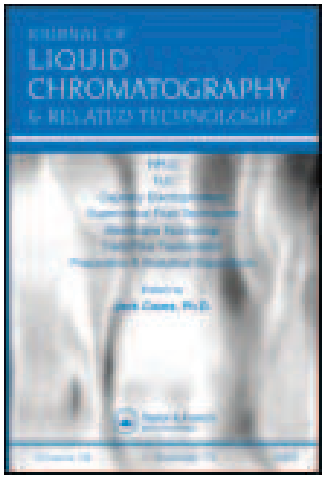

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Separation of Enantiomers of Selected Sulfur-Containing Amino Acids by Using Serially Coupled Achiral-Chiral Columns

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Homocysteine is an important indicator of human health. Increased levels of plasma homocysteine are associated with a higher risk of cardiovascular, cerebrovascular, and peripheral arterial disease. Homocysteine is naturally occurring amino acid and it is formed during the methionine metabolism. It can be either transsulfurated to cysteine or remethylated to methionine. Therefore, the simultaneous monitoring of homocysteine, cysteine, and methionine is often desirable. Many studies are focused on the achiral separations of these amino acids, but no one has considered their chiral separations. In this work, HPLC method has been developed for enantiomeric separations of homocysteine, cysteine, and methionine by using teicoplanin columns.

Keywords: amino acids, columns, electrochemical detection, enantiomers, HPLC, teicoplanin

Introduction

Macrocyclic antibiotics are popular chiral selectors in HPLC[1,2,3] and capillary electrophoresis.[2,4] They include the ansamycins, glycopeptides, and polypeptides. More chiral analytes have been resolved by using the glycopeptides. The most frequently utilized glycopeptides are vancomycin, ristocetin, avoparcin, and teicoplanin.[2] One important property of the glycopeptides is that they are complementary to one another.[5,6] Especially, teicoplanin columns have unique enantioselectivity for amino acids.[6,7]

The presence of D-cysteine (Cys) or D-homocysteine (HCy) in the human body has not been recorded yet. Some studies have indicated that D-methionine (Met) is poorly utilized and excreted in the urine.[8] Met is an essential amino acid that participates in protein synthesis. Met is continuously converted to HCy and adenosine. Significant amounts of Met are subsequently regenerated via the remethylation of HCy. This enzymatic reaction is catalyzed by methionine synthase. The majority of HCy is not remethylated but catabolized with serine to form cystathionine. This reaction is catalyzed by cystathionine-β-synthase. Cystathionine is metabolized to Cys and α-ketobutyrate.[9101] The conversion of Met to Cys is an irreversible process. Cys is essential for glutathione production in human cells.[12]

Enhanced levels of HCy are associated with increased lipid peroxidation and aging. Also, it is an independent indicator of cardiovascular diseases and Alzheimer's disease.[13,14] The participation of HCy in cardiovascular risk is not clearly understood. Many studies are focused on the chiral separations of these amino acids, but no one has considered their chiral separations. Chiral aspect of methionine metabolism may be required. Enantiomeric separations of HCy, Cys, and Met have not been the subject of many studies. However, the simultaneous enantioseparations of these amino acids have not been reported yet.

The aim of this work was to propose HPLC method with serially coupled achiral–chiral columns using electrochemical detection for enantioseparation of HCy, Cys, and Met. C18 column was used for amino acid separation and enantiomers were separated by using teicoplanin columns in on-line coupled system.

Experimental

Chemicals

Acetonitrile, sodium phosphate monobasic monohydrate, 1-octanesulfonic acid sodium salt (OSA), L-Met, D-Met, DL-Met, L-HCy, DL-HCy, L-Cys, D-Cys, and DL-Cys were purchased from Sigma-Aldrich (USA). Methanol and triethylamine were obtained from Merck (Germany). Ortho-phosphoric acid was purchased from Fluka Biochemika (Switzerland).

Preparation of Standard Solutions

All standard solutions were prepared in water. Doubly deionized water (<18 M Ohm cm^{-1}) was used. Working solutions (0.1 mg mL^{-1}) of these analytes were obtained by mixing the stock solutions (1 mg mL^{-1}). Stock solutions were stored at

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−80°C but left to equilibrate at room temperature (25°C) before use. Working solutions were prepared weekly.

Instrumentation and Chromatographic Conditions

The chromatographic system consisted of two isocratic pumps (DeltaChrom SDS 030, Watrex, Praha, Czech Republic) and electrochemical detector (Coulochem II, ESA, Chelmsford, UK). Detector was composed of guard cell Model 5020 and analytical cell Model 5010A (ESA, Chelmsford, UK). Analytical cells had potential +0.9 V (E1) and +1.2 V (E2), while the potential of the guard cell was +1.4 V (E0). The detector gain was set to 5 µA. However, the initial potentials were set to +0.65 V (E1), +0.9 V (E2) and +1.4 V (E0) according to Garaiova et al.[15]

Enantioseparations of selected amino acids were realized by using serially coupled achiral-chiral columns. Achiral separations were performed on column Purosher RP-18 endcapped 250–254 mm (5 µm) (Merck, Darmstadt, Germany) used together with a guard column Purospher STAR RP-18e (5 µm) (Merck, Darmstadt, Germany). Enantiomeric separations were performed on teicoplanin (Chirobiotic T) or teicoplanin aglycone (Chirobiotic TAG) columns (ASTEC, USA). Pump 1 delivered a mobile phase A through the achiral column and pump 2 sent a mobile phase B to a mixing chamber where they were mixed one another. The mixed mobile phase flowed through the chiral column. A mobile phase A contained 50 mmol L^{-1} phosphate buffer, 1 mmol L^{-1} OSA (pH 2.5) and acetonitrile with ratio 94:6 (*v*/*v*). A mobile phase B contained triethylamine and methanol with a ratio 9:1 (*v*/*v*). The flow rate was 0.3 mL min^{-1} (pump 1) and 0.3 mL min^{-1} (pump 2). A nylon filter (47 mm in diameter) was used for the mobile phases filtering. The columns were thermostated with an JET STREAM II Plus HPLC Column Thermostat (WO Industrial Electronics, Austria) at 25°C. All measurements were performed in replicate (3x). The elution of L-form was followed by D-form in all cases. The resolution (*Rs*) of first and second eluting enantiomers is calculated by ratio of difference between retention times t_{R1} and t_{R2} to peak widths at half peak height w_{051} and w_{052} as follow: $Rs = 1.18 * (t_{R2}\text{-}t_{R1})/(w_{051} + w_{052})$.

Results and Discussion

Direct combined enantiomeric separations of HCy, Cys, and Met are complicated because of their similar chemical structures and the absence of any chromophore or fluorophore. Their chemical structures are shown in Figure 1. In this work, we focused mainly on the selection of suitable chromatographic conditions necessary to HCy, Cys, and Met enantiomers separation.

Choice of Chromatographic Conditions

Enantiomeric Separations of Amino Acids in Water/Organic Solvent

Initially, enantiomeric separations of the individual amino acids were optimized in a mobile phase (MP) composed of water and organic solvent (methanol or acetonitrile). This was first

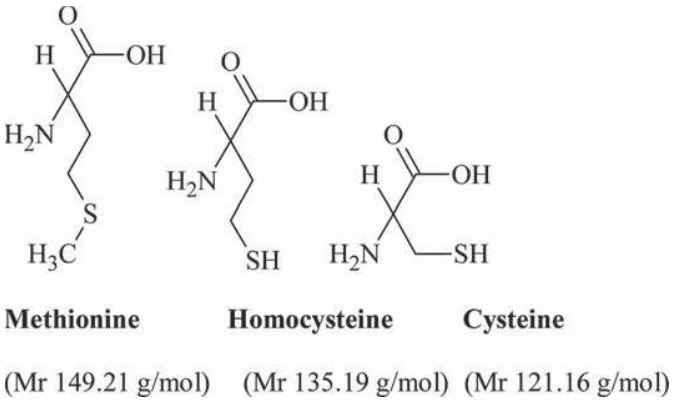

Fig. 1. Chemical structure of methionine, homocysteine and cysteine.[16]

performed on the Chirobiotic T column. Table 1 shows that retention of enantiomers increased with increasing methanol (MeOH) content in the MP. This occurs because of the higher solubility of the selected amino acids in water in comparison to organic solvent. Data of DL-Cys are not shown because the standard had not been delivered yet. Figure 2 shows the dependence of retention factor *k* on the amount of MeOH and acetonitrile (ACN) for Met enantiomers. The resolution of D- and L-Met was higher in the MP containing MeOH in comparison to ACN. This is presumably due to differences in the chemical properties of the organic solvents (MeOH as proton-donor and ACN as proton acceptor). It can be supposed that the solvation properties of MeOH and the formation of hydrogen bond may increase the selectivity. Retention increased when the content of organic solvent was higher than 60%. The retention of the HCy and Met enantiomers on the Chirobiotic T column was similar and this necessitated the addition of a means to separate these two amino acids from one another. Secondly, the

Table 1. Retention factors (*k*), Separation factors (α), and Resolution (*Rs*) as a function of methanol content in the mobile phase

	Methanol %						
	5	10	20	40	50	70	85
Cysteine							
k_L	1.6	1.6	1.7	1.8	1.9	2.5	3.9
Homocysteine							
k_L	1.9	2.0	1.9	2.0	2.1	2.5	–
k_D	2.4	2.5	2.6	3.0	3.4	4.8	–
α	1.2	1.2	1.3	1.5	1.6	1.9	–
Rs	1.3	1.0	1.9	3.3	4.0	6.1	–
Methionine							
k_L	2.4	2.3	2.1	2.1	2.2	2.6	3.5
k_D	2.9	2.9	2.8	3.2	3.5	4.5	7.1
α	1.2	1.2	1.3	1.5	1.6	1.7	2.0
Rs	0.7	1.0	5.3	5.1	6.4	6.8	8.8

Conditions: MP: water and methanol; SP: Chirobiotic T; column temperature: 25°C; flow rate: 0.6 mL min^{-1}.

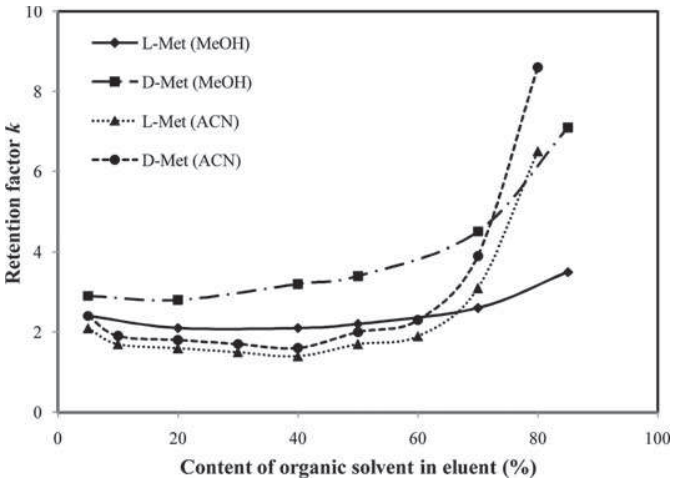

Fig. 2. Dependence of retention factor on methanol (MeOH) or acetonitrile (ACN) content for methionine enantiomers (0.1 mg mL^{-1}). Conditions: MP: water and organic solvent (MeOH or ACN); SP: Chirobiotic T; column temperature: 25°C; flow rate: 0.6 mL min^{-1}.

Chirobiotic TAG column was used. Chirobiotic TAG has complementary separation properties to Chirobiotic T.[2] We assumed that the mixture of amino acids could be separated, but it could not. The MP composed of water and organic solvent were not suitable for the separation of amino acid mixture. Therefore, the connection of achiral column was considered.

Enantiomeric Separations of Amino Acids in Phosphate Buffer/Organic Solvent

Choice of a Mobile Phase for Achiral Column. The separation of both the amino acids and their enantiomers a complex achiral–chiral column system was considered. A mobile phase for the achiral column separation was composed according to Garaiova et al.[15] The mobile phase was tested at different pH values (2.5, 2.7, 3.8, 4.5, 5.4, 6.4). The amino acid mixture was separated in all cases. However, the amino acids eluted too close to each another at pH ≥ 3.8. The pH 2.5 was selected for subsequent experiments. The necessity of an ion-pairing reagent was also considered. The mixture of amino acids was separated better when an ion-pairing reagent was present. The mobile phase composed of 50 mmol L^{-1} phosphate buffer (pH 2.5), 1 mmol L^{-1} OSA, and ACN (94:6, *v/v*) was selected for optimal results. The total time of analysis was 20 min.

Choice of a Chiral Stationary Phase. Chirobiotic T and TAG columns can be used at pH range 3.8 (3.0)–6.8.[7] In this step, only the *off-line* separations were performed on the chiral stationary phases (SPs). In an effort to increase the pH of the element on the chiral column, triethylamine (TEA), and MeOH (9:1, *v/v*) was added into the MP after the achiral column separation. The role of MeOH was tested on amino acid enantioseparation. Measurements were also made in the MP containing ACN. Figure 3 shows the comparison of enantioseparation of HCy and Met in the two MPs. Enantiomers were separated better (higher Rs value) in the MP containing MeOH in comparison to ACN. Resolution of HCy (Rs 1.5) and Met (Rs 3.0) enantiomers was achieved.

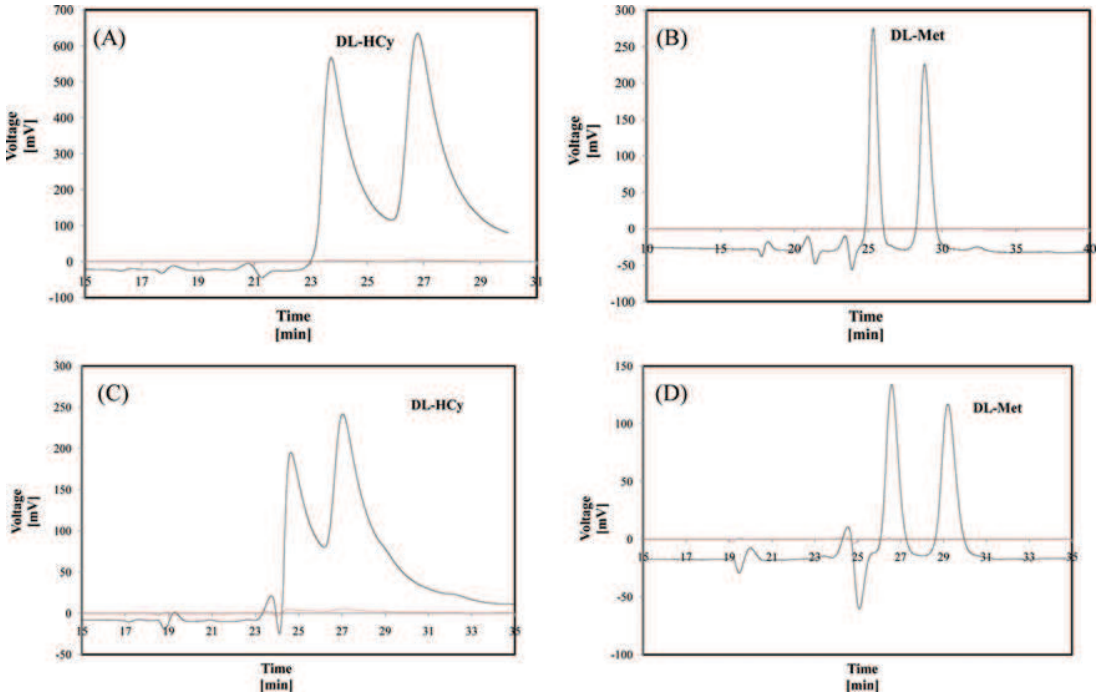

Fig. 3. Chiral separation of amino acids in the mobile phase 1 (*A, B*) and in the mobile phase 2 (*C, D*). Conditions: MP_1:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA, acetonitrile (94:6 *v/v*) and TEA in water (pH 8), *methanol* (9:1 *v/v*) with 1:1 (*v/v*); MP_2:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA, acetonitrile (94:6 *v/v*), and TEA in water (pH 8), *acetonitrile* (9:1 *v/v*) with 1:1 (*v/v*); SP:Chirobiotic T; column temperature: 25°C; flow rate: 0.6 mL min^{-1}; mass concentration of standards: 0.1 mg mL^{-1}.

Enantiomeric separations on Chirobiotic TAG column were also realized. Figure 4 shows the comparison of amino acid enantioseparations by using Chirobiotic T and TAG columns. Baseline separation of HCy (Rs 3.2), Met (Rs 3.0), and Cys (Rs 1.4) enantiomers were recorded by using a Chirobiotic TAG column. Enantiomers of Cys were not separated by using the Chirobiotic T column and, therefore, TAG was preferred for the next set of experiments.

Influence of TEA Addition and Methanol Content on the Enantiomeric Separation. The pH of water was measured after addition of TEA. Different pH values (pH 8–10) were tested. This had not a significant influence on the enantiomeric separations (Rs values were not changed). However, the TEA addition made the peaks narrower. The pH value of water with TEA addition (pH 8) was chosen. The MP_1 and MP_2 ratio was also monitored. Table 2 shows that the best separations of Cys enantiomers and the baseline separations of HCy and Met enantiomers were achieved in the MP_1: MP_2 (1:1, *v/v*). The amount of added methanol was also tested (Table 3). From the results it can be conclude that the best separations of Cys enantiomers and baseline separations of HCy and Met enantiomers were obtained in the MP containing 10% MeOH.

Enantiomeric Separations in the Achiral-Chiral System

After those chromatographic conditions were selected *in off-line* mode, *on-line* enantioseparation of amino acids was realized. Figure 5 shows the chromatogram of amino acid mixture enantiomeric separations by using serially coupled achiral–chiral columns. The amino acids were eluated in the order of Cys (1), HCy (2), and Met (3). The total time of analysis was 40 min.

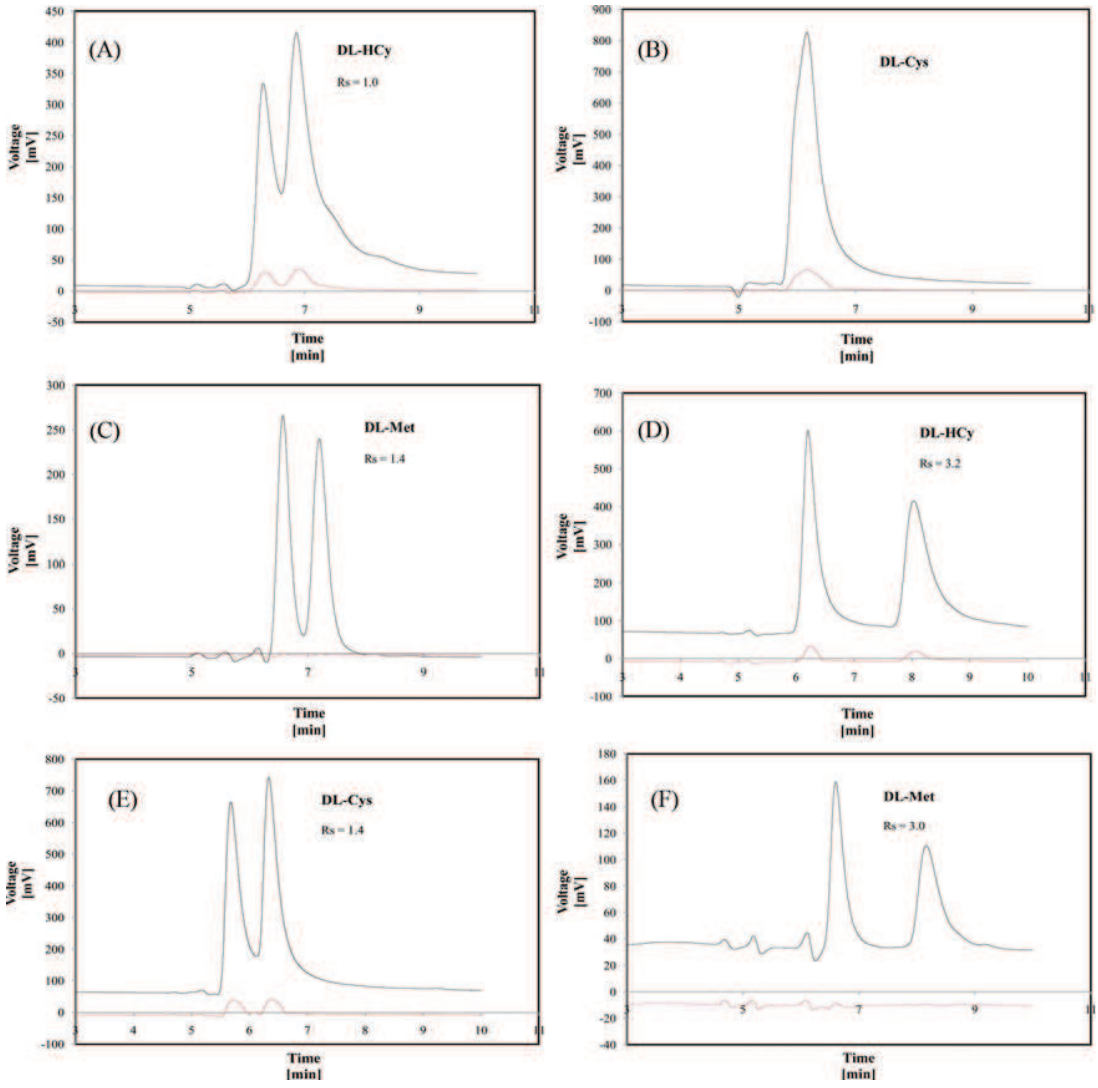

Fig. 4. Comparison of DL-HCy, DL-Cys, and DL-Met enantiomeric separations by using Chirobiotic T (*A, B, C*) and TAG columns (*D, E, F*). Conditions: MP_1:MP_2 (1:1 *v/v*); MP_1:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA:ACN (94:6 *v/v*); MP_2:TEA in water (pH 8):MeOH (9:1 *v/v*); column temperature: 25°C; flow rate: 0.6 mL min^{-1}; mass concentration of standards: 0.1 mg mL^{-1}.

Table 2. Effects of mobile phase's ratio on resolution of amino acid enantiomers

	A			B			C			D		
Compound	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs
DL-Cys	14.1	0.4	0.6	14.9	0.6	0.6	14.1	0.6	0.9	14.0	0.7	0.7
	14.6	0.6		15.6	0.8		14.9	0.5		14.9	0.9	
DL-HCy	21.9	0.7	1.1	20.9	0.7	1.4	21.1	0.8	1.5	20.4	0.8	1.6
	23.5	0.9		23.0	1.0		23.5	1.1		23.2	1.3	
DL-Met	32.6	0.6	1.3	30.9	0.6	1.5	30.6	0.6	1.8	29.1	0.6	2.1
	33.9	0.7		32.7	0.7		32.6	0.7		31.5	0.7	

A -MF1; B - MF1:MF2 (2:1); C - MF1:MF2 (1:1); D - MF1:MF2 (1:2).
Conditions: MP_1:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA:ACN (94:6 *v/v*); MP_2:TEA in water pH 8:MeOH (9:1 *v/v*).
SP: Chirobiotic TAG; mass concentration of standards: 0.1 mg mL^{-1}; column temperature: 25°C; flow rate: 0.6 mL min^{-1}.

Table 3. Effects of methanol content in the MP_2 on resolution of amino acid enantiomers

	A			B			C			D			E		
Compound	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs	t_R [min]	w_{05} [min]	Rs
DL-Cys	14.5	0.4	0.8	14.3	0.5	0.8	13.9	0.5	0.9	13.7	0.6	0.7	13.2	0.6	0.8
	15.3	0.6		15.0	0.6		14.7	0.6		14.4	0.7		14.1	0.7	
DL-Hcy	23.2	0.7	1.5	22.1	0.7	1.5	20.5	0.7	1.6	18.9	0.7	1.6	17.6	0.7	1.8
	25.3	1.0		24.3	1.0		22.7	1.0		21.3	1.0		20.0	1.0	
DL-Met	37.4	0.8	1.3	34.8	0.7	1.4	30.6	0.6	1.7	26.6	0.5	1.9	23.3	0.5	2.2
	39.2	0.9		36.6	0.8		32.4	0.7		28.5	0.7		25.3	0.6	

MP_1:MP_2 (1:1 *v/v*); MP_1:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA:ACN (94:6 *v/v*).
MP_2: A: TEA in water pH 8:MeOH (98:2 *v/v*); B: TEA in water pH 8:MeOH (95:5 *v/v*); C: TEA in water pH 8:MeOH (9:1 *v/v*); D: TEA in water pH 8:MeOH (85:15 *v/v*); E: TEA in water pH 8:MeOH (8:2 *v/v*).
Conditions: SP: Chirobiotic TAG; mass concentration of standards: 0.1 mg mL^{-1}; column temperature: 25°C; flow rate: 0.6 mL min^{-1}.

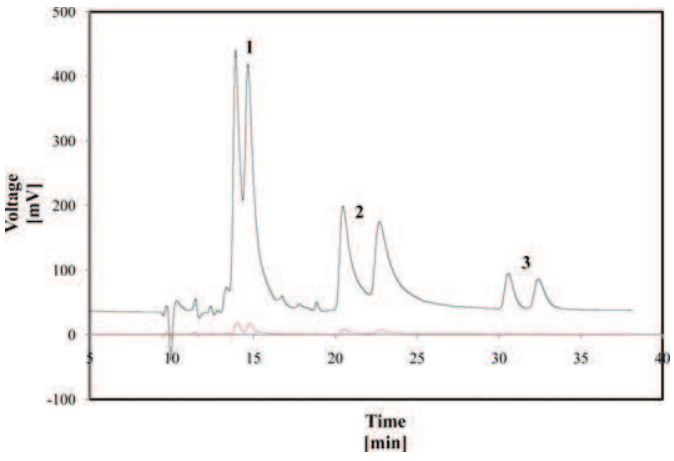

Fig. 5. Enantiomeric separations of amino acid standards: (1) DL-Cys, (2) DL-HCy, and (3) DL-Met. Conditions: MP: mixture MP_1:MP_2 (1:1 *v/v*) (MP_1:50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA:ACN (94:6 *v/v*) and MP_2:TEA in water pH 8:MeOH (9:1 *v/v*)); SP:C18 and Chirobiotic TAG; mass concentration of standards: 0.1 mg mL^{-1}; column temperature: 25°C; flow rate: 0.3 mL min^{-1} (pump 1) and 0.3 mL min^{-1} (pump 2).

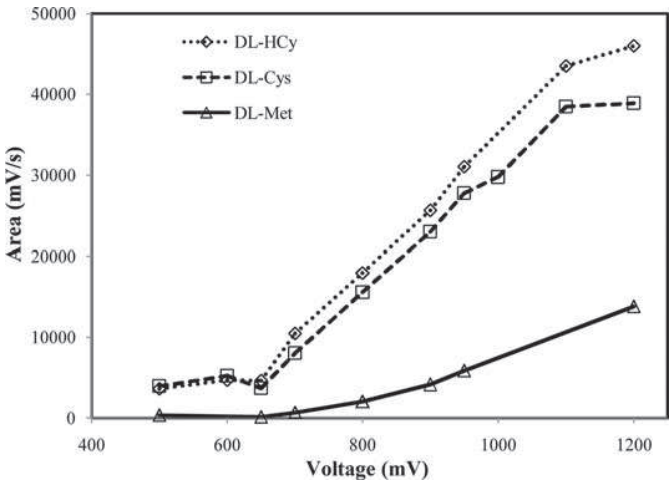

Fig. 6. Peak area of DL-HCy, DL-Cys, and DL-Met as a function of voltage. Conditions: MP: mixture MP_1:MP_2 (1:1 *v/v*) (MP_1: 50 mmol L^{-1} phosphate buffer pH 2.5, 1 mmol L^{-1} OSA:ACN (94:6 *v/v*) and MP_2:TEA in water pH 8:MeOH (9:1 *v/v*)); SP:C18; mass concentration of standards: 0.1 mg mL^{-1}; column temperature: 25°C; flow rate: 0.6 mL min^{-1}; potential of guard cell: +1.4 V.

Choice of Electrode Potentials

The choice of cell potentials was obtained from the peak area versus potential curves. To select appropriate potentials, the standard mixture of amino acids (0.1 mg mL^{-1}) and achiral column were used. The guard cell that oxidized the MP was set to +1.4 V. The different potentials of analytical cell were tested (from +0.5 to +1.2 V with increment 0.05 V). The response of HCy, Cys, and Met increased with increasing cell potential. The response of Met enantiomers was observed from +0.7 V. The hydrodynamic voltammogram reached the plateau for Cys (HCy) under +1.1 V. The maximum response of Met was achieved at +1.2 V (Figure 6). Therefore, potential of +0.9 V (E1) and +1.2 V (E2) were chosen for detection.

Conclusions

In this work, HPLC method with electrochemical detection was proposed for simultaneous separation of HCy, Cys, and Met enantiomers. Chiral columns (Chirobiotic T and Chirobiotic TAG) could not separate amino acid mixtures in a single run without the achiral column connection. Enantiomeric separations were achieved by using serially coupled achiral–chiral columns. The amino acid mixtures were separated by using the achiral column. Their enantiomers were separated on a chiral column. Chirobiotic TAG column was more suitable than Chirobiotic T for enantiomeric separation of selected amino acids. This work mainly focused on the determination of suitable separation conditions.

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