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Streptavidin chiral stationary phase for the separation of adenosine enantiomers

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Abstract

In this paper, a microbore column packed with streptavidin particles was used, at various temperatures (0–24 °C), to separate the adenosine enantiomers by HPLC. Using an aqueous mobile phase, the apparent enantioseparation was high for a small molecule, varying from 11.5 at 0 °C to 6.2 at 24 °C. From the experiments carried out with a streptavidin–biotin complex stationary phase, it was demonstrated that the blockage of the biotin sites of the immobilized streptavidin was responsible for a strong decrease in the enantioselectivity via a direct and/or an indirect effect. From the analysis of the concentration dependencies of the solute retention factor, it was also shown that a reduction of the D-adenosine specific binding sites occurred at the lowest temperature. The thermodynamic parameters determined from the van't Hoff plots indicated that the D-adenosine binding to the streptavidin specific sites was enthalpically driven.

Keywords: Enantiomer separation; Chiral stationary phase, LC; Adenosine; Streptavidin

1. Introduction

Chiral separation is of crucial importance in various fields such as drug and food analysis, biochemistry or clinical pharmacology. Various classes of chiral selectors have been used during the last decades, including oligo and polysaccharides [1], crown-ethers [2], aminoacids [3], macrocyclic antibiotics [4] and proteins. Proteins such as serum albumins [5], α1-acid glycoprotein [6], cellobiohydrolase [7], ovomucoid [8] or avidin [9] have been used successfully as chiral selectors in HPLC or capillary electrophoresis. Avidin and streptavidin have very similar primary structure and can be isolated from hen egg white and from the bacterium *Streptomyces avidinii*, respectively. They are both tetrameric proteins, but differ in their overall charge, with streptavidin being acidic and avidin being basic. Although basic avidin has been widely used as chiral selectors for the resolution of acidic analyte enantiomers in HPLC or capillary electrophoresis, only one paper from Tanaka and Terabe [10] has reported a study on the enantioselective properties of streptavidin. They demonstrated, using a partial filling technique in capillary electrophoresis, that streptavidin was able to discriminate the enantiomers of some acidic (warfarin, dansylated amino acids) and basic (chlorpheniramine, trimipramine, primaquine) analytes [10].

Intensive research efforts are carried out for the discovery of new nucleosidic drugs [11–13]. Furthermore, important papers have focused on the usefulness of "non-natural" L-nucleoside derivatives or L-nucleic acid ligands for the development and identification of potential diagnostic reagent or drugs [14–18]. Therefore, several papers have been reported for the chiral separation of nucleosides and analogues. For example, Thomas and Surber [19] described the first separation of the enantiomers of a nucleoside by chiral high-performance liquid chromatography. They examined several chiral stationary phases to separate the enantiomers of A-69992, a chiral HIV anti-infective nucleoside. The only CSP found to be effective was Nucleosil Chiral-1, a ligand-exchange CSP. Furthermore, Magora et al. [20] reported the resolution of 10 racemates of structurally related uridine analogs, potentially anti-viral agents, under various mobile phase compositions and tempera-

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tures. In addition, Lipka-Belloli et al. [21] reported the use of silica-based cellulose tris-3,5-dimethylphenylcarbamate or tris-methylbenzoate for the determination of enantiomeric purity of nucleoside analogs related to d4T and acyclovir.

In this paper, we described for the first time the use of the streptavidin protein as a chiral stationary phase for HPLC. A microbore column was packed with the commercially available streptavidin POROS particles. This column was able to resolve the enantiomers of adenosine with an important enantioselectivity. A streptavidin–biotin complex column was also used in order to evaluate the role of the biotin sites of streptavidin in the adenosine retention and chiral discrimination. The temperature effects on the binding capacity, solute retention and separation factor were investigated. In addition, thermodynamic parameters for the D-adenosine binding to specific sites were determined from a van't Hoff plot.

2. Experimental and methods

2.1. Reagents and materials

D-adenosine was obtained from Sigma–Aldrich (Saint-Quentin, France). L-Adenosine was purchased from Chemgenes (Ashland, USA). Na_2HPO_4, NaH_2PO_4, $MgCl_2$ and KCl were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The streptavidin POROS bulk media (20 μm polystyrene porous particles) was purchased from Applied Biosystems (Courtaboeuf, France).

2.2. Stationary phases and column packing

Streptavidin particles were packed into a 370 mm × 0.76 mm microbore column. Nut and ferrule were placed on one end of the column body which was attached to an end fitting containing a microbore frit (column outlet). The other end of the column (column inlet) was attached to the outlet of the packing device using nut and ferrule. The particle slurry was added to the packing device. The remaining volume of the packing device was filled with an aqueous buffer (phosphate buffer 20 mM, KCl 25 mM, $MgCl_2$ 1.5 mM, pH 6.0). The top of the packing device was connected to the HPLC pump. The pump was started at about 0.1 ml/min and the flow rate was slowly adjusted to operate at a pressure of about 1500 psi. Twenty microliters of aqueous buffer were pumped through the column. The full packed column was then attached (column inlet) to another end fitting with a microbore frit. In order to explore the role of the protein biotin sites in the solute retention and enantioselectivity, the streptavidin column was modified as follows: around 1.2 μmol of biotin, dissolved in the aqueous buffer, was applied to the microbore column using a pump fixed at a flow rate of 100 μl/min during 2 h, at room temperature. When not used, the microbore column was stored at 4 °C in the aqueous buffer.

2.3. 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, a Shimadzu SCL-10A system controller with Class-VP software (Shimadzu) and an oven Igloocil (Interchim).

2.4. Chromatographic operating conditions

Solute samples were prepared in the mobile phase and injected at least three times (100 nl). The dead time was determined using the sodium nitrate. The mobile phase flow rate was 50 μl/min. The linear elution conditions (very low solute concentrations where adsorption isotherms behave linearly) were tested by injecting analytes at several concentrations varying from 0.9 to 9.4 mM. Solute retention factor as well as enantioselectivity were determined as previously described [22], using the lowest solute concentrations (below the point where the retention was sample size dependent). For the analysis of the temperature effects, the column temperature varied from 0 to 24 °C. In order to assess the column binding capacity change with the temperature varying, the concentration dependencies of the solute retention factor were measured at the different column temperatures, as previously described [23].

3. Results and discussion

3.1. Adenosine retention, apparent enantioselectivity and resolution using a streptavidin stationary phase

The enantioseparation properties of the streptavidin stationary phase were analyzed at various column temperatures using an aqueous buffer as mobile phase (phosphate buffer 20 mM, KCl 25 mM, $MgCl_2$ 1.5 mM, pH 6.0). In all cases, the D-enantiomer was significantly more retained by the column than the L-enantiomer. Retention factors, apparent enantioselectivity and resolution are presented in Table 1 for all the operating conditions. A very high apparent enantioselectivity was obtained varying from ∼11.5 to ∼6.2 over the temperature range studied (Table 1). Such chiral discrimination is due to the fact that the L-enantiomer is weakly retained by the CSP. The chromatographic resolution varied from ∼3.0 to ∼1.1 between 0 and 24 °C. The asymmetry factor A_s of the two enantiomers (determined at 10% of the peak maximum, in linear conditions) ranged from 0.94 to 1.03, indicating that the peak distortion was not significant in this chromatographic system. Representative chromatograms are shown in Fig. 1 for column temperatures of 24, 12 and 0 °C for a solute concentration of 2.34 mM.

Table 1
Adenosine retention, apparent enantioselectivity and resolution using a streptavidin stationary phase[a]

T (°C)	k_D	α	R_s
0	2.89 (0.03)	11.54 (0.17)	3.05 (0.21)
4	1.85 (<0.01)	10.15 (0.59)	2.17 (0.34)
8	1.40 (0.02)	8.85 (0.40)	1.97 (0.12)
12	1.02 (0.01)	7.59 (0.71)	1.70 (0.13)
16	0.80 (0.01)	7.65 (0.82)	1.53 (0.01)
20	0.61 (<0.01)	6.10 (0.40)	1.48 (0.01)
24	0.47 (<0.01)	6.18 (0.04)	1.11 (0.03)

[a] Experiments in linear conditions. Retention factor for the more retained enantiomer: k_D = (retention time − dead time)/dead time. Apparent enantioselectivity: α = retention factor for the more retained enantiomer/retention factor for the less retained enantiomer. Resolution: R_s = (2 × distance of the two peak positions)/sum of bandwidths of the two peaks. Standard deviations of the solute retention factor, enantioselectivity and resolution are in parentheses.

3.2. Role of the protein biotin sites in the enantioselectivity

In order to investigate the role of the protein biotin sites in the adenosine enantioselectivity, a racemic mixture was injected onto the streptavidin–biotin complex column in the same operating conditions (see the experimental section). The D-adenosine retention factor strongly decreased while L-adenosine retention did not change significantly so that the enantioselectivity was diminished. As an example, the adenosine separation factor on the streptavidin–biotin complex stationary phase was found to be ∼6.0 at 0 °C. This decrease in the enantioselectivity was responsible for a complete loss of the enantiomeric resolution over the 12–24 °C column temperature range. Such result is consistent with the previous data from Tanaka and Terabe [10] who have observed, by capillary electrophoresis, that the chiral recognition ability of avidin is lost when biotin is added in the background electrolyte. Three hypothesis can be proposed to explain this behavior (i) the specific sites are the biotin sites which govern *directly* the chiral recognition, (ii) other specific sites at the protein surface are responsible for the enantioselectivity and the blockage of the biotin sites affects *indirectly* this enantioselective interaction via a protein conformational change and (iii) a combination of the two phenomena listed above. Furthermore, as the L-adenosine retention is not affected significantly by the formation of the streptavidin–biotin complex in the stationary phase, it is expected that the protein specific sites are able to interact only with the D-enantiomer of adenosine.

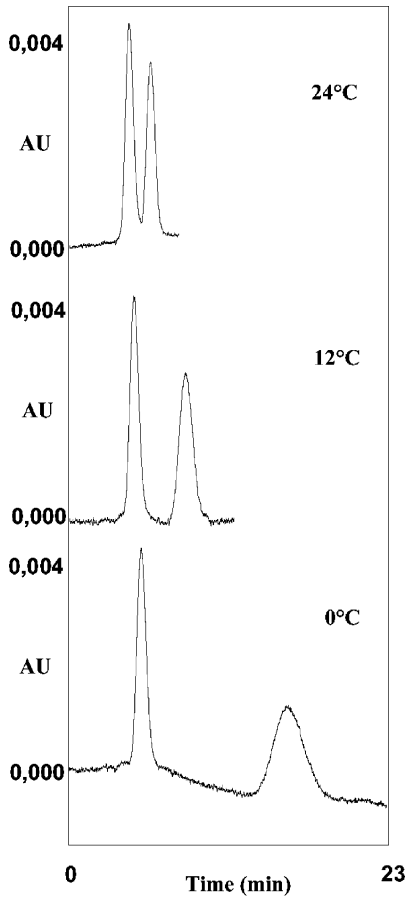

Fig. 1. Chromatographic separation of adenosine enantiomers using an immobilized streptavidin as CSP at various column temperature. First peak: L-adenosine; column: 370 mm × 0.76 mm (i.d.); D,L-adenosine concentration: 2.34 mM; injection volume: 100 nl; flow rate: 50 μl/min; detection at 260 nm.

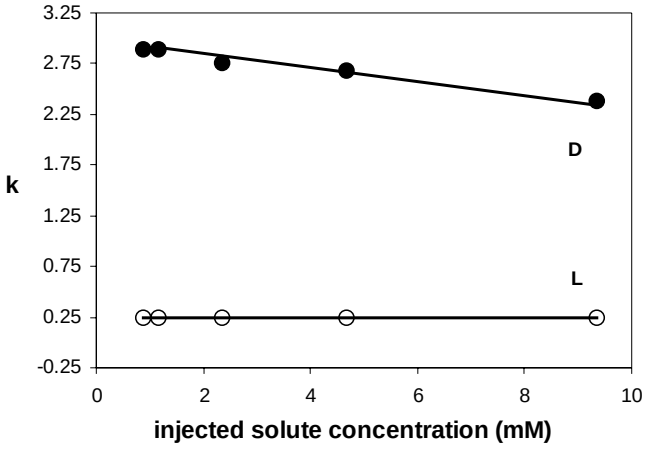

Fig. 2. Solute concentration dependencies of the retention factor for D-adenosine (●) and L-adenosine (○) adenosine using streptavidin as CSP at T = 0 °C. Column: 370 mm × 0.76 mm; flow rate: 50 μl/min; injection volume: 100 nl. Error bars are within the experimental points.

3.3. The concentration dependencies of the solute retention factor. Difference in the binding capacity of the streptavidin column for the adenosine enantiomers

In order to further investigate the difference in the column binding capacity for D- and L-adenosine, the concentration dependencies of the solute retention factor were measured at 0 °C. For D-adenosine, the retention factor varied from 2.89 at 0.9 mM of injected solute solution to 2.38 at 9.4 mM of injected solute solution (Fig. 2). On the other

hand, as can be seen in Fig. 2, the retention factor for L-adenosine was constant over the 0.9–9.4 mM solute concentration range (the L-enantiomer retention factor was equal to 0.25 whatever the injected solute concentration). This indicates that the binding capacity for L-adenosine was significantly higher than this one observed for D-adenosine. So, a diminution of the apparent enantioselectivity was observed when the injected solute concentration increased, varying from ~11.5 at 0.9 mM to ~9.5 at 9.4 mM. This difference in the binding capacity between D- and L-adenosine confirms that the D-adenosine retention is governed mainly by interactions with specific sites [23] which are characterized by an high interaction energy (see below) and a low saturation capacity. In contrast, L-adenosine interacts only with the non-specific sites (characterized by a low interaction energy and an higher saturation capacity) at the streptavidin and/or chromatographic support surface. Such a result is of interest since it indicates that the specific sites of streptavidin have, in principle, quasi-infinite enantioselective properties for adenosine. Similar results have been obtained with conventional or target-specific CSPs. Using immobilized bovine serum albumin, Gilpin and Tittelbach [23] have shown that L-tryptophan is retained mainly by a specific binding site at the protein surface while D-tryptophan does not interact with this specific binding site. Gavazzini et al. [24] have reported also that the L-enantiomers of *N*-Ac-amino acids are totally excluded from the D-enantiomer specific binding site (the aglycone pocket) of the teicoplanin CSP. For the target-specific CSPs such as imprinted polymers [25], antibodies [26] or DNA aptamers [22,27], the specific binding sites of the chiral selector frequently bind with high affinity the target enantiomer without significant affinity for the non-target enantiomer.

From these observations, it was possible to estimate the D-adenosine retention contribution due to the specific sites. It was considered that D-adenosine can be adsorbed on two different kinds of sites (enantioselective and non-enantioselective) with constant and independent affinities and saturation capacities for the solute, as classically described by a bi-Langmuir isotherm model. Therefore, the retention factor of the D-enantiomer can be described as follows:

$$k_D = k_{Ds} + k_{ns} \quad (1)$$

where k_{Ds} is the retention contribution for the D-enantiomer due to specific sites and k_{ns} is the retention contribution due to the non-specific sites. As L-adenosine does not interact significantly with the specific sites (see above), the L-adenosine retention factor can be reduced to the following relation:

$$k_L = k_{ns} \quad (2)$$

Thus, k_{Ds} can be evaluated as follows:

$$k_{Ds} = k_D - k_L \quad (3)$$

At 0 °C, the relative retention contribution due to the specific sites (k_{Ds}) was estimated to be very important, around 90% of the overall retention (k_D).

Furthermore, the number of D-adenosine specific active sites in the column was evaluated. Under moderate non-linear conditions, Snyder and co-workers [28,29] have established that the apparent solute retention factor k can be described by the following single function:

$$\frac{k}{k_{low}} = f\left\{\left[\frac{k_{low}}{k_{low}+1}\right] N_{low}^{1/2} \frac{Q_s}{m_L}\right\} = f\{w_k\} \quad (4)$$

where k_{low} and N_{low} are, respectively, the retention factor and the number of theoretical plate (at the lowest solute concentration used), m_L the number of active sites in the column, Q_s the amount of solute injected and w_k is the loading function. From the overloading experiments, the m_L values can be approached using the empirical dependence of k/k_{low} versus the loading function w_k (Table IV of [29]). Using a polynomial fitting function as described [28], the best-fit D-adenosine m_L value for the data of Fig. 2 was found to be around 20 nmol which corresponds to ~12 nmol per 100 μl of POROS media. As shown by Eble et al. [29], the binding capacity estimated by this approach reflects the number of high-energy/low-capacity sites in the column for an heterogeneous surface. Thus, in our chromatographic system, such value represents roughly the number of specific sites for D-adenosine. From the k_{Ds} value (at the lowest solute concentration) and the estimate of the specific site number in the column, it was possible to evaluate the association constant [22] between D-adenosine and the specific sites (K_{Ds}). The K_{Ds} value was found to be relatively high, around 20 mM^{-1} at 0 °C.

3.4. Temperature effects on the D-adenosine binding capacity

In order to evaluate a possible change in the D-adenosine binding capacity with the temperature increasing, the concentration dependencies of the solute retention factor were measured for all the column temperatures. As can be seen in Fig. 3, the D-adenosine capacity curve over the 4–24 °C temperature range was significantly different from this one observed at $T = 0$ °C. It was not possible to fit the experimental data by the empirical polynomial function as described below for $T = 0$ °C. This was due to the too weak variation of the retention factor with the solute concentration increasing. However, The 4–24 °C curve exhibits a plateau between 0.88 and 2.34 mM followed by a very slight fall-off above 2.34 mM, indicating that the saturation capacity of the column is higher than this one observed at the lowest temperature. As shown previously at $T = 0$ °C, the capacity curve for L-adenosine was still linear over the 0.9–9.4 mM solute concentration range for the column temperatures comprised between 4 and 24 °C (data not shown). The modification of the binding capacity for the D-enantiomer with the temperature varying has been previously observed in some

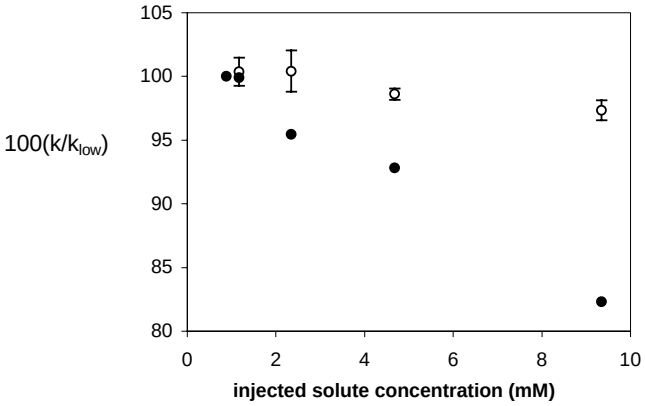

Fig. 3. Solute concentration dependencies of the retention factor for D-adenosine using streptavidin stationary phase at various column temperature. In order to compare the retention data, the normalized parameter $100(k/k_{low})$ was used where k_{low} represents the retention factor at the lowest solute concentration. Average and standard deviation of the retention data obtained over the 4–24 °C temperature range (○). Retention data for $T = 0$ °C (●). Column: 370 mm × 0.76 mm; flow rate: 50 μl/min.

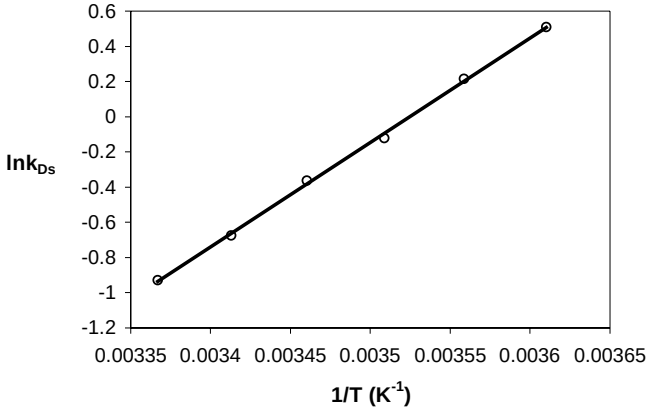

Fig. 4. Van't Hoff plots $\ln k_{Ds}$ vs. $1/T$ using the D-adenosine aptamer stationary phase. Temperature range: 4–24 °C; column: 370 mm × 0.76 mm; flow rate: 50 μl/min.

cases with other immobilized proteins. For example, Guiochon and co-workers [30] showed, by measuring adsorption isotherms, that the bovine serum albumin saturation capacity for the aminoacid derivative enantiomers decreased with increasing temperature. On the other hand, Loun and Hage [31] reported that the human serum albumin binding capacity for warfarin enantiomers increased when the temperature varied from 4 to 45 °C. As proposed by these authors [30,31], such behavior could be consistent with some conformational changes of protein at the lowest temperature, which would affect the saturation capacity of the D-adenosine sites.

3.5. Determination of the thermodynamic parameters for the D-adenosine binding to the streptavidin specific sites

From the temperature experiments, the thermodynamic parameters for the D-adenosine binding to specific sites were determined. In linear conditions, the temperature dependence of retention factor k_{Ds} is given by the following relation:

$$\ln k_{Ds} = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} + \ln \frac{m_{LDs}}{V_M} \quad (5)$$

ΔH and ΔS are, respectively, the enthalpy and entropy of transfer of solute from the mobile phase to the streptavidin specific sites, m_{LDs} the number of active specific sites in the column, T the absolute temperature and R is the gas constant. If the number of active specific sites, solute and solvent properties are independent of temperature and ΔH and ΔS are temperature invariant, a linear van't Hoff plot is obtained.

In our chromatographic system, the D-adenosine m_L value changed at 0 °C. So, in order to provide valuable values of the thermodynamic parameters (see Eq. (5)), the van't Hoff plot was constructed over the 4–24 °C temperature range, for which the D-adenosine binding capacity is roughly constant (see Fig. 3). A linear van't Hoff plot $\ln k_{Ds}$ versus $1/T$ was obtained. This plot is shown in Fig. 4. The linear regression coefficient R^2 was 0.9990. Using Eq. (5), the ΔH and ΔS^* ($\Delta S/R + \ln[m_{LDs}/V_M]$) values were determined. Negative values were obtained, i.e., $\Delta H = -49.4 \pm 0.4$ kJ/mol and $\Delta S^* = -21.0 \pm 0.1$. Such values are consistent with an association process between D-adenosine and the streptavidin specific sites enthalpically driven [32].

4. Conclusion

In summary, the present results demonstrate that streptavidin is a useful chiral stationary phase for the separation of the adenosine enantiomers. At low temperatures, the apparent enantioselectivity is important for a small molecule. It is shown that the blockage of the biotin sites of the immobilized streptavidin is responsible for a strong reduction of the chiral recognition ability via a direct and/or an indirect effect. In addition, the study on the column temperature influence on the retention and chiral recognition shows that the D-adenosine saturation capacity of the streptavidin column decreases at 0 °C. Finally, the solute binding to the streptavidin specific sites is enthalpically governed.

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