Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 804 (2004) 43–51
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Synthesis and evaluation of molecularly imprinted polymers for enalapril and lisinopril, two synthetic peptide anti-hypertensive drugs

Georgios Theodoridis*, Georgia Konsta, Christina Bagia

Department of Chemistry, Aristotle University Thessaloniki, Thessaloniki 54124, Greece

Abstract

Molecularly imprinted polymers (MIPs) for the recognition of enalapril and lisinopril were prepared using 4-vinylpyridine as the functional monomer. Following thermal polymerisation the resulting materials were crushed, ground and sieved. First generation MIPs were produced in protic polar porogenic solvents (mixture of methanol (MeOH) and acetonitrile (ACN)). These MIPs were used and validated as sorbents for solid phase extraction and binding assays. Second generation MIPs were produced with polar aprotic porogenic solvent (DMSO). These polymers were packed in HPLC columns in order to investigate their molecular recognition properties in a dynamic mode. The study of the mobile phase composition included two major parameters: organic modifier content and pH value. Retention factors illustrate selective binding of the template from the imprinted polymers, compared to structurally related compounds.

Keywords: Molecularly imprinted polymers; Enalapril; Lisinopril

1. Introduction

Angiotensin-converting enzyme (ACE) inhibitors are highly specific drugs, which have found extensive use in therapies of hypertension and lately in the treatment of heart failure. ACE inhibitors represent a family of structurally analogous compounds. Since the development of captopril in 1977, many other synthetic peptides of improved properties have found their way to the market: lisinopril, enalapril, ramipril, quinapril, benazepril and, so forth. Their increasing use has pushed analytical chemists to develop analytical methods for their determination in pharmaceutical preparations or biological samples. Hence, spectrophotometric methods, HPLC, capillary electrophoresis, flow injection analysis or combination of them have been reported [1–3].

Enalapril is a prodrug that is not itself highly active. When administered to humans, enalapril is hydrolysed by serum esterase to the active parent dicarboxylic acid enalaprilate (Fig. 1). Enalapril is considered to be a tripeptide analogue rather than a dipeptide. Lisinopril is another synthetic tripeptide exhibiting similar activity and thus therapeutic use. The chemical structure and the properties of the compounds provide simultaneously an attractive model and a challenge for molecular imprinting.

Molecular imprinting is a fast growing research field with significant interest for the separation scientist. Among the applications reported, solid phase extraction (SPE) and chromatographic separations seem to be the most promising [4–10]. The benefits from a stationary phase selective for a given analyte are immense for chromatographic method development. Originating from various starting points an increasing demand for more efficient separation methods drives many chromatographers towards the optimisation of stationary phase selectivity. The most profound example can be seen in the need of the pharmaceutical industry (joined lately by the agrochemical industry) for chiral purification of their products. Molecularly imprinted polymers (MIPs) have shown promise as separation media of predetermined selectivity despite problems such as peak asymmetry, tailing and low plate number. In the majority of the reported works, molecular imprinting occurs in aprotic non-polar environment. However, recent publications report the production of MIPs in protic solvents [11–13].

Molecular imprinting has been mainly applied to rather small molecules of certain characteristics (polarity, functionalities and solubility). Templating larger entities represents a technical challenge due to the multitude of functionalities, the large size and the diversifying shape

* Corresponding author. Tel.: +30-2310-997718; fax: +30-2310-997719.
E-mail address: gtheodor@chem.auth.gr (G. Theodoridis).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.12.035

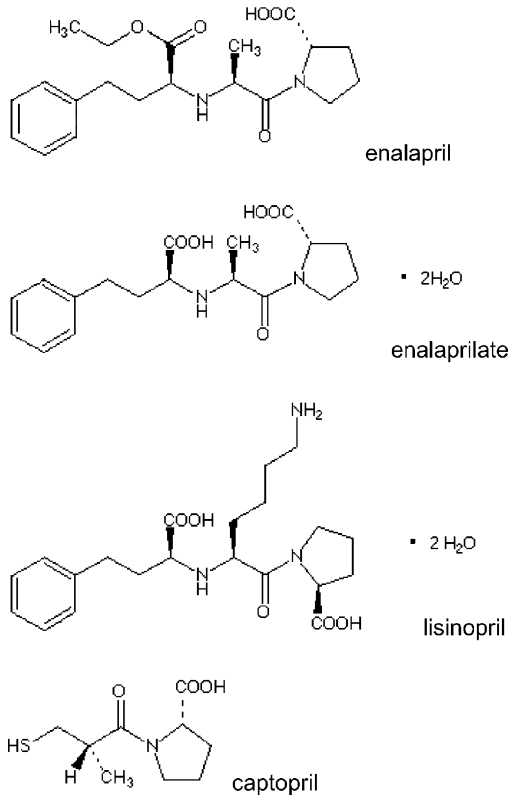

Fig. 1. Molecular structures of the synthetic peptide ACE inhibitors.

of the target molecules. Especially biomolecules represent a difficult target as they do not show tolerance to typical polymerisation conditions (organic solvents, increased temperature, etc.). Template polymerisation of large peptides and proteins is limited also in the final utilisation step: bulky molecules exhibit slow kinetics and cannot interact effectively with molecular recognition pockets in the polymer network (mainly due to steric hindrance). Thus, limited work has been so far performed for the imprinting of peptides and proteins, despite the advantageous features that such a polymeric receptor could offer. Apart from a few exceptions, the majority of the reported works on amino acid and peptide imprinting utilised entities protected with appropriate groups: Boc protected [13–16], *Z*-protected [16,17], acetyl protected peptides [18] or peptide anilides [19]. Peptide modification is generally selected in order to accomplish satisfactory template solubility in apolar solvents (an environment favourable for hydrogen bonding interactions during template polymerisation). Whitcombe and coworkers [20] utilised the "semi-covalent" approach to specifically position selected methacrylic binding sites towards the amino end-groups of a Lys–Trp–Asp tripeptide. The authors reported exceptional discrimination of similar amino acid sequences from acetonitrile (ACN)–water mixtures. Hart and Shea [21,22] exploited Ni(II)–His interactions to manufacture functionalised polyacrylamides for the recognition of His terminal peptides in aqueous solutions. The above works required considerable synthetic effort for the production of polymerisable units carrying the recognition element. Lately, Rachkov and Minoura [23,24] coined the epitope approach, probably inspired by the natural counterparts of MIPs the antibodies. A short peptide sequence representing an exposed fragment of the whole protein is used as the template. The templated polymer is used later for the recognition of the larger entity.

The objective of the present research was the production of MIPs for lisinopril and enalapril two synthetic peptide pharmaceuticals. The authors' intention was to investigate the possibility and the potential of using plain (non-protected) peptides as templates to raise affinity sites in polymeric networks. 4-Vinylpyridine was chosen as the functional monomer and ethyleneglycol dimethylacrylate as the crosslinker. Polymerisation was initiated thermally using both protic and aprotic solvents as the porogens. The obtained polymers were evaluated in binding assays, solid phase extraction (SPE) and HPLC protocols.

2. Materials and methods

2.1. Materials

Enalapril and lisinopril were a kind gift from Farmathen Pharmaceuticals (Athens, Greece), Captopril was offered from Elpen Pharmaceuticals (Athens, Greece). Ethylene glycol dimethacrylate, 4-vinylpyridine and α,α′-azobis-isobutyronitrile (AIBN) were from Fluka (Buchs, Switzerland). All organic solvents used (acetonitrile (ACN), methanol (MeOH), ethanol) were of HPLC quality and were obtained from Merck (Darmstadt, Germany). Water was double de-ionised and filtered though a 20 μm filter (Schleicher & Schuel, Dassel Germany).

2.2. Polymerisation

Polymerisation was initially performed in mixtures of acetonitrile–methanol. Polymers P1, B1, P2, B2 for enalapril and P1 L, B1 L, P2 L, B2 L, P3 L, B3 L for lisinopril. At a later stage polymerisation was performed in DMSO (Polymers P3 P4, P5, B3). The corresponding quantities of the chemicals employed in each polymerisation mixture are summarised in Tables 1 and 2.

For the polymerisation procedure the template was dissolved in appropriate volume of the porogen. 4-Vinylpyridine (4-VPy, functional monomer) and ethyleneglycol dimethacrylate (EGDMA, cross linker) and α,α′-azobis-isobutyl nitrile (AIBN, initiator) were added to the solution. The mixture was purged with N_2 for 5 min for degassing. The tube was subsequently sealed and transferred to a thermostated water bath (60 °C) for 24 h. Next, the tubes were smashed and the polymers obtained, were crushed, ground and then extracted using a Soxhlet apparatus in order to remove the template thus releasing the binding sites created during the imprinting process. As extraction solvent a

Table 1
Composition of the polymerisation mixtures for the enalapril imprinted and the control polymers

	Template (M)	4-VPy (M)	EGDMA (M)	AIBN (M)	Porogen (ml)
P1	0.094	0.940	3.746	0.047	2.8 [ACN–MeOH (7.6:1 (v/v))]
B1	0.000	0.940	3.746	0.047	2.8 [ACN–MeOH (7.6:1 (v/v))]
P2	0.068	0.763	2.703	0.043	3.2 [ACN–MeOH (10:6 (v/v))]
B2	0.000	0.763	2.703	0.043	3.2 [ACN–MeOH (10:6 (v/v))]
P3	0.019	0.877	4.373	0.108	5.2 DMSO
P4	0.192	0.877	4.373	0.108	5.2 DMSO
P5	0.327	0.877	4.373	0.108	5.2 DMSO
B3	0.000	0.877	4.373	0.108	5.2 DMSO

Table 2
Composition of the polymerisation mixtures for the lisinopril imprinted and the control polymers

	Template (M)	4-VPy (M)	EGDMA (M)	AIBN (M)	Porogen (ml)
P1 L	0.033	0.334	1.338	0.046	6.8 [ACN–MeOH (3:5 (v/v))]
B1 L	0.000	0.334	1.338	0.046	6.8 [ACN–MeOH (3:5 (v/v))]
P2 L	0.034	0.669	3.346	0.046	13.3 [ACN–MeOH (3:10 (v/v))]
B2 L	0.000	0.669	3.346	0.046	13.3 [ACN–MeOH (3:10 (v/v))]
P3 L	0.033	0.334	1.672	0.046	6.8 [ACN–MeOH (3:5 (v/v))]
B3 L	0.000	0.334	1.672	0.046	6.8 [ACN–MeOH (3:5 (v/v))]

mixture of methanol–acetic acid 9:1 (v/v) was used. Soxhlet extraction lasted for 24 h resulting in more than 30 solvent cycles. To estimate the effectiveness of the template removal, the concentration of enalapril in the extracts was determined spectrophotometrically by UV and HPLC. The polymers were then processed through a series of grinding and sieving steps to be finally sized between 20 and 71 μm. Fine particles produced during the crushing procedure were removed by repeated sedimentations in methanol–water 1:1 (v/v). Finally, the particles were dried under vacuum and stored at ambient temperature until use. Control (non-imprinted polymers, NIP) were prepared following the same procedure (Soxhlet extraction included), but with the omission of the template.

2.3. Use of MIPs for SPE

Polymers produced in protic environment (Tables 1 and 2) were packed in SPE cartridges, and were validated as media for selective sample preparation. 200 mg of each polymer (20–71 μm) were packed in a cartridge and next the SPE microcolumn was conditioned with appropriate solvents. The analytes were applied on the cartridge in aqueous solutions and next the cartridge was washed with a series of selected solutions (see Table 3 for conditions). These fractions were collected and evaporated to dryness; the residues were reconstituted in an aliquot of the mobile phase and the resulting solutions were analysed by HPLC (conditions in paragraph for UV and HPLC determinations), in order to calculate the recoveries of each protocol and each wash–elution step.

2.4. Binding assays

Binding assays were performed using the fine particles obtained from the sedimentation of the polymers. One milligram quantities of each polymer was weighed in Eppendorf tubes and 1 ml of solutions of the peptides in the binding solvent (0.1–20 μg/ml) were added in the tubes. The tubes were shaken for 30 min and left to incubate at room

Table 3
Extraction recoveries (%) for enalapril obtained at the elution fraction of MI–SPE

Extraction protocol	Recovery (%)											
	P1			B1			P2			B2		
	Load	Wash	Elute	Load	Wash	Elute	Load	Wash	Elute	Load	Wash	Elute
A	80	5	20	83	5	12	63	18	21	63	15	18
B	11	2	85	7	27	62	2	19	76	12	5	75
C	1	2	97[a]	34	7	59[a]	26	7	77[a]	2	1	84[a]

Protocol A: Conditioning with water and polymerisation solvent (Table 1). Loading 2 μg/ml in polymerisation solvent, wash with water. Elute with acetic acid–MeOH (1:9 (v/v)). *Protocol B*: Conditioning with water and buffer PO_4^{3-} (10 mM, pH 3). Loading 2 μg/ml in buffer PO_4^{3-} (10 mM, pH 3), wash with water and elute with acetic acid–MeOH (1:9 (v/v)). *Protocol C*: Conditioning with water and buffer PO_4^{3-} (10 mM, pH 3). Loading 2 μg/ml in buffer PO_4^{3-} (10 mM, pH 2), wash with water and elute with acetic acid–MeOH (1:9 (v/v)) and aq. NH_4OH–MeOH (1:99).

[a] Total combined recovery of the two elution steps.

temperature for 24 h. The binding solvent was a mixture of the polymerisation solvent mixture (methanol–acetonitrile 7.5:1 or 3:1 (v/v)) with water in ratios: organic–water 1:1 and 9:1 (v/v). Next the polymer particles were separated by centrifugation at 3500 rpm for 5 min. One millilitre of the solution was transferred to a cuvette and its concentration in the respective peptide was measured using a UV spectrophotometer.

2.5. UV and HPLC determinations

In order to enable the determination of the peptide fraction bound to the polymers, a fast and simple procedure was necessary. UV measurements at 207 nm were used to determine the amount remaining free in solution. Initially the effect of solution pH and analyte concentration on the UV spectrum of the peptides were studied. The solvent chosen for this study was the polymerisation solvent since this was the same solvent to be used for binding assay. However, studies were also performed with methanolic peptide solutions. A calibration curve was constructed for each peptide by measuring the absorbance of seven analyte concentrations in the range of 0.1–50.0 μg/ml.

For HPLC analysis a Jasco BIP pump was used to deliver the eluent to a MZ Analysen Technik (Mainz, Germany) Inertsil ODS-2 column (5 μm, 250 mm × 4 mm). The column was thermostated at 40 °C within a Hichrom Limited thermojacket column oven. Detection was performed on a Jasco UVIDE C-100 UV detector operating at 207 nm and chromatograms were recorded on a HP 4690 integrator. A Rheodyne (Cotati, CA) 7125 injection valve was used for manual sample introduction of 20 μl (loop volume). The mobile phase used for the determination of the peptides after MIP–SPE was a mixture of acetonitrile–aqueous phosphate buffer (15 mM KH_2PO_4, pH 3) with a flow rate of 1 ml/min.

2.6. Preparation of MIP-HPLC columns and MIP-HPLC

Polymers P3, P4, P5 and B3 were validated as HPLC stationary phases. An amount of polymer particles (20–71 μm particle size) was suspended in MeOH and slurry packed in stainless steel HPLC columns (150 mm × 4 mm i.d.). The operating pressure was measured to provide an indication of the packing quality. Typical pressures were in the range of 400–600 psi with the mobile phases used in this study.

Chromatographic analysis of the template and the related peptide molecules was utilised for the evaluation of the recognition properties of the produced polymers. To study the effect of the mobile phase composition on the retention of the peptides on the polymeric phases, acetonitrile–water mixtures were used as mobile phases (organic modifier content varying from 0 to 100%). The columns packed with the polymer particles, were equilibrated with the corresponding mobile phase until a stable baseline was observed at 207 nm. Injections of two different concentrations of the peptides were performed. To study the effect of the mobile phase pH value, a phosphate buffer (15 mM KH_2PO_4, pH 3.1) was adjusted to the selected value with the addition of small amounts of 0.1 M NaOH solution. Twenty microliter of peptide solution were injected on the HPLC. A solution of 0.1% (v/v) acetone in the mobile phase was used as a void marker. Each analysis was repeated at least once to ensure chromatographic reproducibility. The retention factor k was calculated as $t_R - t_0/t_0$, where t_R is the retention time of the respective analyte and t_0 the retention time of the void marker, corresponding to the void volume of the system. The Imprinting factor, I_f, for the template was calculated as the ratio k_{MIP}/k_{NIP}.

3. Results and discussion

3.1. Imprinted polymer preparation

As can be seen in Fig. 1, the synthetic peptide molecules encompass functional groups that may form hydrogen bonds with the functional monomer, during the pre-organisation of monomer and template in the pre-polymerisation solution. A major limitation faced in the preparation of the MIPs was the low solubility of the peptides in non-polar organic solvents. The peptides are polar molecules readily soluble in water and methanol but, unfortunately, insoluble to most organic solvents. Therefore, initially a polymerisation in protic environment (ACN–MeOH 7.5:1 (v/v) for enalapril and ACN–MeOH 1:3 (v/v) for lisinopril) was performed (details in Tables 1 and 2). The presence of polar protic solvents in the polymerisation mixture is reported to be disadvantageous for imprinting by non-covalent interactions. Such solvents hinder the formation of hydrogen bonds between the template and the functional monomers, desired for the arrangement of selective binding sites. However, polymerisation in similar protic environments has been reported by Haupt et al. [11,25], Kempe and Mosbach [13] and Baggiani et al. [26] for the production of MIPs to be used in binding assays for herbicides. This approach is based on the anticipation that a hydrophobic template will generate hydrophobic binding sites in the polymer. Polymers obtained this way, often function similarly to a reversed phase chromatographic medium with molecular recognition properties. However, in the present study the investigated peptide molecules demonstrate high polarity and hydrophilicity, thus giving little hope for the exploitation of such a mechanism. Therefore, as described in the following paragraphs MIPs produced in protic environment did not show a prominent imprinting effect. Hence, further polymerisations were performed in aprotic environment. DMSO was found to be the only aprotic solvent to provide adequate solubility for the peptide enalapril as a template. Thus, second generation MIPs were produced only for enalapril (polymers P3, P4, P5 and control polymer B3 in Table 1).

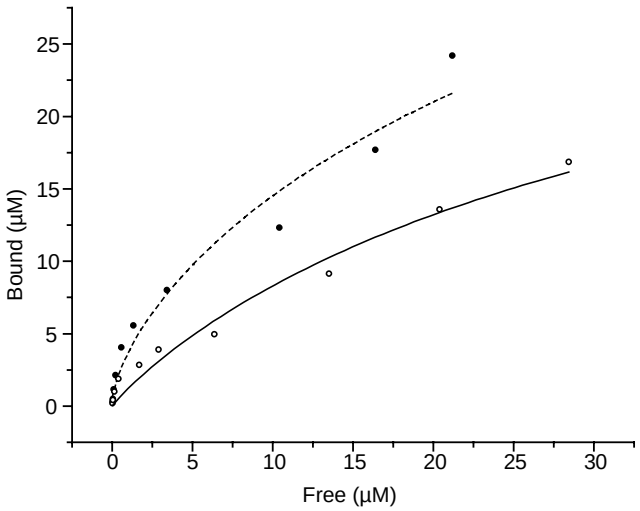

Fig. 2. Binding isotherms obtained from batch rebinding experiment of lisinopril from the polymer P1L (MIP; dashed line solid marks) and B1L (NIP; solid line with open marks). Binding solvent: organic:water 90:10, (organic: ACN–MeOH, 1:3 (v/v)).

3.2. Binding assays

The polymers produced in protic environment were evaluated as materials for SPE and binding assays. Throughout the study these MIPs exhibited high non-specific binding. Yet after optimisation of the binding conditions, the MIPs revealed recognition properties, binding fairly strongly the template in both solvent mixtures. An example of binding isotherm curves showing the amount of lisinopril bound by the MIP and the control polymer as a function of the remaining free lisinopril concentration is seen in Fig. 2. Stronger binding of the template occurred on the imprinted phases compared to the control polymers. As a rule, the graphical representation of the experimental results (binding isotherms and Scatchard plots) revealed the existence of two types of binding sites: high affinity sites and low affinity sites. Such phenomena have been described for imprinted polymers and are mainly attributed to the non-homogeneity of the pre-polymerisation complexes formed by self-assembly of the template with the monomers [27].

In the present study, due to the low UV absorptivity of the peptides, the detection limits observed were much higher than the peptide therapeutic levels. Hence, such a binding assay could not be used as an analytical tool for such aspects. To facilitate such goals other methodologies should be sought, e.g. utilising a labelled peptide analogue as a tracer in a competitive assay. Additionally, for such analytes it seems advantageous to intend towards the production of more selective polymeric hosts using more advanced imprinting technologies such as the semi-covalent approach utilising a sacrificial spacer [20], e.g. a hierarchical imprinting mode recently described [28]. Such polymers could provide more selective and specific analyte binding, a parameter necessary for bio-analytical applications.

3.3. Utilisation of MIPs as SPE media

The MIPs that were used for SPE were those produced in protic solvents (P1, P2, B1, B2). Different extraction protocols were used to evaluate retention and recognition on the MIPs. In general, they exhibited high non-specific interactions with their templates and other related analytes. However, in selected environments indications of the existence of template recognition were observed. Table 3 depicts the results of the three characteristic SPE assays using the MIPs for the extraction of enalapril. The same conditions were applied for the extraction of lisinopril from the lisinopril imprinted polymers (P1 L, B1 L, P2 L, B2 L). As seen in Table 3 using protocols A and B, the imprinted polymers did not provide recoveries higher than the control polymers. Loading of enalapril in the polymerisation solvent resulted in breakthrough of the peptides (protocol A). To overcome this problem SPE was performed in the “reversed phase mode” loading in acidic aqueous medium (pH 3). This resulted in analyte binding by non specific interactions (most likely ionic but also hydrophobic) and thus similar recoveries on the MIPs and the NIPs (protocol B). Lowering the pH of the loading fraction polymer provided the best overall molecular imprinting effect in polymer P1 (protocol C). Similar results were obtained for the SPE assay on the lisinopril imprinted phases. However, these differences were not profound to justify further usage of the polymers as sorbents for selective extraction and analyte isolation in bio-analytical assays.

3.4. Chromatographic characterisation of the MIPs

For the chromatographic characterisation of MIPs frontal chromatography [29], zonal chromatography [29,30] and MIP–HPLC have been proposed [13,14,30]. In the present research, initial studies aimed at the optimisation of the organic solvent content in the mobile phase using as stationary phases polymers prepared in aprotic solvents (P3, P4, P5, B3). Acetonitrile being an aprotic solvent molecule was chosen as the organic solvent. The solvents used for polymerisation could not be an option for such studies, due to incompatibilities with PEEK tubing and low UV detection (DMSO has a UV cut-off at 268 nm and DMF at 368 nm). Mobile phases ranging from pure acetonitrile to pure water were tested for the three imprinted and the non-imprinted control phase. Chromatographic data obtained from such analyses was further analysed to obtain insight in the retention and recognition mechanism. An example is shown in Fig. 3, where a plot of k is given for the elution of the peptides in the imprinted polymeric phase P3. Strong binding of the synthetic peptides was observed in 100% water as the mobile phase. This was attributed to non-specific hydrophobic retention of the peptides on the polymeric phases. Increasing the acetonitrile content decreased retention, as also observed by Baggiani et al. [30]. A reversed phase mechanism seemed to govern retention in water rich mobile phases. However, increasing the acetonitrile

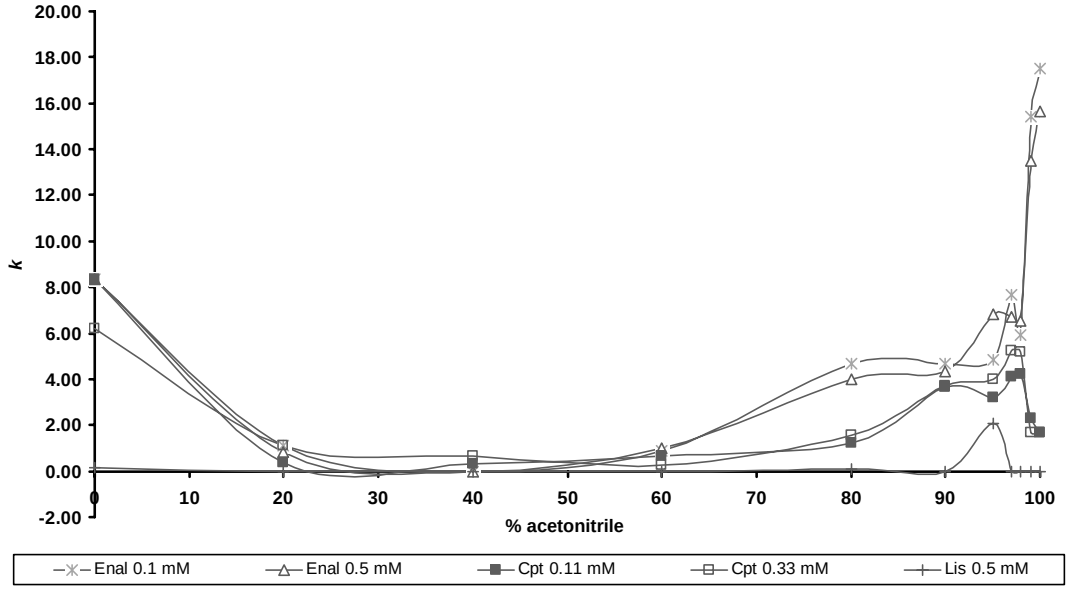

Fig. 3. Effect of mobile phase composition on the retention the three synthetic peptide drugs on P3 imprinted phase (MIP 10 enalapril).

content to higher than 60% resulted in increased retention and selectivity. Especially in pure organic phases (100% acetonitrile), retention and selectivity were optimal: strong binding of the templates was observed in the imprinted polymer, whereas related peptides and especially a not related compound tested (caffeine) exhibited much weaker retention. Such great differences were not observed in the control polymer, where the retention was practically in the same order for all the peptides and could not be attributed to any specific interaction with the stationary phase.

Fig. 4 illustrates a much stronger retention of enalapril from its imprinted phases (especially phases P4 and P3) compared to the other peptides. It is also seen that the non-imprinted phase (B3) exhibits behaviour similar to the imprinted phases for captopril and lisinopril, whereas for the template enalapril the retention is much weaker.

This is more clearly understood when examining the imprinting factors ($I_f = k_{MIP}/k_{NIP}$). Fig. 5 illustrates this perspective for another mobile phase system (acetonitrile–water, 99:1 (v/v)). Much higher imprinting factors are observed for the template compared to related peptides. Again better recognition properties are observed in phases P4 and P3 rather than in phase P5, although the latter was the one produced with the highest template molar ratio. The limited solubility of the template could be a cause of this behaviour. In higher template molar concentrations agglomeration of peptide template in the organic solvent environment could occur. This way a limited

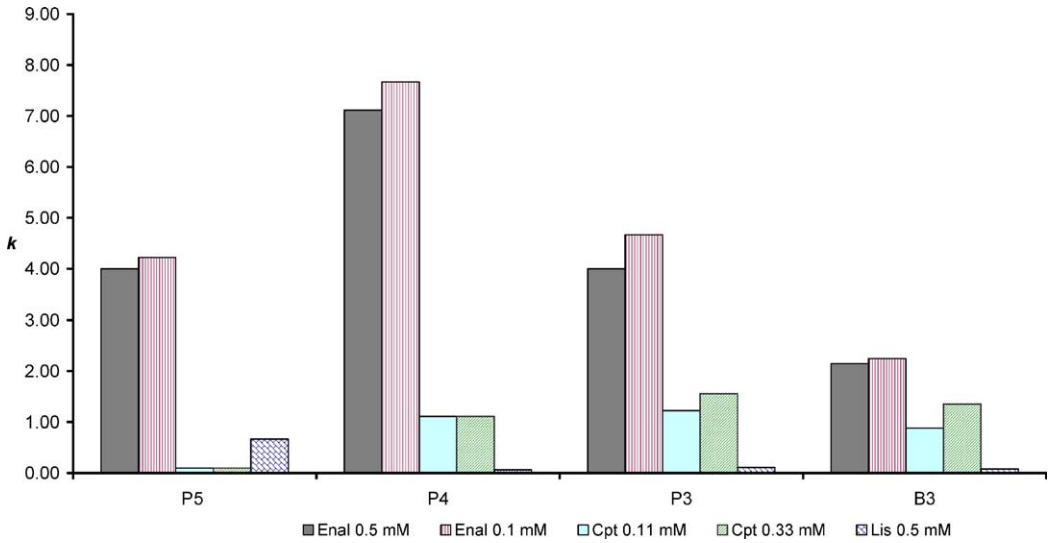

Fig. 4. Retention factors of the three synthetic peptide drugs on three imprinted and the non-imprinted control polymeric phase. Elution with a mobile phase of 80:20 acetonitrile:water v/v. MIP identities as in Table 1.

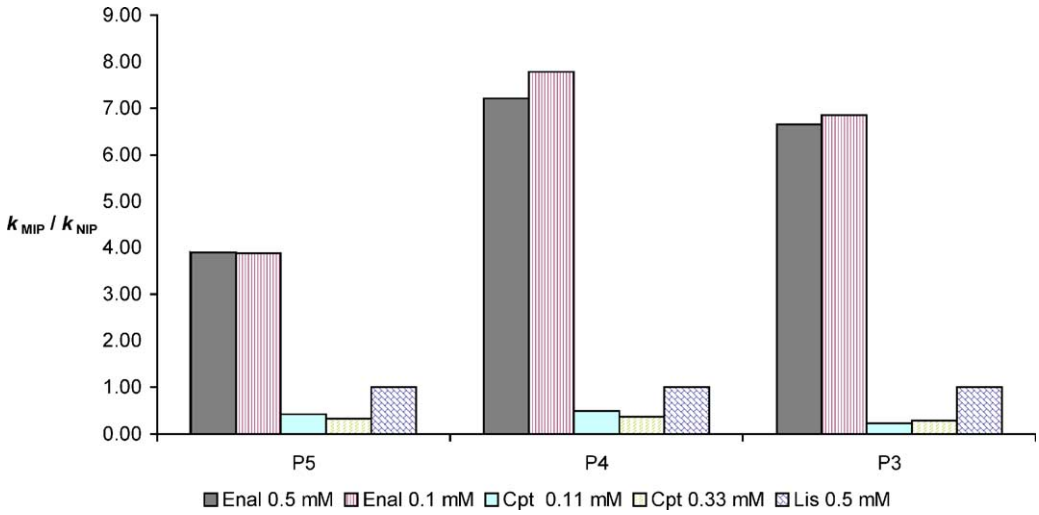

Fig. 5. Imprinting factors ($I_f = k_{MIP}/k_{NIP}$) obtained when eluting enalapril imprinted materials with a mobile phase of ACN–water 99:1 (v/v). Polymer (MIP) identities in Table 1.

amount of the template would be available for incorporation of the template molecules for self organisation and assembly with the monomers. Thus, the number of high affinity recognition sites would be actually limited in the final polymer despite the highest molar ratio of the template in the pre-polymerisation mixture.

Examples of the chromatographic analysis on the polymeric sorbents can be seen in Fig. 6. With a mobile phase of 95:5 (v/v) ACN–water much stronger retention is observed for enalapril (template) in polymers P3 and P4 compared to the control B3 and the imprinted phase P5. Such fingerprints clearly demonstrate a stronger interaction between the analyte and the imprinted polymeric phases. Broad and rather asymmetric peaks were observed. To investigate the source of peak dispersion, the effect of each of the three basic parameters of the van Deemter equation was investigated. Eddy diffusion was not found to be the dominating mechanism since non-related analytes were eluted in sharp symmetric peaks. Also concerning the B term in van Deemter equation (longitudinal diffusion), altering the flow rate did not significantly change the peak shapes. Thus, peak asymmetry for the peptide analytes was attributed to poor mass transfer properties of the polymeric sorbents and to the heterogeneity of the affinity sites. Such phenomena are common in MIPs prepared by conventional bulk polymerisation [31,32].

It is widely accepted that the pH value of the mobile phase affects the chromatographic separation of ionised or ionisable species. This has also been described for imprinted phases [23,29,33,34]. Alteration of pH value of the eluent affects the ionisation status of both the analytes and the stationary phase. Baggiani et al. [30] calculated the degree of ionisation of a methacrylic polymer and the template theophylline in a pH range from 3 to 9. The authors proposed the theoretical presence of more than one carboxyl group in the binding sites which was later supported by Chen et al. [34]. They reported that electrostatic interactions

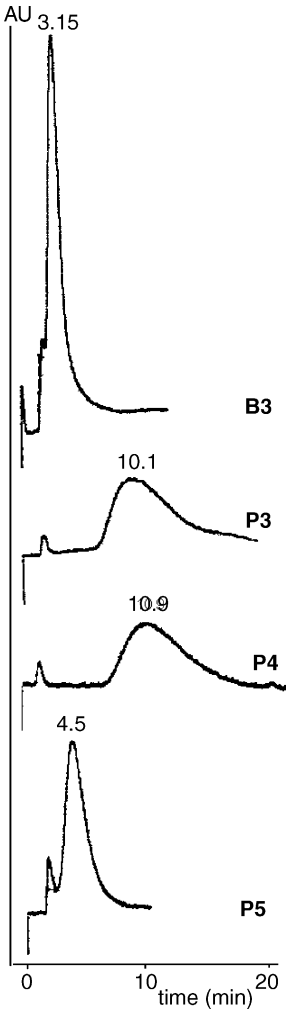

Fig. 6. Chromatographic profiles of 0.5 mM enalapril on the enalapril imprinted polymers: P3–P5 and the control polymer B3 (compositions in Table 1). Mobile phase: ACN–water 95:5 (v/v); flow rate: 1 ml/min; detection at 207 nm.

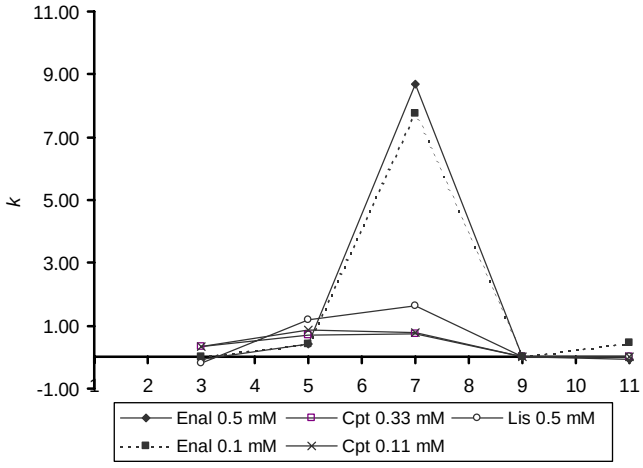

Fig. 7. Effect of mobile phase pH value on the retention of the three synthetic peptide drugs on P4 imprinted phase.

do not play a prominent role in the separation mechanism, which was dominated by the formation of hydrogen bonds (also affected by pH alteration). In the present study, the model compounds exhibit a multiplex of ionisable moieties; lisinopril exhibits four pK values ranging from 2.5 to 10.1. Therefore, it is rather safe to assume that this analyte will be charged in the whole pH working range (pH 3–11, within a mixture of 10% water in acetonitrile). In all the imprinted phases (P3–P5) best conditions were found at pH values of 7. In this pH range, it is assumed that the stationary phase is to a great extent still positively charged. The analytes are also charged: enalapril acidic moiety is dissociated and negatively charged ($pK_{a1} = 3.0$, $pK_{a2} = 5.5$) enabling attractive electrostatic interactions towards the polymeric sorbent. Acidic pH (3 and 5) of the mobile phase suppressed retention and selectivity. This could be attributed to the ionisation and positive charging of both the stationary phase and the analyte molecules and thus repulsive electrostatic forces between them. In alkaline mobile phases (pH 9 and 11) again retention and selectivity were suppressed, resulting to insignificant differences between the analysed peptides. An example of the effect of the pH value on retention behaviour is shown in Fig. 7 for the imprinted polymeric phase P4.

4. Conclusions

Molecular imprinting of synthetic peptides in protic environment produced media where non-selective binding dominated the recognition/retention mechanism. Polymers produced using methanolic mixtures as the porogen exhibited comparable recognition of the template and other related peptides. Additionally similar behavior was observed in the control non-imprinted polymers. Imprinting in aprotic environment (DMSO) provided higher molecular imprinting efficiency. Polymers imprinted using enalapril as the template molecule showed stronger interaction with their template than with other related peptides. Chromatographic evaluation of the polymers provided interesting conclusions on the nature of the recognition mechanism. In pure aqueous mobile phases, a “reversed phase mechanism” resulted in non-selective strong retention of the peptides. Increasing the organic phase content in the mobile phase resulted in decreased retention. Pure organic mobile phases however, facilitated strong selective binding of the template enalapril on the imprinted polymers. Alteration of the pH of the mobile phase was found to greatly affect analyte retention. Electrostatic forces are thus believed to play an essential role in the interaction mechanism especially in polar or aqueous-rich solvents. In such environments and analytes a combination of ionic, and hydrophobic interactions with a stereochemical fit of the template could provide molecular recognition phenomena.

Acknowledgements

The authors are grateful to Prof. M. Georgarakis for supplying the synthetic peptide drugs used in this study and to Prof. H. Tsoukali for HPLC instrument loan.

References

[1] A. Tsakalof, K. Bairachtari, M. Georgarakis, J. Chromatogr. B 783 (2003) 425.
[2] A. Economou, D.G. Themelis, G. Theodoridis, P.D. Tzanavaras, Anal. Chim. Acta 463 (2002) 249.
[3] S. Hillaert, W. Van den Bossche, J. Pharm. Biomed. Anal. 21 (1999) 65.
[4] P.K. Owens, L. Karlsson, E.S.M. Lutz, L.I. Andersson, TRAC Trends Anal. Chem. 18 (1999) 146.
[5] F. Lanza, B. Sellergren, Chromatographia 53 (2001) 599.
[6] B. Sellergren, Anal. Chem. 66 (1994) 1578.
[7] R.J. Ansell, O. Ramstrom, K. Mosbach, Clin. Chem. 42 (1996) 1506.
[8] P.T. Vallano, V.T. Remcho, J. Chromatogr. A 887 (2000) 125.
[9] L.I. Andersson, J. Chromatogr. B 745 (2000) 3.
[10] V.T. Remcho, Z.J. Tan, Anal. Chem. 71 (1999) 248A.
[11] K. Haupt, A. Dzgoev, K. Mosbach, Anal. Chem. 70 (1998) 628.
[12] J. Mathew, O. Buchardt, Bioconj. Chem. 6 (1995) 524.
[13] M. Kempe, K. Mosbach, Anal. Lett. 24 (1991) 1137.
[14] M. Kempe, Anal. Chem. 68 (1996) 1948.
[15] K. Yano, T. Nakagiri, T. Takeuchi, J. Matsui, K. Ikebukuro, I. Karube, Anal. Chim. Acta 357 (1997) 91.
[16] M. Kempe, K. Mosbach, J. Chromatogr. A 691 (1995) 317.
[17] M. Kempe, Lett. Pept. Sci. 7 (2000) 27.
[18] I.A. Nicholls, O. Ramstrom, K. Mosbach, J. Chromatogr. A 691 (1995) 349.
[19] L. Anderson, R. Muller, G. Vlatakis, K. Mosbach, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4788.
[20] J.U. Klein, M.J. Whitcombe, F. Mullholland, E.N. Vullfson, Angew. Chem. Int. Ed. 38 (1999) 2057.
[21] B.R. Hart, K.J. Shea, J. Am. Chem. Soc. 123 (2001) 2072.
[22] B.R. Hart, K.J. Shea, Macromolecules 35 (2002) 6192.
[23] A. Rachkov, N. Minoura, J. Chromatogr. A 889 (2000) 111.
[24] A. Rachkov, N. Minoura, Biochim. Biophys. Acta 1554 (2001) 255.
[25] K. Haupt, A.G. Mayes, K. Mosbach, Anal. Chem. 70 (1998) 3936.
[26] C. Baggiani, G. Giraudi, C. Giovannoli, F. Trotta, A. Vanni, J. Chromatogr. A 883 (2000) 119.

[27] T. Takeuchi, T. Mukawa, J. Matsui, M. Higashi, K.D. Shimizu, Anal. Chem. 73 (2001) 3869.
[28] M.M. Titirici, A.J. Hall, B. Sellergren, Chem. Mater. 14 (2002) 21.
[29] C. Baggiani, F. Trotta, G. Giraudi, G. Moraglio, A. Vanni, J. Chromatogr. A 786 (1997) 23.
[30] C. Baggiani, G. Giraudi, F. Trotta, C. Giovannoli, A. Vanni, Talanta 51 (2000) 71.
[31] G. Masci, G. Casati, V. Cresenzi, J. Pharm. Biomed. Anal. 25 (2001) 211.
[32] V.T. Remcho, Z.J. Tan, Anal. Chem. 71 (1999) 248A.
[33] B. Sellergren, K. Shea, J. Chromatogr. A 654 (1993) 17.
[34] Y. Chen, M. Kele, I. Quinones, B. Sellergen, G. Guiochon, J. Chromatogr. A 927 (2001) 1.