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Multiresidue method for the detection of tranquillisers, xylazine, and a β-blocker in animal production by liquid chromatography-tandem mass spectrometry

Philippe Delahaut*, Pierre-Yves Brasseur, Michel Dubois

CER, Laboratoire d'Hormonologie, Rue du Point du Jour, 8 B-6900 Marloie, Belgium

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Abstract

Tranquillisers are often used in animal production, especially in pigs to calm them before transport to the slaughterhouse. The use of certain substances (derived from phenothiazine) is totally prohibited, whilst other compounds (butyrophenone and β-blockers) are regulated through the establishment of MRLs. A physico-chemical detection method based on liquid chromatography-tandem mass spectrometry is described. Validation was carried out according to the criteria laid down in Directive 2002/657/EC. The method was also used to detect and quantify these substances in treated animals.
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Keywords: Tranquillisers; Xylazine; β-Blockers

1. Introduction

Intensive livestock production has made it possible to increase meat production and reduce its cost in industrialised countries. Pork represents nearly 50% of the meat consumed in some European countries. Pigs are particularly sensitive to stress during handling and transport to the abattoir. This phenomenon leads to high mortality and to poor-quality meat qualified as "Pale Soft Exudative" (PSE) [1].

This is why the use of tranquillisers has become generalised since the 1970s. Phenothiazine derivatives (acepromazine, chlorpromazine and propionlypromazine) were the first to appear on the market. Currently their use is totally prohibited in animal production. Stresnil®, the active principle of which is azaperone, is partially metabolised to azaperol. It is authorised provided its concentration does not exceed the imposed minimum residue limit (MRL) of 100 $g\,kg^{-1}$ in kidney and muscle.

Carazolol is an inhibitor of β-adrenergic receptors (a β-blocker). It avoids the problem of tachycardia in stressed animals. The MRL for this substance is 25 $g\,kg^{-1}$ for kidney and 5 $g\,kg^{-1}$ for muscle.

Xylazine has been used for many years in cattle, horses, dogs, and cats. It causes a hypnotic state with sedation (varying according to the dosage), accompanied by muscle relaxation and a variable degree of analgesia.

Apart from these well-known molecules, other compounds have recently made their appearance in illegal cocktails. Examples include chlorprotixene and cyamemazine, not initially intended for food-producing animals.

Many techniques are currently available for detecting the possible presence of these residues. Immunological methods [2] allow efficient screening, but positive results have to be confirmed by a physico-chemical method. Among the techniques that have been reported are thin-layer chromatography [3] and liquid chromatography extraction coupled with electrochemical detection [4] or ultraviolet and fluorescence detection [5,6]. These methods require extensive clean-up and do not allow sufficiently sensitive detection. Liquid chromatography-tandem mass spectrometry, on the other hand, has the advantage of being sensitive and of providing information on molecular structure. It is, therefore, increasingly becoming the method of choice. The first

* Corresponding author. Tel.: +32 84 310 019; fax: +32 84 316 108.
E-mail address: delahaut@cer-dha.be (P. Delahaut).

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LCMS methods [7–9] could detect only a limited number of molecules. In this paper, we present a validated method that detects all molecules in this group with a very low limit of detection.

2. Experimental

2.1. Materials

2.1.1. Reagents

The reagents used were HPLC-grade acetonitrile (Acros Organics, Geel, Belgium), HPLC-grade H_2O (Acros Organics, Geel, Belgium), analytical-grade ethanol (Baker Analyzed Reagent, Deventer, Holland), sodium chloride (Vel, Leuven, Belgium), and ammonium acetate (Vel, Leuven, Belgium).

2.1.2. Reference compounds

Azaperol (batch V 8972-24) and azaperone (batch V 810-88) were provided by Janssen (Beerse, Belgium). Haloperidol (Internal Standard, IS) (batch 18H0408) was from Sigma (St. Louis, USA). Carazolol (batch 43839300) was purchased from Boehringer Mannheim (Mannheim, Germany). Isobutcar 61 (4-(3-isobutylamino-2-hydroxypropoxy)carbazol—IS purity >95%) (product synthesised by Professor Moore, Geel, Belgium). Chlorpromazine (batch 48H1403), propionylpromazine (batch 34H0942), acepromazine (batch 47F0403), and chlorprothixene (batch 40H0346) were purchased from Sigma (Bornem, Belgium). Chlorpromazine-d3 (batch 24543-45D) was purchased from Radian (Austin, USA). Xylazine was from Bayer (West Germany). Cyamemazine (purity >95%) was a gift from Larime (Lagord, France). Stock solutions ($1\,mg\,ml^{-1}$) were prepared in ethanol and stored at $-20\,°C$ in the dark.

2.1.3. Biological specimens

Biological specimens were taken from animals treated with one of the following: Largatil® (chlorpromazine, Rhône Poulenc Roerer), Combistress® (acepromazine, Phoenix), Combelene® (propionylpromazine, Bayer), Stresnil ® (azaperone, Janssen Pharmaceutica), or Suacron® (carazolol, Upjohn). The doses administered were: chlorpromazine, $11\,mg\,10\,kg^{-1}$; acepromazine, $2\,mg\,10\,kg^{-1}$; propionylpromazine, $5\,mg\,10\,kg^{-1}$; azaperone $0.25\,mg\,10\,kg^{-1}$; carazolol, $0.1\,mg\,10\,kg^{-1}$. The animals were slaughtered 2, 6, or 24 h after product injection. This timing was chosen to mimic field conditions as closely as possible (2 h for a farm located near the abattoir, 6 h for transport over a medium distance, and 24 h for slaughter postponed to the next day).

2.1.4. Instrumentation

The centrifuge used (an RC-3B Refrigerated Centrifuge) was from Sorvall Instruments (Wilmington, USA). The stirring system (HS250 basic) was from Ika Labortechnick (Staufen, Germany). The HPLC system was a Hewlett Packard 1100 series (Waldbronn, Germany). The mass spectrometer was a Quattro Ultima Pt (triple quadrupole, Micromass, Altrincham, Cheshire, UK). The MS system was controlled by version 4.0 of the Masslynx Software.

2.2. Methods

2.2.1. Extraction

Kidney, liver and muscle samples collected from untreated animals were used as blank samples. All test samples were spiked with the same amount of the same cocktail of internal standards just before extraction, and results were expressed with respect to the internal standard response so as to correct for extraction yield fluctuations. The internal standards were: chlorpromazine-d_3 (for promazine derivatives and for xylazine, haloperidol (for azaperone and azaperol), isobutcar 61 (for carazolol). To 5 g homogenised muscle, kidney, or liver was added 20 ml acetonitrile. The mixture was stirred for 15 min and centrifuged at $4\,°C$ for 10 min at 4700 g.

One millilitre of extract was then evaporated to dryness in a bath thermostated at $60\,°C$ and under nitrogen flow. The dried extract was dissolved in 0.8 ml acetonitrile/water (20:80, v/v). A 100 μl aliquot of this solution was injected into the LC-MS-MS system.

2.2.2. HPLC conditions

The mobile phase consisted of two eluents. Eluent A was filtered HPLC-acetonitrile and Eluent B was a 0.1 M ammonium acetate solution. The gradient is described in Table 1.

The column was a Purospher model RP18125 × 3 mm, 5 μm (Merck, Darmstadt, Germany) equipped with a guard column Purospher RP18 (Merck, Darmstadt, Germany) preceded by a Biomatrix column cat. 29230 (Chrompack, Middleburg, Holland) equipped with a pre-column cat. 28128 (Chrompack, Middleburg, Holland). The columns were thermostated at $50\,°C$.

2.2.3. Mass spectrometry conditions

The analytes were detected and identified by tandem MS after electrospray ionisation (ESI) in the positive ion mode. Nitrogen gas flows of 230 and $800\,l\,h^{-1}$ were used, respectively, for nebulising the LC element and for drying the sol-

Table 1
RP-HPLC conditions for detecting and quantifying tranquillisers and a β-blocker

Time (min)	Acetonitrile (%) A	Amonium acetate 0.1 M B
0.0	0	100
3.0	0	100
5.0	30	70
6.0	45	55
7.0	60	40
9.0	70	30
10.0	100	0
11.0	100	0
11.1	0	100
12.0	0	100

vents. The probe temperature was 300 °C and the source temperature was 120 °C. The pressure in the collision cell was 2×10^{-6} bar. The photomultiplier was adjusted to 600 V. The molecular precursor ion was fragmented in the collision cell with argon gas. Analytes were detected by multiple reaction monitoring, after transition of the precursor ion into two product ions. The mass spectrometer was tuned by optimising the specific cone voltage and the collision energy so as to maximise the ion current of the three induced ions by infusion of a standard solution (1 $\mu g\, ml^{-1}$).

2.2.4. Calibration and quantification

Azaperone, azaperol and carazolol were quantified by an internal standard procedure based on matrix calibration curves. The calibration curves were constructed from six points spanning the concentration range from 0 $\mu g\, kg^{-1}$ to twice the MRL. The results were calculated automatically by Masslynx Software version 4.0. For other analytes, i.e. those unauthorised, no quantification was necessary.

3. Results and discussion

3.1. Analytical method

3.1.1. Extraction and purification

In order to estimate the extraction and purification yields, we added an internal standard representative of each family of compounds at the start of the analysis (see Section 5.2.1). The extraction level (recovery) was determined in muscles and kidney spiked with the various tranquillisers at MRL level for carazolol, azaperone, azaperol and at 5 ppb for the other molecules. The values ranged from 86 ± 8% for muscle to 90 ± 10% for kidney. These high recovery values are mainly due to the fact that there was only one extraction step before LC-MS/MS. The detection sensitivity of the Platinum spectrometer made it possible to reduce considerably the number of extraction steps required. For example, purification on an SPE column proved unnecessary.

3.1.2. Liquid chromatography

The chromatography conditions were adjusted with two aims in mind: to develop a quick method and to improve sample purification in order to minimise the frequency of mass spectrometer cleaning.

We placed a Biomatrix® column upstream from the analytical column (C_{18}) in order to eliminate most macromolecules remaining in the sample. ChromSpher BioMatrix columns are packed with silica-based material designed specifically for direct injection of biological matrices onto the column. The stationary phase consists of a combined phase; a hydrophobic part encapsulated by a hydrophilic outer layer. The latter rejects proteins by exclusion while drugs, because of their small size, can penetrate this layer and interact with the hydrophobic part. 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 50 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 100 samples before having to clean the instrument. Fig. 1 shows chromatograms of the analytes and internal standards.

3.1.3. Mass spectrometry

For each substance to be detected, the full-scan spectrum showed an intense protonated molecule. In each case this was the ion chosen as precursor ion for obtaining the product ion spectrum of the molecule concerned. Fig. 2 shows the product-ion spectra of carazolol and haloperidol. The spectra obtained for azaperol and azaperone were as described by

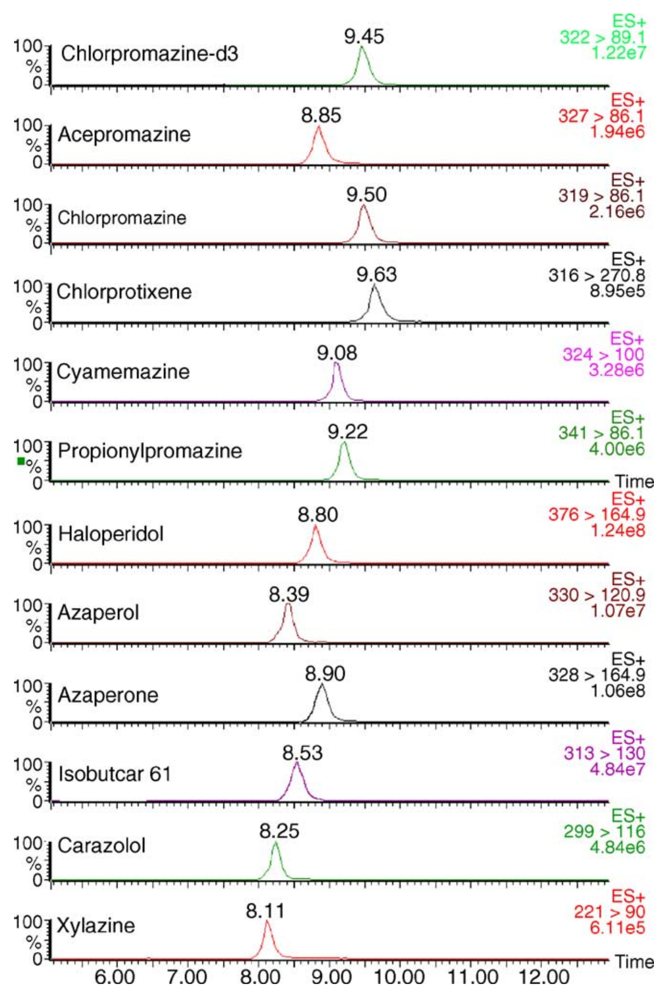

Fig. 1. Chromatograms of analytes and internal standards (chlorpromazine-d3: 10 $\mu g\, kg^{-1}$; haloperidol: 65 $\mu g\, kg^{-1}$; isobutcar 61: 100 $\mu g\, kg^{-1}$; MRL for carazolol and azaperone + azaperol: 5 $\mu g\, kg^{-1}$).

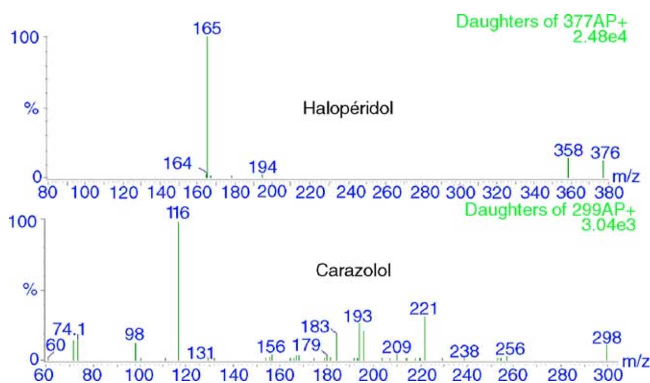

Fig. 2. Product ion spectra for haloperidol (precursor ion 377 *m/z*) and carazolol (precursor ion 299 *m/z*).

Chui et al. [10]. The chosen detection mode was multiple reaction monitoring (MRM).

Table 2 presents the MRM program used. Molecule detection programs based on one product ion are often used to quantify substances in a sample. Yet to increase selectivity, it is useful to detect several characteristic ions. We, therefore, developed an MS-MS program for each individual molecule, based on detection of two product ions issued from the same precursor ion. For the molecules used as internal standards we monitored only one transition, as these are always added to samples at the outset and are thus necessarily present. The platinum instrument enabled us to detect all these transitions in a single MRM program. This technique shows good sensitivity and, thanks to our precursor-product ion detection strategy, high selectivity.

3.2. Validation

The method was validated according to Directive 2002/657/EC [11]. A correspondence between the chromatographic retention time of the analyte in the samples and in the standard solution was checked according to performance criteria for confirmation of contaminants. The Directive introduces the 'identification point' notion for confirming the potential presence of substances. MS/MS-type detection requires two transitions (one precursor ion and two product ions). Acquisition of two MRM transitions made it possible to obtain a total of four identification points for the analysed compounds. It should be reported that this is particularly useful in the case of unauthorised substances, i.e. those belonging to Group A as listed in the text of the Decision, as is the case of compounds derived from phenothiazine. For the other substances, only three IPs are required.

For promazine derivatives and xylazine, validation means reaching a detection limit as low as possible. In the case of substances for which an MRL has been set, the criteria for quantifying these products at MRL level are applicable. The decision limit (CCα) means the limit at and above which it can be concluded with an error of probability of α that a sample is not compliant. The α-error is the probability to obtain a non-compliant result for a sample which is truly compliant. For banned substances the CCα equals the former limit of detection, for substances with a permitted limit, the limit of detection as well as the limit of quantification are of minor interest provided that those limits are much lower than the permitted limit. The decision limit (CCα) was determined by analysing 20 samples spiked at a concentration for which a signal-to-noise ratio of 3 was obtained for the compounds without MRL (promazines), and at MRL level for azaperone, azaperol, and carazolol.

The detection capability (CCβ) means the lowest content of a substance that may be detected, identified and/or quantified in a sample with an error of probability of β. CCβ is a quality parameter of method and not a limit. It depends on the standard deviation: the higher the standard deviation is, the higher the concentration will be.

The detection capability (CCβ) was determined by quantification of 20 samples at CCα level. CCβ equals CCα plus 1.64 times the standard deviation.

3.2.1. Spiked samples from untreated animals

First, biological samples from untreated animals were spiked with one or more target compounds and the cocktail of internal standards, then analysed according to our method. The results presented in Table 3 shows that the method meets EU requirements [11] for validation criteria.

Table 2
Detection and confirmation programs for different tranquillisers, xylazine, and a β-blocker

Molecule	Precursor ion (*m/z*)	Product ion 1 (*m/z*)	Product ion 2 (*m/z*)	Cone voltage (V)	Collision energy (eV)
Chlorpromazine-d_3	322	89	–	50	17
Acepromazine	327	86	58	50	18
Chlorpromazine	319	86	58	50	18
Chlorprotixene	316	271	231	50	17
Cyamemazine	324	100	58	50	15
Propionylpromazine	341	86	58	50	18
Haloperidol	376	165	–	50	20
Azaperol	330	121	149	50	20
Azaperone	328	165	121	50	18
Isobutcar 61	313	130	–	50	18
Carazolol	299	116	222	50	18
Xylazine	221	90	147	50	18

Table 3
Validation results obtained for the different molecules in muscle and kidney

Matrix	Molecule	CCα (μg kg^{-1})	CCβ (μg kg^{-1})	Accuracy (%)	Precision (%)
Muscle	Chlorpromazine	0.5	0.9		
	Acepromazine	0.4	0.6		
	Chlorprothixene	0.6	1.0		
	Propionylpromazine	0.3	0.5		
	Cyamemazine	0.8	1.0		
	Xylazine	1.1	1.8		
	Carazolol	5.6	6.1	104.0	5.0
	Azaperol	56.1	63.0	99.0	7.6
	Azaperone	56.3	64.0	102.4	6.2
Kidney	Chlorpromazine	0.5	0.9		
	Acepromazine	1.0	1.6		
	Chlorprothixene	0.5	0.9		
	Propionylpromazine	0.9	1.6		
	Cyamemazine	1.0	1.7		
	Xylazine	1.5	2.6		
	Carazolol	28.3	30.5	94.8	6.2
	Azaperol	57.6	65.9	97.4	10.9
	Azaperone	55.4	61.6	97.6	8.0

Insofar as there is no reference material available for these substances, the accuracy has been determined with spiked samples at MRL level. As regards the precision, it has been calculated on the basis of the coefficient of variation within-laboratory on these samples. The values range from 5 to 10.9%.

In the case of authorised substances, the CCα and CCβ values were determined on the basis of the values obtained for accuracy and precision (mean value: + 1.64 S.D.). This allows us to explain that the values obtained are relatively high for the substances at MRL.

As far as unauthorised substances are concerned, the CCα and CCβ values appear to be low because the aim was to have a measurement as sensitive as possible.

Compounds with an MRL were readily quantified when present at MRL concentration. Other compounds were easy to detect thanks to the selectivity and sensitivity of mass spectrometry.

3.2.2. Samples from treated animals

To further validate the method, we treated 30 pigs (of about slaughter weight) with veterinary specialities intended for calming animals before transport (a male and a female for each combination of compound administered and time of slaughter). The values obtained for muscle, liver, and kidney tissue are shown in Figs. 3–7.

The figures show that for most of the products tested, the highest levels appeared in the kidneys. The only exception is chlorpromazine (Fig. 3), the liver appearing as a major target organ for this compound.

Acepromazine (Fig. 4) and carazolol (Fig. 6) appeared to be rapidly eliminated. Twenty-four hours post-injection, the levels found were well below the limit of quantification.

Azaperone (Fig. 7) was eliminated more slowly than carazolol. Six hours post-injection, levels of both carazolol and azaperone + azaperol were below their respective MRLs in muscle, but levels of the latter remained near or above the MRL in kidney and liver at this time. Conversion of azaperone to azaperol was rapid, as evidenced by higher levels of azaperol than azaperone in the various tissues (not shown). Two

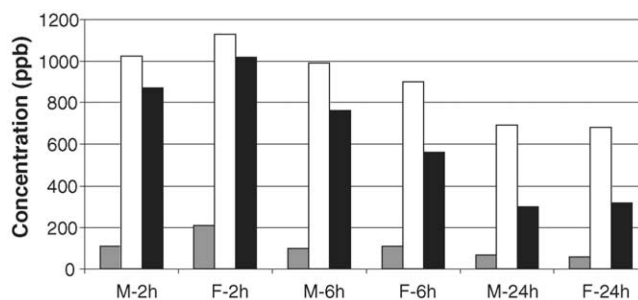

Fig. 3. Evolution of the chlorpromazine concentration in muscle (grey rectangles), liver (white rectangles), and kidney (black rectangles) in pigs slaughtered 2, 6, and 24 h after administration of 11 mg/10 kg chlorpromazine (M = male, F = female).

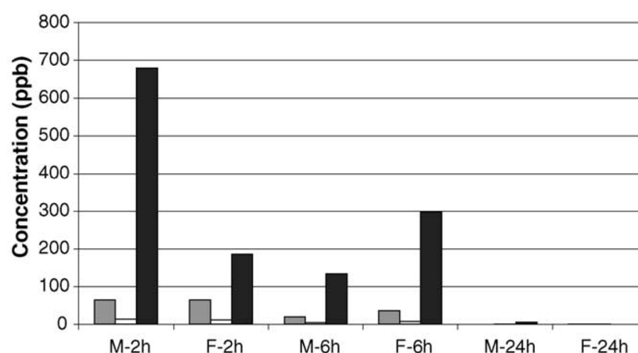

Fig. 4. Evolution of the acepromazine concentration in muscle (grey rectangles), liver (white rectangles), and kidney (black rectangles) in pigs slaughtered 2, 6, and 24 h after administration of 2 mg/10 kg acepromazine (M = male, F = female).

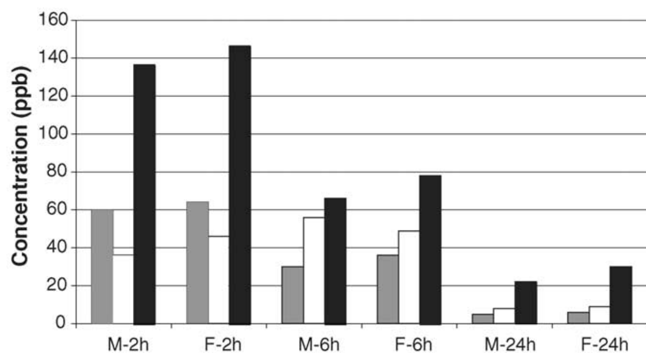

Fig. 5. Evolution of the propionylpromazine concentration in muscle (grey rectangles), liver (white rectangles), and kidney (black rectangles) in pigs slaughtered 2, 6, and 24 h after administration of 5 mg/10 kg propionylpromazine (M = male, F = female).

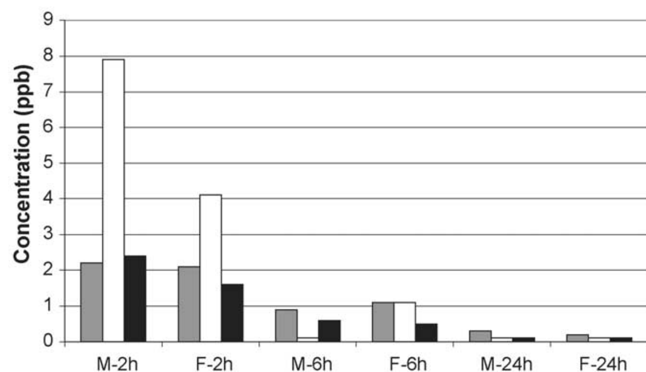

Fig. 6. Evolution of the carazolol concentration in muscle (grey rectangles), liver (white rectangles), and kidney (black rectangles) in pigs slaughtered 2, 6, and 24 h after administration of 0.25 mg/10 kg carazolol (M = male, F = female).

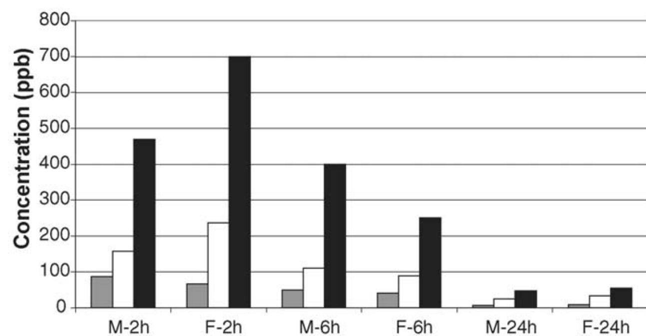

Fig. 7. Evolution of the summed concentrations of azaperone and azaperol in muscle (grey rectangles), liver (white rectangles), and kidney (black rectangles) in pigs slaughtered 2, 6, and 24 h after administration of 0.25 mg/10 kg azaperone (M = male, F = female).

hours post-injection, for instance, the azaperol/azaperone ratio was 2.3 in muscle and kidney and 15.4 in liver tissue.

When cocktails of two or more substances were injected, the behaviour of individual compounds was unaffected (not shown). Elimination of each compound remained similar to that observed in animals treated with a single product.

4. Conclusion

The method described was based on the use of the LC-MS/MS technique for confirmation and, in the case of authorised substances, for quantitation of tranquillisers in pork meat. The method was validated according to the criteria of Directive 2002/657/EC. The validation data obtained with samples from treated animals show that elimination of tranquillisers is very rapid in treated animals. Six hours post-injection, levels of most molecules have fallen below the MRL, at least in muscle tissue.

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