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Synthesis and LC characterization of clenbuterol molecularly imprinted polymers

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Abstract

Highly selective non-covalent clenbuterol (CL) imprinted polymers were prepared using methacrylic acid as monomer and ethylene glycol dimethacrylate as cross-linking agent. HPLC experiments with columns packed with this material showed that CL are selectively recognised with respect to all other adrenergic substances studied using a phosphate buffer/acetonitrile eluent. The separation was strongly dependent on pH and the organic/aqueous phase ratio. An important contribution to the recognition mechanism from hydrophobic interactions was found at higher water content. These results demonstrate that a novel family of absorbents with high selectivity for CL was obtained which can be exploited in solid phase extractions or as recognition elements for selective sensors. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Molecularly imprinted polymers; Clenbuterol; Adrenergic drugs; Hydrophobic interactions

1. Introduction

Clenbuterol (CL) (4-amino-3,5-dichloro-α(*tert*-butylamino) methylbenzyl alcohol, Fig. 1) is a potent respiratory stimulant used in human and animal medicine being recommended for treatment of chronic obstructive pulmonary disease and bronchospasms associated with allergies, infections and exercise [1,2]. This drug, as many other β-adrenergic agents, is also illegally used as a growth promoter in farm animals, leading to a considerable reduction in fat deposits and favouring protein synthesis [3]. Considering that β-adrenergic agents are enough thermoresistant to remain active after ordinary cooking treatments, CL residues in liver and other tissues might be harmful to the consumer, especially to people with cardiac deficiencies and in the late stages of pregnancy [4,5].

This makes particularly useful the development of new materials selective for this class of substances to be used as chromatographic stationary phases for analytical purposes as well, in particular, for the pre-treatment (e.g. solid phase extraction, SPE) of biological samples. The same material could be potentially exploited as a highly specific recognition element for immunoassay like techniques or sensors.

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Until now, high performance thin-layer chromatography (HPTLC) and enzyme immunoassay (EIA) have been used for the screening of CL [6,7] while sample pre-treatment has usually been performed by liquid–liquid partition or SPE cartridges, also with analyte-specific immobilised antibodies [8–10]. Confirmation and quantitative analysis have been made with liquid chromatography–mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatographic methods (HPLC) [11–19].

To develop new recognition systems for CL, the molecular imprinting approach appeared promising to us since highly selective materials used as chromatographic absorbents (especially for SPE) as well as in immunoassay like techniques and selective sensors can be easily prepared [20–25]. Molecularly imprinted polymers (MIPs) are synthesised by means of free radical cross-linking reactions with a high cross-linker/monomer ratio in the presence of a template (the analyte). The functional monomer interacts with the template before polymerization (for example by hydrogen bonding or electrostatic interactions) giving rise to a specific co-ordination which is retained at the end of the reticulation process in the final highly rigid material. After removal of the template, specific cavities complementary in size, shape and chemical functionality to the template are obtained.

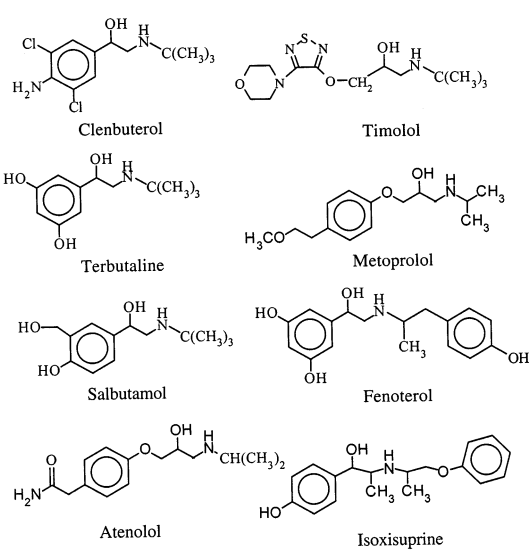

Fig. 1. Structures of CL and other β-adrenergic agents.

The aim of this work was the development of MIPs for CL highly selective with respect to other β-adrenergic substances. The number and nature of functional groups present in a CL molecule seemed to be suitable to design specific recognition cavities with the non-covalent approach using methacrylic acid (MAA) as interacting monomer. Recognition characteristics were evaluated by HPLC measurements using organic and aqueous eluents. To better address the exploitation of new techniques using this MIP, the contribution of electrostatic, hydrogen bonding and hydrophobic forces to template-cavity interactions was investigated.

2. Experimental section

2.1. Materials

MAA, ethylene glycol dimethacrylate (EGDMA), 2,2′-azobis(isobutyronitrile) (AIBN), acetonitrile (AcCN) and acetic acid (AcOH) were obtained from Fluka. MAA and EGDMA were distilled under vacuum before use. AcCN was dried over molecular sieves. AIBN was used as received. Timolol (Tim), Atenolol (Ate), Isoxsuprine (Iso), Metoprolol (Met), Terbutaline (Ter) and Fenoterol (Fen) were purchased from Sigma and were used without further purification. CL was obtained by Resfar (Italy) as the hydrochloride. CL free base for the imprinting process was obtained by extraction of the salt from an alkaline aqueous solution with chloroform. A free sample of Salbutamol hydrochloride (Sal) kindly supplied by Chiesi Farmaceutici (Italy) was used as received. 2-(*tert*-butylamino) ethanol (TBE), (methylaminomethyl) benzyl alcohol (MBA), 4-aminophenethyl alcohol (APA), (3,4 - dichlorophenyl) - 2 - isopropylaminoethanol (DPIAE) were obtained from Aldrich.

2.2. Synthesis of clenbuterol imprinted polymers

Polymerization experiments with a fixed cross-linker to monomer ratio (5:1) were carried out at two different monomer to template ratios (3:1 and 6:1, samples CLMIP1 and CLMIP2, respectively).

In a typical polymerization experiment, 0.5 mmol of AIBN as initiator were added to 2 mmol of CL, 12 mmol of MAA, 60 mmol of EGDMA and 20 ml of AcCN (as porogen) in a thick-walled glass tube. The solution was purged with argon for 10 min, sealed and polymerised under UV irradiation (366 nm in a Rayonet photochemical reactor) at 4°C for 24 h. Blank samples (BMIP) were prepared using the same reaction mixtures but without the template.

2.3. Preparation of HPLC particles and packing of columns

The bulk cross-linked polymer was crushed in a mortar and grounded with a mechanical mill (Retsch S1). The powder was then wet sieved under acetone through a 25 μm sieve. The polymer fraction with particles larger than 25 μm was ground again until all the material passed through the sieve. The sieved polymer was than repeatedly suspended in acetone, sonicated and sedimented to eliminate fine particles.

One to two grams of polymer were suspended in chloroform, sonicated and, finally, packed into an HPLC column (stainless steel 150×4.6 mm i.d., 0.2 μm frits) under pressure (200 bar) using isopropanol as solvent. The template was removed washing with methanol:acetic acid 9:1 until a stable baseline was obtained.

2.4. HPLC analysis of MIPs

HPLC analysis (Varian LC 5020 Chromatograph equipped with a Varian UV 50 variable wavelength detector and a HP 3395 integrator) was performed isocratically using AcCN–AcOH at different ratios and sodium phosphate buffer (PBS) (10^{-3} M)-AcCN at different ratios and pH. The pH value quoted is the pH of the original aqueous component. Flow-rate was 1 ml/min and UV detection wavelength was selected depending on the analytes and the eluent. 10 μl solutions containing the β-adrenergic compound (1 mg/ml) dissolved in the mobile phase were injected. The void volume of the column was determined by injection of acetone.

Capacity factors (k'), separation factors (α) and retention indices (RI) were calculated by the standard equations $k' = (t - t_0)/t_0$; $\alpha = k'_{\text{print molecule}}/k'_{\text{test molecule}}$; $\text{RI} = \alpha_b/\alpha_p$, where t and t_0 are the retention times of analyte and acetone, and subscripts p and b refer to the imprinted and blank polymer, respectively.

3. Results and discussion

Attention has been focused on two CL imprinted polymers prepared at different monomer to cross-linker ratio (CLMIP1 and CLMIP2, Section 2).

Chromatographic performances of HPLC columns packed with these materials were initially tested using organic mobile phases with different AcCN–AcOH composition. A blank polymer prepared in the same manner as the imprinted one but without the template (BMIP), was also tested using the same elution conditions. The specificity of CL imprinted polymers towards many different β-adrenergic substances (Fig. 1) illegally used as growth promoters in farm animals was checked. Some preliminary experiments were carried out using AcCN–AcOH eluent at different compositions. With a 90:10 eluent retention of CL was quite weak ($t = 3.2$ min, $t_0 = 1.8$ min) while with at 98:2 CL was no more eluted (at least after 80 min) and the other β-adrenergic agents are also significatively retained. With a 95% AcCN eluent no baseline separation of CL with respect to the other test substances was obtained.

Considering that two potentially ionizable groups are present in CL we also tried aqueous mobile phases at different pHs. In this way, a better optimisation of the ionic interaction contribution should be possible controlling the ionization of the carboxylic functions on the imprinted polymer and amino groups on the analytes. It has also been demonstrated that hydrophobic forces can be important in determining the selectivity of

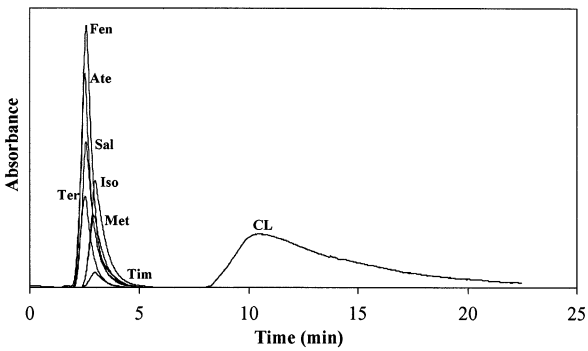

Fig. 2. Chromatographic profiles of β-adrenergic agents on a CL imprinted polymer (CLMIP1). Mobile phase: PBS (pH 3.4, 10^{-3} M)-AcCN (30:70, v/v). Flow 1 ml/min. (The height of each peak was adjusted only for graphic purposes).

MIPs when aqueous eluents are used [26–28]. This contribution can be expected to be quite relevant with CL whose hydrophobic character is quite strong (log $P = 2.46$) [29]. The potential use of these MIPs in water based samples often encountered in food analysis further addressed this choice.

PBS–AcCN at different pHs and aqueous/organic phase ratios have been used as eluents. At a constant ratio (30:70, v/v), different pHs have been tried starting from 2.8. A remarkable and progressive increase of CL retention time was observed by increasing the pH ($k' = 0.79$ and 5.88 at pH 2.8 and 3.4, respectively), while retention time of the other test substances slightly increases. This was expected as a consequence of increased electrostatic interactions due to a progressive dissociation of MIP carboxyl groups. The best selectivity without the drawback of exceedingly long analysis time was obtained at pH 3.4, while at pH 3.7 CL was no eluted even after 80 min while the other β-adrenergic substances showed high retention times. At pH 3.4, almost all the test substances were eluted quite close to the dead volume and a complete baseline separation with CL was obtained (Fig. 2). It must be pointed out that the selectivity obtained with the PBS (pH 3.4, 10^{-3} M)-AcCN (30:70, v/v) eluent was remarkably better than that obtained with the organic mobile phase.

The effectiveness of the imprinting process is confirmed comparing the data obtained with the imprinted and the blank polymer (Table 1). Capacity factors for CL on the blank polymer are quite similar to those obtained for the other β-adrenergic substances while are considerably higher using the imprinted polymer. Such data assess that aspecific interactions do not give an important contribution to CL retention.

Though electrostatic forces were demonstrated to be relevant for the recognition phenomenon in imprinted polymers, the contribution of hydrophobic interactions in aqueous eluents were also found to play an important role [26]. Several experiments using different aqueous to organic phase ratios (30:70, 50:50 and 70:30, v/v) at pH 3.4 have been performed. The dependence of the retention indices on the eluent composition for each adrenergic drug (Table 1) clearly shows that the better separation is achieved with the 30:70

Table 1
HPLC results obtained using a CL imprinted polymer (CLMIP1) with different aqueous mobile phase compositions (PBS (pH 3.4, 10^{-3} M)-AcCN (30:70, 50:50 and 70:30, v/v))

		CL	Tim	Ate	Met	Ter	Iso	Fen	Sal
30:70	k'_b	0.42	0.29	0.24	0.31	0.24	0.38	0.26	0.24
	k'_p	5.88	0.84	0.51	0.79	0.55	0.78	0.57	0.55
	RI	1.00	0.21	0.15	0.18	0.16	0.15	0.15	0.16
50:50	k'_b	0.65	0.22	0.16	0.30	0.18	0.53	0.22	0.18
	k'_p	3.52	0.30	0.12	0.51	0.17	1.12	0.23	0.18
	RI	1.00	0.26	0.14	0.32	0.18	0.39	0.19	0.18
70:30	k'_b	0.96	0.40	0.11	0.44	0.15	1.16	0.35	0.12
	k'_p	8.01	0.85	0.11	1.04	0.16	2.39	0.70	0.15
	RI	1.00	0.26	0.12	0.28	0.13	0.25	0.24	0.14

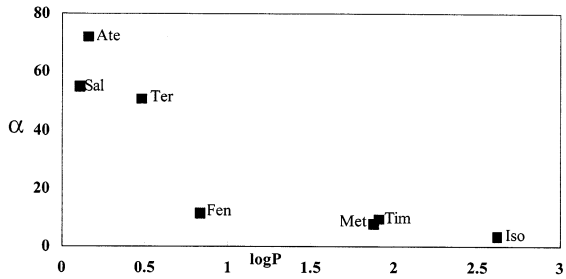

Fig. 3. Separation factors for test substances as a function of log P [13] obtained on a CL imprinted polymer (CLMIP1). Mobile phase: PBS (pH 3.4, 10^{-3} M)-AcCN (70:30, v/v). Flow 1 ml/min. Timolol (Tim), Atenolol (Ate), Isoxsuprine (Iso), Metoprolol (Met), Terbutaline (Ter), Fenoterol (Fen), Salbutamol (Sal).

mobile phase. For all the test substances analysed retention indices (0.15–0.21) were lower than the reference (CL). No noticeable differences are detectable among the whole group of adrenergic drugs. At the highest content of aqueous phase (70:30, v/v) a slight but significative reduction of separation of some of the other analytes with respect to CL was observed. In particular, capacity factors for Tim, Met, Fen and, especially, Iso are higher than those measured with a lower content of aqueous phase. These results can be explained considering the degree of hydrophobicity of these molecules. In fact, the highest capacity factor on the imprinted polymer (k'_p) was observed for isoxsuprine, which is the most hydrophobic molecule analysed (log $P = 2.62$) [29]. The opposite behaviour is observed for the less hydrophobic ones (Ate, Sal, Ter) whose capacity factors with the 70:30 eluent are lower then with the 30:70 ratio. Fig. 3 shows that these considerations can be extended in a semi-quantitative manner to all the other β-adrenergic agents for which a decreasing trend of capacity factors as a function of log P was observed. Isoxsuprine capacity factor on the blank polymer ($k'_b = 1.16$) using the 70:30 eluent was significatively higher than the other adrenergic drugs (0.11–0.40), while was quite similar ($k'_b = 0.38$) when the 30% AcCN eluent was used. This means that strong, aspecific hydrophobic interactions with isoxsuprine are present in the blank polymer and are mainly responsible for the strong retention of this molecule. In fact, the

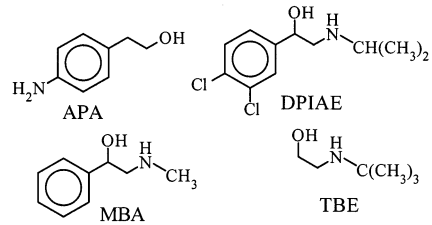

Fig. 4. Chemical structure of test substances analysed with CL MIPs. TBE, MBA, APA, DPIAE.

influence of the aqueous/organic ratio almost completely disappears when the retention indices, which include also the contribution of aspecific interactions with the blank MIP, are considered. To further clarify the mechanism of recognition, some substances (Fig. 4) having selected chemical features in common with CL were analysed with the 30% AcCN eluent (Table 2). 3,4-Dichlorophenyl-2-isopropylaminoethanol (DPIAE) was the only molecule strongly retained on the imprinted column ($k'_p = 2.85$). Besides, no retention is evident with the blank column for all the test substances ($k'_b = 0.20–0.31$), including DPIAE. Therefore, the separation ability toward this molecule must be due to a certain degree of complementarity with CL selective cavities rather than to aspecific interactions. It is worth underlining that DPIAE capacity factor on the imprinted column is also considerably higher than the other adrenergic drugs previously reported with the same eluent (Table 1). Some interesting considerations stem from these results. The only important structural difference between DPIAE and CL

Table 2
HPLC results obtained for test substances (Fig. 4) on a CL imprinted polymer CLMIP1 (p) and a blank polymer BMIP (b). α values are referred to CL[a]

	APA	DPIAE	MBA	TBE
k'_b	0.25	0.31	0.20	0.23
α_b	1.68	1.35	2.10	1.83
k'_p	0.61	2.85	0.42	0.53
α_p	9.64	2.06	14.00	11.09
RI	0.17	0.66	0.15	0.16

[a] Eluent: PBS (pH 3.4, 10^{-3} M)-AcCN (30:70, v/v) Flow 1 ml/min.

is the lack of the aromatic amino group. This leads to a higher retention of CL but DPIAE is much more retained than the other adrenergic drugs studied in this work. In contrast, the presence of two hydroxyl groups in the aromatic moiety in Ter and Sal causes a dramatic decrease of recognition ability, even though the rest of the molecule is the same. This points out a remarkable importance of the aromatic moiety hydrophobicity in the recognition. Despite the hydrophobic aromatic moiety is present in Met, this molecule is probably too flexible (due to the C–O–C bond) and too bulky to properly fit in the cavity. Similar considerations can be extended to the other adrenergic drugs. Besides, the results obtained with MBA suggest that the hydrophobic contribution of the *tert*-butyl group must also be important. In fact, the only difference between MBA and CL is the lack of the *tert*-butyl group.

4. Conclusions

Our results demonstrate the feasibility of the preparation of highly selective absorbents for CL based on the molecular imprinting approach. The selectivity obtained using aqueous eluents based on PBS–AcCN allowed to obtain a complete baseline separation with the other drugs used in this study. The pH and the aqueous/organic composition of the eluent can be adjusted to optimise the contribution of electrostatic, hydrogen bonding and hydrophobic forces to the recognition phenomenon. These results seem to be particularly interesting if the use of this material for SPE in the pre-treatment of biological samples is sought. The chemical synthesis for the preparation of MIPs is quite easy when compared to the procedure to obtain antibodies based affinity columns. The robustness of these imprinted stationary phases makes them particularly suitable for this application, as well as for the use as recognition elements in selective sensors.

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