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Extraction of clenbuterol from calf urine using a molecularly imprinted polymer followed by quantitation by high-performance liquid chromatography with UV detection

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Abstract

A method for the extraction of clenbuterol from calf urine samples using a molecularly imprinted polymer (MIP) has been developed. The aim was that the final extracts from the MIP should allow quantitation of clenbuterol down to 0.5 ng/mL urine using HPLC with UV detection. The MIP was produced using brombuterol as a template and the selectivity of the MIP, for clenbuterol, was tested against a non-imprinted polymer (produced without template) and was found to be high. After loading of 5 mL diluted centrifuged urine, selective binding was established in acetonitrile–acetic acid (98:2). For further elution of interferences, 0.5 *M* ammonium acetate buffer pH 5 and 70% acetonitrile in water was used. Clenbuterol was eluted using 1% trifluoroacetic acid in methanol, which was evaporated and reconstituted in buffer. Results from the HPLC analyses showed that the extraction of clenbuterol using MIP is linear in the range 0.5–100 ng/mL with good precision (4.3% for 0.6 ng/mL and 2.1% for 6.0 ng/mL) and accuracy (96.7% for 0.6 ng/mL and 96.7% for 6.0 ng/mL). The recoveries were 75%. The results show that the method offers a selectivity and sensitivity that make the quantitation of 0.5 ng clenbuterol/mL urine by HPLC–UV possible and a competitive alternative to state-of-the-art routine analytical methods.

Keywords: Molecular imprinting; Solid-phase extraction; Clenbuterol; Beta-agonists

1. Introduction

Clenbuterol (Fig. 1) is a synthetic β_2-agonist, which is used for the treatment of asthma both in humans and animals. In high doses, clenbuterol exhibits a metabolic effect which results in an increase in muscle mass and a decrease in adipose tissue. This has led to misuse both in humans (doping) and in livestock (breeding). Cattle have been fed clenbuterol for the promotion of the growth process in countries all over the world. The use of clenbuterol for this purpose is forbidden in the European Union [1,2] and other countries due to the

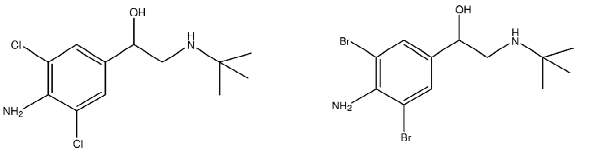

Fig. 1. Clenbuterol (analyte) and brombuterol (template).

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potential health risks associated with β_2-agonist residues in meat products. A number of cases of food poisoning have been reported from the consumption of contaminated meat [3].

The determination of β_2-agonists in biological samples is a difficult analytical task because of the low concentrations (typically <1 ng/mL) of the drugs and the complexity of the matrices. Historically, liquid–liquid extraction has been the preferred technique for cleanup of biological samples [4,5]. These extractions resulted in relatively clean extracts with good recoveries, but were also time-consuming and the solvents used have often involved environmental and health hazards. In recent years, solid-phase extraction (SPE) [6–8] and immunoaffinity chromatography (IAC) [9–11] have become the methods of choice. SPE is cheap, quite fast, gives good recoveries, and can be automated, but does not provide the selectivity needed for very clean extracts. Selectivity is achieved by using IAC, but this technique is expensive, often time-consuming and has to be performed under very specific conditions to keep the affinity sites intact.

A molecularly imprinted polymer (MIP) is produced by polymerisation of a solution containing a functional monomer, a crosslinker and a template [12–17]. Before polymerisation, the functional monomer interacts with the template by, for example, hydrogen, polar, hydrophobic and/or ionic bonds. After polymerisation, the template is removed and the final material contains cavities that can selectively bind compounds very similar in structure, with regard to functional groups and conformation, to the template used (Fig. 2).

One application for the MIP material is as a sorbent in SPE [12–17], where it offers a highly selective binding of the analyte compared with silica-based and other polymeric sorbents. Compared with IAC phases the production time for MIPs is short, production is easy and the material stable under various conditions [18,19]. After binding of the analyte, the MIP material can be washed for the elution of interfering substances. Theses washes can involve rather harsh conditions because the polymers used are often not susceptible to concentrated acids and bases, high ionic strength or different solvents. The selective binding of the analyte can then be broken and the analyte eluted by a change of solvent or pH. Using MIP phases, very clean extracts can be obtained, allowing quantitation to be performed using more cost-effective instrumentation, but still with relatively low limits of detection.

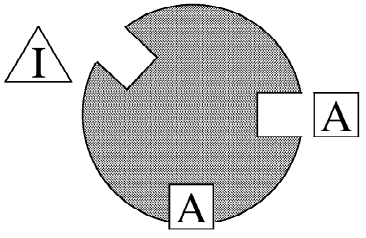

Fig. 2. Schematic diagram of selective binding to the MIP particle. White areas correspond to selective cavities for binding of the analyte (A), while interfering compounds (I) are not bound.

We previously demonstrated the feasibility of using MIPs to achieve selective extraction of clenbuterol from urine samples [20]. Since clenbuterol itself was used as the template the sensitivity was severely limited due to template bleeding. Although new wash protocols may in some cases lead to acceptable bleeding levels [21], it is generally preferred to use a structural analogue as template [22,23]. It was shown that bromoclenbuterol could be used as template and that the resulting MIPs were able to efficiently extract clenbuterol from acetonitrile extracts of bovine liver samples using a matrix solid-phase dispersion approach [23].

Here we demonstrate that MIPs, using brombuterol (Fig. 1) as a template, can be applied as sorbents for the effective cleanup and enrichment of clenbuterol in urine samples.

2. Experimental

2.1. Chemicals

Clenbuterol hydrochloride was purchased from Sigma (USA) and the purity was at least 95%. Brombuterol, free base, was manufactured by Synthelec (Lund, Sweden) and the purity was at least 95%. Acetonitrile (MeCN) and methanol (MeOH) were HPLC-grade from Riedel-de Haën (Germany). Ammonium dihydrogenphosphate (Merck, Germany), ammonium acetate (Merck), diammonium hydrogenphosphate (Merck), acetic acid (HAc;

Riedel-de Haën), and trifluoroacetic acid (TFA; Riedel-deHaën) were all of analytical grade. All water used was distilled and then purified using an ultrapure water system from Elga (UK).

2.2. Materials

MIP4SPE columns for clenbuterol, 25 mg, 10 mL, internal diameter 5 mm, particle size 56 μm, pore size unknown [International Sorbent Technology (IST), UK]. The MIP was produced at MIP Technologies (Lund, Sweden). The polymer was of acrylic type containing a monomer with an acidic functional group, pK_a 4.4. Brombuterol, a structural analogue of clenbuterol, was used as the template molecule. Non-imprinted polymer (MIP Technologies). It was produced like the MIP polymer, except that no template was present during the polymerisation. Calf urine was purchased from Swedish Meats (Kävlinge, Sweden).

2.3. Selectivity test of the MIP

The selective binding of clenbuterol to the MIP was tested by loading 1 mL of acetonitrile, spiked with 100 ng of clenbuterol, onto the MIP and also onto a polymer which was produced, using the same recipe as for the MIP, without the presence of template (non-imprinted polymer). Both columns were then eluted with 1 mL of acetonitrile containing increasing amounts of acetic acid.

2.4. SPE of calf urine using MIP

All sample cleanup was performed using MIP4SPE columns together with a VacMaster Sample Processing Station (IST, UK). All columns were used for only one extraction and then discarded. For all SPE steps a flow-rate of 0.5 mL/min was used, if possible by gravity, otherwise by a slight vacuum. Half a minute of vacuum (−0.7 bar, measured by the vacuum meter in the VacMaster) was applied after each washing solution to allow the columns to semi-dry. The SPE columns were conditioned using the following solutions in sequence: 1 mL of methanol, 1 mL of water and 1 mL of 25 m*M* ammonium acetate pH 6.7.

Five millilitres of centrifuged (3000 *g* for 10 min) calf urine (spiked with clenbuterol or blank), diluted with 5 mL of 25 m*M* ammonium acetate pH 6.7, was used for each sample and applied to the columns. The columns were then washed using the following solutions in sequence: 1 mL of water (2 min of vacuum), 1 mL of acetonitrile–acetic acid (98:2), 1 mL of 0.5 *M* ammonium acetate buffer, pH 5, and 1 mL of 70% acetonitrile in water.

Elution of clenbuterol was performed using 2×1 mL of methanol–trifluoroacetic acid (99:1). The eluate was evaporated under vacuum at +50 °C and reconstituted in 150 μL of 50 m*M* ammonium phosphate buffer, pH 3.0, before analysis.

2.5. Analytical equipment

Analyses were performed using a Merck–Hitachi LaChrom HPLC system consisting of an L-7100 pump, an L-7200 autosampler, an L-7455 diode-array detector, a D-7000 interface, an L-7360 column oven and an L-7612 vacuum degasser. All equipment and data were controlled/evaluated by LaChrom HPLC system manager, version 4.0.

The column used was a BetaBasic C_{18}, 5 μm, 150×2.1 mm, with a guard column, 10×2.1 mm (ThermoHypersilKeystone, UK), and the temperature of the column was 20 °C. The mobile phase used was 50 m*M* ammonium phosphate buffer pH 2–methanol (75:25) pumped at a flow-rate of 0.25 mL/min. One hundred microlitres of each sample was injected and clenbuterol was detected using a single wavelength of 210 nm.

2.6. Performance of the method

The linearity of the method was evaluated by analysing extracts of spiked urine samples at concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 100 ng clenbuterol/mL (n=2).

The absolute recovery of clenbuterol was determined by analysing extracts of spiked urine samples at 0.5, 2.5, 5.0, 25.0 and 100 ng clenbuterol/mL (n=2) and comparing the results with those for extracts of blank urine samples to which corresponding amounts of clenbuterol were added after the evaporation step.

The precision and accuracy of the method were

determined by analysing extracts of spiked urine samples at 0.6 and 6.0 ng clenbuterol/mL (n=6).

3. Results and discussion

3.1. Production of the MIP

The template, crosslinker (difunctional acrylic monomer), acidic monomer (acrylic, pK_a 4.4), initiator and solvent were carefully mixed together. Brombuterol (Fig. 1), a structural analogue of clenbuterol, was chosen as template. The final solution was purged with nitrogen and then polymerised by radical polymerisation. The resulting highly crosslinked polymer was milled, sieved and washed extensively in several steps to minimize bleeding of the template. A non-imprinted polymer was produced in the same manner, except that no template was present during the polymerisation. SPE columns (diameter 5 mm) were packed with 25 mg of each polymer.

3.2. Selectivity test of the MIP

Clenbuterol can bind to the polymer with a variety of bonds (ionic, hydrophobic and hydrogen). In the selective cavities on the MIP, these binding possibilities are sterically arranged to fit clenbuterol. This arrangement is not likely to occur on the non-imprinted polymer because no template molecules were present during the polymerisation. The results (Fig. 3) show that elution with 1% acetic acid elutes all clenbuterol from the non-imprinted polymer while none is eluted from the MIP. The difference in retention of clenbuterol on the MIP and the non-imprinted polymer indicates that there are sites (selective cavities) on the MIP that can bind clenbuterol stronger than the non-imprinted polymer, where the monomers have a random arrangement.

3.3. SPE procedure

The mass transfer of the polymer is slower due to smaller pore sizes [12] compared with a silica-based material and therefore the flow-rate has to be slower than 1 mL/min, which is recommended by IST for, for example, C_{18} columns of the same size. The mass transfer depends on the pore size and the pore size of the polymer changes depending on which solution is used (swelling and shrinking) [12]. The polymer swells 10% when MeCN is used compared with water. The highest recoveries were obtained if the flow-rate did not exceed 0.5 mL/min.

Conditioning consists of wetting the MIP using methanol and water and then adjusting the pH to 6.7 so that the acidic monomer is in a negatively charged state for ionic bonding. Methanol mixed with formic

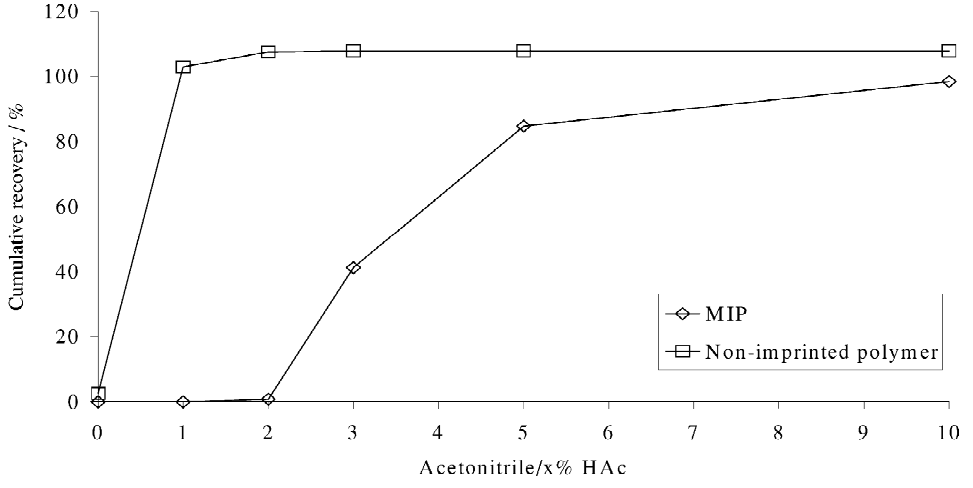

Fig. 3. Selectivity test. One millilitre of acetonitrile, spiked with 100 ng of clenbuterol, was loaded onto a MIP and a non-imprinted polymer. Elution was performed with 1 mL of acetonitrile containing increasing amounts of acetic acid.

acid was tested as a first conditioning solution in order to minimise bleeding of the template from the polymer. This conditioning did not reduce the bleeding and pure methanol was chosen instead because of the risk of losing capacity due to residual acid on the polymer.

During sample loading, clenbuterol is non-selectively retained, together with substances from the urine matrix. In a water environment, not all interactions between the selective cavities of the MIP and clenbuterol are established. Bonding of clenbuterol occurs not only in the selective cavities, but all over the polymer. Non-selective binding enables a high total loading capacity of the material. First, undiluted urine was used as the sample, but the method suffered from low recoveries (<65%) and variations were too large. Therefore, recovery was tested with various dilutions of urine with 25 m*M* buffer pH 6.7 (2:1, 1:1, 1:2 and 1:4). A dilution of 2:1 did not increase the recovery compared with undiluted urine, but all other dilutions both increased the recovery (>70%) and reduced the variation (see below). The lowest of these dilutions (1:1) was chosen. A probable explanation for the lower recoveries is that the high ionic strength of the calf urine reduces the amount of clenbuterol adsorbed to the polymer during sample loading. After sample loading the MIP was washed with water to elute salts and matrix components that were not bonded or absorbed.

For all the possible interactions between the selective cavities of the MIP and clenbuterol to occur the polymer has to be in a MeCN environment. This was achieved by using a selective wash of acetonitrile–acetic acid (98:2). As can be seen in Fig. 3, 2% is the highest concentration of acetic acid that can be used without any loss of clenbuterol from the MIP. For the elution of interfering substances, for example matrix components, the selective wash was very effective, but not effective enough to enable quantification of low concentrations of clenbuterol using UV detection (Fig. 4C).

During method development it was observed that some of the clenbuterol was lost if the water content on the polymer was too high and therefore a few minutes of vacuum was necessary to semi-dry the MIP before the selective wash in order to obtain recoveries of over 70%.

Buffers of various concentrations (0.1, 0.25, 0.5 and 1 *M*) and pH values (3, 5 and 7) were tested for the elution of interferences that were ion and/or hydrogen bonded, but not selectively bonded. No differences were observed between the pH values.

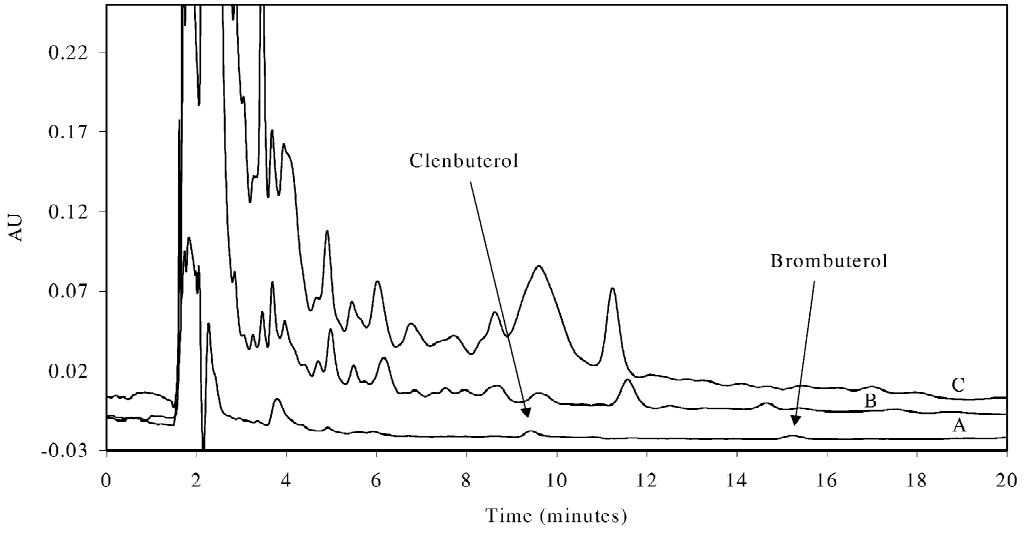

Fig. 4. Significance of the column washes. (A) Washed according to the described method [water, MeCN–HAc (98:2), 0.5 *M* NH_4Ac pH 5 and 70% MeCN used]. (B) Washed without 70% MeCN [water, MeCN–HAc (98:2) and 0.5 *M* NH_4Ac pH 5 used]. (C) Washed without buffer and 70% MeOH [water, MeCN–HAc (98:2) used]. Chromatograms from the analyses of extracts of 5 mL calf urine samples spiked with 2.0 ng clenbuterol/mL.

With 0.1 and 0.25 *M* buffers there were still interferences present that made the quantitation of clenbuterol at low levels difficult. The use of 0.5 and 1.0 *M* buffers resulted in cleaner extracts compared with the wash using buffers of lower concentrations, but the quantitation of low levels of clenbuterol remained difficult due to interferences or residues thereof (Fig. 4B).

In search of a solution that could break both hydrophobic and hydrogen bonds, acetonitrile mixed with water was tested. Water contents above 50% did not give any improved wash effect, but when the water content was lowered to 30% the last of the interferences was eluted. Fig. 4 illustrates the significance of the different washings. Chromatograms from three different spiked urine samples are compared. The first sample was washed using all described washes. For the second sample the wash with 70% MeCN in water was omitted and for the third sample both the wash with 70% MeCN and the wash with buffer were omitted. The chromatograms clearly show that all washes are needed for the evaluation of clenbuterol. Most of the interferences are washed out from the column, which can be seen by the reduction of the front in the chromatograms.

For the elution of clenbuterol, methanol mixed with acids was tested. It was observed that, during the evaporation of some combinations of methanol and acids, clenbuterol was degraded. With high contents of formic acid (>25%) and TFA (≥5%) up to 60% of the clenbuterol was degraded. As a compromise between elution strength and degradation of clenbuterol, methanol with 1% trifluoroacetic acid was chosen for elution. With this solution, no degradation of clenbuterol occurs during evaporation and the recoveries were over 70%.

Figs. 5–7 show chromatograms from the analysis of 5 mL calf urine samples: a blank sample and samples spiked with clenbuterol at 0.5 and 5.0 ng/mL.

During the extraction procedure, clenbuterol molecules bind to sites on the polymer surface, but they also diffuse into the polymer and bind to sites within the polymer. It was observed that the slow diffusion and binding of clenbuterol within the polymer makes it difficult to elute all clenbuterol from the column. During early method development, higher recoveries were observed, but the variations were also greater. All MIP columns were reused during that stage and the increased recovery and variation was due to elution of the remaining clenbuterol from previous extractions. When low concentrations of clenbuterol were analysed the recoveries varied from 75 to 150%, but this variation was not observed at high concentrations of clenbuterol. Using the MIP columns only once eliminated this problem.

3.4. Performance of the method

The linearity of the method was evaluated by

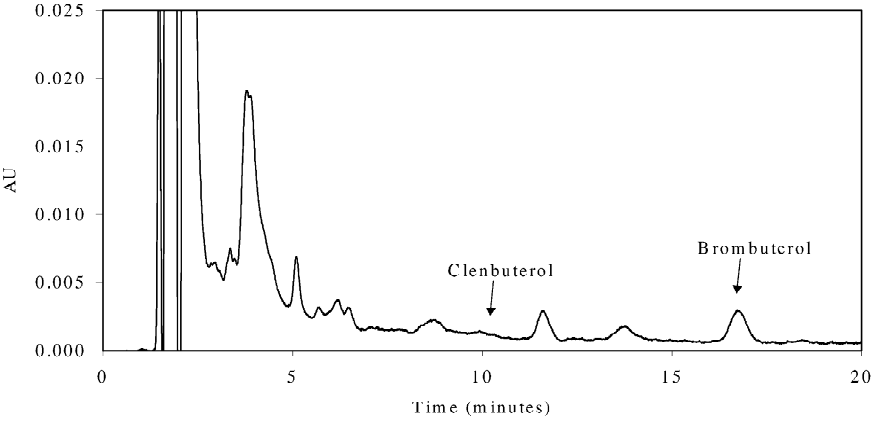

Fig. 5. Chromatogram from the analysis of an extract of a 5 mL blank calf urine sample. The expected retention time of clenbuterol is marked with an arrow. Bleeding of brombuterol (template) is 12 ng/mL in the eluate from the MIP column.

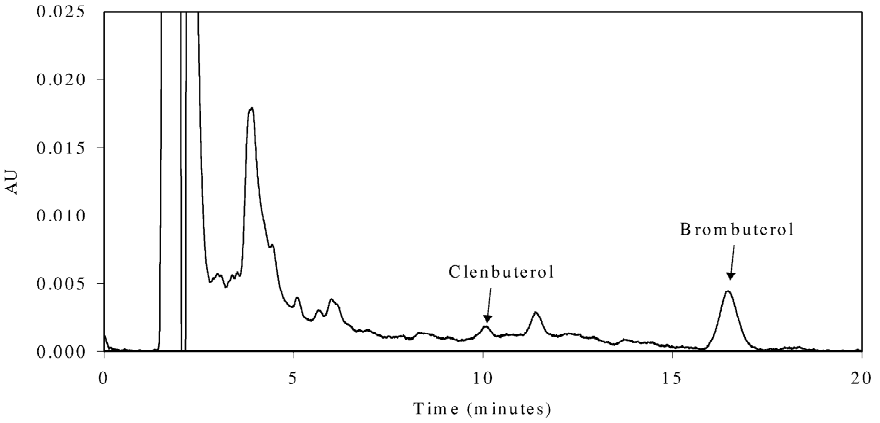

Fig. 6. Chromatogram from the analysis of an extract of a 5 mL calf urine sample spiked with 0.5 ng clenbuterol/mL. Bleeding of brombuterol (template) is 20 ng/mL in the eluate from the MIP column.

analysing spiked urine samples at concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 100 ng clenbuterol/mL ($n=2$). The equation for the resulting calibration curve was $y = 20\,656x - 8770$ with a correlation factor of 0.999.

The absolute recovery of clenbuterol was determined by analysing spiked urine samples at 0.5, 2.5, 5.0, 25.0 and 100 ng clenbuterol/mL (two at each concentration) and comparing the results with those for extracts of blank urine samples to which corresponding amounts of clenbuterol were added after the evaporation step. Matrix effects on the LC response were thereby taken into account. The absolute recovery was measured to be 75% ($\pm$2.1%) and no differences were observed between the concentrations.

The precision and accuracy of the method were determined by analysing spiked urine samples at 0.6 and 6.0 ng clenbuterol/mL ($n=6$). The results are presented in Table 1. The results show that the

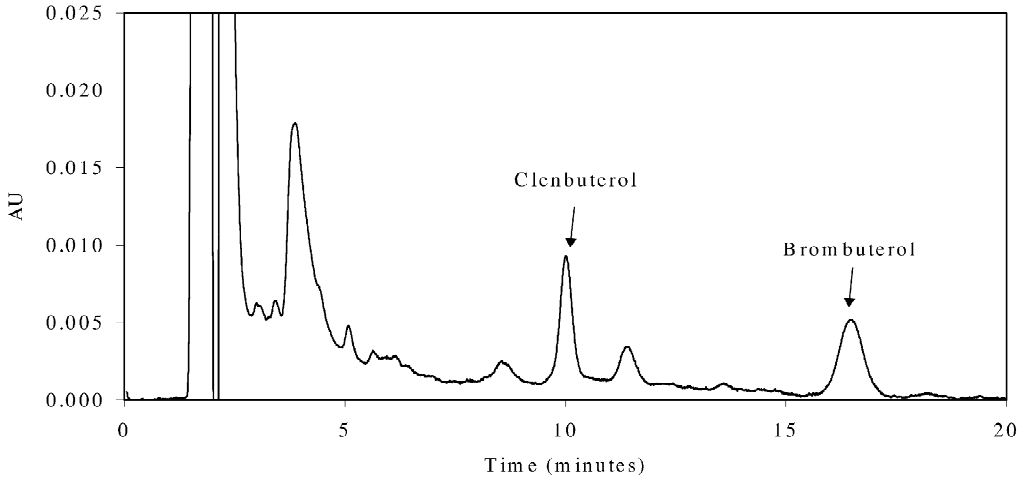

Fig. 7. Chromatogram from the analysis of an extract of a 5 mL calf urine sample spiked with 5.0 ng clenbuterol/mL. Bleeding of brombuterol (template) is 20 ng/mL in the eluate from the MIP column.

Table 1
Precision and accuracy for the analysis of clenbuterol in 5 mL spiked calf urine samples

	Within-day		Between-day	
	0.6 ng/mL	6.0 ng/mL	0.6 ng/mL	6.0 ng/mL
n	5	6	9	10
Mean±SD	0.58±0.025	5.8±0.12	0.61±0.039	5.9±0.24
SD (%)	4.3	2.1	6.4	4.1
Accuracy (%)	96.7	96.7	101.7	98.3
No. analyses	1	1	3	3

performance of this MIP-based method is well within the limits of that expected of current bioanalytical methods.

4. Conclusion

The MIP exhibits high selectivity for clenbuterol. When the selective binding of clenbuterol is established the MIP can be washed harshly to elute interfering compounds from the matrix without any loss of clenbuterol. This results in very clean extracts that makes quantitation by HPLC–UV down to 0.5 ng clenbuterol/mL urine possible.

This work shows that sample cleanup using MIP extraction is well suited for bioanalysis at trace levels and that the resulting methods can be robust with good precision and accuracy. Although different MIP materials exhibit very good selectivity, optimisation of the SPE procedure is needed to achieve the cleanest extracts possible. The strong binding and the slow diffusion within the polymer hamper recovery and there will always be a small proportion of clenbuterol that cannot be eluted from the column. During this work new columns were used for each sample, which was essential to avoid contamination from previous samples.

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