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Short communication

Use of molecularly imprinted solid-phase extraction for the selective clean-up of clenbuterol from calf urine

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Abstract

A feasibility study was performed in order to study the possibilities in using molecularly imprinted polymers (MIPs) as sorbent material in solid-phase extraction (MISPE) for clean-up of clenbuterol from urine. A binding study of clenbuterol in several solvents was performed on a clenbuterol imprinted polymer as well as on a blank polymer. These binding experiments were used to find suitable loading, washing and elution solvents for the MISPE procedure. Extraction of clenbuterol from calf urine was performed by directly loading a 10-ml urine sample onto the MIP column. Thereafter the column was washed with 10 ml of acetonitrile containing 1% acetic acid, and finally clenbuterol was eluted with 6 ml of methanol containing 10% acetic acid. A recovery of 65% was obtained. This recovery could be increased up to 75% if a sample volume of 1 ml was used or up to 100% if urine was freeze-dried and the residue was dissolved in acetonitrile and spiked with clenbuterol prior to analysis. Chromatograms of the wash and eluate solutions show an efficient clean-up, which supports the potential of MISPE for clean-up of trace amounts of clenbuterol from calf urine.

Keywords: Molecular imprinting; Solid-phase extraction; Clenbuterol; Growth hormones

1. Introduction

β-Agonists are used in asthma treatment as they produce relaxation of bronchial smooth muscle and thereby decrease airway resistance. However, at higher doses side effects, which influence protein synthesis and lipolysis, occur. If β-agonists are given to cattle during the fattening process the produced meat will consist of more muscle mass and less adipose tissue. However, the produced meat might contain residues of β-agonists, which can lead to food poisoning [1]. Such growth promoters have therefore been forbidden in the European Union [2,3]. To control the misuse of these drugs, analysis of biofluids is necessary, like urine or plasma. This puts high demands on the analytical procedure, especially as the limit of quantitation (LOQ) is set to 0.5 ng/ml by the European Union. The determination of β-agonists in biofluids has recently been reviewed by Boyd et al. [4]. Prior to analysis the sample has to be cleaned-up to remove interfering matrix components and preferably also to pre-concentrate the analyte. Classical liquid–liquid extraction methods have shown good recoveries [5] but due to the laborious handling, solid-phase extraction (SPE) [6] as well as immunoaffinity chromatography

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(IAC) [7] have in recent years become the methods of choice. For the latter a higher degree of selectivity and thus a lower LOQ can be expected.

Another way to gain selectivity, and thus a more efficient clean-up, is to use molecularly imprinted polymers (MIPs) as sorbent material in solid-phase extraction. MIPs are artificial recognition systems normally based on non-covalent forces, thus trying to mimic nature [8]. A template, the analyte itself or a structurally related analogue, is first allowed to form bonds with functional monomers in a solvent. Subsequently the monomers are crosslinked and the template extracted from the polymer, leaving specific cavities in regard to shape and functionality. These cavities can rebind the analyte of structurally related compounds and thus a selective clean-up can be obtained. Recently the use of SPE with MIPs as sorbent material (MISPE) has been described for various drugs [9–13], drugs of abuse [14] and pollutants [15,16].

The work described in this paper was undertaken in order to show the feasibility of the use of MISPE for the selective clean-up of trace amounts of clenbuterol from calf urine samples.

2. Experimental

2.1. Chemicals

Clenbuterol hydrochloride was a gift from Karl Thomae (Biberach an der Riss, Germany). Acetonitrile and methanol were both HPLC grade and were purchased from Labscan (Dublin, Ireland). Glacial acetic acid, ethanol, 1-propanol, ether, heptane, toluene, hexane and dichloromethane were of analytical quality and were all from Merck (Darmstadt, Germany). Methacrylic acid, ethylene glycol dimethacrylate and azobis(isobutyronitrile) (for synthesis) came from Aldrich Chemie (Steinheim, Germany). 1-Octanesulfonic acid, sodium salt monohydrate 98% and tetrahydrofuran (pa, THF) came from Acros Organics (Geel, Belgium). Blank calf urine was provided by RIVM (Community Reference Laboratory/Laboratory for Analytical Residue Research, National Institute of Public Health and the Environment, Bilthoven, Netherlands). All other chemicals used were of analytical grade. The water was demineralized in the laboratory and purified with a Maxima ultrapure water system (Elga, Salm & Kipp BV, Breukelen, Netherlands).

2.2. Preparation of MISPE columns

A molecularly imprinted polymer was prepared with clenbuterol as template and 60 mg of the material packed into an SPE column, as described elsewhere [17]. As a control a blank polymer was prepared in the same way but without clenbuterol present during polymerization. The template from the clenbuterol imprinted polymer was extracted by using ultrasonication in 100% glacial acetic acid for 4 h with fresh acetic acid being added every 15 min. Just enough glacial acetic acid to wet the sorbent was sucked through the column by placing it in a Vac Elut SPS 24 unit from Varian (Palo Alto, CA, USA), kept at a vacuum of 10 in.Hg (1 in.Hg=338.638 Pa). The column was thereafter placed in an Erlenmeyer flask containing glacial acetic acid and the flask placed in the ultrasonic bath. After washing the column was dried under vacuum in an Univapo 150 H (Genevac, Ipswich, UK) and thereafter was ready for use.

2.3. Analysis equipment

Analysis was performed off-line with a reversed-phase HPLC-system consisting of a pump (Pharmacia/LKB Model 2150, Uppsala, Sweden) and a WISP 710 A (Waters, Milford, MA, USA) automatic sample injector. An electrochemical detector (Amor, Antec Leyden, Zoeterwoude, Netherlands) with a glassy carbon working electrode set at +800 mV vs. an Ag/AgCl in situ reference electrode was used. The column was a LiChroCART 250-4 HPLC cartridge Superspher 60 RP-select B (250×4 mm I.D., Merck) with a LiChroCART 4-4 LiChrospher 60 RP-select B guard column (5 μm, Merck). Peak heights were recorded with an integrator HP 3396 A (Hewlett-Packard, Avondale, PA, USA). The mobile phase consisted of buffer–acetonitrile (70:30). The buffer contained 25 m*M* potassium phosphate, 2.5 m*M* NaCl, 0.17 m*M* EDTA and 2.7 m*M* octanesulfonic acid sodium salt and had a pH of 5.5. Prior to use it was filtered (0.2 μm, Schleicher & Schuell, Dassel, Germany) and degassed. The mobile phase

flow rate was set at 0.8 ml/min and the sample injection volume was 20 μl.

2.4. Binding study

A stock solution of clenbuterol (1 mg/ml) was prepared in ethanol. Standard solutions of clenbuterol (100 ng/ml or 10 ng/ml) in different solvents were prepared from this solution. A dry MISPE column was conditioned with 10 ml of solvent followed by loading of 1 or 10 ml of the same solvent, spiked with 100 or 10 ng/ml clenbuterol. The total amount of clenbuterol loaded on the column was always 100 ng. Controls, where no clenbuterol was added to the solvent, were made in order to correct for template bleeding. A fraction, either 0.2 or 2 ml, of the solvent passing through the column was transferred to a new test tube and evaporated to dryness in a vacuum centrifuge (Univapo 150 H, Genevac). Thereafter, it was re-dissolved in 200 μl of the mobile phase prior to analysis by RP-HPLC.

2.5. Extraction of calf urine

A dry MISPE column was conditioned with 10 ml water before 10 ml calf urine, spiked with 100 ng/ml clenbuterol, was loaded on the column. The column was then washed with 10×1 ml acetonitrile containing 1% acetic acid and finally 6×1 ml methanol–acetic acid (9:1) was used to elute clenbuterol. Each fraction was evaporated to dryness in a vacuum centrifuge (Univapo 150 H, Genevac) and thereafter re-dissolved in 200 μl of the mobile phase prior to analysis by RP-HPLC.

3. Results and discussion

3.1. Binding study

Binding of clenbuterol to the imprinted and the blank polymers was tested with a large variety of solvents spiked with clenbuterol in order to select loading, washing and elution conditions. The percentage of clenbuterol bound is given in Table 1. A

Table 1
Binding of clenbuterol to clenbuterol imprinted and blank polymers in different solvents

Solvent	Bound (%)			
	Clenbuterol MIP 1 ml/100 ng/ml clenbuterol	Blank polymer 1 ml/100 ng/ml clenbuterol	Clenbuterol MIP 10 ml, 10 ng/ml clenbuterol	Blank polymer 10 ml, 10 ng/ml clenbuterol
Water	100	100	100	100
Methanol	100	100	95	42
Ethanol	100	100	100	53
Acetonitrile	100	100	100	100
1-Propanol	100	100	88	44
Heptane	100	100	100	100
Toluene	98	98	100	100
Hexane	100	100	100	100
Dichloromethane	97	100	91	89
THF	76	27	55	26
Acetonitrile with 1% acetic acid	n.d.[a]	n.d.	100	33
Acetonitrile with 2% acetic acid	n.d.	n.d.	98	14
Acetonitrile with 3% acetic acid	n.d.	n.d.	78	4
Acetonitrile with 5% acetic acid	n.d.	n.d.	11	5
Acetonitrile with 10% acetic acid	n.d.	n.d.	2	0
Methanol with 10% acetic acid	n.d.	n.d.	0	0

[a] Not determined.

sample volume of 1 ml was first investigated. As the MIP was prepared with acetonitrile as porogen it was anticipated that the best binding would be obtained in this solvent and as can be seen in Table 1 surprisingly the binding of clenbuterol was complete for both the imprinted and the blank polymers in this solvent. Also many other solvents showed complete binding to both polymers. It was expected that by increasing the sample volume to 10 ml but keeping the total amount of clenbuterol loaded on the column constant, i.e. 100 ng, more pronounced differences between imprinted and blank polymers and between solvents would appear. This behavior was observed in some solvents, e.g. methanol, ethanol and 1-propanol. However, by adding acetic acid to the acetonitrile differences in binding of clenbuterol to the imprinted and the blank polymers were increased. The presence of 1% acetic acid reduced the binding of clenbuterol to the blank polymer with 67% whereas the binding to the imprinted polymer was still complete. By increasing the amount of acetic acid the binding to the blank polymer was reduced further but so was the binding to the imprinted polymer. At an acetic acid content of 10% the binding of clenbuterol was zero or close to zero for both polymers.

3.2. Extraction of clenbuterol from calf urine

A MISPE procedure consists of four steps: conditioning of the column material, loading of the sample, washing to remove non-specifically bound molecules and finally elution of the analyte. In Table 1 suitable solvents for loading, washing and elution can be found. Preferably the urine samples should be loaded directly onto the column. In Table 1 it is seen that binding of clenbuterol to the clenbuterol MIP was complete in water. Therefore 10 ml calf urine, spiked with 100 ng/ml clenbuterol, was loaded directly after conditioning the column with 10 ml of water. Due to interferences it was not possible to measure if any clenbuterol passed through the column. The binding in urine is not selective as 100% clenbuterol in water also binds to the blank polymer, but it was reasoned that the binding could be converted from non-selective to selective by using a selective washing step. Acetonitrile containing 1% acetic acid was chosen for this purpose. The column was washed with 10×1 ml fractions of acetonitrile containing 1% acetic acid. In this medium the non-selective binding was reduced significantly while the binding of clenbuterol to the imprinted polymer was still complete (see Table 1). Clenbuterol was eluted with 6×1 ml methanol containing 10% acetic acid, as no binding occurred in this solvent. The content of clenbuterol was measured in each individual wash and eluate fraction and the result is shown in Table 2. The total loss of clenbuterol in the wash fractions amounted to 3% and the recovery in the elution step to 65%. Corrections have been made for template bleeding by loading a sample without any clenbuterol present. When 1 ml of urine, spiked with 1000 ng/ml clenbuterol, was loaded on the column a recovery of 75% was obtained, which indicates that clenbuterol is most likely lost in the loading step. Chromatograms of the washing and the elution solutions after extraction of blank calf urine, are shown in Fig. 1. The clenbuterol peak is indicated by an arrow in both chromatograms. These peaks corre-

Table 2
Evaluation of the MISPE procedure for clenbuterol in calf urine. Ten ml urine containing 100 ng/ml was applied. Ten 1-ml aliquots washing solvent (1% acetic acid in methanol) were applied and the eluate was analyzed. Six 1-ml aliquots eluting solvent (10% acetic acid in methanol) were applied and the eluate was analyzed

SPE-step	Fraction number	Clenbuterol (ng/ml)
Application	1	[a]
Washing	1	[a]
	2	5
	3	3
	4	2
	5	2
	6	10
	7	3
	8	2
	9	3
	10	2
Elution	1	423
	2	109
	3	65
	4	24
	5	15
	6	15

[a] Not possible to detect clenbuterol due to matrix interferences.

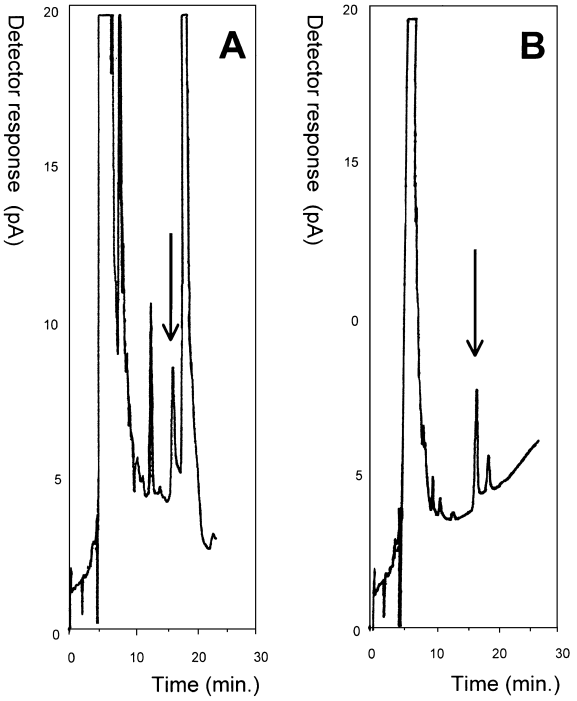

Fig. 1. Chromatograms of (A) washing solution and (B) eluate. 10 ml blank calf urine was loaded on the column. Thereafter it was washed with 10 ml acetonitrile containing 1% acetic acid and finally treated with 10 ml methanol containing 10% acetic acid. The clenbuterol peaks are indicated by an arrow in the chromatograms. These peaks correspond to the bleeding of clenbuterol from the column. When clenbuterol spiked urine samples were used the peak in the eluate increased in relation to the spiked amount, whereas the clenbuterol peak in the wash solution remained constant.

spond to the bleeding of clenbuterol from the column. When clenbuterol spiked urine samples were used, the peak in the eluate increased in relation to the spiked amount, whereas the clenbuterol peak in the wash solution remained constant. The cleanliness of the eluate appears promising for the use of MISPE for the determination of trace amounts of clenbuterol in urine.

To see if the recovery of clenbuterol could be increased, 10 ml of calf urine was freeze-dried and thereafter re-dissolved in 10 ml acetonitrile and spiked with clenbuterol. Not all constituents of the freeze-dried urine re-dissolved and therefore the sample was centrifuged at 4000 *g* for 10 min and the clear supernatant was spiked with 100 ng/ml of clenbuterol and used for extraction with MISPE. The recovery of clenbuterol was in this case 100%. This experiment was done to assess whether competition between matrix components and clenbuterol for binding to the selective binding sites causes recovery losses.

The results obtained in this paper show the potential use of MISPE as an efficient clean-up method for clenbuterol from urine. As clenbuterol has to be measured at trace levels it is not suitable to use clenbuterol itself as template during the polymerization, as approximately 6 ng/ml bleeds from the polymer in the elution solution, methanol containing 10% acetic acid [17]. In the future a structural analogue of clenbuterol will be used as template during polymerization, and optimization of the loading, washing and elution steps will be performed. Indications from this work show that 100% recoveries can be obtained if sample loading is performed in acetonitrile. This can be done either by freeze-drying the samples or by performing a solvent switch prior to loading. The approach is only effective in case clenbuterol can re-dissolve from the lyophilized sample matrix. Considering the polar character of the analyte it is anticipated that this approach can be effective. A recovery of 65% though might not be a problem if it is constant for the entire concentration range. A drawback of MIPs is the high affinity of the MIP for the analyte. It is important that the elution is rapid and quantitative and performed in a small volume, especially if determination is to be performed at trace levels.

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