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ORIGINAL ARTICLE

Chromatographic separation of enantiomers of non-protein α-amino acids after derivatization with Marfey's reagent and its four variants

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Abstract Some non-protein α-amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (Marfey's reagent, MR, FDNP-L-Ala-NH_2,) and four of its structural variants FDNP-L-Phe-NH_2, FDNP-L-Val-NH_2, FDNP-L-Leu-NH_2 and FDNP-L-Pro-NH_2. The resultant diastereomers were separated by normal and reversed phase thin layer chromatography (TLC) and reversed phase HPLC. In normal phase TLC, best resolution was obtained with solvent combination of phenol-water (3:1) while in reversed phase TLC mixtures of acetonitrile with triethylammonium phosphate buffer were found successful for resolution of diastereomers. The separation behavior of diastereomers prepared with different reagents was compared. The diastereomers of most of the amino acids prepared with FDNP-L-Leu-NH_2 were best separated while those prepared with FDNP-L-Pro-NH_2 failed to separate in most of the cases. The diastereomers were also separated on a reversed phase C_8 column with gradient elution using mixture of aqueous-trifluoroacetic acid (TFA) and acetonitrile and with detection at 340 nm. The effects of TFA concentration, flow rate and run time on HPLC separation were studied.

Keywords Chiral separation · Non-protein α-amino acids · FDNP-L-Ala-NH_2 · FDNP-L-Phe-NH_2 · FDNP-L-Val-NH_2 · FDNP-L-Leu-NH_2 · FDNP-L-Pro-NH_2 · Normal and RPTLC · RPHPLC

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Introduction

A large number of amino acids are found in nature, most of which are α-amino acids. These occur in free form or as constituents of other biomolecules like, peptides, proteins, coenzymes, hormones, etc. There are about 20 α-amino acids (nineteen amino and one imino acid), which constitute an alphabet for all proteins and differ only in structure of the side chain *R*. Non-protein amino acids are those amino acids which are neither found in proteins assembled during protein biosynthesis nor generated by post-translational modifications. This may be due to the lack of a specific codon (genetic code) and t-RNA. Hundreds of such amino acids are known and a large number of these are α-amino acids. The non-coded amino acids are found mostly in plants and microorganisms and arise as intermediates or as the end product of the metabolic pathways. Both categories of amino acids play important role in food and pharmaceutical industry. Non-protein amino acids are useful as building blocks for the synthesis of analogs of biologically active peptides, antibiotics, hormones, and enzyme inhibitors (Roberts and Vellaccio 1983). They are also versatile chiral starting materials or chiral auxiliaries in many organic syntheses (Coppola and Schuster 1987).

Chiral separations enjoy a great analytical importance in various fields and both from academic and industrial points of view. Liquid chromatography continues to be the method of choice with both direct and indirect approaches. TLC provides direct resolution of enantiomers of a variety of compounds including amino acids and their different derivatives (Bhushan and Martens 1997, 2003; Martens and Bhushan 1990; Günther and Möller 2003) and is used for its several advantages. Separation of diastereomeric pair via indirect technique is sometimes simpler to perform and often has better resolution than with a direct method

because chromatographic conditions are much easily optimized. In cases requiring sensitive determination in biological samples such as blood and urine, indirect methods using chiral derivatizing reagents can provide highly sensitive detector response. Literature survey shows that very few chiral reagents have been used for indirect chiral separation on TLC plates.

Direct enantiomeric resolution of non-protein α-amino acids has been reported on a column packed with octadecylsilanized silica coated with chiral 18-crown-6 ether (Miyazawa et al. 1991), and by chiral ligand-exchange column (Miyazawa et al. 2004). Chiral reagents like 2,3,4,6-tetra-O-acetyl-*β*-D-glucopyranosyl isothiocyanate (GITC) (Péter et al. 2000), *O*-phthalaldehyde along with isobutyryl-L-cysteine (Hess et al. 2004), and (*S*)-N-(4-nitrophenoxycarbonyl)-phenylalanine methoxy ethyl ester ((*S*)-NIFE) (Olajos et al. 2001) have been used for indirect separation of non-protein amino acids.

Marfey's reagent (MR) was introduced in 1984 and used for preparation of diastereomers of five amino acids followed by reversed phase HPLC resolution (Marfey 1984). Of the various methods available for indirect HPLC resolution of enantiomers of amino acids, use of MR has been very successful. There have appeared two review articles on use of Marfey's reagent to HPLC resolution of amino acids (B'Hymer et al. 2003), and its application including comparison with other derivatizing agents, for resolution of complex mixtures of DL-amino acids and a large number of other compounds, and mechanism of separation (Bhushan and Brückner 2004). MR reacts quantitatively with primary and secondary amino groups and provides a structural feature to replace L-Ala-NH_2 with other L- or D-amino acid amides yielding a variety of chiral derivatizing agents suggesting a wide scope for their application for resolution of a variety of chiral compounds.

Three variants of chiral Marfey's reagent namely, FDNP-L-Val-NH_2, FDNP-L-Phe-NH_2 and FDNP-L-Pro-NH_2 were used by Brückner and Keller-Hoehl (1990) to resolve 19 pairs of proteinogenic DL-amino acids by HPLC as diastereomers. Diastereomers of 22 DL-amino acids (DL-ethionine, DL-citrulline and 20 proteinogenic amino acids) prepared with FDNP-L-Ala-NH_2,were separated on C_{18} silica layers though it was not possible to separate all 44 individual compounds in a single run because of the large number of overlapping R_F values (Ruterbories and Nurok 1987). Both normal and reversed phase TLC have recently been reported from this laboratory for separation of protein DL-amino acids (Bhushan et al. 2007a) and RPTLC and RPHPLC for chiral separation of penicillamine (Bhushan et al. 2007b) by preparing their diastereomers with FDNP-L-Ala-NH_2 and its Phe and Val variants.

Diastereomeric derivatives of MR and structural variants have strong absorbance at 340 nm due to highly absorbing dinitrophenyl chromophore which makes it suitable for chromatographic applications where highly sensitive detection and quantification is required. Amino acids with their simple structures and easy availability of both enantiomers serve as a ready and inexpensive pool for enantiomeric studies.

Keeping in view the characteristics of MR, literature reports on its application and importance of non-protein amino acids, new chiral variants of MR having L-Phe-NH_2, L-Val-NH_2, L-Leu-NH_2 and L-Pro-NH_2 (in place of L-Ala-NH_2) were synthesized and used as derivatizing agents for chiral separation of eight non-protein DL-α-amino acids. The forty pairs of diastereomers were separated by reversed phase TLC and HPLC, and normal phase TLC. Results for the five reagents have been compared. To the best of author's knowledge this is the first report on the liquid chromatographic resolution of diastereomers of non-protein DL-amino acids prepared from structural variants of MR.

Experimental

Chemicals, reagents and instrumentation

DL- and L-α-amino acids, L-alaninamide hydrochloride (L-Ala-NH_2.HCl), L-phenylalaninamide hydrochloride (L-Phe-NH_2.HCl), L-valinamide hydrochloride (L-Val-NH_2.HCl), L-leucinamide hydrochloride (L-Leu-NH_2.HCl), L-prolinamide hydrochloride (L-Pro-NH_2.HCl) and 1,5-difluoro-2,4-dinitrobenzene (DFDNB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other analytical grade chemicals and solvents used were from E. Merck (Mumabi, India). Precoated normal phase TLC plates Alugram® SIL G/UV_{254} and RP-TLC plates RP Alugram® RP-18 W/UV_{254} were from Macherey-Nagel (Düren, Germany) as a gift. The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, photodiode array (PDA) detector 2600, Knauer manual injection valve and Eurochrom operating software was from Knauer (Berlin, Germany). The column used for reversed phase HPLC studies was Agilent BDS C_8, (150 × 4.6 mm, 4 μm particle size). Filtration assembly, with filters of pore size 0.45 and 0.50 μm, and the Milli-Q system used to purify double distilled water were from Millipore (Bedford, USA). Other equipments used were, Polarimeter, model Krüss P3002 (Germany), pH meter Cyberscan 510 (Singapore) and Incubator CI-65 (Remi, Mumbai, India).

Preparation of stock solutions

Stock solutions of DL-, and L-amino acids (0.2 M) were prepared by dissolving calculated amount of each in

hydrochloric acid (1 M). Solutions of all chiral reagents (0.1 M) were prepared in acetone and stored in refrigerator. Stock solutions of sodium hydrogen carbonate (1 M) and hydrochloric acid (2 M) were prepared in deionised water. Triethylammonium phosphate (TEAP) buffers (0.025–0.1 M, pH 3–7) and sodium acetate buffers (0.025–0.1 M, pH 5.5) were prepared and filtered through a 0.45 μm filter. For HPLC studies aqueous-TFA (0.01–0.05 M) solutions were prepared, filtered through a 0.45 μm filter.

Derivatization

Before reaction with the five chiral reagents to form diastereomers the purity both of the DL-amino acids and of the enantiomerically pure compounds was tested by TLC with different mobile phases and by determination of melting point and specific rotation. Derivatization of DL-2-phenylglycine with FDNP-L-Phe-NH_2 (Fig. 1) as a representative example, is described below.

Stock solution of DL-2-phenylglycine (25 μL, 5 μmol) was placed in a 2 mL plastic tube. A solution of FDNP-L-Phe-NH_2 (60 μL, 6 μmol, in acetone; the molar ratio of phenylalanine to FDNP-L-Phe-NH_2 was 1:1.2) was added, followed by addition of a solution of $NaHCO_3$ (1 M, 60 μL). The contents were mixed, heated at 30–40°C for 1 h with frequent shaking, and then cooled to room temperature. The reaction was ended by adding HCl (2 M, 20 μL) and, after mixing, the contents were dried in a vacuum desiccator over KOH pellets. The yellow colored residue of diastereomeric derivatives was then dissolved in CH_3CN (200 μL) and the solution was used for subsequent experiments. All solutions, at every stage of the reaction, were protected from exposure to light.

The same procedure was used for derivatization of other DL- and L-amino acids. Derivatization of the amino acids with FDNP-L-Val-NH_2, FDNP-L-Leu-NH_2, FDNP-L-Pro-NH_2 and FDNP-L-Ala-NH_2 was performed in the same way.

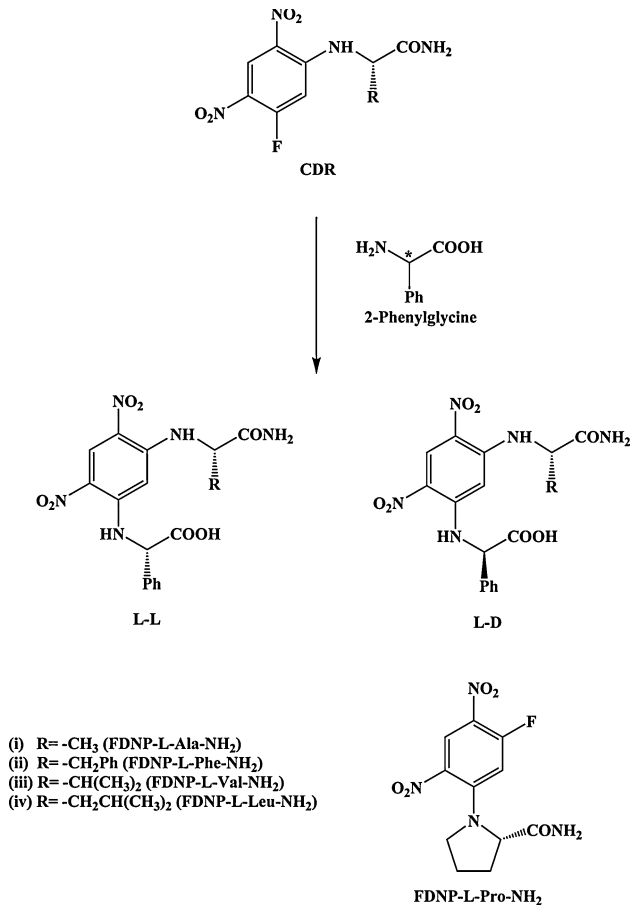

Fig. 1 Representative reaction of derivatization of 2-Phenylglycine and structures of CDRs

TLC

Solutions of the diastereomeric derivatives (2 μL, 50 nmol) of the DL- and L-amino acids were applied on precoated normal and reversed phase TLC plates as small round spots by use of a 10 μL Hamilton syringe and developed in pre-equilibrated rectangular glass chamber with mobile phase at 25°C. The experimental conditions were controlled and pre-equilibration of the chamber was done for nearly 15 min. The temperature was maintained inside an incubator and the chromatographic chambers were placed inside and allowed to attain the specific temperature, before the development. The developed plates were dried with hair-dryer and the separated derivatives were visible as bright yellow spots.

HPLC

Binary mobile phase of CH_3CN with aqueous-TFA (0.01 M) was used at a flow rate of 1.0 mL/min with UV detection at 340 nm. A linear gradient of CH_3CN from 25 to 65% in 45 min was employed. For HPLC studies, 10 μL aliquots of derivatized samples were used and a tenfold dilution was made with CH_3CN and 20 μL was injected onto column. Chromatographic conditions for best resolution of diastereomers were optimized by changing the flow rate and run time.

Validation procedures

All method validation procedures were carried out with diastereomers of DL-2-phenylglycine (Phg) prepared with the chiral reagent, FDNP-L-Val-NH_2. Calibration curves were plotted by derivatizing different standard solutions to find the linearity of the response. Recovery studies were carried out by derivatizing standard solutions of different

known concentrations and mean recovered values (five replicate runs) were represented as percentage of calculated values. Inter-day and intra-day stability studies were performed to find out precision and results are represented in % RSD. Limit of detection (LOD) and limit of quantification (LOQ) were also evaluated to find out the sensitivity of these reagents for detection and quantification.

Results and discussion

Synthesis

MR was prepared by reaction of 1,5-difluoro-2,4-dinitrobenzene (DFDNB) with L-Ala-NH_2 as described in literature (Brückner and Keller-Hoehl 1990). Marfey's reagent (MR) can safely be considered as a chiral variant of Sanger′s reagent (2,4-DNFB). Sanger′s reagent (1945) was unique because it provided DNP derivatives of amino acids that were identifiable by chromatography to establish amino acid sequence in peptides. The structural variants of MR, FDNP-L-Phe-NH_2, FDNP-L-Val-NH_2, FDNP-L-Leu-NH_2 and FDNP-L-Pro-NH_2, were prepared by nucleophilic substitution of one of the fluorine atoms in DFDNB with corresponding amino acid amides under basic conditions, and characterized. The DL- and L-amino acids under study were then derivatized with MR and these structural variants. In the Marfey's reagent (and in its structural variants) the remaining reactive aromatic fluorine atom undergoes nucleophilic substitution by the free amino group on the stereogenic centre of L-and D-amino acids (in the racemic mixture) to create diastereomers.

The amino acid amides were considered to be enantiopure as the Sigma-Aldrich catalogue has mentioned their specific rotation values which can be considered as the literature values for enantiopure samples. Nevertheless, the chiral purity of these amides was further verified by the following method. In all, amides of five amino acids were used to prepare the chiral derivatizing reagents (CDR), i.e., MR and its chiral variants. The CDRs were reacted with enantiopure L-Leu, $[\alpha]_D^{25} = +15 (c = 2, 5\ N\ HCl)$, when the corresponding diastereomers of only L-Leu were obtained. A single HPLC peak (using different mobile phases tried for this purpose including CH_3CN-*aq* TFA described under experimental above) corresponding to the diastereomer of optically pure L-Leu prepared with each of the five CDRs confirmed the enantiopurity of amides. Any impurity of the other antipode in the amide (i.e., of the corresponding D-amino acid) would have provided a mixture of diastereomers of L-Leu with each of the CDR, and thus two peaks in HPLC. Further, the absence of impurities in the same retention time of the peak of interest was confirmed with the help of PDA detector.

The diastereomeric derivatives of the amino acids under study, prepared as above, were separated by normal phase TLC and reversed phase TLC and HPLC.

TLC

Separation of the diastereomers by normal phase TLC

The diastereomeric derivatives (forty pairs) prepared, for the eight non-protein DL-amino acids, by reaction with MR and its four variants were stable for at least one week at 4°C. Chromatograms were developed in solvent systems comprising of CH_3CN-MeOH-H_2O in various proportions. It was observed that the spots reached the solvent front and none of the combinations was successful in resolving the diastereomeric mixtures. Efforts were made to work out other solvent systems. Phenol–water (3:1) was found to be successful to resolve twenty-eight diastereomeric pairs (out of forty); the diastereomeric pair for each of the eight DL-amino acids obtained with FDNP-L-Pro-NH_2 did not resolve and the diastereomers of DL-pipecolic acid prepared with any of the five chiral reagents did not resolve too. Bright and compact spots of diastereomers were obtained with this mobile phase. Representative photograph of actual chromatogram showing the resolution of diastereomeric pairs of amino acids prepared with FDNP-L-Phe-NH_2 is shown in Fig. 2.

Table 1 contains the hR_F values ($R_F \times 100$) and shows that resolution (R_S; calculated by dividing the distance between the two spot centers by the sum of their radii (Bhushan and Martens 2003) for majority of the resolved diastereomers was greater for FDNP-L-Leu-NH_2 derivatives; the order of difference between the R_S of resolved diastereomers is as follows: FDNP-L-Ala-NH_2 < FDNP-L-Phe-NH_2 < FDNP-L-Val-NH_2 < FDNP-L-Leu-NH_2. For those pairs of diastereomers that were separated on silica gel layer in the normal phase mode, hR_F values were D > L. The diastereomer with more intermolecular hydrogen bonding would be expected to have lesser interaction with the silica surface and would thus have the larger hR_F.

Separation of the diastereomers by reversed phase TLC

There was no separation for any of the diastereomers by use of mixtures of CH_3CN or methanol and sodium acetate buffers (0.025–0.1 M, pH 5.5). Enantiomer resolution was based on several trials with different amounts of acetonitrile (10–70%) in TEAP buffer (0.05 M, pH 5.5). Representative photograph of actual chromatogram showing the resolution of diastereomeric pairs of amino acids prepared with FDNP-L-Phe-NH_2 is given in Fig. 3. The hR_F values ($R_F \times 100$) are given in Table 2. A 50:50 ratio of the mobile phase CH_3CN-TEAP buffer (0.05 M, pH 5.5)

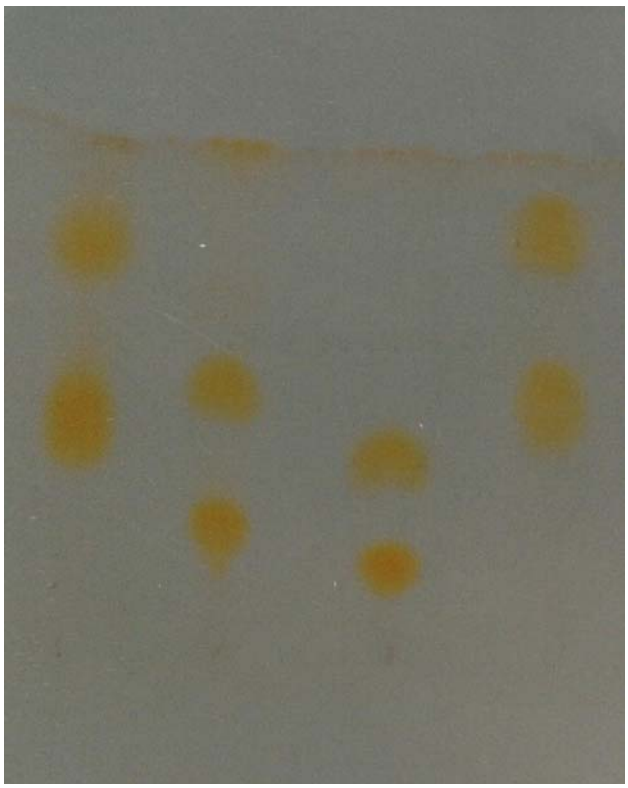

Fig. 2 Chromatogram obtained by normal phase TLC showing separation of the diastereomers of DL-amino acids derivatized with FDNP-L-Phe-NH_2. From *left* to *right* norvaline, aminoadipic acid, cysteic acid and isovaline. The *upper spot* is the D diastereomer and the *lower spot* is the L diastereomer. The mobile phase was phenol-water, 3:1, and the distance to the solvent front was 5.0 cm

was found to be successful to resolve thirty-three diastereomeric pairs (out of forty). It was interesting to observe that FDNP-L-Pro-NH_2 was successful as a CDR to the extent of resolving the diastereomeric pairs only of norvaline and 2-aminobutyric acid prepared with it (though with a poor resolution), and that D-pipecolic acid could be resolved in the form of its diastereomers prepared with only FDNP-L-Val-NH_2 and FDNP-L-Leu-NH_2, out of its derivatives prepared with five CDRs. Resolution (R_S) under reversed phase conditions was found to be less than that under normal phase conditions for almost all the diastereomeric pairs.

In reversed phase TLC the spots of the L-diastereomers moved faster than those of the corresponding D-isomers. This was ascribed to greater intramolecular hydrogen bonding in the D-diastereomers, which would result in the former being more hydrophobic and interacting more strongly with the nonpolar stationary phase.

The reagent FDNP-L-Leu-NH_2 was found to be the best as a derivatizing agent because the diastereomers of all the amino acids prepared with it resolved very well on reversed phase TLC plates, as shown in Table 2. Table 1 and 2 show that the separation of diastereomers of all the eight amino acids prepared with FDNP-L-Pro-NH_2 was largely unsuccessful under both normal and reversed phase TLC. The diastereomers of DL-2-aminooctanoic acid (obtained with all the five chiral reagents) had higher hR_F values in normal phase mode in comparison to those in the reversed phase mode that is because of the long alkyl chain and thus the observed behavior is in agreement with the expected one.

HPLC

Binary mobile phase of CH_3CN with 0.01 M trifluoroacetic acid (TFA) with a linear gradient of acetonitrile from 25 to

Table 1 hR_F values of diastereomers in normal phase TLC

Sr. No	DL-amino acids	Chiral derivatizing reagents and hR_F											
		FDNP-L-Ala-NH_2			FDNP-L-Phe-NH_2			FDNP-L-Val-NH_2			FDNP-L-Leu-NH_2		
		hR_F		R_S	hR_F		R_S	hR_F		R_S	hR_F		R_S
		L	D		L	D		L	D		L	D	
1	Norvaline	32	51	2.3	42	62	2.5	26	60	4.3	31	73	4.7
2	2-Aminoadipic acid	15	22	0.8	29	37	0.9	14	24	1.3	23	37	1.4
3	Cysteic acid	10	18	1.0	22	32	1.3	12	28	2.0	16	31	1.2
4	Isovaline	34	58	3.0	42	64	2.8	24	57	4.1	31	69	5.1
5	Pipecolic acid	NR			NR			NR			NR		
6	2-Aminobutyric acid	32	54	2.5	35	62	3.3	33	64	3.8	37	68	4.3
7	2-Phenylglycine	35	62	3.4	40	70	3.8	35	72	4.6	39	75	5.2
8	2-Aminooctanoic acid	37	65	3.5	42	76	4.3	45	83	4.8	38	84	5.4

Diastereomers of the amino acids prepared with FDNP-L-Pro-NH_2 did not resolve, therefore not shown

NR Not resolved

Solvent system: phenol–water (3:1); Solvent front: 5 cm

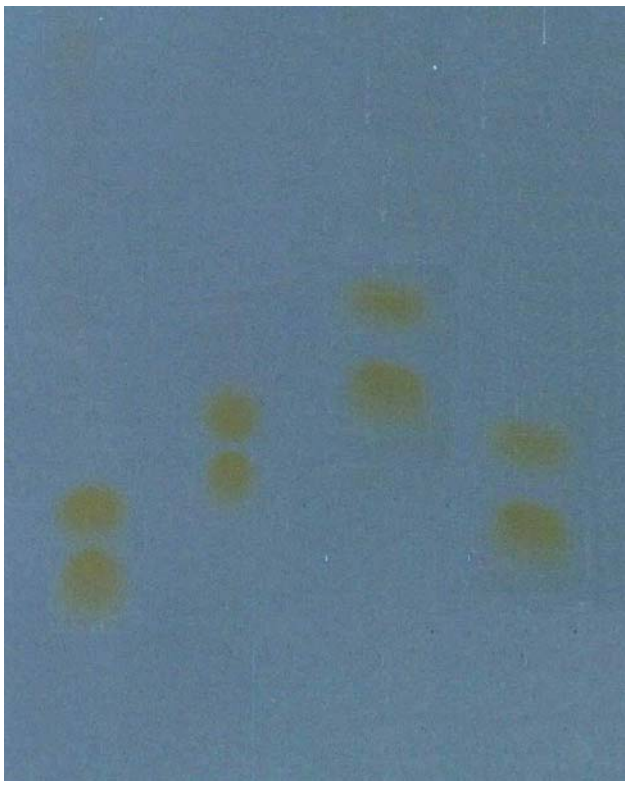

Fig. 3 Chromatogram obtained by reversed phase TLC showing separation of the diastereomers of DL-amino acids derivatized with FDNP-L-Phe-NH_2. From *left* to *right* norvaline, aminoadipic acid, cysteic acid and isovaline. The *upper spot* is the L diastereomer and the *lower spot* is the D diastereomer. The mobile phase was 50:50 CH_3CN in TEAP buffer (0.050 M, pH 5.5) and the distance to the solvent front was 6.5 cm

65% in 45 min was found successful for resolution at a flow rate of 1 mL/min with UV detection at 340 nm.

Sections of representative chromatograms showing resolutions are shown in Fig. 4. The difference between the retention times of diastereomeric derivatives in most of the cases was found to be in the following order FDNP-L-Val-NH_2 > FDNP-L-Phe-NH_2 > FDNP-L-Ala-NH_2 > FDNP-L-Pro-NH_2. Diastereomers of 2-aminoadipic acid and cysteic acid prepared with FDNP-L-Ala-NH_2 and FDNP-L-Pro-NH_2 eluted with solvent peak while very little resolution was achieved for diastereomers obtained with FDNP-L-Phe-NH_2 and FDNP-L-Val-NH_2. The retention times and difference between retention times of diastereomers are given in Table 3. As expected, because of its long alkyl chain, the diastereomers of DL-2-aminooctanoic acid (obtained with four chiral reagents) showed considerably large retention time in RP-HPLC in comparison to the diastereomers of other amino acids.

Flow rate of the mobile phase was varied between 0.5–1.5 mL/min. With increase in flow rate (up to 1.5 mL/min), retention times (and Δt_R, difference in retention time) decreased. TFA concentration was varied from 0.005 M to 0.02 M and there was observed a slight variation in separation factor and resolution with a change in TFA concentration (up to 0.03 M). A gradient (CH_3CN from 35 to 65%) was applied to obtain chromatograms for 35, 45, 55 min. It was found that retention time increased with increase in run time. The effects of all these different HPLC conditions on the separation of diastereomeric pairs of 2-phenylglycine and 2-aminooctanoic acid prepared with FDNP-L-Phe-NH_2 and FDNP-L-Val-NH_2 are reported in Table 4.

Separation mechanism

Marfey (1984) attributed the reason for the L-L diastereomer eluting before the L-D diastereomer to a stronger intramolecular H-bonding in D-isomer than in L-isomer. He

Table 2 hR_F values of diastereomers in reversed phase TLC

Sr. No	DL-amino acids	Chiral derivatizing reagents and hR_F														
		FDNP-L-Ala-NH_2			FDNP-L-Phe-NH_2			FDNP-L-Val-NH_2			FDNP-L-Leu-NH_2			FDNP-L-Pro-NH_2		
		hR_F		R_S	hR_F		R_S	hR_F		R_S	hR_F		R_S	hR_F		R_S
		D	L		D	L		D	L		D	L		D	L	
1	Norvaline	22	27	1.1	6.0	12	1.3	12	23	2.4	8.7	21	2.8	23	26	0.5
2	2-Aminoadipic acid	24	30	1.3	12	22	2.2	13	28	3.2	13	31	3.7	NR		
3	Cysteic acid	24	29	1.1	14	23	1.9	15	32	3.6	12	32	3.9	NR		
4	Isovaline	20	26	1.2	7.0	15	1.7	13	28	3.2	12	33	3.5	NR		
5	Pipecolic acid	NR			3.8	4.6	0.2	14	20	1.3	09	23	1.8	NR		
6	2-Aminobutyric acid	18	24	0.9	5.3	12	1.5	14	28	2.9	07	23	3.1	17	20	0.6
7	2-Phenylglycine	16	20	1.1	6.1	10	0.9	12	25	2.8	10	27	3.4	NR		
8	2-Aminooctanoic acid	3.0	5.8	0.6	2.8	6.1	0.8	3.1	7.8	1.1	3.3	8.3	3.3	NR		

NR Not resolved

Solvent system: CH_3CN in TEAP buffer (0.050 M, pH 5.5) (50:50); Solvent front: 6.5 cm

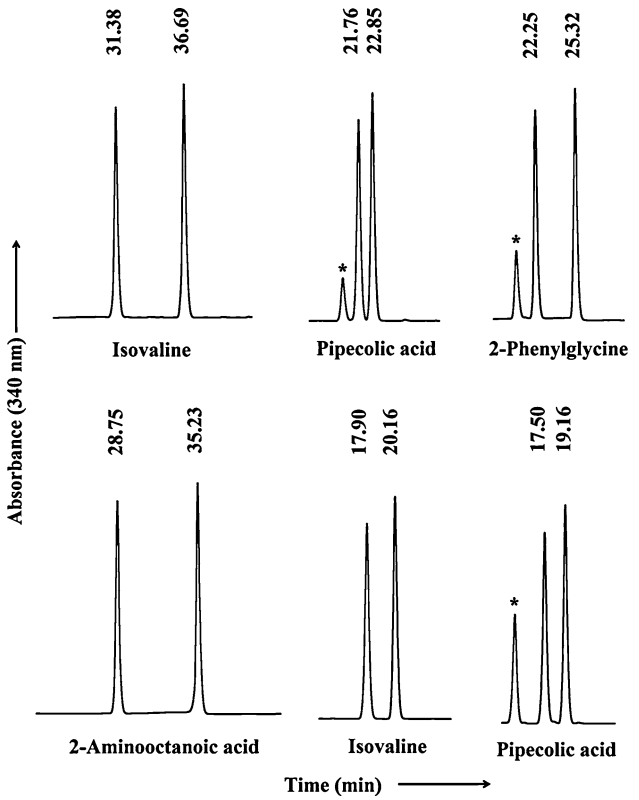

Fig. 4 Sections of chromatograms showing separation of diastereomers of non-protein α-amino acids obtained by reaction with FDNP-L-Phe-NH_2 (upper row) and FDNP-L-Val-NH_2(*lower row*); HPLC conditions: see experimental. *Asterisks* indicates the reagent related peak. First eluting peak correspond to L-L diastereomer

suggested that the carboxyl group can H-bond either to an *ortho* situated nitro group producing a nine member ring or, more likely, to the carbonyl oxygen of the *meta*-situated L-Ala-NH_2 forming a 12-membered ring. Stronger hydrogen bonding in a D-diastereomer would produce a more hydrophobic molecule which would be expected to interact more strongly with the RP column and thus have a longer retention time than an L-diastereomer. It was also suggested that the nature of the amino acid side chain was responsible for the differences in elution times of the diastereomers; e.g., the ionisable side chains of Asp and Glu decrease the separation while neutral and hydrophobic side chains increase it. The resolution mechanism of L-L and L-D diastereomers by RP-HPLC explained by Brückner and Gah (1991) shows that in the L-L diastereomers the carboxy group of the analyte is located extremely close to the carboxamide group of the reagent FDNP-L-Ala-NH_2 which facilitates formation of an intramolecular hydrogen bridge.

Therefore, the hydrophobic character of MR was modified by using various amino acid amides instead of L-Ala-NH_2 (used in Marfey's reagent) and the behavior in HPLC was investigated. The elution order of diastereomers was confirmed using derivatives of single enantiomers. It was observed that diastereomers of L-enantiomers eluted earlier than D-enantiomer. When compared to the methyl group of alanine the isopropyl moiety of valine provides more hydrophobicity and results in greater resolution of the diastereomers and increased retention time (Table 3). It may mean that more hydrophobic derivative is held more strongly by reversed phase HPLC column. This is in agreement with hydrophobicity scale of amino acids reported by Bull and Breese (1974) by calculating their apparent partial specific volume. Accordingly, the amino acids can be arranged in the decreasing order of their hydrophobicity as Val (0.777) > Ala (0.691); the values in parenthesis represent the apparent partial specific volume.

Table 3 Reversed phase HPLC data of diastereomers of non-protein amino acids prepared with MR and its variants

Sr. No	DL-amino acids	Chiral derivatizing reagents and retention time											
		FDNP-L-Ala-NH_2			FDNP-L-Phe-NH_2			FDNP-L-Val-NH_2			FDNP-L-Pro-NH_2		
		t_1	t_2	Δt_R	t_1	t_2	Δt_R	t_1	t_2	Δt_R	t_1	t_2	Δt_R
1	Norvaline	12.42	15.63	3.21	21.51	26.15	4.64	17.53	23.14	5.61	12.55	14.82	2.27
2	2-Aminoadipic acid	[a]	[a]	[a]	15.22	15.39	0.17	11.45	12.06	0.61	[a]	[a]	[a]
3	Cysteic acid	[a]	[a]	[a]	11.03	11.35	0.32	07.33	08.11	0.78	[a]	[a]	[a]
4	Isovaline	11.92	13.18	1.26	31.38	36.69	5.31	17.90	20.16	2.26	11.59	12.33	0.74
5	Pipecolic acid	11.09	12.31	1.22	21.76	22.85	1.09	17.50	19.16	1.66	11.05	12.03	0.98
6	2-Aminobutyric acid	09.13	11.60	2.47	18.57	22.45	3.88	14.74	19.17	4.43	9.47	10.94	1.47
7	2-Phenylglycine	12.35	16.55	4.20	22.25	25.32	3.07	18.48	23.53	5.05	12.67	15.76	3.09
8	2-Aminooctanoic acid	24.23	27.96	3.73	31.59	36.86	5.27	28.75	35.23	6.48	23.94	27.04	3.10

t_1 and t_2 are the retention times of first and second eluting diastereomer respectively. Δt_R is the difference between retention times of diastereomers

t_1 and t_2 are the retention times of L-L and L-D diastereomers respectively

[a] Diastereomeric peaks eluted with solvent peak

Mobile phase: CH_3CN-aqeous TFA (0.01 M) (linear gradient of CH_3CN from 25 to 65% in 45 min)

Table 4 Effect of changes in run time and flow rate on resolution of diastereomers of 2-Phenylglycine and 2-Aminooctanoic acid prepared with FDNP-L-Phe-NH_2 and FDNP-L-Val-NH_2

Effect		FDNP-L-Phe-NH_2								FDNP-L-Val-NH_2							
		2-Phenylglycine				2-Aminooctanoic acid				2-Phenylglycine				2-Aminooctanoic acid			
		Δt	k_1	α	R_S	Δt	k_1	α	R_S	Δt	k_1	α	R_S	Δt	k_1	α	R_S
Run Time (min)	35	2.95	5.73	1.17	8.42	5.16	8.91	1.19	14.74	4.95	4.51	1.36	14.14	6.41	7.84	1.27	16.02
	45	3.07	6.41	1.16	8.77	5.27	9.53	1.18	15.05	5.05	5.16	1.32	14.40	6.48	8.58	1.25	16.20
	55	3.12	7.55	1.13	8.91	5.32	10.48	1.16	15.20	5.10	5.57	1.29	14.57	6.54	9.32	1.16	16.35
Flow Rate (mL/min)	0.5	3.54	7.56	1.15	10.11	5.68	10.16	1.18	16.22	5.73	5.98	1.31	16.37	7.05	9.18	1.25	19.05
	1.0	3.07	6.41	1.16	8.77	5.27	9.53	1.18	15.05	5.05	5.16	1.32	14.40	6.48	8.58	1.25	16.20
	1.5	2.46	5.32	1.15	7.02	4.69	8.24	1.18	13.40	4.38	4.11	1.35	12.50	5.38	7.72	1.23	15.37

Δt_R is the difference between retention times of diastereomers, k_1 capacity factor for first eluting peak, α separation factor, R_S resolution

The two substituents, namely the side chain R group on the amino acid (say, 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 (Fujii et al. 1997). The resolution between the L- and D-amino acid diastereomers can be attributed to the difference in their hydrophobicity, which is derived from such *cis* or *trans*-type arrangement so that the derivative having the *cis*-type arrangement interacts more strongly with the ODS and has a longer retention time than that of the *trans*-type arrangement. Thus, it can be concluded that the diastereomer of the L-amino acid has the *trans*-type arrangement. The observation in the present studies that the diasteromeric pair of the eight DL-amino acids obtained with FDNP-L-Pro-NH_2 showed either poor or no resolution confirms the explanation offered by Brückner and Gah (1991) that steric hindrance due to proline ring, as the side chain, in the diastereomers adversely influences the resolution.

Method validation

Linearity

In TLC, calibration curves were plotted for the diastereomers of Phg prepared with FDNP-L-Val-NH_2 in the range 0.5–5.0 nmol. After the development of chromatogram, each spot corresponding to one diastereomer was scratched, dissolved in 1 mL of methanol and absorbance was determined using spectrophotometer at 340 nm. The amount of derivative extracted from TLC plate was found to be linearly related to that spotted onto the plate within range studied.

Linearity studies in HPLC were carried out using correlations between the peak areas and amounts of derivatives injected onto column in the range 0.05–0.5 nmol. Results showed that linearity was acceptable in this range for (D)-, ($R^2 = 0.997$) and (L)-Phg ($R^2 = 0.999$) respectively.

Accuracy and precision

Inter-day (3 days) and intra-day run precision was assessed by calculating the relative standard deviation of the control sample concentration measured in each validation run. Intra-day and inter-day precision of the proposed method was investigated by fivefold HPLC assay of DL-Phg at five concentrations (20, 40, 60, 80, 100 ng/mL) and the %RSD were less than 2%. RSD for D-Phg varied from 0.32 to 0.76% for intra-day precision and 0.60–1.24% for inter-day precision and for L-Phg 0.38–1.08% and 0.64–1.62% for intra-, and inter day precision respectively. The recoveries of D-, and L-Phg were found to be between 98.5 and 99.7%.

The accuracy of the TLC and HPLC methods was determined by investigating the recovery of D-enantiomer from the samples of pure L-enantiomer. For these studies samples were prepared by spiking D-Phg with fixed amounts of L-Phg. The results indicate that the method can be applied for the detection of D-Phg in L-Phg up to 0.05% by TLC and 0.005% by HPLC.

Limit of detection

In TLC method was found capable to detect 0.34 µg (0.17 µg of each enantiomer) of DL-Phg using FDNP-L-Val-NH_2. The HPLC methods using FDNP-L-Val-NH_2 was capable of detecting 2.21 ng (1.11 ng of each enantiomer) of DL-Phg. Thus, samples containing such small amounts of any of these enantiomers could be separated and detected. The method, although indirect, is sensitive and simple.

Conclusion

MR provides a possibility to replace the chiral selector L-Ala-NH_2 of the reagent with other suitable moieties for resolution of enantiomers. The CDRs in the form of variants of MR can be easily prepared in laboratory, in µmolar

quantity, and with less expenses while their commercial samples are very costly. The reagents have ability to react with all α-amino acids, provide stable derivatives with quantitative yield of the reaction at ease, sensitive detection and visibility of spots on TLC plates in ordinary light requiring no spray reagent. The described procedures can be applied for the identification and quantification of non-protein α-amino acid enantiomers in nanomole range.

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