

Indirect reversed-phase high-performance liquid chromatographic and direct thin-layer chromatographic enantioresolution of (*R,S*)-Cinacalcet

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ABSTRACT: Enantioresolution of the calcimimetic drug (*R,S*)-Cinacalcet was achieved using both indirect and direct approaches. Six chiral variants of Marfey's reagent having L-Ala-NH₂, L-Phe-NH₂, L-Val-NH₂, L-Leu-NH₂, L-Met-NH₂ and D-Phg-NH₂ as chiral auxiliaries were used as derivatizing reagents under microwave irradiation. Derivatization conditions were optimized. Reversed-phase high-performance liquid chromatography was successful using binary mixtures of aqueous trifluoroacetic acid and acetonitrile for separation of diastereomeric pairs with detection at 340 nm. Thin silica gel layers impregnated with optically pure L-histidine and L-arginine were used for direct resolution of enantiomers. The limit of detection was found to be 60 pmol in HPLC while in TLC it was found to be in the range of 0.26–0.28 µg for each enantiomers. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: (*R,S*)-cinacalcet; reversed-phase HPLC; Marfey's reagent; L-histidine; L-arginine; impregnated TLC.

Introduction

Cinacalcet (Cin) is the first drug of a new class of therapeutic agents known as calcimimetics. It is a principal negative regulator of parathyroid hormone release. It is chemically named [1-(*R,S*)-(–)-(1-naphthyl) ethyl]-3-[3-(trifluoromethyl) phenyl]-1- amino-propane (Fig. 1). Cinacalcet is currently used for primary and secondary hyperparathyroidism (HPT) (Balfour and Lesley, 2005). It binds to the calcium-sensing receptors (CaR) of the parathyroid glands, lowers the sensitivity for receptor activation by extracellular calcium, and thus parathyroid hormone release is diminished (Franceschini *et al.*, 2003). Cinacalcet has also proved effective in a broad range of chronic kidney disease patients on dialysis with uncontrolled HPT (Sorbera *et al.*, 2002). The active part of Cinacalcet is its (*R*)-enantiomer. The literature reveals only one report on the separation of (*S*)-Cin from (*R*)-Cin using a Chiralpak-IA column (Ravinder *et al.*, 2009).

Both the direct and indirect approaches are applied for enantioseparation of different classes of compounds. The two approaches have their own advantages depending upon the nature of the sample, the functional group of analyte and the method of detection available. Of the various chiral derivatizing reagents (CDRs) available for indirect enantioseparation of a variety of compounds containing a 1° or 2° amino group, Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDNP-L-Ala-NH₂, MR) has been reported to be one of the most successful (Bhushan and Brückner, 2004; B'Hymer *et al.*, 2003).

MR was first prepared by substituting one of the fluorine atoms in DFDNB by L-Ala-NH₂ (Marfey, 1984). The diastereomers of different racemates prepared with MR show strong absorbance at 340 nm. The DFDNB moiety has been used to prepare a variety of CDRs by substituting one of the fluorine atoms with different

amino acid amides, amino acids and other chiral amines as chiral auxiliaries. In the recent years several such chiral derivatizing reagents have been used in this laboratory for reversed-phase high-performance liquid chromatographic enantioseparation of penicillamine (Bhushan *et al.*, 2007), baclofen (Bhushan and Kumar, 2008a), protein and non-protein amino acids (Bhushan and Kumar, 2008b, 2009a; Bhushan *et al.*, 2009), β-blockers (Bhushan and Tanwar, 2008, 2009a) and mexiletine (Bhushan and Tanwar, 2009b).

Thin-layer chromatography is one of the most simple, economical and versatile techniques for direct enantioseparation. Literature reveals that TLC has been successfully applied for direct enantioseparation of DL-amino acids (Guenther and Moeller 2003; Bhushan and Martens, 1997, 2003, 2010) and NSAIDs (Bhushan and Martens, 2007). Application of optically pure amino acids as suitable chiral impregnating reagents in TLC for direct enantiomeric separation and determination of enantiomeric purity of certain pharmaceutically important compounds has been discussed and reviewed (Bhushan and Martens, 2010). L-Arg (Bhushan and Parshad, 1996; Sajewicz *et al.*, 2006) and other basic amino acids were the most investigated ones as chiral selectors.

Taking into account the above-cited literature and the presence of a secondary amino group in the drug Cinacalcet, it was

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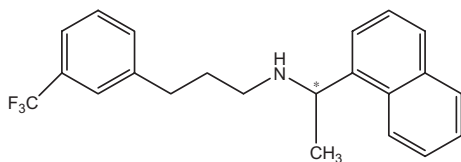


Figure 1. Structure of (*R,S*)-Cinacalcet.

considered worthwhile to apply indirect HPLC and the directly impregnated TLC methods for separation of its enantiomers. Six CDRs based on DFDNB moiety were synthesized that had L-Ala-NH₂, L-Phe-NH₂, L-Val-NH₂, L-Leu-NH₂, L-Met-NH₂ and D-Phg-NH₂ as the chiral auxiliary; these were then used for diastereomerization of (*R,S*)-Cin using microwave (MW) irradiation. The diastereomers were separated by RP-HPLC. L-Histidine and L-arginine were used as chiral impregnating reagents for direct TLC resolution. To the best of the authors' knowledge this is the first report on indirect enantioresolution as well as on direct TLC enantio-resolution of (*R,S*)-Cin.

Experimental

Equipment

The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, fixed wavelength UV detector 2500, Knauer manual injection valve and Eurochrom operating software was from Knauer (Berlin, Germany). Other equipment used was a microwave (Multiwave 3000, Perkin-Elmer, Shelton, CT, USA), a pH meter (Cyberscan 510, Singapore), a Polarimeter P-3002 (Kruss, Hamburg, Germany), a Milli-Q system (Millipore, Bedford, MA, USA), a Perkin Elmer 1600 FT-IR spectrometer (Boardman, OH, USA), a Vario EL III elemental analyzer and a Shimadzu UV-1601 spectrophotometer (spectra were recorded in acetonitrile). ¹H NMR spectra were recorded on a Bruker 500 MHz instrument using [²H₆] dimethyl sulfoxide (DMSO-*d*₆) as deuterated solvent.

Materials

The racemic and chirally pure Cinacalcet was obtained from Manus Aktteva (Ahmedabad, India). DFDNB, L-alanine amide hydrochloride (L-Ala-NH₂HCl), L-phenylalanine amide hydrochloride (L-Phe-NH₂HCl), L-valine amide hydrochloride (L-Val-NH₂HCl), L-leucine amide hydrochloride (L-Leu-NH₂HCl), L-methionine amide hydrochloride (L-Met-NH₂HCl), D-phenylglycine amide (D-Phg-NH₂), L-histidine and L-arginine were obtained from Sigma-Aldrich (St Louis, MO, USA). Silica gel G with 13% calcium sulfate as binder, having chloride, iron and lead impurities up to 0.02% and with pH 7.0 in a 10% aqueous suspension, and all the other analytical-grade chemicals and HPLC-grade solvents were from E. Merck (Mumbai, India). Double-distilled water purified with a Milli-Q system (18.2 MΩ cm²) was used throughout all experiments. Stock solutions of NaHCO₃ (1 M), triethylamine (TEA, 6%) and HOAc (5%) were prepared in purified water.

Synthesis of CDRs and diastereomers

Synthesis of CDRs based on DFDNB moiety. As per the previously reported procedure (Bhushan and Kumar, 2008a, 2009a, b; Brückner and Keller-Hoehl, 1990), six CDRs were synthesized having amino acid amides, viz. L-Ala-NH₂, L-Val-NH₂, L-Phe-NH₂, D-Phg-NH₂, L-Met-NH₂ and L-Leu-NH₂, as chiral auxiliaries. The reaction was carried out under MW irradiation. The CDRs were characterized with the help of UV, IR, CHN and ¹H NMR.

Synthesis of diastereomers of (*R,S*)-Cin. Solution of Cin (11 mM) was prepared in NaHCO₃ (1 M)–MeCN (90:10, v/v). To a solution of (*R,S*)-Cin

(100 μL, 1.1 μmol) in a Teflon tube were added the solutions of CDR1 (110 μL, 1% in acetone, 1.7 μmol) and TEA (60 μL, 6%). The resulting mixture was irradiated under MW for 3 min using 75% power. After cooling to room temperature, HOAc (60 μL, 5%) was added to quench the reaction. Ten microliters of the resulting solution, containing diastereomers, was diluted 10 times with MeCN, and 20 μL of it was injected onto column.

The above-described method was followed for the synthesis of *diastereomers* of (*R,S*)-Cin using the remaining five CDRs. The reaction conditions for derivatization were optimized by derivatizing (*R,S*)-Cin with CDR5. Solutions of chiral derivatizing reagents and their diastereomers with Cin were found to be quite stable for up to 1 week when they were kept in dark at 4°C.

HPLC and TLC

HPLC operating conditions. Reversed-phase HPLC was performed on a Waters Spherisor ODS (250 × 4.6 mm i.d., 5 μm) column (from Parker-Style Fittings, Ireland) with the mobile phase consisting of aqueous TFA (0.01 M)–MeCN in a linear gradient of MeCN from 30 to 65% in 45 min at a flow rate of 1.0 mL/min and UV detection at 340 nm.

TLC. Stock solutions (25 mg/mL) of (*R,S*)-Cin and (*S*)-Cin were prepared in dilute HCl (pH 4). Solutions of L-His and L-Arg were prepared in distilled water (50 mL) and a few drops of ammonia were added to bring the pH to 9, 10, 11, 11.5 and 12. Slurry of silica gel G (25 g) was prepared in these solutions. The TLC plates (10 × 5 cm × 0.5 mm) were prepared by spreading the slurry with a Stahl-type applicator; the plates were activated overnight at 60 ± 2°C. Solutions of racemic and pure isomers of Cin were applied side by side on the plates with a 25 μL Hamilton syringe. Cleaned, dried and paper-lined rectangular glass chambers were used for developing the chromatograms. These were pre-equilibrated with mobile phase MeCN–MeOH–H₂O in different proportions at 18 ± 2°C for 10–15 min. Chromatograms were dried at 45°C in an oven for 10–15 min and cooled to room temperature; spots were located in an iodine chamber.

Validation Procedures

The method validation was done according to ICH guidelines (ICH, 1996) using diastereomers of (*R,S*)-Cin prepared with CDR 5. Slopes and regression equations were determined by plotting calibration curves for peak areas vs concentrations and using linear regression equations. Recovery studies were carried out by derivatizing standard solutions of different known concentrations and mean recovered values (five replicate runs) were represented as percentage of calculated values. Intra-day assay and inter-day assay (5 days) stability studies were carried out to find out precision and coefficient of variation (CV). Limit of detection (LOD) and limit of quantification (LOQ) were also evaluated.

Results and Discussion

Optimization of Conditions for Derivatization

Role of pH. The use of triethyl amine (60 μL, 6%) at a pH around 10.0 was found to be optimum to obtain the best yield for derivatization of (*R,S*)-Cin with CDR5. No derivatization was observed in the absence of triethyl amine. These conditions were, therefore, used for all the derivatization reactions for quantitative yields.

Effect of excess CDR. CDR5 was used in 1–3-fold molar ratio to find the optimum reagent concentration for derivatization of (*R,S*)-Cin. The derivatization was complete when 1.5-fold molar excess of the reagent was used in 3 min using MW (at 75% power). Further increase in reagent concentration (2–3-fold) had little effect on reaction time and yield of derivatization. Therefore,

all the CDRs were used in 1.5 molar excess for quantitative derivatization and to overcome kinetic resolution.

Microwave irradiation. (*R,S*)-Cin was irradiated for 2–4 min with MW (at 75% power); a time of 3 min was found successful. According to the literature (Bhushan and Kumar, 2009b), derivatization of β -amino alcohols took place within 1 min using Marfey's reagent and its chiral variants as the CDRs, but in the present case the reaction time was relatively high, which may be because Cin was overcrowded at just next to the stereogenic center.

HPLC Analysis

Six pairs of diastereomers of (*R,S*)-Cin were synthesized as mentioned above. (*R*)-Isomer was found to elute before the (*S*)-isomer in the case of diastereomers prepared with CDR 1–5. The elution order was reversed for the diastereomers prepared with CDR6 as the chiral auxiliary has a D-configuration in this reagent. Sharp peaks showing baseline separation for the separation of diastereomers prepared with CDRs 3–6 and slightly broader peaks for those prepared with CDRs 1–2 were obtained. The chromatograms showing the separation of respective pairs are given in Fig. 2.

MeCN was found to be a better organic solvent in comparison to methanol as broader peaks (and no separation in a few cases) were obtained with the latter. Linear gradients of MeCN of 20–65, 25–65, 30–65, 35–65, 40–65, 45–65 and 50–60% in 45 min were applied. The experiments showed that both the retention times and R_s decreased with the increasing amount of MeCN. Thus, a linear gradient of MeCN from 30 to 65% in 45 min was found to be successful. Effect of change of flow rate revealed that the retention times increased with broadening of peaks as the flow rate was decreased from 1.0 to 0.5 mL/min. On the other hand, an increase in flow rate from 1.0 to 2.0 mL/min resulted into a decrease in both the retention times and R_s . Thus the optimized flow rate of 1.0 mL/min was used throughout the run.

Structure–Retention Relationship

The chromatographic data for separation of diastereomers is given in Table 1. It shows that the diastereomeric pairs prepared with all six CDRs (having amino acid amides as the chiral auxiliaries) were well separated. The R_s for the diastereomeric pair prepared with FDNP-L-Met-NH₂ (CDR5) is 2.27 and is thus better resolved than the remaining five diastereomeric pairs. The R_s for the diastereomeric pair prepared with FDNP-D-Phg-NH₂ (CDR6) is 1.61, the lowest among the six.

Impregnated TLC

Successful solvent systems were obtained for direct enantiomeric resolution of (*R,S*)-Cin on plates impregnated with L-His and L-Arg, after trying a number of solvent systems. These were, 4:3.5:1 and 5:4:1 combinations of acetonitrile–methanol–water for plates impregnated with L-His and L-Arg, respectively. The spots of the two enantiomers were visualized by exposure to iodine vapors. R_f values obtained with these solvent combinations are given in Table 2. The resolution (R_s) was calculated by dividing the distance between two spots by the sum of the two spot radii. The results are the averages of at least five runs under identical conditions. It was observed that the (*S*)-Cin eluted before the (*R*)-Cin, in both the cases. Actual photographs of chromatograms are shown in Fig. 3.

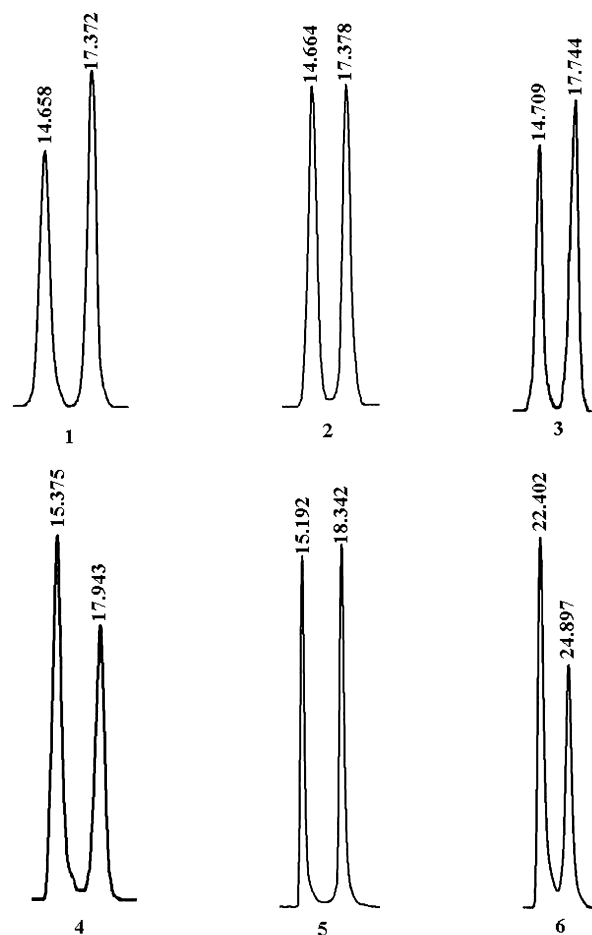


Figure 2. Sections of chromatograms showing resolution of (*R,S*)-Cin as their diastereomers prepared with CDR 1–6; RP column (250 \times 4.6 mm i.d., 5 μ m); detection, 340 nm; (*R*)-enantiomer elutes first prior to (*S*)-enantiomer for the diastereomers prepared with CDR 1–5; mobile phase was aqueous TFA (0.01 M)–MeCN in a linear gradient of MeCN from 30 to 65% in 45 min.

Both the plates impregnated with L-His (at pH 11) and L-Arg (at pH 11.5) existed in the anionic form under the experimental conditions while the enantiomers in the (*R,S*)-Cin existed as protonated cations. At least three interactions must be involved (Dalgliesh, 1952) for enantioresolution to occur. Each of these interactions can be hydrogen-bonding, π – π , steric, hydrophobic or dipole–dipole. Thus with the use of L-His and L-Arg, columbic/charge–charge interaction, π – π , steric and hydrophobic interactions occurred that favored formation of diastereomers *in situ* and hence enantioresolution.

Effect of pH and Temperature

Studies with respect to effect of change of concentration of the impregnating reagents, pH and temperature were made for the successful solvent systems only. The optimized concentration was 1.0% for each of the selectors. There was no resolution below this concentration (1.0%).

Resolution studies were carried out on plates prepared in solutions of pH 9, 10, 11, 11.5 and 12. This ensured that the racemic analyte (*R,S*)-Cin at the time of spotting on each of the impregnated plates was in the cationic form and the chiral selector was

Table 1. HPLC data for separation of (*R,S*)-Cin

Parameters	Diastereomers prepared with					
	CDR1	CDR2	CDR3	CDR4	CDR5	CDR6
k_R	4.33	4.36	4.33	4.71	3.91	7.19
k_S	5.32	5.36	5.43	5.66	4.92	8.11
α	1.23	1.22	1.25	1.20	1.26	1.23
R_s	1.93	1.77	2.11	1.75	2.27	1.61

k_R and k_S are retention factors of the diastereomers of (*R*)-Cin and (*S*)-Cin respectively; α is stereoselective factor; R_s is the resolution of diastereomers of (*R,S*)-Cin.

Table 2. Enantioseparation of (*R,S*)-Cin on L-His and L-Arg impregnated plates

Impregnating reagents	Solvent ratio	R_f from racemic (<i>R,S</i>)-Cin		R_f pure (<i>S</i>)-Cin	R_s
		(<i>S</i>)-Cin	(<i>R</i>)-Cin		
L-His	4:3.5:1	0.62	0.26	0.62	2.18
L-Arg	5:4:1	0.87	0.63	0.87	1.70

Solvent: MeCN–MeOH–H₂O (v/v); R_s is resolution factor; development time, 10 min; temperature, $16 \pm 2^\circ\text{C}$; detection, iodine vapors.

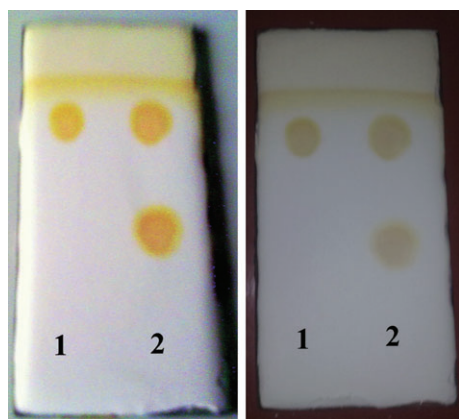


Figure 3. (a) Actual photograph of chromatograms showing resolution of (*R,S*)-Cin using L-His impregnated plates. From left to right: spot 1, pure (*S*)-Cin; spot 2, lower spot for (*R*)-Cin and upper spot for (*S*)-Cin resolved from (*R,S*)-Cin. Development time, 10 min; temperature, $18 \pm 2^\circ\text{C}$; detection, iodine vapors. (b) Actual photograph of chromatograms showing resolution of (*R,S*)-Cin using L-Arg impregnated plates. From left to right: spot 1, pure (*S*)-Cin; spot 2, lower spot for (*R*)-Cin and upper spot for (*S*)-Cin resolved from (*R,S*)-Cin. Development time, 10 min; temperature, $16 \pm 2^\circ\text{C}$; detection, iodine vapors.

in the anionic form. Resolution of enantiomers was observed at pH 11 (using L-His) and at pH 11.5 (using L-Arg). With the variation of these pH values, poor resolution with tailing or even no resolution was obtained.

Additional experiments were carried out with a successful solvent system within the temperature range $10\text{--}30^\circ\text{C}$ within 10 min. For this purpose the chromatographic chambers were placed inside an incubator to attain the specific temperature. The best resolution was obtained at $18 \pm 2^\circ\text{C}$ with both the impregnating reagents. Increase of temperature to 25 or 30°C resulted in tailing of spots and a decrease in temperature to 10°C showed no resolution.

Linearity, Accuracy and Precision

Linearity. The peak area response of (*R*)-Cin and (*S*)-Cin, the first and the second eluting diastereomers prepared with CDR5, was plotted against the corresponding concentration ($100\text{--}500$ pmol) and the linear regression was computed by the least square method using Microsoft Excel. A good linear relationship was obtained over this range. The regression equations were $y = 0.679x + 1.213$ ($R^2 = 0.999$) and $y = 0.647x - 2.43$ ($R^2 = 0.998$) for the diastereomers of (*R*)-Cin and (*S*)-Cin prepared with CDR5, respectively.

Accuracy and precision. The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis ($n = 5$) of (*R,S*)-Cin at five concentrations ($25, 30, 35, 40$ and 50 ng/mL) as shown in Table 3. The coefficients of variation (%) for (*R*)- and (*S*)-Cin were $0.10\text{--}1.39$ and $0.40\text{--}1.28$ for intra-day assay precision and $0.44\text{--}1.38$ and $0.40\text{--}1.43$ for inter-day assay precision. The percentage recoveries for (*R*)- and (*S*)-Cin were $99.0\text{--}99.7$ and $98.6\text{--}99.1$ for intra-day assay and $97.8\text{--}98.9$ and $97.6\text{--}97.9$ for inter-day assay (Table 3).

For an indirect approach, using CDR5, the LOD was found to be 60 pmol and the LOQ was found to be 180 pmol. The LODs were found to be 0.28 μg for each enantiomer of (*R,S*)-Cin using L-His and 0.26 μg using L-Arg for direct approach in the impregnated TLC.

Conclusion

The experiments provided simple and convenient means for enantioseparation of (*R,S*)-Cin by both the indirect and direct approaches. CDRs prepared from DFDNB having L-Ala-NH₂, L-Phe-NH₂, L-Val-NH₂, L-Leu-NH₂, L-Met-NH₂ and D-Phg-NH₂ as chiral auxiliaries were successful and provided diastereomers easily under MW irradiation without racemization. The possibility to obtain (*R*)-Cin in the native form within 10 min using L-Histidine and L-Arginine as readily available and cost-effective chiral selectors proves the direct approach to be an excellent

Table 3. Intra-day assay and inter-day assay precision of diastereomers of (*R,S*)-Cin prepared with CDR5

Concentration (ng/mL)	First eluting diastereomer			Second eluting diastereomer		
	Mean \pm SD	Recovery ^a	CV	Mean \pm SD	Recovery ^a	CV
<i>Intra-day</i>						
25	24.92 \pm 0.06	99.6	0.24	24.65 \pm 0.16	98.6	0.64
30	29.90 \pm 0.03	99.6	0.10	29.69 \pm 0.12	98.9	0.40
35	34.89 \pm 0.08	99.7	0.23	34.63 \pm 0.38	98.9	1.09
40	39.61 \pm 0.34	99.0	0.86	39.64 \pm 0.51	99.1	1.28
50	49.55 \pm 0.69	99.1	1.39	49.34 \pm 0.54	98.7	1.09
<i>Inter-day</i>						
25	24.71 \pm 0.11	98.8	0.44	24.39 \pm 0.12	97.6	0.49
30	29.63 \pm 0.32	98.7	1.07	29.31 \pm 0.39	97.7	1.33
35	34.62 \pm 0.26	98.9	0.75	34.22 \pm 0.49	97.7	1.43
40	39.52 \pm 0.40	98.8	1.02	39.17 \pm 0.16	97.9	0.40
50	48.94 \pm 0.68	97.8	1.38	48.91 \pm 0.57	97.8	1.16

^aPercentage values. CV is coefficient of variation (%); SD is standard deviation (%)

choice for enantioresolution. This method can be utilized not only for enantioseparation but also to check the impurity of (*S*)-Cin in the pharmacologically active (*R*)-Cin.

Acknowledgements

The authors are grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for the award of a junior research fellowship (to R.D.). Thanks are due to the Alexander von Humboldt Foundation, Bonn, Germany, for donating Knauer HPLC equipment (to R.B.). Financial assistance (to R.B.) from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, grant no. 01(2334)/09 EMR-II, is also gratefully acknowledged.

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