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Detection of zilpaterol (Zilmax®) in calf urine and faeces with liquid chromatography–tandem mass spectrometry

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Abstract

Zilpaterol is a new powerful beta-agonist, which is officially registered for fattening purposes in cattle in Mexico and South Africa. Its chemical structure is different from the well-known beta-agonists. Therefore, the routinely used screening methods are not likely to be suited for the analysis of zilpaterol. Also gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry (LC–MS) methods need to be adapted to enable detection of zilpaterol.

In this study, a LC–MS^3 confirmatory method was developed for the simultaneous detection of zilpaterol and di-aromatic beta-agonists in urine samples. A LC–MS^2 method was optimised for the detection of zilpaterol in faeces. To study the excretion profile in urine and faeces, a male veal calf was orally treated with daily doses of Zilmax® during 2 weeks. Zilpaterol was mainly excreted via urine.

Keywords: Zilpaterol; Beta-agonists; LC–MS^n; Excretion profile

1. Introduction

Beta-agonists belong to the group of catecholamines. They can be catalogued in three chemical classes according to their structure: anilines, resorcinols and phenols.

Beta-agonists can bind to beta-2-receptors. Stimulation of these receptors results in relaxation of smooth muscles. Beta-agonists are therefore frequently used as bronchodilator for the treatment of pulmonary diseases in humans and animals. In addition, they also improve carcass composition as they decrease fat in favour of a higher percentage of muscle (repartitioners) [1]. In the European Union, the use of beta-agonists as growth promoters is banned by the council directive 96/23/EC [2].

The growth-promoting effect of beta-agonists in slaughter animals (increase of live weight gain, improvement of feed conversion and increase of the ratio of muscle to fat tissue) is of economical importance. The treated animal shows these (side) effects when the applied dose is five to ten times higher than necessary for therapeutic treatment [1]. The danger of residues in edible animal matrices due to the abuse of beta-agonists is underscored by several human poisoning incidences [3]. The most notable incident occurred in Spain in 1990. There was an outbreak of food poisoning caused by consumption of bovine liver from animals treated with clenbuterol [4].

Zilpaterol (Fig. 1) is a new powerful beta-adrenergic agonist developed as growth promoter for cattle. Its chemical structure is different from the well-known *N*-alkyl-beta-agonists (clenbuterol and salbuterol) as well as the di-aromatic beta-agonists (ractopamine and isoxsuprine).

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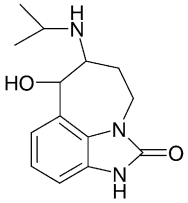

Fig. 1. Chemical structure of zilpaterol.

The efficacy as growth promoter has been sparsely published. Some results from feeding studies demonstrated an additional benefit in combination with an estradiol + trenbolone treatment [5]. Zilpaterol is capable of redirecting the cellular metabolism in favour of protein synthesis. Zilpaterol is used as a 'repartitioning agent' in cattle feed to enhance weight gain, feed efficiency and carcass yield. Zilmax® has been licensed as feed additive in Mexico and South Africa. The dossier has been submitted to the FDA for approval.

Due to its chemical structure, zilpaterol was, until recently, not detected with commonly applied screening and confirmatory methods [6–9].

In this study, a LC–MS^3 confirmatory method was developed for urine that is able to identify simultaneously zilpaterol, ractopamine, isoxsuprine and other di-aromatic beta-agonists. For faeces, an LC–MS^2 method was optimised for detection of zilpaterol and cimaterol (used as internal standard). To study the excretion profile, a male veal calf was orally treated with therapeutic (for growth-promoting purposes) daily doses of Zilmax® during 2 weeks [5]. During this period urine and faeces samples were collected. Without a withdrawal period, the animal was sacrificed and different matrices were collected for later research.

2. Experimental

2.1. Reagents and chemicals

Chemicals and solvents were obtained from Merck (Darmstadt, Germany) and Biosolve (Volkenswaard, The Netherlands). The enzymatic deconjugation was performed with Helix Pomatia juice (β-glucuronidase > 100,000 FU ml^{-1} and sulphatase > 1000,000 FU ml^{-1}) from Bioserpa (Marlborough, MA). Standards and internal standards were obtained from Sigma (St Louis, MO) or RIVM (Bilthoven, The Netherlands). Zilmax and zilpaterol were gifts from Intervet (Schwabenheim, Germany).

2.2. Animal experiment

For the animal experiment, a male veal calf (3–4 months, ± 162 kg) was orally treated with recommended (for growth-promoting purposes) daily doses of Zilmax® during 2 weeks. The dose given was 0.15 mg zilpaterol per kg bodyweight per day, which is equal to 3.13 mg Zilmax® per kg per day. During this period, urine and faeces samples were taken. Without a withdrawal period, the animal was sacrificed and different samples (urine, liver, kidney, muscle, etc.) were collected for later research.

2.3. Extraction and clean-up

To the urine samples (0.5–5 ml) D_5-isoxsuprine and D_5-ractopamine were added as internal standards (5 ng ml^{-1}). The urine was hydrolysed with Helix Pomatia at 37 °C for 16 h. After adjustment of the pH to 9.6, the analytes were extracted with 10 ml isobutanol. After centrifugation (10 min, 2000 rpm, 4 °C) and evaporation under nitrogen, the residue was dissolved in 2 ml of phosphate buffer (pH 6). The clean up was carried out using a 130 mg BondElut Certify (mixed C_8 and SCX) SPE column (Varian Inc.). The column was conditioned with methanol, water and 0.1 mol l^{-1} of phosphate buffer (pH 6). The columns were washed subsequently with 1 mol l^{-1} of acetic acid and methanol. Elution was carried out using 3 ml of ethylacetate containing ammonia (0.57 mol l^{-1}). Following evaporation of the solvents, the residue was dissolved in 150 μl of methanol:water (5:95, v/v) with 10 mmol l^{-1} ammonium acetate and 50 μl were injected on the column.

For faeces 1 g cimaterol was added as internal standard at a level of 100 ng g^{-1}. After addition of 40 ml of hydrochloric acid 2 mol l^{-1}, the sample was shaken for 15 min. After centrifugation (15 min, 3600 rpm, 5 °C), 20 ml of the extract was decanted in a new centrifuge tube. One millilitre of carbonate buffer (10%, pH 9.8) was added and the pH was adjusted to 9.8 using sodium hydroxide (32%, 5N). The extract was shaken for 1 min and after centrifugation (15 min, 3600 rpm, 5 °C) 9 ml of the upper layer was applied on a Chem Elut column (Varian Inc.). Elution was carried out using 40 ml of diethyl ether. A volume of 500 μl of pentafluorpropionic acid (PFPA) (0.3 mol l^{-1}) was added to the tube. The sample was placed in an ultrasonic bath for 10 min. After centrifugation for 15 min, the lower layer of PFPA containing the analyte was formed. Approximately 400 μl of the drop was taken up with a syringe and brought into a pyrex tube. The pyrex tube was placed into a water bath for 5 min and later on taken over in a vial for injection into the LC–MS system.

2.4. Instrumentation and chromatographic conditions

For urine samples, chromatographic separation was achieved using an Inertsil ODS C_{18} column (3 μm, 3.0 mm × 100 mm, Varian Inc.). To separate the different compounds, a linear gradient was used using a mixture of water and methanol with ammonium acetate (Table 1). The flow rate was 0.6 ml min^{-1}. The mass spectrometer was operated in MS^3-mode operating in five segments. Each analyte was evaluated based on the productions present in the mass spectra (Table 2).

For faeces samples chromatographic separation was achieved using an Alltima C_{18} column (5 μm, 250 mm ×

Table 1
Mobile phase and gradient used to separate the non-*N*-alkyl beta-agonists

Time (min)	Methanol:water (5:95, v/v) + 10 mM ammonium acetate	Methanol:water (80:20, v/v) + 30 mM ammonium acetate
0	100	0
1	100	0
15	0	100
20	0	100
22	100	0
25	100	0

3.2 mm, Alltech Associates). The mobile phase consisted of a mixture of pentafluorpropionic acid 10 mM (87%) and acetonitrile (13%). This mobile phase was pumped at a rate of 0.5 ml min^{-1} for 4 min. The mass spectrometer was operated in MS^2-mode.

In both experiments, an 1100 series quaternary pump and an autosampler from Hewlett-Packard (Palo Alto, CA, USA) were used. The MS detector was a ThermoFinnigan LCQ ion trap MS (San José, CA, USA) equipped with an atmospheric pressure chemical ionisation (APCI) interface in positive ion mode.

3. Results and discussion

3.1. Chemical structure of zilpaterol

In evaluating the chemical structure of zilpaterol, the LC–MS^n mass spectra in APCI positive mode were recorded. In MS-full scan, the pseudo-molecular ion with *m*/*z* 262 appeared but also a fragment with *m*/*z* 244 (Fig. 2). This fragment was due to the loss of water (Fig. 5). MS^2-full scan of the pseudo-molecular ion only showed the product ion with *m*/*z* 244 (Fig. 3). Fragmentation of this product ion gave rise to two fragments, one with *m*/*z* 202 and the other with *m*/*z* 185 (Fig. 4). The fragment with *m*/*z* 202 was due to the loss of CH_3CCH_3, a subsequent loss of NH_3 led to the fragment with *m*/*z* 185 (Fig. 5).

3.2. The use of LC–MS^3

The use of beta-agonists as growth promoter is forbidden in the European Union. Beta-agonists are therefore group A substances. The minimum number of identification points (IP) for such forbidden compounds is set to four [2]. LC–MS^n precursor ions earn 1 IP and LC–MS^n product ions earn 1.5 IP. MS^2-full scan of the pseudo-molecular ion only showed one ion with *m*/*z* 244 (Fig. 3). So 2.5 IP (one precursor ion and one product ion) were earned when using MS^2-full scan of zilpaterol. Moreover, the fragmentation of beta-agonists in MS^2-full scan is not specific (loss of water). To create more specificity and to get enough identification points, MS^3-full scan of the product ion was checked. The mass spectrum of MS^3-full scan of the product ion with *m*/*z* 244 showed the ions with *m*/*z* 185 and *m*/*z* 202 (Fig. 4). This led to 5.5 IP (one precursor ion, one product ion and 2 second transition product ions).

Therefore, zilpaterol and the other di-aromatic beta-agonists were identified in urine samples using LC–MS^3 analysis.

3.3. Method for different beta-agonists

The standards zilpaterol, ritodrine, ractopamine, formoterol, isoxsuprine and the two internal standards D_5-ractopamine and D_5-isoxsuprine were spiked to blank calf urine in a concentration of 1 μg l^{-1}. Fig. 6 shows the ion chromatograms of the different beta-agonists. All the beta-agonists could be detected at a level of 1 μg l^{-1} with exception of formoterol which was only detectable at 5 μg l^{-1}. The two chromatographic peaks of D_5-isoxsuprine were two possible isomers of the molecule. Levels of zilpaterol, ritodrine and ractopamine were calculated using D_5-ractopamine as internal standard. Levels of formoterol and isoxsuprine were calculated using D_5-isoxsuprine.

For faeces a LC–MS^2 method was optimised for the detection of zilpaterol. The goal was to study the excretion profile of zilpaterol in faeces samples and not to develop a confirmatory method for beta-agonists in faeces. Therefore, MS^2 fragmentation of zilpaterol was enough since the identity of the compound in the faeces samples was well known in this experiment. In addition, no deuterated internal standard was used. For faeces a level of 1 μg kg^{-1} zilpaterol could be detected.

Both methods were developed in two different laboratories, therefore different chromatographic conditions and different internal standards were used to analyse zilpaterol. For urine, zilpaterol was incorporated in the qualitative multi-residue method for the detection of di-aromatic beta-

Table 2
Instrument method for the detection of beta-agonists in urine samples and the productions used for the evaluation of the beta-agonists

			Analyte	Productions
Segment 1	Scan event 1	262.0 → 244.0; 65.0–265.0	Zipaterol	202, 185
Segment 2	Scan event 1	288.0 → 270.0; 70.0–290.0	Ritodrine	150, 121
Segment 3	Scan event 1	302.0 → 284.0; 75.0–305.0	Ractopamine	164, 121
	Scan event 2	307.0 → 289.0; 75.0–310.0	D_5-ractopamine	167, 121
Segment 4	Scan event 1	345.0 → 327.0; 90.0–350.0	Formoterol	149, 121
Segment 5	Scan event 1	302.0 → 284.0; 75.0–305.0	Isoxsuprine	190, 150
	Scan event 2	307.0 → 289.0; 75.0–310.0	D_5-isoxsuprine	190, 150

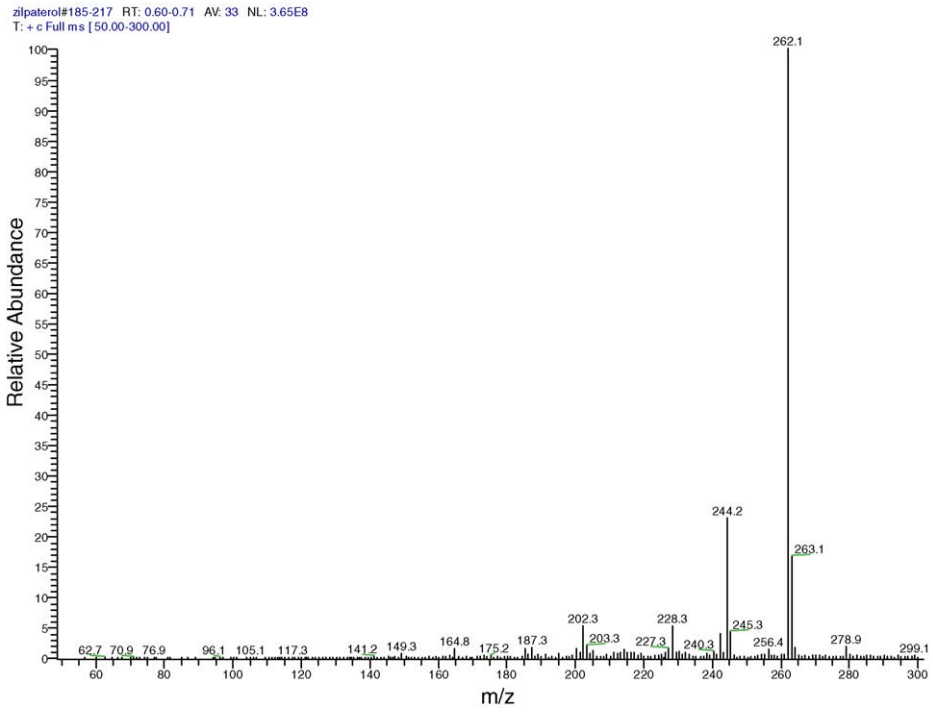

Fig. 2. MS-full scan of zilpaterol.

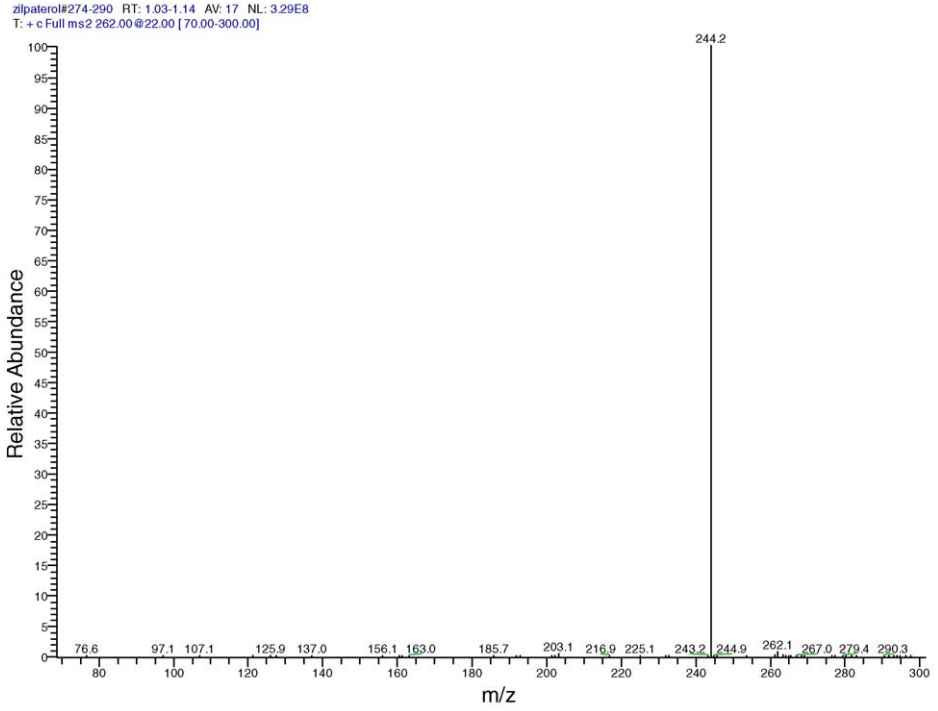

Fig. 3. MS^2-full scan of zilpaterol.

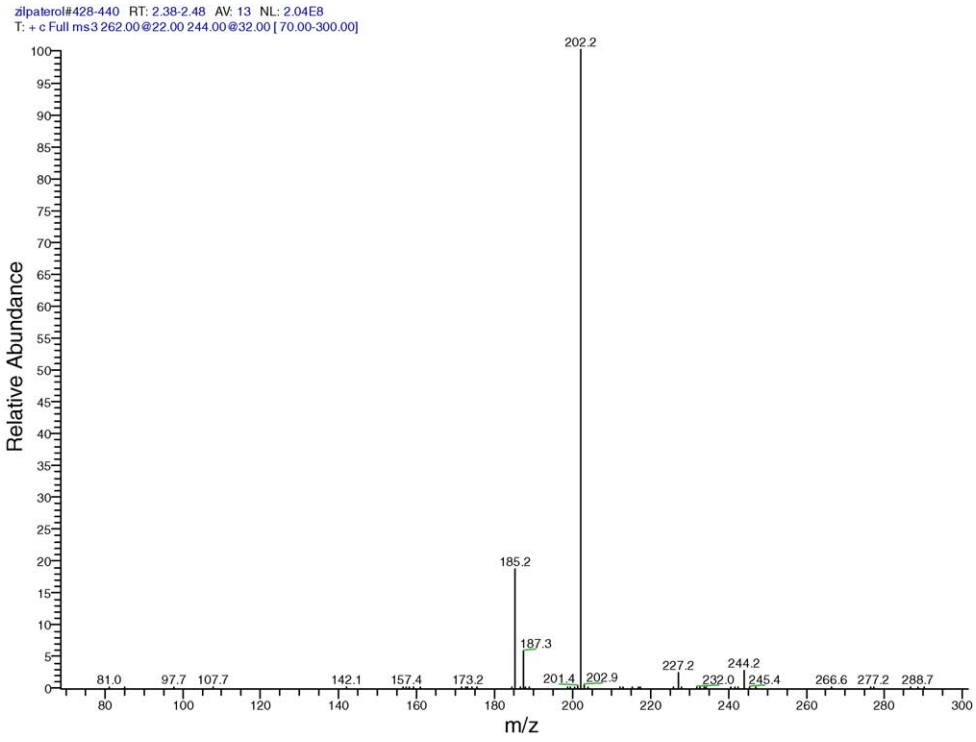

Fig. 4. MS^3-full scan of zilpaterol.

agonists. For faeces no attempts were made to incorporate zilpaterol in the existing method for the detection of beta-agonists and optimise this method, since the goal was to study the excretion profile of zilpaterol in faeces and not to develop a qualitative multi-residue method for beta-agonists in faeces. Cimaterol was chosen as internal standard because of its good response and good reproducibility. Since both methods were developed independent of each other, no attempts were made to extract and analyse di-aromatic beta-agonists from faeces and cimaterol from urine.

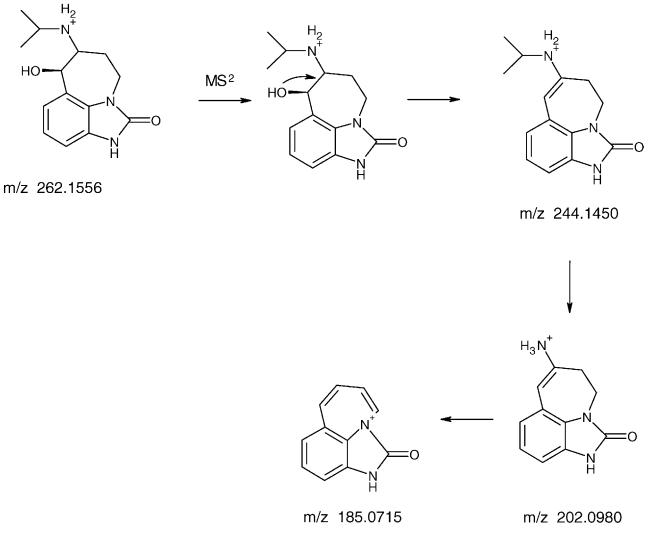

Fig. 5. Fragmentation of the beta-agonist zilpaterol.

3.4. Excretion profile

A male calf was orally treated with 0.15 mg zilpaterol per kg bodyweight per day (3.13 mg Zilmax®). Each day urine and fecal sample was collected and the calf was sacrificed after 14 days.

Fig. 7 shows the excretion profile of zilpaterol in urine and faeces. The levels of zilpaterol in the urine samples were relatively high. Already after 2 days the concentration of zilpaterol exceeded 1000 $\mu g\,l^{-1}$. A steady-state concentration of about 1200 $\mu g\,l^{-1}$ was quickly reached. Also in faeces, a steady-state concentration of 83 $\mu g\,kg^{-1}$ was quickly reached (first measurement was already 71 $\mu g\,kg^{-1}$ on day 2) (Fig. 7). A minimum value of 49 $\mu g\,kg^{-1}$ was detected on day 8, after 5 days a maximum value of 126 $\mu g\,kg^{-1}$ was reached. It could be concluded that zilpaterol was mainly excreted via urine.

As the animal was sacrificed after the last treatment, no data were available for the final elimination of zilpaterol. Based on the results, the assumption could be made that detection of zilpaterol in urine and faeces, after application as a feed additive, could be easily achieved.

3.5. Phase I metabolites of zilpaterol

To study the presence of any co-extracted metabolites of zilpaterol in urine, also MS-full scan analysis of a number of urine samples (days 2, 4, 8 and 12) was performed. The multi-residue method of di-aromatic beta-agonists in urine was used

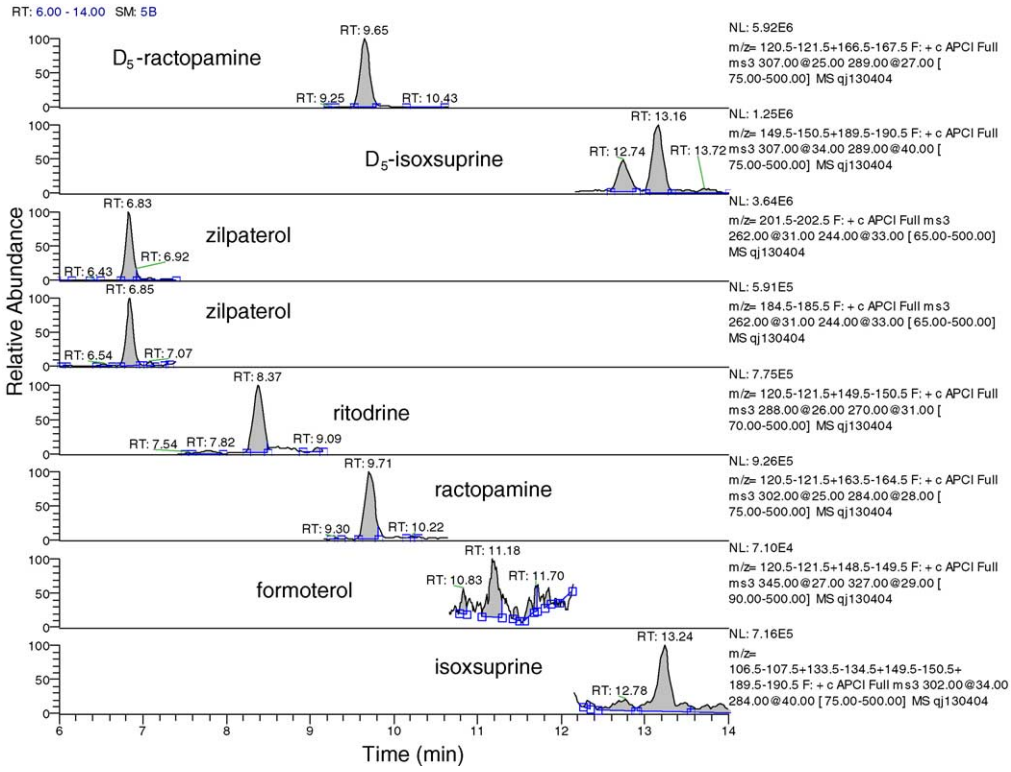

Fig. 6. Ion chromatograms of D_5-ractopamine, D_5-isoxsuprine, zilpaterol, zilpaterol, ritodrine, ractopamine, formoterol and isoxsuprine of a spiked calf urine sample (1 $\mu g\,l^{-1}$).

to analyse possible co-extracted metabolites of zilpaterol. In each sample, a de-isopropyl metabolite was found. Fig. 8 shows the chromatograms of zilpaterol and its de-isopropyl metabolite and Fig. 9 shows MS-full scan of de-isopropyl zilpaterol. The pseudo-molecular ion of de-isopropyl zilpaterol was *m*/*z* 220.

The amount of de-isopropyl zilpaterol was calculated compared to the concentration of zilpaterol in each sample. The ratio de-isopropyl zilpaterol/zilpaterol ranged between 2.45% and 5.66%.

3.6. Phase II metabolites of zilpaterol

To study the phase II metabolites of zilpaterol, MS-full scan analysis of two urine samples and MS^3-full scan analysis of 10 urine samples with and without initial hydrolysis were performed. The extraction of zilpaterol and its possible second phase metabolites was based on cationic exchange between the SCX sorbent present in the BondElut certify SPE columns and the positively charged ammonium groups present in zilpaterol (Fig. 1). No standards of conjugates of zilpaterol were available to test if the described method is suitable for the extraction of possible second phase metabolites. Zilpaterol had a pseudo-molecular ion of *m*/*z* 262 in positive mode, so a glucuronide-conjugate would show an ion *m*/*z* 438 and a sulphate-conjugate would show an ion *m*/*z* 359 in MS-full scan [10]. Fig. 10 shows the different chromatograms and mass spectra of zilpaterol and its possible conjugates. The LC–MS method of di-aromatic beta-agonists in urine was used to analyse these possible phase II metabolites. Some minor adaptions in the gradient were performed; therefore zilpaterol elutes more rapidely compared to Fig. 6. Zilpaterol was mainly excreted unconjugated in urine. No glucuronide-conjugates were detected. The chromatographic peak at 26 min revealed the ion *m*/*z* 359 and its Na-adduct *m*/*z* 381, indicating the presence of a sulphate-conjugate of zilpaterol. Although, the formation of a glucuronide or sulphate-conjugate would create a more hydrophilic behaviour of the compound, a later retention time of the possible sulphate-conjugate of zilpaterol was observed compared to the retention time of the parent compound zilpaterol. Mabuchi et al. (2004) described the retention behaviour of morphine and its glucuronide-conjugate on a C_{18} analytical

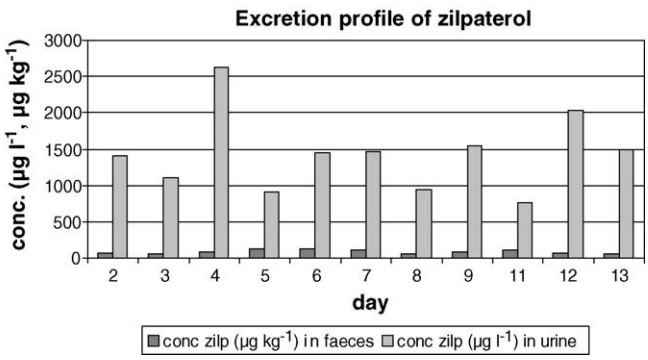

Fig. 7. Excretion profile of zilpaterol in urine and faeces.

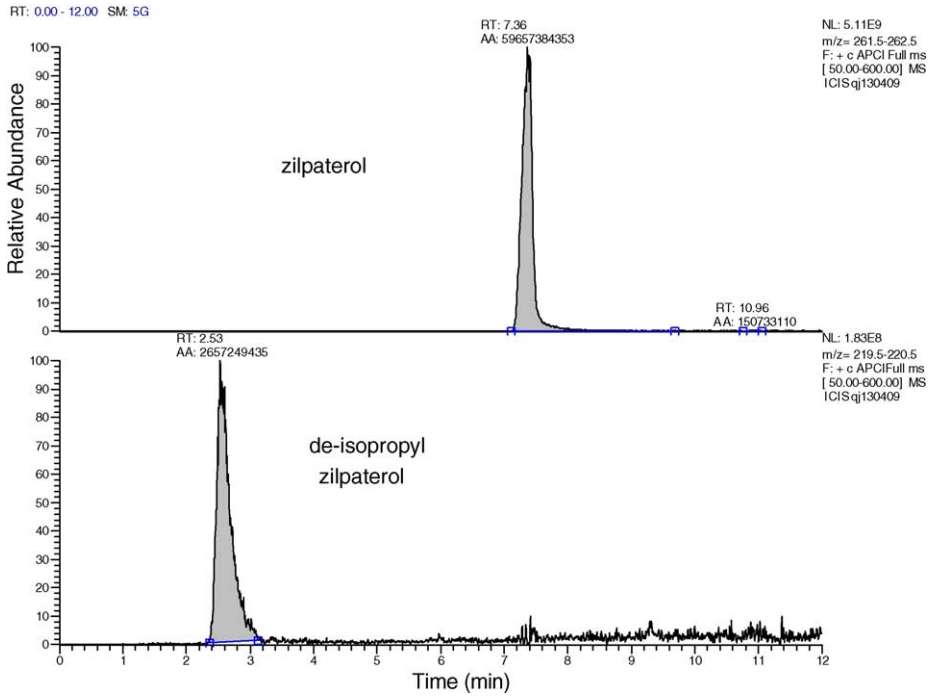

Fig. 8. Chromatograms of zilpaterol and its de-isopropyl metabolite.

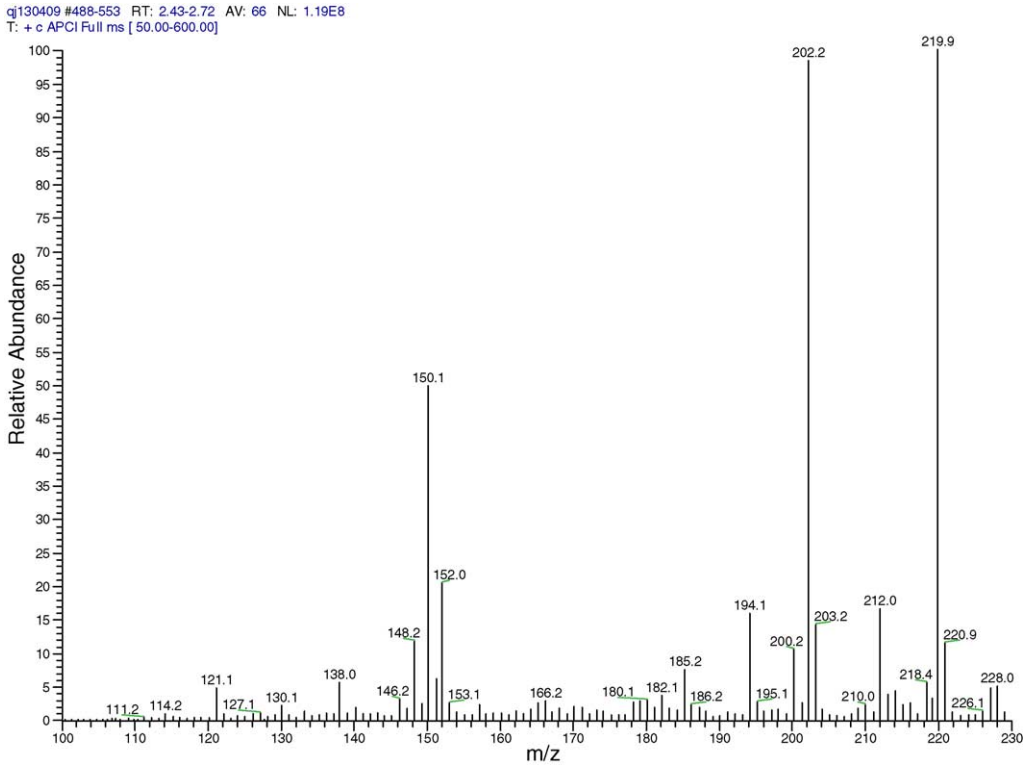

Fig. 9. MS-full scan of the de-isopropyl metabolite of zilpaterol.

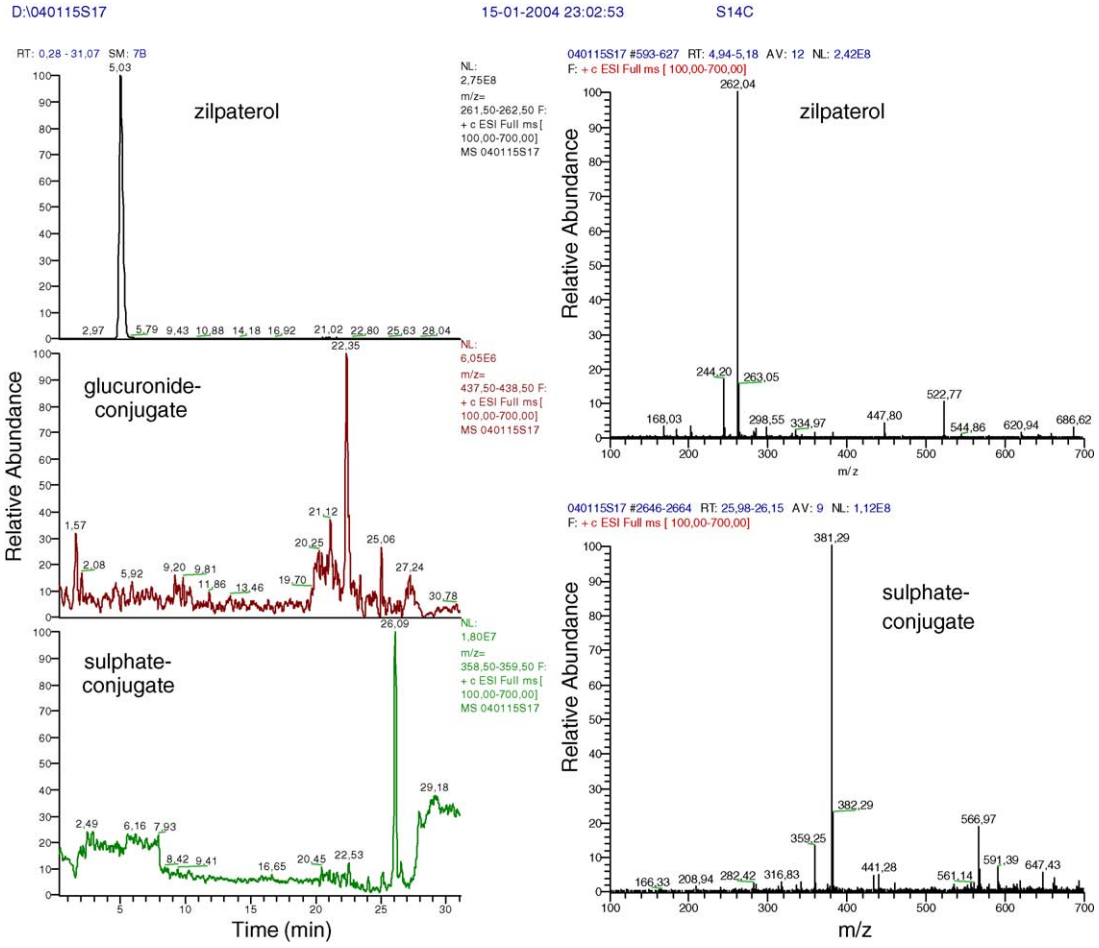

Fig. 10. Chromatograms and mass spectra of zilpaterol and its conjugates.

column. Morphine-3-glucuronide eluted at a later retention time than the parent compound morphine [11]. So not only the hydrophilic character of a compound, but also other characteristics, such as molecular weight will influence the retention time of an analyte. Since there are no standards of the conjugates of zilpaterol, these observations could not be confirmed. On the other hand 10 urine samples taken on the same day were analysed with and without initial hydrolysis. The average of the area's ratio (area of the chromatographic peak of zilpaterol divided by the area of the internal standard) of the five replicates with and without hydrolysis were compared. There was a minor increase in area's ratio of the samples with hydrolysis, indicating the presence of conjugated zilpaterol in urine. Since the area's ratio of the samples without hydrolysis was already significant, it can be concluded that zilpaterol was mainly excreted unconjugated in urine. These experiments only give an indication about the phase II metabolites of zilpaterol, since no conjugated standards are available to confirm these observations. Further research concerning the phase II metabolites of zilpaterol will be necessary. Previous studies revealed that other beta-agonists (clenbuterol and salbutamol) were mainly excreted as glucuronide-conjugates [12]. So it is important to add glucuronidase/sulphatase to urine samples to convert the conjugates of all beta-agonists.

3.7. Quantification

Although the LC–MS^3 method for the detection of beta-agonists in urine is primarily a qualitative method, some quantitative data were examined. Three series, each containing two blank urine samples spiked at a concentration of 1 $\mu g\,l^{-1}$ were analysed at three different days. Table 3 shows the calculated concentrations of zilpaterol, ritodrine, ractopamine, formoterol and isoxsuprine.

The detection limit (CCβ) is the smallest content of a compound that may be detected and identified with an error probability of β. Fig. 6 shows that the CCβ of zilpaterol, ritodrine, ractopamine and isoxsuprine was lower or equal to 1 $\mu g\,kg^{-1}$. For formoterol, the CCβ was lower or equal to 5 $\mu g\,kg^{-1}$.

For samples spiked at a concentration of 1 $\mu g\,l^{-1}$, the accuracy should range from 50% to 120% [2]. The accuracies of all beta-agonists lay within this acceptable range, except for

Table 3
Quantitative data for zilpaterol, ritodrine, ractopamine, formoterol and isoxsuprine analysed with the LC–MS^3 method for beta-agonists in urine

	Concentration ($\mu g\,l^{-1}$)				
	Zilpaterol	Ritodrine	Ractopamine	Formoterol	Isoxsuprine
Day 1	0.92	0.92	1.12	0.72	0.97
	1.04	0.95	1.09	0.48	0.82
Day 2	0.90	0.82	1.02	1.02	1.07
	1.07	1.19	1.01	1.02	1.03
Day 3	1.00	0.99	1.04	1.09	0.94
	1.06	1.05	1.06	0.82	1.04
Average	1.00	0.99	1.05	0.86	0.98
S.D.	0.07	0.13	0.04	0.23	0.09
CV (%)	7.1	13.1	3.8	27	9.5

one analysis of formoterol. Fig. 6 shows that formoterol is not well detectable at $1\,\mu g\,l^{-1}$, so quantification for formoterol should be done at $5\,\mu g\,l^{-1}$, like already mentioned above. The precision of this method was evaluated by the coefficients of variation (CV), which should not exceed the level calculated by the Horwitz equation [2]. For mass fractions lower than $100\,\mu g\,l^{-1}$, the application of the Horwitz equation gave unacceptable high values. Therefore, the CV should be as low as possible; 23% (CV at $100\,\mu g\,l^{-1}$ = 23%) was taken as a guideline. All the coefficients of variation were lower than 14% except for formoterol (27%). So these quantitative data for the LC–MS^3 method for the detection of beta-agonists in urine were very good, even though this method was primarily a qualitative method.

4. Conclusion

A LC–MS^3 confirmatory method was developed that was able to simultaneously identify zilpaterol, ractopamine, isoxsuprine and other di-aromatic beta-agonists in calf urine at a level of $1\,\mu g\,l^{-1}$. For faeces, a LC–MS^2 method was optimised for the detection of zilpaterol in this experiment.

When Zilmax® was administered orally to a male veal calf, the detection of zilpaterol in urine and faeces could be easily achieved. Zilpaterol was mainly excreted via urine.

The method described for urine samples is used in routine control since 2001 within the framework of self-control of veal calves in The Netherlands in order to extend the scope of beta-agonist screened. So far no positive samples were found in this exclusive approach.

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