Acta Chromatographica 26(2014)4, 625–636
DOI: 10.1556/AChrom.26.2014.4.5

High-Performance Liquid Chromatography for Analytical and Small-Scale Preparative Separation of (*R*,*S*)-Mexiletine Using (*S*)-(−)-(N)-Trifluoroacetyl-Prolyl Chloride and (1*S*)-(−)-Camphanic Chloride and Recovery of Native Enantiomer by Detagging

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Summary. A reversed phase high-performance liquid chromatographic method has been established for enantioseparation of (*R*, *S*)-mexiletine. Two volatile and thermally stable acyl chlorides, *viz*., (*S*)-(−)-(*N*)-trifluoro acetyl prolyl chloride and (1*S*)-(−)-camphanic chloride, were used as chiral derivatizing reagents. Binary composition of aqueous trifluoroacetic acid (0.1%)-acetonitrile as mobile phase was successful with ultraviolet (UV) detection at 210 nm. The method was optimized and validated for accuracy, precision, and limit of detection. The limit of detection was found to be 45 ng mL^{-1} and 80 ng mL^{-1} for the two types of diastereomers. Besides, kinetic resolution was achieved, and the experimental conditions optimized for this purpose provided diastereomeric excess up to 74% for (*R*)-isomer. On achieving a resolution value greater than 2, the optimized method for analytical enantioseparation was scaled-up to small-scale preparative level, and the native (*R*)-mexiletine was recovered by acid hydrolysis of the diastereomer.

Key Words: reversed-phase high-performance liquid chromatography, mexiletine, (*S*)-(−)-(*N*)-trifluoro acetyl prolyl chloride, camphanic chloride, chiral derivatizing reagent

Introduction

Mexiletine (1-(2,6-dimethylphenoxy)-2-amino-propane, MEX) *(Fig. 1)* is a Class IB antiarrhythmic, antimyotonic, and analgesic agent in its racemic form [1] and used in the treatment of ventricular arrhythmia [2]. The enantiomers of (*R*, *S*)-MEX have different pharmacokinetic, pharmacodynamic, and receptor binding properties [3, 4]. (*R*)-Enantiomer has more potential for protein binding [5] than the (*S*)-enantiomer.

Different rates of reaction of enantiomers with certain chiral derivatizing reagents (CDRs) and/or catalyst are known to lead to kinetic resolution

of a racemic mixture [6, 7]. Kinetic resolution of pharmaceutically active compounds, containing amino or alcoholic group, by reaction with anhydride of optically active acids has been reported [7]. Literature showed that acid chloride-based chiral derivatizing reagents, such as (*S*)-naproxen acyl chloride [8] and *N*-tosyl-(*S*)-prolyl chloride [9], have been used for kinetic resolution of secondary amino group containing pharmaceuticals of fluoroquinolone series. The use of such enantiomerically pure acylating reagents for the resolution of alcohols and amines provides an alternative to the methods involving enzymes and chiral catalysts.

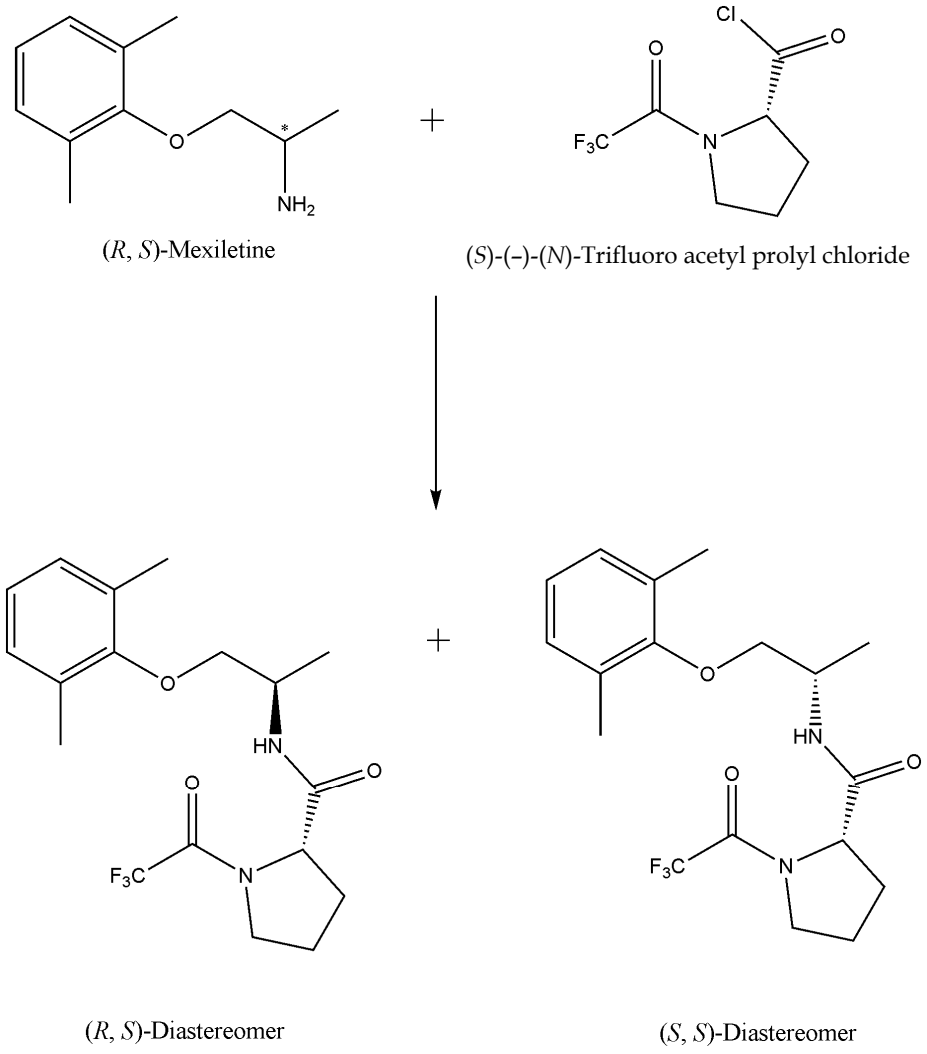

Fig. 1. Synthesis of diastereomers of (*R*, *S*)-MEX using (*S*)-(−)-(*N*)-trifluoro acetyl prolyl chloride as the representative CDR

The chiral reagents, (*S*)-(−)-*N*-trifluoro acetyl prolyl chloride ((*S*)-TFPC) and camphanic chloride, are volatile in nature and thermally stable. (*S*)-TFPC has been utilized as CDR for enantiomeric resolution of amphetamine and methamphetamine in biological fluids such as urine and blood [10–12], and hair [13] by gas chromatography–mass spectroscopy. Camphanic chloride has been utilized to synthesize diastereomers of different alcohols [14–17]; the diastereomers, so formed, were separated by column chromatography and were hydrolyzed to individual enantiomers. Recovery of enantiomers from such derivatives was successful because the said CDRs *viz.* (*S*)-TFPC and camphanic chloride could be easily detached from the product. Derivatization, particularly, to introduce a chromophore in low UV absorbing molecules may not be fully exceptional even for direct approach of enantioresolution.

In this paper, we report reversed-phase high-performance liquid chromatographic (HPLC) enantioseparation of (*R*, *S*)-MEX via an indirect approach using (*S*)-TFPC and camphanic chloride as CDRs. The analytical separation was scaled-up to small-scale preparative level using the same mobile phase and the same reversed phase column; the samples so injected were drawn from diastereoselective synthesis setup. The native enantiomers were recovered by detagging the chiral reagent under mild reaction condition with enhanced yield of (*R*)-MEX. To the best of authors' knowledge, this is the first report of its kind on enantioseparation of (*R*, *S*)-MEX and recovery of its (*R)*-enantiomer.

Experimental

Chemicals and Reagents

(*R*, *S*)-Mexiletine, (*S*)-(−)-(*N*)-trifluoro acetyl prolyl chloride, and (1*S*)-(−)-camphanic chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other analytical-grade chemicals and HPLC grade solvents were obtained from E. Merck (Mumbai, India). Distilled water was further purified using a Milli-Q water purification system of Millipore (Bedford, MA, USA).

Equipments

Waters HPLC system consisting of a 515 HPLC pump, high-pressure binary gradient pump control module II, a Waters 2489 UV/Vis dual wavelength detector, a manual injection valve, an Empower2 operating software (Build number, 2154), and Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm) col-

umn (from Parker-Style Fittings, Ireland), were used. Other equipments used were a pH meter Cyberscan 510 (Singapore) and a Polarimeter P-3002 (Krüss, Hamburg, Germany).

Synthesis of Diastereomers of (*R*, *S*)-MEX

The following stock solutions were prepared:

1. Twenty-five millimolar solution of (*S*)-TFPC was prepared by dissolving 43 μL of (*S*)-TFPC (MW 229.59; density 1.308 g mL^{-1}) in 10 mL dichloro methane (DCM).
2. Twenty millimolar solution of (*R*, *S*)-MEX was prepared by dissolving 35.8 mg of (*R*, *S*)-MEX (MW 179.26) in 10 mL DCM.
3. Twenty-five millimolar solution of (1*S*)-(−)-camphanic chloride was prepared by dissolving 54.10 mg of (1*S*)-(−)-camphanic chloride (MW 216.66) in 10 mL of DCM.

Synthesis of diastereomers of (*R*, *S*)-MEX was carried out under the following reaction conditions:

(A1): Five milliliters of stock solution of (*R*, *S*)-MEX (containing 17.90 mg of (*R*, *S*)-MEX, 0.1 mmol) and 2 mL of stock solution of (*S*)-TFPC (containing 11.5 mg of (*S*)-TFPC; 0.05 mmol) were mixed together (a mole ratio of 2:1). The reaction mixture was vortexed for 1 h at room temperature, without pyridine or any other base.

(A2): Sixty microliters of stock solution of (*R*, *S*)-MEX (containing 215.10 μg of (*R*, *S*)-MEX, 1.2 μmol) and 52 μL of stock solution of (*S*)-TFPC (containing 298.50 μg of (*S*)-TFPC; 1.3 μmol) were mixed (a mole ratio of 1:1.1) in Teflon tube of 1.5 mL, and pyridine (60 μL) was added. The reaction mixture was vortexed for 20 min at room temperature. The reaction was quenched by addition of HOAc (1 M, 60 μL).

(A3): Sixty microliters of stock solution of (*R*, *S*)-MEX (containing 215.10 μg of (*R*, *S*)-MEX, 1.2 μmol) and 72 μL of stock solution of (*S*)-TFPC (containing 413.30 μg of (*S*)-TFPC; 1.8 μmol) were mixed (a mole ratio of 1:1.5) in Teflon tube of 1.5 mL, and pyridine (60 μL) was added. The reaction mixture was vortexed for 20 min at room temperature. The reaction was quenched by addition of HOAc (1 M, 60 μL).

(A4): Sixty microliters of stock solution of (*R*, *S*)-MEX (containing 215.10 μg of (*R*, *S*)-MEX, 1.2 μmol) and 96 μL of stock solution of (*S*)-TFPC (containing 551 μg of (*S*)-TFPC; 2.4 μmol) were mixed (a mole ratio of 1:2) in Teflon tube of 1.5 mL, and pyridine (60 μL) was added. The reaction mixture was vortexed for 20 min at room temperature. The reaction was quenched by addition of HOAc (1 M, 60 μL).

Similar sets of experiments were carried out for the synthesis of diastereomers of (*R*, *S*)-MEX using (1*S*)-(−)-camphanic chloride and denoted as B1, B2, B3, and B4, respectively.

HPLC

A 10-μL volume of each of the resulting solutions (of A1–A4 and B1–B4), containing diastereomers, was diluted 10-fold with MeCN, and 20 μL of it was injected onto the column.

Reversed phase HPLC separation was performed for all the samples of the diastereomers (i.e., A1–A4 and B1–B4) with the same mobile phase consisting of aqueous trifluoroacetic acid (TFA)–acetonitrile (MeCN) both in a linear gradient and isocratic mode and UV detection at 210 nm. TFA was used in the range of 0.01–0.2% to establish its optimum concentration for resolution of diastereomers. The effect of change of flow rate was examined in a range of 0.5–1.5 mL min^{-1}.

Chromatographic conditions of column and mobile phase that were optimized for analytical enantioseparation were applied for small-scale preparative HPLC. The solutions containing diastereomeric excess (samples A1 and B1) were injected onto the column (without dilution) using 200 μL injection loops. This process was repeated for 10 times. After 10 repeated injections, approximately 25 mL fraction of the mobile phase, containing first eluting diastereomer, was collected for sample A1. It was concentrated *in vacuo*. The concentrate was refluxed in a mixture of *conc* HCl and glacial AcOH (2 mL each) for 4 h. The reaction mixture was then evaporated to dryness under vacuum. The residue was dissolved in water (5 mL). It was cooled in an ice bath and then treated with sodium carbonate (1 M, 5 mL) under ice-cooling and the precipitate of the corresponding enantiomer, i.e., (*R*)-MEX was obtained.

Similarly, after the same number of repeated injections, approximately 40 mL fraction of the mobile phase, containing first eluting diastereomer, was collected for sample B1. The collected fraction was subjected to the aforementioned set of experiments, and the enantiomer (*R)*-MEX was obtained.

Results and Discussion

The diastereomers of (*R*, *S*)-MEX were synthesized using the two CDRs in different mole ratios targeting analytical and small-scale preparative separation. A total of eight samples (A1–A4 and B1–B4), containing diastereomers of (*R*, *S*)-MEX, were prepared and investigated. The reaction of (*R*, *S*)-MEX

and (*S*)-TFPC, as the CDR, for the synthesis of diastereomers is shown in *Fig. 1*. The diastereomers were designated as (*R*, *S*)- and (*S*, *S*)-diastereomer; the first letter represents the absolute configuration of MEX, and the second represents configuration of CDR as both the CDRs have (*S*)-configuration.

Analytical Separation

Binary mobile phase consisting of *aq* TFA (0.1%)–MeCN in a linear gradient of MeCN from 30 to 70% in 45 min at a flow rate of 1.0 mL min^{-1} and UV detection at 210 nm was found successful for the separation of diastereomers. Sharp peaks were obtained under gradient elution. MeCN was found to be a better organic modifier in comparison to methanol as broader peaks were observed with the use of methanol.

The retention factor (k), separation factor (α), and resolution (R_S) for the diastereomers of the sample (A2) (using (*S*)-TFPC as the CDR, in presence of pyridine) and the sample (B2) (using camphanic chloride as the CDR, in presence of pyridine) are given in *Table I*. The two pairs of diastereomers were well separated. A slight excess of CDR (mole ratio of (*R*, *S*)-MEX:CDR being 1:1.1) in the samples (A2) and (B2) prevented kinetic resolution and provided mixture of (*R*, *S*)- and (*S*, *S*)-diastereomers in 1:1 ratio; sections of chromatograms showing resolution of diastereomers are shown in *Fig. 2*. In both cases, sharp peaks with good separation factor values ($\alpha > 1.20$) and resolution values ($R_S > 2$) were obtained, and the diastereomer corresponding to (*R*)-(−)-MEX was eluted prior to that of (*S*)-(+)-MEX *(Table I)*.

Table I. Chromatographic data for resolution of diastereomers of (*R*, *S*)-MEX synthesized with CDRs (samples A2 and B2)

CDR	Resolution of diastereomers			
	k_1		α	R_S
(*S*)-(−)-(*N*)-Trifluoro acetyl prolyl chloride	4.50		1.22	3.59
(1*S*)-(−)-Camphanic chloride	7.41		1.32	4.82

k_1 is the retention factor of first eluted diastereomer, α is stereoselective factor, and R_S is the resolution of the diastereomers.

The values of separation factor (α) and resolution (R_S) for diastereomers synthesized with camphanic chloride are higher than those synthesized with (*S*)-TFPC *(Table I)*. Hence, it can be said that camphanic chloride was better as a CDR for HPLC enantioseparation in the present studies.

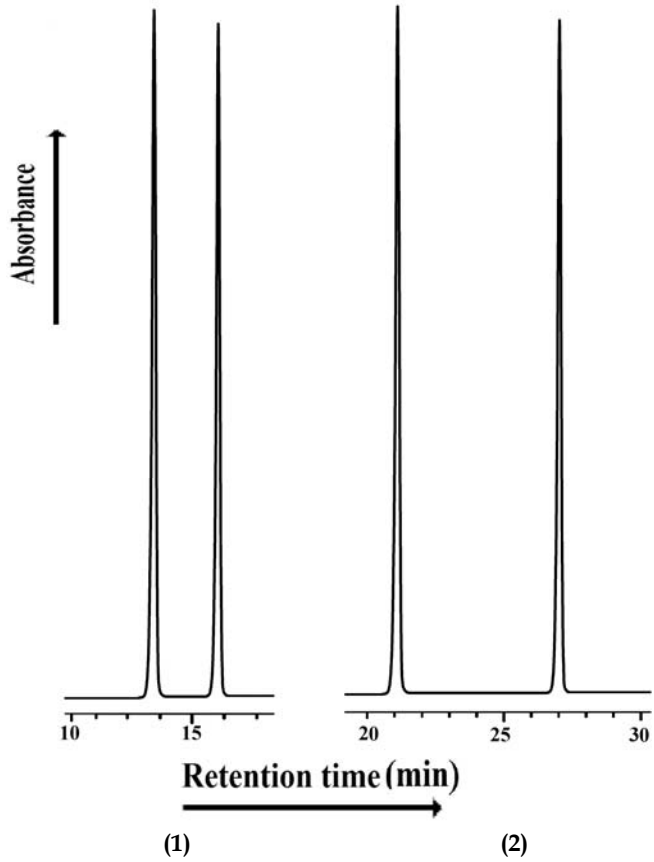

Fig. 2. Sections of chromatograms showing HPLC resolution of diastereomers of (*R*, *S*)-MEX (samples A2 and B2) prepared with (*S*)-TFPC (1) and camphanic chloride (2); column, Waters Spherisorb ODS2 (250 × 4.6 mm I.D., 5 μm); diastereomeric peak corresponding to (*R*)-MEX was eluted prior to that of (*S*)-MEX; mobile phase, aq TFA (0.1%)–MeCN in a linear gradient of MeCN from 30 to 70% in 45 min at a flow rate of 1.0 mL min^{-1} and UV detection at 210 nm

Kinetic Resolution of (*R*, *S*)-MEX

The samples (A1) and (B1) were set up for diastereoselective synthesis based on kinetic resolution and to investigate them for small-scale preparative level separation under the HPLC conditions successful for analytical separation.

The HPLC experiments concluded that the reaction of (*R*, *S*)-MEX with (*S*)-TFPC in a mole ratio of 2:1, in the absence of pyridine (sample A1), resulted into a diastereoselective reaction and the product was found to be

significantly enriched with (*R*, *S*)-diastereomer with diastereomeric excess (*de*) of 71%. The peak areas ccorresponding to the two diastereomers (obtained from the system software) became constant (under the same HPLC mobile phase conditions that were optimized for analytical approach of enantioseparation). The peak areas of the first and second eluted diastereomers from the sample (A1) were 454.141 mAU min and 77.018 mAU min, respectively. The exact ratio of peak areas corresponding to the two diastereomers was 1.71:0.29. It means a kinetic resolution took place. The recovery studies of the two eluted diastereomers served as a measure of their yields of the order of 98%, since recovery of all eluted diastereomers was found to be nearly 98%.

HPLC experiments, under the identical conditions for sample B1 (reaction of (*R*, *S*)-MEX with camphanic chloride in a mole ratio of 2:1 in the absence of pyridine) showed that the diastereoselective products were obtained with *de* up to 74%. The peak areas of the first and second eluted diastereomers from sample (B1) were 462.108 mAU min and 69.050 mAU min, respectively.

Small-Scale Preparative Separation

The HPLC results for analytical enantioresolution (i.e., k_1 and $k_2 < 10$; $\alpha > 1.20$ and $R_S > 2$; *Table I*) inspired us for scaling up of the method to small-scale preparative level. For this purpose, samples (A1) and (B1), which were constituted for diastereoselective synthesis based on kinetic resolution, were used for this purpose.

Literature suggests that an optimized scale-up strategy should be employed to small-scale preparative separation for avoiding time consumption and wastage of materials [18]. Therefore, the same HPLC conditions, which were optimized for analytical enantioresolution of diastereomers of (*R*, *S*)-MEX, were applied for small-scale preparative separation. It was attempted by increasing the load volume of sample on to the same column and the fractions for the peaks corresponding to the two diastereomers were collected. Detagging experiments were performed only on (*R*, *S*)-diastereomer keeping in mind the recovery of (*R*)-MEX, which is the therapeutically important enantiomer. Nevertheless, detagging could be successful on (*S*, *S*)-diastereomer under the identical conditions. The pure enantiomer, (*R*)-MEX, was obtained by acid hydrolysis of the fractions containing (*R*, *S*)-diastereomers. The two CDRs were easily removed by acidic hydrolysis since they are thermally stable in nature. The particle size of the column used for small-scale preparative separation is of 5 μm average diameter which is cost effective in comparison to the smaller and more expensive particles of 1.8 μm or 3.5 μm average diameters [19].

The recovered amounts of (*R*, *S*)-diastereomers were 4.09 mg and 4.22 mg from samples A1 and B1, respectively, while the amounts of native (*R*)-MEX obtained by the hydrolysis were 1.27 mg and 1.50 mg, respectively.

Reaction Enantioselectivity

The reaction carried out is a typical reaction of synthesizing the amides, and it needs the presence of amine (a base), which removes HCl from the reaction medium through formation of a quaternary amino or pyridine salt. The reaction was investigated for different mole ratio of MEX:CDR and both in presence and absence of pyridine (a base).

The effect of pyridine in the present reaction can be explained by taking into account the mechanism proposed for amine-catalyzed esterification [20] and acylation [21] in the earlier studies. In the presence of pyridine, the reaction proceeds *via* acyl ammonium intermediate salt, which reacts with MEX, and the intermediate is converted to amide (the product) along with elimination of 1 mol of HCl; thus, a base-catalyzed mechanism is followed in which pyridine is activating the CDR and facilitating elimination of Cl. These types of acylation reactions are quite tedious to study [22].

To examine the effect of self catalysis role of amine (the (*R*, *S*)-MEX, in this case), samples A1 and B1 were worked up in the absence of pyridine, so the mole ratio of amine:CDR was 2:1. The excess 1 mol of MEX mops up 1 mol of HCl during the reaction, i.e., 1 mol of MEX acts as a base and the other gets converted to amide. Hence, the reaction is complete with respect to CDR. During preparative HPLC separation, the peak related to the excess (*S*)-MEX eluted prior to (*R*, *S*)-diastereomer (and diastereomer of (*R*)-(−)-MEX eluted prior to that of (*S*)-(+)-MEX, *Table I*). So the sample loading did not require purification action. All the components are getting separated by HPLC. Thus, it can be stated that the desired diastereomer is getting purified automatically.

Results, as discussed above, clearly show the difference in the rates of reactions of individual enantiomers of (*R*, *S*)-MEX with (*S*)-TFPC (or camphanic chloride). In the present work, (*R*, *S*)-MEX (primary amine; a nucleophilic reagent) reacted with CDR, i.e., (*S*)-TFPC (an acyl chloride), either in the presence or absence of pyridine (base, a catalyst) in dichloromethane (an aprotic, less polar solvent).

In the absence of pyridine, reaction of MEX with each of the two CDRs yielded amide *via* acyl ammonium intermediate salt. Thus, a nucleophilic mechanism was followed. An intermediate salt is formed by direct replacement of chlorine of the CDR to give a tetrahedral intermediate of higher po-

larity in comparison to initial CDR molecule, i.e., acylation occurs with charge separation.

Method Validation

The experimental method for analytical resolution was validated with respect to linearity, accuracy, and precision for the diastereomers of (*R*, *S*)-MEX synthesized with both CDRs following International Conference of Harmonization (ICH) guidelines [23]; the data related to the diastereomers synthesized with (*S*)-TFPC are summarized here in *Table II*. The accuracy and precision studies were carried out by replicate HPLC analysis ($n = 5$) of diastereomers of (*R*, *S*)-MEX synthesized with (*S*)-TFPC at three concentrations (50–150 ng mL^{-1}). The slopes and correlation coefficients were computed by the calibration graphs between the peak area responses of (*R*, *S*)-diastereomer and (*S*, *S*)-diastereomer and the corresponding concentration (50, 100, and 150 ng mL^{-1}). The regression equations were $y = 0.0085x - 0.904$ ($r^2 = 0.997$) and $y = 0.0081x + 0.981$ ($r^2 = 0.998$) for the (*R*, *S*)-diastereomer and (*S*, *S*)-diastereomer, respectively. The relative standard deviation (%) for (*R*)-MEX and (*S*)-MEX varied from 0.65 to 1.04 and 0.40 to 0.75 for intra-day assay precision and 0.80 to 1.44 and 0.83 to 0.96 for inter-day assay precision.

Table II. Intra-day assay and inter-day assay precision of diastereomers of (*R*, *S*)-MEX synthesized with (*S*)-TFPC

Concentration (ng mL^{-1})	First eluting diastereomer			Second eluting diastereomer		
	Mean ± SD	Recovery[a]	RSD[a]	Mean ± SD	Recovery[a]	RSD[a]
Intra-day						
50	24.58 ± 0.27	98.3	1.04	24.70 ± 0.19	98.8	0.75
100	49.45 ± 0.44	98.9	0.88	49.85 ± 0.20	99.7	0.40
150	75.83 ± 0.49	101.1	0.65	76.20 ± 0.43	101.6	0.57
Inter-day						
50	24.40 ± 0.36	97.9	1.44	24.34 ± 0.24	97.4	0.96
100	48.95 ± 0.48	97.9	0.96	49.15 ± 0.46	98.3	0.92
150	73.58 ± 0.60	98.1	0.80	73.67 ± 0.62	98.2	0.83

[a]Represents percentage values.
SD is standard deviation and RSD is relative standard deviation.

Results of validation studies indicated that the solutions of diastereomers of MEX were quite stable up to 1 week under refrigerated condition (4°C). Limit of detection (LOD) corresponding to the signal-to-noise ratio of 3, of the diastereomers of (*R*, *S*)-MEX synthesized with (*S*)-TFPC

and camphanic chloride, was found to be 45 ng mL^{-1} and 80 ng mL^{-1}, respectively.

Conclusion

The method developed for analytical resolution of (*R*, *S*)-MEX could be scaled up for small-scale preparative separation of diastereomers from the samples constituted for diastereoselective synthesis based on kinetic resolution. (*R*)-MEX reacted nearly six times faster than the (*S*)-enantiomer under the given conditions of diastereomeric synthesis. The native (*R*)-isomer can then be recovered, in good yield, by hydrolysis of the diastereomer since the CDRs are thermally stable. Camphanic chloride was found to be better as a CDR in comparison to (*S*)-TFPC with respect to *R*s and (α) of the diastereomers. The method can be applied in various situations requiring control of enantiomeric purity. The method may be successfully applied to other pharmaceutically important compounds having primary amino group.

Acknowledgments

The authors are grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi, for the award of a senior research fellowship (to R.D.).

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Accepted by TK