ENANTIOSEPARATION OF ORCIPRENALINE, USING HPLC AND CHIRAL REAGENT BASED ON DFDNB

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ABSTRACT

Orciprenaline is a moderately selective β-adrenergic receptor agonist and used in the treatment of asthma. Generally, the (S)-(−)-enantiomer of β-blockers is pharmacologically effective, show about 50–500-fold higher activities than its (R)-(−)-enantiomer. Diastereomers of orciprenaline were synthesized using a chiral derivatizing reagents that was prepared by substituting one fluorine atom in 1,5-difluoro-2,4-dinitrobenzene with chirally pure L-amino acid; L-Phenylalanine. Characterization of the chiral derivatizing reagent was made by using UV, IR, CHN, and ^{1}H NMR. Diastereomers were synthesized under microwave irradiation 45 s at 80% (of 800W) and also by stirring for 50 min at 45 °C. Furthermore, the diastereomers were separated by high-performance liquid chromatography in reversed phase condition on a C_{18} column with detection at 340 nm. Acetonitrile and aqueous trifluoroacetic acid (TFA) were used as the mobile phase components. The conditions of derivatization and chromatographic separation were optimized to achieve good results. The method validation was made for accuracy, precision, limit of detection and limit of quantification.

KEYWORDS Orciprenaline, 1,5-Difluoro-2,4-dinitrobenzene, Enantioseparation, Liquid Chromatography,

INTRODUCTION

Orciprenaline (Orc) is a moderately selective β_2-adrenergic receptor agonist and a bronchodilator used in the treatment of asthma. Generally, the (*S*)-(−)-enantiomer of β-blockers is pharmacologically effective, than its (*R*)-form and shows about 50–500 fold higher activities (Lee and William 1990). But in most cases β-blockers are administered as racemic mixtures.

Many clinical pharmacologists and biologists tend to deal the drug with mixtures of isomers as if one compound were involved and also a physician, presented with such a drug under a brand name is unaware of isomers and generally he then makes mistakes (Ariëns 1984). It is mostly because of non availability of single enantiomer as in enantiomerically purified form. In most of cases the undesired enantiomer can have side effects or even toxic effects. So it is prime importance and challenging task to determine optical purity of the drugs and if it is not enantiomerically pure then to resolve racemic mixtures in to its different enantiomers.

In literature, for separation of such drugs (β-blockers) there have been developed and used two approaches one is direct (no any type of chemical derivatization of enantiomers) and other is indirect approach (derivatization of enantiomers with chiral derivatizing reagent (CDR) to form their diastereomers) to achieve enantiomeric separation of β-blockers using the high performance liquid chromatographic (HPLC) techniques. HPLC techniques have been also utilised previously via indirect method for enantiomeric separation of various active pharmaceuticals; amino acids (Bhushan and Nagar 2013) and selenomethionine (Bhushan and Nagar 2014a) using CDRs based on (*S*)-naproxen moiety, isoxsuprine (Bhushan and Nagar 2015) and selenomethionine (Nagar and Bhushan 2014) using CDRs based on difluorodinitrobenzene moiety.

In literature, certain chiral stationary phases (CSPs) have been used for direct separation of enantiomers of Orc are; sulfobutyl ether β-cyclodextrin (SBECD) (Ngim et al. 2012), methyl-β-cyclodextrin (met-β-CD) (Amini et al. 2000) and sulfated-β-cyclodextrin (S-β-CD) (Yang et al. 2005) as chiral selector on C_{18} column, by direct approach in HPLC. L-Glutamic acid was used as chiral inducing reagent (CIR) for resolution of enantiomers of Orc (Bhushan and Nagar 2015) by direct approach in thin layer chromatography (TLC).

Besides above literature, Bhushan and Nagar (2014b) have been carried out enantioseparation of Orc by using 1,5-difluoro-2,4-dinitrobenzene (DFDNB) based chiral derivatizing reagents (CDRs) having amino acid and amines as chiral moiety.

DFDNB namely as Sanger's reagent a bifunctional variant was first time used for cross linking of proteins such as wool, silk and insulin (Zahn and Meienhofer 1958). Marfey (1984) carried out the reaction of DFDNB with L-alanine amide and formed its chiral variant namely 1-fluoro-2,4-dinitrophenyl-L-alaninamide (FDNP-L-Ala-NH$_2$, Marfey's reagent), in this reaction one fluorine atom in DFDNB was substituted with L-alanine amide. This chiral derivatizing reagent (CDR) was used for HPLC enantioseparation of five DL-amino acids. DFDNB based CDRs have also been used in Bhushan laboratory for the separation of β-blockers (Bhushan and Tanwar 2008; 2009; Bhushan and Dixit 2012) other than the Orc, α-amino acids (Bhushan and Kumar 2008) and α-amino alcohols (Bhushan and Kumar 2009a). There have appeared certain review articles on use of Marfey's reagent (B'Hymer et al. 2003; Bhushan and Brückner 2004; 2011) as CDR.

In view of the characteristics of DFDNB, the literature cited above and the references cited therein on the synthesis of DFDNB based CDRs, L-Phe was chosen as chiral amino acid for synthesis of DFDNB based CDR because the non bonding electron pair of amino group of L-Phe would show resonance by direct conjugation, with the para positioned –NO$_2$ group and would be expected to further increase molar absorptivity of dinitrobenzene moiety. The chiral auxiliary in this CDR contain –CH$_2$C$_6$H$_5$ as the bulky hydrophobic group which enhance the hydrophobicity of the CDR and are thus expected to influence the separation.

The CDR was used for liquid chromatographic enantioseparation of Orc compound since there are no reports on its enantioseparation using this DFDNB based CDR. The method was validated for linearity, accuracy, limit of detection (LOD) and limit of quantification (LOQ). To the best of the authors' knowledge, this is the first report on use of this CDR for enantioseparation of Orc compound (Fig. 1).

EXPERIMENTAL
Chemicals and Reagents

1,5-Difluoro-2,4-dinitrobenzene, L-Phenylalanine (L-Phe), Orciprenaline sulphate as Alupent tablets (Zydus

Healthcare, East Sikkim, India) was obtained from the local market. Trifluoroacetic acid (TFA), phosphoric acid (H_3PO_4), triethylamine (TEA), sodium hydrogen carbonate ($NaHCO_3$) and hydrochloric acid (HCl) of analytical reagent grade, acetonitrile (MeCN) and methanol (MeOH) of HPLC grade were obtained from E. Merck (Mumbai, India). Aqueous–TFA (0.1%) and TEAP (50 mM) buffers were prepared in Millipore water.

Apparatus

The C_{18} column (Promosil, 250 × 4.6 mm I.D., 5 μm) was from younglin. HPLC consisting of a 10 mL pump head, vaccum degasser, Acme UV/Vis detector, younglin manual injection valve and Autochro-3000 operating software was from younglin (Gyeonggi-do, korea). UV-1601 spectrophotometer (Shimadzu), FT-IR spectrometer 1600 (Perkin-Elmer, USA), NMR spectrometer 500 MHz (Bruker, Germany), pH meter Cyberscan 510 (Singapore) and Microwave-Multiwave 3000 (800W, Perkin-Elmer, USA) equipments were also used for experimental studies.

Extraction of Orc from Commercial Tablets

Orc was extracted from commercially available "Alupent tablets" in local market. The extraction process was carried out as per the method described in literature (Bhushan and Nagar 2014b). The purity of the product was confirmed by determination of its melting point and UV absorption (λ_{max}) spectra which were matching with literature reports. The recovery of compound was found in order of 93–95% of the quantities reported on the commercial labels. The purified compound was used as racemic standard.

Preparation of Stock Solutions

Solutions of $NaHCO_3$ (1 M) and HCl (2 M) were prepared in purified water. Stock solution of racemic Orc was prepared by dissolving appropriate amount in 1 M $NaHCO_3$ and used for

derivatization reactions. Solutions and samples were filtered by using 0.45 μm filters. Acetone was used as solvent to prepare solutions of CDR (25 mM) and then CDR stored in refrigerator at 0–4 °C for further uses.

Synthesis of CDR

Due to sensitivity of the reagent synthetic procedures were protected from light. The CDR was synthesized by introducing optically pure L-Phe via substitution of one fluorine atom in DFDNB as per method described in literature (Bhushan and Kumar 2009b). These were characterized and stored at 4 °C. A representative procedure for synthesis of CDR is given below. Following solutions were prepared.

(i) 1 M NaHCO$_3$ in distilled water

(ii) 0.1 M solution of L-Phe in (i), and

(iii) 0.2 M solution of DFDNB in acetone-water (6:4, v/v); it was maintained at 0–4 °C

Synthesis of CDR (1-Fluoro-2,4-dinitrophenyl-L-phenylalanine; FDNP-L-Phe): 20 mL of (ii) was added to 20 mL of (iii)) with constant stirring, in a round bottom flask. The reaction mixture was constantly stirred at 40 °C; after 1 h, the resulting solution was kept at room temperature for 10 min and then 40 mL water was added to it. Further addition of 20 mL HCl (1 M) yielded yellow crystals; these were filtered and washed with cold water. The crystals were dehydrated in desiccators over P$_2$O$_5$.

The structure of the synthesized CDR (FDNP-L-Phe) is shown in Fig. 2 and its characterization data are in agreement with earlier reports (Bhushan and Kumar 2009b).

Synthesis of Diastereomers

The diastereomers of Orc with FDNP-L-Phe were synthesized according to the literature report (Bhushan and Nagar 2014b) using MW irradiation as well as conventional heating.

Following solutions were prepared for the purpose of synthesis of diastereomers.

(iv) 1 M NaHCO$_3$ in distilled water

(v) 50 mM solution of Orc in (iv), by dissolving 10.56 mg of Orc in 1 mL of (iv).

(vi) 25 mM solution of *FDNP-L-Phe* in acetone by dissolving 8.73 mg of *FDNP-L-Phe* in 1 mL acetone; it was maintained at 0–4 °C.

Solution of Orc (as at (v) above) was added to the solution of FDNP-L-Phe (as at (vi) above) in a teflon tube. Following reaction conditions were tried to obtain an optimum reaction yield. Orc and FDNP-L-Phe were allowed to react in different ratios such as, 1:1, 1:1.5, 1:1.7 and 1:2. The reaction mixtures were microwave-irradiated in oven for 30, 40, 45, 50, 55, 60 and 70 s at each of the three different power settings, viz, 70, 80 and 90%. Separate sets of reaction mixture were incubated at 40, 45 and 50 °C with

constant stirring for 40, 50, 60, 70 and 80 min (at each temperature). The scheme for synthesis of daistereomers by the reaction of CDR with (*R/S*)-Orc is given in Fig. 2. The formed daistereomers were processed through the HPLC experiment, the peak areas of each pair of the daistereomers obtained, were taken as a diagnosis for the completion of derivatization reactions of formation of the diastereomers and samples were taken as standards for separation.

A 10 µL of resulting solution of daistereomers was diluted to 100 µL with MeCN, degassed and filtered. Now 20 µL of it was injected onto the column through injector.

HPLC

Following mobile phases were tried;

Mobile phase 1, MeCN with TEAP buffer (10 mM) in linear gradient (30 to 80, 30 to 65, 35 to 60, 25 to 60, 25 to 70, 20 to 60, 20 to 70 and 10 to 90%) in 45 min run.

Mobile phase 2, MeCN with TFA buffer (0.1%) in linear gradient (30 to 80, 30 to 65, 35 to 60, 25 to 60, 25 to 70, 20 to 60, 20 to 70 and 10 to 90%) in 45 min run.

Mobile phase 3, MeOH with TFA buffer (0.1%) in linear gradient (30 to 80, 30 to 65, 35 to 60, 25 to 60, 25 to 70, 20 to 60, 20 to 70 and 10 to 90%) in 45 min run.

Chromatographic runs were carried out and optimized the conditions by varying concentration of buffer (TEAP, 5 to 20 mM and TFA 0.05% to 0.15%), pH (in the range 2.0 to 5.5), and flow rate (0.5 to 1.5 mL/min) of mobile phase. Before using, the mobile phases were filtered through a 0.45µm filter and degassed by sonication and by passing nitrogen.

On increasing the concentration of TFA from 0.05 to 0.10%, an increase in separation factor was observed. There was no significant increase in separation factor when its concentration was increased from 0.10 to 0.15%. High concentration of TFA, due to its strong acidic nature could be harmful to the column; therefore, 0.10% TFA was taken as the optimized concentration. A decrease in flow rate from 1 to 0.5 mL/min resulted in an increase in retention time with slight broadening of peaks while on the other hand, an increase in flow rate from 1 to 1.5 mL/min resulted in decrease of retention time and Δt.

Method Validation

Method was validated using diastereomers of Orc prepared with CDR (FDNP-L-Phe) according to ICH guidelines (ICH 1996).

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The peak areas were plotted against concentrations over the range 20 to 100 ng mL^{-1} to obtain the calibration curve and, slope and correlation coefficients were determined by using regression equation.

RESULTS AND DISCUSSION

Diastereomers

DFDNB is very good moiety in terms of detection and reactivity to form its derivatives. This is due to chromophoric effect of nitro group in DFDNB which enhances molar absorptivity while its strong electron withdrawing nature (-M/-R and –I effect) facilitates nucleophilic substitution of one of the fluorine atoms with L-Phe to yield the CDR (FDNP-L-Phe). Furthermore, a pair of diastereomers of good molar absorptivity was produced by substitution of remaining fluorine in CDR (FDNP-L-Phe) with chiral analyte. Better yield was obtained in the following optimised conditions: CDR (FDNP-L-Phe) and Orc are taken in the mole ratio of 1.7:1; heating the reaction mixture at 45 °C in an incubator for 50 min with constant stirring for conventional heating while irradiation of mixture for 45 s (at 80% of 800 W) under microwave condition in a separate set. The reaction was terminated by addition of HCl (2 M, 20 μL) after cooling to room temperature.

Synthesis of diastereomers of Orc with FDNP-L-Phe was performed under the said optimized conditions. The 1.7 fold molar excess of CDR was successful to prevent kinetic resolution. The two diastereomers formed are of the type [(*R*)-L]-, and [(*S*)-L]- where the first letter refers to the configuration of the analyte (Orc) while the second letter to that of the L-Phe of the CDR (FDNP-L-Phe).

HPLC

The data for resolution (R_s) and separation factor (α) for separation of diastereomers are; 11.76 and 1.12, respectively. Sharp peaks were observed with the mobile phase under gradient conditions with MeCN used as organic modifier in mobile phase 1 and 2 while with MeOH in mobile phase 3 broader peaks were obtained with larger retention time. This is due to lower dielectric constant (33 D) and higher viscosity (0.59 cP at 25 °C) of MeOH in comparison to acetonitrile having dielectric constant (37.5 D) and viscosity (0.343 cP at 25 °C). With TFA buffer (mobile phase 2) sharper peaks were observed in comparison to those obtained using TEAP buffer (mobile phase 1). Thus, the mobile phase 2, in which MeCN used as organic modifier and 0.10% TFA as buffer, with gradient elution (30 to 65%

of MeCN) in 45 min at a flow rate of 1.0 mL/min and detection at 340 nm was found successful for separation of daistereomers of Orc. The chromatogram showing separation of diastereomers of the Orc prepared with CDR (FDNP-L-Phe) is given in Fig. 3.

In HPLC run the peak areas obtained for corresponding daistereomers in each change of the above mentioned derivatization conditions, were calculated by system software; these values taken as a measure of completion of reaction and yield of derivatization. The characterization and chromatographic data of diastereomers synthesized in the two conditions (using conventional heating and microwave irradiation (MWI)) were found to be identical.

Comparison with literature reports

In the present study, derivatization of Orc using DFDNB based CDR (FDNP-L-Phe) in which L-Phe was introduced as chiral auxiliary under MWI required less time (45 s at 80% power of 800 W). Diastereomers of Orc have been separated with resolution (R_s) 11.76 which is better as compared to the resolution reported in literature (Table 1) where certain CSPs, and CDRs used in HPLC and CIR used in TLC.

Separation mechanism

Certain literature reports (Marfey 1984; Bhushan et al. 2009; Fujii et al. 1997) have been explained the mechanism for HPLC separation of diastereomers of certain amino acids, prepared with DFDNB based CDRs, along with explanation for difference in retention time and elution sequence for daistereomers.

Bhushan and Nagar (2014b) also confirmed the elution sequence of daistereomers of β-blockers prepared with DFDNB, experimentally and also theoretically by optimising the structures of the diastereomers of Orc using the Gaussian 09 Rev A. 02 program and hybrid density functional B3LYP with 6-31G (d, p) basis set, which supported the experimental results. The mechanism in the present work is in accordance with literature (Bhushan and Nagar 2014b) that is the [(R)-L]-diastereomer retained for lesser time in comparison to that of the [(S)-L]-diastereomer which means previous one eluted first from the column.

A graphical representation for these diastereomers is shown in Fig. 4a and 4b. Fig. 4a, in [(S)-L]-diastereomer, the hydrophobic groups $-CH_2C_6H_5$ on the stereogenic centre (C-1) of CDR (FDNP-L-Phe) and $-C_6H_3(OH)_2$ on stereogenic

centre (C-2) of Orc (in rectangle) are oriented on the same side with respect to the plane of dinitrobenzene moiety (rectangle) and are considered to be *cis* to each other. Fig.4b, in [(*R*)-L]-diastereomer, the –CH$_2$C$_6$H$_5$ and –C$_6$H$_3$(OH)$_2$ groups are oriented in space opposite to the plane of dinitrobenzene moiety and thus have *trans*-type arrangement.

In other words, the [(*S*)-L]-diastereomer having *cis*-type arrangement (Fig 4a) due to greater hydrophobicity interacts more strongly with ODS material of C$_{18}$ column than the *trans*-type arrangement (Fig 4b) having lesser hydrophobicity, hence [(*S*)-L]-diastereomer has a longer retention time. Besides the different hydrophobic nature of these daistereomers, rheological properties of the mobile phase are also responsible for different partition coefficients and different retention times of [(*R*)-L]-and [(*S*)-L]-diastereomers. Therefore, the diastereomers elute one after another due to these different physical properties.

Advantages/novelty of present work

The advantages in terms of less reaction time for synthesis of diastereomers and in terms of higher R_s (for the separation of diastereomers) have

been described under **'Comparison with literature reports'.** The developed CDR (FDNP-L-Phe) has high molar absorption properties with the capability of detecting diastereomers at low limits; limit of detection was found good (20 to 21 pg mL^{-1}) for diastereomers of Orc.

Method Validation

Method validation studies were performed using diastereomers of (*R*,*S*)-Orc prepared with CDR (FDNP-L-Phe).

Linearity

The peak area response (on Y-axis) of the diastereomers of (*R*)-(first eluting diastereomer) and (*S*)-(second eluting diastereomer) enantiomer prepared with CDR were plotted against the corresponding concentration (20 to 100 ng mL^{-1}; on X-axis). Least square method was used to complete linear regression equation. The relative standard deviation (RSD) values of slope, intercept and correlation coefficient were obtained (less than 1.4%) as a evidence of good linear relationship over this range. The regression equations were y = 0.912x –10.29 (R^2 = 0.998) and y = 0.941x–11.77 (R^2 = 0.999) for the diastereomers of (*R*)- and (*S*)-Orc, respectively (Table 2).

Accuracy, precision and limit of detection

Inter-day (5 days) and intra-day assay studies were carried out by replicate analysis (n = 3) of four standard solutions of diastereomeric mixtures (20, 40, 60, 100 ng mL^{-1}) for determination of accuracy and precision. The recovery and mean SD (standard deviation) for each of the diastereomers were calculated on the basis of peak areas of first and second eluting diastereomer from the slope and intercept of the calibration plots. These are shown in Table 2. The values of relative standard deviation for [(*R*)-L]- and [(*S*)-L]-diastereomers, respectively, were 0.52% to 1.21% and 0.58% to 1.20% for intra-day precision, and 0.64% to 1.11% and 0.67% to 1.17% for inter-day precision. The recovery values for the [(*R*)-L]- and [(*S*)-L]-diastereomers were 99.81% to 101.15% and 99.35% to 101.12% for intra-day assay and 99.83% to 101.23% and 98.70% to 101.16% for inter-day assay, respectively. Limit of detection (LOD), corresponding to signal-to-noise ratio of 3, was found to be 20 pg mL^{-1} and 22 pg mL^{-1} for [(*R*)-L]- and [(*S*)-L]-diastereomers, respectively and limit of quantitation (LOQ) corresponding to signal-to-noise ratio of 10, was found to be 60 pg mL^{-1} and 66 pg mL^{-1} for [(*R*)-L]- and [(*S*)-L]-diastereomers **Table 2**.

CONCLUSION

The presence of strong electron withdrawing two -NO$_2$ groups on DFDNB platform facilitate nucleophilic substitution of its F atom by amino group of chiral amino acid. While, the resonance of the lone pair of electrons of chiral auxiliaries with the -NO$_2$ groups of the DFDNB platform resulted into high molar absorptivity of CDR, FDNP-L-Phe. Thus, the CDR so obtained is capable of detecting at concentration lower than the limit of 1% prescribed for pharmaceutical industry. It is proved that results are better in terms of resolution in comparison to certain other CSPs, CDRs and CIR reported in literature. Under MW irradiation, daistereomers synthesized with shorter reaction time in comparison to that required by other CDRs reported in literature and reaction conditions were easily optimized.

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Table 1 Literature reports with present study on chromatographic separation (in terms of R_s) of enantiomers of Orc using different CSPs[a]/CDRs[b]/CIR[c]

S. no.	CSPs[a]/CDRs[b]/CIR[c]	R_s	Reference
1	Methyl-β-cyclodextrin[a]	3.95	Amini et al. (2000)
2	Sulfobutyl ether β-cyclodextrin	0.80	Ngim et al. (2012)
3	Cellulose tris(3,5-dimethylphenylcarbamate) [a]	4.68	Ullrich et al. (2001)
4	Sulfated-*β*-cyclodextrin[a]	2.03	Yang et al. (2005)
5	(*R*)-2-(5-fluoro-2,4-dinitrophenylamino)-3-(methylthio)propanoic acid[b]	9.79	Bhushan and Nagar (2014b)
6	(*S*)-N-(1-cyclohexylethyl)-5-fluoro-2,4-dinitrobenzenamine[b]	10.22	Bhushan and Nagar (2014b)
7	(*R*)-5-fluoro-2,4-dinitro-N-(1-phenylethyl)benzenamine[b]	8.26	Bhushan and Nagar (2014b)
8	L-Glutamic acid[c]	2.10	Bhushan et al. (2015)

Table 2 Summary of HPLC method validation containing linearity, intra- and inter-day precision, and recovery studies

	First eluting diastereomer			Second eluting diastereomer		
Linearity						
Range	20 to100 ng mL^{-1}			20 to 100 ng mL^{-1}		
Slope	0.912			0.941		
Intercept	−10.29			−11.77		
Correlation Coefficient (R^2)	0.998			0.999		
Actual concentration	Mean±SD (measured)	Recovery (%)	RSD (%)	Mean±SD (measured)	Recovery (%)	RSD (%)

ng mL^{-1}	ng mL^{-1}			ng mL^{-1}		
Intra-day precision (n = 3)						
20	10.109 ± 0.122	99.93	1.21	10.116 ± 0.131	99.97	1.20
40	19.843 ± 0.175	101.105	0.88	19.789 ± 0.162	101.12	0.82
60	30.116 ± 0.174	99.81	0.58	30.215 ± 0.195	99.35	0.65
100	49.450 ± 0.255	101.05	0.52	49.612± 0.288	101.08	0.58
Inter-day precision (n = 3)						
20	9.929 ± 0.110	100.05	1.11	10.168 ± 0.118	101.16	1.17
40	19.956 ± 0.196	99.89	0.98	19.923 ± 0.189	99.96	0.95
60	30.158 ± 0.236	99.83	0.75	30.105 ± 0.217	100.09	0.72
100	50.853 ± 0.325	101.23	0.64	49.790 ± 0.334	98.70	0.67
Sensitivity						
Limit of detection (pg mL^{-1})	20			22		
Limit of quantification (pg mL^{-1})	60			66		

Figure Captions

Fig. 1 Structures of Orciprenaline (* represents chiral centre).

Orciprenalinre
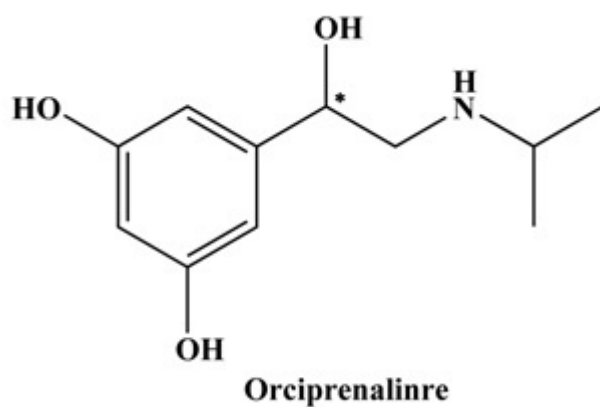
Fig.2 Scheme for synthesis of daistereomers of (*R/S*)-Orc with CDR (FDNP-L-Phe)

DNP-L-Phe-*S*-Orc
(A)

DNP-L-Phe-*R*-Orc
(B)

*Represents chiral centre, C-1 and C-2 are chiral centers of CDR and Orc, respectively). (A) and (B) are [(*S*)-L-]-diastereomer and [(*R*)-L-]-diastereomer, respectively.
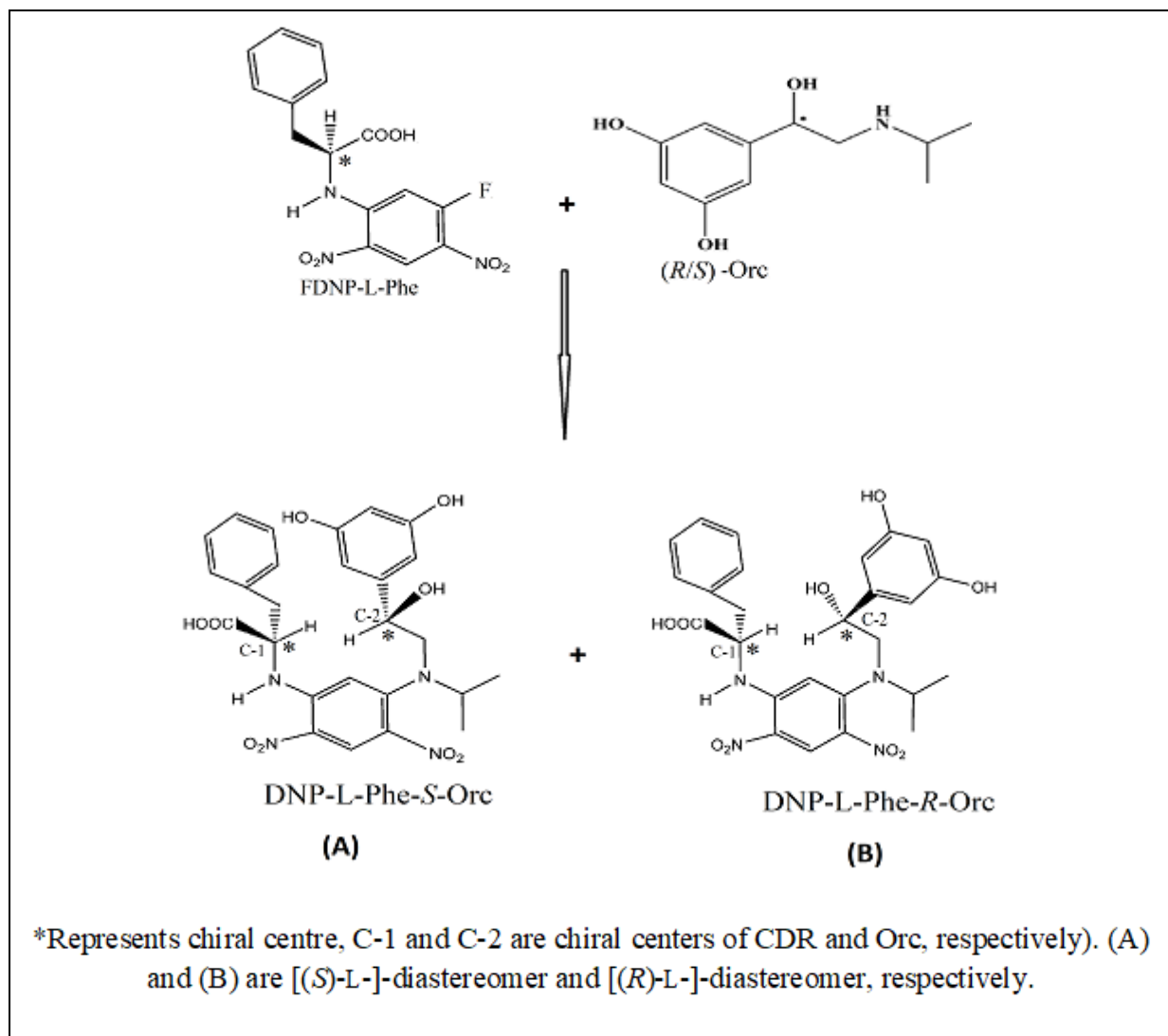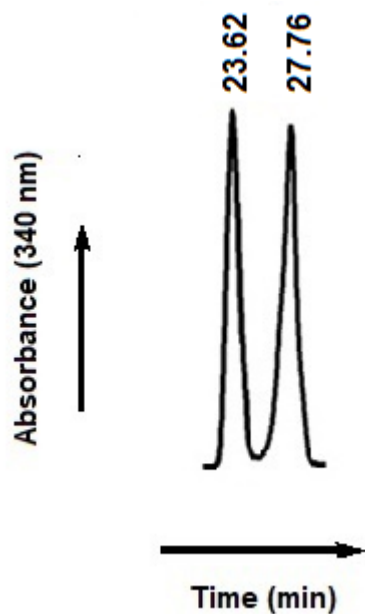
Fig. 3 Sections of chromatograms showing resolution of diastereomers of Orc prepared with CDR (FDNP-L-Phe).

Chromatographic conditions: Promosil C_{18} (250 × 4.6mm i.d., 5 μm particle size); mobile phase 2, linear gradient (30 to 65%) of MeCN with 0.10% TFA for diastereomers of Orc in 45min; flow rate, 1.0mL/min; detection, 340 nm. From left to right, first and second peaks are for [(*R*)-L]- and [(*S*)-L]-diastereomers, respectively.

Fig. 4 In [(*S*)-L]-diastereomer, the hydrophobic groups, -CH$_2$C$_6$H$_5$ on the stereogenic centre of CDR (C-1) and -C$_6$H$_3$(OH)$_2$ on stereogenic centre of Orc (C-2), are appearing on the same side with respect to the plane of dinitrobenzene moiety and thus the two groups are oriented in a *cis*-type arrangement (Fig. 4a). In [(*R*)-L]-diastereomer, the -CH$_2$C$_6$H$_5$ and -C$_6$H$_3$(OH)$_2$ groups are oriented, in space, opposite to the plane of dinitrobenzene moiety and thus have *trans*-type arrangement (Fig.4b).
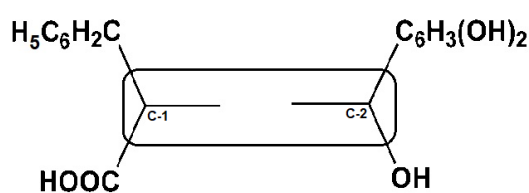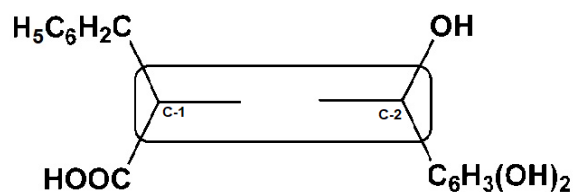
Fig 4a

Fig 4b