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Modification of Monolithic Stationary Phase Using Human Serum Albumin as Chiral Separation

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Abstract. Enantiomers can have different pharmacological effects. Therefore, it is necessary to be able to separate pure enantiomers that have a beneficial biological activity. The purpose of this study was to modify a monolith column in stationary phase with Human Serum Albumin (HSA) for use in separating chiral compounds by HPLC. In this work, poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) (poly(GMA-co-EDMA)) and poly(glycidyl methacrylate-co-trimethylolpropane trimethacrylate) (poly(GMA-co-TRIM)) columns were in-situ co-polymerized inside a silicosteel tubing. A total monomer ratio (% T) and crosslinking agent (% C) 40:25 and 28:12 were applied for the preparation of poly(GMA-co-EDMA) and poly(GMA-co-TRIM), respectively. The porogen used was 1-propanol, 1,4-butanediol, and water in a ratio of 7:4:1 (v/v) and AIBN as an initiator (1% of the total monomer). The column was polymerized at 60 °C for 12 h, then immobilized with various concentrations of Human Serum Albumin (HSA). The monolithic poly(GMA-co-TRIM) column modified with HSA 1 mg/ml was successfully applied to separation of the enantiomers of citronellal using an acetonitrile and water (50:50, v/v) as the mobile phase at 0.05 ml/min. The permeability of this optimized monolithic column was 0.0018 Darcy.

Keywords: albumin, chiral separation, monolith column, stationary phase.

INTRODUCTION

The recent rapid development of the monolith column has resulted in a variety of applications, including use as a separator column for chiral compounds. Chiral compounds can be separated by gas chromatography,[1-3] high performance liquid chromatography (HPLC),[4-7] and capillary electrophoresis.[8-10] Polymer-based monolith columns can be used as separator columns for chiral compounds. The advantages of organic polymer columns include their ability to separate biomolecules stability over a wide pH range and their ease of manufacture and modification.[11] The monolith column has been used in HPLC and has the advantages of having low back pressure and being easy to use. It can be applied for the selective separation of complex samples, including the separation of chiral compounds.[12,13]

Monolith columns for the separation of chiral compounds have been modified with chiral selectors such as a chiral molecule or a molecule with a chiral surface structure.[14] Chiral selectors included cyclodextrin derivatives, polysaccharides, and proteins.[15] The use of cyclodextrins as chiral selectors requires long, complicated synthetic procedures making it inefficient.[16] Proteins have the potential to be chiral selectors because proteins are chiral in their primary structure (they are made up of L-amino acids) and so will interact differently with different binding sites and possess different affinities with chiral molecules.[17] Human Serum Albumin (HSA) was employed as the selector in this study. HSA is an abundant protein in blood plasma comprising up to 60% and has a molecular mass of 66,500 Da and isoelectric point (pI) 4.8. Isocratic conditions can be adopted for immobilization of HSA onto columns.[18-19]

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Essential oils can possess important properties, for example citronellal acts an anti-inflammatory.[20] Others have antimicrobial properties or can be used as pesticides.[21-22] However, not all the components in essential oils are beneficial and so it is important to be able to separate out the beneficial compounds.

The objective of this study was to prepare a monolith-based methacrylate column using a monomer of GMA with two kinds of cross-linker (EDMA and TRIM) and a ternary porogen so that the sample separation process becomes selective and the columns are stable over a wide pH range. Several parameters affecting the performance of the monolith columns, including the solvent composition, porogen composition, flow rates, and HSA concentrations, were investigated in detail. The performance of the columns in separating enantiomeric compounds from citronellal was tested.

MATERIAL AND METHODS

Materials and Instrumentation

The following chemicals were obtained from Sigma Aldrich: gycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EDMA), 1-propanol 1,4-butanediol 99%, aquademin, 2,2- azobis(isobutylonitrile) (AIBN) Human Serum Albumin (HSA), tris(hydroxymethyl) aminomethane, pyridine, NaOH, HCl, sodium carbonate > 99%, ammonium bicarbonate > 99%, 3-(trimethoxysilyl) propyl methacrylate (MAPS) 98%, citronellal. Other chemicals were trimethylolpropane trimethacrylate (TRIM, TCl), AIBN (Indiamart), acetonitrile (Merck), ethanol, and acetone (Smart Lab). Fused silica capillary (silicosteel columns; 10 cm in length, outer diameter 1/16 inch and 1 mm inner diameter, Supelco) were used. All materials had p.a purity and were used directly without further purification.

The chromatographic system used in this work was a HPLC prominence 20 from Shimadzu equipped with LabSolution software, consisting of degasser units (DGU-20A), pumps (LC-20AD), oven (CTO-20A), UV-VIS detector (SPD-20A), a semi-micro flow cell (2.5μL), a Rheodyne 7125 injector with 2 μL loop sample, and controller (CNM-20A).

Monolith Preparation

Prior to use, the silicosteel columns were first silanated using the procedure as described by Shu *et al.*[23] with minor modification. Silicosteel columns was pretreated by filling the column with 0.2 M NaOH for 30 min twice, washing with water, filling with 0.2 M HCl for 30 min twice, and finally rinsing thoroughly with water and acetone. MAPS solution (MAPS:acetone:pyridine = 30:65:5) was used to fill the activated column. Thereafter, the silicosteel column was placed at room temperature for 12 h twice, with both ends sealed. After the process was complete, the silicosteel columns were washed with acetone and cut into 10 cm long pieces, ready to be filled with polymer solution. The synthesis process is shown in Fig. 1.

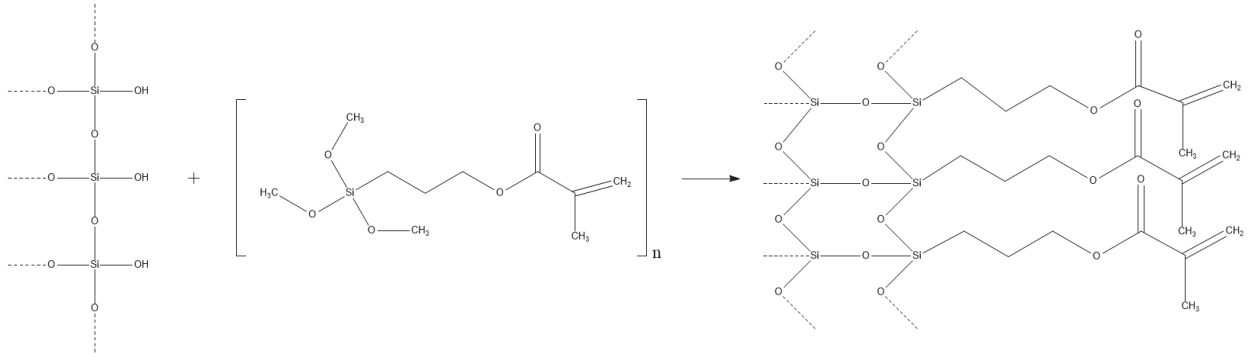

FIGURE 1. Modification of a fused-silica column wall with MAPS for surface modification.

In this study, two types of monolith columns were made, (1) the monolith poly(GMA-co-EDMA) column using the procedure of Tasfiyati *et al.*[24] consisted of a mixture of 40% GMA and 25% EDMA and (2) the monolith poly(GMA-co-TRIM) column using the procedure of Pfaunmiller *et al.*[25] (Fig. 2) with a slight change in porogen

composition, this monolith consisted of a mixture of 28% GMA and 12% TRIM. The porogen used was 1-propanol, 1,4-butanediol, H_2O (7:4:1, v/v) and AIBN 1 % (w/v) as the initiator.

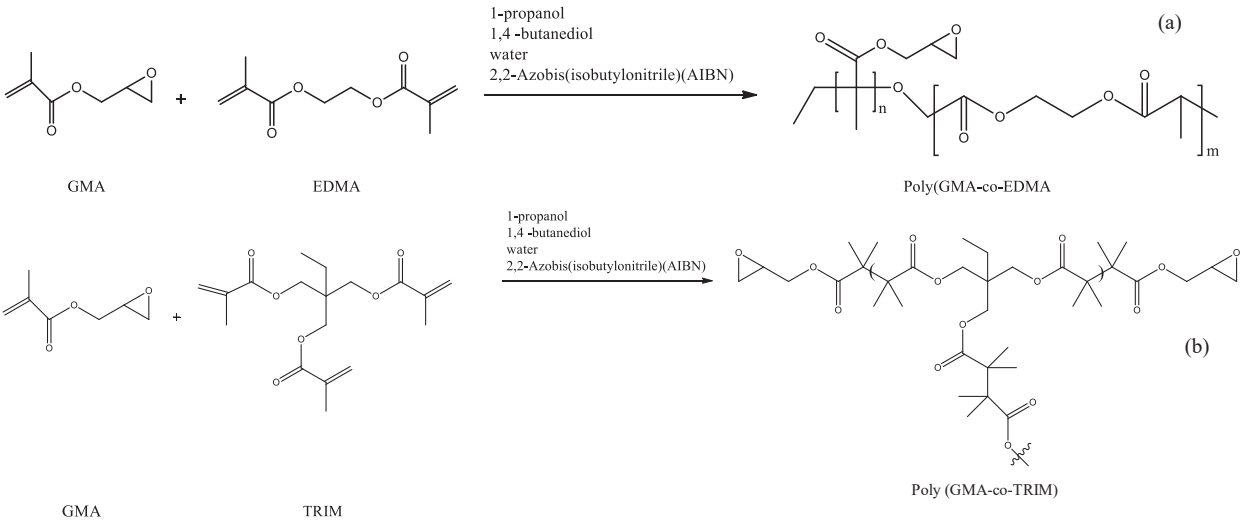

FIGURE 2. The polymerization reaction of the monoliths (a) poly(GMA-co-EDMA) and (b) poly(GMA-co-TRIM).

The mixture of monomers, crosslinking agents, porogen, and initiator was left to react for 30 min at room temperature before being injected into a silanated column, both ends of each column were closed using end plugs, and then polymerized at 60 °C for 12 h. After polymerization, the monolith poly(GMA-co-EDMA) was washed with ethanol and water for about 1 h with a flow rate of 0.05 ml/min. While the monolith poly(GMA-co-TRIM) column was washed using acetonitrile and H_2O for 1 h with a flow rate of 0.05 ml/min.

Immobilized Human Serum Albumin (HSA)

HSA was immobilized using the epoxy method of Malik *et al.*[19] (Fig. 3). Each monolith column was carried out by circulating solution containing 1-2 mg/ml HSA in pH 9.0, 0.2 M carbonate buffer through the monolith column for 6 h at room temperature and 0.05 ml/min. The epoxy group was blocked by passing through the column using pH 8 0.2 M Tris buffer for 2 h at room temperature. The monolith column was then washed for 4 h using pH 9 0.2 M a carbonate buffer at flow rate of 0.05 ml/min. Columns were stored at 4 °C before use.

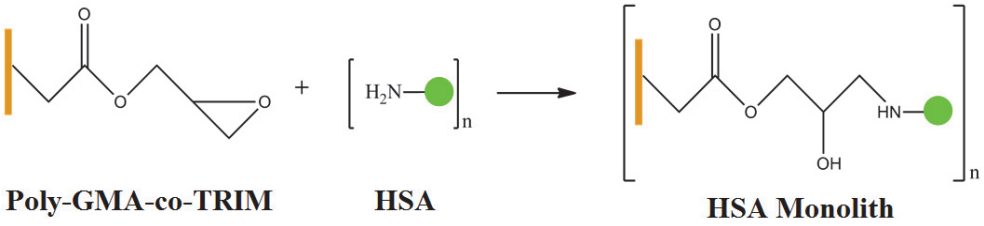

FIGURE 3. HSA immobilization reaction by the epoxy method.

Monolith Column Permeability

The permeability of each monolith column was tested for use in HPLC with a mobile phase of acetonitrile: H_2O (50:50, v/v). The backpressure was recorded at flow rates from 0.01 ml/min to 0.1 ml/min and the permeability calculated using the Darcy equation.[24] Fig. 4 shows the setup of the HPLC.

$$K = \frac{\eta \times L \times u}{\Delta p} = \frac{\eta \times L \times F_m}{\Delta p \times \pi \times r^2} \quad (1)$$

in which

K : Permeability (m^2)
L : Column length (cm)
u : Linear motion phase ($m\ s^{-1}$)
r : Column radius (m)
η : Motion phase viscosity (Pa.s)
Δp : Backpressure (Pa)
F_m : Flow rate of the mobile phase ($m^3 s^{-1}$)

Separation Procedure of Chiral Compounds with HPLC

The modified monolith columns with immobilized HSA were applied to separate the enantiomers present in citronellal. Up to 2.5 ppm (2µl) of sample was injected into the HPLC system and then separated using the chiral monolith column with the optimized mobile phase composition of acetonitrile: H_2O and the UV detector was set at 274 nm wavelength.

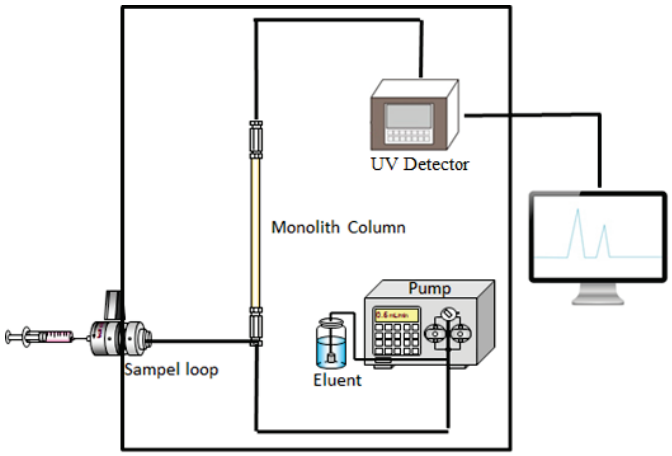

FIGURE 4. HPLC Instrument.

RESULT AND DISCUSSION

Preparation of Monolithic Columns

The silanization process using MAPS was performed prior to the in-situ copolymerization, to modify the surface of the silicosteel column and provide functional groups that would bind covalently with the monolith polymer.

Two types of monolith columns were made with various crosslinking agents namely poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) (poly (GMA-co-EDMA)) and poly(glycidyl methacrylate-co-trimethylolpropane trimethacrylate)(poly (GMA-co-TRIM)). The poly(GMA-co-EDMA) column was made from GMA and EDMA in a ratio of 40:25, while for poly(GMA-co-TRIM) the ratio of GMA:TRIM was 28:12. The porogen used was a mixture of 1-propanol, 1,4 butanadiol, water (7:4:1, v/v), and AIBN as a radical initiator (1% w/v). The porogen forms pores in the monolith columns. The use of AIBN as an initiator may affect the porosity of the monolith. AIBN forms radicals at a temperature of 40 °C to 80 °C. The polymerization reaction is an addition polymerization and is also a chain growth reaction between two different monomers, forming a random copolymer chain. Polymerization was carried out at 60 °C for 12 h. TRIM has the potential to form a higher density of crosslinks than EDMA, thus increasing the functional groups formed on the surface of monolith. The polymerization reaction is shown in Fig. 2.

The monolith columns were modified using Human Serum Albumin (HSA) as a chiral selector. Each column was immobilized by HSA with a concentration of 1-2 mg/ml dissolved in a pH 9 carbonate buffer. The epoxy group of the immobilized column was blocked by a continuous flow of pH 8 Tris buffer through the column. The immobilized reaction for attaching HSA onto the monolith column is presented in Fig. 3.

Monolith Column Permeability

The backpressure for each monolith column composition used in the permeability tests are shown in Fig. 5.

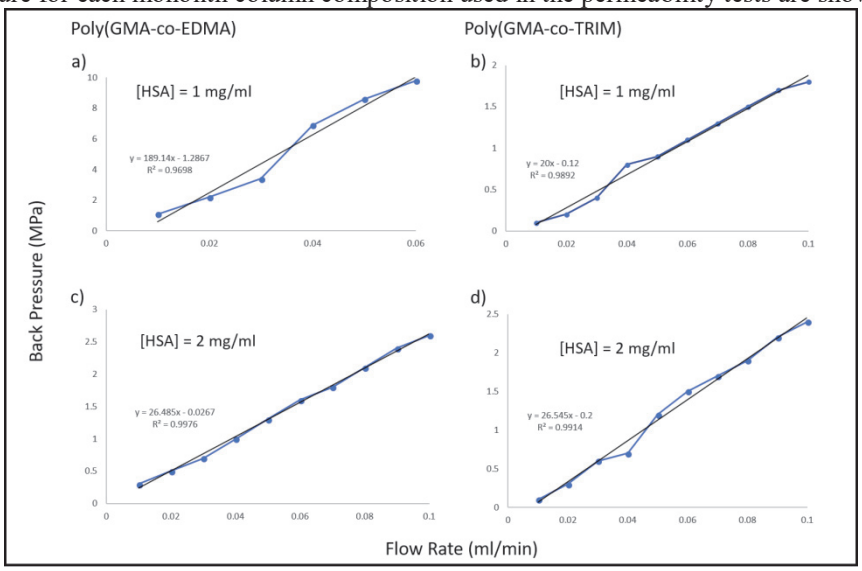

FIGURE 5. Backpressure linearity versus flow rate: (a) poly(GMA-co-EDMA) column with [HSA] = 1 mg/ml; (b) poly(GMA-co-TRIM) column with [HSA] = 1 mg/ml; (c) poly(GMA-co-EDMA) column with [HSA] = 2 mg/ml; and (d) poly(GMA-co-TRIM) column with [HSA] = 2 mg/ml.

Both poly(GMA-co-EDMA) monolith columns had higher backpressure than the poly(GMA-co-TRIM) columns. This is presumably because the poly(GMA-co-EDMA) monolith columns were microporous in character, causing rather poor mass transfer. Consequently, poly(GMA-co-EDMA) monolith columns were not investigated further. In contrast, the poly(GMA-co-TRIM) monolith columns had a low backpressure, so it is likely that this column was more mesoporous in character than the poly(GMA-co-EDMA) columns. The permeability values for each monolith column are shown in Table 1.

TABLE 1. Monolith column permeability

No.	Column	Concentration of HSA (mg/ml)	Permeability (m^2)	Permeability (darcy)
1	GMA-co-EDMA	1	2.1×10^{-15}	0.0021
2	GMA-co-EDMA	2	9.9×10^{-15}	0.0099
3	GMA-co-TRIM	1	1.8×10^{-15}	0.0018
4	GMA-co-TRIM	2	1.5×10^{-15}	0.0015

Application of Monolith Columns for Separation of Chiral Compounds

Only the performance of poly(GMA-co-TRIM) monolith columns in separating citronellal was investigated. The poly(GMA-co-EDMA) column was not used due to high backpressure (more than 10 MPa).

It can be seen in Fig. 6 that the most effective separation of citronellal by poly(GMA-co-TRIM) columns with immobilized HSA occurred with an acetonitrile:water ratio of 50:50 (v/v) for the mobile phase. The polarity of the mobile phase was 7.6 for acetonitrile and water ratio (50:50) and 7.8 for acetonitrile and water ratio (40:60). S(-) citronellal appeared at a retention time of 2.76 min while R(+) citronellal had a stronger interaction with the column and appeared at a retention time 7.28 min.

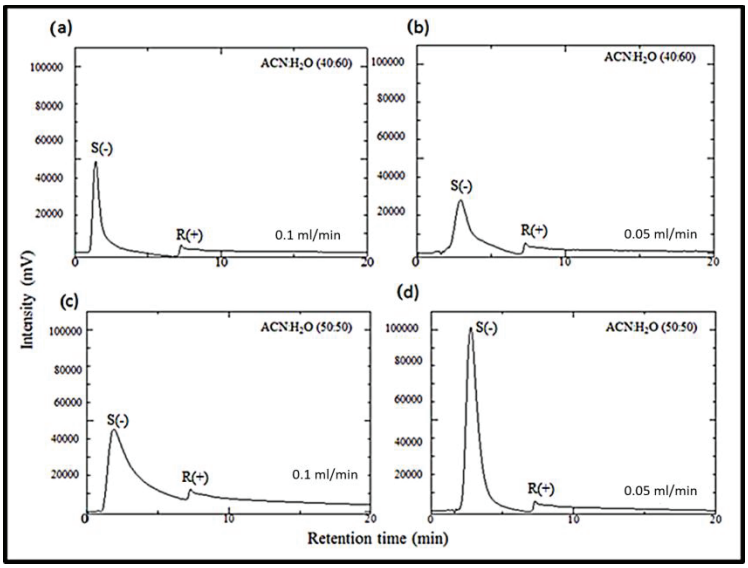

FIGURE 6. The citronellal chromatogram separated using a poly(GMA-co-TRIM) modified by 1 mg/ml HSA monolith column with a mobile phase of (a) acetonitrile and water ratio (40:60) with a flow rate of 0.1 ml/min; (b) acetonitrile and water ratio (40:60) with a flow rate of 0.05 ml/min; (c) acetonitrile and water ratio (50:50) with a flow rate of 0.1 ml/min; and (d) acetonitrile and water ratio (50:50) with a flow rate of 0.05 ml/min.

CONCLUSION

The monolith column poly(GMA-co-TRIM) was successfully prepared by in situ polymerization in silicosteel columns with compositions of %T:%C 28:12 using 1-propanol, 1,4-butanediol, water (7:4:1, v/v), and AIBN initiator 1% (w/v) and subsequently modified with HSA. The column had a permeability of 0.0018 Darcy and was used for the separation of enantiomeric compound from citronellal.

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