NJC

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c7nj02898a

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

Synthesis of diastereomeric anhydrides of (*RS*)-ketorolac and (*RS*)-etodolac, semi-preparative HPLC enantioseparation, establishment of molecular asymmetry and recovery of pure enantiomers†

Poonam Malik and Ravi Bhushan *

Herein, enantioseparation of two anti-inflammatory drugs, namely, (*RS*)-ketorolac and (*RS*)-etodolac, commonly marketed and administered as racemates, was achieved by RP-HPLC. This method provided very low limit of detection values (3.69 and 3.02 ng mL^{-1} for diastereomeric derivatives of (*R*)- and (*S*)-Ket, respectively) as compared to those reported in literature. (*S*)-Naproxen benzotriazole ester, which was used as a chiral reagent, was synthesized and characterized by UV, IR, and ^{1}H NMR spectroscopies, elemental analysis, and polarimetry. The diastereomeric derivatives were synthesized *via* microwave irradiation, separated on an analytical scale by RP-HPLC, and then isolated by preparative HPLC. The use of a mobile phase containing methanol and aqueous triethylamine phosphate (TEAP) in the isocratic mode was found to be successful for the separation of diastereomeric derivatives, and the separation conditions with respect to pH, flow rate, and buffer concentration were optimized. The diastereomeric derivatives were characterized, and their absolute configuration was established. Hydrolysis of the derivatives provided native enantiomers under mild reaction conditions. This study describes the successful enantioseparation of the above mentioned two analytes by semi-preparative HPLC with easy recovery of the native enantiomers without racemization and with the establishment of molecular asymmetry.

Received 13th August 2017,
Accepted 5th October 2017

DOI: 10.1039/c7nj02898a

rsc.li/njc

1. Introduction

Both ketorolac (Ket) and etodolac (Etd) are widely used as non-steroidal anti-inflammatory drugs (NSAIDs). The physiological activity of both drugs is almost exclusively confined to their (*S*)-(−)-enantiomers, whereas the drugs are marketed and administered as racemic mixtures. Without emphasizing the importance of enantiomeric resolution, the control of enantiomeric purity and the determination of individual enantiomeric drug molecules remain subjects of potential importance from the clinical, analytical, and regulatory points of view. Enantioseparation by an indirect approach of derivatization of a variety of racemates including several common pharmaceuticals has been limited to the synthesis, detection, and separation of diastereomeric derivatives, which are generally amides because of the functional group compatibility of the chiral reagent and this type of analytes. To the best of our knowledge, there is no report in the literature that deals with the recovery of native enantiomers from diastereomeric derivatives after their separation, with the exception of one report on the detagging of diastereomeric amides of (*RS*)-propranolol (Prl) that have been prepared using a (*S*)-levofloxacin (Lfx)-based chiral reagent.[1] Although the ^{1}H NMR spectra of diastereomeric amides of (*RS*)-Etd prepared using (*R*)-(−)-1-cyclohexylethylamine and separated by preparative TLC were obtained to establish their configuration, the recovery of native enantiomers was not achieved.[2]

Ketorolac (Ket) has been introduced as a safer intravenous alternative to opioid analgesics.[3] It is chemically designated as (*RS*)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid (Fig. 1) and has a stereogenic centre located within the pyrrolizine ring.

Etodolac has the systematic chemical name (*RS*)-1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole-1-acetic acid (Fig. 1). It is used as an analgesic and for the treatment of rheumatoid arthritis and osteoarthritis. It is considered relatively better as a therapeutic agent as it produces less gastrointestinal toxicity in comparison with certain other NSAIDs.[4]

Lal and Bhushan reported an efficient, simple, validated, analytical, and semi-preparative HPLC method for the direct enantioresolution of (*RS*)-Ket using chiral columns of mono-chloromethylated derivatives of cellulose and amylose.[5] The enantioseparation of (*RS*)-Etd has also been reported by this group by different methods, which include (i) HPLC enantioseparation by

Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee 247667, India. E-mail: rbushfcy54@gmail.com, rbushfcy@iitr.ac.in, pmalik2289@gmail.com; Fax: +91-1332-286202; Tel: +91-1332-285795

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7nj02898a

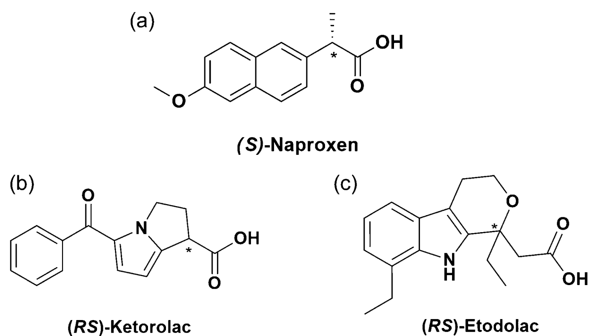

Fig. 1 Structures of (*S*)-naproxen and racemic drugs.

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

pre-column formation of chiral ligand-exchange complexes;[6a] (ii) direct enantioresolution involving both achiral phases in TLC, where a few L-amino acids are used as chiral inducing reagents;[6b] (iii) LC-MS separation of diastereomeric derivatives that have been synthesized using three different enantiomerically pure amines[6c] ($[M + H]^+$ or $[M]^+$ have been obtained for confirmation of the formation and structures of the diastereomers); and (iv) TLC enantioseparation of diastereomeric amide derivatives synthesized using enantiomerically pure (*R*)-(−)-1-cyclohexylethylamine.[2] A few other studies have been reported on the direct enantioseparation of Ket using a polysaccharide-based CSP[7,8] and a teicoplanin-based stationary phase.[9] Starek *et al.* reviewed TLC methods for the determination of NSAIDs.[10] The enantioseparation of Etd has been achieved on a chiral AGP (α1-acid glycoprotein) column[11] and chiral stationary phases such as CHI-DMB [chemically known as *O*,*O*′-di(3,5-dimethylbenzoyl)-(2*R*,3*R*)-diallyltartardiamide] and (*R*,*R*)-DNB-DPEDA [*i.e.*, (*R*,*R*)-*N*-3,5-dinitrobenzoyl-1,2-diphenylethane-1,2-diamine],[12] which are π-electron acceptor/donor stationary phases of broad applicability.

Literature related to the enantioseparation of Ket and Etd by both direct and indirect approaches has been cited and discussed in the abovementioned reports; therefore, the same have not been mentioned herein. However, some of the drawbacks and limitations based on relevant literature reports have been briefly mentioned hereinafter.

(*R*)-(+)-l-(l-Naphthyl)ethylamine was used as a derivatizing reagent for (*RS*)-Ket, and the separation was carried out using an α-acid glycoprotein chiral column,[13] but the experimental procedure of sample preparation and analysis was very cumbersome. Thus, there seems to be no reasonable justification for using a chiral derivatizing reagent (CDR) and then performing separation on a chiral column. The other methods reported in the literature suggest that there are several major limitations such as: (a) there have been a few reports on the direct enantioresolution of Ket and Etd using HPLC and chiral columns, but the latter are very expensive, have low durability and limited applicability, and are very sensitive to the chromatographic conditions; (b) the method described by Ing-Lorenzini *et al.*[14] for the determination of enantiomers of Ket involves a tedious and time-consuming extraction procedure using liquid–liquid extraction; (c) the HPLC separation of diastereomeric amides[15] involves the derivatization of (*RS*)-Ket with thionyl chloride/(*S*)-1-phenylethylamine, but the use of thionyl chloride has been discouraged in chemical methodology for a long time; (d) the pre-column derivatization of (*RS*)-Ket reported by Vakily *et al.* in 1995[16] has the drawback of rapid and complete racemization due to the strongly basic conditions required for derivatization; and (e) an indirect approach using (*S*)-(−)-α-(1-naphthyl)ethylamine as the derivatizing reagent for the HPLC enantioseparation of (*RS*)-Etd[17] involves a very cumbersome derivatization reaction and multistep sample preparation before the sample is loaded onto the column although it has been claimed to be a simplified method with a shortened derivatization time.

Taking into account the abovementioned literature, this study was aimed at reporting a sensitive HPLC method for the enantioseparation of (*RS*)-Ket and (*RS*)-Etd with the establishment of the configuration of the diastereomeric derivatives, followed by easy recovery of the native enantiomers for their possible use. Therefore, (*S*)-(+)-naproxen (Npx, structure shown in Fig. 1) was chosen as the chiral moiety owing to its significant characteristics, helpful in enantioseparation by a derivatization approach, and a chiral reagent (CR) was synthesized and characterized.

(*S*)-Naproxen (Npx) was chosen because of its large conjugated naphthyl ring having a higher molar absorptivity ($\varepsilon > 100\,000$) as compared to that of certain other chromophoric molecules such as (*S*)-levofloxacin ($\varepsilon \sim 24\,000$), *s*-trichlorotriazine ($\varepsilon \sim 36\,000$), and the dinitrophenyl moiety ($\varepsilon \sim 30\,000\ mol^{-1}\ cm^{-1}$). Moreover, it is easily available as a pure (*S*)-enantiomer and does not require any other chiral auxiliary as is required by dinitrophenyl or *S*-trichlorotriazine moieties to convert them into chiral reagents. The carboxylic acid group of Npx is easily activated by the introduction of different nucleophilic moieties, which serve as effective leaving groups in a subsequent nucleophilic substitution reaction for the synthesis of diastereomeric derivatives. Its higher molar absorptivity facilitates the sensitive detection of the derivatized analyte.

A (*S*)-naproxen-based CR was synthesized as its ester by a simple straightforward reaction with 1*H*-benzotriazole and characterized by UV, IR, and ^{1}H NMR spectroscopies, elemental analysis, and polarimetry. It was used to synthesize diastereomeric derivatives of the two NSAIDs *via* microwave irradiation. The CR thus provided a pair of diastereomeric anhydrides for each of the two analytes, and hydrolysis of the anhydrides provided the native enantiomers under mild reaction conditions. The presence of carboxylic acid groups in both (*RS*)-Ket and (*RS*)-Etd is advantageous in the overall approach and in the success of the experiments and results presented in this study. The present study is novel and different from the existing literature as it reports the semi-preparative HPLC enantioseparation of two analytes with easy recovery of the native enantiomers without racemization and the establishment of molecular asymmetry using ^{1}H NMR and polarimetric studies.

2. Materials and methods

2.1 Chemicals and equipment

N-Hydroxybenzotriazole (OH-Btz), (*S*)-(+)-naproxen [$[\alpha]_D^{25}$ = +66°, (c = 1, $CHCl_3$), assay ≥ 98.0%], dicyclohexylcarbodiimide

This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2017

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

(DCC), and other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). Pharmaceutical tablets of (*RS*)-ketorolac marketed as Ketorol DT (Dr Reddy Laboratories Ltd, Hyderabad, India) containing 10 mg of an active pharmaceutical ingredient and (*RS*)-etodolac marketed as Etova (Ipca Laboratories Ltd, Mumbai, India) containing 400 mg of an active pharmaceutical ingredient were purchased from a pharmacist's shop in the local market. All the reagents [concentrated HCl, sodium bicarbonate ($NaHCO_3$), and phosphoric acid (H_3PO_4)] and solvents [ethyl acetate (EtOAc), acetonitrile (MeCN), and methanol (MeOH)] of HPLC grade were purchased from E. Merck (Mumbai, India).

The HPLC system (LC-20 AD) was obtained from Shimadzu (Kyoto, Japan), the LiChrospher C_{18} column (length × i.d., 25 cm × 4.6 mm, 5 μm particle size) was obtained from Merck (Darmstadt, Germany); the preparative HPLC system (LC-20AP Prominence preparative liquid chromatograph) was obtained from Shimadzu (Kyoto, Japan).

Other equipment/instruments used in the present investigations were as follows: a microwave (Multiwave 3000, 800 W, PerkinElmer, Shelton, CT, USA), a pH meter (CyberScan 510, Singapore), an FTIR spectrometer (Nicolet 6700, Thermo Scientific, USA), an elemental analyzer (Vario EL III, Hanau, Germany), and a 400 MHz NMR spectrometer (Jeol Inc., Peabody, USA). UV spectra were obtained in MeOH using the Shimadzu UV-2450 spectrophotometer. Double-distilled water was further purified using a Milli-Q system from Millipore (Bedford, MA, USA) to obtain purified water (18.2 MΩ cm), which was used throughout the experiments.

2.2 Isolation and purification of the title compounds as reference standards

(*RS*)-Ket (M.W. 255 g mol^{-1}) was extracted, isolated, purified, and characterized as reported earlier.[5] (*RS*)-Etd (M.W. 287 g mol^{-1}) was also extracted from the commercial tablets, isolated, purified, and characterized in a similar manner as described in an earlier report.[6c] The λ_{max} values, melting points, and characteristic IR peaks of the purified compounds were not included because the focus of this study was on enantiomer resolution. The recovery was nearly 97% against the amounts reported on the commercial labels in each case. The two compounds thus-obtained were used as standard racemates. Calibration plots were developed using the standard references.

2.3 Standard solutions

(a) (*RS*)-Ket and (*RS*)-Etd (1 mM each) were dissolved in 0.1 M $NaHCO_3$, and the stock solutions were diluted with methanol to prepare the required working solutions.

(b) Triethylammonium phosphate (TEAP) buffer (10 mM) was prepared, and its pH was adjusted to 3.5.

(c) A solution of the CR (10 mM) was prepared in MeCN.

2.4 Synthesis and characterization of CR

Npx ester (Npx-OBtz). The CR was synthesized by the reaction of (*S*)-(+)-Npx (5 mL, 0.2 M) with OH-Btz (5 mL, 0.2 M) in THF with the dropwise addition of DCC solution (3 mL, 0.36 M). The reaction mixture was stirred under a nitrogen atmosphere for 3 h. Dicyclohexylurea was precipitated and removed by filtration. The product in the filtrate was extracted with ethyl acetate and washed with brine, water, and saturated $NaHCO_3$ separately. Then, it was recrystallized from hot EtOH to obtain the reagent as an off-white solid. Characterization data of the CR are given in the ESI.†

2.5 Microwave-irradiated synthesis of diastereomeric derivatives

Separate sets of reaction mixtures were prepared by taking solutions of (*RS*)-Ket or (*RS*)-Etd (1 mM, 100 μL). To each mixture, a solution of the CR (1 mM, 200 μL) and triethylamine (20 μL) was added. The syntheses of the diastereomeric derivatives were carried out independently. The experimental conditions were optimized for best separation by varying the pH, ratio of racemic drug: CR (1 : 1, 1 : 1.5, 1 : 2, and 1 : 2.5), and microwave irradiation (MWI) time (in the range from 60 s to 150 s at an interval of 30 s) at 80% power (800 W). The diastereomeric mixture was subjected to HPLC separation in each case.

2.6 Preparative synthesis of diastereomeric derivatives

The diastereomeric derivatives were synthesized on a preparative level by scaling up the optimized synthesis conditions. (*RS*)-Ket (150 mg, 59 mM) or (*RS*)-Etd (150 mg, 52 mM) was dissolved in 10 mL of 0.1 M $NaHCO_3$, and to each solution, a solution of the CR (200 mg, 58 mM) in 20 mL acetonitrile was added. The yield was 320 mg (92%).

2.7 RPHPLC of diastereomeric derivatives

The diastereomeric mixture obtained from the reaction vial was diluted 10 times using a solvent system consisting of MeOH and TEAP buffer (10 mM, pH 3.5), and 20 μL of the above-mentioned mixture was injected onto the simple C_{18} column. The solvent system was degassed, sonicated, and filtered through 0.45 μm filters before use.

To optimize the separation conditions, the following variations were carried out: the mobile phase was used in isocratic elution mode with (i) varying strengths of TEAP buffer (*i.e.*, from 25 to 75% with a difference of 10% each time) for a period of 5–25 minutes, (ii) buffer concentrations of 5 mM, 10 mM, and 15 mM, and (iii) flow rates of 0.5 and 1.0 mL min^{-1} at each stage of the experiment. The oven temperature was fixed at 40 °C. Detection was carried out at 320 nm using a PDA detector.

2.8 Validation of the method

Validation studies were performed to assess recovery, linearity, accuracy, and precision using diastereomeric derivatives of (*RS*)-Ket (as representatives) in accordance with the International Council for Harmonisation (ICH) guidelines.[18] Peak areas (given by the system software) were used to determine stability and recovery. The concentrations of the diastereomeric mixtures were in the range of 5–5000 ng mL^{-1}. Linear plots of peak area *vs.* concentration were drawn, and the linear regression equation was used to determine slopes and intercepts. The LOD and LOQ were calculated. To determine the precision, inter-day and intra-day assay studies have been carried out, and the results are presented as the relative standard deviation (RSD).

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

2.9 Isolation of diastereomeric derivatives by preparative HPLC

The separation conditions were optimized for preparative HPLC by employing separation conditions similar to those used for analytical RP-HPLC. Experiments were carried out using both isocratic and gradient modes to achieve preparative enantioseparation using MeOH and TEAP (pH 3.5) as a solvent system. Elution strength was varied from 25% to 75% of TEAP buffer in a gradient over 30 min. The flow rate was 5 mL min^{-1}. The mixture of diastereomers (as obtained from the preparative synthesis) was diluted with MeOH, and 500 μL of the mixture was injected onto the C_{18} achiral column. Then, two vials were placed in rack 1 in the sample collector block for preparative HPLC. The time interval over which two peaks were obtained was selected for the collection of separated fractions from the fraction collector. A fraction of 2 mL was obtained for each injection. Further injections were performed twenty times, and fractions of nearly 40 mL were obtained during all the runs. The fractions containing single and identical diastereomers were combined and concentrated in vacuum. The diastereomeric derivatives separated in this way were recovered, and their purity was checked. These derivatives were designated as Ds-I and Ds-II for the first and second eluted diastereomeric derivatives of Ket and Ds-III and Ds-IV for the diastereomeric derivatives of Etd, respectively. The characterization data of the two separated diastereomers are given below.

2.10 Detagging of diastereomeric derivatives and isolation of enantiomers

Ds-I and Ds-II (100 mg each) were first dissolved individually in a small amount of MeOH, and then, H_2SO_4 (10 mL, 2 N) was added. The solution(s) was irradiated under sealed vessel conditions using MW for hydrolysis. The MWI system operated at a frequency of 2.45 GHz with an 80% continuous microwave irradiation power of 800 W. The experiment was performed in the following manner.

(i) With each change (60, 90, 120, 150, and 180 s) in the MWI time, samples were analyzed by HPLC as follows: 10 μL of each solution was diluted ten times with the mobile phase, and 20 μL of the diluted solution was applied to the simple C_{18} column. Because the retention times of the diastereomeric derivatives of Ket and Npx were different, two corresponding peaks were observed until complete hydrolysis occurred. On the basis of the peak areas (as given by the system software), it was observed that after a few optimization runs, complete hydrolysis or conversion of the individual diastereomeric anhydrides to their respective acids was achieved within 6 min. (This was a simple achiral RPHPLC column; therefore, one peak was obtained for an enantiomeric pair.)

(ii) The hydrolysates corresponding to Ds-I to Ds-IV were freeze-dried, and the residues were extracted with water. It is expected that only (*R*)- and (*S*)-Ket would go into the solution because (*S*)-Npx is insoluble in water. The solution was centrifuged, and (*S*)-Npx remained as a residue; the residue was further shaken with water and centrifuged. The combined supernatant was lyophilized, and the residue was dissolved in a small amount of MeOH.

The methanolic solutions corresponding to the four enantiomers were concentrated and allowed to crystallize after the addition of a few drops of water. Crystals were obtained by decantation and then air-dried and characterized.

3. Results and discussion

The recovery of native enantiomers from diastereomeric derivatives constitutes a difficult proposition because the experimental conditions of hydrolysis may lead to either decomposition of the enantiomers or racemization. Most of the time, the pure enantiomer (and thus the corresponding diastereomer) of the analyte is not available. Therefore, additional supporting investigations, such as isolation of diastereomers and recovery of enantiomers (ensuring no racemization), would be required for the determination of absolute configuration and confirmation of the elution order; this has been achieved in the present case by obtaining the ^1H NMR spectra and specific rotation. We feel that the success of the synthesis of diastereomeric derivatives and their separation is also a subject to the recovery of native enantiomers and verification of their configuration.

3.1 Chiral reagent

The reaction of organic amines with esters of carboxylic acids has been the simplest method of acylation in organic synthesis. However, with the use of DCC as a coupling reagent, both activation and coupling proceed concurrently. The rate of reaction of an amine with DCC is much lower in comparison with the rate of addition of a carboxylic acid to one of the double bonds of carbodiimide.[19] Therefore, the synthesis of the CR as an ester comprised the simple straightforward reaction of (*S*)-(+)-Npx with OH-Btz in the presence of DCC. The CR has been characterized (the relevant data are included in the manuscript) and used for the synthesis of diastereomeric derivatives.

The sample of (*S*)-(+)-Npx obtained from Sigma-Aldrich was first purified by recrystallization, and its enantiomeric purity was ascertained polarimetrically. Because the reaction of achiral OH-Btz took place only at the terminal carboxyl group of (*S*)-(+)-Npx and not directly at the stereogenic centre, it was considered that the CR synthesised in this way was enantiomerically pure (supported by the characterization data). Since no additional peaks were observed in the HPLC separation chromatograms for the diastereomeric derivatives, it was further inferred that the CR used for the synthesis of the diastereomeric derivatives was enantiomerically pure. If there was any impurity in the CR that corresponded to the (*R*)-(−)-isomer of naproxen, additional peaks would have been obtained in the HPLC separation chromatograms for the diastereomeric derivatives that would have been formed from a small amount of the (*R*)-(−)-isomer of the CR.

3.2 Diastereomeric derivatives

The optimized reaction conditions for the completion of the reaction in the synthesis of the diastereomeric derivatives were as follows: a 1 : 2 molar ratio of the analyte *vs.* CR and an MWI time of 150 s. It is known that the rates of reaction of

This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2017

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

enantiomers with chiral molecules are different. The two enantiomers of the (*RS*)-analyte may react at different rates with the CR, which is a chiral molecule. The derivatization reaction of (*RS*)-Ket or (*RS*)-Etd with the CR at different mole ratios exhibited slight kinetic resolution at a 1 : 1 ratio of (*RS*)-Etd:CR. The completion of the derivatization reaction was inferred from the identical peak areas (as provided by the system software). The 94% recovery of the eluted diastereomers provided an estimate of the yields. The effect of the MWI time on the completion of the derivatization reaction of (*RS*)-Ket is shown in Fig. S1 in the ESI.†

A representative scheme for the synthesis of the CR and diastereomeric derivatives of (*RS*)-Ket is shown in Fig. 2. Since derivatization involved a reaction between the activated terminal group of (*S*)-(+)-Npx and the carboxyl terminal group of the analyte [for example, (*RS*)-Etd], in neither of the two reactants, a direct attack at the stereogenic centre was involved, and no racemization was detected throughout the study. Thus, the following types of diastereomeric derivatives were considered to have been formed: (a) [(*R*)-Ket-(*S*)-Npx], (b) [(*S*)-Ket-(*S*)-Npx], (c) [(*R*)-Etd-(*S*)-Npx], and (d) [(*S*)-Etd-(*S*)-Npx]. The first letter [capital (*R*) or (*S*)] represents the absolute configuration of the enantiomer of the corresponding analyte, whereas the second letter represents the absolute configuration of (*S*)-Npx.

Earlier reports in the literature[20–22] have shown that a chiral amide prepared from (*S*)-(+)-Npx and 1*H*-benzotriazole acts as a CR for the formation of diastereoisomeric derivatives of various pharmaceuticals and other compounds containing an amino group; the amino group acts as a nucleophile, attacks the CR, and substitutes the benzotriazole moiety. The overall reaction results in the formation of a pair of diastereomeric derivatives containing an amide bond. However, in none of these reports, native enantiomers were obtained.

Moreover, there has been no report on the formation of diastereoisomeric derivatives for any of the chosen analytes [(*RS*)-Ket or (*RS*)-Etd] with a CR based on (*S*)-(+)-naproxen and 1-hydroxybenzotriazole. It may be argued that the present CR, being an ester with (*S*)-(+)-naproxen as the chiral moiety, is more reactive (in comparison with an amide) in forming the diastereomeric derivatives of the chosen analytes under mild reaction conditions, and because of the mild conditions, the formation of diastereomeric derivatives is faster and the chances of racemization are reduced.

A possible mode of bond formation leading to diastereomeric derivatives in the form of anhydrides (as shown in Fig. 3) has been discussed hereinafter. In general, anhydrides and esters have intermediate reactivity.

The electronegativities of N, C, and O atoms are 3.04, 2.55, and 3.44, respectively, on the first scale developed by Linus Pauling.[23,24] Moreover, the electronegativity of the carbonyl group (>C=O) calculated by the revised Lewis-Langmuir equation is 2.706 ± 0.01.[25] The difference between the electronegativities of carbon and oxygen is large enough to make the C=O bond moderately polar. In the CR, there is a N atom (of the benzotriazole moiety) at the position of the hydroxyl hydrogen atom of the carboxyl group of the parent naproxen moiety. Thus, the carbonyl carbon atom (>C=O) becomes more electron-deficient owing to the presence of highly electronegative elements

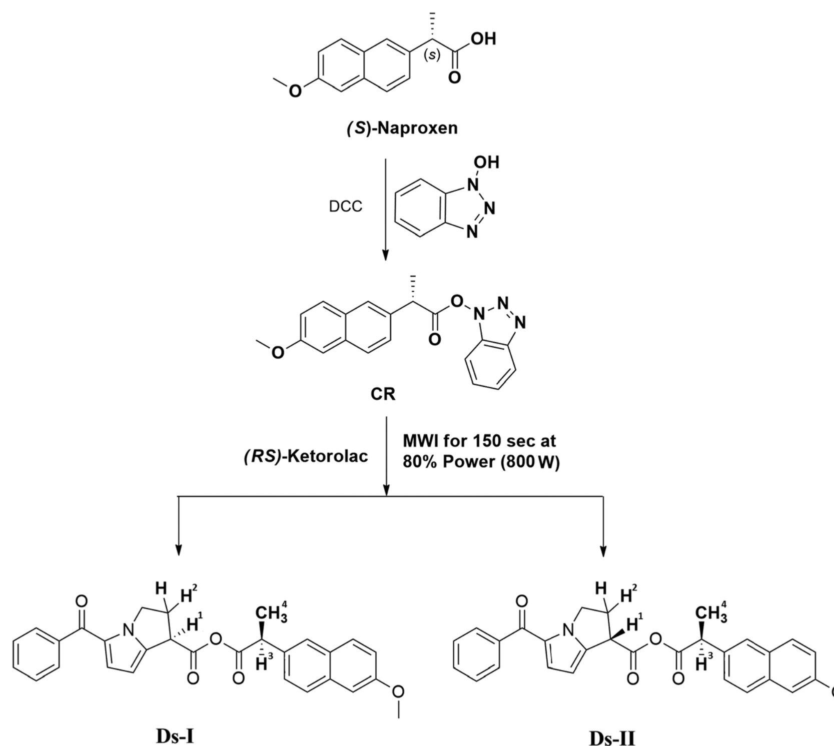

Fig. 2 Representative scheme of the synthesis of the CR and diastereomeric derivatives of (*RS*)-Ket.

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

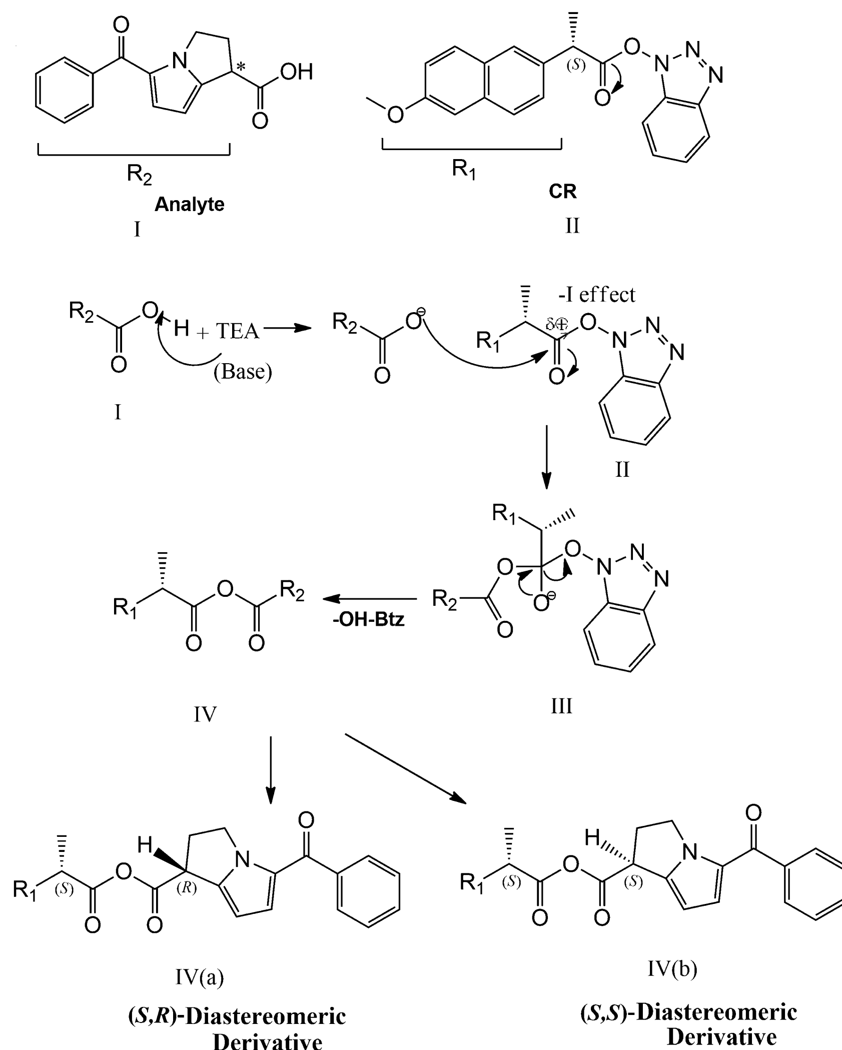

Fig. 3 Plausible mechanistic route of anhydride synthesis.

(O and N) in its close vicinity (II in Fig. 3). The literature suggests that the electronegativity depends on the environment of a particular atom within a molecule as well as on its degree of hybridization.[26–28]

In the present situation of the synthesis of diastereomeric derivatives, the COO^- group of the analyte acts as a nucleophile and attacks the electrophilic carbon atom of the CR, and the pi-bond electrons undergo resonance to the more electronegative oxygen atom to form a tetrahedral adduct (as a transitional intermediate III shown in Fig. 3) *via* a carbon-nucleophile bond with a change in hybridization from sp^2 to sp^3. These inductive and resonance effects result in the carbonyl carbon atom having a large δ^+ charge and high reactivity, and it is thus easily attacked by a nucleophile (the COO^- group in the (*R*)- or (*S*)-enantiomer of Ket or Etd in the racemic analyte). Moreover, the OH-benzotriazole moiety acts as a good leaving group and is displaced very easily.

Thus, the overall reaction leads to the formation of the product (**IVa** and **b** in Fig. 3) as a pair of diastereomeric derivatives, which are structurally anhydrides. This structure is more stable owing to stronger resonance or greater delocalization (with two oxygen atoms); therefore, its formation is favored. The presence of a weak base (triethylamine) during the derivatization reaction would facilitate the formation of the abovementioned diastereomeric derivatives by making the analyte more nucleophilic in nature, and excess substrate molecules would not react with each other to form naproxen anhydride.

3.3 HPLC separation of diastereomeric mixtures

It was observed that the same chromatographic conditions were successful for the separation of diastereomeric pairs of both analytes; these included a mobile phase that comprised a MeOH–TEAP buffer (pH 3.5) at a flow rate of 1.0 mL min^{-1} in the isocratic mode (from 75% to 25%) using a C_{18} column and detection at 320 nm.

Actual chromatograms (obtained from the system) showing the baseline separation of the pairs of diastereomeric derivatives of (*RS*)-Ket and (*RS*)-Etd are given in Fig. 4(i) and (ii), respectively. The diastereomeric derivatives of both racemic analytes displayed very good separation. Table 1 summarizes the values of the retention factor *k*, separation factor *α*, and

 This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2017

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

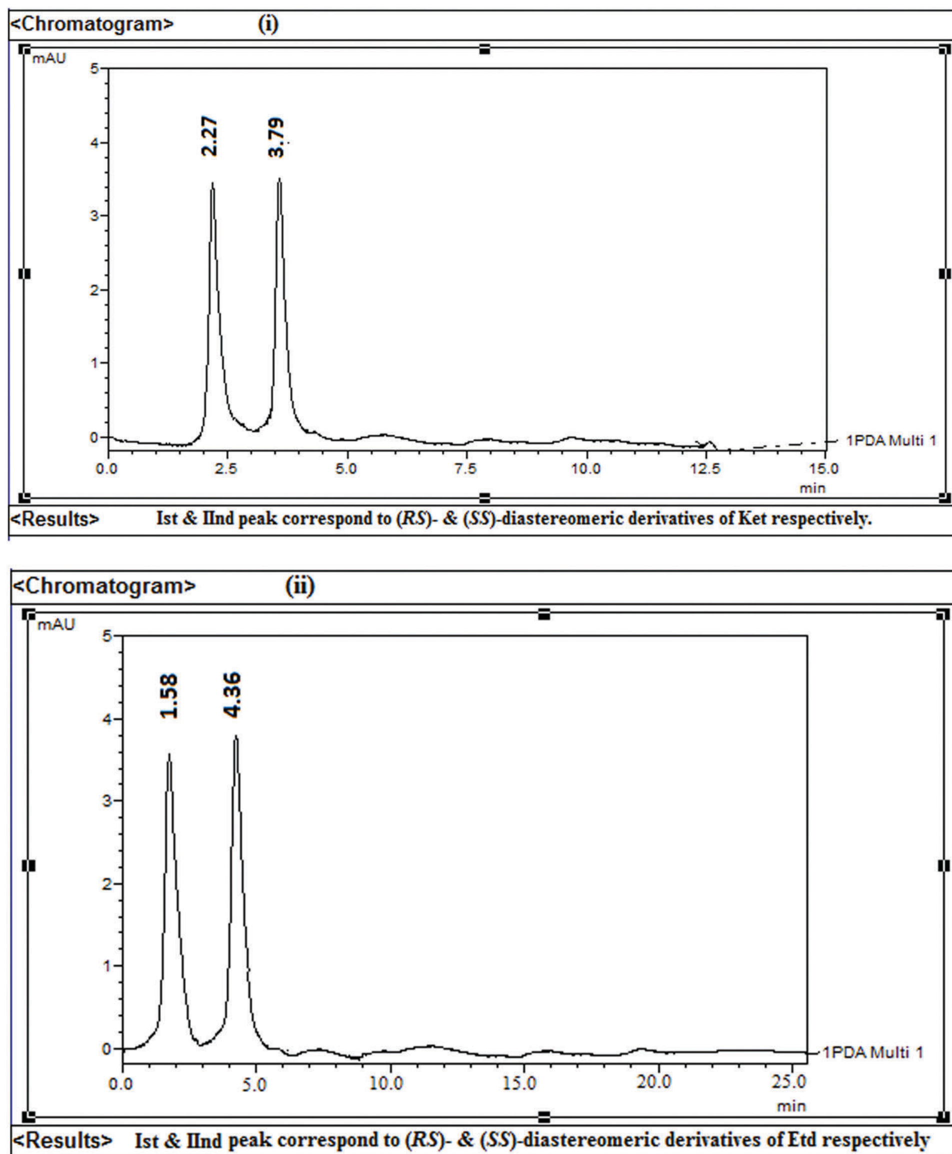

Fig. 4 Actual chromatograms (obtained from the system) showing the separation of the diastereomeric derivatives of (i) (*RS*)-Ket and (ii) (*RS*)-Etd. Chromatographic conditions: column: LiChrospher C_{18} (250 mm × 4.6 mm I.D., particle size 5 μm); mobile phase: MeOH–TEAP buffer (pH 3.5), binary gradient (from 75% to 25%); flow rate: 1 mL min^{-1}; detection: 320 nm; and injection volume: 10 μL. The first elution peak corresponds to the (*R*,*S*)-diastereomeric derivative in both the cases. The *x*-axis shows the time in min, and the *y*-axis shows the absorbance in mAU.

Table 1 HPLC separation data for diastereomeric derivatives of racemic analytes

	Characteristics of the separated diastereomers			
Racemic analyte	k_1	k_2	α	Rs
(*RS*)-Ketorolac	0.89	2.16	2.42	6.1
(*RS*)-Etodolac	0.41	1.90	4.63	7.2

resolution Rs for the diastereomeric derivatives separated under the optimized HPLC conditions. It was observed that the resolution in the separation of the diastereomeric derivatives of Etd was higher than that for the diastereomeric derivatives of Ket, whereas the retention time of the diastereomeric derivatives of Ket was higher.

The peak areas, as obtained from the system software, were similar, namely, 236 390 and 236 274 for the peaks of the two diastereomeric derivatives of Ket and 288 726 and 288 364 for the peaks of the two diastereomeric derivatives of Etd. An equimolar mixture of the diastereomers, *i.e.*, [(*R*)-Ket-(*S*)-Npx] and [(*S*)-Ket-(*S*)-Npx], which was separated and isolated by preparative HPLC, was run independently under the same optimized HPLC separation conditions as the diastereomeric derivatives. The results were in agreement in terms of the retention time and peak area.

A comparison of the present results with the literature shows that the indirect approach is better in terms of selectivity, sensitivity, lower costs, and easier experimental optimization. In contrast, CSPs are very expensive, the optimization of the separation conditions is cumbersome and time-consuming,

View Article Online

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

and the detection of the underivatized enantiomers by UV spectroscopy is less sensitive in the absence of a strong chromophore.

The retention times were compared with those of the diastereomers Ds-I and Ds-II, which were separated by preparative HPLC and run independently and as an equimolar mixture. On the basis of the data obtained from the system software, the resolution (Rs) of the diastereomeric derivatives was calculated as per the standard formula (as described in the ESI†).

Mobile phase: MeOH–TEAP buffer of a pH of 3.5 (75:25, v/v) in the isocratic mode at a flow rate of 1 mL min^{-1}; k_1 = retention factor of the diastereomeric derivative (Ds-I); k_2 = retention factor of the diastereomeric derivative (Ds-II); α = separation factor; and Rs = resolution.

3.4 Isolation by preparative HPLC and characterization of diastereomeric derivatives

The chromatographic separation conditions optimized for analytical separation were adopted for preparative-scale separation and adjusted suitably. A mobile phase comprising MeOH-TEAP buffer in a ratio of 75 : 25 in the isocratic mode at a flow rate of 5 mL min^{-1} was found to be optimal for the isolation of the derivatives of (*RS*)-Ket.

The diastereomeric derivatives isolated by preparative HPLC were characterized by obtaining the ^{1}H NMR, UV, and IR spectra, specific rotation, and melting point, and CHN analysis results. The characterization data of the two diastereomeric derivatives of (*RS*)-Ket are given in the ESI.†

3.5 Structures and configurations of diastereomeric derivatives

The three-dimensional chemical structures and configurations of the diastereomeric derivatives of (*RS*)-Ket (as representatives), together with the spatial orientation of groups with respect to the anhydride bond (as shown in Fig. 3), were examined and confirmed by developing molecular models using the orbital molecular building system (Cochranes of Oxford Ltd, Leafield, Oxford OX8 5NT, England).

3.5.1 ^{1}H NMR spectra of the diastereomeric derivatives.

The chemical shifts of four (α and β) protons, *i.e.*, H^1, H^2, H^3, and H^4, in the two diastereomeric derivatives (as marked in Fig. 2) were observed in their ^{1}H NMR spectrum (Fig. 5). The relative orientation of the nucleus with respect to the external field changes its chemical shift (δ) and the ring current induced by the delocalized π-electrons in the aromatic rings. It is clearly seen that in the first eluted diastereomer (Ds-I), the H^1, H^2, and H^3 protons (shown in Fig. 2) are more deshielded, and the H^4 proton is more shielded, as evidenced by the chemical shift observed for the second diastereomer (Ds-II). The protons that lie outside the ring experience deshielding because the induced magnetic field has the same direction outside the ring as the external field. In contrast, the protons lying in the vicinity of the aromatic ring experience shielding because both fields are in opposite directions.

By observing the shielded and deshielded chemical shift values, the conformations were drawn in a 3D view using the Chem3D Pro 12.0 software. Using these structures, all the δ-values could be explained well. Fig. 6 shows the 3D structures of different conformations of the (*R*,*S*)-diastereomeric derivative of Ket prepared with the CR. In Fig. 6a, the H^1 proton (as already marked in Fig. 2 and correlated with the NMR spectrum shown in Fig. 5) lies in the plane of π-electron-containing rings (but outside the rings), and thus experiences a deshielding effect, whereas the H^2 proton in Ket (in Fig. 6b) and the H^4 proton in the CR (in Fig. 6d) point towards the ring current of the pyrrole moiety in Ket. The H^3 proton on the asymmetric carbon atom of the CR (Fig. 6c) points away from both the π-electron-containing rings.

On the other hand, the 3D structures of different conformations drawn for the (*S*,*S*)-diastereomeric derivative are shown in Fig. 7. The H^2 proton (Fig. 7b) lies in the ring current of the naphthalene ring, whereas the H^3 proton (Fig. 7c) points towards the ring current of the pyrrole ring. The H^1 and H^4 protons (Fig. 7a and d, respectively) point away from all the rings.

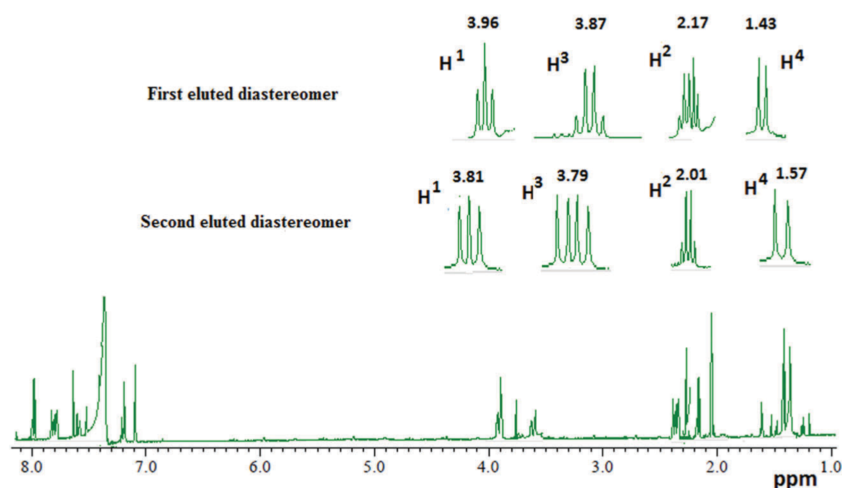

Fig. 5 Sections of ^{1}H NMR (400 MHz using $CDCl_3$) spectra of diastereomeric derivatives, illustrating the differences in δ values for α and β protons corresponding to asymmetric carbon atoms.

This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2017

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

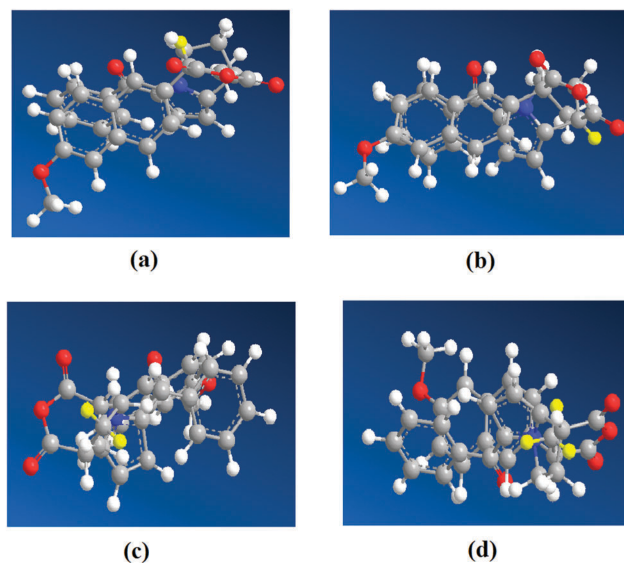

Fig. 6 3D structures of different conformations (drawn using Chem3D Pro 12.0 software) of the (*R*,*S*)-diastereomeric derivative of Ket prepared with the CR. In (a) the H^1 proton (as already marked in Fig. 2 and correlated with the NMR spectrum shown in Fig. 5) lies in the plane of the rings that contain π-electrons, but outside the rings, and thus experiences a deshielding effect, whereas the H^2 proton in Ket (in (b)) and the H^4 proton in the CR (in (d)) point toward the ring current of the pyrrole moiety in Ket. The H^3 proton on the asymmetric carbon atom of the CR ((c)) points away from both rings that contain π-electrons.

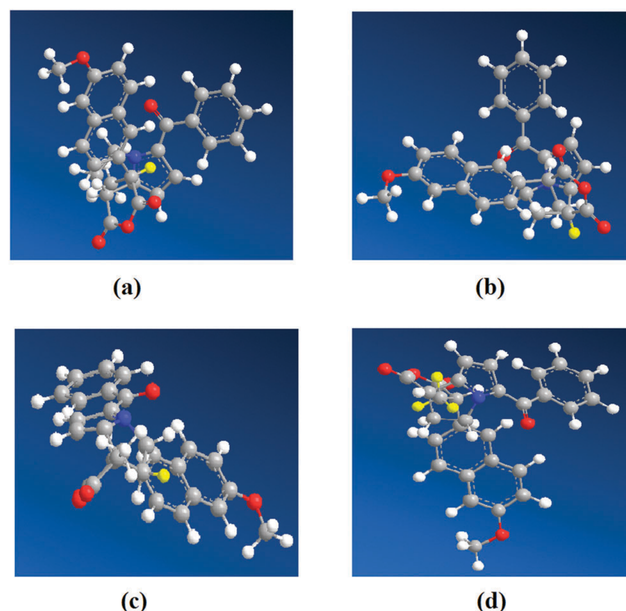

Fig. 7 3D structures of different conformations of the diastereomeric derivative of Ket with the (*S*,*S*)-configuration prepared with the CR, as drawn using Chem3D Pro 12.0 software. The H^2 proton (b) lies in the ring current of the naphthalene ring, whereas the H^3 proton (c) points toward the ring current of the pyrrole ring. The H^1 and H^4 protons (a and d, respectively) point away from all the rings.

Both pyrrole and naphthalene rings have strong anisotropic shielding effects in the presence of an external magnetic field that result in a chemical shift difference of 0.15 δ for H^1 protons between the two diastereomeric derivatives. Similarly, the differences for H^2, H^3, and H^4 were observed to be 0.16, 0.08, and −0.14 δ, respectively (Fig. 5). Hence, the pyrrole ring shields the H^2 and H^4 protons (as shown in Fig. 6b and d) and the H^3 proton (in Fig. 7c), whereas the H^2 proton (Fig. 7b) experiences a shielding effect due to the naphthalene ring. Because the naphthalene ring contains more π-electrons, its anisotropic effect will be greater, and it will shield to an even greater extent, as seen in Fig. 7b with respect to Fig. 6b.

On the basis of these figures and values, it is inferred that the first eluted diastereomeric derivative corresponds to [(*R*)-Ket-(*S*)-Npx], and the second eluted diastereomeric derivative corresponds to [(*S*)-Ket-(*S*)-Npx]. These results are in agreement with the calculated values of the specific rotation of the pure enantiomers obtained by detagging of the corresponding diastereomeric derivatives.

3.6 Detagging of the diastereomeric derivatives

One of the objectives of the present investigations was to obtain the native enantiomers from the diastereomeric derivatives after their separation by HPLC. Therefore, experiments were designed to obtain the derivatives that could be detagged under relatively mild conditions and thus anhydrides as these are reactive and undergo easy hydrolysis. Acid hydrolysis did not involve any reaction at the stereogenic centre. Therefore, racemization was neither theoretically expected nor experimentally observed. The recoveries of the enantiomers of both analytes [(*RS*)-Ket and (*RS*)-Etd] and that of (*S*)-(+)-Npx were in an appropriate molar ratio and in good yields.

All the native enantiomers and (*S*)-(+)-Npx that were isolated and purified were characterized by obtaining their specific rotation and spectroscopic techniques such as ^{1}H NMR, HRMS, and IR spectroscopies. The characterization data of the four purified enantiomers and (*S*)-Npx are presented in the ESI.†

3.7 Elution order

The determination of the elution order of the species being separated in a chromatographic experiment is an important criterion for establishing the success of the analytical procedure. The diastereomeric derivatives were obtained by preparative HPLC as the first and second eluted species. Furthermore, the native enantiomers were isolated by the hydrolysis of each of these derivatives and characterized; thus, the elution order was very clearly established.

3.8 Method validation

Validation studies were carried out using ketorolac as a representative. Sample solutions of both diastereomeric derivatives of Ket were prepared in the concentration range from 45 to 5000 ng mL^{-1}. Replicate samples were analyzed (n = 5) by HPLC using concentrations (45, 500, 2000, 3500, and 5000 ng mL^{-1} of the individual diastereomeric derivatives) of (*RS*)-Ket prepared with the CR.

Data for the accuracy and precision of the method were determined according to ICH guidelines. The acceptance criterion for accuracy is ±15% of the reference value, and the found mean

value is within 15% of the actual value taken. In addition, the precision determined at each concentration is within 15% of the coefficient of variation (CV). Regression equations were obtained by plotting graphs of the concentration of the diastereomeric derivative (x) against the peak area (y) and found to be $y = 4.97x + 4721.4$ ($r^2 = 0.99$) and $y = 4.96x + 4723$ ($r^2 = 0.99$) for the first and second eluted diastereomeric derivative, respectively. The slope, intercept, relative standard deviation (RSD), recovery, and linearity were determined using these regression equations.

The calculated RSD values for the (*R*,*S*)- and (*S*,*S*)-diastereomeric derivatives were 0.25–0.33% and 0.17–0.25%, respectively, for inter-day assay precision and 0.24–0.41% and 0.22–0.48%, respectively, for intra-day assay precision. The solutions were considered stable if the deviation from the nominal value was within ±10.0%. The calculated recoveries for the first and second eluted diastereomeric derivatives were 99.4–100.6% and 99.2–100.1%, respectively, for the inter-day assay and 99.4–102.3% and 99.3–102.7%, respectively, for the intra-day assay, respectively (Table S2, given in the ESI†). The method was inferred to be accurate owing to the very low RSD values and high percentage recoveries.

The limits of detection and quantification, *i.e.*, the LOD and LOQ, values were also determined to perform validation studies. LOQs of 11.19 and 9.15 ng mL^{-1} and LODs of 3.69 and 3.02 ng mL^{-1} were found for the diastereomeric derivatives of (*R*)- and (*S*)-Ket, respectively. Herein, the LODs are much lower than the prescribed LOD (1%) in the pharmaceutical industry.

4. Novelty of the present work and conclusion

The enantioseparation of two commonly used drugs, namely, (*RS*)-Ket and (*RS*)-Etd, has been achieved by both analytical and preparative HPLC. Although a preparative HPLC setup may be considered expensive in comparison with the preparative separation by conventional open silica gel column chromatography, the latter is more cumbersome and time-consuming because it requires verification/analysis of each fraction that is collected manually.

The present method provides a successful approach for the easy recovery of the native enantiomers of (*RS*)-Ket and (*RS*)-Etd after their enantioseparation *via* derivatization. It does not require a reference standard for comparison of the retention times and is capable of establishing the true elution order. The confirmation of the molecular asymmetry of the diastereomeric derivatives, followed by the verification of the configuration of the enantiomers recovered from the derivatives, makes this method superior to those reported in literature in the area of enantioseparation, where the use of a CR remains limited to derivatization followed by separation of the diastereomeric derivatives.

This method and approach are useful for both academic and industrial scientists in all areas of chemical science. The method is self-contained and comprehensive and also endorses the future usefulness of the reagents involved. It opens up areas (i) for the development of new CRs that would yield diastereomeric derivatives that can be easily hydrolysed to the native enantiomers without racemisation; (ii) for measuring the success of enantioseparation methods and the synthesis of diastereomers; (iii) for controlling the enantiomeric purity of chiral drugs in industrial and analytical laboratories (in particular, those associated with regulatory agencies); and (iv) for use in different stages of enantioselective synthesis as there is generally a chance of error in establishing the ee of a sample because a purification step that employs conventional chromatography prior to loading the sample onto a chiral column may lead to unobserved enantiomeric enrichment.[29]

Although the reported method represents an indirect approach to enantioseparation, it is simpler, as separation is possible on an achiral C_{18} column with easy optimization of the chromatographic conditions, and the CR provides highly sensitive detector response in comparison with chiral columns, which are highly expensive.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors gratefully acknowledge the financial assistance received from the Council of Scientific and Industrial Research, New Delhi, India, in the form of a senior research fellowship (to Poonam Malik).

References

1 S. Alwera and R. Bhushan, *Biomed. Chromatogr.*, 2016, **30**, 1223–1233.
2 A. Dalal and R. Bhushan, *J. Planar Chromatogr.*, 2016, **29**, 366–371.
3 A. Macario and A. G. Lipman, *Pain Med.*, 2001, **2**, 336–351.
4 *Modern Pharmacology with Clinical Applications*, ed. R. Craig and R. E. Stitzel, Lippincott Williams and Wilkins, Philadelphia, USA, 6th edn, 2004.
5 M. Lal and R. Bhushan, *Biomed. Chromatogr.*, 2016, **30**, 1526–1534.
6 (*a*) M. Singh and R. Bhushan, *Biomed. Chromatogr.*, 2016, **30**, 1728–1732; (*b*) M. Singh and R. Bhushan, *J. Planar Chromatogr.*, 2016, **29**, 184–189; (*c*) M. Singh and R. Bhushan, *Biomed. Chromatogr.*, 2015, **29**, 1330–1337.
7 K. S. S. Dossou, E. Farcas, A.-C. Servais, P. Chiap, B. Chankvetadze, J. Crommen and M. Fillet, *J. Chromatogr. A*, 2012, **1234**, 56–63.
8 I. Matarashvili, D. Ghughunishvili, L. Chankvetadze, N. Takaishvili, T. Khatiashvili, M. Tsintsadze, T. Farkas and B. Chankvetadze, *J. Chromatogr. A*, 2017, **1483**, 86–92.
9 O. H. Ismail, A. Ciogli, C. Villani, M. D. Martino, M. Pierini, A. Cavazzini, D. S. Bell and F. Gasparrini, *J. Chromatogr. A*, 2016, **1427**, 55–68.

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

 This journal is © The Royal Society of Chemistry and the Centre National de la Recherche Scientifique 2017

View Article Online

Published on 06 October 2017. Downloaded by University of Reading on 23/10/2017 18:19:33.

10 M. Starek, M. Dabrowska and J. Skucinski, *Recent Pat. Chem. Eng.*, 2010, **3**, 49–73.
11 H. P. Jadhav and D. B. Pathare, *Int. J. Pharmacol. Pharm. Sci.*, 2015, **7**, 77–80.
12 X. Zhang, Z. Li, B. Shen, J. Chen and X. Xu, *J. Anal. Sci., Methods Instrum.*, 2012, **2**, 18–23.
13 I. Tsina, Y. L. Tam, A. Boyd, C. Rocha, I. Massey and T. Tarnowski, *J. Pharm. Biomed. Anal.*, 1996, **15**, 403–417.
14 K. R. Ing-Lorenzini, J. A. Desmeules, M. Besson, J. L. Veuthey, P. Dayer and Y. Daali, *J. Chromatogr. A*, 2009, **1216**, 3851–3856.
15 P. Hayball, J. Tamblyn, Y. Holden and J. Wrobel, *Chirality*, 1993, **5**, 31–35.
16 M. Vakily, B. Corrigan and F. Jamali, *Pharmacol. Res.*, 1995, **12**, 1652–1657.
17 Y. X. Jin, Y. H. Tang and S. Zeng, *J. Pharm. Biomed. Anal.*, 2008, **46**, 953–958.
18 International Conference on Harmonization, Q2B Document, Validation of Analytical Procedures, International Conference of Harmonization, Geneva, 1996.
19 M. Bodanszky, *Peptide Chemistry: A Practical Textbook*, Springer-Verlag, New York, 1993, 2nd edn, pp. 58–68.
20 R. Bhushan and R. Dubey, *J. Chromatogr. A*, 2011, **1218**, 3648–3653.
21 R. Bhushan and H. Nagar, *Biomed. Chromatogr.*, 2013, **27**, 750–756.
22 S. Batra and R. Bhushan, *Biomed. Chromatogr.*, 2014, **28**, 815–825.
23 http://www.webelements.com.
24 *CRC Handbook of Chemistry and Physics*, ed. D. R. Lide, CRC Press, Boca Raton, Florida, 2003, 84th edn, Section 9, Molecular Structure and Spectroscopy, Electronegativity.
25 D. Leah, G. O'Neale, F. B. Alcindor, T. L. Meek and B. G. Patrick, *THEOCHEM*, 2003, **639**, 151–156.
26 J. E. Huheey, *J. Phys. Chem.*, 1965, **69**, 3284.
27 A. D. Walsh, *Discuss. Faraday Soc.*, 1947, **2**, 18–24.
28 L. Pauling, *Nature of the Chemical Bond*, Cornell University Press, 1960, pp. 88–107.
29 J. Martens and R. Bhushan, *Helv. Chim. Acta*, 2014, **97**, 161–187.