Talanta 115 (2013) 418–421

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fast enantioseparation by HPLC on a modified carbon nanotube monolithic stationary phase with a pyrenyl aminoglycoside derivative

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ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form
28 May 2013
Accepted 31 May 2013
Available online 6 June 2013

Keywords:
Enantioseparation
Carbon nanotube
Pyrenyl aminoglycoside

ABSTRACT

This work deals with the development of a carbon nanotube (CNT) monolithic column coated with a pyrenyl derivative as chiral selector. For this, a solution of pyrenyl neomycine A was pumping through a monolithic CNT column previously developed by our group. This coating was stable against the desorption for months when aqueous mobile phases were used. This column was applied to the chiral separation of underivatized amino acids. As well, ultra fast separations in the range of seconds were achieved using high flow-rates.

1. Introduction

During the past three decades, high performance liquid chromatography (HPLC) has been used to a great extent for enantioseparation. Ligand exchange chromatography (LEC) has been introduced by Davankov [1]. The separation mechanism of LE was based on the formation of diastereoisomeric ternary mixed metal complexes between the chiral selector ligand and the analytes. Among different metal ions, Cu (II) gave the best results. The chiral selector can be chemically bonded or coated on a non-chiral HPLC phase. The great interest of monolithic phases in LEC was that very fast separation in the range of seconds can be obtained. For example, such chiral monolithic columns were applied to the chiral separation of amino-acids or glycyl dipeptides [2,3]. A recent trend is the use of carbon nanotubes (CNTs) as stationary phase in HPLC [4–8] due to the non-covalent interaction established between the analyte and these nanostructured materials including electrostatic interactions (e.g. dipole–dipole), hydrogen bonds, π–π stacking, dispersion forces, dative bonds and the hydrophobic effect. Our group described a silica-based CNT column for the separation of polychlorinated biphenyls (PCBs), terpenes [5] and peptides [6]. As well, we demonstrated the advantages of the entrapment of CNTs and boron nitride nanotubes (BNNTs) into a monolithic support to enhance the performance of an enzymatic reactor [9] and the separation of a mixture of small molecules [10]. Recently, our group described an effective and simple method for the immobilization of ultra short size single wall carbon nanotubes (CNTs) on a monolithic HPLC material [11]. Due to the ultra short size of the tubes (average diameter 1 nm and length $<$ 10 nm (average length 7.5 nm)) inferior to the size of the macropores (2 μm) and mesopores (13 nm) of the monolithic structure, the tubes can penetrate these two kind of pores. In this manuscript, we report a simple approach to non covalent functionalization of the sidewalls of the immobilized single-walled carbon nanotubes with a pyrenyl derivative of neomycine A an aminoglycoside antibiotic chiral selector. This novel chiral stationary phase was then tested successfully for the enantioseparation of a series of 10 amino-acids.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Waters HPLC pump 501 (Saint-Quentin, Yvelines, France), an Interchim rheodyne injection valve, Model 7125 (Interchim, Montluçon, France), fitted with a reverse 20 μL sample loop, a Merck L4000 variable-wavelength UV spectrophotometer detector, and a Merck D2500 chromato—integrator (Nogent sur Marne, France). The silica chromolith performance column (100 mm × 4.6 mm) was obtained from Merck KGaA (Darmstadt, Germany) and coated with the ultra short size CNTs

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http://dx.doi.org/10.1016/j.talanta.2013.05.073

(average diameter 1 nm and length <10 nm (average length 7.5 nm)) as described in [11].

2.2. Reagents

Water was obtained from an Elgastat water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge. All organic solvents and test solutes were of analytical grade. Copper (II) sulfate was obtained from Sigma (Saint Quentin, France). All racemates and enantiomers were obtained from Sigma (Saint Quentin, France). The chiral selector was a pyrenyl derivative of the aminoglycoside neomycine A (PNA) and was synthesized as described by Hamasaki [12].

3. Method

3.1. PNA immobilization process on the silica-based monolithic CNT column

The pyrenyl group, being highly aromatic in nature, is known to interact strongly with the basal plane of graphite via π stacking and also found to interact with the sidewalls of CNTs in a similar manner [13–15]. The monolithic CNT stationary phase was thus coated using an aqueous PNA solution (30 mL) containing PNA at a concentration of 10 mM. This solution was pumped through the CNT monolithic column at a flow rate of 0.1 mL/min for one night at room temperature. Unbound PNA was then removed by washing the CNT column extensively with a total aqueous mobile phase. When not in use, the column was stored at room temperature in water—ACN (97-3, v/v).

3.2. Chromatographic data determination

The retention of the compounds on the chiral chromatographic support was evaluated using the retention factor $k=(t-t_0)/t_0$, where t was the retention time of the injected solute on the chromatogram and t_0 the column dead time determined using the mobile phase peak. The efficiency of the column reflecting the band broadening was characterized by estimating the height equivalent to a theoretical plate $h=L/(5.54(t/\delta)^2)$ where L was the column length and δ was the peak width at half-height. Moreover, the asymmetry factor A, reflecting the peak distortion, was determined by calculating the ratio of the second part of the peak over the first part of the peak at 10% of the peak height. The enantioselectivity α was determined using the following equation $\alpha=k_2/k_1$ where k_2 was the retention factor of the most retained enantiomer and k_1 for the less retained enantiomer on the chromatogram. The corresponding enantiomeric resolution was calculated using the equation $Rs=1.18(t_2-t_1)/(\delta_2+\delta_1)$.

4. Result and discussion

The amount of PNA adsorbed onto the chromatographic support was estimated by subtracting the UV absorbance, at 254 nm, of the unbound PNA solution (corresponding to both the unbound ligand fraction of the coating solution and the washed fraction) from the initial solution. The immobilization yield of PNA solution on the CNT monolithic surface was thus determined and corresponding to 120 μmol of PNA/column. To evaluate the column to column reproducibility, three columns were prepared as described above. Three different batches of PNA and CNT were used. The retention factor (k), the peak asymmetry (A) and the enantioresolution (Rs) were obtained with Ala, Glu and Tryp used as tested analytes. The mobile phase was $H_2O/CuSO_4$ 1 mM with a flow rate of 9 mL/min and a column temperature equal to 293 K. The results given in Table 1 showed that the technique was reliable and reproducible. As well, typical reproducibility of this column in retention time, peak asymmetry and resolution measured as relative standard deviation was $<0.4\%$. After half a year and more than 1000 injections, the decrease for the values of retention factor, peak asymmetry and resolution on this column was $<1.8\%$. An endurance test concerning the column stability over a wide mobile phase flow-rate was also carried out. The chromatographic parameter tested was the retention factor of the D-Tryp. For all these experiments the mobile phase was an aqueous mobile phase containing 1 mM of $CuSO_4$. As can be seen in Fig. 1, the column stability is excellent over a wide flow-rate range. The effect of long-term storage (for six months) under the mobile phase

Table 1
Evaluation of retention factor k (A), peak asymmetry A (B) and enantioresolution Rs (C) for column to column reproducibility. Mobile phase: $H_2O/CuSO_4$ 1 mM, flow rate: 9 mL/min, column temperature: 293 K. Standard deviations in parantheses.

Column (A)	Retention factor k		
	D-Ala	D-Glu	D-Tryp
1	0.73 (0.02)	5.19 (0.03)	8.86 (0.03)
2	0.72 (0.01)	5.17 (0.02)	8.85 (0.04)
3	0.71 (0.02)	5.18 (0.02)	8.87 (0.03)
(B)	Peak asymmetry A		
	D-Ala	D-Glu	D-Tryp
1	1.00 (0.01)	1.02 (0.01)	1.07 (0.01)
2	1.01 (0.01)	1.01 (0.01)	1.07 (0.02)
3	1.00 (0.01)	1.02 (0.01)	1.06 (0.02)
(C)	Enantioresolution Rs		
	Ala	Glu	Tryp
1	1.48 (0.02)	1.70 (0.01)	1.20 (0.03)
2	1.49 (0.01)	1.69 (0.03)	1.22 (0.03)
3	1.48 (0.02)	1.72 (0.01)	1.20 (0.04)

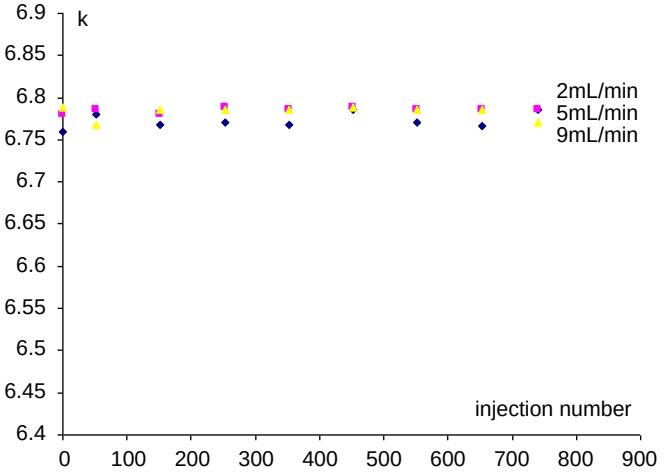

Fig. 1. k versus the injection number of D-Trp at 2 mL/min, 5 mL/min and 9 mL/min. Mobile phase: $H_2O/CuSO_4$ 1 mM, column temperature: 303 K, detection wavelength: 235 nm.

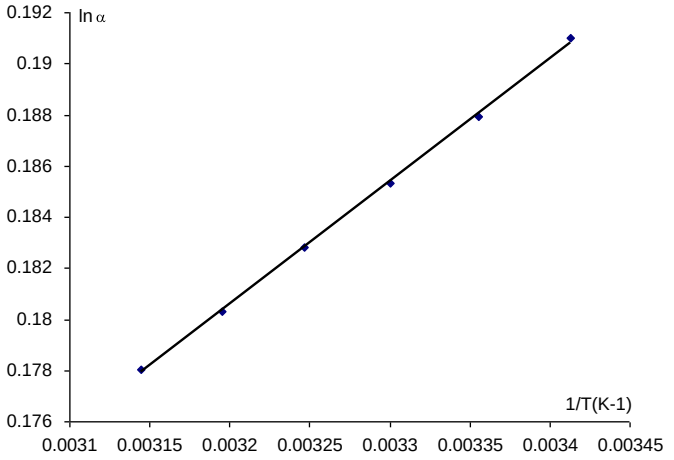

Fig. 2. Plots of ln α versus $1/T$ (K^{-1}) for DL-Tryp. Mobile phase: $H_2O/CuSO_4$ 1 mM, flow rate: 9 mL/min, detection wavelength: 235 nm.

Table 2
Separation factors α (at $T=293$ K) and thermodynamic parameters $\Delta(\Delta H)$ (kJ/mol) and $\Delta(\Delta S)$ (J/mol/K) with standard deviation in parentheses for DL-Tryp at different $CuSO_4$ concentrations (mM) in the mobile phase $H_2O/CuSO_4$ (mM). Flow rate: 9 mL/min, detection wavelength: 235 nm.

$[CuSO_4]$ (mM)	α	$\Delta(\Delta H)$ (J/mol)	$\Delta(\Delta S)$ (kJ/mol/K)
0.10	1.04	−85.4 (0.2)	34.6 (0.1)
0.15	1.10	−205.1 (0.3)	92.9 (0.2)
0.25	1.15	−287.4 (0.2)	181.9 (0.3)
0.50	1.20	−370.4 (0.4)	252.5 (0.2)
1.00	1.21	−389.9 (0.2)	255.2 (0.1)
1.50	1.19	−375.6 (0.3)	212.3 (0.2)
2.00	1.16	−320.8 (0.2)	139.9 (0.3)
2.50	1.13	−289.8 (0.2)	27.8 (0.1)

conditions was also determined. A long-term storage did not significantly affect the retention properties of the PNA-coated CNT column. All these results demonstrated this coating was stable against desorption for months when aqueous mobile phases were used.

To gain further insight into the enantioselectivity mechanism the previous experiments were carried out at six other temperatures (i.e., 20 °C, 25 °C, 30 °C; 35 °C, 40 °C and 45 °C) and for different eluent $CuSO_4$ concentrations in the mobile phase (i.e., 0.10 mM, 0.15 mM; 0.25 mM, 0.50 mM, 1 mM; 1.5 mM, 2 mM and 2.5 mM). The differences between the dissolution enthalpies $\Delta(\Delta H)$ and dissolution entropies $\Delta(\Delta S)$ of the D and L enantiomers were calculated using the slopes (P) and intercept (I) of the linear plots ln α vs ($1/T$) (van'T Hoff plots; $r^2 > 0.9991$) i.e., $\Delta H^\circ = -R.P$ and $\Delta(\Delta S) = R.I$ where $R = 8.32$ J/mol/K. An example of plot was given in Fig. 2 and α, $\Delta(\Delta H)$ and $\Delta(\Delta S)$ versus $CuSO_4$ concentration were presented in Table 2 for the tryptophan racemate. As can be seen in this Table 2, when the Cu(II) concentration was less than or equal to ~1 mM, $\Delta(\Delta H)$ became increasingly negative and $\Delta(\Delta S)$ became increasingly positive as $[CuSO_4]$ was increased, indicating that the increase in enantioselectivity α (see Table 1) was enthalpically and entropically controlled. When the $CuSO_4$ concentration was > 1 mM $\Delta(\Delta H)$ were negative values but increased as $[CuSO_4]$ was increased, $\Delta(\Delta S)$ were positive values but decreased as $[CuSO_4]$ was increased and the enantioselectivity α decreased. Data given in Table 3 confirmed that the maximum of enantioselectivity was obtained for a copper concentration of 0.5–1 mM for the 10 amino acids. Dative bonds were characterized by negative enthalpies and positive entropies changes [16]. This variation can be thus explained by a favorable contribution of the dative stereoselective bonds between the enantiomers and the –PNA

Table 3
Separation factors α (at $T=293$ K) for all amino acids at different $CuSO_4$ concentrations (mM) in the mobile phase $H_2O/CuSO_4$ (mM). Relative standard deviation of the enantioselectivity was typically less than 1%. Flow rate: 9 mL/min, detection wavelength: 235 nm.

$CuSO_4$ (mM)	Tryp	Tyr	Phe	Glu	Asp	Gln	Asn	Val	Ser	Ala
0.10	1.04	1.07	1.01	1.30	1.20	1.14	1.12	1.02	1.01	1.14
0.15	1.10	1.15	1.05	1.35	1.27	1.20	1.16	1.06	1.04	1.21
0.25	1.15	1.19	1.10	1.41	1.34	1.26	1.25	1.10	1.06	1.28
0.50	1.20	1.23	1.12	1.47	1.38	1.28	1.32	1.14	1.09	1.30
1.00	1.21	1.25	1.09	1.43	1.40	1.32	1.30	1.12	1.13	1.32
1.50	1.19	1.22	1.07	1.38	1.37	1.30	1.28	1.10	1.10	1.28
2.00	1.16	1.18	1.06	1.35	1.32	1.27	1.25	1.09	1.07	1.26
2.50	1.13	1.15	1.04	1.32	1.28	1.24	1.22	1.06	1.05	1.22

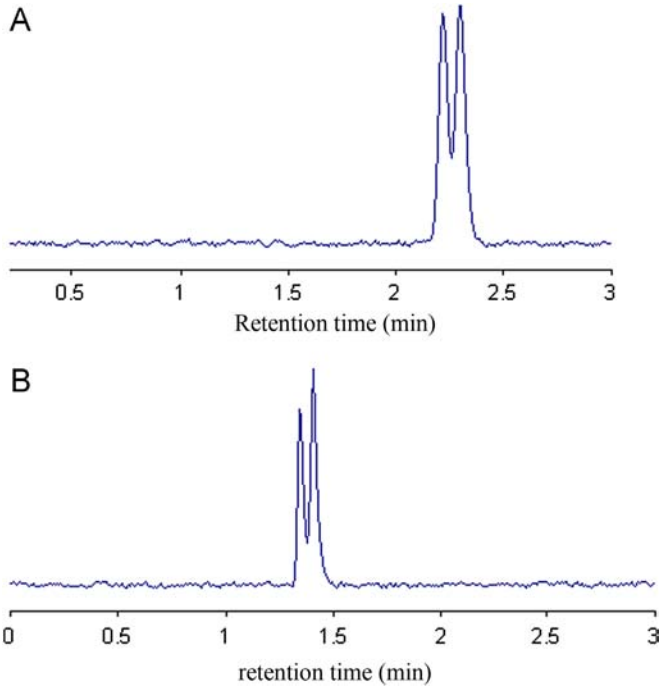

Fig. 3. HPLC chromatograms for the enantioseparation of: (A) DL-Tryp for $[CuSO_4]=0.1$ mM, (B) DL-Tryp for $[CuSO_4]=1$ mM. Flow rate: 9 mL/min, column temperature: 293 K, detection wavelength: 235 nm (The first peak on the chromatogram corresponded to the L-enantiomer).

ligand/Cu(II) coated on the CNT chromolith stationary phase i.e., the diastereoisomeric ternary complex formation when the $[CuSO_4]$ was < 1 mM. An example illustrating the use of such behavior for optimizing the chiral separation of DL-Tryp was shown in Fig. 3. For $[CuSO_4]=0.1$ mM, the α value was 1.04, an analysis time around 2.3 min and a low resolution was observed (Fig. 3A). For $[CuSO_4]=1$ mM, the α value was 1.21 with a better resolution and in addition the analysis time was reduced around 1.5 min (Fig. 3B). Later in this paper the $CuSO_4$ concentration was thus equal to 1 mM to obtain highest enantioselectivity. The retention factor (k) of the most strongly retained enantiomer (i.e., the D enantiomer), the asymmetry factor (A) and the resolution Rs of the two enantiomers on the chromatogram were evaluated using the ten amino acids as probe solutes (Table 4). The retention factor obtained was in the sequence: Trp > Tyr > Phe > Glu > Asp > Gln > Asn > Val > Ser > Ala. The amino-acids with aromatic rings in their structure presented the highest retention due to their strong dispersive interactions with the sidewalls of CNTs and with the pyrenyl group of the adsorbed neomycine A. The acidic amino acids Glu ($pK_a=3.86$) and Asp

Table 4
k for the D enantiomer, *A* and *Rs* values. Mobile phase: $H_2O/CuSO_4$ 1 mM. Flow rate: 9 mL/min, column temperature: 293 K, detection wavelength: 235 nm.

Solute molecule	*k*	*A*	*Rs*
Tryp	8.86	1.07	1.20
Tyr	7.95	1.08	1.30
Phe	7.11	1.09	0.98
Glu	5.19	1.02	1.70
Asp	4.02	1.00	1.58
Gln	3.12	1.00	1.47
Asn	1.33	1.00	1.50
Val	1.02	1.00	0.94
Ser	0.94	1.00	0.97
Ala	0.73	1.00	1.48

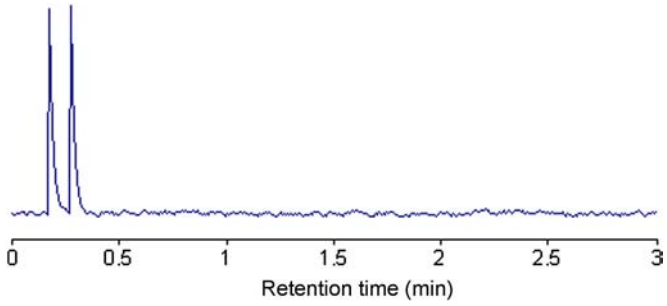

Fig. 4. HPLC chromatogram for the enantioseparation of DL-Ala for $[CuSO_4]$= 1 mM. Flow rate: 9 mL/min, column temperature: 293 K, detection wavelength: 235 nm (The first peak on the chromatogram corresponded to the L-enantiomer).

(pK_a=4.07) were also well retained due to their ionic interaction between their anionic forms and the protonated amino groups of neomycine A. As well, due to mass transfert properties of the monolithic skeleton the 10 enantiomers can be baseline separated in the range of seconds by use of flow-rates of 9 mL/min. An example of enantioseparation was given in Fig. 4 for the less retained amino acid i.e., DL-Ala.

5. Conclusion

This paper described a controlled and in-situ method for immobilizing a pyrenyl neomycine A derivative on a chromolith CNT chromatographic support. This simple immobilization method enabled the development of a stable stationary phase which exhibited efficient enantioseparation at high flow-rate values with no loss of peak resolution.

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