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High-performance affinity monolith chromatography for chiral separation and determination of enzyme kinetic constants

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ABSTRACT

A new kind of immobilized human serum albumin (HSA) column was developed by using the sub-micron skeletal polymer monolith based on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) [poly(GMA-EDMA)] as the support of high-performance affinity chromatography. Using the epoxide functional groups presented in GMA, the HSA immobilization procedure was performed by two different means. The affinity columns were successfully adopted for the chiral separation of D,L-amino acids (AAs). Then this method was shown to be applicable to the quantitative analysis of D-tryptophan, with a linear range between 12.0 μM and 979.0 μM, and a correlation coefficient above 0.99. Furthermore, it was used for the analysis of urine sample. This assay is demonstrated to be facile and relatively rapid. So it allows us to measure the enzyme catalytic activity in the incubation of D,L-AAs with D-AA oxidase and to study the kinetics of the enzyme reaction. It implied that the affinity monolithic columns can be a useful tool for studying DAAO enzyme reaction and investigating the potential enzyme mechanism requirement among chiral conversion.

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1. Introduction

High-performance affinity chromatography (HPAC) has held an impressively strong position in the separation and analysis of chemicals. It has many advantages, such as high specificity, ease of automation, and reuse of the same ligand for multiple applications [1–4]. In this approach, an HPLC column contained an immobilized ligand capable of specifically binding the analyte or group of analytes. Up till now, the new protein- and biomolecule-based stationary phases have been widely investigated for special recognition sites and biocompatible properties [5]. Among those biomolecules, human serum albumin (HSA) has been frequently used in silica-based HPLC columns to separate various chiral analytes [3,6] and study drug–protein binding processes [7]. Furthermore, it was also proved to be effective for the separation of amino acid enantiomers (AAs) [8].

AAs are of high biological interest, both as single components and constituents of peptides and proteins. Especially, D-AAs play an important role in the regulation of many processes, such as aging, neural signaling, and hormone secretion [9]. According to pharmacokinetics studies, oxidative determination of D-AAs can be catalyzed by D-AA oxidase (DAAO) to yield hydrogen peroxide and an imino acid. The latter is further non-enzymatically hydrolyzed to an α-ketoacids and ammonium [10]. An assay was established for analysis the DAAO activity in mammalian tissues with the substrate D-tryptophan (D-Trp) analogs by a RP-HPLC system with a fluorescence detector [11,12]. In addition, chiral ligand-exchange capillary electrophoresis of D,L-AAs were studied to determine the enzyme kinetic constants in our lab [13]. However, as a valuable technology of enantiomer separation, HPAC has not been used in the application of quantitative analysis of the real D-AA samples in DAAO enzyme reaction.

Among the various supports of HPAC, polymer monoliths are of particular interest because of good biocompatibility, lack of diffusion resistance during mass transfer and excellent pH stability [14–16]. They have been employed in several previous studies to create affinity monoliths [17,18] and used in such applications as sample purification [19], chiral separation [8] and ultrafast immunoextractions [20]. However, conventional polymer monoliths made by free radical polymerization are often constructed by irregular microglobules and aggregated clusters, which easily lead to low permeability and limited surface area [21,22]. In order to solve this problem, many different types of polymerization have been developed. High internal phase emulsion (HIPE) is a promising method for the preparation of highly porous materials [23]. A novel kind of sub-micron skeletal polymer monolith was devel-

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oped in our lab based on HIPE and block copolymer chemistry [24]. These new monoliths presented good permeability, larger surface area and subsequently larger protein binding capacity [25]. Their unique properties make these supports superior as supports for HPAC.

In this work, the sub-micron skeletal polymer monoliths based on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) [poly(GMA-EDMA)] were explored for the preparation of high-performance affinity monolithic columns containing HSA as a chiral stationary phase. Then it was successfully used for the enantioseparation of D,L-AAs. In addition, the new kind of affinity column was applied to the measurement of DAAO enzyme kinetic constants and analysis of the real D-AA samples in enzyme reaction for the first time. It also hinted that the method is potentially adaptable to the study of enzyme mechanism.

2. Experimental

2.1. Materials

Glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) were purchased from Acros Company (New Jersey, USA). Pluronic F127 (PF127) was obtained from Chuang Qi Company (Beijing, China). Tris hydroxymethyl aminomethane (Tris) was from Fuchen Chemical Plant (Tianjin, China). DAAO (from porcine kidney), all D- and L-AA standards and D,L-Trp, D,L-phenylalanine (Phe), D,L-tyrosine (Tyr) were purchased from Sigma Chemical (St. Louis, USA). HSA was from Beijing Xin Jing Ke Biotechnology Company (Beijing, China). Potassium persulfate, tetrahydrofuran (THF), anhydrous calcium chloride, diethylamine and ethanol were from Beijing Chemical Company (Beijing, China). Other reagents were all of analytical reagent grade. Water was obtained from a triple distilled water system and solutions were filtered through a 0.45-μm membrane before use. A human urine sample was provided by a healthy volunteer. Then 20-fold volumes of MeOH was added and mixed for 2 min. These mixtures were centrifuged at 4500 × *g* for 5 min and the supernatants were collected.

2.2. Equipment

Chromatographic investigations were carried out with a Shimadzu LC-10A HPLC system (Shimadzu, Japan) consisting of a binary LC-10AT HPLC pump and a SPD-10A UV–vis detector. Data processing was performed with a HW-2000 chromatography workstation (Nanjing Qianpu Software, China).

2.3. Preparation of sub-micron skeletal polymer monoliths

In the preparation procedure, the reactor was charged with GMA 2.1 mL, EDMA 1.2 mL, and PF127 (5.5%, v/v), followed by stirring at 400 rpm. To this mixture, an aqueous solution containing potassium persulfate (the initiator, 0.2%, w/v based on H_2O) and anhydrous calcium chloride (the electrolyte, 1.0%, w/v based on H_2O) in deionized water was added dropwise. Stirring was continued for 30 min whereupon a white, milky emulsion was formed. The emulsion was purged with nitrogen for 5 min, transferred to stainless-steel column and cured at 60 °C for 36 h. After cooling to room temperature, the column was connected to the HPLC system to remove the PF127 template and the unreacted monomers by pumping deionized water (50.0 mL) and ethanol (30.0 mL) through the column.

2.4. Immobilization of HSA on sub-micron skeletal polymer monoliths

HSA was immobilized on a 5.0 cm long sub-micron skeletal monolithic column using two different immobilized means. Before immobilization, the columns were equilibrated for 10 min by washing with immobilization buffer (50 mM Tris–HCl buffer at pH 8.7 for epoxy mean and 50 mM Tris–HCl buffer at pH 7.6 for EDA mean containing no HSA). In the dynamic method, the immobilization solution was pumped through the monoliths using a syringe.

2.4.1. Epoxy means

At first, HSA was covalently immobilized on the monolith for 24 h. A 2.0 mg mL^{-1} solution of trypsin was freshly prepared in 50 mM Tris–HCl buffer (pH 8.7). After equilibrating the monoliths with this buffer for 1 h, the protein solution was pumped through the monolith at a flow rate of 0.05 mL min^{-1}. After 1–6 days, the monolith conjugated with HSA was washed with 50 mM Tris–HCl buffer including 0.5 M NaCl to eliminate nonspecific physically adsorbed protein. The residual epoxide groups were blocked by 1 mg mL^{-1} aspartic acid in 50 mM Tris–HCl buffer for 1 h. The immobilization process was performed at room temperature.

2.4.2. EDA means

A mixture of diethylamine and THF (1/1, v/v) was continuously pumped through the monoliths for 7 h at 80 °C, 0.2 mL min^{-1}. Thereafter, the column was washed routinely with THF and deionized water. A solution of 10% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 8.0) was flushed through the monolithic column for 12 h at room temperature. Then HSA was immobilized on the activated support by continuously pumping 2 mg mL^{-1} HSA in 50 mM Tris–HCl buffer (pH 7.6) containing 5 mg mL^{-1} sodium cyanoborohydride ($NaCNBH_3$) for 1–6 days at room temperature. Subsequently, the nonspecifically adsorbed protein and the residual aldehyde groups on the surface of the support were depleted by pumping with 50 mM Tris–HCl buffer (pH 7.6) for 5 h. When not in use, the affinity column should be stored in 50 mM Tris–HCl buffer (pH 7.6) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 4 °C.

2.5. Permeability properties of the affinity column

The permeability behavior of the affinity column was described by pressure drop of monolithic column at different flow rates using pure water as mobile phase. The values of the system pressure were measured at each flow rate without and with the column, and the difference between the two values was calculated as the pressure drop across the column.

3. Results and discussions

3.1. Investigation of immobilization means

For coupling of proteins onto the surfaces, epoxy and EDA means were tested for covalent binding, which eliminated any leakage of the immobilized enzyme. In certain condition, the epoxide functionalities could react directly with the amino groups of the protein molecule (epoxy means). Furthermore, epoxide group also could be modified with a diamine followed by activation using a glutaraldehyde and then protein molecule was grafted (EDA means). The procedures of HSA immobilization on the monolithic column are shown in Fig. 1.

For comparing the efficiency of two immobilization means, affinity columns were applied in chiral separation of D,L-AAs. During the pilot experiments with both immobilization means, no any significant difference have been observed either in the separation results or stability of the prepared protein columns. However, the

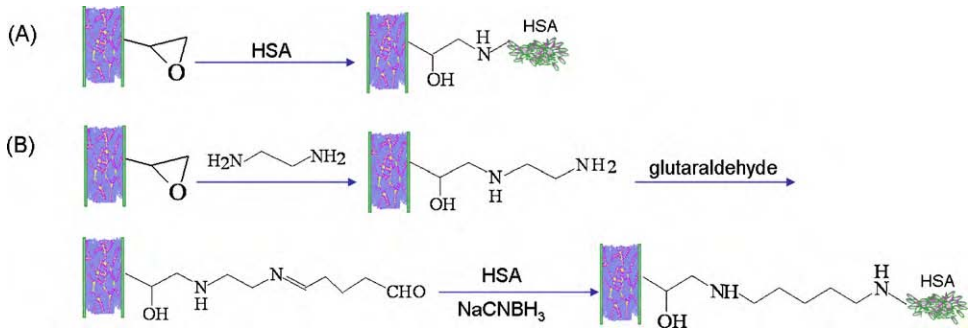

Fig. 1. Schematic diagram of the two preparation means based on the epoxide functional groups: (A) epoxy mean; (B) EDA mean.

process of EDA means was quite complicated and time-consuming. Moreover another disadvantage of this immobilization means was a potential for production of undesirable by-products, *e.g.*, homoconjugates and various polymers [26]. Therefore, the simpler epoxy mean was applied for the preparation of the affinity column.

3.2. Effect of immobilization time

In order to evaluate the efficiency of the affinity columns, three pairs of D,L-AAs (D,L-Trp, D,L-Phe, and D,L-Tyr) were chosen as the test analytes. Enantiomer separation chromatograms of D,L-Trp and D,L-Phe were obtained (Fig. 2 and Fig. 3), while D,L-Tyr was not separated. It has been reported [23] that the interaction between D,L-AAs and HSA occurred at a series of well-defined binding sites on the surface of the protein. The chiral separation efficiency also depends on the strength and the number of sites to which a given molecule binds varying from different compounds or even between different chiral forms of the same compound [27]. It should be mentioned that the mechanism of enantiomer separation was actually far more complicated. Thus the reason of the unsuccessful separation of D,L-Tyr was still not very clear and the mechanism was needed further study.

Furthermore, Fig. 2 displays the separation chromatogram of D,L-Trp in different affinity columns prepared under the different immobilization time for 33 h, 66 h and 100 h, respectively. Additionally, Fig. 3 displays that of D,L-Phe in columns prepared by 33 h and 100 h. It was found that with increasing the immobilization time, the chiral separation efficiency was more obvious. When the immobilization time was 33 h, D,L-Trp was not separated at all. Once it was extended to 66 h, the chirally separation efficiency was improved. Additionally, the best result was obtained under the immobilization time for 100 h. This was presumably because HSA is relatively large protein molecule with the molecular weight of 66.5-kDa [28]. So protein molecules could hardly diffuse into the channels between skeletons and reach adsorption equilibrium in short time. This phenomenon was also quite similar with that

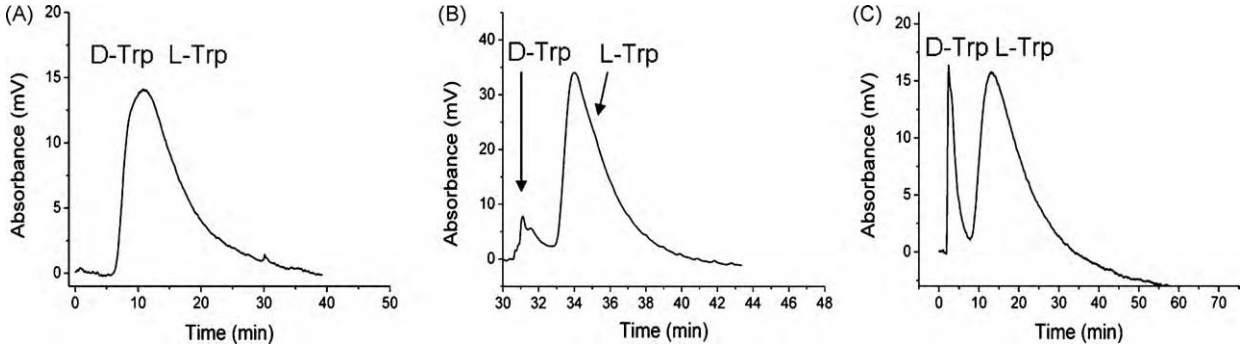

Fig. 2. Effect of immobilization time on the separation of D,L-Trp on the affinity column: (A) 33 h; (B) 66 h; (C) 100 h.

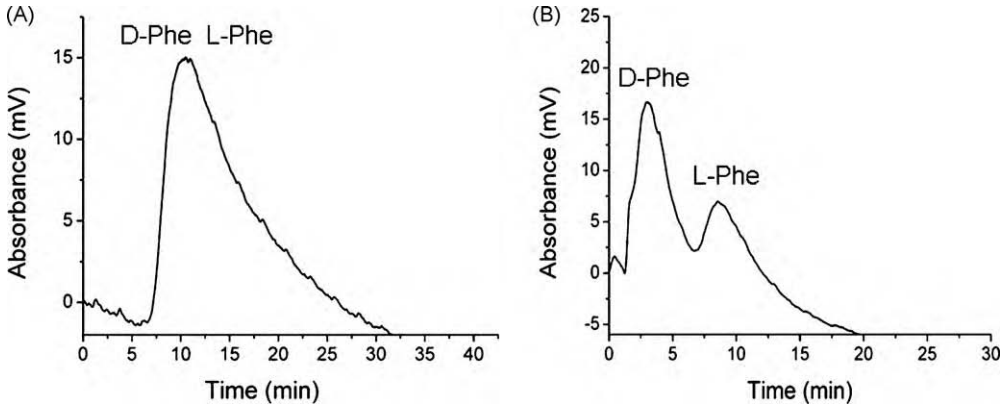

Fig. 3. Effect of immobilization time on the separation of D,L-Phe on the affinity column: (A) 33 h; (D) 100 h.

Table 1
Influence of velocity in the chiral separation of D,L-Trp.

Velocity (cm h^{-1})	t_D (min)	t_L (min)	R_s[a]
3.61	7.74	39.77	0.62
7.22	2.41	13.06	1.01
14.44	1.47	4.71	0.70
18.05	0.63	3.73	0.69

[a] $R_s = 2(t_L - t_D)/(W_L + W_D)$, where t_i is the retention time and W_i is the peak width.

reported in Ref. [8]. In addition, the affinity column under the immobilization time for 133 h was also prepared. Although the immobilization time was further extended, the chirally separation efficiency was not further improved and the result was similar with using the column by 100 h. Furthermore, if the immobilization time was even longer, the more reagent and protein should be used. Thus the immobilization time of 100 h was applied for the preparation of the affinity column in further study.

3.3. Influence of velocity

To investigate the influence of hydrodynamic features of the monolithic support, chiral separation of D,L-Trp were performed by affinity column at different velocity. As shown in Table 1, at the velocity of 3.61 cm h^{-1}, D,L-Trp was not completely separated. Then best separation was obtained at the velocity of 7.22 cm h^{-1}. With the velocities increasing to more than 14.44 cm h^{-1}, the chiral separation efficiency became worse. It had been demonstrated that in the crystal structures of HSA, site I is formed as a pocket in subdomain IIA and involves the lone of the protein [29]. Velocity determines the contact time between protein and D,L-AAs. So if velocity was too high, the contact time was relatively short and the separation efficiency would be decreased. On the other hand, if velocity was lower, the retention time became longer. Therefore the appropriate velocity of 7.22 cm h^{-1} was employed for further study.

3.4. Permeability of the affinity monolithic columns

As a new type of stationary phase for high-performance liquid chromatography, these polymer-based monoliths realized good separation efficiency with a low back-pressure drop, due to a combination of well-defined nanometer-sized skeleton, high porosity, and micrometer-sized macropores. The flow-through properties of affinity monolithic columns were evaluated by determining the chromatographic permeability (K, the superficial velocity based column permeability), which is defined as [30]:

$$K = \frac{\mu_F \eta L}{\Delta P}$$

where L is the length of the column, η the viscosity of the mobile phase, ΔP the backpressure. In the above equation, μ_F is the superficial velocity, which is defined as:

$$\mu_F = \frac{\varepsilon_T L}{t_0}$$

where ε_T is the total porosity of the monolith, t_0 is the dead time of the void marker.

The chromatographic permeability for the affinity monolithic column was found to be ca. 4.6×10^{-13} m^2. The large K value observed implies the presence of larger through-pores and high porosity in the monolith, which result in their superior mass transfer characteristics at higher flow rate. Furthermore, the micrometer-sized macropores and nanometer-sized skeleton reduce the diffusion path length, providing low backpressure. It proved that the affinity monolithic column after immobilization was still stable and not compressed.

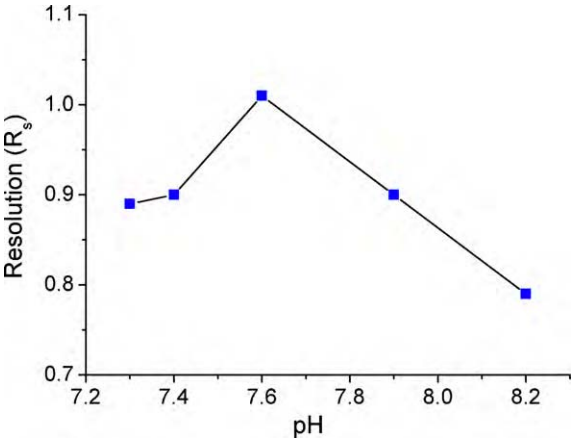

Fig. 4. Effect of buffer pH on the resolution.

3.5. Characterization of the affinity monolithic columns

Reproducibility is a basic requirement with respect to broader application of a novel kind of affinity monolithic column of HAPC. So in order to evaluate the column-to-column reproducibility, we prepared five affinity columns under the same polymerization composition. Then they was evaluated by four injections of D,L-Trp solutions. The relative standard deviation (RSD) of retention time was less than 4.0% and that of peak area was less than 5.0%. Meanwhile, the separation efficiency almost kept the same and the RSD of resolution was less than 4.0%. Additionally, the run-to-run analysis was determined by five injections of D,L-Trp samples. The RSD of retention time was less than 2.0% and that of peak area was less than 5.0%. Moreover, the RSD of resolution was less than 3.0%.

Standard working equations for D-Trp were constructed between peak area (y) and concentration (x). The linear range of the working equations was chosen only when the correlation coefficient reached 0.99, giving a linear range between 12.0 and 979.0 μM. The value of limit of detection (LOD) was 3.0 μM.

We also studied pH effect on chiral separation of D,L-Trp at pH 7.3 to 8.2. As shown in Fig. 4, the R_s increased slightly with the increasing of buffer pH. When pH reached at 7.6, the highest value of the resolution could be gotten. Then, the separation became a bit worse at pH 7.7–8.4. Additionally, when pH was at 7.1 or at 8.5, D,L-Trp was not separated. The reason might be due to the slight change of HSA stereo configuration. However, the process of enantiomer separation was complicated, so the mechanism needed further study. Thus the buffer pH at 7.6 was employed in this work.

3.6. Determination of enzyme kinetic constants

For extending the application of HAPC, the high-performance affinity monoliths were used in the separation of the real samples and determination of enzyme kinetic constants. Fig. 5 shows the typical chromatography related to D,L-Trp samples incubating with DAAO for different time. The effect of the DAAO catalytic reaction was obviously observed by monitoring DAAO incubation time on the production. It could be found that with increasing the incubation time from 5 min to 30 min, the peak area of D-Trp became less and less (Fig. 5). Meanwhile, the peak area of L-Trp remained the same. It was due to that in the DAAO catalytic reaction, D-AAs was catalyzed to yield the products, such as α-ketoacids, which had been mentioned in Ref. [31] and confirmed by the decreasing amount of D-Trp.

High-performance affinity monolithic column showed potential in application to change D- and L-AAs concentrations in the catalytic reaction and kinetic constants determination. To demonstrate this

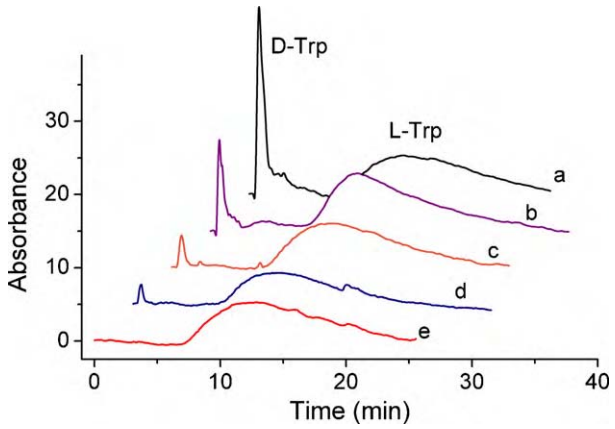

Fig. 5. Chromatograms measured from D,L-Trp incubated with DAAO for different time: (a) 0 min; (b) 5 min; (c) 10 min; (d) 15 min; (e) 30 min.

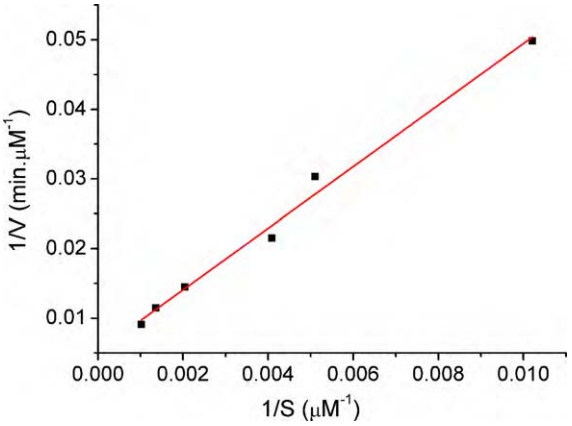

Fig. 6. Determination of DAAO enzymatic kinetic constants by high-performance affinity monolithic column. A Lineweaver–Burk plot is shown. Reactions at each substrate concentration were repeated three times. Points were fit using a linear regression model (R^2 = 0.98). Kinetic constants were K_m = 845.0 μM, and V_{max} = 191.2 μM min^{-1}.

capability, the apparent kinetic parameters, Michaelis–Menten constant (K_m) and maximum velocity (V_{max}), were estimated and D-Trp was used as the substrate. The kinetic study of the enzyme reaction was carried out at various substrate concentrations ranging from 24.4 to 979.3 μM. DAAO (5.0 units/mL) was incubated with those substrates at 37 °C for 5 min. The velocities of DAAO-catalyzed reaction were calculated from the amounts of D-Trp decreased in the enzymatic reaction. Then the velocities were plotted as a function of the D-Trp concentrations. The double-reciprocal plots (Lineweaver–Burk plots) of enzymatic activity were acquired with good linearity (R^2 = 0.98) (Fig. 6). The apparent K_m and V_{max} were extrapolated plotting the initial reaction velocity to the injected D-Trp concentrations and fitting the experimental points with the Michaelis–Menten equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

where v is the velocity of the enzymatic activity, K_m is the Michaelis constant, V_{max} is the maximum velocity, and [S] is the concentration of substrate.

It was calculated that the K_m value was 845.0 μM from the decreased amount of D-Trp versus time graphs for reaction containing one of five different D-Trp concentrations. The V_{max} was determined to be 191.2 μM min^{-1}. These results were in agreement with published methods for evaluating the enzyme kinetic constants [32]. This suggested high-performance affinity monolithic column could be applied in enzyme research.

To assess the applicability of this method, it was used for the analysis of urine sample collected from a volunteer. As shown in Fig. 7(A), no D,L-Trp in urine sample was detected by the affinity monolithic column. Then, D,L-Trp as substrate was added into the urine sample and incubated with DAAO for 15 min or 30 min. Fig. 7(B) shows the peak area of D-Trp became less while the peak area of L-Trp remained the same. At last, D-Trp was totally catalyzed at 30 min. The result demonstrated the potential of this method in real application.

4. Conclusion

This study has shown the preparation of HSA high-performance affinity monolithic columns based on a sub-micron skeletal polymer monolith. In terms of repeatability of the immobilization reaction and long-term stability, the material was demonstrated to be suitable for the immobilization of protein. Two immobilization means were explored for the preparation of affinity columns and the epoxy mean was chosen because of its simplicity and reproducibility. Additionally, influence of immobilization time and velocity was also studied on the separation efficiency of D,L-AAs.

The superior permeability property could be attributed to the high cross-linking homogeneity and larger pore size of the sub-micron skeletal monolith. Furthermore, the affinity monolithic column was shown to work well in the quantitative analysis of real D-AA samples and urine samples in enzyme reaction and determination of enzyme kinetic constants. These results indicated that affinity monolithic columns could be the useful tool for enzyme kinetic study and potential enzyme mechanism exploration.

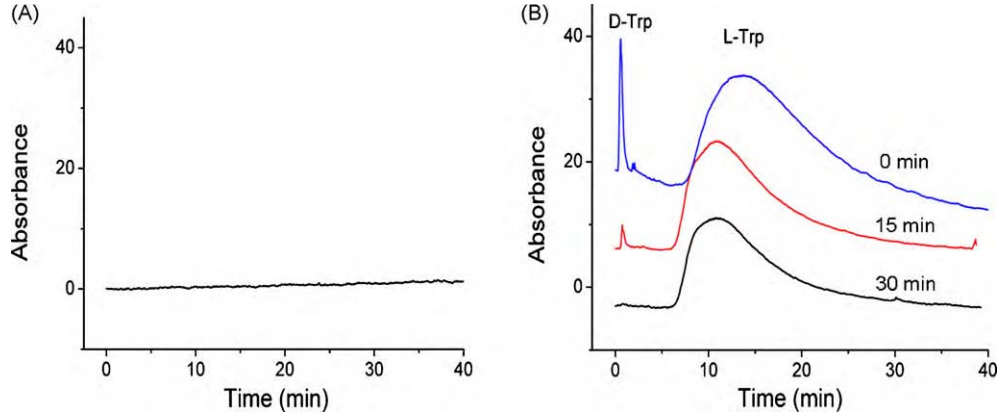

Fig. 7. Chromatograms of urine samples: (A) without adding D,L-Trp; (B) with adding D,L-Trp incubated with DAAO for different time.

Acknowledgements

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