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Use of molecularly imprinted polymers in the solid-phase extraction of clenbuterol from animal feeds and biological matrices

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Abstract

Clenbuterol molecularly imprinted polymers (MIPs) as chromatographic stationary phase for the solid-phase extraction (SPE) of the drug from biological samples have been prepared. Propylene columns filled with 500 mg of clenbuterol MIPs have been tested with respect to their loading capacity, memory effects, selectivity toward related drugs (mabuterol, clenproperol, clenisopenterol, ritodrine) and specificity toward interferences arising from heterogeneous matrices such as animal feeds, bovine urine and liver. Analytes were concentrated on Extrelut 20 columns and the residues resuspended in 70% acetonitrile. Application, washing and elution fractions were collected and analyzed by HPLC–diode array detection. Results indicate this MIP approach in SPE is extremely selective for clenbuterol, mabuterol, clenproperol and clenisopenterol (>95% found in the eluate), with a loading capacity of about 20 μg/100 mg of stationary phase. Ritodrine showed a recovery rate of 51%. The molecular recognition mechanism is so specific to allow clenbuterol detection and identification by conventional detectors at level of interest (ppb) also from complex matrices such as feeds, urine and liver.

Keywords: Molecularly imprinted polymers; Clenbuterol

1. Introduction

Clenbuterol (CLE) (4-amino-3,5-dichloro-α(*tert.*-butylamino)methylbenzyl alcohol, Fig. 1) is a β_2-adrenergic agonist recommended for the treatment of chronic obstructive pulmonary disease and bronchospasms associated with allergies, infections and exercise [1,2]. Recently, as with many other β-adrenergic agents, clenbuterol has been proposed as a growth promoter in beef production, leading to a considerable reduction in fat deposits and enhancing protein synthesis [3]. Several cases of intoxication recorded in humans after meat consumption [4,5] have strengthened the demand for very selective and specific analytical procedures able to confirm clenbuterol presence in biological samples.

Molecular-based recognition techniques based on immunoaffinity chromatography (IAC) [6,7] and on acceptor affinity chromatography [8] have been proposed for the clean-up of clenbuterol and related drugs. Nowadays, the molecular imprinting approach appears promising to us since highly selective ma-

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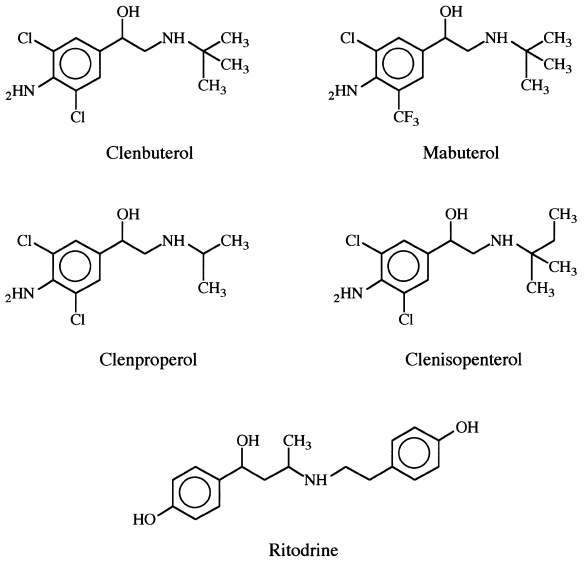

Fig. 1. Structures of clenbuterol and other β-adrenergic agents.

terials used as chromatographic absorbents for solid-phase extraction (SPE) can be easily prepared [9–14]. Recently, we proved that HPLC columns packed with clenbuterol molecularly imprinted polymers (MIPs) are able to completely separate the template from many other adrenergic drugs [15]. Furthermore, a feasibility study was performed by other authors in order evaluate the possibilities in using MIPs as sorbent material in SPE for the clean-up of clenbuterol from urine using an organic eluent [16].

The aim of this work was the development of molecularly imprinted polymer SPE columns (SPE MIP) for CLE and the evaluation of their performances within the frame of an analytical strategy to monitor the illegal use of this drug in animal productions at residue level [17]. Cross-reactivity with other adrenergic drugs was also studied.

2. Experimental

2.1. Materials

Methacrylic acid (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. Sulfamethazine sodium salt, flumequine, tylosin tartrate, oxytetracycline from Sigma, CLE, mabuterol (MAB), ritodrine (RIT), clenproperol (CPR), clenisopenterol (CIP) as hydrochloride salts (Fig. 1) were kindly provided by the European reference laboratory for β-agonists residues in food (Berlin, Germany).

2.2. Synthesis of clenbuterol imprinted polymers

Clenbuterol molecularly imprinted polymers were prepared as previously described [15] using a fixed crosslinker:monomer:template ratio (60:12:4). In a typical polymerization experiment, 0.5 mmol of AIBN as initiator was added to 4 mmol of CLE, 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 polymers (BMIP) were prepared using the same reaction mixtures but without the template.

2.3. Preparation of particles and packing of SPE columns

The bulk cross-linked polymer was crushed in a mortar, grounded with a mechanical mill (Retsch S1) and wet sieved with acetone through a 63-μm sieve (ASTM series). The polymer fraction above 63 μm was ground again until all the material passed through the sieve. The sieved polymer was then repeatedly suspended in acetone, sonicated and sedimented to eliminate fine particles.

SPE MIP columns were prepared by packing 500 mg into 6-ml polyethylene reservoirs (Mallincrodt Baker), putting two wool–glass frits at the bottom and on the top of the columns.

The elution of the template was performed by repeated washings of the SPE MIP columns with 25 ml methanol–trifluroacetic acid (TFA) (99:1, v/v) at boiling temperature.

2.4. SPE procedure

The choice of application, washing and elution solutions was derived by previous experience on the

use of MIPs for high-performance liquid chromatography (HPLC) columns [15]. Briefly, phosphate buffer (pH 3.4; 3 m*M*)–acetonitrile (30:70, v/v) solution was chosen for application (AS) and washing (WS) steps. Methanol–TFA (99:1, v/v) was used for the elution (ES).

To calculate SPE MIP columns loading capacity and selectivity, clenbuterol and related drugs were dissolved in the application solution, to final concentrations of 1.0, 10, 100 μg/ml, respectively.

To account for any possible non-specific contribution in the molecular recognition, 100 μg/ml solutions of most in use veterinary drugs such as sulfamethazine, flumequine, oxytetracycline and tylosin were applied.

After column conditioning (2 ml of the AS), 1 ml of the standards solution was applied onto different columns with a flow of 0.5 ml/min. After column drying, 4 ml of WS was applied. Finally, the elution was performed by 10 ml of ES.

Fractions (1 ml) of the application, washing and elution step solutions were collected and analysed by HPLC–UV–diode array detection (DAD) to calculate losses and recoveries for each analyte.

2.5. Memory effects

To evaluate the presence of memory effects and of the possible progressive bleeding of the template, SPE columns were washed with five fractions of 25 ml (TFA-MeOH, 1:99, v/v). Each fraction, collected in a round bottom flask and evaporated on a rotary evaporator, was resuspended in 0.5 ml of mobile phase and analyzed by HPLC, with a detection limit of 2 ng/ml on the concentrated fraction.

2.6. Matrix interference

To evaluate possible matrix interference affecting the molecular recognition mechanism, 20 different blank samples of milk replacers for veal calves (20 ml), bovine urine (20 ml) and livers (5 g) were spiked with clenbuterol at residue levels of 5 (milk and urine) and 20 ppb (liver), respectively. Livers were extracted in 20 ml of 0.1 *M* HCl, sonicated and centrifuged 30 min at 3000 *g*, 4 °C. The liquid phases from the different matrices were then brought to pH>11 with 200 μl of 10 *M* NaOH under vortexing and then adsorbed on Extrelut 20 columns; compounds of interest were eluted with 60 ml *n*-hexane–dimethyl chloride (8:2, v/v), according to the procedure of Degroodt et al. [18]. Residue concentrated on the rotary evaporator was resuspended in 1 ml of the AS and applied onto the SPE MIP column according to the previously described procedure. Elution fractions (5 ml) from blank and spiked samples, concentrated to a final volume of 200 μl were injected into the HPLC system to assess the recoveries and specificity.

2.7. HPLC analysis of β-agonists and non-related veterinary drugs

HPLC analysis (Beckmann Analytical Model 126 pump equipped with a Model 168 DAD system) of solutions eluted from the SPE MIP columns was performed using 0.017 *M* H_3PO_4, pH 3.5 (DEA) and AcCN as solvents. Analyses were carried out using a linear gradient from 10% to 100% of AcCN in 20 min (10 min equilibration time), with a flow-rate of 1 ml/min. A 100-μl loop and a LiChrosphere RP C_{18} 250×4 mm column (5 μm) from Merck were used. The detector was set at 245 and 275 nm for clenbuterol and related agonists, 275 nm for sulfamethazine and tylosin and 360 nm for flumequine and oxytetracycline, respectively; UV spectra were recorded in purity mode from 220 to 400 nm. Calibration curves were built for each compound considered in the range 25–1.000 ng injected and concentrations calculated with the external standard method. Detection limits, calculated as 3:1 signal-to-noise ratio were 10 ng for CLE, MAB, CPR, CIP and 20 ng for RIT.

Detection limits for non-related drugs were 20 ng for sulfamethazine and tylosin and 50 ng for oxytetracycline and flumequine.

3. Results and discussion

SPE columns were packed with clenbuterol MIP as obtained by the procedure given in the Experimental section. The template used in the synthesis of the polymer was extracted directly on the MIP loaded SPE cartridges. Different extraction solvents and techniques were used. The best results

Table 1
Elution profile of 100 μg clenbuterol on 500 mg SPE MIP and blank (BMIP) columns

	Fractions (1 ml)														
	A1	W1	W2	W3	W4	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
SPE MIP	N.D.	N.D.	N.D.	N.D.	N.D.	31	63	3	2	1	N.D.	N.D.	N.D.	N.D.	N.D.
BMIP	80	18	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Flow: 1 ml/min (A1=application, W1–W4=washing, E1–E10=elution, N.D.=not detected, <100 ng/ml; each step is 1 ml, see the Experimental section for composition).

were obtained washing the SPE columns with methanol–TFA (99:1, v/v) at the boiling temperature. No clenbuterol was detected in the washing solvent after the MIP was treated three times with 25 ml of eluent (see detection limit reported in the Experimental section).

500 mg SPE MIP columns were loaded with different amounts of clenbuterol to determine the specific loading capacity. This was found to support up to 100±2.7 μg (n=12). Such a feature has been reassessed on three different trials, over 6 months, thus assuring the reproducibility of the performance. After loading with 100 μg, the elution profile of clenbuterol as mean recovery (n=12) calculated on each 1-ml fraction collected from application, washing and elution steps, was determined (Table 1). It is worthwhile to underline that clenbuterol, completely retained during the wash, is efficiently removed only in the elution steps. The same experiment was done by packing the column with a blank polymer synthesized with all the reagents used for the imprinted polymer without the template. The elution profile (Table 1) clearly shows that the retention obtained with the SPE MIP column is due to an imprinting effect rather than to non-specific interactions.

The results of the selectivity study with respect to CLE, MAB, CPR, CIP are reported in Fig. 2. According to the molecules considered (Fig. 1), SPE MIP columns for clenbuterol show a very good selectivity towards other drugs structurally correlated. The substitution of the chlorine atom with a CF_3 group on the aromatic ring (mabuterol) or the substitution of the *tert*.-butyl group with an isopropyl one (clenproperol) or iso-pentyl one (pentilclenbuterol) does not greatly affect the molecular recognition mechanism. In fact, we have demonstrated [15] that clenbuterol recognition is mainly based on electrostatic interactions of the aliphatic

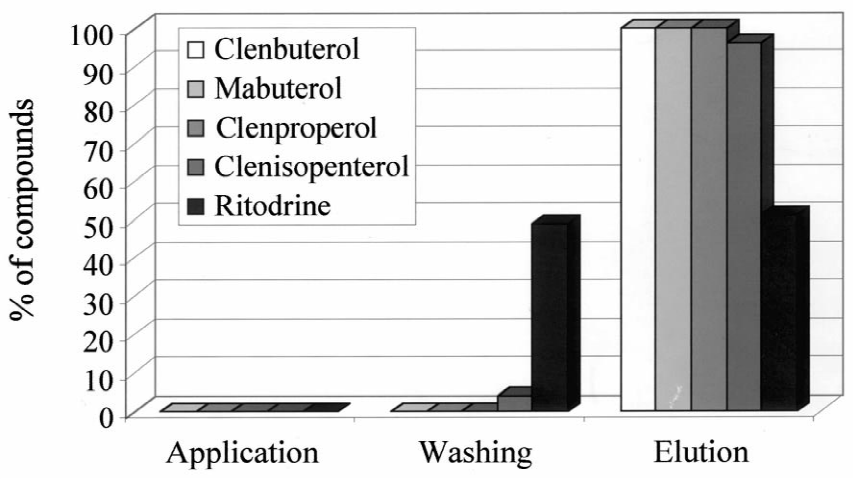

Fig. 2. Chromatographic profiles of β-adrenergic agents on an SPE cartridge packed with a clenbuterol imprinted polymer (see the Experimental section for eluents composition).

amino group, on hydrogen bonds, as well as hydrophobic interactions of the aromatic and *tert*.-butyl portion of the molecule. These features are shared among all the molecules cited above. By contrast, a phenolic compound such as ritodrine, is not as retained as clenbuterol probably as a consequence of the lack of hydrophobicity of the terminal part of the molecule due to the presence of a *p*-hydroxyphenylethyl group instead of the *tert*.-butyl group.

In similar experiments made with the blank polymer (data not reported) all the analytes were eluted within the A1, W1 and W2 steps (see Table 1) thus indicating the effectiveness of the molecular imprinting recognition.

On the other hand, the non-related veterinary drugs considered, sulfamethazine, tylosin, flumequine, and oxytetracycline applied at 100 μl/ml on the CLE MIP columns were almost lost in the application step (96%, data not reported).

After the elution of 100 μg clenbuterol from the SPE MIP columns, the analysis of the 25-ml subsequent fractions methanol–TFA (99:1, v/v), concentrated to a final volume of 1 ml does not show any bleeding (1<40 pg/ml, limit of detection of the procedure) (data not shown). This allows their re-use, under an appropriate quality control scheme, thus reducing time and analytical costs.

Matrix interference was evaluated using SPE MIP for the purification of clenbuterol at residue levels from milk replacers for veal calves, bovine urine and liver. The recoveries of clenbuterol are shown in Table 2.

The HPLC–UV chromatograms of a blank urine and a urine spiked at 5 ppb are reported in Fig. 3. In relation to the retention time of clenbuterol and anilino-like related drugs, no interfering peak is present, even if the amount of the extracted sample was quite relevant (20 ml for urine and milk replacers, 5 g for livers). Matrix interference seems not to affect greatly the specificity and recovery rates of clenbuterol, at residue level. According to such results, the application of SPE MIP columns to heterogeneous biological matrices has proved to be effective and practicable. Moreover, the specificity of the SPE procedure on MIP columns allowed us to use conventional UV–DAD. This finding suggests that the use of SPE MIPs in routine analysis could be a real tool to improve analytical specificity, even if a mass spectrometer device is not available in the laboratory. To this purpose, a cross-validation study was performed by gas chromatography–tandem mass spectrometry (GC–MS–MS) [5] on 32 incurred samples (7 feed, 25 urine, 10 liver), previously screened as positive for CLE. After MIPs clean-up, all the HPLC–UV peaks collected in relation to CLE retention time, showed the unambiguous presence of the analyte as a trimethylsilane (TMS) derivative.

Table 2
Recoveries of clenbuterol in different matrices after extraction with the SPE MIP cartridge

Matrix	*n*	Added (ng)	Found (ng)
Liver (5 g)	20	100	91±5
Urine (20 ml)	20	100	93±3
Milk replacer (20 ml)	20	100	93±3

Residues resuspended in 200 μl HPLC mobile phase, 100 μl injected.

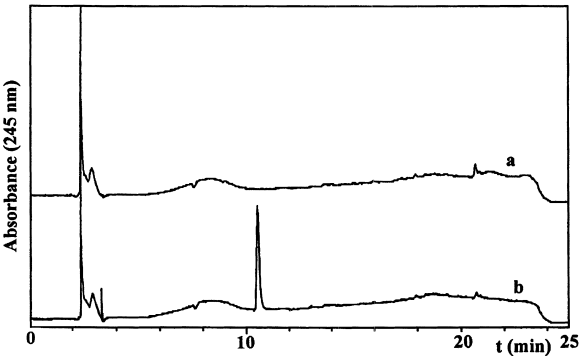

Fig. 3. HPLC–UV chromatograms of a blank urine (a) and a urine spiked with 5 ppb of clenbuterol (b) extracted with SPE MIP.

4. Conclusions

Our study indicates that clenbuterol SPE MIPs are effective for the multi-residue purification of clenbuterol and anilino-like β_2-agonists. The results on biological matrices suggest such purification can be considered a practicable solution for sample preparation in routine analysis. Further work is in progress to broaden the spectra of molecularly recognized compounds, by using a blend of MIPs, imprinted also with phenolic-like agonists, such as ritodrine, ractopamine, fenoterol, salbutamol.

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