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Determination of a method for detecting and quantifying azaperone, azaperol and carazolol in pig tissues by liquid chromatography–tandem mass spectrometry

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Abstract

A quick, simple method for quantifying carazolol, azaperol and azaperone is described. Liquid extraction was followed by a clean-up on an Oasis® SPE cartridge. The analytes were separated by HPLC and analysed by MS–MS with atmospheric pressure chemical ionisation in the positive mode. The method was applied to muscle and kidney from untreated pigs, the samples being spiked with the three molecules of interest. Recovery was between 70 and 106%. Quantification parameters were also good: the accuracy was between 80 and 110% and the coefficient of variation did not exceed 16%, being below 8% for 90% of the samples. Linearity was good from MRL/4 to 2MRL. For unequivocal identification of each analyte, four ions were detected. The method proved very suitable for routine analysis.

Keywords: Azaperone; Azaperol; Carazolol

1. Introduction

Over the last decades, intensive farming has made it possible to increase the meat production capacity at a lower cost per head. These improvements have had many drawbacks, such as increased vulnerability to diseases and stress. Swine, principally of the Landrace and Pietrain breeds, are particularly sensitive to stress.

The stress factor causes high mortality rates, notably during transport of swine from the farm to the slaughterhouse. Furthermore, stressed pigs yield meat of poor quality called PSE (pale soft exudative). Stress can thus cause non-negligible financial losses for pig farmers, who are therefore led to use medicinal preparations to fight stress and its effects.

One frequently used medicine is Stresnil®, of which azaperone is the active molecule. It acts similarly to aminobutyric acid, causing aggressiveness and motor activity to decrease. Azaperone is metabolised principally to azaperol.

Suacron® is another medicine used in this context. It contains carazolol, an inhibitor of β-adrenergic receptors. It is used to control tachycardia due to hyperactivity of the sympathetic system. Eating meat containing high levels of these substances can be harmful to consumer health. This is why the EU has set maximum residue limits (MRLs) for azaperone and carazalol [1]. The MRLs of azaperone and azaperol are 100 μg/kg in kidney tissues and 50 μg/kg in muscle tissues. The MRLs of carazolol are

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lower: 25 μg/kg in kidney tissues and 5 μg/kg in muscle tissues.

In the framework of analysing animal tissues destined for human consumption, it is imperative to develop methods for identifying and quantifying unequivocally all substances for which an MRL exists. Several techniques have been used to analyse azaperone, azaperol and carazolol, notably TLC [2,3], EIA [4], HPLC–UV [5–8], HPLC–fluorimetry [9,10], and HPLC coupled to electrochemical detection [11].

The disadvantage of these techniques is a lack of information on the structure of the detected molecules. On the other hand, liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) yields structural data on the molecules detected. This technique is used to study metabolism [12–14]. Since it is also a sensitive method, we applied it to detecting carazolol, azaperone and azaperol in pig kidneys and muscle.

2. Materials and methods

2.1. Reagents

HPLC-grade acetonitrile (Acros Organics, Geel, Belgium), methanol (Acros Organics), and acetic acid (Baker Analyzed-HPLC Reagent, Deventer, The Netherlands) were used. Ethanol (Baker Analyzed Reagent) petroleum ether 40–65° (Acros Organics) and hexane (Baker Analyzed Reagent) were of analytical grade. Sodium hydroxide (Vel, Leuven, Belgium), ammonium hydroxide (Vel) ammonium acetate (Vel) and sodium tungstate (Merck, Darmstadt, Germany) were also of analytical grade. Synthesis-grade succinic anhydride (Merck) was used.

2.2. Reference compounds

Azaperol (batch V 8972-24) and azaperone (batch V 810-88) were provided by Janssen (Beerse, Belgium). Carazolol (batch 43839300) was purchased from Boehringer Mannheim (Mannheim, Germany) and haloperidol (batch 18H0408) from Sigma (St. Louis, MO, USA).

Stock solutions (1 mg/ml) were prepared in ethanol.

2.3. Instrumentation

The centrifuge (RC-3B Refrigerated Centrifuge) was from Sorvall Instruments (Wilmington, DE, USA). The stirring system (HS250 basic) was from Ika (Staufen, Germany).

The SPE columns were 6-cm^3 Oasis HLB columns (WAT 10622 02) and were purchased from Waters (Milford, USA). The HPLC chain was a Hewlett-Packard 1100 series (Waldbronn, Germany) and the mass spectrometer was a Quattro II (Micromass, Manchester, UK).

2.4. Tissue extraction (muscle and kidney)

Kidney and muscle samples from untreated animals were used as blanks. Each sample was spiked with 325 ng internal standard (I.S.; haloperidol) before extraction. To correct for extraction yield fluctuations, all results were adjusted according to the I.S. response. To 5 g homogenised tissue were added 17.5 ml succinate buffer (0.05 mol/l), 7.5 ml acetonitrile, and 10 ml petroleum ether. The pH of the succinate buffer was adjusted to ~4 by adding droplets of sodium hydroxide solution (5 mol/l). The mixture was stirred for 15 min and centrifuged for 10 min at 4700 *g* and 4°C. The petroleum ether was discarded and the remaining liquid phase recovered. Succinate–acetonitrile extraction was repeated and the two extracts were pooled. To the resulting extract was added 5 ml sodium tungstate solution (5% w/v). The mixture was allowed to rest for 1 h, then centrifuged at 4700 *g* for 10 min. The centrifuged extract was loaded onto an SPE column (Oasis HLB®) preconditioned with 10 ml methanol and 10 ml water. The column was washed with 10 ml water, 20 ml methanol–water (20:80 v/v), and 5 ml hexane. Elution was with 5 ml methanol. The extract was evaporated to dryness in a thermostated bath under a nitrogen flow. The dried extract was dissolved in 350 μl acetonitrile–water (20:80 v/v). A 70-μl aliquot of this solution was injected into the LC–MS–MS system.

2.5. HPLC conditions

The mobile phase consisted of two eluents. Eluent A was filtered HPLC-acetonitrile and eluent B was a

0.1 mol/l ammonium acetate solution. We used a two-linear-step elution gradient. The initial conditions, maintained for 1 min, were eluent A 100%, eluent B 0% (v/v). During the first gradient step (7 min), the percentage of eluent A was lowered to 30% and that of eluent B increased to 70% (v/v). During the second gradient step (maintained for 1 min), eluent A was set at 0%, eluent B at 100% (v/v). A 2-min post-run was used to return to the initial conditions.

The column was a Purospher model RP18 125×3 mm, 5 μm (Merck) equipped with a guard column Purospher RP18 (Merck) preceded by a Cat. 29230 Biomatrix column (Chrompack, Middleburg, The Netherlands) equipped with a Cat. 28128 pre-column (Chrompack). The columns were thermostated at 50°C.

2.6. Mass spectrometry conditions

The source used was an atmospheric pressure ionisation (API) model. Because the electrospray ionisation interface gave good intensity but unstable results, the APCI ionisation interface in positive mode was preferred. The negative mode was also tested but gave satisfactory results only for carazolol.

The probe temperature was 400°C and the source temperature 150°C. The drying gas flow-rate was 300 l/h and the sheath gas flow-rate was 120 l/h. The pressure in the collision cell was $2.4 \cdot 10^{-3}$ mbar. The photomultiplier was adjusted to 850 V. The data were collected in the multiple reaction monitoring mode (MRM), using a specific acceleration voltage and specific collision energy for each molecule.

2.7. Calibration

Azaperol, azaperone and carazolol were quantified by means of standard curves constructed from eight points spanning the concentration range from 0 ppb to twice the MRL. Haloperidol was used as the I.S.

Each standard solution was prepared by dissolving the desired amount of each substance (see below) and 325 ng haloperidol in ethanol. The solvent was then evaporated to dryness in a thermostated bath and under a nitrogen flow. The dry residue was redissolved in 350 μl acetonitrile–water (20:80, v/v).

For azaperol and azaperone, the following amounts were dissolved in ethanol: 0, 50, 125, 200, 250, 250, 375 and 500 ng for muscle tissue and 0, 125, 375, 500, 500, 750 and 1000 ng for kidney tissue. For carazolol, the following amounts were dissolved in ethanol: 0, 5, 12.5, 20, 25, 25, 37.5 and 50 ng for muscle tissue and 0, 37.5, 75, 112.5, 150, 225 and 300 ng for kidney tissue.

A 70-μl aliquot of each solution was injected into the LC–MS–MS system.

2.8. Chromatogram integration

As the calibration curves were prepared with pure standard solutions and not with extracted samples, a corrective factor was introduced into the integration procedure. Three blank samples, each spiked with all three analytes (in known amounts) and haloperidol (325 ng), were extracted, analysed by LC–MS–MS, and the responses compared with the corresponding calibration curve responses.

2.9. Validation of the method

Over 3 consecutive days, seven blank samples and nine spiked samples were extracted. Three samples were spiked with an amount corresponding to half the MRL, three with an amount corresponding to the MRL, and three with an amount corresponding to twice the MRL. Haloperidol (325 ng) was included in each spiked sample. All samples were injected twice into the LC–MS–MS system.

The following parameters were studied on the basis of the results obtained: the limit of detection (LOD), the limit of quantification (LOQ), the intra- and inter-day coefficients of variation, the accuracy and the extraction yield.

3. Results and discussion

3.1. Extraction

The liquid–solid extraction in itself posed no problems. The determining step was the solid-phase extraction (SPE), which depended on the method chosen to extract the substances. This step was carried out initially on columns of silica-bound C_{18}.

The problem encountered with such columns was elution. When the eluent was methanol, the extraction yields were poor: 50, 26 and 0% for azaperol, azaperone, and carazolol respectively. Isopropanol–dichloromethane–30% ammonium hydroxide (78:20:2, v/v/v) proved to be a better eluent. The problem here was that many unwanted molecules eluted with the substances to be analysed, precipitating when the sample was stored in a refrigerator and rendering injection impossible.

The performance of Oasis HLB® columns was better. Methanol elution gave good yields and cleaner samples. Fig. 1 shows an example of a blank sample spiked with the I.S. only.

Lastly, we studied the influence of the loading pH on the extraction yields obtained with an SPE column containing a polymer sorbent. Using spiked blank samples we made sure that no matrix interferences appeared after pH modifications. Loading at high pH (pH 10) is best, as the molecules are not protonated and this increases their affinity for the column. The yields recorded after solid–liquid extraction followed by purification on an Oasis HLB column were between 70 and 106%.

3.2. Liquid chromatography

The chromatographic conditions were adjusted with two aims in mind:

1. to develop a quick method
2. to improve sample purification in order to minimise the frequency at which the mass spectrometer had to be cleaned.

Use of a gradient enabled us to achieve both aims. An isocratic method can be used to separate effectively the three analytes, but the analysis time is relatively long (>15 min). The gradient proved more

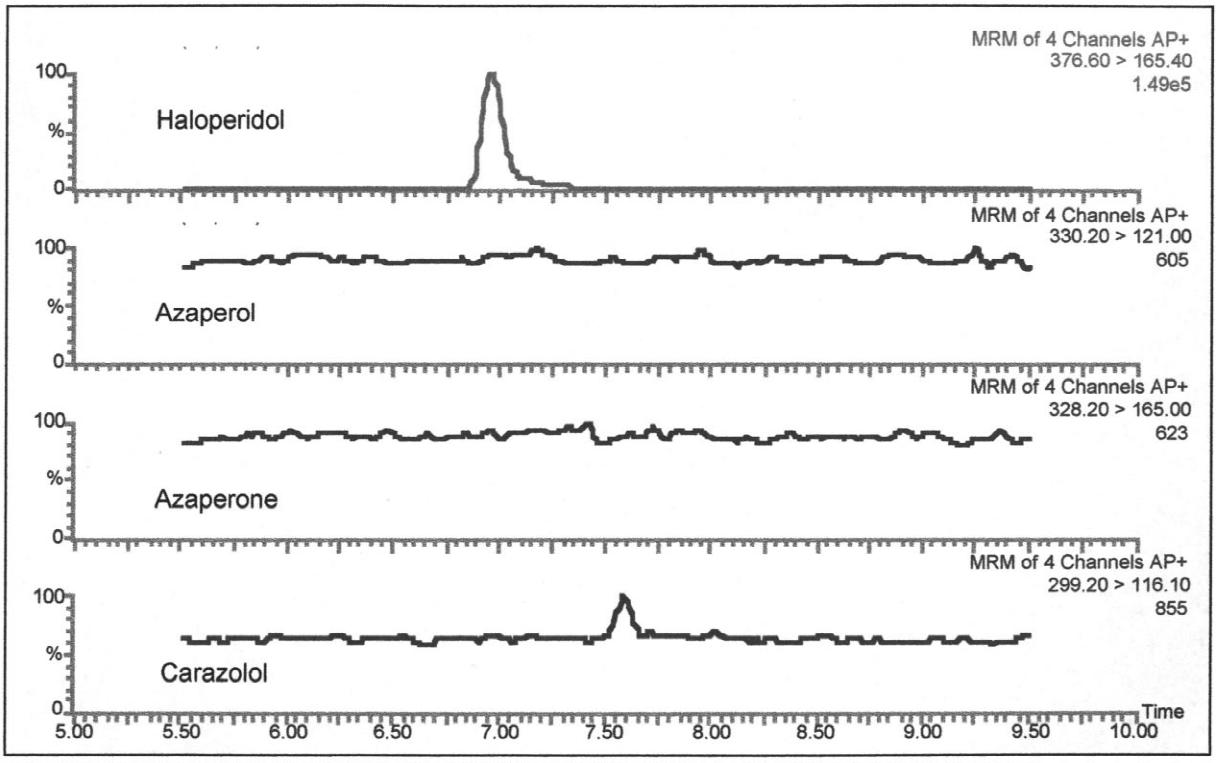

Fig. 1. Chromatogram of a blank sample spiked with haloperidol as I.S.

efficient, as it took only 9 min to separate the analytes. Taking into account the 2-min post-run, the total analysis time was 11 min. Yet an additional purification step proved necessary: when the solvent flowed through the mass spectrometer throughout the run, the instrument displayed decreased sensitivity after only about twenty samples. To improve this situation, two elements were added to the LC system.

First, upstream from the analytical column (C_{18}), we placed a Biomatrix® column in order to eliminate most of the macromolecules remaining in the sample. A 'switch column' allowed the flow to pass solely through the Biomatrix column for 1 min, then through the analytical column and mass spectrometer. The sample was thus purified before reaching the analytical column. This additional purification made it possible to inject about fifty samples before having to clean the source of the mass spectrometer. It also extended the lifetime of the analytical column. Lastly, a switching valve was placed between the analytical column and the mass spectrometer, allowing the flow to pass through the mass spectrometer only during analyte elution. These combined measures made it possible to analyse about a hundred samples before having to clean the instrument. Fig. 2 shows chromatograms of the analytes and I.S.

3.3. Mass spectrometry

For each analyte and haloperidol, the full-scan spectrum showed an intense peak corresponding to a pseudo-molecular ion. In each case this was the ion

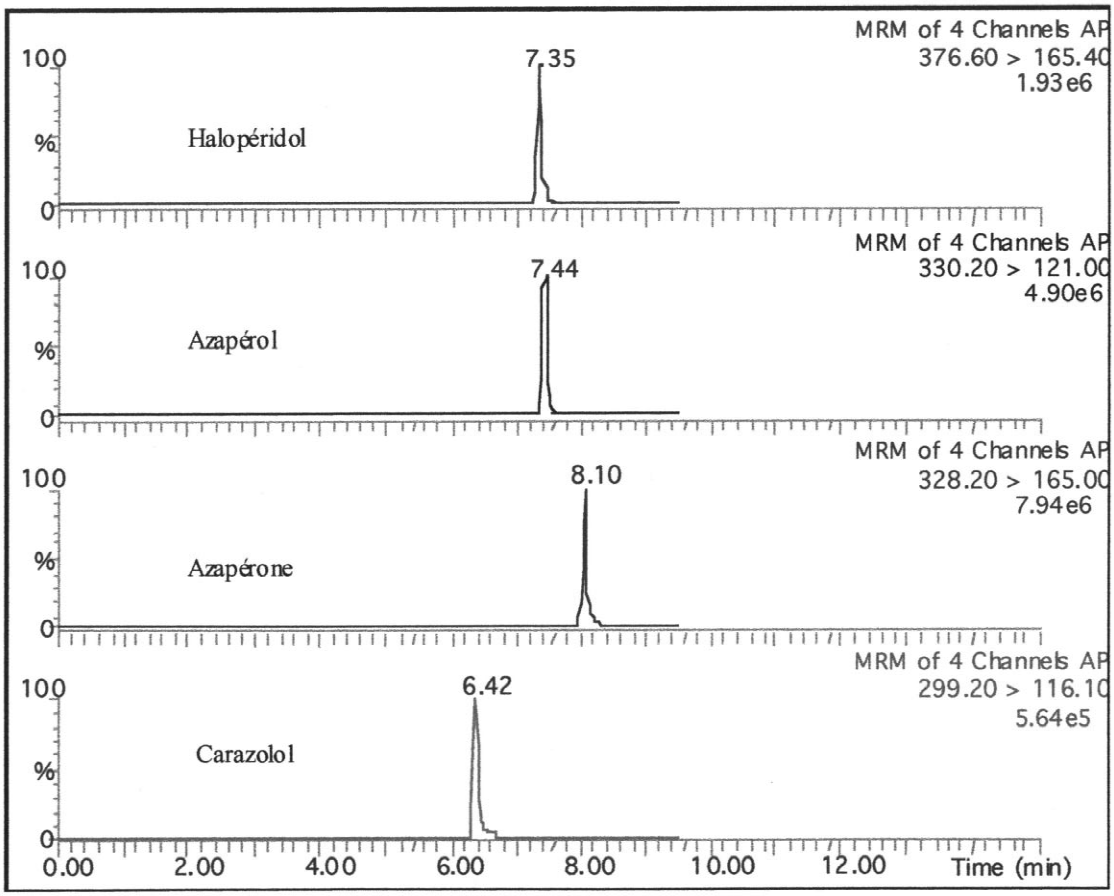

Fig. 2. Chromatogram of a muscle sample spiked with the molecules at a concentration equal to the MRL/2.

Table 1
Molecular masses and pseudo-molecular ions for azaperol, azaperone, and carazolol

Molecule	M_r	Pseudo molecular ion (m/z)
Carazolol	298.2	299.2
Azaperone	327.2	328.2
Azaperol	329.2	330.2
Haloperidol	375.6	376.6

chosen as parent ion for obtaining the product ion spectrum of the molecule concerned. Table 1 shows the original molecular mass (M_r) and the pseudo-molecular ion of each molecule. Fig. 3 shows the product-ion spectra of carazolol and haloperidol. The spectra obtained for azaperol and azaperone were as described by Chui et al. [13].

The chosen detection mode was MRM. Molecule detection programs based on one product ion (see Table 2) were used to quantify the substances in a sample, but to increase the selectivity, it was useful to detect several characteristic ions. We therefore developed an MS–MS program for each individual molecule, based on detection of four product ions issued from the same parent ion (Table 3).

This technique has good sensitivity and, thanks to parent–daughter ion detection, high specificity. Other tranquillisers, mainly promazine derivatives, were

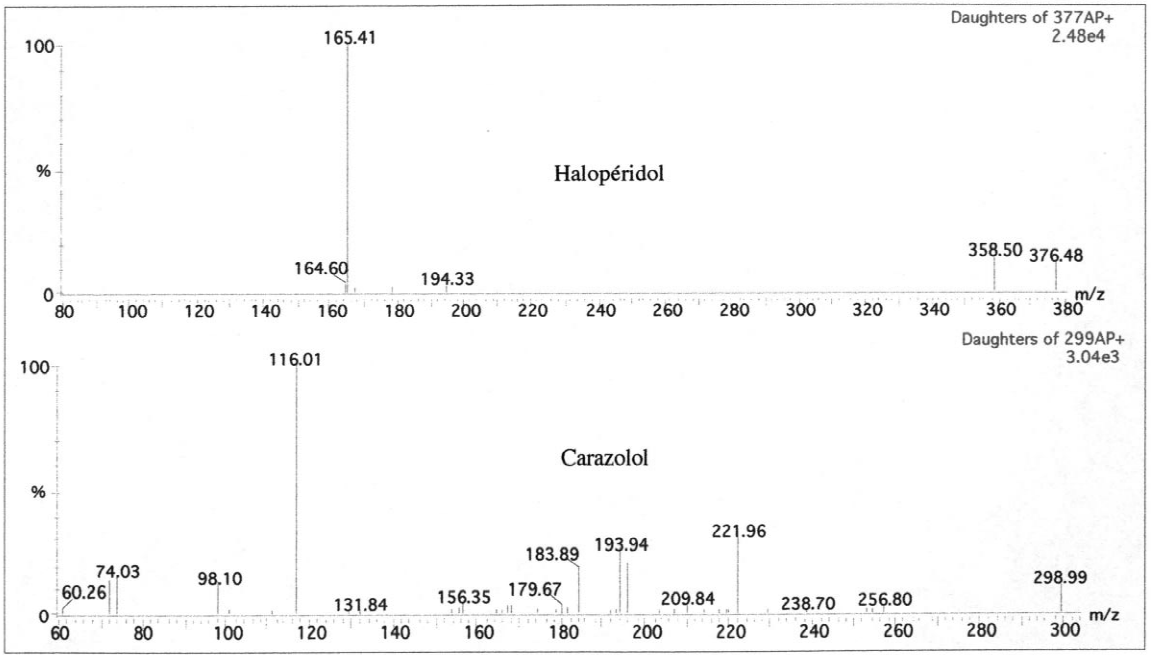

Fig. 3. Product ion spectra for haloperidol (parent ion 377 m/z) and carazolol (parent ion 299 m/z).

Table 2
Detection programs used in MS–MS

Molecules	Parent ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Carazolol	299.2	116.1	25	19
Azaperone	328.2	165.0	25	15
Azaperol	330.2	121.0	25	15
Haloperidol	376.6	165.4	20	35

Table 3
Parent ions and product ions used for confirmation in MS–MS

Molecule	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (eV)
Carazolol	299.2	116.1	20
		194.0	20
		183.9	20
		222.0	20
Azaperone	328.2	165.0	22
		121.0	22
		123.0	22
		147.0	22
Azaperol	330.2	149.0	19
		121.0	19
		192.0	19
		312.0	19

checked for their ability to interfere, but no interferences appeared.

The sensitivities achieved allow detection of the four product ions in samples spiked with the substance at a concentration corresponding to MRL/2 (Fig. 4).

3.4. Limits of detection and quantification

The LOD was calculated as the mean baseline value of 42 blanks plus three times the standard deviation. The LOQ was calculated as the mean baseline value plus six times the standard deviation (Table 4).

3.5. Accuracy and precision

The aim of this study was to develop a method for simultaneous determination of azaperol, azaperone and carazolol. The most important parameters for assessing a quantitative method are its accuracy and precision. In our case, the aim was to conform to EU accuracy and precision standards (Regulation 93/256/EEC). Tables 5 and 6 show, respectively, the values obtained for muscle and kidney extracts.

Our method proved quite accurate for all three molecules. As for the C.V.s the only values to be somewhat high were those for carazolol — present in muscle a concentration of MRL/2 or MRL. This

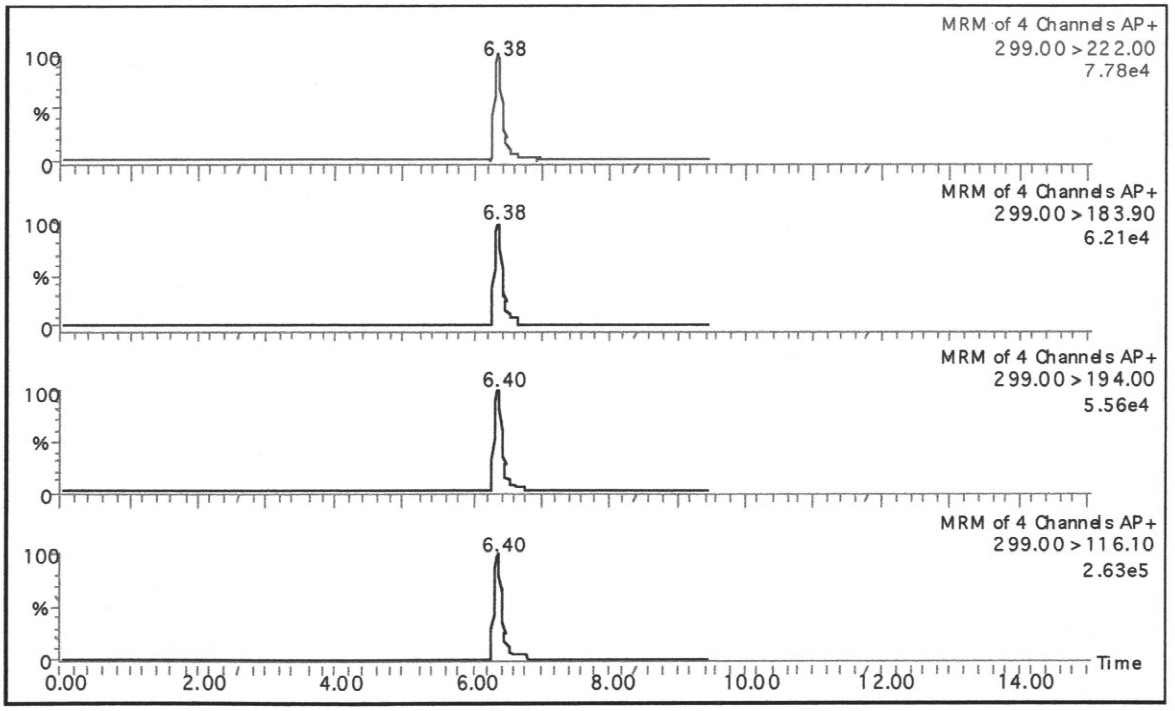

Fig. 4. Detection of carazolol based on four specific ions.

Table 4
Detection limits and quantification limits of azaperol, azaperone and carazolol in muscle and kidney tissues

Matrix	Azaperol		Azaperone		Carazolol	
	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
Muscles	0.43	0.73	1.41	2.55	0.06	0.13
Kidneys	2.14	3.51	1.05	1.76	0.75	1.23

Table 5
Precision and accuracy of the quantification method applied to muscle samples

Molecule	Amount added (μg/kg)	Mean amount determined (μg/kg)				C.V. (%)			
		Day 1	Day 2	Day 3	Mean value	Day 1	Day 2	Day 3	Mean value
Azaperol	25	24.2	24.7	25.4	24.8	6	7	3	6
	50	53.0	47.8	50.8	50.6	4	4	4	6
	100	107.5	104.8	104.9	105.7	2	4	4	4
Azaperone	25	24.6	24.9	23.8	24.4	5	2	6	5
	50	51.9	50.0	48.6	50.2	4	5	5	5
	100	106.8	106.1	102.7	105.2	2	3	2	3
Carazolol	2.5	2.29	2.70	2.34	2.44	11	5	10	11
	5	3.89	5.22	4.88	4.66	16	4	8	15
	10	10.30	10.43	9.63	10.12	5	6	4	6

may be due in part to the structural difference between carazolol and the I.S.: haloperidol is a butyrophenone derivative whereas carazolol is derived from carbazol. Such structural differences can lead to differences in behaviour upon extraction. Another explanation may be the low MRL of carazolol. Because the concentrations are lower, integration of the chromatographic peaks is more strongly affected by background variations.

The correlation coefficient for the standard curves between 0 and twice the MRL was above 0.99 for all three molecules.

4. Conclusions

The present results show the advantage of using LC–MS–MS to detect drug residues. The technique

Table 6
Precision and accuracy of the quantification method applied to kidney samples

Molecule	Amount (μg/kg)	Mean amount determined (μg/kg)				C.V. (%)			
		Day 1	Day 2	Day 3	Mean value	Day 1	Day 2	Day 3	Mean value
Azaperol	50	48.5	50.1	45.2	47.9	4	4	8	7
	100	93.0	97.0	90.4	94.1	3	4	2	5
	200	207.4	214.7	208.4	210.2	2	10	2	6
Azaperone	50	51.9	52.5	43.7	49.3	3	5	5	9
	100	101.7	103.3	94.8	99.9	1	4	5	5
	200	210.1	206.7	207.9	208.2	2	8	2	5
Carazolol	12.5	11.71	11.40	11.09	11.40	6	8	5	6
	25	23.40	25.01	24.64	24.35	2	4	4	5
	50	52.72	49.71	53.27	51.90	4	4	4	5

allows several sample analysis within 1 day. The LODs and LOQs are well below the MRL/2 for each molecule and the selectivity is high. Furthermore, sample extraction is quick, making this method a powerful tool for routine analysis of azaperol, azaperone and carazolol.

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