>11</sub>H<sub>14</sub>NO<sub>2</sub>: C, 69.45 (69.07); H, 7.42 (7.72); N, 14.73 (14.52).

#### Synthesis of cyanuric chloride based chiral reagents:

A solution of CH<sub>3</sub>-O-L-proline (260 mg, 2 mmol) and Na<sub>2</sub>CO<sub>3</sub> (0.5 g) was prepared in 20 mL of acetone. Then under stirring, the solution of cyanuric chloride (368 mg, 2 mmol) in 10 mL acetone was added. The reaction mixture was then stirred for 1 h at 20 °C. The reaction was quenched by adding 15 mL water. The crystallize product was obtained on removal of the acetone and the product was filtered and washed with ice-cold water [36]. The dichloromethane was used to extract the filtrate and concentrated under reduced pressure to obtain **CR-2**. Similarly, chiral reagents of cyanuric chloride with L-proline, Ph-O-L-proline and Ph-N-L-proline were prepared (Figs. 3 and 4).

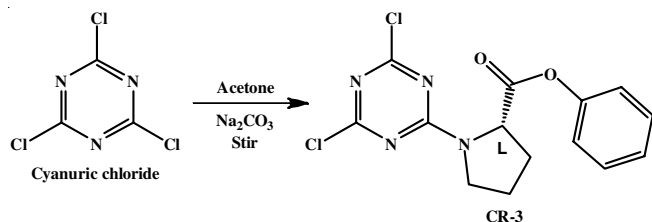


Fig. 3. Synthesis of chiral reagent of cyanuric chloride

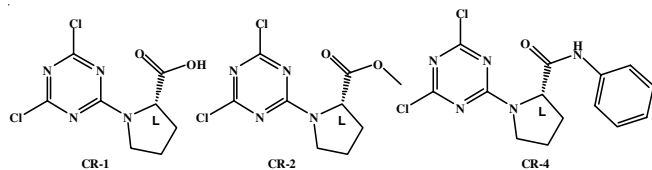


Fig. 4. Structure of CR-1, CR-2 and CR-3

**CR-1:** Yield: 94%, colour: white, [α]<sub>D</sub><sup>25</sup> = -68° (c = 0.5, MeOH), UV (nm, in MeCN): 232 (λ<sub>max</sub>), IR (KBr, cm<sup>-1</sup>): 3300, 3000, 2936, 2872, 2812, 1710, 1660, 1480, 1460, 1410, 1340, 1260, 1070, 905 and 730. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.0-2.15 (m, 3H, -CH<sub>2</sub>-), 2.25-2.33 (m, 1H, -CH-), 3.75-3.95 (m, 2H, -CH<sub>2</sub>-) and 4.30-4.35 (m, 1H, -N-CH<sub>1</sub>-). HRMS: Calcd for C<sub>8</sub>H<sub>8</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: 263.9995 (M<sup>+</sup>+H), found 263.9205; Anal. calcd. (found) % for C<sub>8</sub>H<sub>8</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: C, 36.52 (36.82); H, 3.07 (3.11); N, 21.30 (21.25).

**CR-2:** Yield: 96%, colour: white, [α]<sub>D</sub><sup>25</sup> = -59° (c = 0.5, MeOH), UV (nm, in MeCN): 234 (λ<sub>max</sub>), IR (KBr, cm<sup>-1</sup>): 3290, 2970, 2910, 2880, 2830, 1770, 1480, 1460, 1370, 1320, 1180, 980 and 745. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.0-2.15 (m, 3H, -CH<sub>2</sub>-),

2.27-2.34 (m, 1H, -CH-), 3.72 (s, 3H, -O-CH<sub>3</sub>), 3.75-3.95 (m, 2H, -CH<sub>2</sub>-) and 4.32-4.36 (m, 1H, -N-CH<sub>1</sub>-). HRMS: Calcd for C<sub>9</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: 278.0181 (M<sup>+</sup>+H), found 278.1168; Anal. calcd. (found) % for C<sub>9</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: C, 39.01 (38.89); H, 3.64 (3.54); N, 20.22 (20.55).

**CR-3:** Yield 92%, colour: white, [α]<sub>D</sub><sup>25</sup> = -62° (c = 0.5, MeOH), UV (nm, in MeCN): 233 (λ<sub>max</sub>), IR (KBr, cm<sup>-1</sup>): 3050, 2970, 2900, 2850, 1760, 1610, 1585, 1490, 1465, 1320, 1240, 1210, 1089, 989, 857 and 750. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 1.96-2.21 (m, 3H, -CH<sub>2</sub>-), 2.32-2.38 (m, 1H, -CH-), 3.70-3.92 (m, 2H, -CH<sub>2</sub>-) and 4.29-4.38 (m, 1H, -N-CH<sub>1</sub>-), 7.1 (dd, 2H, Ar), 7.4 (t, 2H, Ar), 7.65 (t, 1H, Ar). HRMS: Calcd. for C<sub>14</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: 339.0337 (M<sup>+</sup>+H), found 339.0854. Anal. calcd. (found) % for C<sub>14</sub>H<sub>12</sub>Cl<sub>2</sub>N<sub>4</sub>O<sub>2</sub>: C, 49.58 (48.98); H, 3.57 (3.71); N, 16.52 (16.85).

**CR-4:** Yield 94%, colour: pale white, UV (nm, in MeCN): 232 (λ<sub>max</sub>), IR (KBr, cm<sup>-1</sup>): 3385, 3300, 2940, 2810, 1730, 1610, 1580, 1530, 1440, 1356, 1240, 1110, 980, 885 and 735. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 2.08-2.19 (m, 3H, -CH<sub>2</sub>-), 2.31-2.39 (m, 1H, -CH-), 3.69-3.87 (m, 2H, -CH<sub>2</sub>-), 4.62-4.65 (m, 1H, -CH-), 4.29-4.38 (m, 1H, -N-CH<sub>1</sub>-), 7.15 (m, 1H, Ar), 7.42-7.47 (m, 4H, Ar), 8.89 (s, 1H, -NH-CO-). HRMS: Calcd. for C<sub>14</sub>H<sub>13</sub>Cl<sub>2</sub>N<sub>5</sub>O: 338.1920 (M<sup>+</sup>+H), found 338.2011. Anal. calcd. (found) % for C<sub>14</sub>H<sub>13</sub>Cl<sub>2</sub>N<sub>5</sub>O: C, 49.72 (49.22); H, 3.87 (3.76); N, 20.71 (20.98).

#### Synthesis of diastereomeric derivatives of (RS)-propranolol:

The diastereomeric derivatives of (RS)-propranolol were synthesized under microwave irradiation. The reaction mixture of (RS)-propranolol (50 μL, 1 mM), **CR-1** (70 μL, 1 mM) and TEA (10 μL) was microwaved for 60 s at 80% power (800 W) [37] (Figs. 5 and 6).

The following variables were used to optimize the experimental conditions for diastereomeric derivatives synthesis: microwave irradiation (MWI) period (30 s to 100 s) at 80% power (800 W); pH 9-11; and (RS)-propranolol:chiral reagents ratio (1:1 to 1:3). The reaction progress and completion was

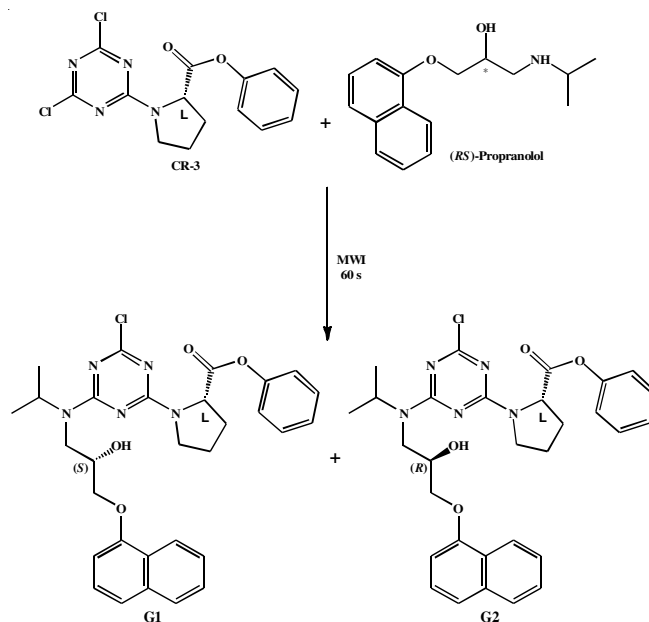


Fig. 5. Diastereomeric derivatives of (RS)-propranolol synthesized by CR-3

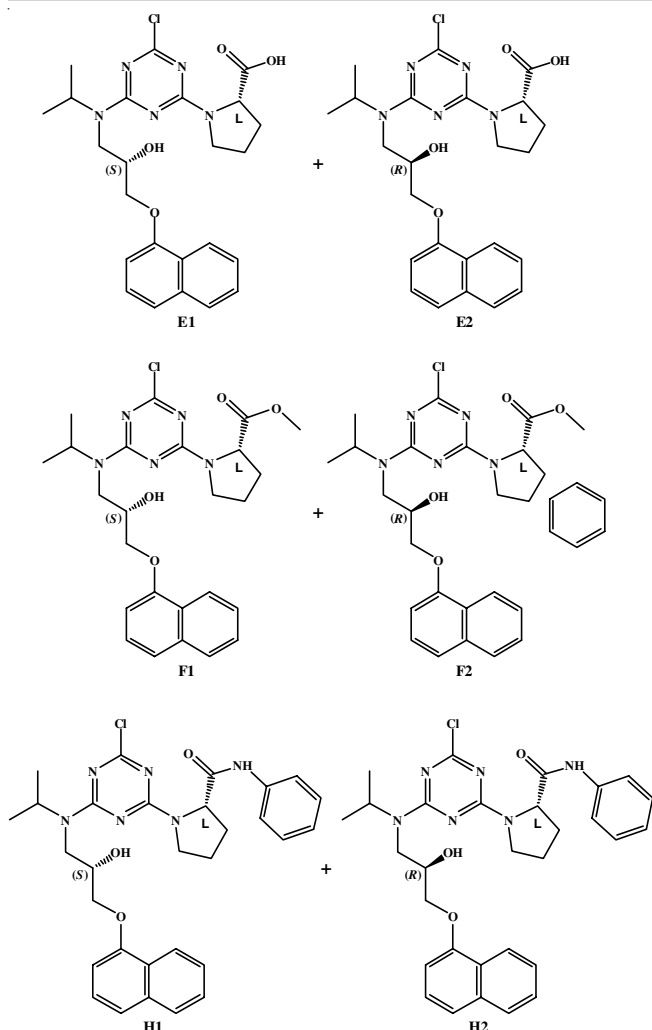


Fig. 6. Diastereomeric derivatives of (*RS*)-propranolol E1 and E2, F1 and F2 and H1 and H2, respectively, synthesized by CR-1, CR-2 and CR-3

monitored by recording peak area of the chromatograms of diastereomeric derivatives, under optimized RP-HPLC separation conditions. The similar approach was also applied for synthesis of diastereomeric derivatives of (*RS*)-propranolol with CR-2, CR-3 and CR-4.

## RESULTS AND DISCUSSION

### Chiral reagents and derivatization of (*RS*)-propranolol:

In the presence of coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), the derivatives of L-proline (A-D) were prepared. EDC, a dehydrating reagent, facilitated the synthesis of esters and amides by removing -H from the amino/alcoholic group and -OH from the carboxylic group [32]. In this work, four new cyanuric chloride-based chiral reagents (CR 1-4) were synthesized by introducing optically pure derivatives of L-proline. The chlorine atoms in the cyanuric chloride are very reactive toward the nucleophile substitution and thus provides an easy synthesis of chiral reagents [17]. The amino group of the synthesized derivatives of L-proline (A-D) acts as very good nucleophiles and substitute one chlorine atom from cyanuric chloride and prepared the chiral

derivatives of cyanuric chloride (CR 1-4). The prepared chiral reagents have two chlorine atoms in their structure, so these are also known as dichloro-*s*-triazine (DCT) [18]. DCTs have been used for enantioseparation applications due to their high molar absorbance and give ease of derivatization [17-21].

After replacing/substituting one chlorine atom from cyanuric chloride, the reactivity of chiral reagents toward the nucleophilic substitution decreases; thus, the second substitution of chlorine needs a high temperature or energy compared to the first substitution [17]. Prepared chiral reagents have two chlorine in their structure. These chlorine atoms show completion for nucleophilic substitution; thus, only one chlorine atom substitutes from chiral reagent at the time of synthesis of diastereomeric derivatives, so no side product from during the reaction [18]. The 60 s of microwave irradiation (MWI) time at 80% (800 W) of power was found sufficient for derivatization of diastereomers of (*RS*)-propranolol. Further heating leads to form side products/impurities in the reaction. In the presence of a high pH reaction, proceed smooth and finish quickly compared to low pH conditions. No racemization was observed during derivatization because the reaction did not occur on the chiral centre [37]. Two peaks of similar intensity in the chromatographic spectrum also indicate that no racemization occurred during the derivatization. The storage conditions and the stability of prepared the chiral reagents were optimized by varying different parameters, such as long-term (at < 5 °C) and short-term (at RT). The chiral reagents were found to be stable for more than six months, but in the basic conditions (pH 7-11) shows poor stability and readily react and deactivate. Also, the derivatization reaction was optimized by varying the reaction time, and it was observed that the 60 s time is sufficient to complete the reaction (Fig. 7).

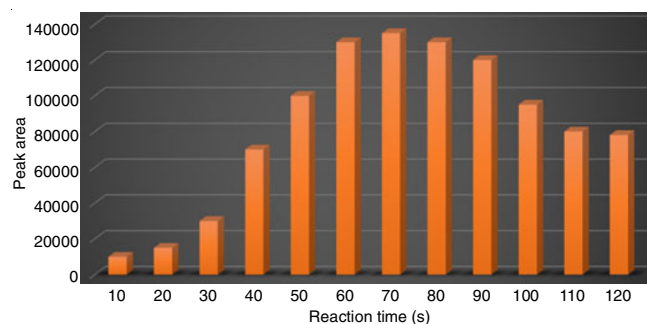


Fig. 7. Effect of microwave irradiation time on the completion of reaction of the formation of diastereomeric derivatives

The structures of diastereomeric derivatives of (*RS*)-propranolol prepared with CR-3 are shown in Fig. 3, while the structures of the remaining diastereomeric derivatives of (*RS*)-propranolol prepared with chiral reagents (1, 2 and 4) are shown in Fig. 4. The diastereomeric derivatives of (*RS*)-propranolol prepared with CR-1, CR-2, CR-3 and CR-4 are designated as E1-E2, F1-F2, G1-G2 and H1-H2, respectively (Fig. 6).

### Separation of prepared diastereomeric derivatives:

Mobile phase (A) consisting of MeCN-TEAP (pH 3.5) in 35 min (gradient, 20% to 80% MeCN) at a flow rate of 1 mL min<sup>-1</sup> was found to be successful in terms of selectivity and reproducibility.

ucibility; values for separation factor ( $\alpha$ ), retention factor ( $k$ ) and resolution ( $R_s$ ) under optimized HPLC conditions are given in Table-1 for the prepared diastereomeric derivatives of (*RS*)-propranolol with chiral reagents. Fig. 8 shows sections of chromatograms showing the resolution G1 and G2 (diastereomeric derivatives prepared with CR-3).

TABLE-1 CHROMATOGRAPHIC SEPARATION DATA OF DDs OF ( <i>RS</i> )-PROPRANOLOL PREPARED WITH CRs (1-4)						
CRs	Separation data for DDs of ( <i>RS</i> )-propranolol					
	$t_1$ (min)	$t_2$ (min)	$k_1$	$k_2$	$\alpha$	$R_s$
CR-1	7.12	9.11	5.47	7.29	1.34	7.96
CR-2	7.65	9.87	5.95	7.97	1.33	7.40
CR-3	12.81	14.92	10.64	11.99	1.12	8.44
CR-4	13.14	15.78	10.94	13.34	1.21	10.56

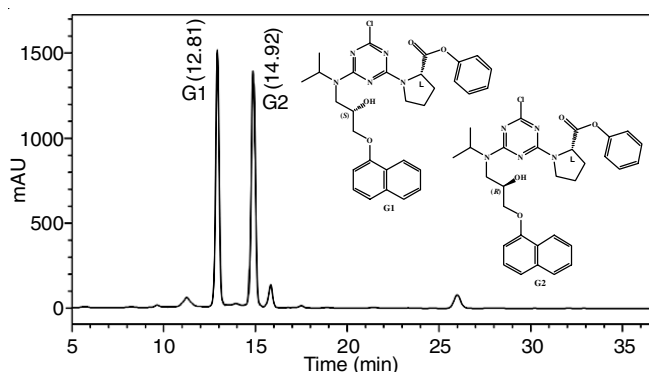


Fig. 8. Chromatogram showing separation of diastereomeric derivatives G1 and G2

The separation of diastereomeric derivatives were investigated using MeOH in the mobile phase as an organic modifier instead of MeCN; under the same HPLC conditions, MeCN provided sharper peaks, improved resolution and shorter retention time. This is due to the low UV cutoff, low viscosity (MeOH viscosity is 0.59 cP while MeCN viscosity is 0.38 cP), and high eluting capacity of MeCN [38,39]. Furthermore, MeCN has unique properties such as dissolving a broad variety of solutes, low acidity and low chemical reactivity; hence, using MeCN as an organic modifier result in a lower retention factor and higher peak heights [17,32]. The UV spectrum corresponding to first and second eluting diastereomeric derivatives was captured (using PDA detector) at 12.81 and 14.92 min (for G1 and G2; as a representative). These were found to be identical.

**Elution order, separation mechanism and lowest energy optimized structures:** The elution order and separation mechanism of the diastereomeric derivatives of (*RS*)-propranolol were investigated using DFT calculations. Gaussian 09 rev. A.02 and hybrid density functional B3LYP with 6-31G\* basis set program was used to create the lowest energy minimized 3D structures of the diastereomeric derivatives (as representative G1 and G2). The optimized structures of G1 and G2 (diastereomeric derivatives of (*RS*)-propranolol prepared with CR-3) are given in Fig. 9. In the generated 3D structures of G1 and G2, it is clearly visible that the optically pure L-proline

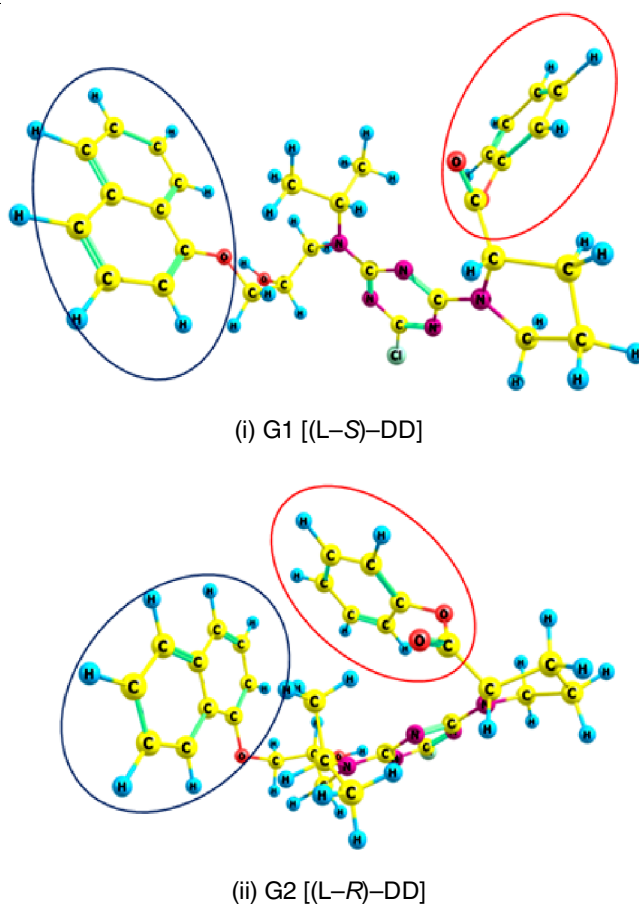


Fig. 9. Structures of diastereomeric derivatives, G1 and G2, optimized for minimized energy, developed using the program Gaussian 09 Rev. A.02 and hybrid density function B3LYP with 6-31G\*

molecule is the main reason to have different molecular structures. In the G1, the aromatic rings of (*RS*)-propranolol and benzene ring of L-proline are arranged at a maximum distance (terminal C-C distance is 13.48 Å), while in the G2 molecule, both aromatic rings are arranged nearby and form a compact structure (terminal C-C distance is 10.89 Å) compared to G1. The hypothesis here is that the bigger diastereomeric derivative (G1) exposes more surfaces to interact with the polar mobile phase. Because of that, G1 having more solubility in the mobile phase and is resultant; it elutes first from the C<sub>18</sub> column compared to G2. The G2 is smaller, showing more hydrophobic properties [32] and interacts comparatively more with the hydrophobic C<sub>18</sub> column, thus eluting in the last. The similar approach was applied to other diastereomeric derivatives and determined their elution order.

Among all prepared diastereomeric derivatives, the lowest elution time was observed for E1 and E2 (respectively, 7.12 and 9.11), while the highest elution time was observed for H1 and H2 (respectively, 13.14 and 15.78). On increasing the carbon ratio in the diastereomeric derivative leads to an increase in elution time. The presence of polar atoms/functional groups with low carbon atoms ratio decreases the hydrophobic interactions, thus elute first. The elution order is given in Table-2.

**Validation:** Validation studies were conducted per ICH guidelines [40] to determine linearity, accuracy and precision.

TABLE-2  
LOWEST ENERGY OPTIMIZED STRUCTURES OF DDs OF (*RS*)-PROPRANOLOL PREPARED WITH CRs AND ELUTION ORDER

CRs	Size of the optimized DDs (terminal C-C distance in Å)		First eluted DD
	( <i>S,L</i> )-DD	( <i>S,D</i> )-DD	
CR-1	13.39 (E1)	8.64 (E2)	E1
CR-2	13.23 (F1)	8.49 (F2)	F1
CR-3	13.48 (G1)	10.89 (G2)	G1
CR-4	12.56 (H1)	10.55 (H2)	H1

Relative standard deviation (RSD), the limit of detection (LOD) and the limit of quantification (LOQ) were established for RP-HPLC separation of diastereomeric derivatives G1 and G2 in a concentration range between 200-2000 ng mL<sup>-1</sup>. The quantification and analysis of stabilities and recoveries were done or calculated using the peak areas. The validation results are shown in Table-3. The calculated recovery values for the first and second eluting diastereomeric derivatives in intra-day assays are 98.47 and 99.72 %, respectively, and 99.09 and 99.81 % in inter-day assays. The LOD and LOQ, respectively, were found to be 0.324 ng mL<sup>-1</sup> and 0.972 ng mL<sup>-1</sup>.

The replicate study of five different concentrations (100, 250, 500, 750 and 1000 ng mL<sup>-1</sup>) of the diastereomeric derivatives (G1 and G2) on three consecutive days was used to determine intra-day precision. Replicate analysis of five concentrations over three days was used to assess inter-day precision. The proposed method's accuracy is demonstrated by high percentage recoveries (> 99%) and low RSD values (1.07-1.39%). Changing the operating conditions, such as mobile phase, flow rate, temperature and detection wavelength, was used to investigate the method's robustness. The validation results do not display any significant changes when the above parameters are modified.

TABLE-3  
METHOD VALIDATION FOR RP-HPLC SEPARATION OF DDs OF (*RS*)-PROPRANOLOL PREPARED WITH CR-3

Linearity	First eluting DD (G1)		Second eluting DD (G2)			
	Range (ng mL <sup>-1</sup> )	Slope	Intercept	Correlation coefficient (R <sup>2</sup> )		
	100-1000	3.871	8.95	0.999		
	100-1000	4.514	7.98	0.998		
Accuracy and precision						
Conc. of each DDs (ng mL <sup>-1</sup> )	First eluting DD (G1)			Second eluting DD (G2)		
	Found conc. mean ± SD (ng mL <sup>-1</sup> )	Recovery (%)	RSD (%)	Found conc. mean ± SD (ng mL <sup>-1</sup> )	Recovery (%)	RSD (%)
Intra-day precision						
100	97.11 ± 0.28	97.11	0.54	98.99 ± 0.36	98.99	0.62
250	246.7 ± 0.37	98.68	0.61	250.1 ± 0.52	100.00	1.24
500	489.5 ± 4.26	97.90	1.16	500.0 ± 4.82	100.00	1.36
750	741.8 ± 6.74	98.90	1.34	748.5 ± 7.15	99.80	1.48
1000	997.8 ± 9.16	99.78	1.71	998.1 ± 10.14	99.81	1.52
	Mean	98.47	1.07		99.72	1.25
Inter-day precision						
100	97.56 ± 0.34	97.56	0.42	100.08 ± 0.18	100.08	0.74
250	248.4 ± 0.61	99.36	1.12	251.2 ± 0.64	100.48	1.22
500	494.3 ± 2.14	98.86	1.28	497.7 ± 3.06	99.54	1.32
750	749.1 ± 5.21	99.88	1.56	742.1 ± 6.28	98.94	1.78
1000	998.2 ± 10.21	99.82	1.82	1000.4 ± 8.61	100.04	1.89
	Mean	99.09	1.24		99.81	1.39

Sensitivity:

LOD (ng mL<sup>-1</sup>): 0.324; LOQ (ng mL<sup>-1</sup>): 0.972; [n (=5) is the number of replicates, SD = standard deviation, RSD = relative standard deviation]

## Conclusion

In summary, this is the successful report on the synthesis of a new chiral reagent based on cyanuric chloride and the synthesis of diastereomeric derivatives of (*RS*)-propranolol. The present method offers a precise, simple, reliable and perfect method for the enantiomers separation and determination of enantiomeric purity of (*RS*)-propranolol with high resolution, low retention times and low LOD and LOQ. The chiral reagents (CRs) successfully facilitated C-N bond formation with racemic (*RS*)-propranolol (under MW irradiation conditions with lower reaction time). The mobile phase MeCN-TEAP was found successful in separating all prepared diastereomeric derivatives. DFT based lowest energy minimized structures of diastereomeric derivatives were found successful in confirming absolute configurations, separation mechanism and elution order. This method can be used to determine trace amounts of amino group containing substances and to regulate their enantiomeric purity.

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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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