Amino Acids (2010) 39:549–554
DOI 10.1007/s00726-010-0472-z

ORIGINAL ARTICLE

Application of (*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester as a chiral derivatizing reagent for reversed-phase high-performance liquid chromatographic separation of diastereomers of amino alcohols, non-protein amino acids, and PenA

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Received: 9 May 2009 / Accepted: 4 January 2010 / Published online: 28 January 2010

Abstract Indirect enantioresolution of 15 primary and secondary amino group containing compounds (amino alcohols, non-protein amino acids, PenA) was done using the reagent (*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(*S*)-NIFE] by reversed-phase high-performance liquid chromatography. The diastereomeric derivatives were analyzed under reversed-phase conditions using linear gradient. The detection was at 205 nm and sharp peaks were obtained. The reagent used is comparatively economic than the other derivatizing reagents. Method validation was also done.

Keywords
(*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester · Diastereomers · Chiral separation · HPLC · DL-PenA · Amino alcohols · Amino acids

Introduction

The diastereomers of aromatic amino acids prepared with (*S*)-*N*-(4-Nitrophenoxycarbonyl) phenylalanine methoxyethyl ester [(*S*)-NIFE] showed the highest enantioselectivity, followed by diastereomers of some synthetic unusual amino acids such as *β*-methoxytyrosine (*β*-MeOTyr), *allo*threonine (*allo*-Thr), and *allo*-isoleucine (*allo*-Ile) prepared with *o*-phthalaldehyde/isobutyryl-L-cysteine (OPA-IBLC), 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC), and Marfey's reagent (MR); MR and its chiral variants have shown a very low sensitivity for resolution of the diastereomers of these unusal amino acids, while (*S*)-NIFE provided approximately twice the signal intensity for the same (Hess et al. 2004). (*S*)-NIFE has been used as a CDR for indirect high-performance liquid chromatograph (HPLC) separation of a variety of compounds containing primary or secondary amino group, such as protein amino acids (Péter et al. 2000), and analogs of phenyl alanine (Olajos et al. 2001), *β*-alkyl-substituted analogs of tyrosine, phenylalanine, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and tryptophan (Vékes et al. 2002) and unusual *β*-amino acids like *N*-MeThr, *allo*-*N*-MeThr, and 3-amino-4-methylpentanoic acid (Árki et al. 2004; Péter et al. 2004). The resolution of diastereomers of amino acids prepared with (*S,S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocynate [(*S,S*)-DANI] was found to be somewhat lower than those for prepared with (*S*)-NIFE (Árki et al. 2004). The derivatives of protein amino acids, unusual *β*-amino acids, and the reagent solutions were also stable for several weeks (Péter et al. 2000) in comparison with GITC-derivatized *β*-amino acids (Hess et al. 2004).

Literature reveals that the time required for derivatization of penicillamine is 40 min at 50°C by fluorescent chiral tagging reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS) (Jin and Toyo'oka 1998) and 30 min at room temperature with 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate (Ito et al. 1992); the time required for derivatization of non-protein amino acids with monosubstituted triazine reagent was 180 min at 50°C while it was 90 min at 80°C with disubstituted triazine reagent (Bhushan and Kumar 2008a); Marfey's reagent and its variants required 60 min at 45°C for amino acids and penicillamine (Bhushan and Kumar 2008b; Bhushan et al. 2007, 2009).

In view of the aforementioned representative literature reports, the work published from this laboratory (Bhushan

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et al. 2009; Bhushan and Kumar 2010) and the literature cited therein, and the characteristic advantages offered by (*S*)-NIFE for enantiomeric resolution of compounds containing primary or secondary amino group (*S*)-NIFE was used in the present studies as a CDR to prepare diastereomers of 13 primary and 2 secondary amino group containing compounds (Fig. 1) belonging to the categories of amino alcohols and non-protein amino acids including penicillamine. The diastereomers were separated by reversed-phase (RP) HPLC. The separation method was validated for linearity, accuracy, and limit of detection. To the best of authors' knowledge there is no earlier report on chiral HPLC assay of enantiomers of these selected racemic amino compounds using (*S*)-NIFE reagent.

Experimental

Chemicals, reagents, and instrumentation

(±)-PenA, (−)-PenA and (*S*)-NIFE were obtained from Sigma–Aldrich (Bangalore, India). All racemic and enantiomerically pure amino alcohols (Homophenylalaninol, Valinol, Prolinol, Leucinol, Alaninol, Phenylglycinol, 2-Amino-1-butanol, Phenylalaninol), and non-protein amino acids (such as Norvaline, 2-Phenylglycine, 2-Aminooctanoic Acid, Pipecolic Acid, 2-Aminobutyric Acid, 2-Aminoadipic Acid) were obtained from Sigma–Aldrich (Bangalore, India). Trifluroacetic acid (TFA), Triethylamine (TEA), and other chemicals of analytical grade were purchased from s.d. fine chemicals Ltd (Mumbai, India). The HPLC system consisting of a 10-mL pump head 1000, manager 5000 degasser, UV detector 2500, manual injection valve and Eurochrom operating software was from Knauer (Berlin, Germany). pH meter Cyberscan 510 (Singapore) was used. The Milli-Q system from Millipore (Bedford, MA, USA) was used to purify double-distilled water to HPLC-grade deionised water.

Preparation of working standard solutions

Amino alcohols (1 mg mL^{-1}) were dissolved in 1 M HCl. Some of the amino acid solutions (1 mg mL^{-1}) were prepared in purified water and some amino acids were dissolved in 1 M NaHCO3. Reagent solution (1 mg 100 μL^{-1}) was prepared in water-free dioxane and stored in refrigerator at 0–4°C. Solution of TFA (0.1%) was prepared in purified water.

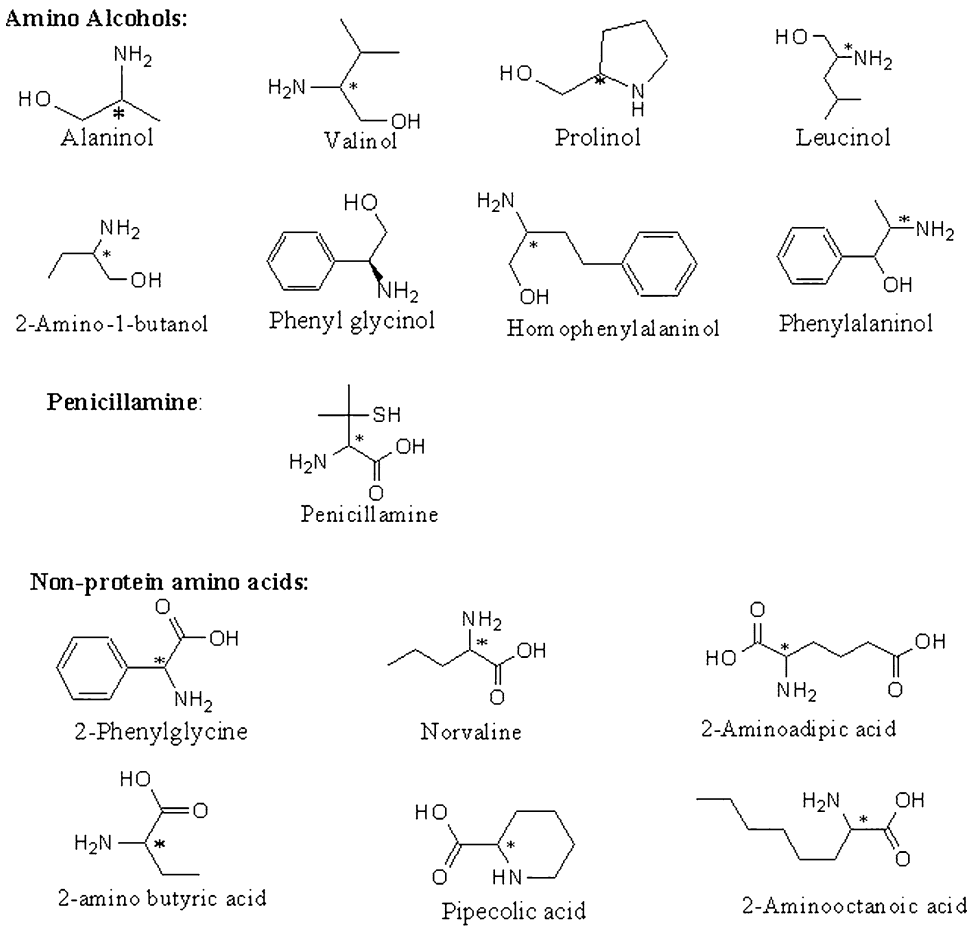

Fig. 1 Chemical structure of racemic 1° and 2° amino compounds

Synthesis of diastereomers

Diastereomers of the non-protein amino acids, (±)-PenA and amino alcohols were prepared with (*S*)-NIFE as per literature (Péter et al. 2000). The resulting solution was degassed and filtered and the diastereomeric mixture was subjected to RP-HPLC for separation.

HPLC conditions

RP-HPLC was performed on C18 column (250 mm × 4.6 mm I.D., 5 μm) from Knauer. Mobile phase: component **A,** 0.1% aq. TFA +0.1% TFA in MeOH (90:10, v/v) and component **B**, 0.1% aq. TFA + 0.1% TFA in MeOH (10:90, v/v) were used under a linear gradient of A from 100 to 0% in 90 min. The flow rate was 1.0 mL min^{-1} with UV detection at 205 nm. The load amount of 20 μL was injected into the column. Mobile phase was filtered through a 0.45-μm filter and degassed before use.

Results and discussion

Synthesis of diastereomers

The general scheme for the synthesis of diastereomers of (±)-norvaline is shown in Fig. 2. In comparison with various other CDRs, as noted in "Introduction", (*S*)-NIFE required less time (only 20 min) for derivatization at ambient temperature and provided lower limit of detection for the diastereomers, with sharp peaks. Under these conditions of temperature and time, the chiral reagent (*S*)-NIFE was found successful for complete derivatization when used with twofold molar excess. At 1.5-fold excess of reagent, the reaction was far from complete, and a peak of unreacted norvaline was detected. Therefore, twofold molar excess of the reagent was used for all the studies. While studying the effect of pH, 0.5 μL TEA (pH >11) was established as the optimized value. Derivatization was carried out in basic medium and no derivatization was observed in the absence of TEA.

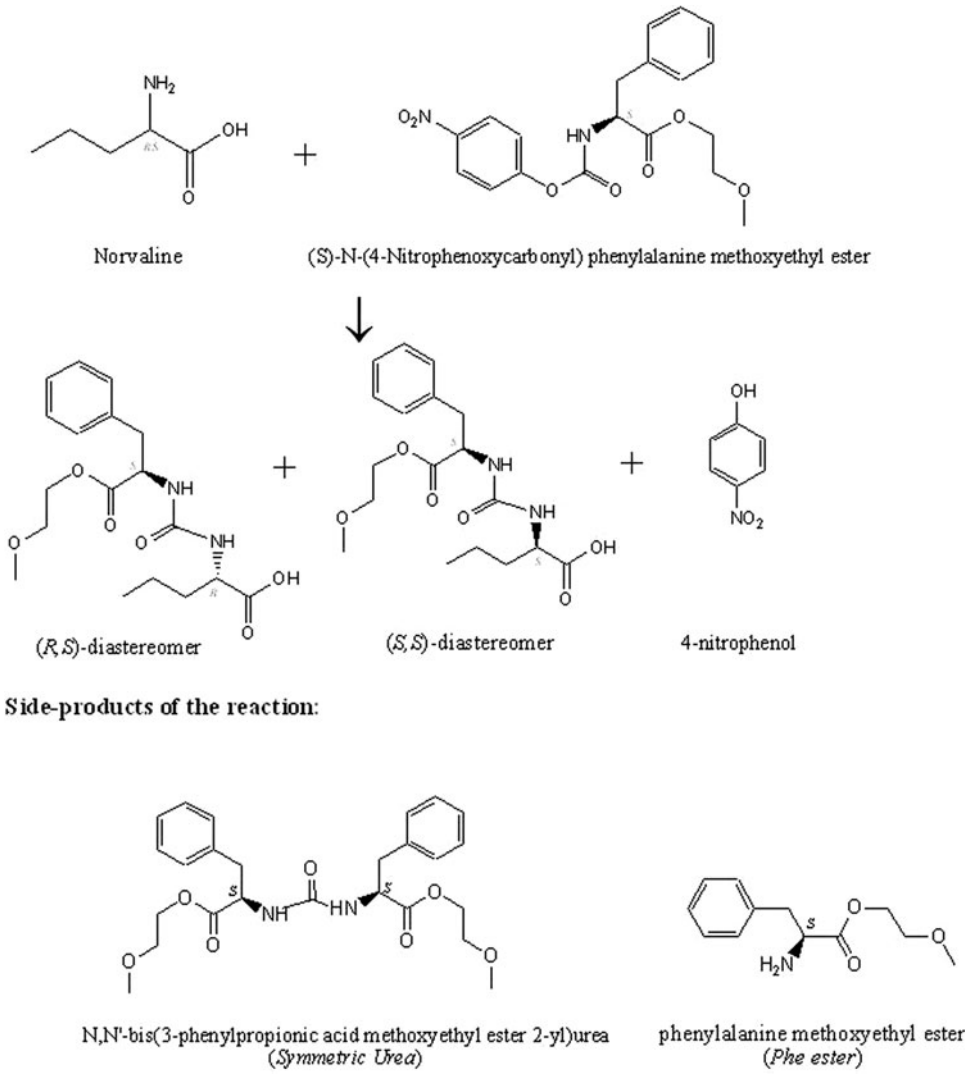

Fig. 2 Scheme of derivatization of norvaline with (*S*)-NIFE and self decomposition of the latter

In accordance with the literature (Péter et al. 2002) there occurs a reaction, in the presence of a base, between (*S*)-NIFE and the amino group of the selected amino compounds when the desired urea diastereomers are formed, accompanied by the formation of an equimolar amount of 4-nitrophenol, along with the formation of three side products by self-decomposition of (*S*)-NIFE (Fig. 2). The peaks of the diastereomers eluted between 4-nitrophenol and the *symmetric urea*. The *Phe ester* had almost the same retention time as 4-nitrophenol on the RP C18 column. The peaks of nitrophenol and *Phe ester* appeared at the retention time of 31.28 min while that for *symmetric urea* was at 78.97 min.

HPLC

The sections of chromatograms showing resolution of diastereomers of the six non-protein amino acids, and PenA prepared with (*S*)-NIFE are shown in Fig. 3. The peak areas, as given by the software, for the diastereomers corresponding to (+) and (−) isomers, respectively, of each of the analytes are given in parantheses as follows: norvaline (44.41, 45.97), 2-phenylglycine (76.01, 77.10), 2-aminooctanoic acid (72.55, 73.21), pipecolic acid (43.15, 41.89), 2-aminobutyric acid (55.02, 55.52), 2-aminoadipic acid (67.22, 68.15), and PenA (79.95, 78.92). Diastereomers of (+)-PenA and (+)-non-protein amino acids were eluted with the mobile phase earlier than the diastereomers of the corresponding (−)-isomers. Chromatograms were obtained by applying the same gradient for 60, 70, 80, and 90 min. The best separation was obtained in 90 min. On varying the TFA concentration from 0.005 to 0.03 M, a slight increase in separation factor was observed. Flow rate of the mobile phase was varied between 0.2 and 2.0 mL/min; retention times and Δt decreased with increase in flow rate up to 2 mL/min. The successful flow rate was 1 mL/min. The chromatographic data with respect to the resolved diastereomers are shown in Table 1. Elution order of diastereomers was confirmed by preparing them from single enantiomer of the analytes and performing HPLC under the identical conditions.

Structure–retention relationship

Fujii et al. (1997) explained the mechanism of separation of the diastereomers of amino acids (say, DL-Ala) prepared with MR and suggested stable and common conformations for both D- and L-Ala derivatives in which each substituent on the α-carbons of the Ala and L-Val-NH_2 (except for their amino groups) was oriented perpendicular to the planar phenyl ring (of Marfey's reagent) so that the side chain R group on the amino acid (D- or L-Ala) and the R group of the amino acid amide moiety (L-Val-NH_2), may be oriented *cis* or *trans* to each other in the diastereomer prepared with the MR; the resolution between the L- and D-amino acid diastereomers was then attributed to the difference in the hydrophobicity of such *cis* or *trans*-type arrangement when the derivative having the *cis*-type arrangement interacts more strongly with ODS material and has a longer retention time than that of the *trans*-type arrangement. In the present studies, such an arrangement of planar six-membered ring is absent with the (*S*)-NIFE reagent, and there are no *cis* or *trans* type arrangements to affect the hydrophobicity of the diastereomers; the hydrophobicity of the alkyl side chain of

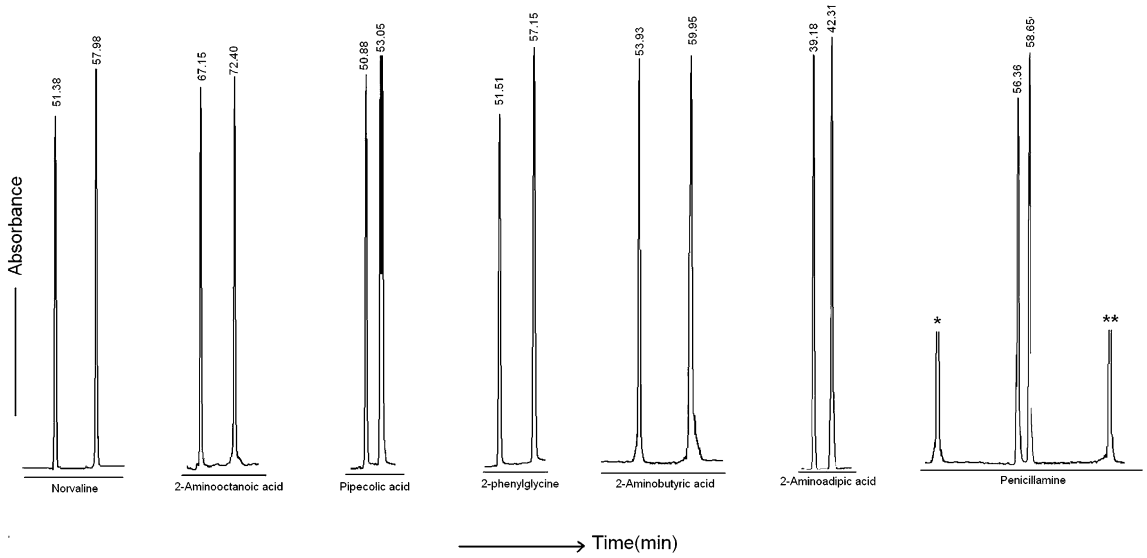

Fig. 3 The sections of chromatograms showing resolution of diastereomers of six racemic non-protein amino acids, and PenA prepared with (*S*)-NIFE. (+)-Diastereomer was eluted earlier than (−)-diastereomer in each case. Mobile phase: as given in experimental; flow rate 1.0 mL min^{-1}; detection at 205 nm. *Peak of nitrophenol and *Phe ester*; **peak for *symmetric urea*

Table 1 Resolution of diastereomers of racemic amino compounds by RP-HPLC

S. no.	Amino alcohols	Chromatographic data			
		k_2	k_1	α	R_S
1	Homophenylalaninol	26.474	24.466	1.082	20.550
2	Valinol	19.625	17.619	1.114	21.729
3	Prolinol	17.791	17.282	1.029	5.164
4	Leucinol	24.578	22.891	1.074	12.159
5	Alaninol	14.858	14.358	1.035	4.406
6	Phenylglycinol	18.190	17.952	1.013	3.599
7	2-Amino-1-butanol	18.307	16.589	1.104	17.552
8	Phenylalaninol	20.769	20.463	1.015	2.672
Non-protein amino acids					
9	Norvaline	59.979	53.087	1.113	24.765
10	2-Phenylglycine	22.645	20.314	1.115	15.913
11	2-Aminooctanoic acid	28.954	26.782	1.081	20.958
12	Pipecolic acid	20.949	20.052	1.044	8.255
13	2-Aminobutyric acid	23.979	21.472	1.117	19.858
14	2-Aminoadipic acid	16.632	15.326	1.085	11.586
15	Penicillamine[a]	23.266	22.321	1.042	8.471

Column: Eurospher C18; mobile phase as given in experimental; flow rate 1.0 mL min^{-1}; load amount, 20 µL; detection at 205 nm. t_0 value was 2.41 min; α is the separation factor

[a] Bis derivatives

amino acids seems to be the only factor to affect the interaction of the diastereomers with the ODS material of the column and the separation. Thus, looking to the alkyl side chain in the structures of analytes as shown in Fig. 1, the following observation was made: with increasing alkyl group in the side chain the retention times of the analytes (Fig. 3) increased, particularly for the following sets: leucinol > valinol; homophenyl alaninol > phenylalaninol > phenyl glycinol > prolinol > 2-aminobutanol > alaninol; 2-aminooctanoic acid > norvaline > phenylglycine > pipecolic acid > 2-aminoadipic acid; 2-aminobutyric acid > 2-aminoadipic acid.

Method development and validation

Linearity, accuracy, precision and limit of detection

Linearity studies were carried out by injecting the derivatives in the range 50–500 pmol and using correlations between the peak areas. Linearity was acceptable in this range for (+)-norvaline ($R^2 = 0.9986$) and for (−)-norvaline ($R^2 = 0.9995$) as diastereomers, respectively. Intra-day and inter-day precision was investigated by fivefold assay of (±)-norvaline at five concentration levels (10, 25, 50, 75, 100 ng mL^{-1}) and the RSD were <2%. RSD for (+)-norvaline varied from 0.10 to 0.28% for intra-day precision and 0.06 to 0.20% for inter-day precision and for (−)-norvaline 0.12 to 0.43 and 0.01 to 0.82% for intra and inter day precision, as diastereomers, respectively. The recoveries of diastereomers of (+)- and (−)-norvaline were found to be between 96.51 and 99.43%. For accuracy studies, samples were prepared by spiking (−)-norvaline with fixed amounts of (+)-norvaline in the range 0.001–0.01%. The results indicate that this method can be applied for the detection of (−)-norvaline in (+)-norvaline up to 0.003% by HPLC in the form of diastereomers. The present HPLC method using (*S*)-NIFE is capable of detecting 0.02 ng (0.01 ng of each enantiomer) of norvaline while the LOD for norvaline with MR was 57.5 µg (28.75 µg of each enantiomer) (Bhushan and Kumar 2008b).

Conclusion

Application of (*S*)-NIFE as the CDR does not require special protection of all synthesis steps from light and the time required for derivatization is less than the other derivatizing reagents. Overall, it is thus more economical. Under the reaction conditions no racemization was observed and the derivatives were stable for several weeks. The method has thus potential application in quality control in pharmaceutical formulations of these pharmaceutically and biologically important compounds.

Acknowledgments The authors are thankful to the Ministry of Human Resources Development, Government of India, New Delhi, for the award of a research assistantship (to C.A.) and to the Alexander von Humboldt Foundation, Bonn, Germany, for donating Knauer HPLC equipment and award of a fellowship (to R.B.)

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