ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1845–1850

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Investigation of chromatographic performances and binding characteristics of BSA-encapsulated capillary column prepared by the sol–gel method

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Received 13 April 2002; received in revised form 27 May 2002; accepted 30 May 2002

Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

We have developed a novel protein-encapsulation technique using the sol–gel method for the preparation of monolithic capillary columns for capillary electrochromatography. Bovine serum albumin (BSA) was encapsulated in tetramethoxysilane-based hydrogel and the enantioselectivity was evaluated. The present work examined the effect of various factors such as running buffer pH and concentration or organic modifier on the chromatographic performances as well as binding characteristics of BSA for D,L-tryptophan (Trp). The retention and enantioseparation dramatically changed depending on the buffer pH and concentrations. These factors influenced EOF and especially binding characteristics of BSA for L-Trp. Under the optimized conditions, the BSA-encapsulated columns revealed the adequate repeatability concerning column-to-column and run-to-run.

Keywords: Sol–gel; Capillary electrochromatography; Protein-encapsulation; Monolithic capillary column; Enantioseparation

1. Introduction

Chiral separation is an important area in pharmaceuticals or biochemicals. In particular, chiral separations using proteins as chiral selectors are widely employed for the assay of drug enantiomers [1]. Because the binding of drug is a part of its physiological role, bovine serum albumin (BSA) [2–6] and human serum albumin (HSA) [7–10] have been thoroughly studied regarding the characteristics of their binding sites. The resulting knowledge has been applied to the chiral separation of various drugs.

Capillary electrochromatography (CEC) has been regarded as a very promising analytical

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00527-7

separation technique that combines the efficiency of capillary zone electrophoresis and the selectivity of liquid chromatography (LC) with the use of a solid stationary phase [11,12]. Several techniques have been reported that use proteins for chiral separation in CEC. At present, there are mainly three techniques to immobilize proteins in a CEC column. The first one is the use of a LC support, such as silica particles, to attach proteins [10,13]. Binding protein onto the silica particle could abolish high background response at the detection region, which was the biggest problem when protein chiral selectors were used as buffer additives. The second technique uses cross-linked gel to immobilize protein, in which protein was cross-linked with glutaraldehyde by covalent bonding and allowed to form a gel in a capillary [4]. A third way is immobilizing protein by forming a dynamic protein coating onto the capillary wall through the chemical or physical adsorption of the protein [9].

Recently, we developed a novel protein-encapsulation technique using the sol–gel method for the preparation of monolithic capillary columns for CEC [14]. The silicate matrix is formed by hydrolysis of alkoxysilane precursors followed by condensation to yield polymeric oxo-bridged SiO_2 networks. Two proteins, BSA and ovomucoid from chicken egg white, were encapsulated in tetramethoxysilane (TMOS)-based silica matrix during the hydrolysis and polycondensation process of silica networks. The protein encapsulation was carried out within a capillary in a single step under mild conditions without exposure to a variety of perturbing conditions, such as acidic pH, organic solvent [15]. The characteristics of gel including gelation speed or mechanical strength were varied associated with gelation conditions such as buffer pH and concentration, and these changes consequently affected EOF and enantioselectivity. The optimized monolithic columns showed adequate chromatographic performance, including mechanical strength, penetration of pressurized flow and chiral separation. BSA-encapsulated column could achieve the enantioseparation of Trp and benzoin and OVM-encapsulated column could separate the enantiomers of benzoin and some basic drugs.

In this paper, we report the influence of various factors including running buffer pH, concentration or organic modifier on the chromatographic performances to investigate the characteristics of this new type of monolithic capillary columns. We used BSA-encapsulated columns and D,L-Trp as the test compounds.

2. Experimental section

2.1. Materials and chemicals

Fused-silica capillary (75 μm i.d.) was obtained from Polymicro Technologies Inc. (Phoenix, AZ). TMOS and methacryloxypropyltrimethoxysilane were purchased from Tokyo Kasei (Tokyo, Japan). D,L-Trp was purchased from Sigma-Aldrich (Milwaukee, WI). BSA (crystallized cold alcohol precipitate 97%) was purchased from Wako Pure Chemicals (Osaka, Japan). Methyltrimethoxysilane was purchased from ShinEtsu Chemicals (Tokyo, Japan). Water was purified by MilliQ apparatus (Millipore, Bedford, MA).

2.2. Monolithic capillary column preparation

The capillary column used in this work was prepared by the procedure in our previous report [14,16].

2.3. Equipment

CEC experiments were carried out on a Beckman P/ACE5510 capillary electrophoresis instrument (Fullerton, CA) with a UV-absorbance detector. Samples were introduced electrokinetically at the anodic side (2 kV, 3 s). In most experiments, the running buffer was 20 mM phosphate buffer (pH 7.0) and a voltage of 2 kV was applied. The temperature was kept at 25 °C in all experiments.

The samples were diluted in the running buffer. Before use, all solutions were filtered through a 0.22-μm membrane (Millipore, Bedford, MA) and degassed by ultrasonication. Thiourea was used as a marker compound of EOF, because the migration time of thiourea was almost the same as that

of methanol and thiourea is often used as EOF marker [14,17,18].

3. Results and discussion

The silicate matrix is formed by hydrolysis of an alkoxysilane precursor followed by condensation to yield polymeric oxo-bridged SiO_2 networks [19]. While the silicate network grows, it traps BSA molecules.

Hydrolysis:

$$Si(OR)_4 + H_2O \rightarrow (RO)_3SiOH + ROH$$

Condensation:

$$2(RO)_3SiOH \rightarrow (RO)_3Si-O-Si(OR)_3 + H_2O$$

$$(RO)_4Si + (RO)_3SiOH \rightarrow (RO)_3Si-O-Si(OR)_3 + ROH$$

3.1. Loadability of BSA-encapsulated column

The effect of sample loading on the retention time and enantioselectivity (Rs) of Trp for the BSA-encapsulated column is shown in Fig. 1. The Trp concentration was varied from 16 to 4 mM. Retention times of DL-Trp were slightly decreased with decreased in DL-Trp concentration. The change is a little larger for D-Trp than that for L-Trp and consequently, enantioselectivity decreased with an increase in DL-Trp concentrations. Theoretical plate number of D-Trp in each concentration is 22 000, 21 000 and 9200 for 4, 8, and 16 mM, respectively. The dramatical decrease in theoretical plate number at 16 mM means saturation of sample volume. Although a sample loading less than 4 mM is favorable to achieve better resolution, this concentration is eventually a lower limit based on the S/N ratio. The relatively poor loading capacity is often observed in protein-based chiral separation columns [20].

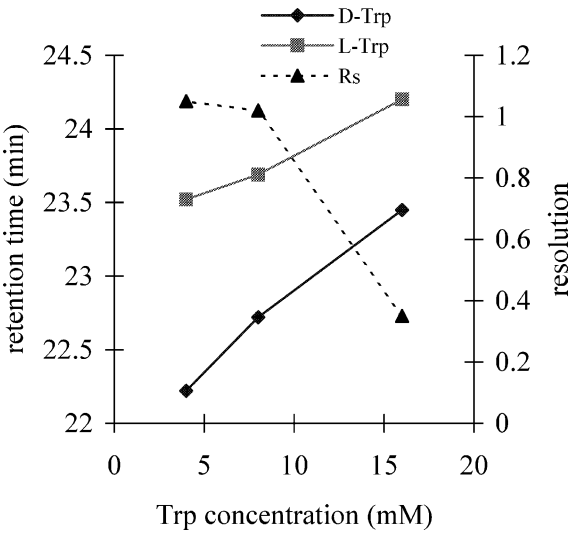

Fig. 1. Effect of sample loading on the retention time and enantioselectivity (Rs). Conditions: sample: D,L-Trp. Mobile phase: 20 mM phosphate buffer (pH 7.0). Applied voltage: 2 kV. Injection: 2 kV, 3 s. Column length: 30 cm. Detection: 254 nm.

3.2. Effect of buffer concentration on retention and enantioselectivity

The concentration of running buffer was varied to investigate the effect on the retention and enantioselectivity of Trp. The running buffer used was 5, 20, 40 and 50 mM phosphate buffer (pH 7.0). Fig. 2 shows the retention time and enantioselectivity (Rs) of D,L-Trp at each buffer concentration. Thiourea was used as EOF marker. The retention times of DL-Trp increased with an increase in the buffer concentration, which was derived from the decrease in EOF.

At 5 mM phosphate buffer, D- and L-Trp was not separated. Probably, fast EOF prevented BSA from interacting with L-Trp. On the other hand, at the buffer concentration more than 20 mM, D- and L-Trp could be separated and the Rs values increased with an increase in the buffer concentration, which is different from other investigations using HPLC previously reported [5,8]. This increase in enantioselectivity is presumably attributed to the decrease in EOF, which allows the more distinguishing interaction between BSA and L-Trp, compared to D-Trp. Binding of L-Trp on BSA

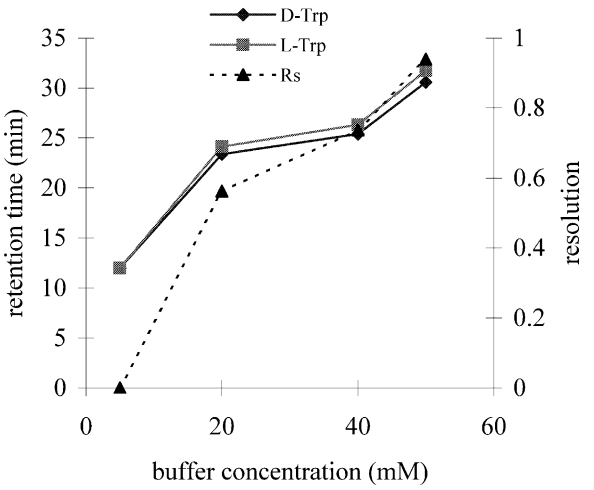

Fig. 2. Effect of running buffer concentration on the retention time and enantioselectivity (Rs). Conditions: Sample: 5 mM D,L-Trp. Mobile phase: phosphate buffer (pH 7.0). Applied voltage: 2 kV. Injection: 2 kV, 3 s. Column length: 30 cm. Detection: 254 nm.

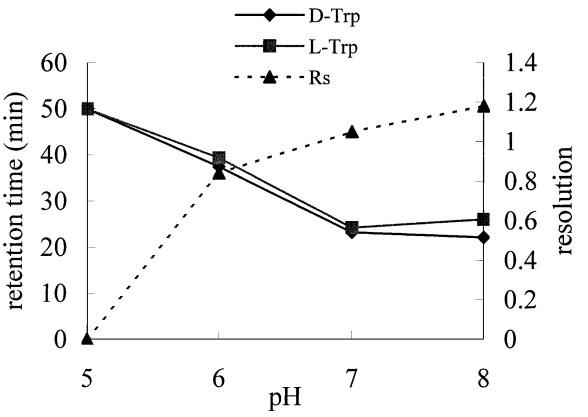

Fig. 3. Effect of running buffer pH on the retention time and enantioselectivity (Rs). Conditions: sample: 5 mM D,L-Trp. Mobile phase: 20 mM phosphate buffer. Applied voltage: 2 kV. Injection: 2 kV, 3 s. Column length: 30 cm. Detection: 254 nm.

involves both charge interactions and hydrophobic interactions [20]. Therefore, another reason for the increase in enantioselectivity is speculated that the increase in the buffer concentration expelled the nonspecific interaction (such as charged interaction) between silanol groups and Trp, whereas it increased the hydrophobic interaction between BSA and Trp. Because 50 mM phosphate buffer gave the large current which causes bubble formation, the buffer concentration ranging from 20 to 40 mM is appropriate for the running buffer.

3.3. Effect of buffer pH

Figs. 3 and 4 show the effect of buffer pH on the retention and enantioselectivity. The pH was varied from 5.0 to 8.0 at a constant concentration of 20 mM phosphate. The retention times of thiourea which indicate EOF velocity decreased at the higher pH, where more silanol groups disassociate. The increase in EOF velocity decreased retention times of DL-Trp. At pH 5.0, Trp eluted with thiourea, which indicates that no or very few, if any, interaction occurred between BSA and Trp. BSA is composed of three globular domains. At neutral pH, the three domains are held together by ionic and electrostatic interactions. Between pH 5 and 3.5, BSA undergoes a conformational changes in which the domains physically separate and slight expansion of the molecule [21–24]. In the light of these facts, the failure in binding of BSA and Trp at pH 5.0 may be explained by the conformational changes of BSA around pH 5.0. Other techniques using BSA or HSA as chiral selector also achieved only poor resolution at pH 5.0 [8,25].

At pH 6.0, 7.0 and 8.0, enantioselectivity increased with an increase in pH value and enantioselectivity was the best at pH 8.0. This tendency was also reported by other investigators [5,25]. These results seems to be reasonable considering the fact that the binding of L-Trp on BSA increases with an increase in pH value and it reaches the maximum at pH 9.0 [20]. However, because alkaline condition is not preferable for silica gel, pH 7.0 would be appropriate in this system.

3.4. Effect of organic modifier

It is known that the addition of organic modifier to the running buffer improves separation efficiency due to some reason, such as decrease in EOF, change in zeta potential or viscosity of mobile phase [26,27]. Five percentage of methanol or 1-propanol was added to running buffer to investigate the effect. In both cases, the retention

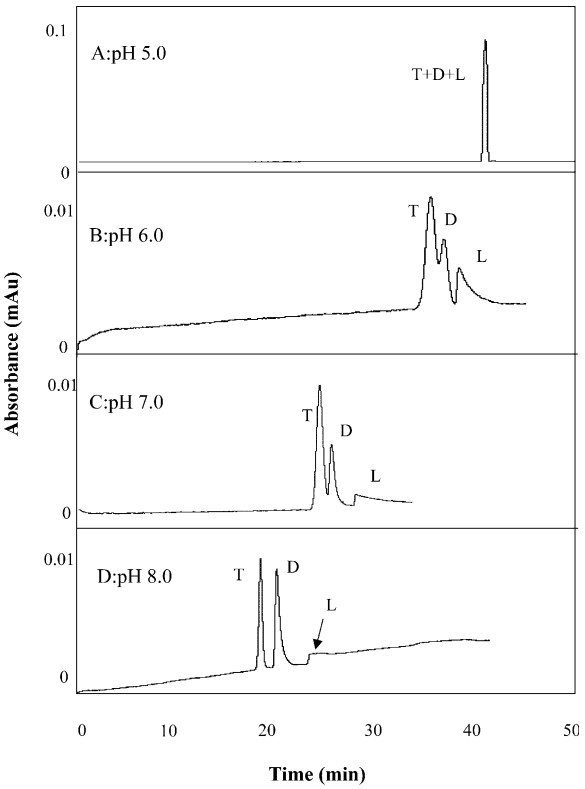

Fig. 4. Electrochromatograms of D,L-Trp and thiourea on BSA-encapsulated column and effect of running buffer pH. T: thiourea. D: D-Trp. L: L-Trp. Conditions: sample: 5 mM D,L-Trp + 10 mM thiourea. Mobile phase: 20 mM phosphate buffer ((A) pH 5.0, (B) pH 6.0, (C) pH 7.0, (D) pH 8.0). Applied voltage: 2 kV. Injection: 2 kV, 3 s. Column length: 30 cm. Detection: 254 nm.

times of Trp slightly decreased and Trp enantiomers were unresolved. In the separation of Trp enantiomers by capillary zone electrophoresis using BSA as chiral complexing agents in the background electrolyte, the addition of 1-propanol increased the retention time, whereas enantioselectivity was decreased [28]. Another HPLC study, in which Trp enantiomers were resolved using BSA-immobilized column, reported the decrease both in the retention time and enantioselectivity with use of 1-propanol [5]. Regarding the effect of organic modifier on the enantioselectivity of Trp, the BSA-encapsulated columns showed the similar characteristics to other techniques using BSA.

Table 1
Repeatability of the BSA-encapsulated column

Repeatability	n	RSD (%) of t_D (min)	RSD (%) of t_L (min)
Column-to-column	4	2.71	3.46
Run-to-run	5	2.61	2.57

Conditions: sample: 5 mM D,L-Trp. Mobile phase: 20 mM phosphate buffer. Applied voltage: 2 kV. Injection: 2 kV, 3 s. Column length: 30 cm. Detection: 254 nm.

3.5. Repeatability

The repeatability of four BSA-encapsulated columns, which were prepared in four different batches was investigated by employing D,L-Trp as test compounds. All columns gave the complete resolution of D,L-Trp (Rs value is 1.13). As shown in Table 1, reproducibility of retention times both for D- and L-Trp was satisfactory.

Run-to-run repeatability using one column was also favorable (Table 1). The relative standard deviations of the retention times were 2.71% for D-Trp and 2.57% for L-Trp, respectively ($N = 5$). These results suggest that this BSA-encapsulated monolithic column can be used for the reproducible routine analysis.

4. Conclusion

A new type of monolithic capillary column which encapsulated protein in the gel matrix was developed using sol–gel method. The chromatographic performances of BSA-encapsulated column for chiral separation were investigated using D,L-Trp as the test compounds. The running buffer condition largely influenced the retention and enantioselectivity of Trp enantiomers. These results were caused by several factors, such as changes in EOF or microenvironment neighboring the binding sites on BSA. The latter effect on the enantioseparation was especially obvious when the buffer pH was varied and our results coincided with others previously reported using HPLC. These results partially suggest that the microenvironment neighboring the binding site of BSA for

Trp was not largely affected by encapsulation in the sol–gel matrix.

Acknowledgements

This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology.

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