Amino Acids (2012) 42:1417–1423
DOI 10.1007/s00726-011-0838-x

ORIGINAL ARTICLE

Application of amino acid amides as chiral auxiliaries in difluoro dinitro benzene and cyanuric chloride moieties for high-performance liquid-chromatographic enantioseparation of selenomethionine and its mixture with methionine and cysteine

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Received: 3 December 2010 / Accepted: 18 January 2011 / Published online: 4 February 2011

Abstract L-Ala-NH_2, L-Val-NH_2, L-Leu-NH_2, and D-Phg-NH_2 were used as chiral auxiliaries to synthesize four chiral derivatizing reagents (CDRs) of each of the three categories, viz., difluoro dinitro benzene (DFDNB) based chiral variants, and cyanuric chloride (CC) based monochloro-s-triazine reagents (MCTs) and dichloro-s-triazine reagents (DCTs). DFDNB based chiral variants were synthesized by substituting one of the fluorine atoms of DFDNB with respective amino acid amides. The MCTs and DCTs were synthesized by substituting chlorine atom with aforesaid amino acid amide moieties in 6-methoxy dichloro-s-triazine and in CC, respectively. In total, 12 CDRs were characterized and used for microwave-assisted synthesis (45 s at 80% of 800 W using DFDNB-based chiral variants, 80 s at 90% of 800 W power using MCTs, and 50 s at 80% of 800 W power using DCTs) of diastereomers of (A) SeMet, and (B) mixture of (1) SeMet and Met, and (2) SeMet, Met, and Cys. The diastereomers were enantioseparated by reversed-phase high-performance liquid chromatography using gradient elution with mobile phases containing aq. TFA (0.1%)—MeCN in different compositions. The method was validated for accuracy, precision, and limit of detection.

Keywords Amino acid amides · DFDNB based chiral variants · MCTs · DCTs · Selenomethionine · Methionine · Cysteine · Diastereomer separation · Reversed-phase HPLC

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Introduction

Enantioseparation of DL-amino acids (by both direct and indirect approaches) using different chromatographic methods continues to be a subject of immense importance since D- and L-amino acids display different absorption kinetics and follow partly different metabolic pathways in humans (Berg 1959). A number of review articles have appeared in literature which deals with indirect applications including those for amino acids (Bojarski 1997; Görög 2000; Nambara 1984; Srinivas 2004). Ilisz et al. (2008) reviewed the chiral derivatizing reagents (CDRs) which were used for chiral analysis of amino acids by high-performance liquid chromatography (HPLC).

L-Amino acids have been used as chiral selectors (both in thin-layer chromatography and high-performance liquid chromatography) for enantioseparation of a variety of compounds (including several pharmaceuticals) and amides of L-amino acids, in particular, have been used as chiral auxiliaries to prepare CDRs by incorporating the former into difluoro dinitro benzene (DFDNB) and cyanuric chloride (CC) moieties (Bhushan and Martens 2010). The two way use of amino acids is mainly because they provide a ready, commercial, cost-effective chiral pool and prospects of simple derivatization of their functional groups.

L-Alaninamide (L-Ala-NH_2) was the first amino acid amide that was placed as chiral auxiliary in DFDNB (Marfey 1984) and in CC (Brückner and Streker 1992); the CDRs so obtained were applied for the high-performance liquid chromatographic (HPLC) resolution of diastereomers of a few amino acids. CDRs based on DFDNB, popularly known as Marfey's reagent and its variants, and those based on CC have been described for their application to enantioresolution of amino acids (Bhushan and

Martens 2010; Bhushan and Brückner 2004; B'Hymer et al. 2003).

In accordance with the theme of the present paper the literature survey showed that the amides of L-Ala, L-Val, L-Leu, L-Met, L-Phe, L-Pro and D-Phg have been used as chiral auxiliaries to prepare CDRs based on DFDNB (Bhushan et al. 2009; Bhushan and Kumar 2008b, 2009a while amides of L-Ala, L-Val, L-Leu, L-Met, L-Phe, and D-Phg have been used as chiral auxiliaries to prepare CDRs based on CC (Brückner and Wachsmann 2003; Bhushan and Kumar 2008a; Bhushan and Dixit 2010) and those were used for enantioresolution of proteinogenic and non proteinogenic amino acids by HPLC. However, it was revealed that there were no reports on indirect enantioseparation of DL-SeMet and/or its mixture with sulphur-containing amino acids using HPLC and UV detector.

Selenomethionine (SeMet; Fig. 1), an amino acid and a major nutritional source of selenium for higher animals and humans (Schrauzer 2000), has the ability to be incorporated into the body proteins in place of methionine; it provides a mechanism of reversible selenium storage in organs and tissues (Schrauzer 2003). Selenium deficiency has adverse impact on immune system leading to higher mortality in HIV-infected patients besides influencing vital human organs such as cardiovascular system (Neve 1996) and central nervous system (Castano et al. 1997). More bioavailability and less toxicity of SeMet compared to inorganic selenium (Thomson and Stewart 1974; Lindemann and Hintelmann 2002) make the former most common source of selenium nutritional supplement.

Literature reveals enantioseparation of SeMet by HPLC using indirect approach supplemented with elemental specific ICP-MS as detector using various CDRs such as, *o*-phthaldialdehyde and *N*-isobutyryl-L-cysteine (Bergmann et al. 2004) and Marfey's reagent (Montes-Bayon et al. 2001). Application of ICP-MS detector is associated with high equipment cost and special method for sample preparation in comparison to the UV–Vis detectors. Direct enantioseparation of SeMet on chiral stationary phases along with their limitations and advantages has been reviewed by Sanz-Medel and Blanco-González (2001). Huang et al. (2005) reported chiral speciation and determination of DL-SeMet on a newly synthesized chiral ligand-exchange stationary phase using HPLC and UV detector.

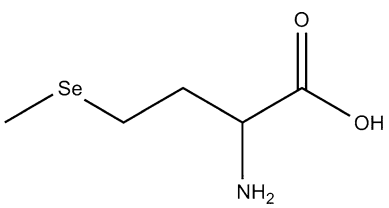

Fig. 1 Structure of DL-Selenomethionine

In view of the literature as cited above and the cross references, and taking into account the importance of SeMet, utility of amino acid amides as chiral auxiliaries in CDRs, the literature reports on their applications, as discussed above, and in search of new faster methods for chiral resolution of SeMet, 12 CDRs were synthesized (based on DFDNB and CC moieties) and used for enantioseparation of (A) SeMet, and (B) mixture of (1) SeMet and Met, and (2) SeMet, Met, and Cys. To the best of authors' knowledge this is the first report on the separation of enantiomers of selenomethionine along with its mixture with methionine and cysteine using the above said CDRs and RP-HPLC using UV detection.

Experimental

Chemical reagents and instrumentation

DL-cysteine, DL-methionine, DL-selenomethionine, L-cysteine, L-methionine and L-selenomethionine, other racemic and chirally pure amino acid amides were obtained from Sigma-Aldrich (Bangalore, India). Difluoro dinitro benzene (DFDNB) and s-triazine were obtained from Sigma-Aldrich (St Louis, MO, USA). All other analytical grade chemicals and HPLC solvents were from E. Merck (Mumbai, India). The HPLC system consisting of a 10 mL pump head 1000, manager 5000 degasser, UV detector 2500, manual injection valve and operating software was from Knauer (Berlin, Germany). Other equipments used were Microwave-Multiwave 3000 (800 W, Perkin-Elmer, Shelton, CT, USA), pH meter Cyberscan 510 (Singapore), 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 spectra were recorded on a Bruker 500 MHz instrument using dimethyl sulfoxide. The Milli-Q system from Millipore (Bedford, MA, USA) was used to purify double-distilled water to HPLC-grade deionised water.

Synthesis of CDRs

L-Ala-NH_2, L-Val-NH_2, L-Leu-NH_2, and D-Phg-NH_2, were used as chiral auxiliaries to synthesize four CDRs of each of the three categories, viz., DFDNB based CDRs 1–4, and CC based MCTs (CDR 5–8) and DCTs (CDR 9–12). These are, CDRs 1–4: FDNP-L-Ala-NH_2, FDNP-L-Val-NH_2, FDNP-L-Leu-NH_2, FDNP-D-Phg-NH_2; CDRs 5–8: *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-alaninamide, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-valinamide, *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-L-leucinamide, and *N*-(4-Chloro-6-methoxy-[1,3,5]-triazine-2-yl)-D-

phenylglycinamide; and CDRs 9–12: *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-alaninamide, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-valinamide, *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-L-leucinamide and *N*-(4,6-Dichloro-[1,3,5]triazine-2-yl)-D-phenylglycinamide, respectively.

The synthesis, characterization and determination of enantiomeric purity of the CDRs were carried out as described elsewhere (Bhushan and Kumar 2008a, b, 2009b).

Microwave-assisted synthesis of diastereomers of DL-SeMet

Using CDRs (1–4)

The diastereomers of DL-SeMet were synthesized as per experimental conditions reported in literature (Bhushan and Kumar 2008b) except that MW irradiation was used in the present case instead of conventional heating. Microwave irradiation for 45 s at 80% power (of 800 W) was found successful for the synthesis of diastereomers of DL-SeMet and the mixtures (1) DL-SeMet and DL-Met, and (2) DL-SeMet, DL-Met, and DL-Cys with the CDRs 1–4.

In comparison to the present studies a time of 2 h was required for previously reported (Montes-Bayon et al. 2001) synthesis of diastereomers of SeMet with Marfey's reagent under conventional heating.

Using CDRs (5–12)

In this case the diastereomers of DL-SeMet were synthesized according to the reported literature (Bhushan and Kumar 2008a). For the three sets of analytes, microwave irradiation was required for 80 s at 90% power using CDRs 5–8 and for 50 s at 80% using CDRs 9–12.

A volume of 10 μL of the resulting solution, containing diastereomers, was diluted ten times with mobile phase, and 20 μL of it was injected onto the column. The reaction conditions for derivatization were optimized by derivatizing DL-SeMet with CDR3 and CDR11. Solutions of CDRs and the diastereomers of SeMet and its mixture with amino acids were found to be quite stable up to 1 week in dark at 4°C.

The diastereomers of DL-SeMet prepared with DFDNB based CDR (e.g., CDR1) are recognized as, dinitrophenyl-L-alanylamide-L-selenomethionine (DNP-L-Ala-NH_2-L-SeMet, the L–L diastereomer) and dinitrophenyl-L-alanylamide-D-selenomethionine (DNP-L-Ala-NH_2-D-SeMet, the L–D diastereomer). The diastereomers of DL-SeMet prepared with MCTs (e.g., CDR5) are recognized as 6-methoxy-triazinyl-L-Ala-NH_2-L-SeMet and 6-methoxy-triazinyl-L-Ala-NH_2-D-SeMet. Similarly, the corresponding diastereomers were prepared with DCTs (e.g., CDR9).

HPLC conditions

RP-HPLC was performed on a Waters Spherisoro ODS (250 × 4.6 mm I.D., 5 μm) column (from Parker-Style Fittings, Ireland). The following mobile phases were used:

Mobile Phase I (*used for the separation of diastereomers of SeMet prepared with CDRs 1–4*). aq. TFA (0.1%)—MeCN in a linear gradient of MeCN from 25 to 65% in 45 min; and

Mobile Phase II [*used for the separation of diastereomers of mixture of* (1) *SeMet and Met, and* (2) *SeMet, Met, and Cys prepared with CDRs 1–4*]. aq. TFA (0.1%)—MeCN in a linear gradient of MeCN from 30 to 65% in 50 min, at a flow rate of 1 mL/min and UV detection at 340 nm, and

Mobile Phase III (*used for the separation of diastereomers of SeMet prepared with CDRs 5–12*). Eluent A: aq. TFA (0.1%)—MeCN (90:10); Eluent B: aq. TFA (0.1%)—MeCN (20:80), a linear gradient from 100% A to 100% B in 45 min at a flow rate of 1 mL/min with UV detection at 230 nm was employed.

Validation procedures

Method validation for analytical separation was done using diastereomers of DL-SeMet prepared with CDR3 following International Conference on Harmonization (ICH) guidelines (1996). Calibration curves were plotted by derivatizing standard solutions to find the linearity of 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.

Results and discussion

Enantioresolution of DL-SeMet

Using CDRs (1–4)

The chromatographic conditions for enantioseparation of diastereomers of DL-SeMet prepared with CDRs 1–4 were optimized for the best resolution. The mobile phase I was found successful and the resolution data are shown in Table 1a. It shows that the diastereomers prepared with CDR3 (FDNP-L-Leu-NH_2) have the highest resolution value (27.10).

Using CDRs (8–15, MCT and DCT reagents)

The chromatographic conditions were optimized and mobile phase III was found successful for enantioseparation of

Table 1 HPLC resolution data for enantioseparation of diastereomers of DL-SeMet prepared with CDR 1–12

CDR no.		Chromatographic parameters			
		k_L	k_D	α	R_S
(a) Chiral auxiliary attached to DFDNB					
1	L-Ala-NH_2	7.61	9.58	1.26	15.66
2	L-Val-NH_2	10.88	13.35	1.22	22.65
3	L-Leu-NH_2	13.01	14.55	1.12	27.10
4	D-Phg-NH_2	12.14	9.62	1.26	17.61
(b) Chiral auxiliary attached to CC					
MCTs					
5	L-Ala-NH_2	5.34	5.91	1.11	4.24
6	L-Val-NH_2	7.74	8.25	1.07	5.38
7	L-Leu-NH_2	7.94	8.39	1.06	6.39
8	D-Phg-NH_2	5.94	5.45	1.09	5.09
DCTs					
9	L-Ala-NH_2	5.57	6.19	1.11	7.46
10	L-Val-NH_2	7.87	8.31	1.06	8.63
11	L-Leu-NH_2	7.98	8.64	1.08	9.40
12	D-Phg-NH_2	6.23	5.58	1.12	7.21

k_L and k_D retention factors of the diastereomers of L-SeMet and D-SeMet respectively, α selectivity, R_S resolution value

diastereomers of DL-SeMet prepared with CDRs 5–12; the resolution data are shown in Table 1b. The highest resolution values were obtained for the diastereomers of DL-SeMet prepared with CDR11. The resolution values of diastereomers of SeMet prepared with DCT reagents (CDRs 9–12) were higher compared to the corresponding diastereomers prepared with MCT reagents (CDRs 5–8). This could be attributed to the difference in electronegativity of oxygen and chlorine; the electronegativity of oxygen is 3.5 and that of Cl is 3.0 on Pauling scale. Thus DCTs with two chlorine atoms may have greater interaction with stationary phase (hence better resolution) in comparison to MCTs which have one oxygen atom of the methoxy group. The higher electronegativity of oxygen may result into weaker interaction with the reversed-phase C18 material of the stationary phase.

Sections of chromatograms showing baseline resolution of the diastereomers of DL-SeMet prepared with CDRs 1–4 and CDRs 5–12 are given in Fig. 2(i) and (ii), respectively.

The diastereomers of L-SeMet were found to be eluted earlier than their D-counterparts except those prepared with CDR 4, 8 and 12 (having D-Phg-NH_2 moiety as the chiral auxiliary). MeCN was found to be the better organic solvent than methanol, as larger retention times and poor resolutions were observed with the latter.

The results (in the present studies) revealed that the CDRs (based on DFDNB and CC) have their own advantages, for example, (1) the diastereomers of SeMet prepared with CDRs 1–4 (based on DFDNB moiety) have better resolution (R_S, 15.66 to 27.10) than those prepared with CDRs 5–12 (based on CC moiety; R_S, 4.24 to 9.24), and (2) at every stage of the reaction the solutions related to CDRs 1–4 (based on DFDNB moiety) were protected from exposure to light, while the solutions related to CDRs 5–12 (CC based) did not require such a protection.

Enantioseparation of the mixture (1) DL-SeMet and DL-Met and (2) DL-SeMet, DL-Met and DL-Cys

Mobile phase II and mobile phase III were found to be successful for the resolution of diastereomeric mixtures (1) DL-SeMet and DL-Met and (2) DL-SeMet, DL-Met and DL-Cys, prepared with each of the CDRs 1–4 and CDRs 5–12, respectively. Sections of chromatograms showing resolution of diastereomers from the mixtures (1) DL-SeMet and DL-Met and (2) DL-SeMet, DL-Met and DL-Cys prepared with CDR3 are given in Fig. 3(i) and (ii), respectively. However, the best resolution was obtained for the

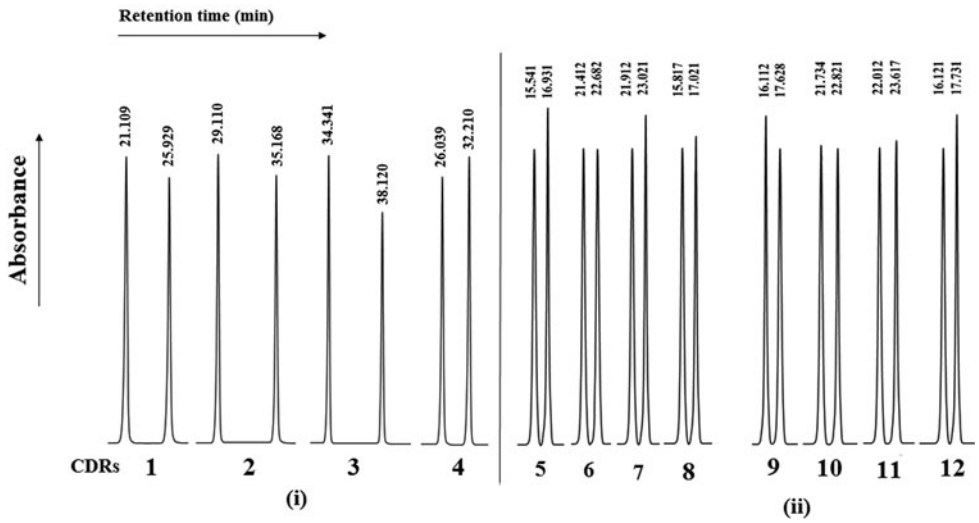

Fig. 2 Sections of chromatograms showing resolution of diastereomers of DL-SeMet prepared with (**i**) CDRs 1–4; mobile phase I (described in "Experimental") and (**ii**) CDRs 5–12; mobile phase III (described in "Experimental"); Waters Spherisoro ODS (250 × 4.6 mm I.D., 5 μm); the first peak corresponds to L–L-diastereomer in each case, except for the diastereomers prepared with CDR 4, 11 and 15

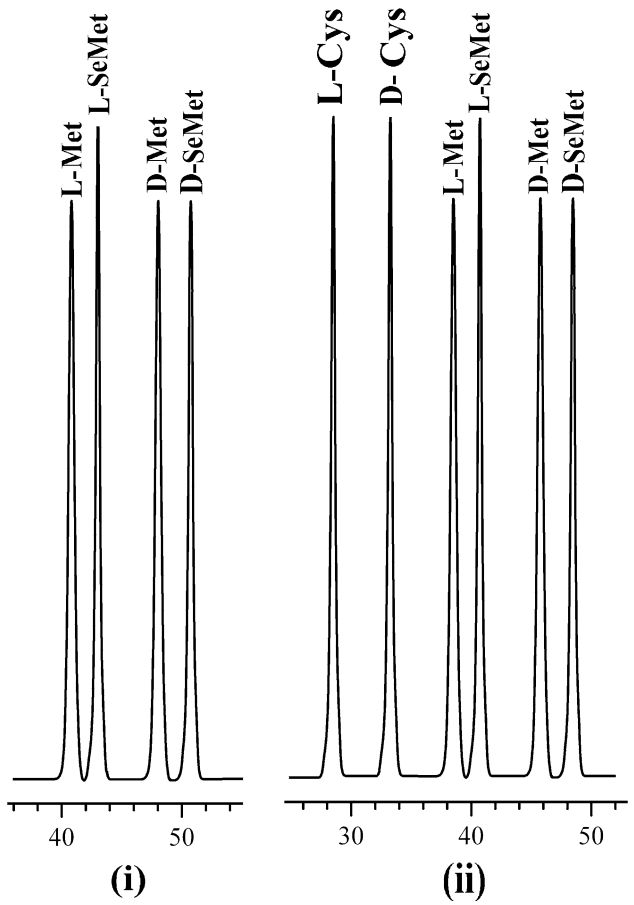

Fig. 3 Sections of chromatograms showing resolution of diastereomers of the mixtures (**i**) DL-SeMet and DL-Met and (**ii**) DL-SeMet, DL-Met and DL-Cys prepared with CDR3; Waters Spherisoro ODS (250 × 4.6 mm I.D., 5 µm); detection at 340 nm for all the diastereomers; mobile phase II (described in "Experimental")

diastereomers prepared with CDR3. The elution order of the diastereomers (prepared with CDR3) from the mixture (1) DL-SeMet and DL-Met, was as follows: L-Met, L-SeMet, D-Met and D-SeMet, respectively, while that from the mixture (2) containing DL-SeMet, DL-Met and DL-Cys was: L-Cys, D-Cys, L-Met, L-SeMet, D-Met and D-SeMet, respectively.

Separation mechanism

The mechanism for separation and elution order of diastereomers of SeMet prepared with CDRs 1–4, can be considered to be the same as already explained for the separation of diastereomers of amino acids prepared with amino acid amide variants of MR (Fujii et al. 1997; Bhushan et al. 2009), while that of SeMet prepared with CDRs 5–12 was in quite good correlation to the reported explanation for the separation of diastereomers of amino acids prepared with MCT and DCT reagents (Bhushan and Kumar 2008a).

Effect of side chain of amino acid amides as chiral auxiliaries present in the CDRs on resolution of diastereomers of SeMet

The amino acid amides present as the chiral auxiliaries in the CDRs 1–12 can be arranged as: L-Leu-NH_2 > L-Val-NH_2 > L-Ala-NH_2 for their decreasing hydrophobicity order based on partial specific volumes (values given in parenthesis) of corresponding amino acids, Leu (0.842) > Val (0.777) > Ala (0.691), calculated by Bull and Breese (1974). Thus the retention times, shown in parenthesis, of L–L-diastereomers of SeMet prepared with the CDRs 1–3 decreased with the decreasing hydrophobicity of the amino acid amide side chain in the corresponding CDRs: DNP-L-Leu-NH_2-L-SeMet (35.34) > DNP-L-Val-NH_2-L-SeMet (29.11) > DNP-L-Ala-NH_2-L-SeMet (21.11). The same behavior was observed for the diastereomers prepared with CDRs 5, 6, 7 and CDRs 9, 10, 11.

Linearity, accuracy and precision

The peak area response of the diastereomers of L-SeMet and D-SeMet prepared with CDR3 was plotted against the corresponding concentration (100–500 pmol), and the linear regression was computed. A good linear relationship was obtained over this range. The regression equations were $y = 1.279x + 0.807$ ($R^2 = 0.999$) and $y = 1.267x - 1.37$ ($R^2 = 0.998$) for the diastereomers of L-SeMet and D-SeMet prepared with CDR3, respectively.

The intra-day assay and inter-day assay studies for accuracy and precision were carried out by replicate HPLC analysis (n=5) of diastereomers of DL-SeMet prepared with CDR3 at five concentrations (25, 30, 35, 40, 50 $ngmL^{-1}$) as shown in Table 2. The coefficient of variation (CV, %) for D-SeMet varied from 0.52 to 1.12 for intra-day assay precision and 0.57 to 1.24 for inter-day assay precision and those values for L-SeMet were from 0.12 to 1.41 and 0.43 to 1.42, respectively. The percentage recovery for D-SeMet varied from 98.6 to 99.3 for intra-day assay and 97.6 to 97.8 for inter-day assay and those values for L-SeMet were from 99.1 to 99.6 and 97.6 to 98.8, respectively.

For the determination of accuracy, the recoveries of D-SeMet from the solution containing excess of L-SeMet were investigated. Solution of L-SeMet was spiked with fixed amount of D-SeMet within the range of 0.001–0.010%. The results indicate the ability of this method for detection up to 0.002% of D-SeMet in L-SeMet by HPLC.

Conclusion

The novelty of the present work lies in the simple, successful and effective analytical enantioseparation of SeMet

Table 2 Intra-day assay and inter-day assay precision for the enantioseparation of diastereomers of DL-SeMet prepared with CDR3

Concentration ($ngmL^{-1}$)	First eluting diastereomer			Second eluting diastereomer		
	Mean ± SD	Recovery[a]	CV[a]	Mean ± SD	Recovery[a]	CV[a]
Intra-day						
25	24.86 ± 0.08	99.5	0.33	24.66 ± 0.16	98.7	0.53
30	29.82 ± 0.06	99.4	0.21	29.64 ± 0.15	98.8	0.52
35	34.86 ± 0.04	99.6	0.12	34.58 ± 0.38	98.8	1.11
40	39.64 ± 0.09	99.1	0.23	39.72 ± 0.44	99.3	1.12
50	49.65 ± 0.70	99.3	1.41	49.30 ± 0.55	98.6	1.11
Inter-day						
25	24.66 ± 0.11	98.7	0.43	24.40 ± 0.14	97.6	0.57
30	29.58 ± 0.34	98.6	1.17	29.28 ± 0.34	97.6	1.15
35	34.58 ± 0.27	98.8	0.78	34.16 ± 0.42	97.6	1.24
40	39.48 ± 0.44	98.7	1.12	39.12 ± 0.24	97.8	0.61
50	48.80 ± 0.69	97.6	1.42	48.85 ± 0.60	97.7	1.23

SD standard deviation *CV* coefficient of variation

[a] Percentage values

and also within the mixtures of sulphur-containing amino acids (Met and Cys) in a single chromatographic run (along with a 0.002% LOD). Microwave assisted synthesis of the diastereomers of SeMet using the CDRs has reduced the derivatization time to seconds. The method can further be applied to the biological samples as well. This method establishes the importance of amino acid amides as chiral auxiliaries attached to UV detectable DFDNB and s-triazine moieties.

Acknowledgments The authors are grateful to the Council of Scientific and Industrial Research (CSIR), New Delhi, for the award of a senior research fellowship (to R.D.). Thanks to Alexander von Humboldt Foundation, Bonn, Germany, for donating Knauer HPLC equipment (to R.B.). Financial assistance (to RB) from Council of Scientific and Industrial Research (CSIR), New Delhi, India, (Grant No. 01(2334)/09 EMR-II) is also gratefully acknowledged.

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