Uniform-Sized Clenbuterol Molecularly Imprinted Polymers Prepared with Methacrylic Acid or Acrylamide as an Interacting Monomer

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ABSTRACT: Clenbuterol molecularly imprinted polymer microbeads were prepared with a two-step swelling and thermal polymerization technique with either methacrylic acid or acrylamide (AAm) as the monomer and ethylene glycol dimethacrylate as the crosslinker at different monomer/crosslinker ratios. The quality of the microbeads, in terms of shape, size distribution, rigidity, and monomer incorporation, was evaluated as a function of the reaction parameters. A good imprinting effect was obtained with both systems, as assessed by high-performance liquid chromatography (HPLC) experiments with phosphate-buffered saline/acetonitrile eluents, with a complete baseline separation of clenbuterol with respect to other β-adrenergic agents obtained. When AAm was used as the monomer, improved control of the polymerization process was achieved, producing microbeads with lower polydispersity and no lack of separation capacity. © 2002 John Wiley & Sons, Inc. J Appl Polym Sci 83: 2660–2668, 2002

Key words: molecularly imprinted polymer; uniform sized microbeads, two-step swelling polymerization; clenbuterol; liquid chromatography; emulsion polymerization; molecular imprinting

INTRODUCTION

Molecularly imprinted polymers (MIPs) are highly crosslinked materials in which a monomer carrying functional group(s) able to interact with a molecule to be recognized (template) is polymerized in the presence of a crosslinker to give a matrix in which selective cavities are formed around the template. When the latter is removed, sites complementary to the template in both shape and functionality are present in the material and are able to selectively recognize the print molecule with respect to other analytes.[1–6] Such materials can be exploited in many different fields, such as liquid chromatography, solid-phase extraction, membranes, sensors, artificial antibodies, and catalysis.[5,6] The coordination of the monomer molecules around the template can be achieved by noncovalent interactions such as hydrogen bonds, ion pairs, and hydrophobic interactions or reversible covalent interactions. Of the two methods, the covalent approach usually provides better defined cavities with higher selectivity, whereas the noncovalent one is more flexible and easier to apply because no chemical derivatization is required. MIPs are normally prepared by a bulk polymerization method in which a rigid rod

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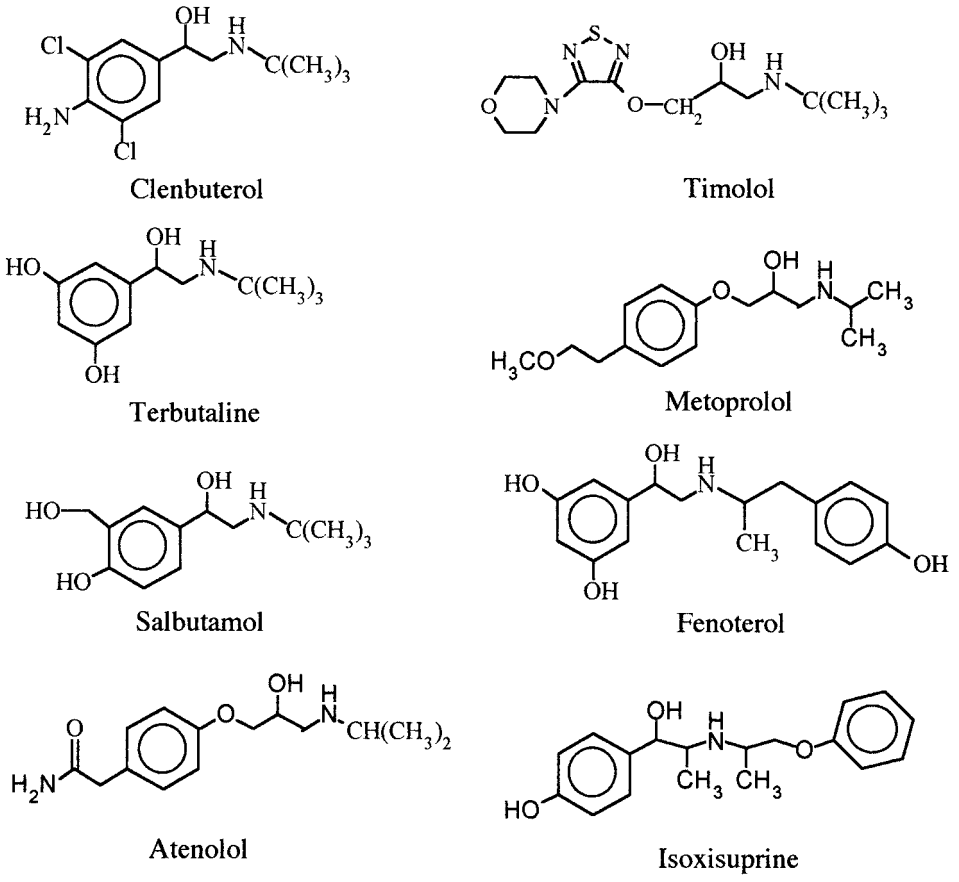

Figure 1 Structures of CL and β-adrenergic compounds used in this study.

of material is synthesized, crushed, ground with a mechanical mill, sieved, and finally sedimented to obtain the final particles. This process has many disadvantages: it is time-consuming and gives polydisperse particles with no defined shape, and the yield of the final material is low (20–30%). To overcome these problems, several attempts have been made to prepare uniform-sized MIPs. A suspension polymerization technique with liquid perfluorocarbons as dispersing agents was used for the preparation of Boc-L-Phe-imprinted polymers; spherical polymeric materials with good recognition ability were obtained.[7,8] In this way, the use of water as a dispersing agent was overcome, thereby preventing the weakening of noncovalent interactions. Moreover, MIPs for (*S*)-naproxen,[9–11] (*S*)-ibuprofen,[12] and propanolol[13] were prepared with a multistep swelling polymerization method[14] with water as a dispersing agent. The recognition ability of these materials was comparable to that of those materials prepared with the nonaqueous bulk polymerization technique.

Recently, we prepared highly selective MIPs for clenbuterol (CL; Fig. 1), with methacrylic acid (MAA) as the monomer, that were able to completely separate the template with respect to many other structurally similar β-adrenergic substances when used as high-performance liquid chromatography (HPLC) stationary phases eluted with a mixture of phosphate-buffered saline (PBS) and acetonitrile.[15,16] We investigated the contribution of the various noncovalent bonds involved in the recognition mechanism, showing the importance of hydrophobic forces coupled with electrostatic and hydrogen-bonding interactions in the overall process.

In this work, we prepared uniform-sized CL molecularly imprinted microbeads with the multistep swelling polymerization approach. Different polymers were prepared either with MAA or acrylamide (AAm) as the monomer at different ratios with the crosslinker [ethylene glycol dimethacrylate (EGDMA)] and with changes in the swelling-step parameters. In particular, AAm

has been reported to provide good imprinting when used to prepare MIP against amino acid derivatives[17] because of its ability to strongly interact with the template via hydrogen bonds. AAm was also chosen because we expected to obtain an improved incorporation of the monomer inside the particles in the second step of swelling on account of its nonionic character. The dependence of the quality of the microbeads on the polymerization conditions is discussed. The molecular recognition ability with respect to other β-adrenergic substances (Fig. 1) was evaluated by the packing of HPLC columns with the imprinted beads.

EXPERIMENTAL

Materials

MAA, EGDMA, AAm, and 2,2′-azobisisobutyronitrile (AIBN) were obtained from Fluka (Milan, Italy). Dibutylphthalate and lauryl sulfate were obtained from Sigma (Milan, Italy). Toluene and sodium chloride were purchased from Carlo Erba (Milan, Italy). Poly(vinyl alcohol) (PVA; 80% hydrolyzed, weight-average molecular weight = 9000–10,000 Da) was obtained from Aldrich (Milan, Italy). 2-*t*-Butylaminoethanol (TBE) was also from Aldrich. All the listed reagents were used as received. Polystyrene microbeads (Prolabo, Pithiviers, France) with an average diameter of 2.967 ± 0.087 μm were exhaustively washed with distilled water before use to eliminate the surfactant. CL, purchased from Resfar (Milan, Italy) as a hydrochloride salt, was extracted with chloroform from an alkaline aqueous solution and finally isolated as a free base by evaporation of the solvent. Timolol (Tim), atenolol (Ate), isoxysuprine (Isox), metoprolol (Met), terbutaline (Ter), and fenoterol (Fen) were obtained from Sigma (Milan, Italy). Salbutamol (Sal) was the kind gift of Chiesi Farmaceutici (Parma, Italy).

For the HPLC analysis, $Na_2HPO_4 \cdot 12H_2O$ and NaH_2PO_4, used as buffers, were obtained from Fluka. Acetonitrile, methanol, acetic acid, and propan-2-ol were supplied by Carlo Erba Reagenti (Milan, Italy). Solvents were analytical-grade or HPLC-grade.

Preparation of the Imprinted Microbeads

CL-imprinted microbeads were prepared by a two-step swelling and polymerization technique (or seed suspension polymerization) technique.[18,19] Polymers with different monomer/crosslinker ratios were synthesized. Corresponding control polymers were also prepared with TBE instead of the print molecule.

First Step of Swelling

The latex was prepared as follows. Distilled water (31 mL), polystyrene microbeads (0.13 g), AIBN (0.55 g), and sodium dodecyl sulphate (SDS) (0.02 g) were placed in a 100-mL, round-bottom flask. The mixture was sonicated for 10 min (UTA 18 Ultrasonic Falc, Bergamo, Italy). Separately, another latex was prepared by the addition of dibutylphthalate (12 mL) and SDS (0.02 g) to 35 mL of distilled water. This mixture was sonicated for at least 10 min and then added to the latex prepared first . The resulting latex was stirred at 200 rpm with an anchor-shaped stirrer for 24 h at room temperature. With an optical microscope, it was possible to evaluate the disappearance of the oil microdroplets in the final suspension, which indicates the end of the swelling process and the complete incorporation of the organic phase into the particles. The diameter of the swollen beads was around 5–6 μm.

Second Step of Swelling

Several different CL-imprinted microbeads were prepared with changes in the monomer (MAA or AAm) and monomer/template ratio (6:1, 12:1, and 18:1). Details on the amounts of the reagents used in each experiment are given in Table I. Toluene (as a porogen), EGDMA, MAA (or AAm), and CL were placed in a round-bottom flask. The mixture was sonicated until CL was completely dissolved. An aqueous solution prepared by the dissolution of PVA in a proper amount of the latex obtained in the first step of swelling was added to the organic phase. The mixture was sonicated to obtain a stable latex and left stirring at 250 rpm and room temperature for 24 h until the swelling process was completed, as judged by the observation of the mixture with an optical microscope.

Polymerization

After the second step of swelling was completed, argon was bubbled through the reaction mixture for 10 min to remove oxygen. Polymerization was then allowed to proceed for 24 h at 70°C with constant stirring (250 rpm). The polymer parti-

Table I Preparation of CL MIPs

	CLMIPMAA-1	CLMIPMAA-2	CLMIPMAA-3	CLMIPAAm
Water (mL)	90	90	90	90
PVA (g)	1.9	1.9	1.9	1.9
MAA (mL)	3	2	1	—
AAm (g)	—	—	—	2.355
EGDMA (mL)	11	11	11	11
Toluene (mL)	10	10	10	10
CL (g)	0.55	0.55	0.55	0.55
1° Step suspension (mL)	13	13	13	13

Control polymers were prepared with the same compositions but with TBE instead of the print molecule.

cles were washed by repeated sedimentation, first in water and then in acetone, to remove the stabilizer and the remaining organic phase incorporated inside the particles. The water washings were collected and used to determine the amount of MAA not absorbed during the swelling process.[20]

The yield of each polymerization was calculated from the weight of the dried polymer after complete removal of the template, with respect to the amount of polystyrene, monomer, and crosslinker used in the experiment.

Imprinted Microbead Characterization

Morphological Characterization

The size distribution and surface characteristics of the polymer microbeads were evaluated with scanning electron microscopy (SEM). An investigation of the rigidity of the porous polymer matrix was performed by the evaluation of the swelling in toluene and in different types of acetonitrile/PBS mixtures used in the chromatographic experiments.

HPLC Analysis

Polymer microbeads (1.5–2 g) were sonicated in chloroform. The suspension was then loaded into a reservoir connected to an HPLC pump, and the HPLC column (stainless steel, 150 × 4.6 mm in inner diameter, fitted with 2-μm frits) was filled under pressure (200 bar) with propan-2-ol as a solvent.

For removal of the template, the column was connected to an HPLC system and washed with 9:1 methanol/acetic acid at a flow rate of 0.5 mL/min until a stable baseline was obtained.

HPLC analysis (Varian LC 5020 chromatograph equipped with a Varian UV 50 variable-wavelength detector and an HP 3395 integrator) was performed isocratically with acetonitrile/PBS ($10^{-3}M$) at different ratios and pHs. The flow rate was 1 mL/min, and the UV detection wavelength was selected on the basis of the analytes and eluent. Solutions (10 μL) of the β-adrenergic compound (1 mg/mL) dissolved in the mobile phase were injected. The void volume of the column was determined by the injection of acetone.

Capacity factors (K'), separation factors (α), and retention indices (RIs) were calculated as follows: $K' = (t - t_o)/t_o$, where t and t_o are the retention times of the analyte and acetone, respectively; $\alpha = K'_{\text{print molecule}}/K'_{\text{test molecule}}$; and RI $= \alpha_b/\alpha_p$, where the subscripts p and b refer to the imprinted and blank polymers, respectively.

RESULTS AND DISCUSSION

To make CL-imprinted polymers suitable for industrial application, some attempts have been made to prepare molecularly imprinted microbeads with the general procedure called the two-step swelling and polymerization technique.[18,21–24]

The main feature of this technique is the initial activation of polystyrene seed particles in an aqueous dispersion. As a result, the activated beads are capable of absorbing the monomer and crosslinker in an amount that far exceeds that of pure polymer beads. The activation of the seeds results from the presence of a highly water-insoluble, low molecular weight compound (dibutylphthalate). An oil-soluble initiator must be

added during the activation step to carry out the final polymerization.

A second step of swelling is subsequently carried out by the addition of the monomer, crosslinker, template, and porogen (which gives macroporous particles) in the form of an aqueous suspension to the activated seed particles.

For the desired results, the choice of components and swelling conditions is of great importance. The optimum swelling process can be achieved if both the monomer and the crosslinker are highly insoluble in the water phase and the suspension is kept fairly stable during the whole process.[22,25,26] Moreover, the choice of the monomer, crosslinker, and porogen should be made with consideration of the imprinting effect.

In a preliminary stage of our study, we demonstrated that the best stabilizer for the second step of swelling was PVA with a molecular weight of about 10,000 Da and a degree of hydrolysis of 80%.

Toluene was selected as the porogen[9,27,28] because it is highly compatible with the imprinting phenomenon and it is incorporated into the organic phase in the particles, resulting in porous microbeads.

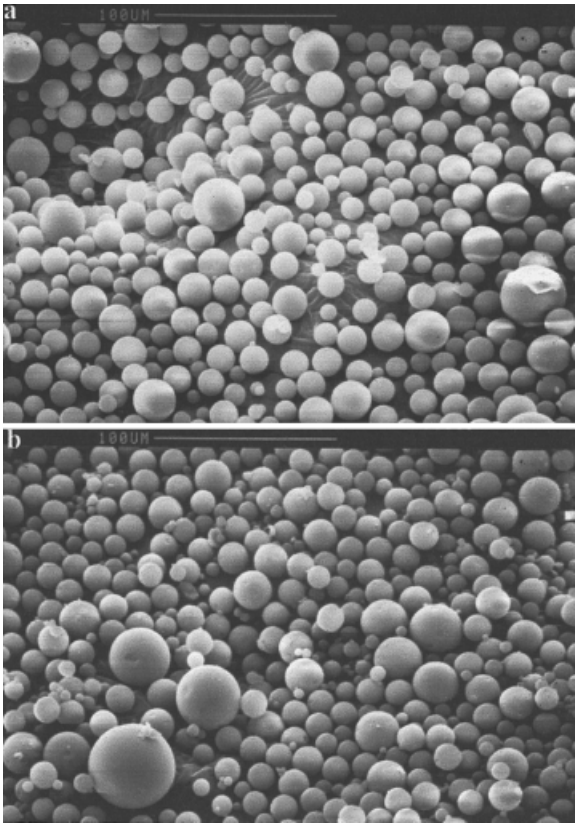

Figure 2 Scanning electron micrographs of (a) CLMIPMAA-1 and (b) CLMIPMAA-2.

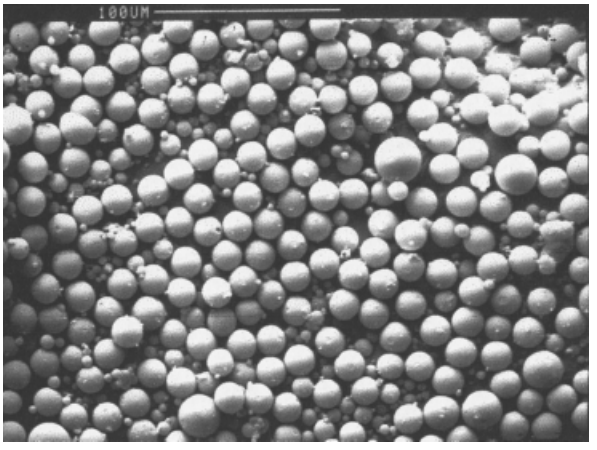

Figure 3 Scanning electron micrograph of CLMIPAAm.

MAA and EGDMA were initially examined as a functional monomer and a crosslinker, respectively, on the basis of the good results we obtained for the bulk polymerization of CL-imprinted polymers.[16] MAA, in fact, may form both ionic and hydrogen bonds with various template functional groups. Furthermore, MAA has already been used successfully as a monomer for the preparation of MIPs with the two-step swelling technique.[9–14] Three samples of MIP microbeads with different compositions were prepared [60:36:2 (CLMIPMAA-1), 60:24:2 (CLMIPMAA-2), and 60:12:2 (CLMIPMAA-3) EGDMA/MAA/CL].

The crosslinker/template ratio was kept constant, whereas the monomer/template ratio was changed. Blank polymers prepared in the same manner but with TBE instead of the template were also synthesized.

The amount of MAA not absorbed by the beads was determined by acid–base titration of the water phase at the end of the second step of swelling. The results confirmed the presence of about 20–25% of the acid in the water phase; therefore, in principle, the amount of MAA absorbed should be enough to guarantee the formation of crosslinker/monomer/template complexes necessary to build up imprinting sites. Actually, as shown later, no separation ability was found at the lower monomer/template ratio (12/2, CLMIPMAA-3). Therefore, this MIP is not further considered in the subsequent discussion.

Unfortunately, the presence of this excess of MAA affected the size distribution of the beads

Table II Chromatographic Results Obtained with CLMIPMAA-1B and the Equivalent Control Polymer

	CLMIPMAA-1B				Control Polymer		
Test Compound	t (min)	K'	α	RI	t (min)	K'	α
CL	17.55	8.62	1	1	3.46	0.90	1
Isoxy	9.28	4.08	2.11	0.53	3.14	0.72	1.25
Ate	8.11	3.44	2.50	0.43	3.36	0.84	1.07
Tim	8.12	3.45	2.49	0.48	3.20	0.75	1.20
Sal	6.12	2.36	3.65	0.41	2.92	0.60	1.50
Ter	5.46	1.99	4.32	0.55	2.78	0.37	2.40
Fen	5.67	2.11	4.10	0.41	2.77	0.52	1.70
Met	8.91	3.88	2.22	0.50	3.29	0.804	1.12

Mobile phase: acetonitrile/PBS (pH 3.9) 70 : 30. Flow = 1 mL/min.

because it had polymerized on the surfaces of the beads themselves. This was shown by the pH value of the aqueous phase after the polymerization step. In the cases evaluated, the pH was around 6, indicating the complete consumption of the acid from the water phase.

The particle size distribution was observed with SEM, which showed an average bead size of 10–15 μm (Fig. 2).

A further attempt to obtain CL-imprinted polymer beads was carried out with AAm as the monomer instead of MAA but with the crosslinker/monomer/template ratios unchanged (60:36:2, CLMIPAAm). The use of this monomer eliminated ionic interactions with CL because of the absence of the carboxylic group but allowed the formation of strong hydrogen bonds with the template molecule.[17]

The polymer beads had a narrower size distribution range than the range obtained with MAA as the monomer. The diameter of the majority of the particles was about 15 μm, although a small percentage of 3-μm-diameter beads were present. (Fig. 3).

In all the syntheses, very good yields were obtained, varying from 75 to 85%, a great improvement over the 20–30% yields obtained with the traditional bulk polymerization method.

SEM images of the different kinds of beads synthesized also showed that highly porous matrices were obtained. The rigidity of the particles was investigated through measurements of the swelling of the porous polymers in toluene and different acetonitrile/PBS eluents. Rigidity is particularly important for chromatographic applications, for which there is a demand for packing

Table III Chromatographic Results Obtained with CLMIPMAA-2 and the Equivalent Control Polymer

	CLMIPMAA-2				Control Polymer		
Test Compound	t (min)	K'	α	RI	t (min)	K'	α
CL	23.20	13.02	1	1	4.70	1.84	1
Isoxy	9.00	4.44	2.93	0.39	4.30	1.60	1.15
Ate	12.38	6.48	2.00	0.40	5.45	2.29	0.80
Tim	6.50	2.93	4.44	0.05	5.41	2.27	0.21
Sal	6.26	2.78	4.68	0.21	4.72	1.85	0.99
Ter	5.57	2.36	5.51	0.17	4.79	1.90	0.97
Fen	5.40	2.27	5.73	0.18	4.67	1.82	1.01

Mobile phase: acetonitrile/PBS (pH 3.4) 85 : 15. Flow = 1 mL/min.

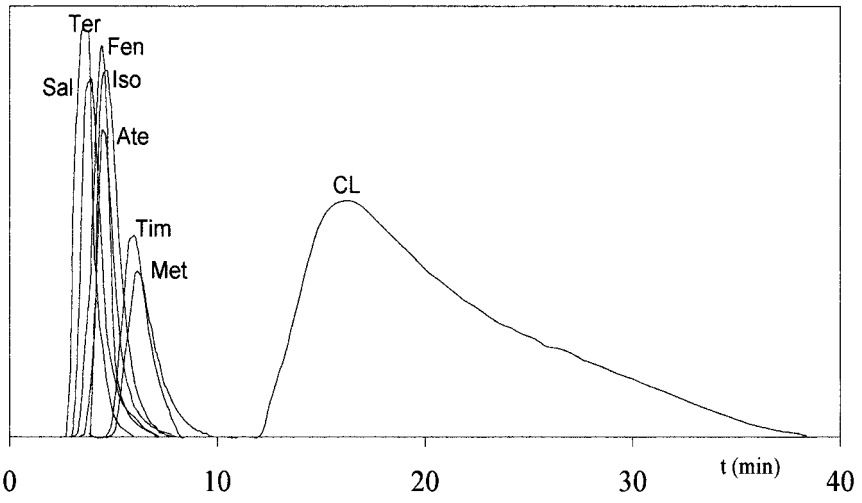

Figure 4 HPLC profiles obtained by the isocratic elution of β-adrenergic agents with the CL-imprinted polymers CLMIPMAA-1A and CLMIPMAA-1B.

materials that are noncompressible and able to withstand high pressures.

As expected, the best results were obtained with CLMIPMAA-2, which had the highest degree of crosslinking. In this case, the increase in the volume of the beads in toluene after 24 h of equilibration was 20%, whereas with CLMIPMAA-1 and CLMIPAAm (18:1 monomer/template), the increase in the volume was 50%. No swelling was shown with the other eluents tested.

The chromatographic characterization of the MIPs was carried out by the fine tuning of the composition of the eluent with respect to the pH and the organic/aqueous ratios.

For the polymers produced with MAA, the best performances were obtained with slightly different eluents for CLMIPMAA-1 and CLMIPMAA-2 [70:30 (pH 3.9) and 85:15 (pH 3.4) acetonitrile/PBS, respectively; Tables II and III, Figs. 4 and 5].

In all cases, good separation comparable to that obtained with bulk polymerization was obtained.

However, with the AAm-based MIP, the optimum eluent to achieve the best selectivity for CL, over the other β-adrenergic molecules, was 50:50 acetonitrile/PBS (pH 2). Results are reported in Table IV and Figure 6.

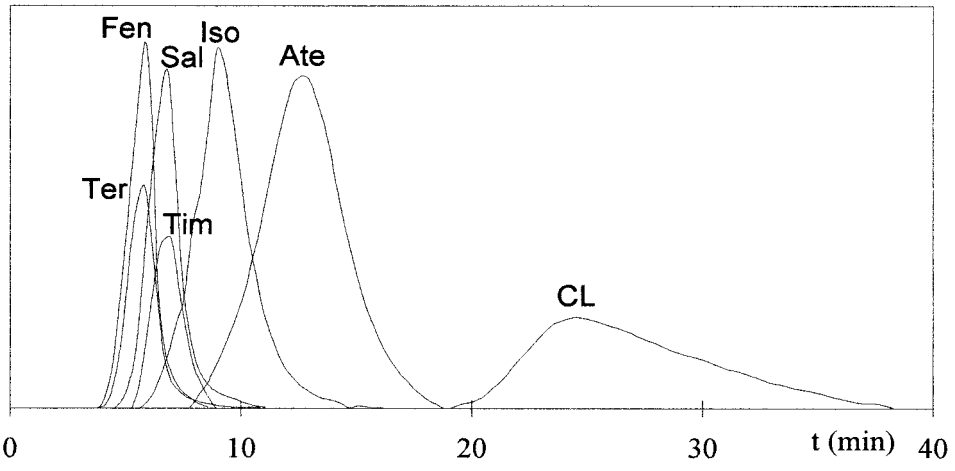

Figure 5 HPLC profiles obtained by the isocratic elution of β-adrenergic agents with the CL-imprinted polymer CLMIPMAA-2.

Table IV Chromatographic Results Obtained with the MIP EGDMA–AAm–CL (60 : 36 : 2) and the Equivalent Control Polymer

	EGDMA–AAm–CL				Control Polymer		
Test Compound	t (min)	K'	α	RI	t (min)	K'	α
CL	6.54	4.18	1	1	2.27	0.90	1
Isox	1.62	0.29	14.43	0.12	1.88	0.58	1.56
Ate	1.38	0.1	41.80	0.60	1.24	0.03	25.11
Tim	2.53	1	4.18	0.55	1.66	0.39	2.3
Sal	1.36	0.08	52.25	0.03	1.84	0.54	1.66
Ter	1.47	0.16	26.12	0.06	1.85	0.55	1.65
Fen	1.48	0.17	23.75	0.09	1.68	0.41	2.2
Met	1.55	0.23	17.94	0.08	1.89	0.58	1.55

Mobile phase: acetonitrile/PBS (pH 2) 50 : 50.

It is evident that with these mobile phases, nonspecific interactions between the polymer and other β-adrenergic substances are minimized, whereas the ones due to the imprinting effect remain. As a result, CL is retained much more than the other molecules tested, providing complete resolution of the template from the other compounds. This was further confirmed by the lack of recognition observed with the blank polymers, for which the retention time of CL was similar to those of the other substances tested.

Furthermore, the complete lack of recognition ability (data not shown) observed when a competing ligand (acetic acid) was used in the eluent (90:10 acetonitrile/acetic acid) clearly confirmed the relevance of hydrogen-bonding interactions in the template recognition.

CONCLUSIONS

CL MIP microbeads were prepared with a two-step swelling and thermal polymerization technique already reported in the literature. MIPs prepared with MAA and EGDMA as the monomer and crosslinker, respectively, at different ratios showed a good separation capacity of CL with respect to other β-adrenergic agents, with a complete baseline separation obtained in HPLC experiments with PBS/acetonitrile eluents. AAm as a new interacting monomer for the two-step swelling technique was also evaluated because of its ability to give stronger hydrogen-bonding interactions than MAA. The lower water solubility of AAm with respect to MAA led to better control of the polymerization process, producing mi-

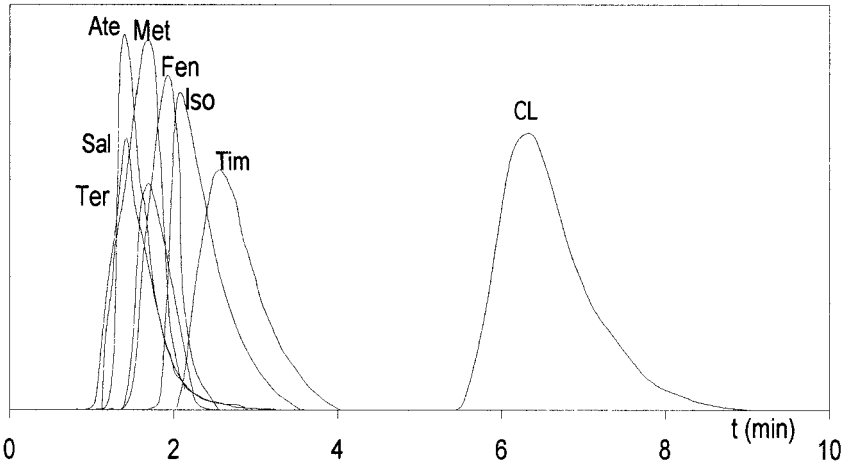

Figure 6 HPLC profiles obtained by the isocratic elution of β-adrenergic agents with the CL-imprinted polymer EGDMA–AAm–CL (60:36:2).

crobeads with lower polydispersity but with no lack of separation capacity with aqueous eluents.

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REFERENCES

1. Mosbach, K.; Ramstrom, O. Biotechnology 1996, 14, 163.
2. Ansell, R. J.; Kriz, D.; Mosbach, K. Curr Opin Biotechnol 1996, 7, 89.
3. Shea, K. J. Trends Polym Sci 1994, 2, 166.
4. Wulf, G. Angew Chem Int Ed Engl 1995, 34, 1812.
5. Sellergren, B. Trends Anal Chem 1997, 16, 310.
6. Mayes, A. G.; Mosbach, K.; Sellergren, B. Trends Anal Chem 1997, 16, 321.
7. Mayes, A. G.; Mosbach, K. Anal Chem 1996, 68, 3769.
8. Ansell, R. J.; Mosbach, K. J Chromatogr A 1997, 787, 55.
9. Haginaka, J.; Takehira, H.; Hosoya, K.; Tanaka, N. Chem Lett 1997, 555.
10. Haginaka, J.; Takehira, H.; Hosoya, K.; Tanaka, N. J Chromatogr A 1998, 816, 113.
11. Haginaka, J.; Takehira, H.; Hosoya, K.; Tanaka, N. J Chromatogr A 1999, 849, 331.
12. Haginaka, J.; Sanbe, H.; Takehira, H. J Chromatogr A 1999, 857, 117.
13. Haginaka, J.; Sakai, Y.; Narimatsu, S. Anal Sci 1998, 14, 829.
14. Hosoya, K.; Fréchet, J. M. J. J Polym Sci Part A: Polym Chem 1993, 31, 2129.
15. Crescenzi, V.; Masci, G.; Fonsi, M.; Casati, G. Polym Prepr 1998, 39, 699.
16. Masci, G.; Casati, G.; Crescenzi, V. J Pharm Biomed Anal, to appear.
17. Yu, C.; Mosbach, K. J Org Chem 1997, 62, 4057.
18. Ellingsen, T.; Sintef, O. A.; Ugelstad, J.; Hagen, S. J Chromatogr 1990, 535, 147.
19. Hosoya, K.; Yoshizako, K.; Shirasu, Y.; Kimata, K.; Araki, T.; Tanaka, N.; Haginaka, J. J Chromatogr A 1994, 728, 139.
20. Baggiani, C.; Trotta, F.; Giraudi, G.; Moraglio, G.; Vanni, A. J Chromatogr A 1997, 786, 23.
21. Ugelstad, J.; Kaggerud, K. H.; Hansen, F. K.; Berge, A. Makromol Chem 1979, 180, 737.
22. Ugelstad, J.; Mork, P. C. Adv Colloid Interface Sci 1980, 13, 101.
23. Ugelstad, J.; Hansen, F. K. J Polym Sci Polym Chem Ed 1978, 16, 1953.
24. Ugelstad, J.; Hansen, F. K. J Polym Sci Polym Chem Ed 1979, 17, 3033.
25. Mark, B.; Overberger, M. Encyclopedia of Polymer Science and Engineering, 2nd ed.; Vol. 6, p 1.
26. Arshady, R. J Chromatogr 1991, 586, 181.
27. Sellergren, B.; Shea, K. J Chromatogr 1993, 635, 31.
28. Svec, F.; Fréchet, J. M. J. Science 1996, 273, 205.