Synthesis of (S)-naproxen-benzotriazole and its application as chiral derivatizing reagent for microwave-assisted synthesis and indirect high performance liquid chromatographic separation of diastereomers of penicillamine, cysteine and homocysteine

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ABSTRACT

(S)-Naproxen-benzotriazole was synthesized by the reaction of (S)-naproxen with 1H-benzotriazole using coupling reagent dicyclohexyl carbodiimide and 4-dimethylamino pyridine (DCC/DMAP). It was used as chiral derivatizing reagent for microwave irradiated synthesis of diastereomers of penicillamine, cysteine and homocysteine. The diastereomers were separated by reversed phase high performance liquid chromatography using gradient elution of triethylammonium phosphate (pH 3.5)–acetonitrile (30–65% within 30 min). The method was validated for accuracy, precision, and limit of detection.

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1. Introduction

Penicillamine (PenA), cysteine (Cys) and homocysteine (Homocys) are thiol group containing amino acids and are easily available pharmaceutically important compounds (Fig. 1). D-Isomer of PenA is pharmacologically active while L-isomer, found naturally, is toxic [1] and hence use of DL-PenA is restricted [2]. Certain pharmaceutical applications and aspects of biological importance of PenA [3–7], Cys [8,9], and Homocys [10] are described in literature.

The development of analytical methods for enantioseparation of pharmaceutically useful compounds becomes important and desirable for control of their enantiomeric purity and to understand their pharmacokinetics and pharmacodynamics. The application of various chiral derivatizing reagents (CDRs) for enantioseparation has been advantageous due to availability of, excellent detection sensitivity and flexibility of chromatographic conditions required for separation of diastereomers on inexpensive achiral columns [11,12]. Different CDRs have been used for enantioseparation of PenA [13–18], Cys [17,19–23] and Homocys [17,20].

Enantiomeric mixture of derivatives of DL-PenA, prepared by cyclization with HCHO, was separated by ligand exchange approach both in thin layer chromatography (using ChiralPlate®) [24] and high-performance liquid chromatography (HPLC) [25]. Separation of spiro-derivatives of DL-PenA, prepared by cyclization with ninhydrin, was made using Cu(II)-L-proline complex as chiral mobile phase additive in HPLC [26], while β-cyclodextrin and α-glycoprotein columns provided analytical and preparative enantioseparation [27] of spiro derivatives of DL-PenA and DL-Cys.

Enantioresolution of Homocys has been achieved by HPLC as homocysteine thiolactone using different CSPs based on L-valine-3,5-dimethylanilide attached to monodisperse poly(glycidyl methacrylate-co-ethylene) beads [28], isopropyl-carbamate functionalized cyclofructan6 (IP-CF6) bonded to silica gel [29] and diphenyl substituted 1,1′-binaphthyl crown ether [30].

Literature reveals that different N-acylbenzotriazoles have been synthesized via reaction of benzotriazole with different unsaturated carboxylic acids [31] and reaction of 1-(1-methanesulfonyl) benzotriazole with different carboxylic acids [32] and N-Boc-α-amino acids [33]. These N-acylbenzotraizoles have been further utilized for N-acylation of different amino compounds [34–36]. (S)-naproxen provides a chiral platform having a UV absorbing chromophore to synthesize CDRs since it has a carboxylic

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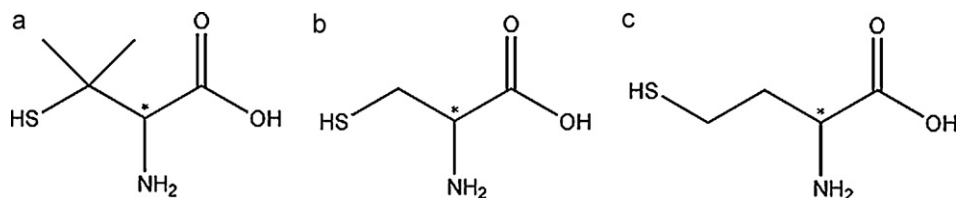E-mail address: rbushfcy@iitr.ernet.in (R. Bhushan).

Fig. 1. Structures of (a) DL-penicillamine (b) DL-cysteine (c) DL-homocysteine. The asterisk (*) indicates stereogenic centre.

acid group. N-Succinimidyl-(S)-2-(6-methoxynaphth-2-yl) propionate was synthesized by the reaction of (S)-naproxen with N-hydroxysuccinimide and was used as a CDR for enantioseparation of DL-PenA [14].

On account of the above cited literature and to search new CDR for enantioseparation of pharmaceutically important amino compounds, a benzotriazole (Btz) derivative of (S)-naproxen (Nap), (S)-1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(6-methoxynaphthalen-2-yl)propan-1-one (Nap-Btz), was synthesized and was used as a CDR for enantioseparation of PenA, Cys and Homocys. The novelty introduced here is the synthesis of a new CDR and its application for microwave assisted synthesis, and reversed phase HPLC separation, of the diastereomers of penicillamine, cysteine and homocysteine.

2. Experimental

2.1. Chemical and reagents

(S)-Naproxen, 1H-benzotriazole, dicyclohexyl carbodiimide (DCC), 4-dimethylamino pyridine (DMAP), D-PenA, D-Cys, DL-PenA, DL-Cys and DL-Homocys were purchased from Sigma–Aldrich (St. Louis, MO, USA). L-Homocys was purchased from AK Scientific Inc. (Ahern Ave Union City, CA, USA). Concentrated acetic acid, sodium hydrogen carbonate and pyridine of analytical grade, and acetonitrile and methanol of HPLC grade were obtained from E. Merck (Mumbai, India). Double distilled water purified (18.2 MΩ cm^3) with Milli-Q system was used throughout.

2.2. Apparatus

The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, fixed wavelength UV detector 2500, manual injection valve and operating software was from Knauer (Berlin, Germany). Other equipment used were Microwave–Multiwave 3000 (800 W, Perkin–Elmer, Shelton, CT, USA), pH meter Cyberscan 510 (Singapore), Polarimeter P-3002 (Krüss, Hamburg, Germany), Milli-Q system (Millipore, Bedford, MA, USA), Perkin Elmer 1600 FT IR spectrometer (Boardman, OH, USA), Vario EL III elemental analyzer, and Shimadzu UV-1601 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brüker 500 MHz instrument using deuterated chloroform.

2.3. Synthesis of chiral derivatizing reagent ((S)-1-(1H-benzo[d][1,2,3]triazol-1-yl)-2-(6-methoxynaphthalen-2-yl)propan-1-one; Nap-Btz)

4-Dimethyl amino pyridine (DMAP, 16 mg; 0.13 mmol) and dicyclohexyl carbodiimide (DCC, 540 mg; 2.60 mmol) were added to a stirred solution of 1H-benzotriazole (1H-Btz, 155 mg; 1.30 mmol) and (S)-naproxen (Nap, 300 mg; 1.30 mmol) in dichloromethane (10 mL). The reaction mixture was stirred vigorously for 2.5 h at room temperature. The reaction mixture was filtered and the residue (dicyclohexyl urea) was rejected. The filtrate was concentrated *in vacuuo* and the residue was re-dissolved

in ethyl acetate (EtOAc, 15 mL). The extract was washed five times with water, five times with brine solution, five times with concentrated NaHCO$_3$ and finally ten times with 1N HCl. The purified extract was dried over MgSO$_4$ and the solvent was removed under reduced pressure. The residue was recrystallized from hexane (as the desired CDR).

Colour: white; yield: 407 mg (94.5%); m.p. 181.0–182.0 °C; $[\alpha]_D^{25}$ (c = 1.880, DMF) = +249.42°; UV (nm, in DMF): 231 (λ_{max}); IR (KBr): 3327, 3125, 3037, 2926, 2851, 2659, 2345, 1625, 1572, 1441, 1311, 1239, 1184, 1086, 1054, 947, 894, 796 and 652 cm^{-1}; ^{1}H NMR (500 MHz, CDCl$_3$) δ 1.85 (d, 3H, CH$_3$–CH<), 3.92 (s, 3H, CH3–O–), 5.57 (q, 1H, >CH–CH$_3$), 7.09 (d, 1H, Nap–5–H), 7.13 (dd, 1H, Nap–3–H), 7.28 (d, 1H, Nap–7–H), 7.49–7.51 (m, 1H, Btz–5–H), 7.62–7.67 (m, 1H, Btz–6–H), 7.72 (d, 2H, Nap–4,8–H), 7.91 (d, 1H, Nap–1–H), 8.08 (d, 1H, Btz–4–H), 8.32 (d, 1H, Btz–7–H); ^{13}C NMR (125 MHz, CDCl$_3$) δ 18.1, 43.8, 55.6, 105.6, 113.9, 118.9, 120.3, 126.2, 126.3, 126.8, 127.9, 128.9, 129.1, 129.9, 131.0, 133.5, 134.1, 145.8, 157.2, 173.0; elemental analysis: calcd for C$_{20}$H$_{17}$N$_3$O$_2$: C, 72.49%; H, 5.17%; N, 12.68%. Found: C, 72.42%; H, 5.20%; N, 12.61%.

2.4. Microwave-assisted synthesis of diastereomers

To the solutions of cysteine (100 μL, 50 nmol, in NaHCO$_3$ (1 M)) and Nap-Btz (150 μL, 85 nmol, in MeCN) in mole ratio of 1:1.7 in a Teflon tube of 1.5 mL, borate buffer (0.1 M, pH 9.5, 60 μL) was added and the reaction mixture was then exposed to microwave irradiation (MWI) for 30 s at 80% (of 800 W) and cooled to room temperature. The reaction was quenched by addition of HOAc (1 M, 60 μL). A 10 μL volume of resulting solution, containing the diastereomers, was diluted 10 fold with MeCN, and 20 μL of it was injected onto the column. The chiral derivatizing reagent Nap-Btz was quite stable for one month while the solution of diastereomers of cysteine was quite stable up to one week under refrigerated condition (4 °C).

The experiments related to synthesis of diastereomers followed by their HPLC resolution under the optimized condition were repeated after the interval of 10, 20, 30 and 40 days, to check the stability of CDR. The results (in terms of percentage peak area of diastereomers and their resolution) showed that the CDR was successful when used within 30 days.

2.5. HPLC

Reversed-phase HPLC was performed on a Waters Spherisorb ODS2 (250 mm × 4.6 mm I.D., 5 μm) column (from Parker-Style Fittings, Ireland) with the mobile phase consisting of aq TEAP (10 mM, pH 3.5) – MeCN in a linear gradient of MeCN from 30 to 65% in 30 min at a flow rate of 1.0 mL/min and UV detection at 231 nm. Triethylammonium phosphate (TEAP) buffer solution was prepared by diluting triethyl amine to 10 mM with ultra-purified water and adjusting to pH 3.5 by addition of phosphoric acid.

2.6. Validation studies

The method validation was done according to ICH guidelines [37] using diastereomers of DL-Cys prepared with Nap-Btz. By plot-
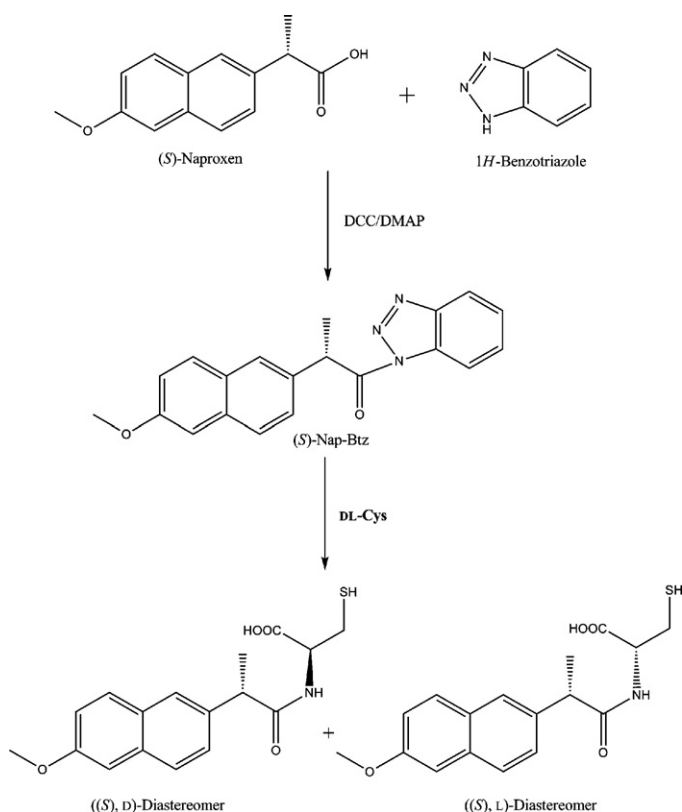
Fig. 2. Synthesis of (*S*)-Nap-Btz chiral derivatizing reagent and diastereomers of DL-Cys.

ting calibration curves for peak areas versus concentrations slopes and regression equations were determined. Recovery studies were carried out by derivatizing standard solutions of different known concentrations and mean recovered values (six 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 relative standard deviation (RSD) and precision. Limit of detection (LOD) was also evaluated.

3. Results and discussion

3.1. Synthesis and characterization of chiral derivatizing reagent (Nap-Btz)

The reaction for the synthesis of chiral derivatizing reagent, Nap-Btz and diastereomers by its reaction with cysteine is shown in Fig. 2. Nap-Btz was formed by nucleophilic attack of 1*H*-benzotriazole on the carbonyl carbon of the carboxylic acid of Nap followed by the removal of dicyclohexylurea. An excellent yield (>94%) of Nap-Btz was obtained by the reaction of (*S*)-naproxen with 1*H*-benzotriazole in the presence of coupling reagent DCC/DMAP under mild conditions. The CDR, Nap-Btz was characterized by IR, UV, CHN and ^{1}H NMR. The characterization data related to Nap-Btz is given in Section 2.3. The chiral purity of Nap-Btz was established according to the earlier reports on determination of chiral purity of different CDRs [11].

Katritzky et al. [38] synthesized benzotriazole activated naproxen in 4 h using hazardous thionyl chloride while in the present work synthesis of Nap-Btz has been achieved under mild conditions using DCC/DMAP coupling reagent. The effect of DMAP on the synthesis of Nap-Btz was also investigated. In the absence of DMAP the reaction took 4 h for completion. The newly synthesized CDR (Nap-Btz) is an amide and is more stable than *N*-succinimidyl-

(*S*)-2-(6-methoxynaphth-2-yl) propionate (SINP) [14], an ester, due to higher thermodynamic stability of amides over esters.

3.2. Synthesis of diastereomers

The experimental conditions for microwave-irradiated synthesis of diastereomeric pairs of Cys, Homocys and PenA with Nap-Btz were optimized to ascertain the best and successful conditions; effect of buffer pH, reagent excess, and reaction time and microwave power was systematically investigated. The mixture of the two diastereomers so formed under each change of experimental conditions was subjected to HPLC resolution and the peak areas corresponding to the two resolved diastereomers, calculated by system software, were taken as a measure of complete derivatization.

3.2.1. Effect of pH of base

Since reaction of Cys (and other analytes) with Nap-Btz follows a nucleophilic substitution, the reaction requires basic medium. Borate buffer was used to facilitate the derivatization. Effect of pH of buffer was investigated within the range of 6.5–10. Borate buffer at pH 9.5 (0.1 M, 60 μL) was found to be optimum to obtain the best yield for derivatization of DL-Cys with Nap-Btz. The derivatization (in terms of peak area) increased with increase in pH from 6.5 to 9.5 and increment in pH up to 10 showed no significant change in derivatization yield. Therefore, a buffer of pH 9.5 was chosen for further experiments on derivatization. No derivatization was observed in the absence of buffer.

3.2.2. Effect of reagent excess

Nap-Btz was used in 1–5 fold molar excess to ascertain complete derivatization. Derivatization of Cys was found optimum at a molar ratio of 1:1.7 (Cys: CDR) using MWI (30 s at 80% power). Slight kinetic resolution was observed at lower ratios of Cys:CDR. Increase in reagent concentration up to 5 fold had no significant effect on yield of derivatization and reaction time. Therefore, the CDR was used in the same ratio for all the three analytes.

3.2.3. Microwave irradiation

Separate sets of reaction mixture were irradiated in the microwave oven for 10, 20, 30, 40, 50, and 60 s at 75–90% power. A derivatization time of 30 s (at 80% power) was found successful for complete derivatization of DL-Cys. The MWI time and power corresponding to maximum diastereomeric peak areas (representing the completion of reaction) were taken as optimized derivatization conditions and were used for the three analytes.

3.3. HPLC

The chromatographic parameters: retention factor (k), separation factor (α) and resolution (R_S) for the resolved diastereomers of PenA, Cys and Homocys prepared with Nap-Btz are given in Table 1. The three pairs of diastereomers were well separated under the reversed-phase HPLC conditions. D-Isomer was eluted prior to L-isomer for all the three cases. Sections of chromatograms showing resolution of diastereomers of PenA, Cys and Homocys prepared with Nap-Btz are shown in Fig. 3.

3.3.1. Effect of pH and concentration of buffer used in mobile phase

The effect of pH of buffer used in the mobile phase on the separation of diastereomers of DL-Cys prepared with Nap-Btz was studied within the pH range of 2.5–6.0 keeping the concentration of buffer at 10 mM. TEAP buffer was used to investigate within the range of pH 2.5–4.0 and the best resolution was observed at pH 3.5. To study the effect of pH within the range 4.0–6.0, triethylammonium

Table 1
Chromatographic data for resolution of diastereomers of penicillamine, cysteine, and homocysteine prepared with Nap-Btz.

Amino Acids	Chromatographic parameters			
	k_D	k_L	α	R_S
DL-penicillamine	9.19	11.86	1.29	24.09
DL-cysteine	8.76	10.25	1.17	20.71
DL-homocysteine	8.98	10.87	1.21	21.23

where k_D and k_L are the retention fators of D- and L-enantiomers respectively, α is stereoselective factor and R_S is the resolution of the diastereomers of corresponding amino acids; mobile phase was *aq* TEAP (10 mM, pH 3.5) – MeCN in a linear gradient of MeCN from 30 to 65% in 30 min at a flow rate of 1.0 mL/min and UV detection at 231 nm.
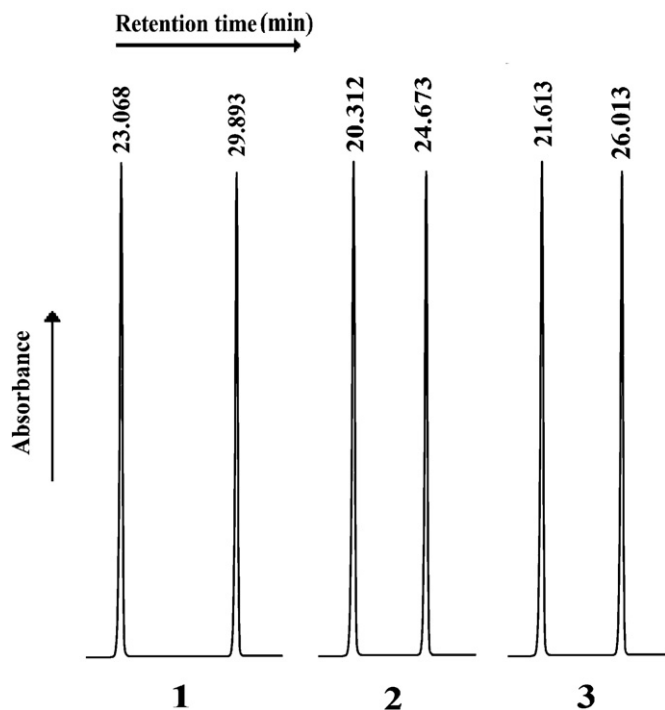

Fig. 3. Sections of chromatograms showing resolution of DL-penicillamine (**1**), DL-cysteine (**2**), and DL-homocysteine (**3**) as their diastereomers prepared with (*S*)-Nap-Btz; Waters Spherisorb ODS2 (250 mm × 4.6 mm I.D., 5 μm); diastereomeric peak corresponding to D-enantiomer was eluted prior to that of L-enantiomer; mobile phase was *aq* TEAP (10 mM, pH 3.5) – MeCN in a linear gradient of MeCN from 30 to 65% in 30 min at a flow rate of 1.0 mL/min and detection at 231 nm for all the diastereomers.

acetete buffer was used and it was found that the diastereomers were not resolved in this pH range. Thus using TEAP buffer of pH 3.5, effect of varying buffer concentration was studied within the range of 05–40 mM. Peak broadening was found with decrease in concentration of TEAP buffer below 10 mM and little difference in resolution was found when buffer concentration was above 10 mM (up to 40 mM; with a change of 5 mM at a time). Since high buffer concentration may harm the column hence 10 mM buffer concentration was taken as optimized concentration.

3.3.2. Effect of organic solvent

A binary mobile phase consisting of MeCN and TEAP buffer was found to be the best. Both the gradient and isocratic elution modes were investigated. Sharp peaks were obtained under gradient elution. MeCN was found to be a better organic modifier in comparison to methanol as broader peaks (and even no separation in a few cases) and larger retention times were observed with methanol.

3.3.3. Effect of flow rate

The effect of change of flow rate on the separation of diastereomers was examined by varying the flow rate in portions of 0.5 mL/min within the range of 0.5–1.5 mL/min. There was observed an increase in retention time along with broadening of the diastereomeric peaks with the decrease in the flow rate from 1.0 to 0.5 mL/min. The retention times and resolution values decreased when flow rate was increased from 1.0 to 1.5 mL/min. Hence flow rate of 1.0 mL/min was used throughout the experiment.

A comparison of present work to the earlier reports with respect to time required for derivatization of penicillamine, cysteine and homocysteine using different CDRs/tagging reagents and their enantioseparation (in terms of resolution) using different HPLC resolution methods is shown in Table 2. It clearly establishes the novelty and superiority of the present report.

3.4. Separation mechanism

Since the pH of the mobile phase is around 3.5 (pH of TEAP buffer, used in the mobile phase) and is considerably acidic the carboxylic group of the analyte will not be ionized, therefore, ionic interaction is likely to play the least role. The factors contributing to the hydrophobicity of diastereomers are then required to be considered. The following factors may contribute to the hydrophobicity of diastereomers: (i) presence of naphthyl group in the reagent platform of the diastereomers; (ii) the sulphur atom in the sulfydryl group with its less electronegativity and large size by its position in periodic table; (iii) the partial double bond character of the amide bond because of lone pair of the amino nitrogen of the analyte that may be undergoing delocalization with the neighboring carbonyl group. It can be interpreted that the hydrophobic interactions of the two diastereomers with the reversed phase material of the column are responsible for their different partition coefficients resulting into different retention times and thus separation. The characteristic feature of diastereomers is that they have different physical properties. Based on the chemical structures, the analytes may also be arranged in their decreasing order of hydrophobic character as PenA > Homocys > Cys because PenA contains two –CH$_3$ groups and Homocys contains an additional –CH$_2$ in comparison to Cys. This is reflected in the observed resolution values of the three sets of analytes, PenA (R_S, 24.09) > Homocys (R_S, 21.23) > Cys (R_S, 20.71).

4. Method validation

The experimental method was validated with respect to linearity, accuracy and precision for the diastereomers of DL-Cys prepared with Nap-Btz and summarized here:

4.1. Linearity

The linear regression was computed by the least square method using Microsoft Excel program to determine the slopes and correlation coefficients for the calibration graphs between the peak area (in AU; absorbance unit) responses of ((S), D)-diastereomer and ((S), L)-diastereomer and the corresponding concentration (0.025–0.100 nmol). A good linear relationship was obtained over this range. The regression equations were $y = 1.085x + 1.447$ ($R^2 = 0.999$) and $y = 1.132x + 1.398$ ($R^2 = 0.998$) for the ((S), D) – diastereomer and ((S), L) – diastereomer, respectively.

4.2. Accuracy and precision

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis ($n = 6$) of DL-Cys at three concentrations (0.025, 0.050, 0.100 nmol). The relative standard deviation (%) for D- and L-Cys varied from 0.46 to 0.80 and

Table 2
Comparison of HPLC enantioseparation of penicillamine, cysteine, and homocysteine using different CDRs/tagging reagents.

Basis of separation	CDRs/tagging reagents used for derivatization of	Derivatization time	Resolution of the derivatives (R_S)	References
DL-penicillamine				
Diastereomers	Nap-Btz	30 s	24.09	Present work
	FDNP-L-val-NH$_2$	1 h	0.815	[13]
	SINP	15–18 min	23.19	[14]
	DBD-PyNCS	60 min	3.33	[17]
Ligand exchange	HCHO	2 h	NG	[25]
CMPA	Ninhydrin	NG	1.31	[26]
CSPs[a,b]	Ninhydrin	5 min	6.92[a], 2.38[b]	[27]
CSPs[a,b]	DNFB	45 min	7.60[a], 1.75[b]	[27]
DL-cysteine				
Diastereomers	Nap-Btz	30 s	20.71	Present work
	OPA and L-val	1 min	3.44	[20]
	(S)-NIFE	20 min	1.15	[22]
	DBD-PyNCS	60 min	1.15	[17]
	FDNP-L-val	50 s	6.83	[23]
CSPs[a,b]	Ninhydrin	5 min	5.95[a], 2.34[b]	[27]
CSPs[a,b]	DNFB	45 min	6.71[a], 1.27[b]	[27]
DL-homocysteine				
Diastereomers	Nap-Btz	30 s	21.23	Present work
	OPA and L-val	1 min	1.13	[20]
	DBD-PyNCS	60 min	NR	[17]
CSPs[c]	Enantiomeric mixture of Homocys thiolactone was used as the analyte	NG	NG	[28]
CSPs[d]		NG	1.1	[29]
CSPs[e]		NG	2.14	[30]

CMPA, chiral mobile phase additive; CSPs, chiral stationary phases; NG, not given; NR, not resolved; FDNP, fluoro dinitro phenyl; SINP, N-succinimidyl-(S)-2-(6-methoxynaphth-2-yl) propionate; DBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N, N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; DNFB, dinitro fluoro benzene; OPA, o-phthalaldehyde and

[a] α-Acid glycoprotien column.
[b] β-Cyclodextrin column.
[c] Based on L-valine-3,5-dimethylanilide attached to monodisperse poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads.
[d] Isopropyl-carbamate functionalized cyclofructan6 (IP-CF6) bonded to the silica gel.
[e] Diphenyl-substituted 1,1′-binaphthyl crown ether.

0.39 to 1.25 for intra-day assay precision and 0.62 to 1.37 and 0.72 to 1.21 for inter-day assay precision. The percentage recovery for D- and L-Cys varied from 98.1 to 99.6 and 98.2 to 99.0 for intra-day assay and 96.0 to 99.1 and 95.9 to 99.0 for inter-day assay.

To determine the limit of detection (LOD), corresponding to the signal-to-noise ratio of 3, the recoveries of L-Cys from the solution containing excess of D-Cys was investigated. Solution of D-Cys was spiked with fixed amount of L-Cys within the range of 0.01–0.10%. The results indicate that this method can be applied for detection of L-Cys in D-Cys up to 0.04% by HPLC.

5. Conclusion

The CDR, (S)-naproxen-benzotriazole (Nap-Btz), an azole derivative of (S)-naproxen, was synthesized under mild conditions within 2.5 h and was found very well with respect to its stability (for one month) and separation characteristics. Its use for microwave assisted synthesis of diastereomers of penicillamine, cysteine and homocysteine required only 30 s (at 80% power). The HPLC separation, using UV detection, of diastereomers showed an LOD of 0.0001–0.0015 nmol. Thus the CDR can successfully be applied to check the enantiomeric purity of commercial D-penicillamine and other amino group containing pharmaceuticals.

The enantioresolution of DL-PenA, for example, after achiral derivatization, has required application of either ChiralPlate® [24] or chiral column (LOD was 0.01%) [27] (both are very expensive, particularly the β-cyclodextrin or polysaccharide based chiral columns are 4–5 times expensive in comparison to reversed phase columns) or use of one or the other chiral ligand exchange reagent in mobile phase (LOD was 0.01%) [25,26] using HPLC. On the other hand, application of Nap-Btz as CDR allowed enantioseparation of diastereomers under less expensive and simple reversed-phase HPLC conditions with a very good LOD.

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