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Reversal of the enantiomeric elution order of some aromatic amino acids using reversed-phase chromatographic supports coated with the teicoplanin chiral selector

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Abstract

In this paper, two chiral stationary phases were prepared by coating the surface of both C8 and C18 high-performance liquid chromatography (HPLC) supports with the teicoplanin chiral selector. The hydrophobic C11 acyl side chain, attached to the D-glucosamine group of teicoplanin, served as anchor moiety for the immobilization of the chiral selector on the apolar support material. The retention and enantioselectivity of these coated stationary phases were studied using some aromatic amino acids as probe solutes and an aqueous solution as mobile phase. It was found that the enantiomer elution order on the modified C8 and C18 stationary phases was reversed (L > D) relatively to that classically observed with a teicoplanin covalently immobilized on a silica support (D > L). Such a dynamic coating on the reversed-phase supports was found to be of interest since the apparent enantioselectivity was not significantly changed by the use during an extended period of time or following a long-term storage of the columns.

Keywords: Chiral separation; Teicoplanin; Coating; Aromatic amino acids

1. Introduction

Coating methodology of apolar chromatographic surfaces with appropriate ligands has been widely exploited in HPLC notably for the separation of ionic species by ion chromatography [1] or the study of the interactions between bioactive compounds and "membrane-like" systems [2]. Such an approach has been also reported for HPLC chiral separation. Previous papers have shown that reversed-phase chromatographic supports such as C18 or porous graphitic carbon stationary phases, coated with chiral selectors covalently bonded to a suitable non-polar anchor molecule, can be used successfully as chiral stationary phases (CSPs). Various chiral selectors such as amino acids derivatives, tartaramide, lasalocid or acylcarnitine have been immobilized via such a methodology and successfully used for the resolution of various racemates [3–7].

During the past decade, the macrocyclic antibiotics have been widely used as chiral selectors in both capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), especially for the resolution of native amino acid racemates. Although these glycopeptides have been used in some cases as chiral mobile phase additives (CMPA) [8–11], the design of chiral stationary phases is the most popular methodology reported for the HPLC applications. To date, the commercially available glycopeptidic CSPs are silica based, with the chiral selector covalently bound [12,13]. The cyclic antibiotics have been attached to silica gel via carboxylic acid or epoxy-terminated organosilanes [12]. Teicoplanin is unique among the glycopeptides in that it has a C11 hydrophobic acyl side chain attached to the glucopyranosyl group. This characteristic is notably responsible for the formation of micelles and specific pharmacological

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properties [14]. It is expected that such hydrophobic tail could be used to immobilize the chiral selector on hydrophobic chromatographic supports and then create a new type of chiral coating.

The aim of this paper was to investigate the feasibility of developing an apolar solid support permanently coated with teicoplanin. Both C18 and C8 reversed-phase chromatographic supports were tested to immobilize the chiral selector. The retention behaviour and enantioselective properties as well as the stability of these modified stationary phases were analyzed under aqueous mobile phase conditions using some aromatic amino acids as probe solutes.

2. Experimental and methods

2.1. Apparatus

The HPLC system consisted of a LC Shimadzu pump 10AT (Sarreguemines, France), a Rheodyne injection valve model 7125 (Interchim, Montluçon, France) fitted with a 20 μL sample loop, a Shimadzu SPD-10A UV–vis detector ($\lambda = 260$ nm for the compound detection and $\lambda = 310$ nm for the detection of the teicoplanin breakthrough curve during the coating procedure). The C18 (250 mm × 4.0 mm) and C8 (250 mm × 4.6 mm) reversed-phase columns (dp: 5 μm, pore size: 100 Å) were purchased from Merck (Darmstadt, Germany) and Macherey-Nagel (Düren, Germany), respectively. These columns were used with controlled temperature (25 °C) in an oven Igloocil (Interchim).

2.2. Reagents

All racemates and enantiomers were obtained from Sigma–Aldrich (Saint-Quentin, France) or Bachem (Weil am Rhein, Germany). Na_2HPO_4 and NaH_2PO_4 were supplied by Sigma–Aldrich. Teicoplanin was provided by Astec (Whippany, USA). Acetonitrile HPLC grade (ACN) was purchased from Fisher Scientific (Leicestershine, UK). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge.

2.3. Coating procedure

The immobilization of the teicoplanin chiral selector was performed in situ, by frontal chromatography on the reversed-phase columns. A 1 mM aqueous solution of teicoplanin was pumped onto the column at a flow rate of 0.1 mL/min and a column temperature of 25 °C until a breakthrough was detected with a stable detector response at $\lambda = 310$ nm. Before the chromatographic experiments, the columns were washed with the aqueous mobile phase (phosphate buffer 5 mM, pH 7.0) until stable baseline was observed. The amount of teicoplanin immobilized on the chromatographic supports was estimated by subtracting the UV absorbance of the unbound teicoplanin solution from that of the initial solution, at 280 nm. It was estimated to be about 0.150 mmol for the two columns each. When not in use for an extended period of time (long-term storage experiment), the columns were stored in the aqueous buffer containing sodium azide (0.05%) in order to prevent microbial contamination.

2.4. Chromatographic operating conditions

The mobile phase consisted of phosphate buffer (5 mM, pH 7.0). The flow rate varied from 0.25 to 1.70 mL/min. Samples were prepared in the mobile phase at a concentration of 2 mM. Twenty microliters was injected in triplicate and the retention times were measured. The apparent retention factor k was determined using the following relation: $k = (t_R - t_0)/t_0$, where t_R is the retention time of the respective enantiomer and t_0 is the retention time of an unretained species. Although this is not the most accurate approach for estimating the retention factor, t_R was determined through the solute peak position. This simplification is justified because no thermodynamic or kinetic data were extracted from this chromatographic parameter. t_0 was determined using methanol as void time marker. The retention times and column void time were corrected for the extra-column void time. They were assessed by injections of solute onto the chromatographic system when no column was present. The apparent enantioselectivity α was calculated as follows: $\alpha = k_2/k_1$, where k_2 is the retention factor for the more retained enantiomer and k_1 is the retention factor for the less retained enantiomer. The efficiency of the column was characterized by calculating the number of theoretical plates $N = 5.54(t_R/\delta)$, where δ is the peak width at half-height. The resolution R_s was calculated using the following relation: $R_s = [1.18(t_{R2} - t_{R1})]/(\delta_2 + \delta_1)$. 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. Chromatographic properties of the reversed-phase supports coated with the teicoplanin chiral selector

Table 1 presents the various aromatic amino acids which were used as probe solutes. The retention and enantioselective properties of the dynamically modified supports were investigated using an aqueous mobile phase which consisted of phosphate buffer adjusted to pH 7.0. Preliminary results showed that such operating conditions were optimal for the enantiomeric separation. A decrease in the mobile phase pH or the addition of an organic modifier (acetonitrile) in the eluent decreased the solute retention and altered significantly the enantioselective properties of the two CSPs. It can be noted that acetonitrile was responsible for the teicoplanin desorption from the column since different solute retention factors were obtained before and after the addition of the

Table 1
Chromatographic data for some aromatic amino acids and derivatives on the C18 and C8 supports coated with the teicoplanin chiral selector using an aqueous mobile phase[a]

Compounds	k_L	k_D	α	N_L	N_D
Modified C18 support					
Tyrosine	1.12	0.90	1.24	185	270
α-Methyltyrosine	2.30	1.32	1.74	150	170
4-Aminophenylalanine	1.39	0.83	1.67	170	245
5-Hydroxyltryptophan	5.82	3.64	1.60	90	160
Tryptophan	17.65	11.30	1.56	140	160
1-Methyltryptophan	76.90	55.63	1.38	50	75
Modified C8 support					
Tyrosine	3.26	2.78	1.17	450	785
α-Methyltyrosine	4.12	3.16	1.51	430	800
4-Aminophenylalanine	4.19	3.8	1.35	445	495
5-Hydroxyltryptophan	7.89	7.89	1.00	335	335
Tryptophan	13.99	11.65	1.20	365	410
1-Methyltryptophan	34.69	34.69	1.00	245	245

[a] Relative standard deviation of the solute retention factors was less than 1%. Mobile phase consisted of phosphate buffer (5 mM, pH 7.0). Flow rate: 1.7 mL/min. Sample concentration: 2 mM.

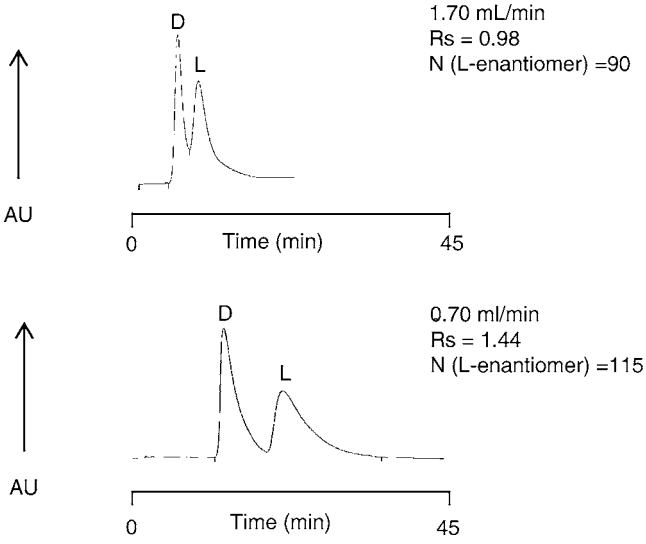

Fig. 1. Chromatographic resolution of 5-hydroxyltryptophan using the teicoplanin-coated C18 stationary phase. Column: 250 mm × 4.0 mm (i.d.); amount of teicoplanin immobilized was about 0.150 mmol; mobile phase: phosphate buffer 5 mM, pH 7.0; column temperature: 25 °C; injected concentration: 2 mM; injection volume: 20 μL; detection at 260 nm.

organic modifier in the mobile phase. At a column temperature of 25 °C, the overall retention depended, at least in part, on the overall hydrophobic character of the substituent (Table 1). At a flow rate of 1 mL/min, the compounds with the more hydrophilic R groups (tyrosine, α-methyltyrosine, 4-aminophenylalanine and 5-hydroxyltryptophan) exhibited relatively low retention times while the compounds having the more hydrophobic substituents such as tryptophan and 1-methyltryptophan displayed a too high retention. So, the experiments were carried out at a relative high flow rate of 1.7 mL/min for the comparison of the retention factor and enantioselectivity of the different compounds. From a chiral recognition point of view, the D-enantiomers were eluted before the L-enantiomers on the two teicoplanin-coated supports (Table 1). Such a result is quite surprising as it is well established that, for the common native amino acids analyzed in this study, a reversed elution order (D > L) was obtained on the "conventional" commercially available teicoplanin CSPs [15–18], i.e. when the chiral selector is covalently immobilized on a gel silica support. This may reflect some drastic orientation or conformation changes of the chiral selector when the teicoplanin hydrophobic tail is embedded into the alkyl chains. For example, a previous study showed that the elution order of amino acids on porous graphitic carbon coated with *N*-alkyl-L-phenylalanine derivatives was L > D while a reversed elution order (D > L) was obtained with *N*-aryl substituted L-phenylalanine phases [3]. To explain this behaviour, the authors proposed a stereoselective binding model based on two distinct spatial orientations of the chiral selectors at the chromatographic surface. Nevertheless, such a behaviour appears to be of great practical interest as it allows to reverse easily the elution order of the solute enantiomers relatively to that obtained with a classical teicoplanin CSP. Some differences were observed between the modified C8 and C18 supports. As can be seen in Table 1, a lower apparent enantioselectivity was obtained with the modified C8 support for all the compounds. Moreover, 5-hydroxyltryptophan and 1-methyltryptophan were resolved on the teicoplanin-coated C18 support while no enantiomeric separation was obtained on the modified C8 support. This can be explained, at least in part, by the fact that the number of immobilized teicoplanin moles by column volume was higher for the modified C18 column than that obtained for the modified C8 column (∼48 μmol/mL versus ∼36 μmol/mL).

Although valuable apparent enantioselectivity values were observed in most cases (Table 1), no baseline resolution was observed on the two chiral stationary phases for any racemate at a flow rate of 1.7 mL/min. This was due to the fact that low efficiency as well as pronounced peak tailing was obtained at this flow rate. For the last eluting enantiomers, *N* varied between 50 and 450 in relation to the compounds analyzed and the type of support used (Table 1). The efficiency values were globally lower than those observed for the common amino acids under reversed-phase conditions with a conventional teicoplanin CSP (*N* for the last eluting enantiomers varying from 180 to 1300) [19]. The A_s value for the L-enantiomers was between 1.5 and 3.5, in accordance with the asymmetry factors reported by Cavazzini et al. for amino acids using a covalently bound teicoplanin [20]. In order to improve the resolution, the flow rate was reduced, except for the more hydrophobic compounds (tryptophan and 1-methyltryptophan). In all cases, a significant improvement of the resolution was achieved. As an example, the resolution for 5-hydroxyltryptophan on the modified C18 support increased when the flow rate varied from 1.7 to 0.7 mL/min (Fig. 1). The resolution enhancement was classically dependent on the increase in the efficiency as the flow rate decreased (see Fig. 1). However, the R_s increase

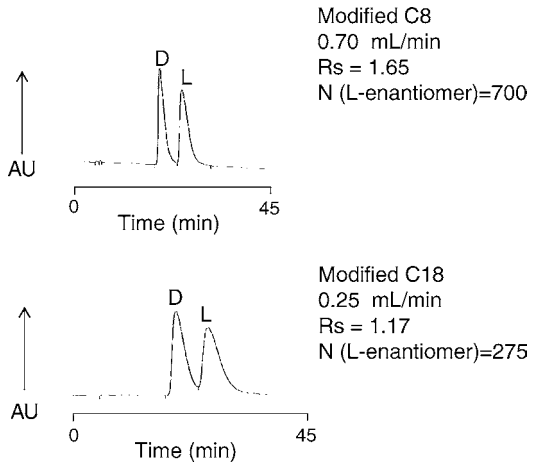

Fig. 2. Chromatographic resolution of 4-aminophenylalanine using the teicoplanin-coated C18 and C8 stationary phases. Column: 250 mm × 4.0 mm or 4.6 mm (i.d.); amount of teicoplanin immobilized was about 0.150 mmol for the two columns each; mobile phase: phosphate buffer 5 mM, pH 7.0; column temperature: 25 °C; injected concentration: 2 mM; injection volume: 20 μL; detection at 260 nm.

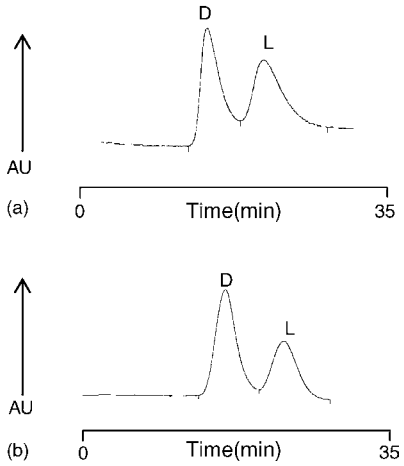

Fig. 3. Chromatographic resolution of tryptophan using the teicoplanin-coated C18 stationary phase: (a) in initial conditions and (b) after the passage of around 2000 column volumes of mobile phase. Column: 250 mm × 4.0 mm (i.d.); amount of teicoplanin immobilized was about 0.150 mmol; mobile phase: phosphate buffer 5 mM, pH 7.0; column temperature: 25 °C; injected concentration: 2 mM; injection volume: 20 μL; detection at 260 nm.

was also due to an increase in the apparent enantioselectivity when the flow rate was reduced (from 1.60 to 1.78). Such data indicate that the flow rate of 1.7 mL/min was too high to establish the local equilibrium conditions within the column. It is also interesting to note that the highest efficiency was obtained using the modified C8 column (Table 1). As an example, although the apparent enantioselectivity for 4-aminophenylalanine was higher with the C18 column (see Table 1), a better resolution was obtained on the C8 column, even at a higher flow rate (Fig. 2).

3.2. Temporal stability and regeneration of the supports coated with the teicoplanin chiral selector

In order to investigate the practical applicability of such a modified support, the stability of the modified C8 and C18 supports over the time was assessed. The properties of the columns were evaluated comparing the tryptophan retention factor and enantioselectivity during an extended period of time under identical mobile phase conditions and at a column temperature of 25 °C. Although a slight increase in the solute retention factors was observed, the tryptophan α value did not change significantly after the passage of around 2000 column volumes of mobile phase (Fig. 3). After an intensive use of the columns during more than two months, some loss of the apparent enantioselectivity was observed. However, it was less than 20%. In such a case, the columns were subjected to a regeneration step by recycling the coating solution through the original columns, as described in Section 2. Such regeneration allowed the complete restoration of the original retention and enantioselectivity characteristics, indicating the usefulness of such a procedure. In addition, the effects of a long-term storage under the mobile phase containing sodium azide (0.05%) were evaluated for a period of about three months. It did not affect significantly the enantioselective and retention properties of the two columns (less than 5% difference in the chromatographic data).

4. Conclusion

In this paper, a very simple coating method of reversed-phase supports for the preparation of a teicoplanin CSP allowing the direct resolution of some aromatic amino acids and derivatives is described. This methodology appears to be of interest since the modified supports exhibit a good stability over the time and can be easily regenerated without loss of enantioselectivity. In addition, this new type of teicoplanin immobilization allows to reverse the enantiomeric elution order relatively to that observed with a conventional covalently immobilized teicoplanin. Such behaviour could be the result of some drastic orientation or conformation changes of the chiral selector when the teicoplanin hydrophobic tail is embedded into the alkyl chains.

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