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Preparation of immunoaffinity columns for direct enantiomeric separation of amphetamine and/or methamphetamine

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Abstract

Immunoaffinity chromatographic columns were prepared for direct enantiomeric determination of racemic methamphetamine and amphetamine in this study. The stationary phase was synthesized by covalently bonding an anti-D-methamphetamine monoclonal antibody onto a pre-activated support (e.g. silica, sepharose 4B). Chromatographic results revealed that the immunoaffinity columns achieved enantiomeric separation of racemic amphetamine and methamphetamine. The immunoaffinity columns also have the ability to directly extract D-methamphetamine from urine by changing the pH of the mobile phase, this ability making it practical for the columns to determine a very low concentration of D-methamphetamine in urine.

Keywords: Immunoaffinity columns; Enantiomer separation; Stationary phases, LC; Amphetamine; Methamphetamine

1. Introduction

Methamphetamine has two optically active isomers due to its chiral center, with the D-(+)-methamphetamine (D-MA) being a stronger central nervous system stimulant than the L-(−)-methamphetamine (L-MA). D-MA is therefore more frequently abused world-wide. In particular, in Asia, where its precursor is easily available, since its precursor ''ephedrine'' is a component in ma-huang (a Chinese medicine). Current guidelines for positive identification of methamphetamine abuse is the simultaneous detection of methamphetamine (MA) and amphetamine (AP) with GC–MS. Some legitimate medicines however are metabolized to MA or AP and excreted in urine [1–7]. The effective anti-Parkinson and anti-depressant drug (+)-selegiline (deprenyl), whose precursor of manufacturing is L-MA, can be metabolized in the human body to L-MA and L-AP [4,5]. Therefore, a method must be developed, capable of distinguishing between the D- and L-forms of MA for judging the illicit use of D-MA.

Many methods previously reported for the enantiomeric separation of racemic MA and AP by high-performance liquid chromatography (HPLC) were carried out by derivatizing the analytes with a chiral reagent, and then separating the diastereomers formed on an achiral stationary phase [8–26]. These methods may cause serious error if any optical impurity in derivative reagents is present, since both the diastereomers of RS′ and SR′ have the same chromatographic character. Other methods for the enantiomeric separation of racemic MA and AP by

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HPLC were to derivatize MA or AP with an achiral reagent and then separate the derivatives formed on a chiral stationary phase [27–34]. These methods mentioned above however are time-consuming and tedious, with low sensitivity detection owing to the complicated procedures involved. Direct enantiomeric separation of MA by HPLC with the β-cyclodextrin immobilized stationary phase has been reported [35–37]. Organic solvents such as acetonitrile or methanol were added to the mobile phase during the separation procedure. These organic solvents are toxic, risking the operator's health and environmental pollution. Yukiko et al. directly separated MA enantiomers by HPLC on a chiral crown ether-coated stationary phase [38], but the mobile phase used (pH 1.8 and containing $HClO_4$) may corrode the HPLC system.

Antibody-coupled or Fab-fragments-coupled Sepharose columns had been used for enantioseparation or stereoselective extraction of abscisic acid, amino acids, diarylalkyltriazole or the active metabolite of loxoprofen in human [39–44], however sepharose is not rigid enough to be packed in an HPLC column. Methods of covalent attachment of functional proteins to a solid support had been explored by Shriver-Lake [45]. Clarke et al. also explored immobilization maximum loading capacity for intact immunoglobulin G to HPLC supports [46]. The present study prepared immunoaffinity columns for both liquid chromatography and HPLC by immobilizing anti-D-MA monoclonal antibody to Sepharose 4B and silica gel, and successfully separated the enantiomers of racemic MA with no prior derivation, and no addition of organic solvent in the mobile phase.

2. Experimental

2.1. Chemicals

An anti-D-methamphetamine monoclonal antibody, purified from mouse ascites, was generously provided by the Development Center For Biotechnology in Taiwan. Silica gel (Nucleosil; pore size 300 Å, particle size 7 μm, surface area 100 m^2/g) was obtained from Macherey-Nagel and pre-dried at 180 °C for 16 h before use. Chemicals used in the synthetic processes and in the chromatographic experiments were reagent-grade. This study used 3-aminopropyltriethoxylsilane (APS) (Sigma), CNBr activated sepharose 4B (Sigma), 50% glutardialdehyde solution and toluene (Riedel Dehaen), diethyl ether (J. T. Baker), trichloroacetic anhydride (Fluka), deuterated racemic MA-D_8, MA-D_{14} and AP-D_5 (Fig. 5a; Radian International) and an amphetamine/methamphetamine assay reagents kit (Syva Company). Aqueous sodium phosphate buffer solution (PB) was employed as the mobile phase.

2.2. Synthesis of the sepharose 4B-antibody stationary phase

By the method of Axen et al. [47], 2.5 g of CNBr activated sepharose 4B was reacted with the D-MA specific antibodies in an ice bath for 24 h. After filtration, the colloidal material obtained was washed with 50 m*M* PB (pH 7.0).

2.3. Synthesis of the silica-antibody stationary phase

The antibodies were immobilized onto the surface of silica by the methods described in Refs. [46,48,49]. APS (5 ml) was added to a suspension of pre-dried silica gel (5 g) in 150 ml of toluene, and refluxed for 15 h. The APS-derived silica gel (Fig. 1A) was filtered, washed consecutively with 100 ml each of toluene, methanol and diethyl ether, and stored at room temperature in a vacuum for further use.

The APS-derived silica (5 g) was added to the solution of 140 ml 0.10 *M* of PB (pH 7.0) containing 5% glutardialdehyde, and stirred for 4 h. The suspension was filtered and washed with de-ionized water to obtain the glutardialdehyde modified silica (Fig. 1B).

A 4.2-g aliquot of glutardialdehyde modified silica was suspended in 140 ml, 0.10 *M* of PB (pH 7.0); 10 mg anti-D-MA monoclonal antibodies was added to the suspension and stirred at 0 °C for 4 h, then the suspension was filtered, and the solid washed with 250 ml, 0.10 *M* of PB (pH 7.0) three times to obtain the antibody immobilized silica (Fig. 1C). The quantity of antibody in the final reaction solution and the wash solutions was determined by UV absorbance spectrophotometry. In total, 69% of the anti-

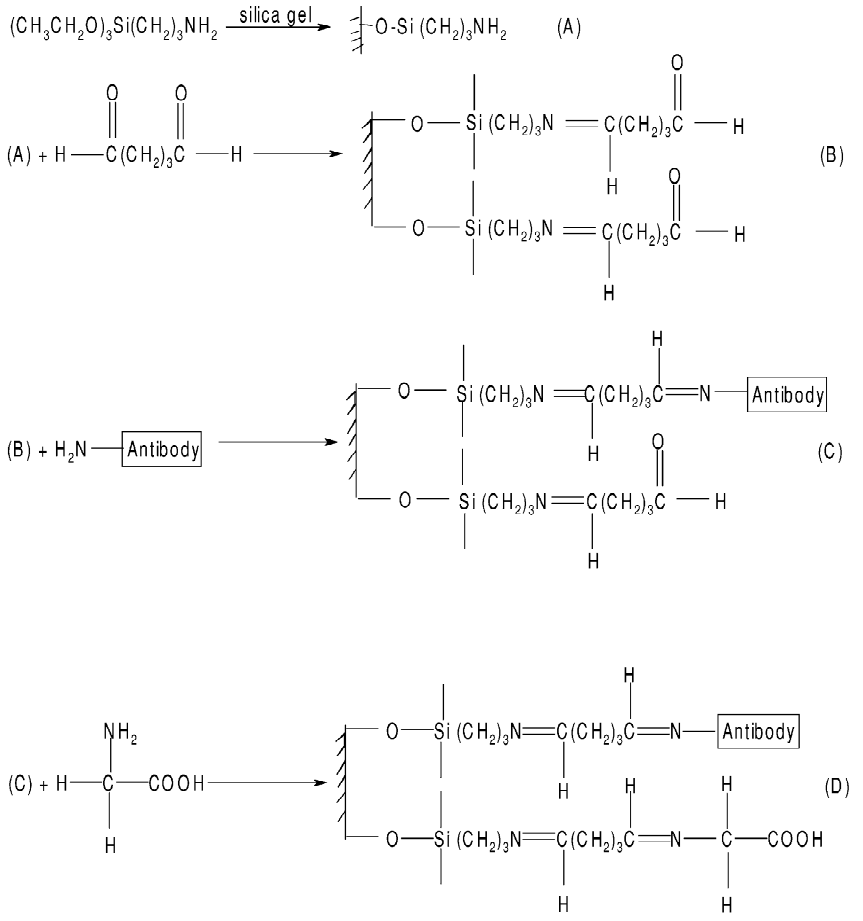


Fig. 1. Preparation of the stationary phase of antibody immobilized silica (ABSP).

bodies added were immobilized onto the surface of silica, 1.6 mg of antibodies were immobilized on 1 g of silica.

The antibody immobilized silica suspended in 20 ml of 0.10 *M* PB (pH 7.0) was mixed with glycine solution (3.7 g dissolved in 50 ml, 0.10 *M* of PB, pH 7.0). The suspension was stirred at 0 °C for 6 h and then filtered, and the resulting solid was washed with 0.10 *M* of PB (pH 7.0) to obtain the antibody immobilized silica stationary phase (ABSP) which had been end capped by glycine (Fig. 1D).

2.4. Column packing

The analytical column for traditional liquid chromatography (LC) was prepared by packing the antibody coupled sepharose 4B manually into a glass column (150 mm×10.0 mm I.D.).

The analytical column for HPLC was prepared by packing ABSP into a stainless-steel tubing (250 mm×4.6 mm I.D.). Approximately 3 g ABSP was suspended in 0.10 *M* of PB (pH 7.0), and poured into a 75-ml packing bomb. The suspended solution was packed into the column under 350 bar with 0.10 *M* of PB (pH 7.0) as the displacing medium.

2.5. Liquid chromatographic studies

The glass column was placed into a LC system, and samples containing racemic amphetamine and/or methamphetamine were loaded into the column, the mobile phase (50 m*M* PB) was poured slowly into the column, gravity gradually causing the mobile

phase to come through the column. The effluent was fractionated with the affinity column by changing the pH of the mobile phase (Fig. 2g). Each fraction collected 0.50 ml of the effluent. All samples except MA addict's urine were prepared by dissolving the solute in pH 7.0, 50 m*M* PB. MA addict's urine was filtered through a 300 Å pore diameter membrane prior to injection. Syva Emit d.a.u. Amphetamine Class Assay (a commercially available EIA kit, employing the EMIT principle) was used to detect the presence of two enantiomers of methamphetamine and amphetamine. The relative rates of fractions measured in each set of experiments carried by LC are exhibited in Fig. 2.

2.6. HPLC chromatographic studies

The characteristics of the HPLC column were investigated with an HP 1050 series HPLC system (Agilent Technologies Taiwan) comprising a solvent pump and auto-sampler. The mobile phase was 50 m*M* PB; its pH was adjusted from 8.0 to 3.0 gradually (Fig. 3b). Effluent was delivered at 0.4 ml/min, and fractions of 0.6 ml were collected. Preparation of samples is the same as described in Section 2.5. Determination of the presence of AP and/or MA in fractions was performed with a Clinical Chemistry Analyzer (Keysys; Boehringer Mannheim) and Syva Emit d.a.u. Amphetamine Class Assay reagents. Different concentrations of D-AP dissolved in normal urine (0, 2000 and 10 000 ng/ml) were used to calibrate the machine prior to the determination. Values obtained (relative rate) of fractions in each set of experiments carried by HPLC are exhibited in Figs. 3 to 8 (except Fig. 5). The cross-reactivities of Syva Emit d.a.u. Amphetamine Class Assay toward D-AP, L-AP, D-MA and L-MA are different and had been experimentally determined in our laboratory. The assay shows preferential reactivity toward D-enantiomers of AP and MA. With EIA method used in this study, it is actually difficult to quantitatively determine simultaneously two enantiomers of AP or MA. Therefore, GC–MS was used to further identify the performance of the HPLC column in enantioseparation of MA.

Deuterated racemic MA (MA-D_8 and MA-D_{14}; Fig. 5a) was separated into respective stereospecific isomers by the HPLC column. Equal volumes of L-MA-D_8 and D-MA-D_{14} (both are 1.1 μg/ml dissolved in pH 7.0, 50 m*M* PB) were mixed, 100 μl of the mixture was injected 20 times into the HPLC column at pH 8.0 of the mobile phase. The column was conditioned at pH 8.0 of the mobile phase for a further 30 min, and then the chromatogram was carried out by changing the pH of the mobile phase as in Fig. 3b. The concentration of L-MA-D_8 and D-MA-D_{14} in each fraction collected was analyzed with GC–MS and the results are shown in Fig. 5b.

To each fraction (0.6 ml) was added 1.0 μg of racemic AP-D_5 (Fig. 5a) dissolved in 100 μl of methanol as internal standard. The solution was made alkaline with the addition of 2.0 ml of 1.0 N NaOH, and extracted with 3.0 ml of ethylacetate. The organic layer was evaporated to dryness with nitrogen at 50–60 °C. The dried samples were derivatized with trichloroacetic anhydride at 50–60 °C for 10 min. Unreacted derivatization reagent was hydrolyzed with bicarbonate buffer. The derivatized product was re-extracted with ethylacetate, dried and reconstituted with 100 μl of ethylacetate and analyzed with GC–MS.

The ions (m/z) monitored were as follows: AP-D_5: 123.1, 194.0, 195.9; L-MA-D_8: 209.0, 92.0, 122.1; and D-MA-D_{14}: 209.0, 98.1, 128.1; underlined ions were used for quantitation, and the results are shown in Fig. 5b.

GC–MS analyses were carried out with a Hewlett-Packard (HP) 6890/5973 GC–MS equipped with HP 6890 autosampler using election impact (EI) ionization at 70 eV. An HP-MS crosslinked 5% diphenyl and 95% dimethyl polysiloxane capillary column (25 m×0.25 mm, 0.33 μm film thickness) was used for GC separation. Samples were injected by the splitless mode. Oven temperature was programmed from 100 °C (held 1 min) to 220 °C at the rate of 20 °C/min, and then to 320 °C at the rate of 4.4 °C/min and held for 5.5 min for column cleaning. The carrier gas was helium with a flow-rate of 0.7 ml/min. The temperature of the injection port was 270 °C and the transfer line set at 315 °C. The screen analysis was carried out in the selective ion mode and the mass spectra were obtained by scanning from m/z 50 to 550.

3. Results and discussion

Fig. 2 displays the chromatograms obtained from

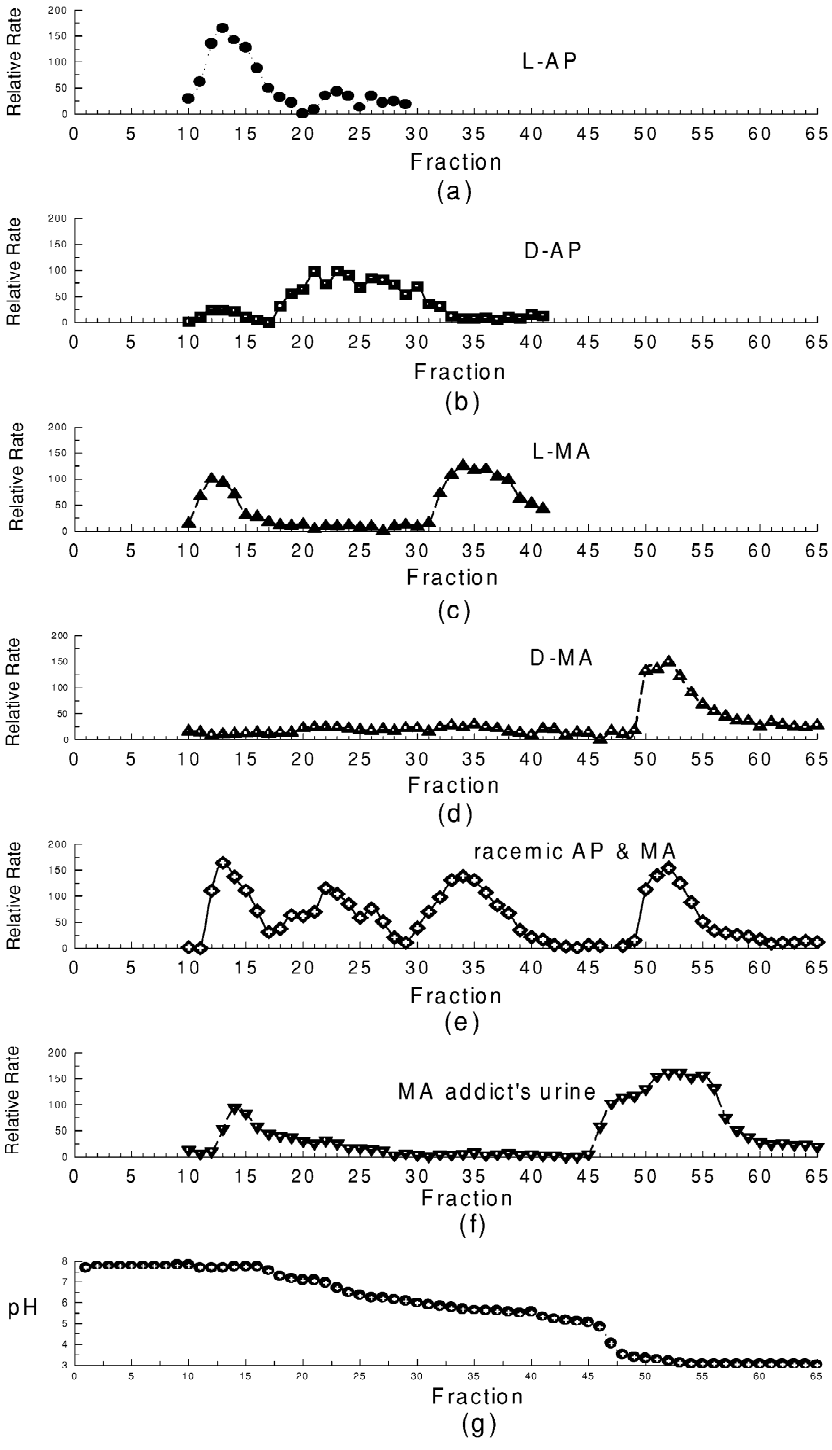


Fig. 2. Chromatograms for samples (a) 1.0 μg/ml of L-AP, (b) 1.0 μg/ml of D-AP, (c) 1.0 μg/ml of L-MA, (d) 1.0 μg/ml of D-MA, (e) a mixture of racemic amphetamine and methamphetamine (the concentration of each component is 1.0 μg/ml), (f) MA addict's urine, and (g) the variation in the pH of the mobile phase during each chromatogram; the sample volume loaded was 0.50 ml.

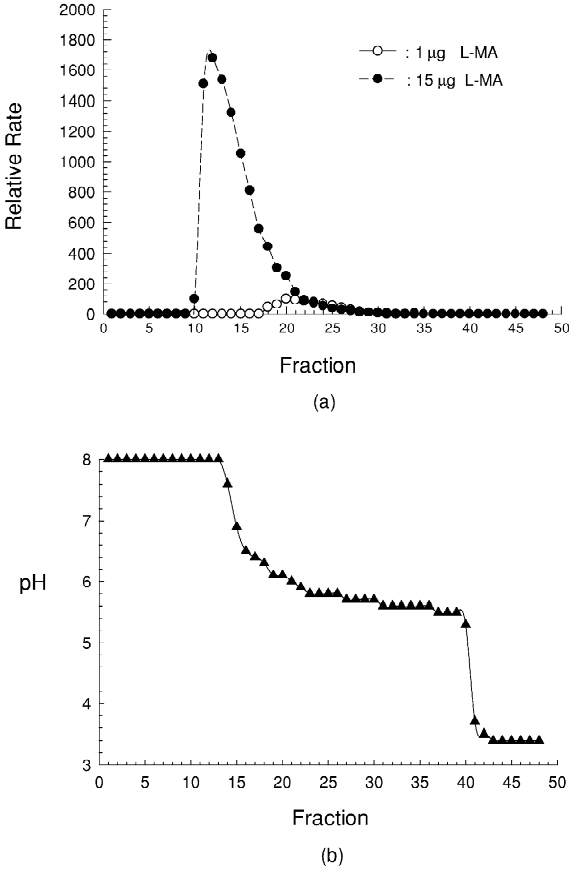


Fig. 3. Chromatograms of various amounts of L-MA. A volume of 10 μl (○) and 150 μl (●) of L-MA (0.10 μg/μl) was injected (a), the variation in pH of the mobile during each chromatogram was displayed in (b). Other chromatographic conditions are detailed in the Experimental section.

traditional LC. The profiles (a), (b), (c), (d), (e), and (f) represent the chromatograms for the sample L-AP, D-AP, L-MA, D-MA, mixture of racemic AP and racemic MA, and MA addict's urine, respectively. Profile (g) exhibits the progressive pH change in the mobile phase. Profile (a) peaked around fraction No.14, corresponding to pH 8.0 of the mobile phase. The dead volume of the affinity column prepared in this study is approximately 7 ml, indicating the column almost has no affinity towards L-AP when the mobile phase pH is approximately 8.0. The peaks of profiles (b) and (c) around fraction No.14 may be caused by the sample's overload. Comparison of profiles (a), (b), (c), (d), and (e) reveals that the four components in the sample of profile (e) are eluted in the order of L-AP, D-AP, L-MA, and D-MA. This illustrates that the affinity of the LC column toward any enantiomer of AP or MA is strongly dependent on the pH of the mobile phase and D-MA was eluted last at about pH 3.0. It is obvious that MA in the MA addict's urine is D-MA exclusively by comparing Fig. 2f to Fig. 2c,d. According to the literature [50], D-MA can be metabolized into D-AP in the human body. Reasonably, the peak around fraction No.14 in Fig. 2f is not caused by the existence of L-AP, therefore, it was caused by D-MA overloading the column.

Figs. 3a and 4 exhibit the HPLC chromatograms of various amounts of L-MA and D-MA, respectively. The front of the peak shifted to lower fraction number as the amount of L-MA or D-MA injected increased, while the final fraction of each peak was not apparently changed with the amount of L-MA or D-MA injected. This may indicate that the affinities of the active sites on this ABSP toward D-MA or L-MA are not homogeneous. The more D-MA or L-MA injected, the more the active sites possessing weaker affinity would be occupied, and the molecules occupying the active sites possessing weaker affinity would be easily eluted. When the injected amount of D-MA reached 15 μg (Fig. 4), an additional peak appeared at around fraction ≠6, this indicates 15 μg of D-MA was greater than the column capacity, and the excess D-MA overflowed

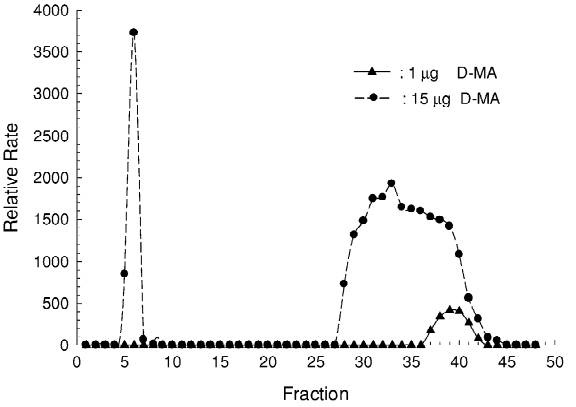


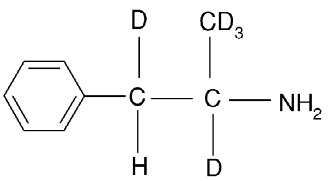
Fig. 4. Chromatograms for various amounts of D-MA. A volume of 10 μl (▲) and 150 μl (●) of D-MA (0.10 μg/μl) was injected. Other chromatographic conditions are detailed in the Experimental section.

at the dead volume of the column. The peak positions for L-MA in Fig. 3a and D-MA in Fig. 4 seldom overlap, revealing that both MA enantiomers interact with the active sites on the ABSP with different affinity. Like the bovine serum albumin, antibodies are proteins consisting of many amino groups and carboxylic acids [51]. Therefore, the ABSP could exhibit the characteristics of zwitterion stationary phase as described in our previous report [48] and provide a multi-interacting point for the chiral binding site's enantiomeric selection. In this study, the moiety of CH_3, $-NH(CH_3)$ and the benzyl group of MA molecule may contribute hydrophobic, electrostatic and $\pi-\pi$ interactions with the active sites on the ABSP. Increasing $[H^+]$ in the mobile phase would increase the density of positive charge on the ABSP and increase the repulsive force between the ABSP and the protonated MA. Since D-MA eluted at a lower pH than L-MA, this can illustrate that the chiral binding sites on the ABSP exhibit more attraction to D-MA than L-MA. This is reasonable since the antibody immobilized on the ABSP was against D-MA, the structure of the active sites on the ABSP are more adapted to grasp the molecular D-MA than to the molecular L-MA.

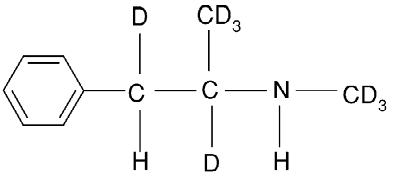
To further prove the enantioseparation and solid-phase extraction (SPE) ability of the HPLC column toward two enantiomers of MA, very low concentrations (1.1 μg/ml) of deuterated racemic MA (the mixture of equal amount of L-MA-D_8 and D-MA-D_{14}) were analysed with the HPLC column as described in the Experimental section. The profiles of L-MA-D_8 and D-MA-D_{14} in Fig. 5b illustrated that the HPLC column could extract and separate both enantiomers of MA.

The ability of the HPLC column to separate both MA enantiomers was also demonstrated by analyzing a sample containing the enantiomeric excess of MA (the molar ratio of D-MA/L-MA was 1:100) (Fig. 6). A small peak of D-MA at around fraction No. 39 in Fig. 6 demonstrates that the HPLC column has high resolution for the separation of both enantiomers of MA.

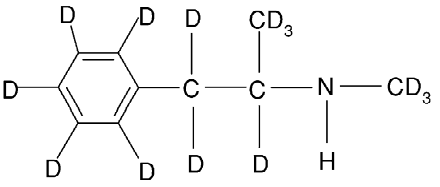
The chromatograms of both MA addict's urine and normal urine are shown in Fig. 7. Since the first peak in the profile of MA addict's urine does not extend to the fraction number around 25 (the position L-MA was eluted as illustrated by Fig. 3a), while the



Amphetamine-D_5 (AP-D_5)

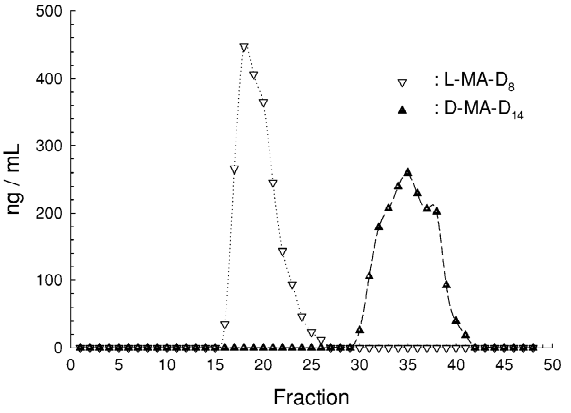


Methamphetamine-D_8 (MA-D_8)



Methamphetamine-D_{14} (MA-D_{14})

(a)



(b)

Fig. 5. The structures of AP-D_5, MA-D_8 and MA-D_{14} are exhibited in (a) and the concentration of L-MA-D_8 (▽) and D-MA-D_{14} (▲) in each fraction collected from the chromatogram of deuterated racemic MA was determined by GC–MS (b); 100 μl of the sample (the mixture of equal volume of 1.1 μg/ml L-MA-D_8 and 1.1 μg/ml D-MA-D_{14}) was injected 20 times into the HPLC column at pH 8.0 of the mobile phase. Other chromatographic conditions are detailed in the Experimental section.

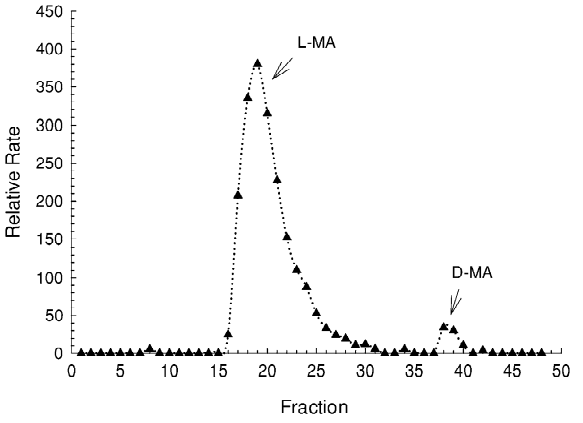


Fig. 6. Chromatogram of the enantiomeric excess of MA (the molar ratio of D-MA/L-MA was 1:100, total concentration of MA is 1.0 μg/μl) by the HPLC column. Injected volume is 3.0 μl and other chromatographic conditions are detailed in the Experimental section.

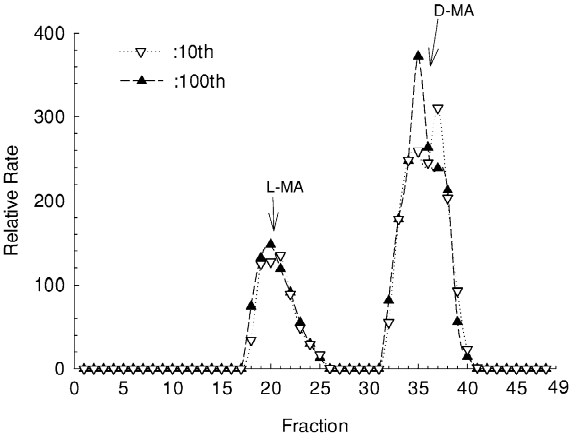


Fig. 8. Overlaid chromatograms showing repeated cycles (cycles 10th "▽" and 100th "▲") of the enantioseparation of racemic MA (0.10 μg/μl); 20 μl of the sample was injected and other chromatographic conditions are detailed in the Experimental section.

second peak covers the fraction number around 40. By comparing Fig. 7 with Fig. 5b, it can be concluded that the MA in MA addict's urine is exclusively D-MA. The fraction number of the first peak in MA addict's urine profile ranged from 14 to 18, which might be caused by the existence of D-AP in the MA addict's urine [50]. The chromatogram of normal urine does not display any peak, demonstrating that this study's method for distinguishing both MA's enantiomers was not hindered by urine impurity. Overlaid chromatograms (Fig. 8) showing repeated cycles (cycles 10th, 100th) of the D/L-enantioseparation of MA with HPLC demonstrated the reusability of the HPLC column. In order to monitor the presence of MA in the effluent on-line, we will endeavor to apply the HPLC column to the HPLC–MS system in the future.

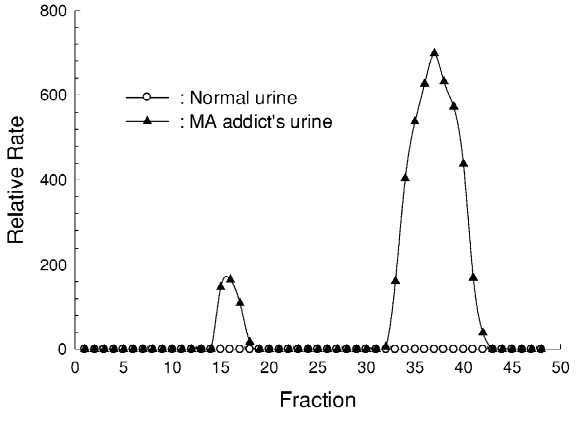


Fig. 7. Chromatograms of MA addict's urine (▲) and normal urine (○) by the HPLC column; 30 μl of urine sample was injected. Other chromatographic conditions are detailed in the Experimental section.

4. Conclusions

A chiral stationary phase was synthesized by immobilizing anti-D-MA monoclonal antibody on the pre-activated polysaccharides or silica. The stationary phase prepared in this report could be used to completely separate two enantiomers of MA without any pre-derivatization of the MA or any addition of organic solvent in the mobile phase. The chromatographic behavior of this stationary phase is strongly dependent on the pH of the mobile phase, and therefore the HPLC column prepared in this study could extract both enantiomers of MA by regulating the pH of the mobile phase; thus it is practical to directly determine very low D-MA concentrations in urine without any prior extraction of MA from the urine sample.

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