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Multi-residue liquid chromatography/tandem mass spectrometric analysis of beta-agonists in urine using molecular imprinted polymers

Nathalie Van Hoof[1], Dirk Courtheyn[2], Jean-Philippe Antignac[3], Mieke Van de Wiele[2], Sofie Poelmans[1], Herlinde Noppe[1] and Hubert De Brabander[1]*

[1]Laboratory of Chemical Analysis, Ghent University, Department of Veterinary Public Health and Food Safety, Salisburylaan 133, B-9820 Merelbeke, Belgium
[2]Federal Food Laboratory, Braemkasteelstraat 59, B-9050 Gentbrugge, Belgium
[3]LABERCA-Ecole Nationale Vétérinaire de Nantes, Route de Gachet, BP 50707, F-44307 Nantes Cedex 3, France

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Ion suppression, a matrix effect that affects quantitative mass spectrometry, is one of the main problems encountered in liquid chromatography/tandem mass spectrometry. Two different clean-up steps for the multi-residue analysis of beta-agonists in urine were evaluated with respect to minimisation of ion suppression, namely, a mixed-phase solid phase extraction (SPE) column, i.e., clean screen Dau (CSD), and a molecular imprinted polymer (MIP) SPE column. Ion suppression experiments revealed that CSD sample clean-up can lead to false negative results for some beta-agonists, and that clean-up using MIP columns is more selective for beta-agonists than the use of CSD columns. Copyright © 2005 John Wiley & Sons, Ltd.

Beta-agonists are used for growth promotion in cattle. They improve carcass composition as they decrease fat in favour of a higher percentage of muscle (repartitioning agents). In the European Union the use of beta-agonists as growth promoters has been banned since 1996,[1] while other countries, like the USA, Mexico and South Africa, have licensed some of them at growth-promoting doses. Therefore, multi-residue methods are necessary to monitor the abuse of beta-agonists.

In this field of residue control, the fulfilment of precise analytical criteria is mandatory, as described in Commission Decision 2002/657/EC.[2] In particular, the requirements in terms of unambiguous identification of the target analytes led to the widespread utilisation of mass spectrometry as a confirmatory technique. While gas chromatography/mass spectrometry (GC/MS) instruments were historically the more widely used for various classes of residues, liquid chromatography/mass spectrometry (LC/MS) today appears as the method of choice and the major actual investment for many laboratories, especially for the analysis of polar compounds. However, after a first period of great enthusiasm shared by most end-users, some problems related to these LC/MS-related techniques started to be reported. One main source of pitfalls was the existence of matrix effects in general, and the ion suppression phenomenon in particular. Therefore, one should adopt a standard practice that acknowledges the necessity of improved sample preparation before measurement in order to minimise problems of this kind.

Methods described in the literature are mainly based on mixed-phase solid-phase extraction (SPE).[3–11] These SPE procedures have proven to be selective not only for beta-agonists, but also for other basic drugs. Therefore, they cannot provide the degree of selectivity needed for each beta-agonist.[12] Therefore, the use of molecular imprinted polymers (MIPs) for the sample clean-up of beta-agonists was investigated in this article. MIPs are cross-linked polymers prepared in the presence of a template molecule. The template molecule may be a particular analyte or drug molecule, or an analogue of it. Functional monomers interact with the template molecule during polymerisation and the template is removed from the polymer afterwards. The cavities thus created in the polymer are complementary to the template both in shape and in chemical positioning of functional groups. These multiple interaction sites lead to cavities with highly specific binding affinity. The beta-agonist MIP product is designed to contain appropriately positioned acidic functional groups capable of forming hydrogen bonds at several positions with the beta-agonists. These polymers can withstand high temperatures, a large pH range, and organic solvents, without losing their recognition properties. As a consequence of these properties they are well suited as selective sorbents in SPE, allowing selective clean-up of compounds prior to analysis.

In this context, the aim of this work was to test whether the clean-up of beta-agonists using MIP columns could improve the overall method performance, not only in terms of analyte recovery, but also in terms of removal of interfering

*Correspondence to: H. De Brabander, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium.
E-mail: Hubert.DeBrabander@Ugent.be
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compounds and reduction of the ion suppression phenomenon. Previous experiments revealed that sample clean-up using MIP extraction is well suited for bioanalysis at trace levels and that the resulting methods can be robust with good precision and accuracy.[12–15] Fiori *et al.*[15] have already compared two different clean-up steps involving SPE using non-endcapped C_{18} and MIPs. The mechanism of C_{18} SPE columns is based on the hydrophobic behaviour of the columns, and therefore can be used for clean-up of a wide range of compounds; better recoveries were observed using MIP columns.[15] Since clean screen Dau (CSD) columns are more selective than C_{18} columns and are used more frequently nowadays for the routine analysis of beta-agonists, it was useful to evaluate these two different clean-up steps, CSD and MIPs, with respect to their ability to minimise ion suppression in liquid chromatography/tandem mass spectrometry (LC/MS/MS).

EXPERIMENTAL

Reagents and chemicals

The beta-agonist standards salbutamol, clenbuterol, isoxsuprine, fenoterol and tulobuterol were obtained from Sigma-Aldrich (St. Louis, MO, USA), while cimaterol, mabuterol, brombuterol, terbutaline, hydroxymethyl clenbuterol, cimbuterol, mapenterol and clenproperol were from the EU Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany); zilpaterol was a gift from Intervet (Schwabenheim, Germany). The internal standard, deuterated clenbuterol, was obtained from RIVM (Bilthoven, The Netherlands). Clean screen Dau columns were from UCT Technologies (Bristol, PA, USA), and MIP4SPE beta-agonist columns were from MIP Technologies (Lund, Sweden). The enzymatic deconjugation was performed with *β*-glucuronidase from Sigma-Aldrich. All chemicals used were of analytical grade from Merck (Darmstadt, Germany) and Acros (Geel, Belgium).

Stock standard solutions of 1000 ng μL^{-1} were prepared in ethanol. For the preparation of working solutions, methanol was used as diluent. All standard and working solutions were stored at −20°C.

Instrumentation

The high-performance liquid chromatography (HPLC) apparatus was comprised of a P4000 quaternary pump and an AS3000 autosampler (ThermoFinnigan, San José, CA, USA). Chromatographic separation was achieved using an Alltima HP C_{18} column (5 µm, 150 × 2.1 mm; Alltech, Deerfield, IL, USA). The mobile phase consisted of a mixture of methanol (A) and water with 5 mM pentafluoropropionic acid (PFPA) (B). A linear gradient was run (20% A for 5 min, increasing to 35% A over 10 min, and finally increasing to 100% A in the next 3 min) at a flow rate of 0.3 mL min^{-1}.

LC/MS^2 detection used a LCQ Deca ion trap (ThermoFinnigan) with an electrospray ionisation (ESI) interface operating in positive ion mode. The instrument parameters are summarised in Table 1. The precursor isolation width was set to 2 Da for each beta-agonist. Each analyte was identified on the basis of at least two product ions present in the MS^2 or MS^3 spectra (Table 2).

Extraction and clean-up

Clean screen Dau SPE

To blank urine samples (5 mL) a spike solution of beta-agonists at their minimum required performance limit (MRPL) concentration and 1 µg L^{-1} clenbuterol-d_6 (internal standard) (Table 3) was added. After addition of 2.5 mL 0.2 M acetate buffer (pH 4.6) the pH of the mixture was adjusted to 4.6 and the sample was centrifuged (8000 rpm, 15 min, 5°C). The clean-up was performed using a 500 mg clean screen Dau (CSD) (mixed C_8 and SCX) SPE column. The column was conditioned with methanol, water and 0.1 mol L^{-1} phosphate buffer (pH 6). After application of the extract, the cartridge was washed first with 1 mol L^{-1} acetic acid, vacuum-dried, and subsequently washed with methanol and vacuum-dried again. Elution used 6 mL ethyl acetate containing concentrated ammonia (97:3). The eluate was evaporated to dryness at 60°C under a stream of nitrogen. The residue was reconstituted in 30 µL methanol and 90 µL 5 mM PFPA solution, before injecting 30 µL on the HPLC column.

Table 1. Instrumental method for the detection of beta-agonists in urine samples

Segments	Scan events	Parent mass and mass range	Analyte
Segment 1	Scan event 1	262.0 → 244.0; 100–270	Zilpaterol
0–6.2 min	Scan event 2	220.0 → 202.0; 100–230	Cimaterol
	Scan event 3	240.0 → 222.0; 100–250	Salbutamol
	Scan event 4	226.0 → 170.0; 100–230	Terbutaline
	Scan event 5	234.0 → 216.0; 100–240	Cimbuterol
Segment 2	Scan event 1	263.0 → 245.0; 100–270	Clenproperol
6.2–13.5 min	Scan event 2	302.0; 100–310	Ractopamine
	Scan event 3	277.0 → 259.0; 100–280	Clenbuterol
	Scan event 4	283.0; 100–290	Clenbuterol-d3 (I.S.)
	Scan event 5	304.0; 100–310	Fenoterol
	Scan event 6	293.0 → 275.0; 100–300	Hydroxymethyl clenbuterol
Segment 3	Scan event 1	228.0; 100–230	Tulobuterol
13.5–22 min	Scan event 2	311.0 → 293.0; 100–320	Mabuterol
	Scan event 3	367.0 → 349.0; 100–370	Brombuterol
	Scan event 4	302.0 → 284.0; 100–310	Isoxsuprine
	Scan event 5	325.0; 100–330	Mapenterol

Table 2. Precursor and product ions (*m/z*) used for the evaluation of the beta-agonists

Analyte	Precursor ion	MS^2 first-generation product ions	MS^3 second-generation product ions
Zilpaterol	262	244	185, 202
Cimaterol	220	202	160
Salbutamol	240	222	148, 166
Terbutaline	226	170	152
Clenproperol	263	245	203
Tulobuterol	228	154, 172, 210	
Ractopamine	302	164, 284	
Clenbuterol	277	259	203
Mabuterol	311	293	237
Brombuterol	367	349	293
Isoxsuprine	302	284	107, 135, 150, 190
Hydroxymethyl clenbuterol	293	275	203
Fenoterol	304	135, 286	
Cimbuterol	234	216	160
Mapenterol	325	237, 307	

Molecular Imprinted Polymer SPE

To blank urine samples (5 mL) a spike solution of beta-agonists at their MRPL concentration and 1 μg L^{-1} clenbuterol-d_6 was added. The urine samples were first diluted 1:1 with water and centrifuged at 9000 rpm for 10 min. The clean-up was performed using a 25 mg MIP4SPE (beta-agonist) SPE column. The column was conditioned with methanol, water and 25 mM ammonium acetate buffer (pH 6.7). After application of the extract, the cartridge was washed with 1 mL water and vacuum-dried, and subsequently with 1 mL 1% acetic acid in acetonitrile, 1 mL 50 mM ammonium acetate buffer and 1 mL 60% acetonitrile in water. Elution used 2 × 1 mL 10% acetic acid in methanol, applying gentle vacuum between the two fractions. The flow rate was 0.5 mL min^{-1}, except for the analyte elution where a lower flow rate was applied. The eluate was evaporated to dryness at 60°C under a stream of nitrogen. The residue was reconstituted in 30 μL methanol and 90 μL 5 mM PFPA, before injecting 30 μL on the HPLC column.

Table 3. MRPL values for beta-agonists in urine proposed by the EU Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany)

Analyte	Proposed MRPL (μg L^{-1})
Zilpaterol	1
Cimaterol	3
Salbutamol	3
Terbutaline	3
Clenproperol	3
Tulobuterol	1
Ractopamine	3
Clenbuterol	1
Mabuterol	1
Brombuterol	1
Isoxsuprine	3
Cimbuterol	3
Mapenterol	1
Fenoterol	3
Hydroxymethyl clenbuterol	1

RESULTS AND DISCUSSION

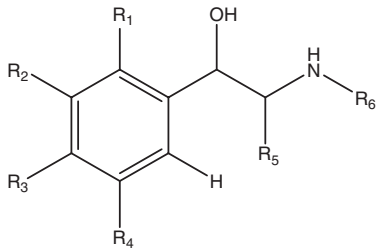
LC/MS^n method

A multi-residue LC/MS^n method was developed for the qualitative analysis of 15 beta-agonists (Fig. 1) in urine. The beta-agonists were spiked into blank calf urine at their MRPL concentrations. Figure 2 shows the extracted ion chromatograms for the beta-agonists after clean-up with CSD columns and without enzymatic hydrolysis. All the beta-agonists could be detected at the MRPL level, but the signals for zilpaterol and terbutaline were weak and subject to significant interferences (low signal-to-noise ratio). Figure 3 shows the ion chromatograms for the different beta-agonists after clean-up with MIP columns and without hydrolysis. All the beta-agonists could be detected at the MRPL level according to the 2002/657/EC decision criteria.[2] Recoveries for the different beta-agonists using MIP clean-up are in the range 40–70%, except for zilpaterol, salbutamol and terbutaline, which have recoveries below 40%.

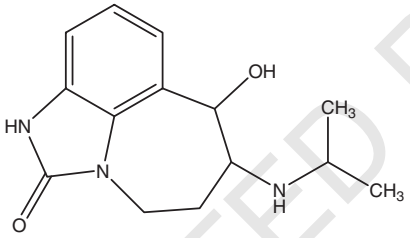
The beta-agonists could also be detected with signals of the same order-of-magnitude as in Figs. 2 and 3 after hydrolysis with glucuronidase at 50°C for 2 h. The aim of this work was to compare the effectiveness of the clean-up using CSD with that using MIP SPE, with respect to removal of interfering compounds and reduction of ion suppression. Since hydrolysis did not seem to interfere with the signals for the analytes, there was no suppression or enhancement of the signals as the result of this hydrolysis (data not shown). Therefore, the subsequent experiments concerning ion suppression were performed without hydrolysis. Of course, for real samples from animals dosed with beta-agonists, in which the analytes could be conjugated and for which quantitative analyses are required, the effect of enzymatic hydrolysis would have to be examined in detail.

Ion suppression

The main analytical problems encountered in LC/MS^n arise from matrix effects, and in particular involve ionisation suppression. This phenomenon affects many aspects of the method performance such as detection capability, repeatability, and accuracy. The cause of ionisation suppression is a change



	R_1	R_2	R_3	R_4	R_5	R_6
Cimaterol	H	CN	NH_2	H	H	$CH(CH_3)_2$
Salbutamol	H	CH_2OH	OH	H	H	$C(CH_3)_3$
Terbutaline	H	OH	H	OH	H	$C(CH_3)_3$
Clenproperol	H	Cl	NH_2	Cl	H	$CH(CH_3)_2$
Ractopamine	H	H	OH	H	H	$CH(CH_3)$-$(CH_2)_2$-PhOH
Clenbuterol	H	Cl	NH_2	Cl	H	$C(CH_3)_3$
Tulobuterol	Cl	H	H	H	H	$C(CH_3)_3$
Mabuterol	H	Cl	NH_2	CF_3	H	$C(CH_3)_3$
Brombuterol	H	Br	NH_2	Br	H	$C(CH_3)_3$
Isoxsuprine	H	H	OH	H	CH_3	CH(CH3)-CH2-O-Ph
Cimaterol	H	CN	NH_2	H	H	$CH(CH_3)_2$
Fenoterol	H	OH	H	OH	H	$CH(CH_3)$-CH_2-PhOH
Hydroxymethyl clenbuterol	H	Cl	NH_2	Cl	H	$C(CH_3)_2$-CH_2-OH
Mapenterol	H	Cl	NH_2	CF_3	H	$C(CH_3)_2CH_2CH_3$



zilpaterol

Figure 1. Structures of beta-agonists considered in this work.

in the spray droplet solution properties arising from the presence of co-eluting non-volatile or less volatile solutes. Polar compounds such as beta-agonists seem to be particularly susceptible to ion suppression. The positive ionisation mode is usually considered as less specific, and consequently more subjected to ion suppression.[16,17]

The typical experimental system used to evaluate ion suppression in LC/MS^n is depicted in Fig. 4.[17] Either clean mobile phase or real samples are injected into the LC system. A standard solution containing the analyte of interest is continuously infused through a T-coupling system, mixed with the LC eluate, and passed into the mass spectrometer interface. The resulting signal recorded by the mass spectrometer is the net result of these two solutions. Because the analyte is introduced into the mass detector at a constant rate, a constant ESI response should ideally be observed. This is the case when pure mobile phase is injected into the LC system. When blank urine is injected into the LC system, the resulting total ion current increases due to the new material arriving in the interface, and the product signal of the analyte decreases in certain retention time regions as a result of the negative influence of interfering compounds eluting at these retention times.[16]

This experiment was performed for the beta-agonists zilpaterol, cimaterol, salbutamol and terbutaline. These beta-agonists elute around the same retention time, and the signals for zilpaterol and terbutaline were weak after clean-up with CSD. First the standard solution and a spiked urine at the MRPL concentration were injected to obtain the retention time of the analytes. Subsequently, pure mobile phase was injected while the analyte was continuously infused. Finally, to evaluate ion suppression blank urine was injected while the analyte was infused. Figure 5 shows the data obtained by the injection of blank urine extracts obtained after clean-up with CSD and or with MIP while continuously infusing zilpaterol and cimaterol. After clean-up with CSD no significant suppression was observed for the product signal of cimaterol near its expected retention time (RT, 3.1 min). However, severe ion suppression appeared for zilpaterol (RT 3.6 min), i.e., in the time window in which zilpaterol elutes there was a serious decrease in the zilpaterol signal due to the interfering compounds that also eluted in this retention time window. In contrast, after clean-up with MIP there was no significant suppression of the signals for either zilpaterol or cimaterol in the time windows in which each analyte elutes. Similar MIP results were obtained for salbutamol and

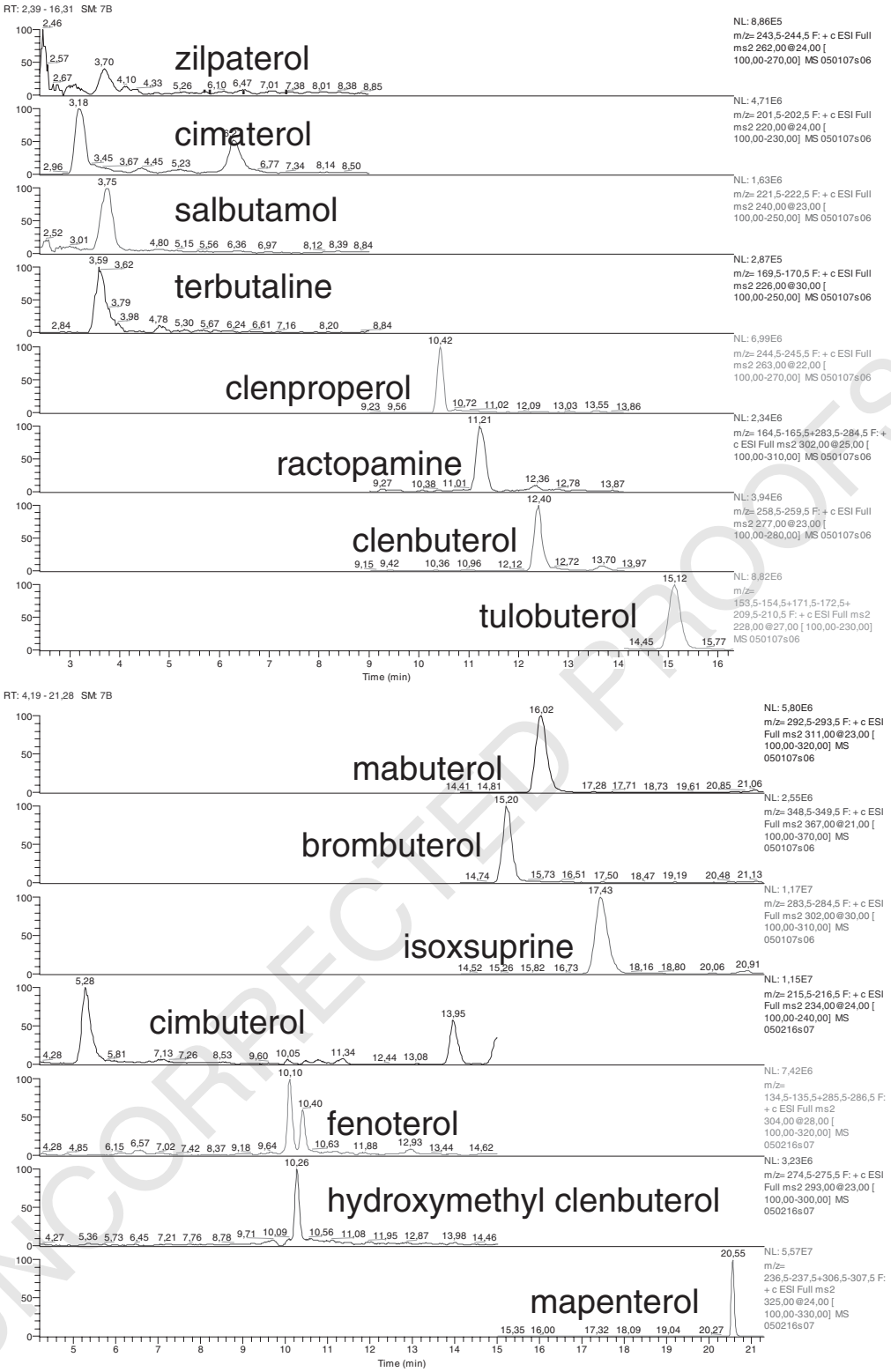


Figure 2. Extracted ion chromatograms for the different beta-agonists at their MRPL concentrations in calf urine using CSD clean-up (no glucuronidase treatment).

terbutaline. However, after clean-up with CSD there was severe suppression of the signal for terbutaline near its expected retention time; no significant suppression was observed for the signal for salbutamol at its retention time.

This experiment shows that CSD sample clean-up could lead to underestimation of the concentrations of some beta-agonists and could lead to a potential risk of false negative results. A possible solution to overcome false negative results is the use of an adequate internal standard, preferably a deuterated internal standard, in order to correct for the ion suppression effect.[16] Of course, this is only possible when the ion signal is not suppressed completely. Since different beta-agonists are suppressed, different adequate internal standards are necessary which can in turn lead to analytical

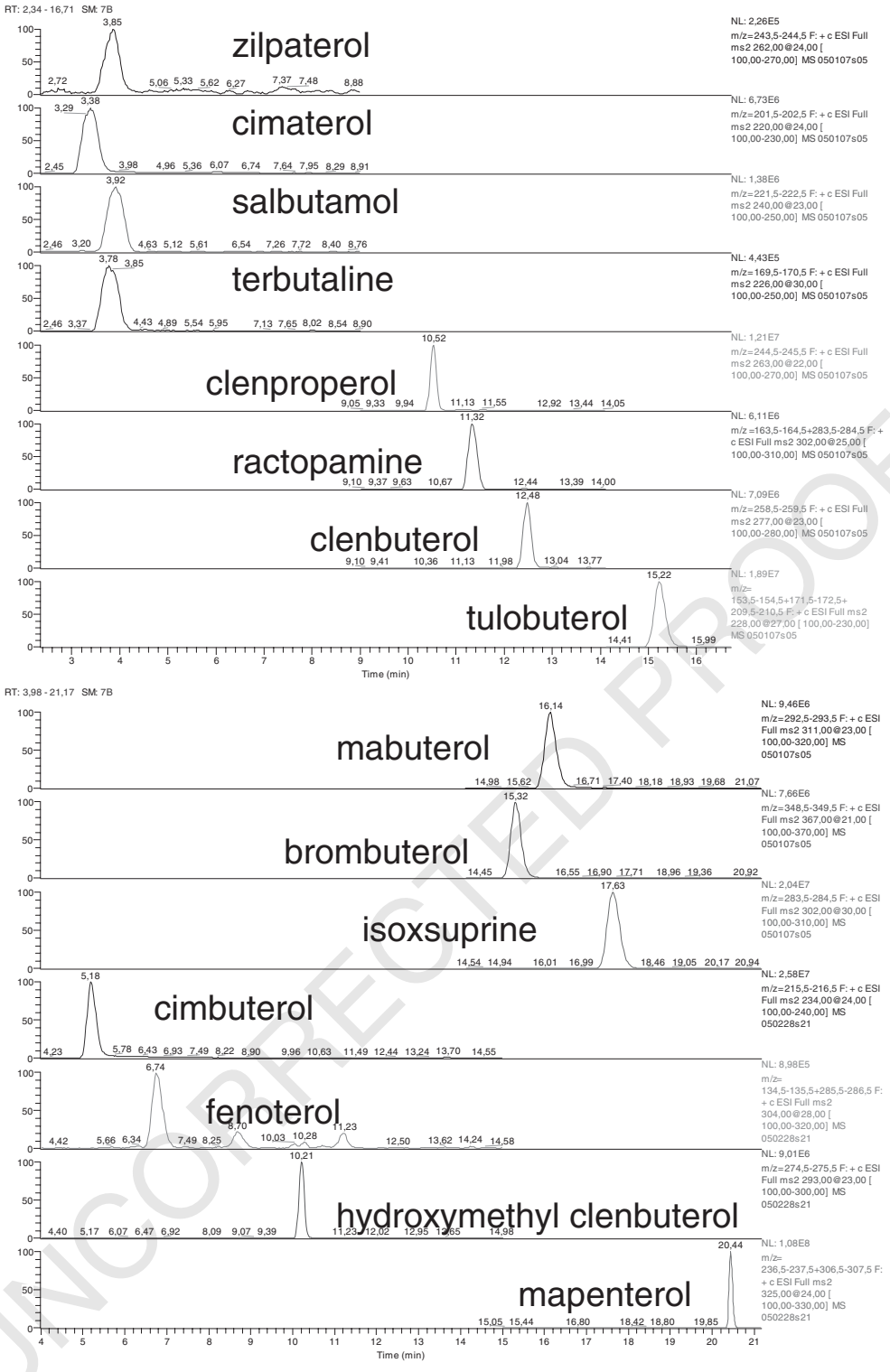


Figure 3. Extracted ion chromatograms for the different beta-agonists at their MRPL concentrations in calf urine using MIP clean-up (no glucuronidase treatment).

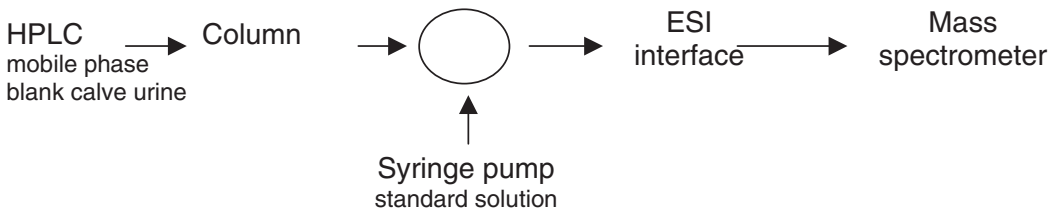


Figure 4. Post-column infusion system used to test for ionisation suppression.

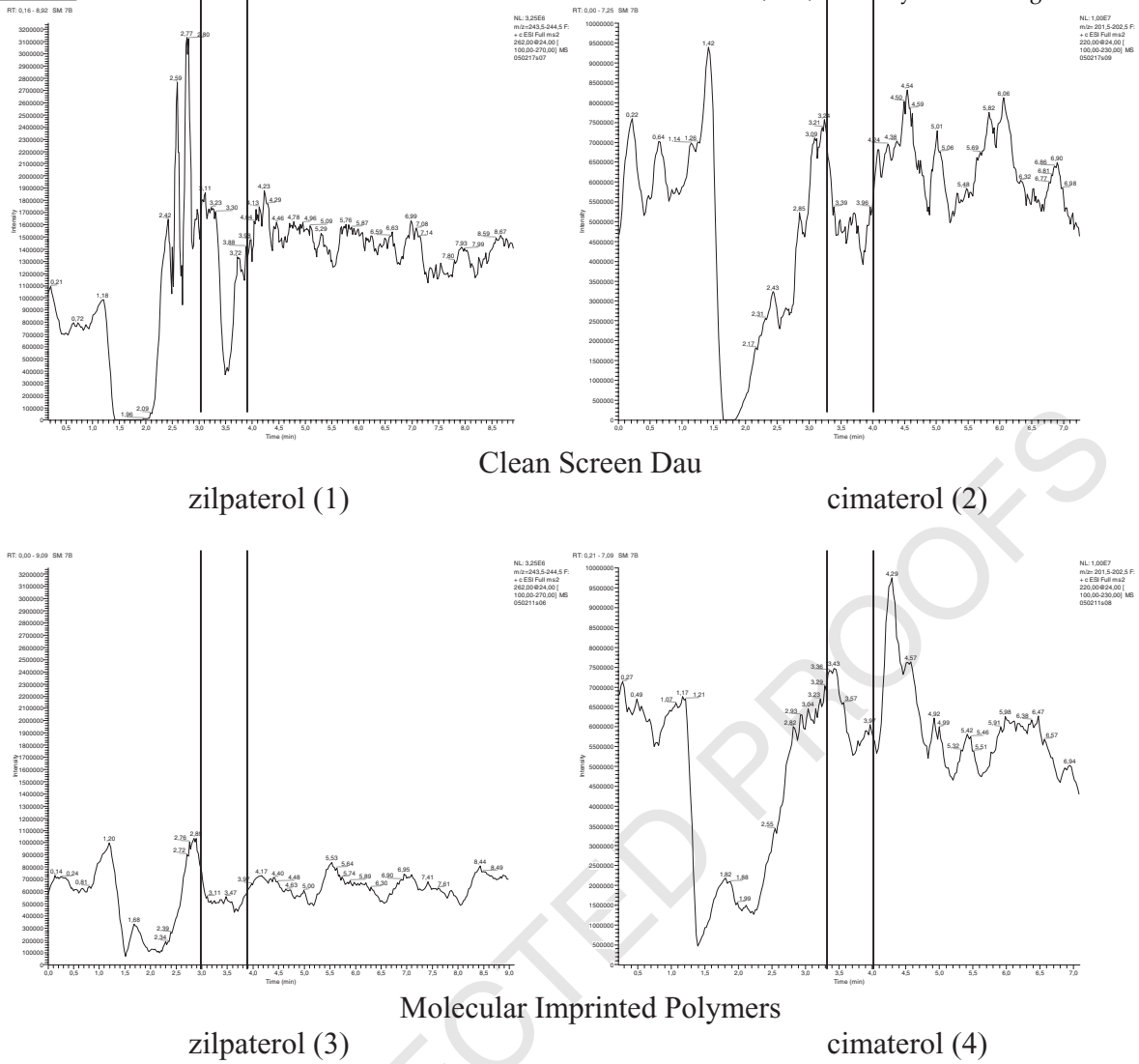


Figure 5. MS/MS signals for zilpaterol (1&3) and cimaterol (2&4) detected using the apparatus shown in Fig. 4, using continuous infusion of zilpaterol or cimaterol and LC injection of blank calf urine samples after clean-up using either CSD (1&2) or MIP (3&4) columns.

problems concerning sensitivity of the multi-residue method. Also, purchase of multiple isotope-labeled internal standards can be expensive for a purely qualitative method.

The percentages of ion suppression for the different beta-agonists are reported in Table 4 as percentages of the expected signal. They were calculated by analysing five post-extraction spiked samples and five pure standards, and calculating the ratio between the two values. The concentrations of the beta-agonists added to the blank urine samples were equal to those present in the standard solutions. If the signal is not suppressed, the percentage of the expected signal is 100%. The values obtained experimentally (Table 4) indicate that clean-up using MIP columns is more selective than that using CSD columns for many beta-agonists (zilpaterol, terbutaline, ractopamine, clenbuterol, brombuterol, isoxsuprine, cimbuterol, mapenterol and hydroxymethyl clenbuterol). Only the beta-agonists clenproperol and fenoterol gave a higher percentage of the expected signal after clean-up with CSD, but even in those cases the percentages after clean-up with MIP are satisfactory.

Table 4. Percentage of the expected signal as an indicator of the percentage of ion suppression

	% of the expected signal	
Analyte	CSD	MIP
Zilpaterol	49 ± 0.63	89 ± 2.40
Cimaterol	112 ± 0.50	111 ± 9.65
Salbutamol	107 ± 0.37	109 ± 4.70
Terbutaline	47 ± 1.18	88 ± 2.18
Clenproperol	123 ± 0.72	75 ± 1.39
Tulobuterol	136 ± 0.80	101 ± 4.59
Ractopamine	40 ± 0.20	80 ± 3.38
Clenbuterol	56 ± 0.34	76 ± 1.62
Mabuterol	85 ± 0.61	107 ± 3.15
Brombuterol	46 ± 1.25	107 ± 4.04
Isoxsuprine	61 ± 1.00	104 ± 1.65
Cimbuterol	55 ± 0.17	102 ± 3.75
Mapenterol	51 ± 0.14	72 ± 0.58
Fenoterol	100 ± 0.95	77 ± 1.29
Hydroxymethyl clenbuterol	53 ± 0.14	81 ± 2.07

Based on the information received from MIP Technologies, the manufacturers of the MIP columns, there should be no ion suppression from the template; the template bleeding level is normally just a few ng/mL. However, the manufacturer did not reveal the nature of the template. A blank water sample was processed by the MIP method and analysed in full-scan mode to check for template bleeding, but no clear chromatographic peak or signal was obtained.

These experiments indicate that sample clean-up using MIP columns is more selective than that using CSD columns. As a result, sample clean-up can influence the repeatability of a method in routine analysis when different samples are run within the same session since the co-eluting interferences are not necessarily reproducible. Consequently, ion suppression experiments should be performed during method development to prevent problems regarding false negative results and problems regarding the repeatability.

Qualitative validation

The multi-residue method using MIP SPE columns presented in this paper is only a qualitative method. The following qualitative validation parameters were tested: specificity, selectivity, decision limit (CCα) and detection capability (CCβ).

The specificity of the method was demonstrated by LC/MS^2 and LC/MS^3 analyses of blank urine (at least 20 blank urine samples were analysed); no interferences were observed in analysis of these blank samples and in analysis of urine spiked with the different beta-agonists.

The minimum number of identification points (IPs) for beta-agonists is set to four. Table 2 shows the MS^2 and MS^3 product ions needed for the identification of each beta-agonist. Most beta-agonists only have one MS^2 product ion (so 2.5 IPs are earned), therefore MS^3 fragmentation is necessary to obtain enough IPs.

The CCβ of each beta-agonist is equal to or lower than the MRPL concentrations, i.e., 1 $\mu g\,kg^{-1}$ for clenbuterol, brombuterol, hydroxymethylclenbuterol, mabuterol, mapenterol, tulobuterol and zilpaterol, and 3 $\mu g\,kg^{-1}$ for cimaterol, cimbuterol, clenproperol, isoxsuprine, fenoterol, ractopamine, salbutamol and terbutaline. The CCα was calculated by subtracting 1.64 times the maximum standard deviation of the CCβ-value. For the calculation of CCα, the maximum standard deviation was derived from the maximum coefficient of variation of 25% ($CC\alpha \leq 0.59$ when $CC\beta \leq 1$ and $CC\alpha \leq 1.77$ when $CC\beta \leq 3$). Additional experiments will be necessary to obtain the standard deviation for each beta-agonist.

For purposes of quantification, the clean-up needs to be optimised for the different beta-agonists in order to be able to obtain reproducible results; also hydrolysis of the spiked and real urine samples is necessary, and more than one internal standard should be added to the method since some of the beta-agonists have rather different chemical structures.

CONCLUSIONS

A multi-residue method was developed for the detection of beta-agonists in urine. Two different SPE clean-up steps were evaluated, using either clean screen Dau (CSD) or molecular imprinted polymer (MIP) columns. Ion suppression experiments revealed that CSD sample clean-up could lead to false negative results for some beta-agonists; the percentages of the expected signal actually observed show that there is less suppression of the signals when urine is pretreated with MIP columns, i.e., clean-up using MIP columns is more selective than that using CSD columns.

A qualitative validation was performed using MIP clean-up; at this point only a qualitative determination/identification of the different beta-agonists is possible. Before quantification can be done, suitable internal standards need to be added to the method and the clean-up needs to be optimised to obtain reproducible results.

This study has shown that MIPs are very promising for sample clean-up for these beta-agonists, but further research is necessary before they can be incorporated into fully validated quantitative assays.

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