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Chiral resolution of histidine using an anti-D-histidine L-RNA aptamer microbore column☆

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Abstract

In this paper, we report a new anti-amino acid aptamer chiral stationary phase (CSP). The enantiomers of histidine were separated using an immobilized histidine-specific L-RNA aptamer (40-mer) and an aqueous buffer as mobile phase. The effects of the variation of different operating parameters, including the mobile phase pH and the $MgCl_2$ concentration as well as the column temperature, on the solute retention were assessed. The results suggested that (i) the protonated form of histidine was involved in the stereospecific RNA binding and (ii) Mg^{2+} was essential for the target enantiomer binding to the specific aptamer sites. From a practical point of view, it appeared that the baseline resolution in a minimum analysis time can be achieved at a column temperature of 35 °C for an eluent containing 10 mM of $MgCl_2$, pH 5.5.

Keywords: Aptamer; Histidine; Chiral separation

1. Introduction

The detection and quantification of amino acid enantiomers is important in many areas of science. For example, it is now well established that D-amino acids, found at a low level in higher order organisms in the form of free amino acids, peptides and proteins, can play a preponderant role in biochemistry and physio-pathology: D-serine functions as an important neuromodulator, D-aspartate is implied in developmental and endocrine functions and D-arginine plays a role in the urea cycle [1]. In addition, in forensic science, racemization methods which are based on the age-dependent non-enzymatic changes of L-form amino acids to D-form are one of the most reliable and accurate methods for the estimation of chronological age [2]. Furthermore, the enantiomeric ratio of amino acids can be used as a reliable parameter to assess food quality [3]. Therefore, it is appeared of very great interest to develop efficient analytical methodologies to separate and quantify the amino acid enantiomers. A variety of chiral selectors such as crown-ethers, macrocyclic antibiotics and ligand-exchange selectors have been successfully used for the HPLC and CE enantiomeric separation of native amino acids.

Single-stranded oligonucleotides obtained from very large combinatorial libraries (DNA or RNA aptamers) have been used successfully as affinity stationary phases in liquid chromatography (LC) or capillary electrochromatography (CEC) for the purification/separation of a variety of molecules, from compounds as large as proteins [4] to compounds as small as amino acids, adenosine and derivatives or flavin mononucleotide [5–7]. The enantioselective properties of DNA or RNA aptamers selected against a target enantiomer were accounted by our group to create a new class of target-specific chiral selectors for use as chiral stationary phases (CSPs) in HPLC or additives in capillary electrophoresis [8–12]. The aptamer CSPs were developed for the chromatographic resolution of the racemates of different analytes [8,9]. At the present time, the anti-amino acid aptamer CSPs have been designed for the enantiomeric separation of various native amino acids, including aromatic species (tyrosine, tryptophan, etc.) [10] and arginine [11].

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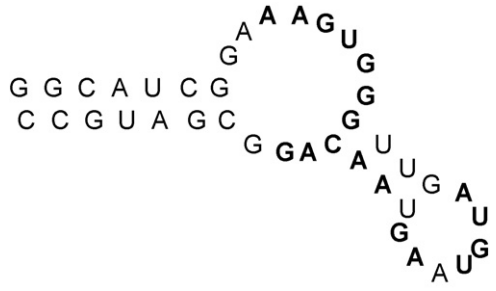

Fig. 1. Sequence and secondary structure of the 40-mer RNA aptamer used as chiral selector. The consensus sequence is shown in bold [12].

In this paper, we describe a new anti-amino acid aptamer CSP which was able to separate the enantiomers of histidine. The sequence of a 40-mer RNA aptamer (Fig. 1), specifically selected against L-histidine, was used [13] and the mirror-image strategy, previously described to design biostable RNA CSPs, was applied [10,11]. The anti-histidine aptamer was immobilized on a chromatographic support via a biotin-streptavidin bridge and the RNA modified particles were packed into a microbore column. The effects of various chromatographic parameter changes (mobile phase pH and magnesium concentration, column temperature) on the solute retention were evaluated and the optimal conditions for the chiral separation were assessed.

2. Experimental and methods

2.1. Reagents and materials

Histidine enantiomers were obtained from Sigma–Aldrich (Saint–Quentin, France). Hepes, NaCl and $MgCl_2$ were supplied by Sigma–Aldrich. Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. L-RNA oligonucleotide was synthesized and 5′-biotinylated by CureVac (Tubingen, Germany). Biotin phosphoramidite containing a 16 atom spacer arm based on triethylene glycol was used for the aptamer biotinylation. The oligoribonucleotide was purified by HPLC. The streptavidin POROS bulk media (20 μm polystyrene particles) was purchased from Applied Biosystems (Courtaboeuf, France).

2.2. Stationary phase preparation and column packing

Prior to immobilization, the biotinylated aptamer was renaturated by heating oligonucleotide at 90 °C for 5 min in an aqueous buffer (50 mM Hepes buffer, 250 mM NaCl, 10 mM $MgCl_2$ adjusted at pH 7.0) used as the in vitro selection buffer and left to stand at room temperature for 30 min. The immobilization of the aptamer was attained by mixing 60 nmol of oligonucleotide (in the aqueous buffer) per 1000 μl of the streptavidin media slurry during 3 h at ambient temperature. Unbound RNA was removed by washing with the same buffer. The amount of oligonucleotide coupled to the chromatographic support was estimated by subtracting the UV absorbance, at 260 nm, of the unbound RNA solution from the initial solution. It was equal to 16 nmol of biotinylated oligonucleotide bound per 100 μl of support media. The RNA modified particles were packed in-house into a PEEK microbore column (260 mm × 0.80 mm) using a previously described slurry-packing procedure [9]. When not in use, the column was stored in the aqueous buffer containing sodium azide (0.05%).

2.3. Control stationary phase

Non-modified streptavidin particles were also packed into a PEEK microbore column of same dimensions (260 mm × 0.80 mm) as a control stationary phase, following the procedure reported previously. It has been shown that streptavidin is able to discriminate the enantiomers of some analytes such as warfarin, trimipramine or adenosine [14,15]. In order to evaluate possible enantioselective properties of the immobilized streptavidin toward the solute, a racemic mixture of histidine was injected onto a microbore column packed with non-modified streptavidin POROS particles. The enantiomers of histidine were not separated and were not retained by the column. This result indicates that the streptavidin chromatographic support was roughly inert toward the various compounds and did not affect the chiral recognition.

2.4. Apparatus

The HPLC system consisted of a LC Shimadzu pump 10AT (Sarreguemines, France), a Shimadzu SIL-10AD auto injector, a Shimadzu SPD-10A UV–vis detector (detection at 217 nm), a Shimadzu SCL-10A system controller with Class-VP software (Shimadzu) and an oven Igloocil (Interchim).

2.5. Chromatographic parameters

Solute samples were prepared in the mobile phase and injected (100 nl) in triplicate. The mobile phase flow rate was 15 μl/min for the L-RNA CSP and the control streptavidin column (non-modified streptavidin particles). The retention factor and the efficiency of the column were estimated through the calculation of the first and second moments of the peaks, as previously described [16]. The resolution R_s was calculated using the following relation: $R_s = [2(t_{R2} - t_{R1})]/(w_2 + w_1)$, where t_R is the retention time determined at the peak apex and w is the peak width. The asymmetry factor A_s was determined by calculating the width ratio of the second (or right) part of the peak over the first (or early) part of the peak at 10% of the peak height.

3. Results and discussion

3.1. The oligonucleotidic sequence used in this study

The minimal sequence of the 40-mer RNA aptamer used in the present work was obtained via a SELEX procedure directed against L-histidine (see Fig. 1 for the secondary structure). This oligonucleotide is known to strongly discriminate between L- and D-histidine [12]. The dissociation constants, determined by isocratic competitive affinity chromatography, are in the

micromolar range: $K_d = \sim 10\ \mu M$ for the L-histidine-aptamer association and $\sim 10{,}000\ \mu M$ for the D-histidine-aptamer association [13]. As previously designed [11], the mirror-image approach was applied to create a biostable L-RNA CSP in order to use such aptamer in a routine chromatographic context.

3.2. Enantioselective properties of the anti-D-histidine L-RNA stationary phase

In the first stage of this work, the chromatographic properties of the L-RNA aptamer stationary phase were evaluated at ambient temperature (25 °C) and using as mobile phase an aqueous buffer similar to that prepared for the SELEX procedure (50 mM Hepes buffer, 250 mM NaCl, 10 mM $MgCl_2$ adjusted at pH 7.0). The sample was injected at an enantiomer concentration of 2 mM, which corresponded to the lowest concentration allowing the target detection. As expected from the data reported by Yarus and co-workers [13], the L-RNA CSP was able to discriminate the enantiomers of histidine, the D-enantiomer being more retained than the L-enantiomer: the retention factors for D-histidine was 0.98 ± 0.04 while the non-target enantiomer was weakly retained by the column (0.19 ± 0.05). This is consistent with the principle of chiral inversion, i.e., the mirror-image of the "natural" aptamer recognizes with the same affinity and specificity the mirror image of the target [10,11].

3.3. Effects of chromatographic condition changes on the solute retention

Firstly, experiments were performed to analyze the role of the eluent pH. As the pK_a of histidine is known to be around 6.0, the mobile phase pH was varied between 5.5 and 7.5 (for a 25 °C column temperature) in order to evaluate the possible role of the solute protonation on the solute retention. The k_L values varied very slightly, if any, over the eluent pH range. In contrast, the target retention factor was roughly constant for the pH values of 5.5 and 6.0 and decreased significantly as the eluent pH was increased from 6.5 and 7.5. No chiral resolution was obtained for an eluent pH equal to 7.5. Fig. 2 shows the variation of the solute retention factor as a function of the eluent pH. Because the k_D value increased as pH dropped near the known pK_a of histidine, it is suggested that the protonated form of histidine was involved in the stereospecific RNA binding and then electrostatic interactions played a role in the target association with the aptamer CSP. Such behavior is in accordance with the previous results reported by Majerfeld et al. [13]. It can be noted that the mobile phase pH change could also modify the active site conformation and/or the binding capacity of the aptamer CSP.

Further experiments were conducted at various column temperatures (20–35 °C), under different mobile phase pH conditions. The data revealed that, whatever the mobile phase pH, an increase in the column temperature was responsible for a decrease in the target retention factor while the k_L value did not change significantly. As an example, Fig. 3 presents the variation of the retention factor for D- and L-histidine in relation to the column temperature, for a mobile phase pH value equal to 6.0. As previously reported [10], the target enantiomer behavior could be explained by the involvement of specific interactions which were enthalpically driven and/or a temperature-dependent change of the conformation/binding capacity of the aptamer CSP.

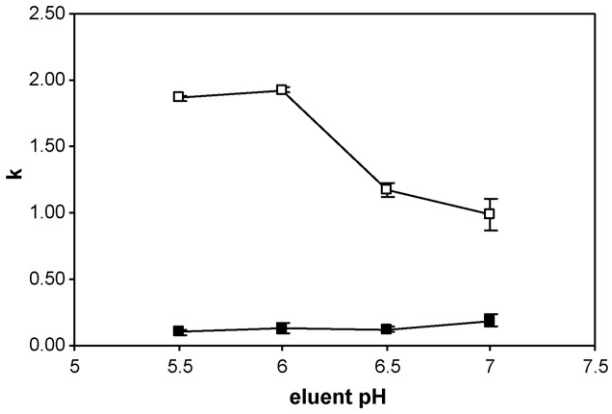

Fig. 2. Plots of k *vs.* mobile phase pH for D-histidine (open symbol) and L-histidine (filled symbol). Column: 260 mm × 0.80 mm (i.d.); mobile phase: Hepes buffer 50 mM, NaCl 250 mM, $MgCl_2$ 10 mM; column temperature: 25 °C; injected concentration: 2 mM; injection volume: 100 nl; flow rate: 15 μl/min; detection at 217 nm.

The effects of the eluent $MgCl_2$ concentration on the target retention were also analyzed. It is well established that divalent cations such as Mg^{2+} are particularly important for inducing and stabilizing RNA tertiary structural motifs [17]. So, in order to evaluate the role of the three-dimensional conformation of the RNA ligand on the target recognition, the $MgCl_2$ concentration in the aqueous eluent was varied from 10 to 0 mM. Fig. 4 presents the k versus $MgCl_2$ concentration plot for the two enantiomers, at a column temperature of 20 °C and for an eluent pH of 6.5. The target retention factor was weakly affected, if any, as the mobile phase magnesium concentration decreased from 10 to 2 mM. However, when the eluent $MgCl_2$ concentration further decreased, a strong reduction of the target retention was observed. On the other hand, the L-histidine retention factor did not changed significantly when the eluent salt concentration

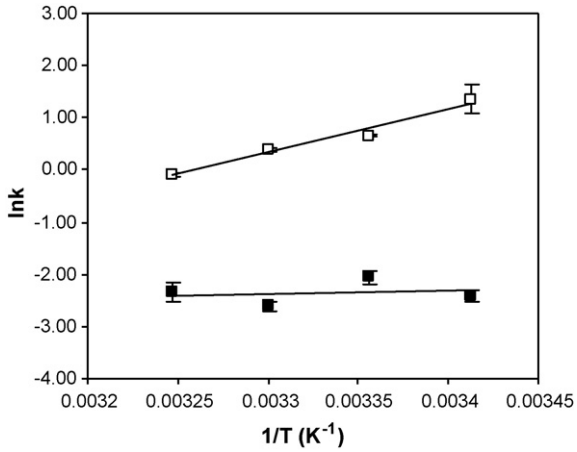

Fig. 3. Plots of ln k *vs.* $1/T$ for D-histidine (open symbol) and L-histidine (filled symbol). Column: 260 mm × 0.80 mm (i.d.); mobile phase: Hepes buffer 50 mM, NaCl 250 mM, $MgCl_2$ 10 mM, pH 6.0; injected concentration: 2 mM; injection volume: 100 nl; flow rate: 15 μl/min; detection at 217 nm.

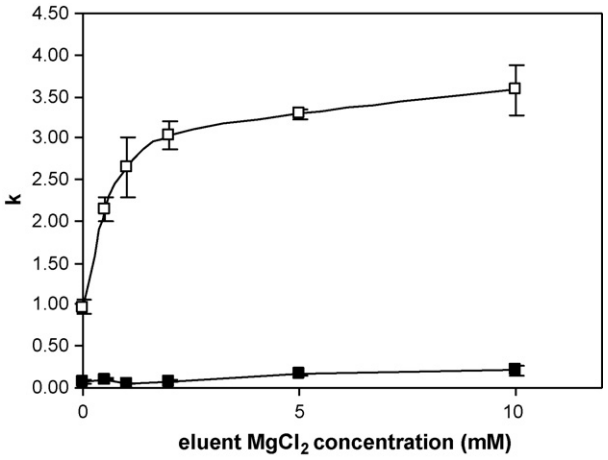

Fig. 4. Plots of k *vs*. mobile phase $MgCl_2$ concentration for D-histidine (open symbol) and L-histidine (filled symbol). Column: 260 mm × 0.80 mm (i.d.); mobile phase: Hepes buffer 50 mM, NaCl 250 mM, pH 6.5; column temperature: 20 °C; injected concentration: 2 mM; injection volume: 100 nl; flow rate: 15 μl/min; detection at 217 nm.

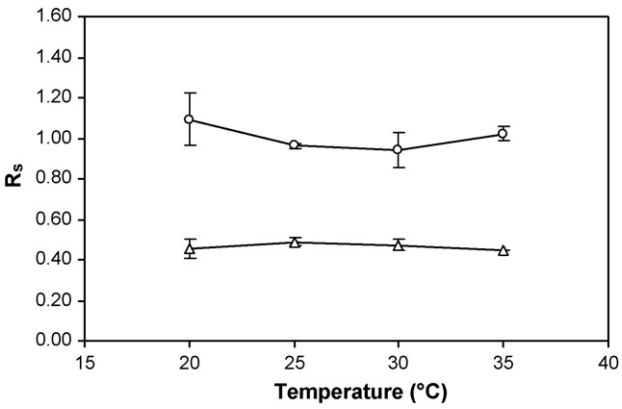

Fig. 5. Plots of R_s *vs*. the column temperature at a mobile phase pH of 7.0 (triangle) and 5.5 (circle). Column: 260 mm × 0.80 mm (i.d.); mobile phase: Hepes buffer 50 mM, NaCl 250 mM, $MgCl_2$ 10 mM; injected concentration: 2 mM; injection volume: 100 nl; flow rate: 15 μl/min; detection at 217 nm.

was modified. Such behavior seems to indicate that a change in the tertiary structure of the RNA occurred at low Mg^{2+} concentration, affecting the target affinity for the RNA through a modification of the three-dimensional shape of the aptamer specific site. This is in accordance with previous results obtained for other aptamer-ligand associations [5,18]. Alternatively, a modification of the affinity constant or the RNA binding capacity could be also involved in the reduction of the target enantiomer retention factor.

3.4. Efficiency, peak asymmetry and chiral resolution

For all the pH or the magnesium concentration conditions, low efficiency and significant peak asymmetry were typically observed for the low temperature range (20–25 °C). As an example, at a pH of 5.5, an $MgCl_2$ concentration of 10 mM and a column temperature equal to 25 °C, $N_L = 144 \pm 42$, $N_D = 31 \pm 5$, $A_{sL} = 2.67 \pm 0.40$ and $A_{sD} = 6.27 \pm 0.25$. Such poor performances can result, at least in part, from extra-column band-broadening effects, the high particle diameter used (20 μm) and concentration overload of binding sites (non-linear effects). Furthermore, the band-broadening and the peak distortion were higher for D-histidine. This indicates that slower mass transfer kinetics were involved in the target-aptamer association [8,10]. Moreover, under the same pH and magnesium conditions, the peak shape and efficiency can be improved for the D-enantiomer when the column temperature was increased ($N_D = 94 \pm 8$, $A_{sD} = 3.45 \pm 0.60$ at $T = 35$ °C). On the other hand, the efficiency performances for the L-enantiomer did not changed significantly with the temperature increasing ($N_L = 179 \pm 50$, $A_{sL} = 2.23 \pm 0.25$ at $T = 35$ °C). The desorption kinetics of the target from the specific binding sites of the aptamer CSP were expected to become faster as the temperature increased so that efficiency and peak shape were improved [19].

Concerning the chiral resolution, it is important to point out that such increase in the efficiency performances was able to counterbalance the temperature-dependent reduction of the target retention factor. This is exemplified in Fig. 5 for two mobile phase pH values: the R_s value did not varied significantly when the temperature increased from 20 to 35 °C. So, from a practical point of view, the temperature increase allowed to reduce the analysis time without alteration of the resolution. Fig. 6 illustrates such column temperature effects on the chiral separation for a mobile phase pH 5.5, at 10 mM $MgCl_2$.

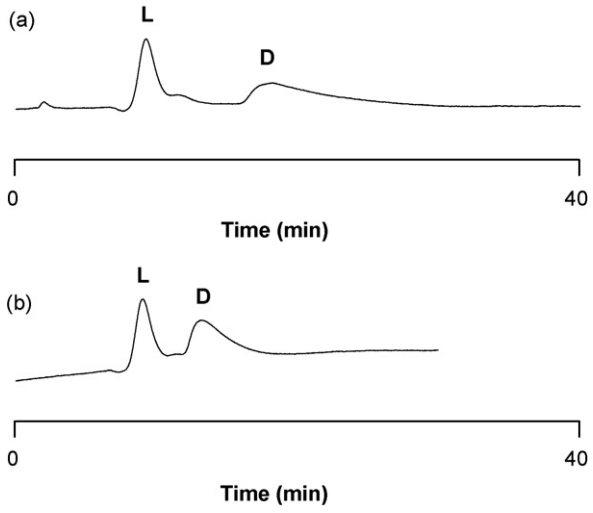

Fig. 6. Chromatograms obtained when a racemic mixture of histidine was injected onto the the histidine-specific L-RNA aptamer CSP. Column: 260 mm × 0.80 mm (i.d.); mobile phase: Hepes buffer 50 mM, NaCl 250 mM, $MgCl_2$ 10 mM, pH 5.5; injected concentration: 2 mM; injection volume: 100 nl; flow rate: 15 μl/min; detection at 217 nm. (a) $T = 25$ °C; (b) $T = 35$ °C.

4. Conclusion

In this paper, we report a new L-RNA aptamer CSP which is able to separate the histidine enantiomers. The effects of some chromatographic parameters on the solute retention have been evaluated. The results suggest that (i) the protonated form of histidine is involved in the stereospecific RNA binding and (ii) Mg^{2+} is essential for the target binding to the specific aptamer

sites. In addition, it appears that both the separation of the histidine enantiomers and the analysis time can be easily modulated by varying the mobile phase nature and the column temperature. The baseline resolution in a minimum analysis time can be achieved at a column temperature of 35 °C for an eluent containing 10 mM of $MgCl_2$, pH 5.5.

References

[1] S.A. Fuchs, R. Berger, L.W.J. Klomp, T.J. de Koning, Mol. Genet. Metab. 85 (2005) 168.
[2] G. Lubec, B. Lubec, Amino Acids 4 (1993) 1.
[3] R. Marchelli, A. Dossena, G. Palla, Trends Food Sci. Technol. 7 (1996) 113.
[4] T.S. Romig, C. Bell, D.W. Drolet, J. Chromatogr. B 731 (1999) 275.
[5] Q. Deng, I. German, D. Buchanan, R.T. Kennedy, Anal. Chem. 73 (2001) 5415.
[6] R.B. Kotia, L. Li, L.B. McGown, Anal. Chem. 72 (2000) 827.
[7] S.L. Clark, V.T. Remcho, J. Sep. Sci. 26 (2003) 1451.
[8] M. Michaud, E. Jourdan, A. Villet, A. Ravel, C. Grosset, E. Peyrin, J. Am. Chem. Soc. 125 (2003) 8672.
[9] M. Michaud, E. Jourdan, C. Ravelet, A. Villet, A. Ravel, C. Grosset, E. Peyrin, Anal. Chem. 76 (2004) 1015.
[10] C. Ravelet, R. Boulkedid, A. Ravel, C. Grosset, A. Villet, J. Fize, E. Peyrin, J. Chromatogr. A 1076 (2005) 62.
[11] A. Brumbt, C. Ravelet, C. Grosset, A. Ravel, A. Villet, E. Peyrin, Anal. Chem. 77 (2005) 1993.
[12] J. Ruta, C. Ravelet, C. Grosset, J. Fize, A. Ravel, A. Villet, E. Peyrin, Anal. Chem. 78 (2006) 3032.
[13] I. Majerfeld, D. Puthenvedu, M. Yarus, J. Mol. Evol. 61 (2005) 226.
[14] Y. Tanaka, S. Terabe, Chromatographia 49 (1999) 489.
[15] C. Ravelet, M. Michaud, A. Ravel, C. Grosset, A. Villet, E. Peyrin, J. Chromatogr. A 1036 (2004) 155.
[16] A.M. Talbert, G.E. Tranter, E. Holmes, P.L. Francis, Anal. Chem. 74 (2002) 446.
[17] P. Brion, E. Westhof, Annu. Rev. Biophys. Biomol. Struct. 26 (1997) 113.
[18] F.M. Jucker, R.M. Philips, S.A. McCallum, A. Pardi, Biochemistry 42 (2003) 2560.
[19] D.V. McCalley, J. Chromatogr. A 902 (2000) 311.