ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1997) 1351–1358

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Capillary electrochromatographic separation of amino acid enantiomers using on-column prepared molecularly imprinted polymer[1]

Jin-Ming Lin *, Tatsuro Nakagama, Katsumi Uchiyama, Toshiyuki Hobo

Department of Industrial Chemistry, Faculty of Engineering, Tokyo Metropolitan University, Minami-Ohsawa 1-1, Hachioji-Shi, 192-03 Tokyo, Japan

Received 21 August 1996; accepted 15 November 1996

Abstract

The use of molecularly imprinted polymer polymerized in capillary for the separation of amino acid enantiomers by electrochromatography is described. The substrate-selective polymers were prepared by using L-phenylalanine anilide as print molecule and methacrylic acid and/or 2-vinylpyridine as the functional monomers, which is believed to interact both ionically and through hydrogen bonding with the print molecule. Several aspects of the polymer preparation were investigated, including the treatment of the inside surface of the capillary, the composition of the polymers and the running conditions of the capillary electrochromatography. Such separation was highly specific and depended on the presence of both the print molecule and the functional monomer in the polymerization mixture. This preliminary report demonstrates a novel and simple method for the development of the capillary electrochromatographic separation of amino acid enantiomers using molecularly imprinted polymer. © 1997 Elsevier Science B.V.

Keywords: Capillary electrochromatography; Molecular imprinting method; Enantiomeric separation; Amino acid

1. Introduction

Optical resolution is one of the important objectives in pharmaceutical and medical fields. Especially, the separation of amino acid enantiomers has been widely investigated because of their commercial significance and ease of availability. Several chromatographic techniques such as high-performance liquid chromatography (HPLC) [1,2] and gas chromatography (GC) [3,4] have be employed for the resolution of racemates. Recently, many reports on the enantiomeric separations by capillary electrophoresis (CE) appeared [5,6], including micellar electrokinetic chromatography (MEKC) or capillary gel electrophoresis (CGE) with chiral selectors. Most of these reports concerning enantiomeric resolution were carried out by using cyclodextrin, chiral crown ethers or chiral functionalized micelles. Almost all of these

* Corresponding author. Fax: +81 426 772821; e-mail: lin@ecomp.metro-u.ac.jp

[1] Presented at the Seventh International Symposium on Pharmaceutical and Biomedical Analysis, August, 1996, Osaka, Japan.

0731-7085/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved.
PII S0731-7085(96)02013-4

classes of additives were macrocyclic molecules having cavities which were available for host–guest type interactions with the solutes. These separations are largely empirical, and the mechanism of chiral recognition is not fully understood. Therefore, it is often difficult to predict the elution order of enantiomers. During recent years the use of molecular imprinting method in the preparation of separation media for enantiomeric resolutions has been considerable [7–9]. A lot of chiral template molecules, for example, L-phenylalanine anilide [10], L-mandelic [11], (*R*)-phenylsuccinic acid [12], etc. were used to reorganize functionalized monomers in the copolymerization mixed solution. This is a technique whereby a polymer is prepared in the presence of a 'guest' or 'print' molecule so that specific chemical interactions occur between the functional monomers and the print molecules. The resulting polymer contains specific sites, which can recognize the print molecules, and this polymer can be freed from the template by hydrolysis or extraction. Subsequently the polymers bind preferentially with the original template molecule in competition with structurally related molecules. After crushing and sieving the obtained network polymer, these polymer particles are used as a selective chromatographic stationary phase for chiral recognition [13,14]. In this research field, Mosbach et al. reviewed a number of papers concerned with the synthesis and application of molecularly imprinted polymers [9]. To our knowledge the study and the application of the molecular imprinting method for the separation of enantiomers in capillary electrochromatography has not been reported. In this study we chose the most well-known and studied functional monomer and cross-linking monomer for the synthesis of an amino acid imprinted polymer.

2. Experimental

2.1. Apparatus

A purpose-built CE system was used to conduct the experiments. A regulated high voltage power supply, 0–30 kV, model HEL-30P2-TTu (Matsusada Precision Devices, Kousatsu City, Japan) was used. Electrochromatography was performed in a 50 cm × 0.075 mm i.d. fused-silica capillary column (GL Sciences, Tokyo, Japan). This capillary consisted of two sections. One part was 25 cm long and filled with molecularly imprinted polymer. Another part was also 25 cm long and had a 0.5 cm detection window near the end of the capillary and this section was filled with buffer. These two parts of the capillary were connected by a Teflon tube. On-column detection was accomplished with a re-built UV-8011 ultraviolet detector (Tosoh, Tokyo, Japan). Each end of the capillary was dipped into separate 5 ml vials. Platinum wire electrodes were inserted into the two vials for connection to the electrical circuit. The electropherograms were recorded on a C-R5A Chromatopac (Shimadzu, Japan). A model SSC 3512 C column oven (Senshu Scientific, Tokyo, Japan) and a model BM-42 water bath (Yamato Scientific, Tokyo, Japan) were used for temperature control of the polymerization process. A W-113 ultrasonic multi cleaner (Honda Electric, Tokyo, Japan) was used for the degassing process.

2.2. Materials

Thionyl chloride (Guaranteed Reagent) was obtained from Nacalai Tesque (Japan). Vinyl magnesium bromide, ammonium acetate, α,α'-azobis(isobutytronitrile) (AIBN), methacrylic acid (MAAA), ethylene glycol dimethacrylate (EDMA) and 2-vinylpyridine were purchased from Tokyo Kasei (Japan). Acetonitrile and chloroform were chromatographic grade and all of them were purchased from Kokusan Chemical Works (Tokyo, Japan). Amino acid enantiomers were purchased from Sigma (St. Louis, MO) and used as received. Before use organic solvents and sample solutions were filtered through a membrane filter unit of 0.1 μm pore size (Advantec, Tokyo, Japan) and then degassed. Sample solutions were prepared from electrolyte buffer solution. The sample concentrations were 5×10^{-4}–1×10^{-3} mol l^{-1}.

2.3. Capillary modification

The capillary treatment procedure is shown schematically in Fig. 1. The fused silica capillaries were first etched with 1.0 mol l^{-1} potassium hydroxide solution for 2 h at room temperature and rinsed with distilled water for 30 min. The capillaries were dried at 60°C with a nitrogen purge overnight.

In order to introduce thionyl chloride into the capillary, the flask was evacuated to ca. 20 mmHg. The introduction flow rate was 5 cm min^{-1} for a 75 μm i.d. capillary. After flowing thionyl chloride for about 30 min, the capillary was filled with thionyl chloride, one end of the capillary near the vacuum pump was sealed using a small silicone rubber cover. The opposite end of the capillary was evacuated for approximately 20 min, during which time the pressure was 60 mmHg or less. Throughout this evacuation process, the capillary was maintained at 60°C by immersing it in a heating bath. The end of the capillary near the vacuum line was then also sealed with a small silicone rubber cover and the sealed capillary was placed in a 60°C heating bath for 12 h. This capillary was then purged with nitrogen for 1 h at 60°C.

Fig. 1. Scheme of the procedure for the preparation of vinyl-bound molecularly imprinted polymer filled capillaries.

2.4. Grignard reaction

Under a nitrogen purge, 5 ml of dry THF was placed in a 10 ml vial fitted with a silicone rubber septum. Nitrogen was bubbled through the THF for several minutes. With a dry syringe, 1 ml of vinyl magnesium bromide was added to the THF and the solution was purged with nitrogen. If the solution becomes cloudy upon addition of the Grignard reagent to the THF the procedure must be repeated. One end of the sealed chlorinated capillary was opened while immersed in dry THF. This open end was quickly placed into the vinyl magnesium bromide–THF solution. The other end of the capillary was immediately connected to a vacuum line. Vacuum was applied to the capillary causing the vinyl magnesium bromide–THF solution to be drawn into the capillary. After the solution passed through the capillary for several minutes, the end of the capillary near the vial septum in the reagent solution was sealed off using a small silicone rubber cover. The capillary was placed in a 60°C heating bath and the vacuum (60 mmHg or less) was maintained for 30 min. The other end of the capillary was sealed near the vacuum line connection, and the sealed capillary was placed in a 60°C heating bath for 12 h.

2.5. Molecularly imprinted polymer filling method

Both ends of the silicone rubber covers were removed and the capillary was rinsed with dry THF for 20 min. The composition of polymerization solution was: 2.62×10^{-3} mol L-phenylalanine anilide (L-PheAN); 5.6×10^{-3} mol MAA and 5.6×10^{-3} mol 2-vinylpyridine; 5.24×10^{-2} mol EDMA; 7.6×10^{-4} mol AIBN; 1.6×10^{-3} mol ammonium acetate and 16 ml chloroform. These reagents were added to a 25 ml ampoule.

Solubilization was achieved by sonication. The solution was degassed for 10 min under vacuum in the sonicating bath and then the ampoule was sealed with a silicone rubber cover and one end of the capillary was inserted into the solution and the other end of the capillary was connected to a vacuum line. For thermal initiation, the ampoule was placed in a water-bath at the decomposition temperature of the initiator (60°C for AIBN). The capillary was placed in a column oven at 60°C. The flow rate of the solution in the capillary was controlled at about 10 μl min^{-1} for about 1 h. After this treatment the capillary was separated from the vacuum line and sealed with a silicone rubber cover. The other end was taken from the polymerization solution and also sealed with silicone rubber cover. The sealed capillary was placed in a 60°C heating bath for 24 h.

2.6. Capillary electrophoretic operation

The applied field strength for CEC was increased step by step. Firstly 5 and then 10 and 15 kV for 20 min, respectively. After the base-line became stable, 20 kV was applied. Sample introduction was performed by the electroinjection method at a constant voltage (10 kV) for a fixed period (5–10 s). Between analysis, the capillaries and the electrodes were rinsed with water and the running buffer. The signal from the detection was recorded by a C-R5A Chromatopac recorder. The resolution Rs; was defined as:

$$Rs = 2 \times [(t_2 - t_1)/(w_1 + w_2)] \quad (1)$$

where t is the migration time and w the peak width at the base.

3. Results and discussion

The reaction scheme that we proposed for the direct synthesis of a polymer in a capillary was performed by the following steps: (1) chlorination of the silica surface through a well-known reaction of thionyl chloride with the surface silanol groups; (2) reaction of the Grignard reagent, vinyl magnesium bromide, with the chlorinated silica results in direct attachment of the vinyl moiety to the silica surface; and (3) the reaction of the bonded vinyl group with MAA and the corresponding cross-linker reagent, starts when AIBN is added. The reaction of the Grignard reagent is the most important part of the sequence. In our initial work we found that without any inner surface treatment it was very difficult to get a completely polymer filled capillary because after polymerization the volume of the MAA and EDMA solution shrinks which will cause the polymer to separate. This is due to the polymer being highly cross-linked. The separated polymer of course has no electronic conduction. The organic group of the Grignard reagent must contain a terminal double bond, which provides a reactive site for subsequent bonding with the MAA and the cross-linker reagents. We found that the vinyl group was the most suitable and vinyl magnesium bromide was a readily available reagent. In this study our primary purpose was to develop a direct recognition method for amino acid enantiomers by capillary electrochromatography using molecularly imprinted polymer. The synthesis of polymer inside the capillary is a key process to optimize the capillary electrophoretic separation of the enantiomers.

3.1. Composition of the polymer

The particular goal of this study was to develop a vinyl polymer, wherein a molecule of specific chirality is separated from the appropriate vinyl monomers, then the chiral moiety is removed by electrophoresis and is replace in the polymer from a mixture of the appropriate racemates in a chemical process. Fig. 1 illustrates schematically the strategy used to prepare phenylalanine selective polymer. The print molecule, L-pheAN, was present at low concentration in the mixture of vinyl monomers, cross-linker, initiator, conducting agent and an inert organic solvent. It is expected that substrate molecules would interact preferentially with carboxyl-containing vinyl monomers due to Coulombic forces between positively charged amino groups of the substrate and opposite charged carboxylates of carboxyl-containing vinyl monomers. Specific complexes of the substrate and vinyl monomers could therefore be

Table 1
Effect of the contents of monomer and print molecule in the polymerization mixture on the separation of D,L-phenylalanine

Polymer	MAA ($\times 10^{-3}$ mol l^{-1})	2-Vinylpyridine ($\times 10^{-3}$ mol l^{-1})	Print molecule ($\times 10^{-3}$ mol l^{-1})	Molar ratio (M:P)	Resolution
1	1.60	0	0.16	10	0.74
2	1.30	0	0.16	8	0.88
3	0.70	0	0.16	5	1.02
4	0.60	0	0.16	4	1.11
5	0.30	0	0.16	2	0.89
6	0.80	0.80	0.16	10	0.75
7	0.60	0.60	0.16	8	0.93
8	0.35	0.35	0.16	5	1.20
9	0.30	0.30	0.16	4	1.25
10	0.15	0.15	0.16	2	0.94

Functional monomer; methacrylic acid and 2 vinylpyridine, print molecule: L-phenylalanine anilide. 5.24×10^{-2} mol of cross-linker monomer (EDMA), 7.6×10^{-4} mol of initiator (AINB), 1.6×10^{-3} mol of ammonium acetate and solvent (chloroform) was added to the mixture to make up to 16 ml in a borosilicate glass ampoule and complete solubilization was achieved by sonication. M:P, molar ratio of monomer to print molecule.

formed due to electrostatic interactions during polymerization. After polymerization, loosely bound print molecules were electrically removed from the polymers. The resultant polymers contain imprints of the print molecules. It has carboxylic groups which can interact specifically with the amino function of the sample molecule. In this work, we also tried to use L-phenylalanine as the print molecule for the preparation of molecularly imprinted polymer, but the dissolution of phenylalanine in chloroform is very low. This causes the polymerization solution to contained some solid phenylalanine which makes it difficult to fill the capillary. On the other hand, too low a concentration of print molecule in the polymer is of no benefit to the process.

Several different kinds of polymers specific for phenylalanine were prepared in this study. From the results shown in Table 1 the different molar ratios of print molecule to monomer give different resolution for the separation of phenylalanine. We also found that the resolution of phenylalanine was improved by using a mixture of MAA and 2-vinylpyridine. The low concentration of print molecule in the polymerization solution was beneficial for the separation. Without the imprint molecule the polymer showed no recognition ability for the chiral samples. In this study, a mixture of 0.35 mol l^{-1} MAA and 0.335 mol l^{-1} 2-vinylpyridine was used and the concentration of the print molecule was 0.16 mol l^{-1}. The molar ratio of mixed monomer to print molecule was about 5:1. The concentration of cross-linking agent was required in the polymerization mixture in order to produce macroporous polymer of high rigidity. Fig. 2 indicates that the optimum molar ratio for cross-linker to monomer is about 5:1.

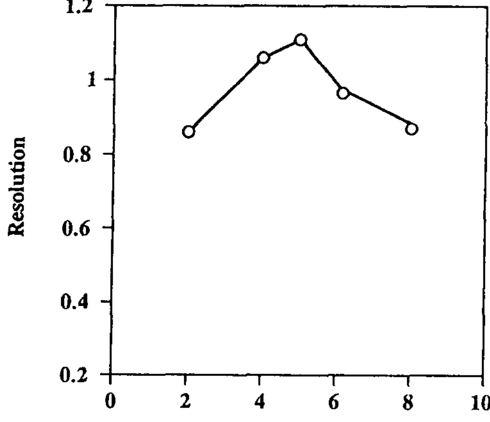

Fig. 2. Effect of the molar ratio of cross-linker to monomer on the separation of phenylalanine.

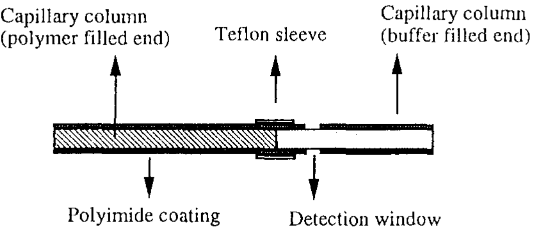

Fig. 3. Schematic diagram of the combination of the polymer filled capillary with the open tubular capillary.

3.2. Design of the polymer filled capillary column

The samples cannot be detected on-line by a UV-VIS spectrophotometric detector because the polymer in the capillary has UV absorptivity. On-column UV detection requires a detection window behind the polymer filled section. As shown in Fig. 3, the column consists of two capillary columns. Two sections of the columns have the same inner and outer diameter connected by a Teflon tube. One end of the polymer filled capillary column was joined to the end of an electrophoretic buffer filled open tubular capillary, which carried the detection window ca. 5 mm behind the connection to the end of the capillary. With this setup, the capillary for electrochromatography is readily prepared. This process must be done very carefully because the organic solvent in capillary is very easily vaporized at room temperature. It should be noted that there must be no gas inside either the polymer filled capillary or the electrophoretic buffer filled column.

3.3. Effect of running electrolyte on the resolution

Electrophoretic separation in nonaqueous or mixed aqueous–organic media have received increased attention in recent years. The advantages of performing capillary electrophoresis in organic solvents or in mixed solvent systems are three: (1) most organic compounds exhibit greater solubility than water, (ii) changes in the effective mobilities may lead to great selectivity and (iii) the electroosmotic flow is reduced [15,16]. In this study we used an aqueous–organic solvent system for the CEC. Although there were a wide variety of organic solvents with properties that could be suitable for CE, only a few studies have been published reporting CE separations in pure organic or in mixed aqueous–organic solvents [17–21]. Most of them were concerned with isotachophoresis. The solvents were the lower alcohols, acetonitrile, dimethylsulfoxide, dimethyformamide, tetrahydrofuran or acetone.

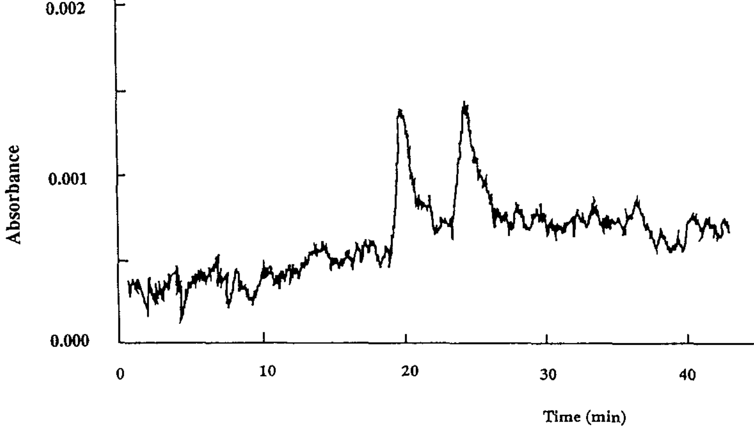

Fig. 4. A typical capillary electrochromatogram of D,L-phenylalanine by molecularly imprinted polymer as the recognition selector. Conditions: print molecule, 0.16 mol l^{-1} PheAN; functional monomers, 0.35 mol l^{-1} MAA and 0.35 mol l^{-1} 2-vinylpyridine; cross-linker, 3.3 mol l^{-1} EDMA; initiator, 0.048 mol l^{-1} AIBN; 0.1 mol l^{-1} ammonium acetate; polymerization temperature is 60°C. Polymer filled capillary, 25 cm × 0.075 mm i.d. (total length 50 cm); applied voltage, 400 V cm^{-1}; detection wavelength, 254 nm; injection was performed for 5 s at 10 kV.

Table 2
Chiral separation of some enantiomers by capillary electrochromatography using a molecular imprinted polymer filled column

Compound	Structure	Resolution
Phenylalanine	H HOOC—C—NH2 CH2	1.22
Tyrosine	H HOOC—C—NH2 CH2 OH	0.86
Phenylglycine	H HOOC—C—NH2	0.73
Tryptophan	H HOOC—C—NH2 N H	0
Serine	H HOOC—C—NH2 CH2OH	0

Considering the dielectric constant and water solubility, acetonitrile was chosen for this study. A relative satisfactory result was obtained when the electrolyte consisted of acetonitrile–acetic acid–water (80:10:10%). The presence of an appropriate amount of acetic acid and water was necessary as there was almost no electronic conduction with acetonitrile on its own.

The effect of the applied field strength on the resolution was also studied. At low voltage almost no separation could be achieved and the peaks were broad. We think this is probably due to the relatively stronger hydrophobic interaction of the analyts with the molecularly imprinted polymer. The influence of applied field strength on the resolution was studied at 5, 10, 15, 20, 25 and 30 kV, corresponding to field strengths of 100, 200, 300, 400, 500 and 600 V cm^{-1}, respectively. The resolution and the peak shape obtained optima at 400 V cm^{-1}. Under these conditions, a typical capillary electrochromatogram of D,L-phenylalanine is shown in Fig. 4. Although the D- and L-type of amino acid can be separated well, the resolutions were not satisfactory because the theoretical plates were low. The mechanism of this separation system will be investigated in the future.

3.4. Enantiomeric resolution of other amino acids

We considered that a polymer prepared using L-pheAN as the print molecule may also be able to separate the other chiral aromatic amino acid. Since the position of functional groups within the polymer was defined, five derivatives of amino acids, including serine, tryptophan, phenylglycine and tyrosine have been tested. From the data shown in Table 2, it is clear that the polymer is able to recognize the samples of similar structure

to the original print molecule. Unfortunately the separation results are not satisfactory, especially the repeat ability is poor for the different capillaries.

4. Conclusion

The successful use of a molecularly imprinted polymer for the separation of chiral amino acids by capillary electrochromatography demonstrates that it is an useful method in enantiomeric separation. The capillary filled with L-pheAN printed polymer was used for the separation of phenylalanine and similar amino acids. The major shortcoming of the polymer prepared in the capillary at present appears to be sometimes pronounced broad peaks, but the potential exists for further improvements in the column performance.

Acknowledgements

Financial support of this research by grant of the Sasakawa Science Research Foundation of the Japan Science Society (Grant No. 8-149) is gratefully acknowledged.

References

[1] W. Lindner, Chromatography, 24 (1987) 97–106.
[2] S. Ahuja, Chiral Separations by Liquid Chromatography, American Chemical Society, Washington DC, 1991.
[3] K. Watabe, E. Gil-Av, T. Hobo and S. Suzuki, Anal. Chem., 61 (1989) 126–132.
[4] S.G. Allenmark, Chromatographic Enantioseparation, Methods and Application, Ellis Horwood, Chichester, UK, 1988.
[5] H. Nishi and S. Terabe, J. Chromatogr., 694 (1995) 245–276.
[6] S.F.Y. Li, Capillary Electrophoresis Principles, Practices and Applications, J. Chromatogr. Library 52, 1992.
[7] B. Sellergren and K.J. Shea, J. Chromatogr., 654 (1993) 17–28.
[8] L. Fischer, R. Muller, B. Ekberg and K. Mosback, J. Am. Chem. Soc., 113 (1991) 9358–9360.
[9] M. Kempe and K. Mosbach, J. Chromatogr., 694 (1995) 3–13.
[10] L.I. Andersson, D.J. O'Shannessy and K. Mosbach, J. Chromatogr., 513 (1990) 167–179.
[11] M. Kempe, L. Fischer and K. Mosback, J. Mol. Recogn., 6 (1993) 25–29.
[12] O. Ramstrom, L.I. Andersson and K. Mosbach, J. Org. Chem., 58 (1993) 7562–7564.
[13] G. Vlatakis, L.I. Andersson, R. Muller and K. Mosbach, Nature, 361 (1993) 645–647.
[14] G. Wulff and M. Minarik, J. Liq. Chromatogr., 13 (1990) 1801–1807.
[15] C. Schwer and E. Kenndler, Anal. Chem., 63 (1991) 1801–1807.
[16] W. Schiitzner and E. Kenndler, Anal. Chem., 64 (1992) 1991–1995.
[17] M. Chiari and E. Kenndler, J. Chromatogr., 716 (1995) 303–310.
[18] M.P. Harrold, M.J. Wojtusik, J. Riviello and P. Henson, J. Chromatogr., 640 (1993) 463–471.
[19] M. Idei, I. Mezo, Zs. Vadasz, A. Horvath, I. Teplan and Gy. Keri, J. Chromatogr., 648 (1993) 251–256.
[20] M. Idei, I. Mezo, Zs. Vadasz, A. Horvath, I. Teplan and Gy. Keri, J. Liq. Chromatogr., 155 (1992) 3181–3187.
[21] S. Fujiwara and S. Honda, Anal. Chem., 59 (1987) 487–490.