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High-resolution chiral separation using microfluidics-based membrane chromatography

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Abstract

A plastic microfluidic system, containing porous poly(vinylidene fluoride) (PVDF) membranes adsorbed with bovine serum albumin (BSA), is demonstrated for high resolution chiral separation of racemic tryptophan and thiopental mixtures. Microfluidic networks on poly(dimethylsiloxane) (PDMS) substrates are fabricated by capillary molding technique. This miniaturized chiral separation system consists of two layers of PVDF membranes which are sandwiched between two PDMS slabs containing microchannels facing the membranes. On-line adsorption of BSA onto the membranes is employed for the preparation of chiral stationary phase and the evaluation of solution conditions in an effort to achieve maximum protein adsorption. Variations in the mobile phase conditions, including solution pH and ammonium sulfate concentration, are studied for their effects on chiral separation. Based on the large surface area to volume ratio of porous membrane media, adsorbed BSA onto the PVDF membranes enables high resolution separation of racemic mixtures with sample consumption of sub-nanogram or less in the integrated microfluidic networks. In addition, the membrane pore diameter in the submicron range eliminates the constraints of diffusional mass-transfer resistance during protein adsorption and chiral chromatographic processes.

Keywords: Enantiomer separation; Microfluidics; Membrane chromatography; Tryptophan; Thiopental

1. Introduction

Non-covalent interactions between a protein and small molecules are phenomena commonly found in various biological processes. Since proteins are intrinsically chiral macromolecules, most of these interactions are enantioselective. As a result, the enantioselectivity of proteins such as bovine serum albumin (BSA) and ovoglycoproteins offers a novel approach to preparing stationary phases toward chiral separation of enantiomeric compounds [1,2]. Soon after the demonstration of applying BSA for chiral separation in liquid chromatography [3], several techniques, mainly based on covalent binding, ionic interaction, and hydrophobic adsorption, have been developed to prepare the stationary phases containing BSA [4]. In addition, various studies were reported for the effects of experimental conditions on the separation performance of these chiral stationary phases [4–15].

Membrane chromatography has been introduced for solving two main issues encountered in scaling up chromatographic separations using fine particulate support media, namely technical challenges in over-

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coming high back pressure and slow solute diffusion kinetics [16]. Subsequently, fast chromatographic separations were achieved without applying extremely high pressure in membrane chromatography [17]. Since then, the applications of membrane chromatography have attracted great attention from pharmaceutical industries [18], particularly for protein separations [19]. Membrane chromatography has also been attempted to perform separations on non-protein target analytes such as DNA plasmids [20], oligonucleotides [21,22], peptides [21,23], amino acids [4,24], and small hydrophobic molecules [21]. Among all these studies, chiral separation of amino acids [4,24] has been demonstrated using the BSA-adsorbed porous membranes as the stationary phase.

The area of microfluidics has developed into one of the most dynamic fields in analytical chemistry during the past decade [25–33]. Miniaturized bioanalytical devices provide several advantages over bench-top instruments, including smaller dead volume, smaller sample consumption, shorter analysis time, lower cost, greater portability, and potentially greater separation resolution for complex mixtures in an integrated format. Materials such as silicon, glass, quartz, and polymers have been explored for the fabrication of microfluidic systems [34–36]. Among all these materials, a silicone elastomeric polymer, poly(dimethylsiloxane) (PDMS), attracts the interests of researchers because it is inexpensive, nontoxic, inert and can be easily processed for the fabrication of microfluidic networks [37].

In this work, microchannels on PDMS substrates are fabricated by capillary molding technique. The as-made microfluidic networks directly connect to the capillaries as the fluid inlets and outlets. By exploiting the use of a hydrophobic and porous poly(vinylidene fluoride) (PVDF) membrane, a PDMS-based microfluidic system containing the BSA-adsorbed PVDF membranes is constructed. Effects of solution conditions on BSA adsorption and chiral separation using the BSA-adsorbed PVDF membranes as the stationary phase are investigated. Chiral separations of racemic mixtures, including tryptophan and thiopental, are performed and demonstrated using this miniaturized membrane chromatography system. Important advantages of miniaturizing membrane chromatography include significantly reduced dead volumes and minimal sample and reagent consumption. In addition, the membrane pore diameter in the submicron range eliminates the constraints of diffusional mass-transfer resistance of racemic compounds to adsorbed BSA.

2. Experimental

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris) and ammonium sulfate were purchased from Bio-Rad (Richmond, CA). D-tryptophan, L-tryptophan, and sodium mono- and diphosphate were acquired from Fisher Scientific (Pittsburgh, PA). BSA and D,L-thiopental were obtained from Sigma (St. Louis, MO). Sylgard 184 silicone elastomer kit for the fabrication of the PDMS slabs and epoxy glue were purchased from Dow Corning (Midland, MI) and Devcon (Danvers, MA), respectively. Fused-silica capillaries (50 μm I.D./100 μm O.D.) were acquired from Polymicro Technologies (Phoenix, AZ). The PVDF membranes with pore diameters of 0.1 μm (Immobilon-P^{SQ}) were obtained from Millipore (Bedford, MA). All solutions were prepared using water purified by a Nanopure II system (Dubuque, IA) and further filtered with a 0.22 μm membrane (Costar, Cambridge, MA).

2.2. Fabrication of PDMS slabs containing microchannels

The procedures involved in a capillary molding technique are outlined in Fig. 1 for the fabrication of PDMS microchannels. First, a hole was punched on one side of an aluminum dish (Fisher) using a syringe needle. A fused-silica capillary was then inserted through the hole and kept at the bottom of the dish. The hole was sealed with epoxy then the PDMS prepolymer was poured into the dish. The capillary was used as a template for the fabrication of microchannel, as well as the fluid inlet/outlet. After the PDMS prepolymer was cured at 200°C for 2 h inside a GC oven, the aluminum dish was carefully peeled off to obtain a PDMS substrate with the embedded capillary. A microchannel was formed

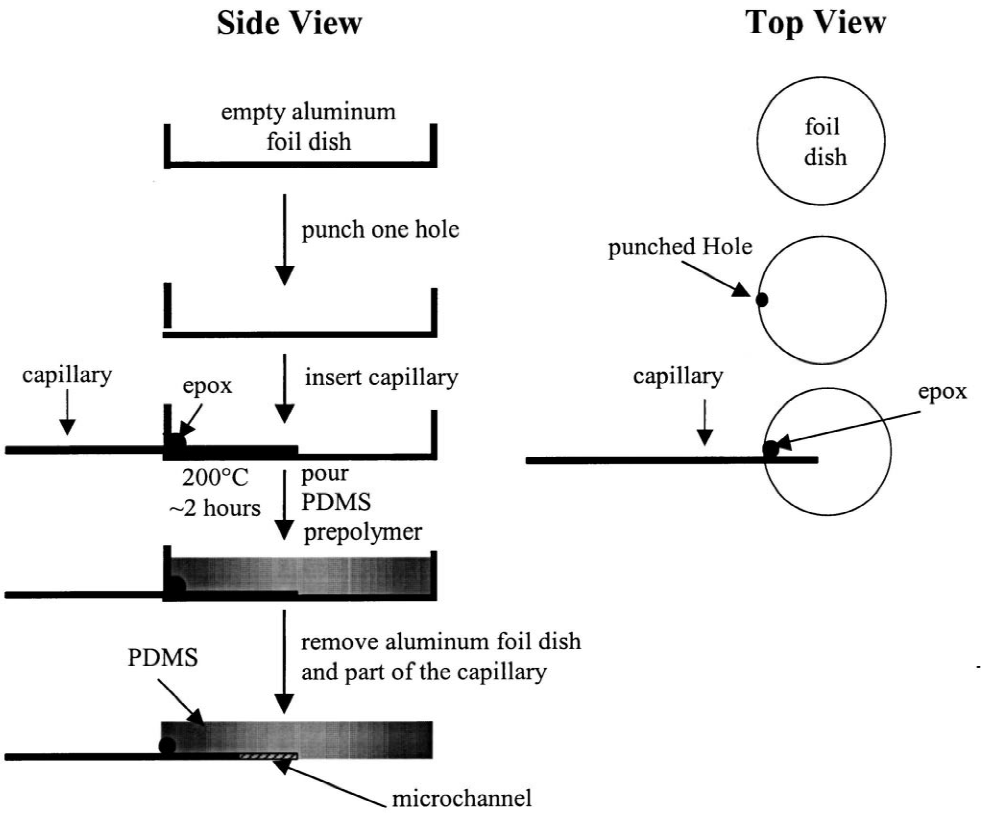

Fig. 1. Procedures for fabricating PDMS microchannels used in miniaturized membrane chromatography for chiral separation.

by the removal of the last 0.5 cm of capillary tubing in the PDMS substrate.

2.3. Assembly of miniaturized membrane chromatography for chiral separation

A symmetrically configured membrane chromatography system (see Fig. 2) consisted of two aluminum plates, two copolyester plates, two PVDF membranes, and two PDMS substrates containing the microchannels and the capillaries. To assemble the system, the membranes were first sandwiched between two PDMS slabs with microchannels facing the membranes. Microchannels were aligned using the capillaries extending out of PMDS substrates. Two copolyester plates were used to provide the additional support and were clamped between two aluminum plates.

2.4. On-line BSA adsorption and chiral separation using miniaturized membrane chromatography

The 10 cm long capillary extending out of the upper PDMS substrate was employed as the solution inlet and was connected to a Harvard Apparatus PHD 2000 syringe pump (Holliston, MA) through a 250 μl Hamilton syringe (Reno, NV). A detection window was created on a 10 cm long capillary extending out of the lower PDMS substrate for on-column monitoring of eluants using a UVIS 200 detector (Linear Instruments, Reno, NV). Both BSA and racemic compounds (including thiopental and tryptophan) were monitored by UV absorbance at 280 nm and 218 nm, respectively.

Four different buffer solutions, including 20 m*M* Tris at pH 8, 20 m*M* Tris at pH 6, 20 m*M* phosphate at pH 6, and 20 m*M* phosphate at pH 6 containing 100 m*M* ammonium sulfate, were employed for the

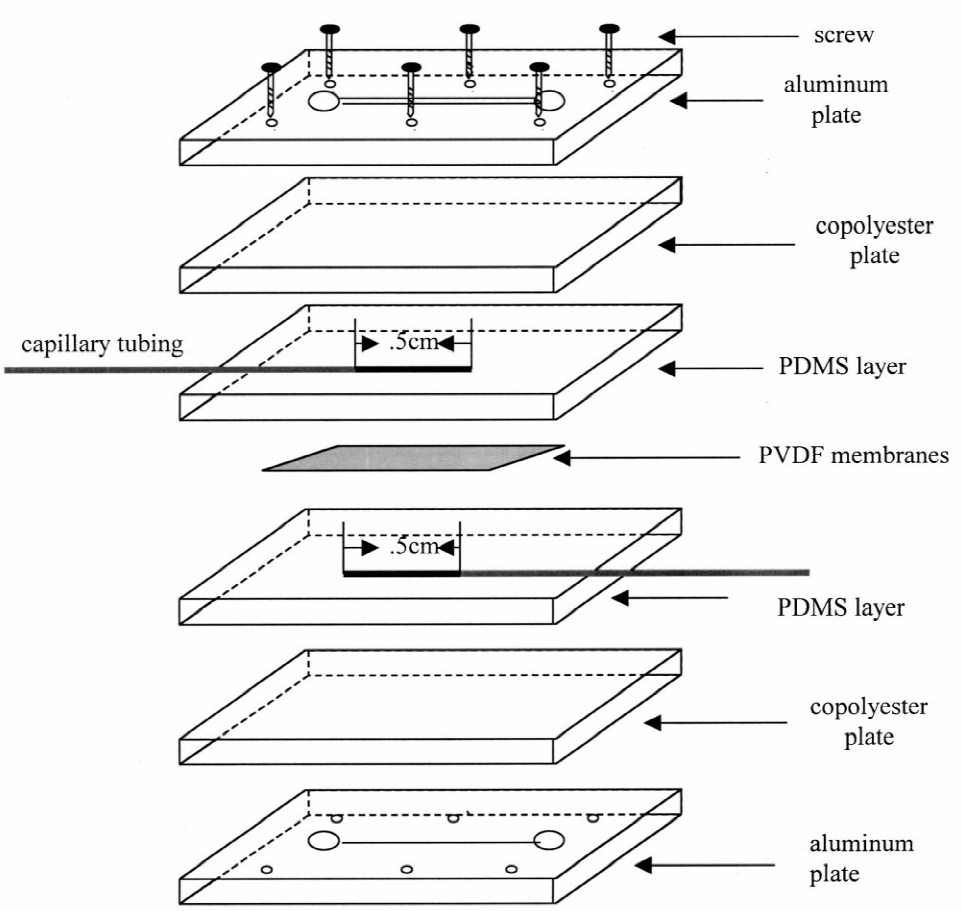

Fig. 2. Assembly of complete miniaturized membrane chromatography containing preformed PDMS microchannels. Channels are fabricated in the PDMS substrates. The copolyester pieces are used to provide structural support to the soft PDMS substrates.

preparation of BSA solutions with a final concentration of 1 mg/ml. The miniaturized membrane chromatography system was presoaked and washed by introducing a 2 h 20 m*M* phosphate buffer flush at a flow-rate of 0.1 μl/min. A BSA solution was then introduced through the inlet capillary and was forced to permeate across the PVDF membranes. The amount of BSA adsorbed onto the membranes was calculated by integrating the concentration difference between the feed and the effluent in the outlet capillary extending out of the lower PDMS substrate. Effects of solution conditions on BSA adsorption were studied by introducing the BSA solutions prepared in various buffers.

The miniaturized membrane chromatography system containing adsorbed BSA was flushed with a 20 m*M* phosphate buffer at a flow-rate of 0.8 μl/min for 5 min to remove any unbound BSA. D,L-thiopental with a final concentration of 0.6 m*M* was prepared using 20 m*M* Tris at pH 6. A 50 nl sample plug containing D,L-thiopental was injected at a flow-rate of 0.1 μl/min using a syringe pump. To study the effect of ammonium sulfate on chiral separation of D,L-thiopental, 20 m*M* Tris buffer at pH 6 was employed as the mobile phase and was introduced at a flow-rate of 0.8 μl/min in the absence or presence of 100 m*M* ammonium sulfate.

Equal molar mixture of D- and L-tryptophan with a total concentration of 0.6 m*M* was prepared using 20 m*M* Tris at pH 6. Immediately after the injection of a 50 nl D,L-tryptophan sample plug, the buffer solution containing 20 m*M* Tris and 100 m*M* am-

monium sulfate was employed at a flow-rate of 0.8 μl/min for the elution of racemic analytes in the miniaturized membrane chromatography system. To study the effect of solution pH on chiral separation of D,L-tryptophan, the solution pH in the mobile phase was varied between 5 and 7.6.

The separation factor, α, can be defined as:

$$\alpha = (t_L - t_O)/(t_D - t_O) \quad (1)$$

where t_O is the void time of the miniaturized membrane chromatography system and t_D and t_L are the retention times of D- and L-analyte in the chromatogram, respectively. The void volume in the miniaturized membrane chromatography system and the void time at a flow-rate of 0.8 μl/min were estimated to be around 0.64 μl and 0.8 min, respectively. The separation factors for the resolution of racemic tryptophan and thiopental mixtures in microfluidics-based membrane chromatography were measured under various mobile phase conditions.

3. Results and discussion

3.1. BSA adsorption onto the PVDF membrane

BSA consists of 583 amino acid residues [38,39] and is a relatively acidic protein with pI around 4.7. The PVDF membrane (Immobilon-P^{SQ}, pore diameter of 0.10 μm) employed in this study is naturally hydrophobic and is designed for Western transfers and protein sequencing procedures. The amount of BSA adsorbed onto two layers of PVDF membrane was measured by integrating the concentration difference between the feed and the effluent in the outlet capillary monitored by UV absorbance at 280 nm. The amounts of adsorbed BSA at four different solution conditions are summarized in Table 1.

The hydrophobic nature of BSA adsorption onto the PVDF membrane was further supported by the increase in the amount of adsorbed BSA with decreasing solution pH from 8 to 6 in Tris buffer. The hydrophobicity of BSA increases by adjusting the solution pH close to its p*I* of 4.7. In contrast to the studies reported by Nakamura et al. [24], the adsorption of BSA onto a chemically modified hollow fiber membrane was optimized at pH 8. The BSA adsorption in their studies was attributed to the ionic interactions between the protein molecules and the diethylamino groups as the anion-exchange moieties on the membrane.

Table 1
Solution condition dependence of BSA adsorption onto PVDF membrane

Solution conditions	Specific amount of adsorbed BSA ($\mu g/cm^2$)[a]
20 m*M* Tris at pH 8	75
20 m*M* Tris at pH 6	150
20 m*M* Phosphate at pH 6	300
20 m*M* Phosphate/100 m*M* ammonium sulfate at pH 6	400

[a] Specific amount of adsorbed BSA ($\mu g/cm^2$) = the amount of adsorbed BSA (μg)/the exposed frontal surface area (cm^2).

Instead of using Tris buffer, the application of phosphate buffer further increased the amount of BSA adsorbed onto the membrane (see Table 1). The addition of ammonium sulfate into the phosphate buffer contributed to the so-called salting-out effect for achieving the maximum adsorbed amount of BSA. The frontal surface area of two PVDF membranes exposed to the PDMS microchannel in the upper PDMS substrate (see Fig. 2) was estimated to be around 5×10^{-3} cm^2 (0.5 cm length × 100 μm width). Thus, the maximum adsorbed amount of BSA was estimated to be around 400 μg per cm^2 of frontal surface area in each of two PVDF membranes.

The measured BSA adsorption capacity was in good agreement with that of 340 $\mu g/cm^2$ reported by the manufacturer (Fisher Catalog 2000/01, page 974). The porous structure of this PVDF membrane provides a large internal surface area (over 400 cm^2 of internal surface per cm^2 of frontal surface) for protein adsorption. By assuming 400 cm^2 of internal surface per cm^2 of frontal surface, the maximum adsorbed amount of BSA was converted from 400 $\mu g/cm^2$ (of frontal surface) to 10 mg per m^2 of pore surface area. Theoretical values of 2.5 mg/m^2 and 8 mg/m^2 were reported for saturated adsorption of BSA with a side-on and an end-on orientation onto the surfaces, respectively [24]. The discrepancy between our estimate and theoretical values could be the result of the underestimation of available pore surface area for BSA adsorption.

3.2. Miniaturized membrane chiral separation of D,L-thiopental

For performing chiral separation in miniaturized membrane chromatography system, on-line BSA adsorption was carried out using a 1 mg/ml BSA solution containing 20 m*M* phosphate buffer (pH 6) and 100 m*M* ammonium sulfate for the preparation of chiral stationary phase. A 50 nl sample plug containing 0.6 m*M* D,L-thiopental was injected at a flow-rate of 0.1 μl/min. The chromatograms of a racemic D,L-thiopental solution are shown in Fig. 3A and B using Tris buffer (pH 6) as the mobile phase at a flow-rate of 0.8 μl/min.

No clear chiral separation of D,L-thiopental was observed using only 20 m*M* Tris at pH 6. By simply adding ammonium sulfate into Tris buffer, significant enhancement in chiral resolution of D,L-thiopental was obtained with a separation factor of 1.74. Similar to the effect of ammonium sulfate on the adsorption of BSA onto the membrane, the addition of ammonium sulfate as a salting-out reagent provides further increase in the hydrophobic interaction between BSA and D,L-thiopental as evidenced by the increase in elution times. Thus, the increase in the interaction between BSA and D,L-thiopental may contribute to the enhanced chiral resolution. The middle peak between D- and L-thiopental shown in Fig. 3 B may be the result of impurities in the sample.

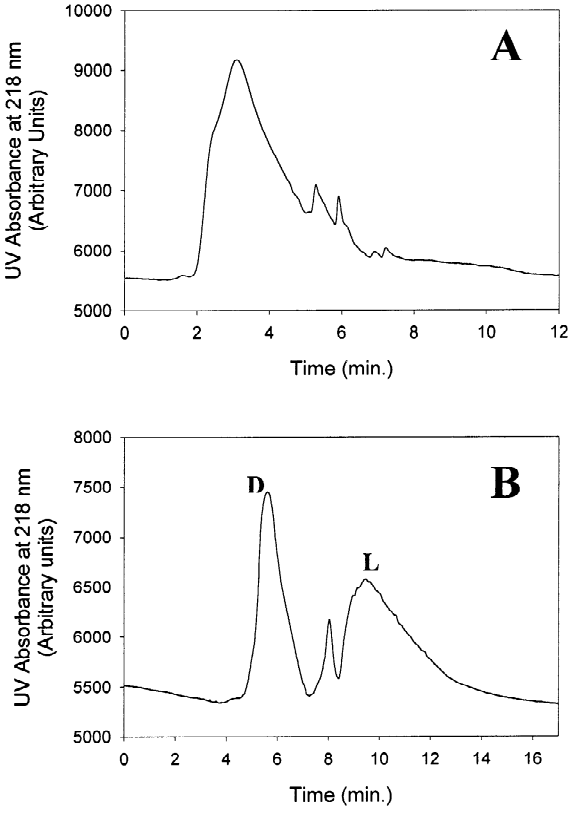

Fig. 3. Chiral separation of D,L-thiopental in the miniaturized membrane chromatography system using (A) 20 m*M* Tris at pH 6 and (B) 20 m*M* Tris and 100 m*M* ammonium sulfate at pH 6 as the mobile phase.

3.3. Miniaturized membrane chiral separation of D,L-tryptophan

A 50 nl sample plug containing 0.6 m*M* of D,L-tryptophan was injected at a flow-rate of 0.1 μl/min. To study the effect of solution pH on chiral separation of D,L-tryptophan in the miniaturized membrane chromatography system, the solution pH in the mobile phase of 20 m*M* Tris and 100 m*M* ammonium sulfate was varied between 5 and 7.6. The chromatograms summarized in Fig. 4 illustrated the baseline resolution of D,L-tryptophan at all three solution pHs.

The use of two layers of PVDF membrane containing adsorbed BSA ensured satisfactory resolution of D,L-tryptophan. The separation factors among D,L-tryptophan increased with increasing solution pH from 1.8 at pH 5 to 4.0 at pH 7.6. BSA was reported to exhibit a 100-fold greater affinity for L-tryptophan over D-tryptophan in solution by McMenamy and co-workers [40]. The separation factor of D,L-tryptophan for BSA immobilized on various supports ranged from 1.2 to 14 under different mobile phase conditions [5–8,11,24]. Moreover, separation resolution, R_s, also increased from 0.55 at pH 5 to 3.73 at pH 7.6. The R_s of D,L-tryptophan using immobilized BSA as chiral stationary phase ranged from 1.96 to 5.75 at pH 7.7 using various eluants [8].

The observed effect of solution pH on chiral separation of D,L-tryptophan using BSA as the stationary phase was consistent with those reported in the literature [6,7]. The well known fast, virtually immediate, and reversible changes in albumin conformation over the whole pH range of the albumin

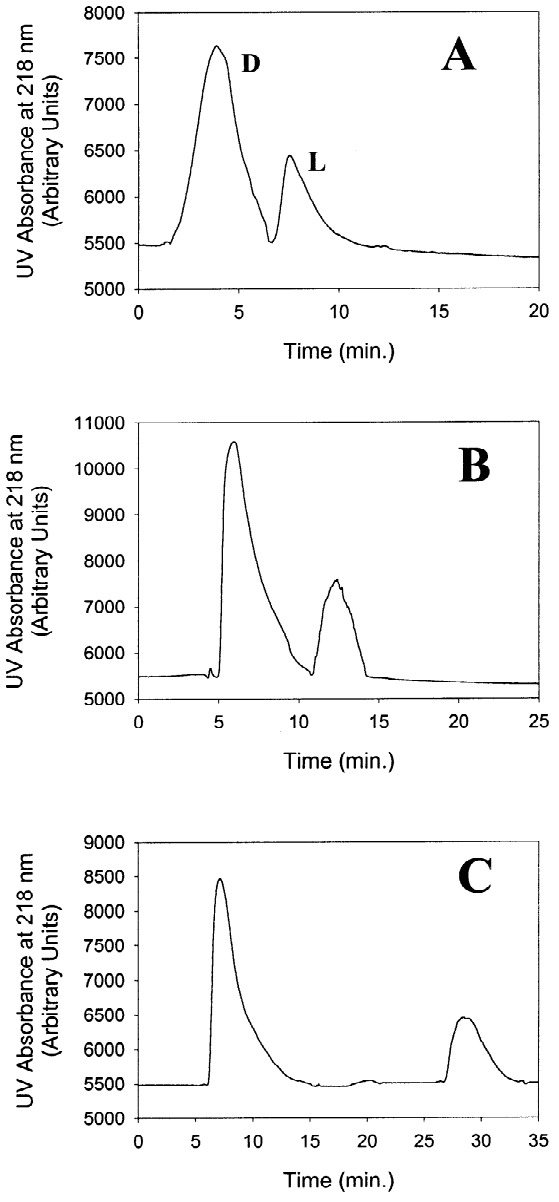

Fig. 4. Chiral separation of D,L-tryptophan in the miniaturized membrane chromatography system using the mobile phase of 20 m*M* Tris and 100 m*M* ammonium sulfate at (A) pH 5, (B) pH 6, and (C) pH 7.6.

titration curve are commonly utilized as the most effective tool in the control of both analyte retentions and chiral separation selectivity [11]. In contrast, slow changes in the albumin enantioselectivity and in its ability to bind analytes of various types occur in weakly alkaline mobile phases in the pH range 7–10.

The robustness of the miniaturized membrane chromatography system is proven to be quite high as evidenced by continuous and repeated analysis of D,L-tryptophan for at least 1 week. The system was stored at 4°C when it was not in use. Run-to-run variation of D,L-tryptophan separation is about 5–10% and is in good agreement with other membrane chromatography-based chiral separations reported in the literature [4,24]. No leakage of BSA and no change in BSA's selectivity toward D,L-tryptophan were detected during repeated separations for at least 1 week. Furthermore, the miniaturized membrane chromatography system can be regenerated by simply replacing the old or clogged membranes with two new PVDF strips, followed by re-adsorption with a fresh BSA solution.

4. Conclusions

A microfabricated membrane chromatography system containing adsorbed BSA as the stationary phase is developed and demonstrated for performing high resolution chiral separation of racemic mixtures of D,L-thiopental and D,L-tryptophan. Capillary molding technique is employed for the construction of PDMS microfluidic network without the need of sophisticated microfabrication facilities in a clean room. The PDMS microchannels containing direct capillary connections allow easy interfacing with external fluid delivery and detection instruments.

Significant reduction in dead volume (0.64 μl), sample consumption (50 nl sample plug containing 0.6 m*M* D,L-thiopental or D,L-tryptophan), and mobile phase usage can be achieved by performing chiral separation in an integrated and miniaturized membrane chromatography system. On-line adsorption of BSA onto two layers of PVDF membrane is employed for the preparation of chiral stationary phase and the studies toward the effects of solution conditions on BSA adsorption. The system further facilitates rapid screening of mobile phase conditions and potentially allows the usage of minute chiral ligands in the preparation and selection of stationary phases for the optimization of chiral separation.

Acknowledgements

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