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Chiral separations of mandelic acid by HPLC using molecularly imprinted polymers

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Abstract: Styrene is used in a variety of chemical industries. Environmental and occupational exposures to styrene occur predominantly through inhalation. The major metabolite of styrene is present in two enantiomeric forms, chiral R- and S- hydroxy-1-phenyl-acetic acid (R-and S-mandelic acid, MA). Thus, the concentration of MA, particularly of its enantiomers, has been used in urine tests to determine whether workers have been exposed to styrene.

This study describes a method of analyzing mandelic acid using molecular imprinting techniques and HPLC detection to perform the separation of diastereoisomers of mandelic acid. The molecularly imprinted polymer (MIP) was prepared by non-covalent molecular imprinting using (+) MA, (-) MA or (+) phenylalanine, (-) phenylalanine as templates. Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were copolymerized in the presence of the template molecules. The bulk polymerization was carried out at 4ºC under UV radiation. The resulting MIP was grounded into 25~44¼m particles, which were slurry packed into analytical columns. After the template molecules were removed, the MIP-packed columns were found to be effective for the chromatographic resolution of (±)-mandelic acid. This method is simpler and more convenient than other chromatographic methods.

Keywords: molecular imprinting technique; enantiomers; (±)-mandelic acid; chromatographic resolution.

Introduction

Styrene is a yellowish oily liquid widely used in the production of various plastics, polyester resins and synthetic rubber. In October 2003 alone, 70439 tons of styrene was used in Taiwan [1], where many workers are exposed to styrene daily. The possible association between the exposure to styrene and it's toxic effect in humans has been reported in recent years [2], and the International Agency for Research on Cancer classifies styrene as a possible human carcinogen [3]. The major metabolite of styrene is found by hepatic cytochrome P-450 to be present in two enantiomeric forms, R-(+)-styrene-7,8-epoxide and S-(-)-styrene-7,8-epoxide (STO). This metabolite can be further hydrated to 1, 2-dihydroxy-1-phenylethane (phenylethylene glycol, PEG). PEG can be metabolized into chiral R-and S- 1-hydroxy-1-phenyl-acetic acid (R-and S-mandelic acid, MA.), benzoylformic acid (phenylglyoxylic acid, PGA), benzoic acid (BA) and hippuric acid (HIA) [4]. Metabolites formed are excreted in urine, with 85% of the absorbed styrene eliminated as mandelic acid [5]. Thus, the concentration of MA, particularly its enantiomers, in urine has been used as a biological indicator of styrene exposure in workers [6]. Gas chromatography [7], capillary electrophoresis [8], high-pressure liquid chromatography (HPLC) [9 - 11], and liquid chromatography/electrospray tandem mass spectrometry [12] have been widely used to determine MA concentration in the urine of workers exposed to styrene solvent. The main disadvantage of these methods is the need for an additional derivatization procedure [12]. In this study, we used molecularly imprinted polymer

(MIP) as the stationary phase for the HPLC analyses. Molecular imprinting is a promising technique for preparing synthetic polymers which possesses highly selective recognition properties and serves specific purposes, e.g., an antibody mimic, recognition in sensors or chiral stationary phase (CSP). MIP-based CSP have been produced for a wide range of chiral compounds, with separation factors typically ranging between 1.5 and 5.

Because of its simple, convenient preparation and its stability under high temperatures, MIP has received much attention in recent years [13, 14]. (±)-Mandelic acid and (±)-phenylalanine (Fig.1), used as the imprinting template in the present work, can interact with functional monomer by non-covalent bonding, which forms a complex. The complex involves polymerization of functional monomers and cross linking monomers around the template. After removing template molecules from the obtained polymer, complementary recognition sites can be found. These sites can rebind the same molecule. The present paper demonstrates that (±)-MA or (±)-phenylalanine imprinted polymers can be readily prepared for use in chromatographic detection of MA in urine.

Experimental

Reagents

Methacrylic acid (MAA, 99%) and ethylene glycol dimethacrylate (EGDMA, 98%) were obtained from Merck. D-(-)-mandelic acid, L-(+)-mandelic acid and 2, 2'-Azobisisobutrionitrile (AIBN) were obtained from TCI (Tokyo, Japan). HPLC grade methanol, alcohol, acetone and acetonitrile were obtained from TEDIA. D-(-)-phenylalanine and L-(+)- phenylalanine were purchased from Sigma (St. Louis, MO, USA). EGDMA and MAA were distilled to remove the inhibitors before polymerization. Water is double de-ionized.

Preparation of molecular imprinting stationary phase

The MIP stationary phase was prepared by bulk polymerization at 4°C. In a 250ml conical Erlenmeyer flask, mandelic acid (0.0152 g) or phenylalanine (0.0165g), methacrylic acid (0.682 ml), EGDMA (4.72ml) and AIBN (0.02g) were dissolved in 5 ml chloroform. After degassing and nitrogen purging for 3 min, the flask was sealed and allowed

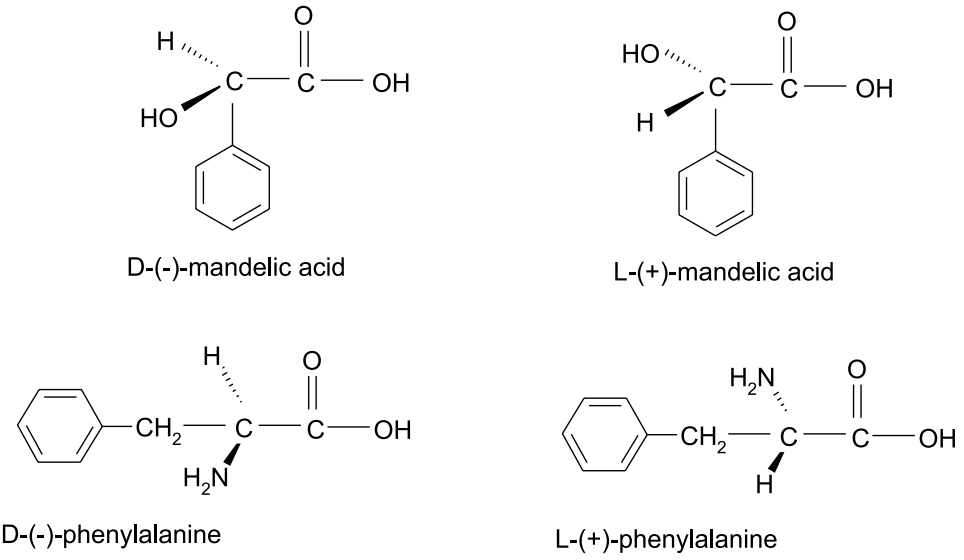

Figure1: Structure of (±)-mandelic acid and (±)-phenylalanine

to polymerize at 4°C for 6 h under UV (365 nm, 100 W lamp) irradiation. Either mandelic acid or phenylalanine was used as the template in each preparation of MIP. Methacrylic acid was used as the functional monomer, EGDMA as the crosslinking monomer and AIBN as the free radical initiator. After polymerization, the chloroform was poured out. In the form of a white solid, the product was dried in a vacuum oven for 12 h at room temperature. The resultant bulk rigid polymer was finally ground into fine particles using a mortar and pestle. Polymer particles were to pass through 25 to 44μm sieve .The shape of mandelic acid-imprinted particle utilizes SEM (JXA-840, Japan) to observe. Template molecules were removed from the particles after they were packed into columns by continuously washing with acetonitrile until a stable baseline was reached.

Liquid chromatography

Liquid chromatography was carried out using a modular HPLC apparatus (JASCO PU-2080 chromatograph) equipped with a JASCO UV -2075 variable wavelength detector, a Rheodyne 7725 injector (assembled with a 20 μl sample loop) and a Peak ABC Chromatography Workstation Ver.2.10integrator.

MIP particles were suspended in methanol by sonication and then slurry packed into 25 cm × 0.46 cm I.D. stainless steel columns using an air-driven fluid pump with acetone as the solvent. The backpressure for packing was 300 bars. Template molecules were removed from the columns by continuously washing with methanol-acetic acid (9:1, v/v) until a stable baseline was reached. For the HPLC analysis, a 10-¼l sample solution was injected by use syringe and eluted isocratically at a flow-rate of 0.5 ml/min. The temperature was kept at 25°C. The effluent solution was constantly monitored by measuring the absorbance at 225 nm. Toluene was used as the non-retained component to determine the void fraction for each column. Capacity factors (k'_- and k'_+) were calculated according to standard chromatographic theory as $k'_- = (t_- - t_0)/t_0$ and $k'_+ = (t_+ - t_0)/t_0$, where t_- and t_+ are the retention times of (-) mandelic acid and (+) mandelic acid, respectively, and toluene retention time was used as the retention time of the non-retained component, t_0. The separation factor (±) was defined as the ratio of these two capacity factors.

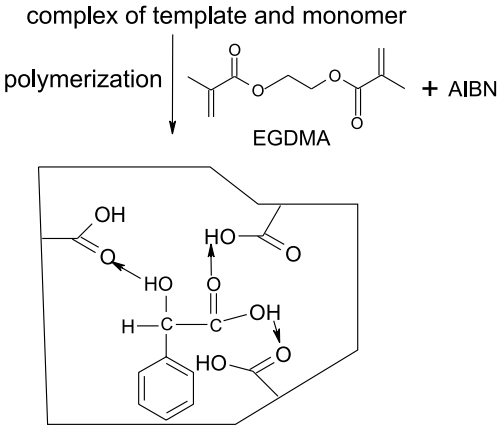

Figure 2: Schematic representation of the molecular imprinting process for non-covalent imprinting of mandelic acid using methacrylic acid as functional monomer.

Results and discussion

Preparation of stationary phase by bulk polymerization

We successfully prepared 4 MIPs using (-)-mandelic, (+)-mandelic acid, (-) phenylalanine and (+) phenylalanine as template molecules. In the preparation of the MIP stationary phase, methacrylic acid was used as the host molecule to the template. Fig. 2 shows the hypothetical

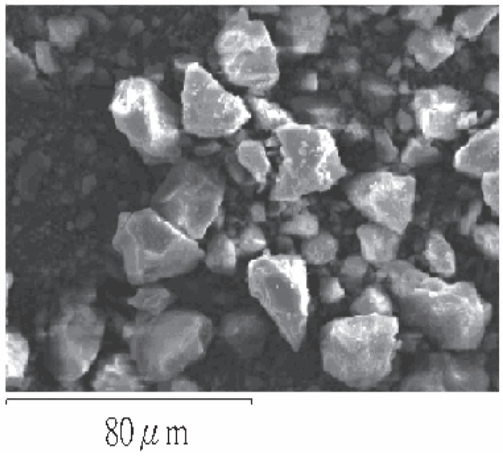

Figure 3:SEM of typical irregularly shaped mandelic acid-imprinted particles

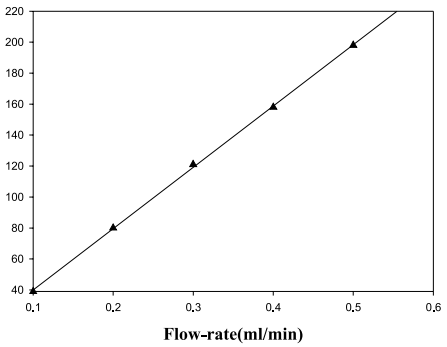

Figure 4: A linear curve of pressure vs. volumetric flow-rate

formation of the mandelic acid-imprinted polymers resulting from the free-radical polymerization in the bulk mode. Depending on the bulk polymerization method used, MIPs can have various physical configurations (see Fig.3) and a wide size distribution (25~44μm). Although the MIPs particles for packing were irregular in shape, the 25cm-long columns possessed a linear curve of volumetric flow-rate vs. pressure (Fig.4). The linearity was proved up to a pressure of ca 200 bar, at which the flow-rate was 0.5 ml/min

Resolution of enantiomers of mandelic acid

Table 1 shows that the composition of mobile phase achieved the resolution of (±)-mandelic acid. The results of resolution are expressed in Table 2 and Figure 5~6. The compounds of interest are a pair of enantiomers:D-(-)-mandelic acid and L-(+)-mandelic acid. Mandelic acid contains an asymmetric center. To this asymmetric center can be bound not only hydrogen but also hydroxyl and carboxyl. Since the template in this study has a simple structure, the composition of mobile phase played a very important role, especially its pH value, on the resolution of template and its enantiomer. In our study, a mobile phase containing polar substances was used to weaken the binding of template molecules and

Buffer 1	Methanol : water : triethyl amine = 19 : 76 : 5 (v/v/v) adjusted with acetic acid to pH 4.0
Buffer 2	Ethanol : water : triethyl amine = 47.5 : 47.5 : 5 (v/v/v) adjusted with acetic acid to pH 6.6
Buffer 3	Methanol : acetonitrile : triethyl amine : acetic acid =52 : 45 : 1:2 (v/v/v/v) pH 6.79

Table1: The composition of mobile phase on the resolution of (±)-mandelic acid(15).

Template	Concentration in sample(g/l) (+)-MA	(-)-MA	Retention time[d] (+)-MA	(-)-MA	Separation factor(α)
(+)-MA	0.3	0.2	44.54	19.97	2.33[a]
	0.6	0.4	46.29	19.82	2.44[b]
	0.7	0.3	52.21	19.21	2.86[c]
(-)-MA	1.0	-	23.94	-	-
	0.7	0.3	23.85	35.26	1.51[c]
	-	0.8	-	35.13	-
(+)-phenylalanine	0.7	0.3	23.14	35.69	1.58[c]
(-)-phenylalanine	0.3	0.2	22.85	37.09	1.66[c]

[a, b, c] used Buffer 1, Buffer 2 and Buffer 3 as mobile phase

[d]Toluene retention time was used as the retention time of the non-retained component, t_0 = 1.52min

Table 2: Composition of solution and resolution of (±)-mandelic acid

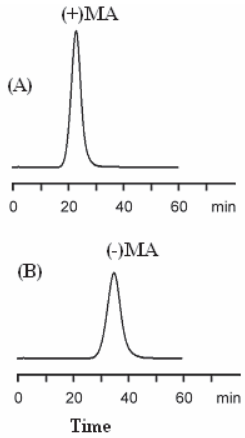

Figure 5: Resolution of pure (+)-mandelic acid and (-)-mandelic acid, using MIP with (-)-mandelic acid as template and Buffer 3 as mobile phase.

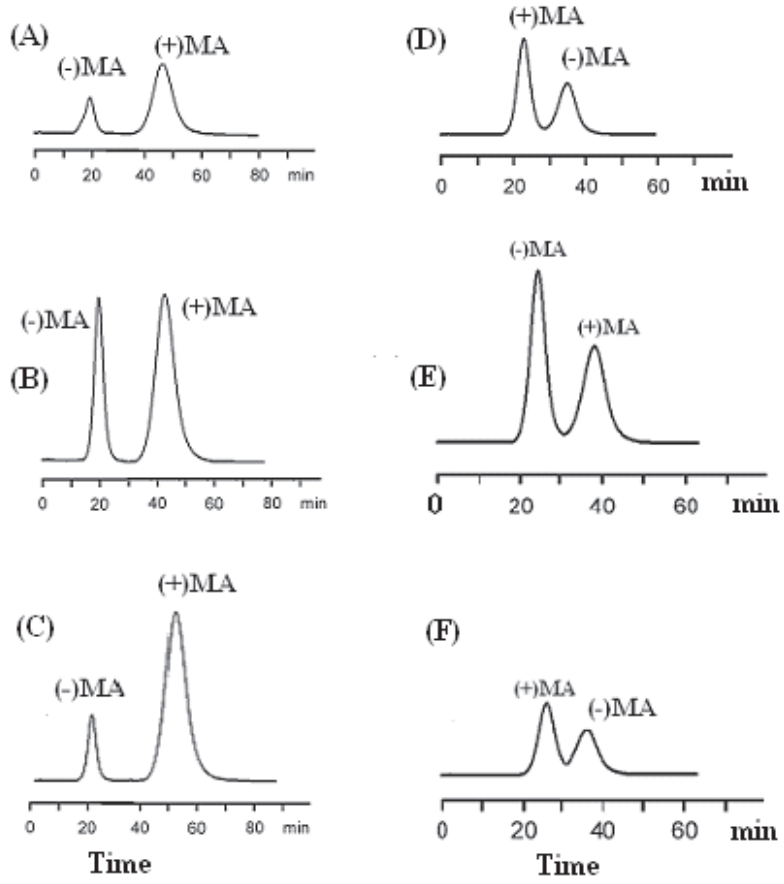

Figure 6: Separation of enantiomers of mandelic acid using MIP with (+)-mandelic acid as template (A~C), (-)-mandelic acid as template (D), (+)-phenylalanine as template (E) and (-)-phenylalanine as template (F).

consequently to release them on the imprinting cavity of the stationary phase.

With the use of (-)-mandelic acid as a template, it can be seen that pure compound eluted at 23.94min and at 35.13min (Fig.5). Figure 6A~6C show that enantiomers of mandelic acid could be separated well using MIP made by (+)-mandelic acid as the template, with the separation factors being 2.33, 2.44 and 2.86 when used buffer 1, buffer 2 and buffer 3 as mobile phase. Use (-)-mandelic acid as template, the concentration of (+)-mandelic acid and (-)-mandelic acid in the solution was 0.7:0.3(g/l), the retention time of (+)-mandelic acid in the column was 23.85 minutes and (-)-mandelic acid was 35.26 minutes (Fig.6 D), thus indicating that no matter use any one, (±)-mandelic acid could be qualitatively analyzed.

The values of separation factor obtained in this study are much greater than the ones reported in a previous study [15]. These results also show that the pH value of mobile phase significantly influenced the retention time of the eluted peaks. The same result has been reported in a system using Chirobiotic T as the column to separate the enantiomers of mandelic acid [15]. Chirobiotic T has 23 chiral centers surrounding four cavities. Hydrogen donor and acceptor sites are readily available close to seven aromatic rings. Chirobiotic T is an excellent alternative to crown ether and ligand exchange for amino acids and hydroxy acids.

Liquid chromatography by using phenylalanine as template

Phenylalanine has one more carbon atom than mandelic acid, and an amino group is bound onto the asymmetric center. Therefore, retention of mandelic acid in the MIP column is higher than that of toluene (a non-retained compound). The concentration of (+)-mandelic acid and (-)-mandelic acid in the solution was 0.7:0.3(g/l), the retention time of (+)-mandelic acid in the column was 35.69 minutes and (-)-mandelic acid was 23.14 minutes (Fig.6E), the value of ±was 1.58 when BOC-L-(+)-phenylalanine was used as the template. Fig.6 F shows the efficiency of chromatograph when used BOC-D-(-)-phenylalanine as template. The successful separation is believed to be dependent on the biospecific adsorption of mandelic acid to the recognition sites left by the removed template. This separation is caused by the hydrogen bonding between the hydroxyl group of mandelic acid and carboxylic groups of immobilized methacrylic acid.

Conclusions

In this study, we have used four molecules, (±)- mandelic acid and (±)- phenylalanine, as the templates for the preparation of MIP. Chromatographic columns packed with MIP particles were effective for the resolution of mandelic acid enantiomers. The recognition and binding of template molecules was based on interactions between hydroxyl groups of the template and the carboxyl group of methacrylic acid, a host molecule in the MIP. The higher separation factor values obtained in the present work suggest that non-covalent molecular imprinting is a promising method for analyzing chiral compound with a simple structure.

Acknowledgement

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Chin-Yin Hung, Han-Hung Huang, Ching-Chiang Hwang. Separação quiral de ácido mandélico por HPLC usando polímeros molecularmente impressos

Resumo: O estireno é muito usado em indústrias químicas. A exposição ambiental e ocupacional ocorre predominantemente através da inalação. O principal metabólito do estireno ocorre em duas formas enantioméricas, quiral-R e ácido aceto-1-fenil-1-hidroxi-S (ácido mandélico R e S, AM). A concentração de AM, especialmente nos enantiômeros, tem sido usada em testes de urina para verificar a exposição de

trabalhadores ao estireno. Este trabalho descreve um método de análise do ácido mandélico utilizando técnicas de impressão molecular e detecção por HPLC para obter a separação possível dos isômeros diaestéreo do ácido mandélico. O polímero molecularmente impresso (PMI) foi preparado por impressão não-covalente, utilizando-se moldes de AM(+), AM(-) ou fenil-alanina (+), fenil-alanina(-). O ácido meta-acrílico (AMA) e o di-metil-acrilato de etileno glicol (DMAEG) foram co-polimerizados na presença das moléculas do molde. A polimerização da amostra foi obtida a 4ºC sob radiação UV. O PIM resultante foi moído até a obtenção de partículas com 25~44 mm, que foram colocadas em forma de pasta em colunas analíticas. Após a remoção das moléculas do molde, as colunas de PIM foram consideradas adequadas para a resolução cromatográfica do ácido mandélico (±). Este método é mais simples e conveniente do que outros métodos cromatográficos.

Palavras-chave: técnica de impressão molecular; enantiômeros; ácido mandélico (±); resolução cromatográfica

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