

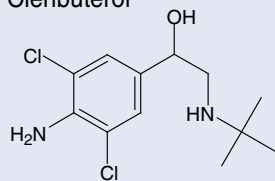
Molecular Imprinted Polymer SPE Increases Sensitivity for the Extraction and Analysis of Clenbuterol from Urine

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Introduction

Clenbuterol (Figure 1) is a beta-agonist known for its growth-promoting properties in which use of the drug induces significant weight gain by increasing the proportion of muscle mass to fat. Although the US Food and Drug Administration, US Department of Agricultural and European Union have banned the use of clenbuterol for humans and livestock, illegal use of the drug still readily

Figure 1. Structure of Clenbuterol



occurs (1). For example, the drug is widely used among body builders and athletes due to its anabolic effects. Two Olympic athletes were banned from the 1992 Barcelona Olympic Games due to the drug, and two swimmers

tested positive for the drug in the 2002 World Championships in Perth, Australia. Clenbuterol is readily used by farmers to give show animals a competitive advantage as well. In 1995, the US Department of Agriculture issued a press release stating that both they and the FDA will be taking enforcement actions against the use of clenbuterol (2). In the 1990s, numerous outbreaks in clenbuterol food poisoning arose throughout Europe (3). As recently as September 2006, over 300 cases of food poisoning occurred in Shanghai due to the consumption of clenbuterol contaminated pork (4).

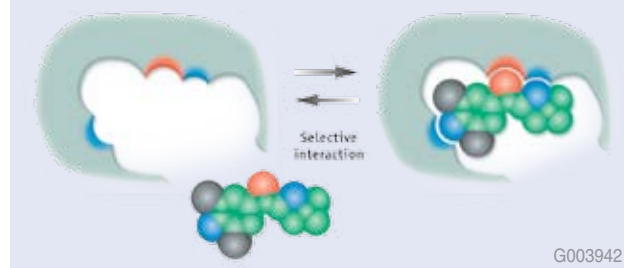
Due to the potential health risks and competitive advantage associated with clenbuterol's use in livestock and human performance enhancement, residue screening programs for the banned drug are conducted worldwide. It is therefore critical to develop a highly selective and sensitive analytical assay to monitor clenbuterol residues in difficult biological matrices such as urine, retina, tissues, etc.

In this report, we discuss the use of a molecular imprinted polymer technology (SupelMIP™) specifically designed for the selective extraction of trace levels of clenbuterol from urine for subsequent LC-MS-MS analysis. The technique was compared against a published method using a conventional hydrophilic polymer SPE phase (5).

Molecular Imprinted Polymers

Molecular imprinted polymers (MIPs) are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related compounds with high selectivity. Selectivity is introduced during MIP synthesis in which a template molecule, designed to mimic the analyte, guides the formation of specific cavities that are sterically and chemically complementary to the target analyte(s). As a result, multiple interactions (e.g., hydrogen bonding, ionic, Van der Waals, hydrophobic) can take place between the MIP cavity and analyte functional groups (Figure 2). The strong retention offered between a MIP phase and its target analyte(s) allows for the use of exhaustive wash procedures during solid phase extraction that results in superior sample cleanup prior to analysis.

Figure 2. Visual Depiction of MIP Binding Site



Extraction of Clenbuterol from Urine

In this study, an extraction method using SupelMIP SPE – Clenbuterol was compared against a published method using conventional hydrophilic polymer SPE phase (5). Table 1 describes the two extraction procedures.

SupelMIP SPE Offers Reduced Background Resulting in Greater Sensitivity

Clenbuterol was extracted from urine using both molecular imprinted polymer SPE and conventional hydrophilic polymer SPE via the procedures described in Table 1. Extracts were further analyzed via LC-MS-MS. From the LC-MS-MS chromatograms (MRM) depicted in Figure 3, we see that blank urine samples extracted with the SupelMIP protocol offered low background. In contrast, the conventional hydrophilic polymer SPE procedure co-extracted matrix interferences resulting in a high background response within LC elution area of

Table 1. Comparison of SupelMIP SPE – Clenbuterol Method and Conventional Hydrophilic Polymer SPE Method

SupelMIP SPE – Clenbuterol Method**Sample Pre-Treatment:**

Human urine samples were spiked with 0.0 (blank), 0.04, 0.1, 0.5, and 1.0 ng/mL clenbuterol. Spiked and blank urine samples centrifuged at 3000 rpm for 10 min., and the resultant supernatant was diluted 1:1 (v/v) with 25 mM ammonium acetate buffer, pH 6.5

SPE Procedure:

SupelMIP SPE – Clenbuterol, 25 mg/10 mL (LRC) (Cat. No. 53201-U)

1. Condition and equilibrate MIP phase with 1 mL methanol, 1 mL DI water, and 1 mL 25 mM ammonium acetate, pH 6.5
2. Load 1 mL pre-treated sample on to the cartridge.
3. Wash (elute interferences) using the following wash scheme: 1 mL DI water followed by 2 min. vacuum; 1 mL 2% acetic acid in acetonitrile; 1 mL 0.5 M ammonium acetate, pH 5; 1 mL 70% acetonitrile followed by 2 min. vacuum
4. Elute clenbuterol with 2 x 1 mL 1% TFA in methanol. Apply gentle vacuum between each fraction.
5. Evaporate under nitrogen and reconstitute with 1 mL LC mobile phase prior to LC-MS-MS analysis

Published Clenbuterol Method Using Conventional Hydrophilic Polymer SPE Phase (5)**Sample Pre-Treatment:**

Human urine samples were spiked with 0.0 (blank), 0.2, 1.0, and 2.0 ng/mL clenbuterol. Spiked and blank urine diluted 1:1 (v/v) with 2% ammonium hydroxide

SPE Procedure:

Conventional Hydrophilic Polymer SPE Phase, 30 mg/1 mL

1. Condition and equilibrate SPE phase with 1 mL methanol and 1 mL DI water
2. Load 1 mL pre-treated sample on to the cartridge.
3. Wash (elute interferences) with 0.5 mL 2% ammonia and 0.5 mL 30% methanol
4. Elute clenbuterol with 2 mL methanol
5. Evaporate under nitrogen and reconstitute with 1 mL LC mobile phase prior to LC-MS-MS analysis

clenbuterol (1-2 min.). This can potentially lead to lower assay reproducibility, accuracy and sensitivity thereby elevating lower limits of quantitation (LLOQ). Table 2 lists recovery values for clenbuterol using both sample prep procedures. Recovery for clenbuterol using the SupelMIP phase was 75% at the spike levels tested (0.1-1.0 ng/mL). 99% recovery was observed at the 0.1 ng/mL spike level. In contrast, reduced response levels were observed across the spike range tested using the hydrophilic polymer SPE protocol with a recovery of 8% at the lowest spike level

(0.1 ng/mL). Note that both extraction procedures were repeated using buffer as a sample matrix, and recovery values were comparable for all three spike levels (data not shown); therefore, the lower response values observed for the polymer phase was primarily caused by matrix effects. To further demonstrate the selectivity and sensitivity differences between the two procedures, Figure 4 compares the linear relation of known spike concentrations vs. calculated concentrations determined from the

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Figure 3. Clenbuterol Spiked Urine Samples Extracted with SupelMIP SPE vs. Conventional Hydrophilic Polymer SPE

column: Ascentis Express C18, 5 cm x 2.1 mm I.D., 2.7 μ m (53822-U)
 instrument: Applied Biosystems 3200 Q-TRAP
 mobile phase: 10% acetonitrile in 10 mM ammonium acetate (pH unadjusted):acetonitrile (80:10)
 temp.: 35 $^{\circ}$ C
 flow rate: 0.2 mL/min.
 detection: MS/MS, MRM transitions (277.2/203.1 and 277.3/168.2 m/z)
 ion mode: Positive
 ion source: Turbospray
 ion spray voltage: 3200 V
 source temperature: 425 $^{\circ}$ C
 collision gas: 45 psi
 injection: 5 μ L

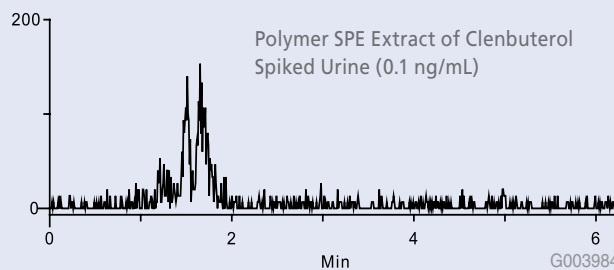
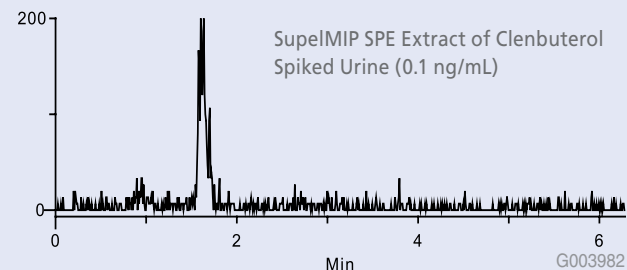
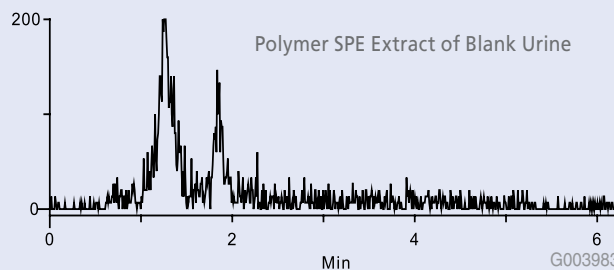
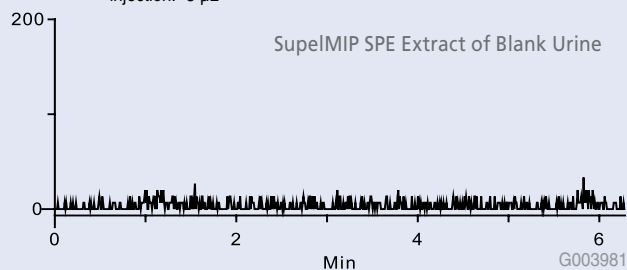


Table 2. Recovery Comparison for Clenbuterol from Urine using SupelMIP SPE and Conventional Hydrophilic Polymer SPE

Spike Level (ng/mL)	% Recovery from Urine	
	SupelMIP SPE - Clenbuterol	Hydrophilic Polymer SPE
0.1	99%	8%
0.5	75%	66%
1.0	75%	69%

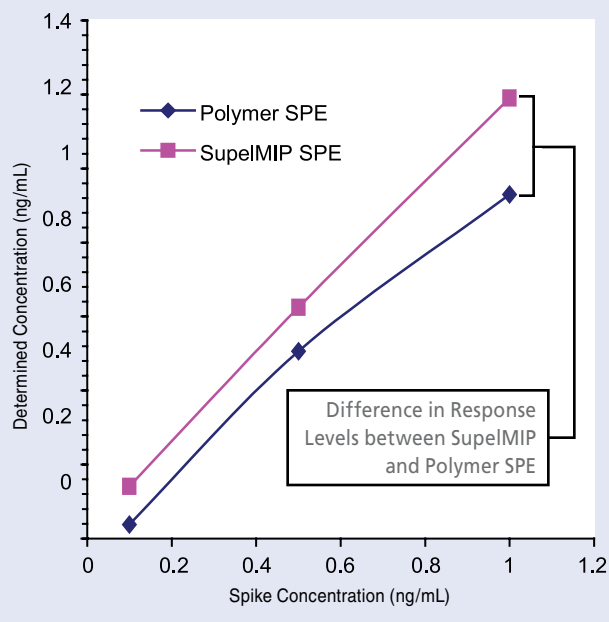
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signal responses obtained from blank urine extracts spiked post-extraction. Post-extraction spiked samples were compared for both the SupelMIP and polymer SPE protocols described. Increased levels of ion-suppression were observed for the polymer SPE protocol relative to the SupelMIP procedure.

Conclusion

In this report, we demonstrated the extraction of SupelMIP SPE – Clenbuterol method against a published hydrophilic polymer SPE method for the trace extraction of clenbuterol from urine for subsequent LC-MS-MS analysis. Because selectivity is introduced during the development of the MIP itself, it allows for a binding site that is sterically and chemical complementary to the target analyte(s). The multiple interactions that take place between the imprint binding site and analyte(s) of interest offer strong interactions enabling the use of exhaustive wash conditions during the SPE process to provide cleaner extracts prior to analysis. This was demonstrated for clenbuterol in which the SupelMIP SPE approach offered greater selectivity resulting in lower limits of quantitation which is critical for the analysis of this banned drug.

Figure 4. Known Spike Concentration vs. Determined Concentration for SupelMIP SPE and Polymer SPE Extracts of Clenbuterol from Urine (Post-Extraction Spike)



Although the conventional hydrophilic polymer SPE method offers less procedural steps, sample prep selectivity was inadequate resulting in increased levels of background ultimately resulting in a less sensitive assay.

References

1. Council Directive 86/469/EEC, European Union, Brussels, 1988
2. Clenbuterol, FSIS Backgrounders, July 1995, <<http://www.fsis.usda.gov/OA/background/clenbute.htm>>
3. GA Mitchell and G Dunnavan, J. Anim. Sci., 1998, 76:208-211
4. D Patton, New Food Poisoning Case Hits China, Sep 2006, <<http://www.foodproductiondaily.com/news/ng.asp?id=70659>>
5. M Josefsson et al., J Chromat. A, 2006, 1120:1-12

Featured Products

SupelMIP SPE Cartridges	Sorbent Mass (mg)	Cartridge Volume (mL)	Cartridges per Box	Cat. No.
Clenbuterol	25	10	50	53201-U
Beta-agonists (class selective)	25	10	50	53202-U
Beta-agonists (class selective)	25	3	50	53225-U
Beta-blockers (class selective)	25	10	50	53218-U
Beta-blockers (class selective)	25	3	50	53213-U
Full Beta Receptor (Beta-agonists and Beta-blockers)	25	10	50	53223-U
Full Beta Receptor (Beta-agonists and Beta-blockers)	25	3	50	53224-U
Chloramphenicol	25	10	50	53210-U
Chloramphenicol	25	3	50	53209-U
NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)	25	10	50	53206-U
NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol)	25	3	50	53203-U
TSNAs (4 different Tobacco specific Nitrosamines: NNK, NNN, NAB, NAT)	50	10	50	53221-U
TSNAs (4 different Tobacco specific Nitrosamines: NNK, NNN, NAB, NAT)	50	3	50	53222-U
Riboflavin (vitamin B2)	25	10	50	53207-U
Triazines (class selective)	25	10	50	53208-U