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Quantitative analysis of levamisole in porcine tissues by high-performance liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry

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Abstract

This work presents the development and the validation of an LC–MS–MS method with atmospheric pressure chemical ionization for the quantitative determination of levamisole, an anthelmintic for veterinary use, in porcine tissue samples. A liquid–liquid back extraction procedure using hexane–isoamylalcohol (95:5, v/v) as extraction solvent was followed by a solid-phase extraction procedure using an SCX column to clean up the tissue samples. Methyllevamisole was used as the internal standard. Chromatographic separation was achieved on a LiChrospher® 60 RP-select B (5 μm) column using a mixture of 0.1 *M* ammonium acetate in water and acetonitrile as the mobile phase. The mass spectrometer was operated in MS–MS full scanning mode. The method was validated for the analysis of various porcine tissues: muscle, kidney, liver, fat and skin plus fat, according to the requirements defined by the European Community. Calibration graphs were prepared for all tissues and good linearity was achieved over the concentration ranges tested ($r>0.99$ and goodness of fit $<10\%$). Limits of quantification of 5.0 ng/g were obtained for the analysis of levamisole in muscle, kidney, fat and skin plus fat tissues, and of 50.0 ng/g for liver analysis, which correspond in all cases to half the MRLs (maximum residue limits). Limits of detection ranged between 2 and 4 ng/g tissue. The within-day and between-day precisions (RSD, %) and the results for accuracy fell within the ranges specified. The method has been successfully used for the quantitative determination of levamisole in tissue samples from pigs medicated via drinking water. Moreover the product ion spectra of the levamisole peak in spiked and incurred tissue samples were in close agreement (based on ion ratio measurements) with those of standard solutions, indicating the worthiness of the described method for pure qualitative purposes.

Keywords: Levamisole

1. Introduction

Levamisole, (*S*)-(−)-2,3,5,6-tetrahydro-6-phenylimidazo-[2,1b]thiazole is an anthelmintic drug. It is widely used for the control of gastro-intestinal parasites in cattle, sheep and pigs, due to its potent broad-spectrum action. It is normally administered by pour-on or by direct subcutaneous or intramuscular injection. The MRL in animal products for consumption is fixed by the European Union at 100

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ng/g for liver, and 10 ng/g for muscle, kidney and fat tissue of cattle, sheep, pigs and poultry [1].

Various methods have been published for the determination of levamisole in milk, biological fluids and meat. They include gas chromatography (GC) with thermionic specific detection [2] or mass spectrometric detection [3], high-performance liquid chromatography (HPLC) with UV detection [4–8] or mass spectrometric detection [9], on-line high-performance liquid chromatography–gas chromatography–tandem ion-trap mass spectrometry [10], supercritical fluid chromatography–mass spectrometry [11] or mass-analyzed ion kinetic energy spectrometry [12].

This paper describes a method for the quantitative determination of levamisole in porcine tissue samples by liquid chromatography combined with atmospheric pressure chemical ionization (APCI) mass spectrometry. To enhance the precision and accuracy of the method, internal standardization was used. A simple extraction and clean-up step, including a liquid–liquid and a solid-phase extraction, was performed to isolate the analytes from the different tissue matrices. The method was validated according to the requirements defined by the European Community [13–15] at the MRL, at half the MRL and at double the MRL levels. Only some of the above mentioned methods are able to detect levamisole levels as low as 5 ng/g for muscle, kidney, liver, fat and skin plus fat and 50 ng/g for liver tissue [2,9], and even then validation results for quantification have scarcely been presented [6]. Finally, the method has been successfully used for residue depletion studies according to the European guidelines [13] after administration of levamisole to pigs via drinking water.

2. Experimental

2.1. Chemicals and standards

Levamisole hydrochloride was a Chemical Reference Substance (CRS) of the European Pharmacopoeia (Strasbourg, France). A stock solution of 1000 μg/ml was prepared in methanol. It was stored at −20°C and replaced by a fresh stock solution every 3 months. Working solutions at levamisole concentrations of 25, 20, 15, 10 and 5 μg/ml for spiking of liver tissues and of 2.5, 2.0, 1.5, 1.0 and 0.5 μg/ml for spiking of the other tissues (muscle, kidney, fat and skin plus fat) were prepared by appropriate dilution of the stock solution with water. By spiking 5 g of each tissue with 50 μl of the respective working solutions, levamisole concentrations of 250, 200, 150, 100, and 50 ng/g for liver and 25, 20, 15, 10 and 5 ng/g for muscle, kidney, fat and skin plus fat were obtained. All the working solutions were stored in a refrigerator (2–8°C) and were replaced every month by a fresh solution.

Methyllevamisole hydrochloride, used as internal standard, was purchased from Janssen Pharmaceutica (Beerse, Belgium). A stock solution of 1000 μg/ml was prepared in methanol. Storage conditions were the same as for the levamisole stock solution. Two working solutions at concentrations of 10 and 1 μg/ml were prepared by appropriate dilution of the stock solution with water. By spiking liver and the other tissues (muscle, kidney, fat and skin plus fat) with 50 μl of the 10 and 1 μg/ml working solutions, respectively, methyllevamisole concentrations of 100 ng/g of liver and 10 ng/g of the other tissues were obtained. The internal standard working solutions were stored in the refrigerator (2–8°C) and were replaced every month by a fresh solution.

All solvents used for the mobile phase (water, tetrahydrofurane (THF) and acetonitrile) were of HPLC grade and were purchased from Acros (Geel, Belgium — water) and from Merck (Darmstadt, Germany — THF and acetonitrile). All other chemicals were of pro analysi or analytical reagent grade and were purchased from Merck (hexane, isoamylalcohol, triethylamine, sulfuric acid, ammonia 25% solution and sodium hydroxide pellets), from Panreac (Barcelona, Spain — methanol) and from Sigma (Bornem, Belgium — ammonium acetate).

2.2. Biological samples

Known levamisole-free tissue samples (muscle, kidney, fat, skin plus fat and liver) were obtained from pigs which did not receive any medication. Incurred tissue samples were obtained from 20 pigs which received levamisole with drinking water at a normal dose of 8 mg/kg body weight during an 8-h period. The animals were slaughtered in five groups

of four after 16 and 40 h, and 4, 8 and 12 days, after cessation of medication. After slaughtering, the tissue samples were minced and homogenized using a Moulinette mixer (Barcelona, Spain) and transferred into a plastic bag and frozen at −20°C until analysis.

2.3. Tissue extraction procedure

Five grams of tissue homogenate were transferred into a capped 50-ml polypropylene centrifuge tube and spiked with 50 μl of a working solution of the internal standard methyllevamisole. After vortex mixing during 15 s, 20 ml of a hexane–isoamylalcohol (95:5, v/v) solution were added. The sample was vortex mixed during another 15 s, followed by the addition of 10 ml of a 1 *M* NaOH solution. The mixture was homogenized by vortex mixing for 15 s. The tube was placed for 5 min in an ultrasonic bath (Vel, Leuven, Belgium). After a settling period of 5 min, the tube was rotated during 10 min on a home made apparatus. After centrifugation at 5000 rev./min during 10 min, the upper organic layer was transferred into a new tube, containing 15 ml of a 0.05 *M* sulfuric acid solution. The tube was rotated again during 10 min and separation of the aqueous and the organic phases was achieved by settling the sample for ~5 min. The upper organic phase was removed with a pipet and the aqueous phase was set aside for further purification by solid-phase extraction. The liquid–liquid back extraction procedure used was based on the method published by Woestenborghs et al. [2].

2.4. Solid-phase clean-up

An SCX column (Strong Cation eXchanger, benzene sulphonic acid) (500 mg/10 ml, Isolute, Sopachem, Brussels, Belgium) was installed on a vacuum-manifold (Alltech, Laarne, Belgium) and preconditioned with, respectively, 3 ml methanol, 3 ml water and 1 ml of a 0.05 *M* sulfuric acid solution. The aqueous tissue extract was allowed to pass slowly through the SCX column. The column was washed with, respectively, 3 ml water, 1 ml of a 0.05 *M* sulfuric acid solution and 3 ml methanol. The analytes were eluted with 3 ml of a solution of concentrated ammonia in methanol (25:75, v/v). The eluate was evaporated to dryness at 60°C, under a gentle stream of nitrogen gas. The dry residue was redissolved in 250 μl of the HPLC mobile phase A and vortex mixed during 15 s. The reconstituted sample was transferred to an autosampler vial and a 100-μl aliquot was injected onto the HPLC column.

2.5. Chromatography

The HPLC system consisted of a quaternary gradient pump P4000, an autosampler AS3000 with cooling device and a degassing kit using helium to sparge the eluents (all from Thermo Separation Products, ThermoQuest, San Jose, CA, USA). Chromatographic separation was achieved using a reversed-phase LiChrospher® 60 RP-select B (5 μm) column (125 mm×4 mm i.d.), in combination with a guard column of the same type (4 mm×4 mm i.d.), from Merck. The mobile phase A was a solution of 0.1 *M* ammonium acetate in water, containing 7.7% (v/v) tetrahydrofurane and 0.3% (v/v) triethylamine, while the mobile phase B was pure acetonitrile. Eluate was delivered to the HPLC column at a constant flow-rate of 1.0 ml/min. A gradient elution was performed to elute some tissue components from the column: 0–7 min: 65% A, 35% B; 9 min: 50% A, 50% B; 9–14 min: 50% A, 50% B; 15 min: 65% A, 35% B; 15–20 min: 65% A, 35% B. This chromatographic procedure was based on the publication of Cannavan et al. [9].

2.6. Mass spectrometry

The HPLC column effluent (1 ml/min) was pumped to an LCQ® mass spectrometer instrument (Finnigan MAT, ThermoQuest), equipped with an APCI ion source, which was used in the positive ion MS–MS mode. A divert valve was used to divert the HPLC effluent to the waste during the first 2 min and the last 14 min of the chromatographic run. The HPLC effluent is directed to the mass spectrometer between 2 and 6 min after the start of the run, when levamisole and methyllevamisole were eluted from the column, respectively, at 3.4 and 4.6 min. The instrument was calibrated with a solution of coffeine, MRFA (L-methionyl-arginyl-phenylalanyl-alanineacetate·H_2O) and Ultramark® 1621, according to manufacturers' instructions. Thereafter, the

instrument was tuned by direct infusion of a solution of 20 μg/ml levamisole in the APCI source. Optimal collision energy in MS–MS mode, corresponding to a (nearly) 100% fragmentation of the protonated molecular ions of both levamisole and methyllevamisole (m/z=205.1 and 219.1, respectively), was found to be 1 V. Under these conditions, typical product ions at m/z=145.3 and 178.0 were obtained for levamisole (m/z=205.1), and at m/z=159.2 and 192.0 for methyllevamisole (m/z=219.1). Quantification was done with the LCQuan® software (ThermoQuest), using for levamisole both product ions at m/z=145.3 and 178.0, and for methyllevamisole at m/z=159.2 and 192.0.

2.7. Validation criteria

The proposed method for the quantitative determination of levamisole was validated by a set of parameters which are in compliance with the requirements as defined in the Rules Governing Medicinal Products in the European Community [13–15]. Moreover, based on Commission Decision No. 93/256/EEC, the method was validated at the fixed MRLs, at half the MRLs (MRL/2) and at double the MRLs (MRL×2) [14]. The following parameters were examined.

Linearity: the linearity of the method was checked using spiked blank tissue samples. For each calibration curve, six different concentration levels were used, including a zero level and the MRL level. Peak area ratios between levamisole and methyllevamisole were plotted against their concentration ratio and a linear regression was performed. The acceptance criteria for linearity were: correlation coefficient $r \geq 0.99$ and goodness of fit coefficient <10% [16].

Accuracy: the accuracy was defined as the closeness of agreement between the true value and the mean results of a series of experiments (n=6). It was determined by analyzing six blank tissue samples spiked at the same concentration level and by comparing the measured concentration to the spiked concentration. The concentrations used were equal to 1/2 MRL, MRL and 2×MRL. The accuracy must be in the range −30% and +10% for levels >1 ng/g to 10 ng/g and −20% to +10% for levels >10 ng/g [13–15].

Precision: the precision was defined as the 'within laboratory repeatability' since it includes the use of the same method on identical test material, in the same laboratory by the same operator using the same equipment within short intervals of time. It is expressed as the relative standard deviation (RSD, in %), being the ratio between the standard deviation (SD) and the mean found concentration (in %). The maximum allowable tolerances for the imprecision (RSDmax) for analyses carried out under repeatability conditions are one half to two thirds of the values calculated according to the Horwitz equation ($RSD = 2^{(1-0.5\ \log c)}$) [15]. The 'within-run or within-day' precision was determined on the same samples as for the accuracy, while the 'between-day' precision was determined on blank tissue samples spiked at the MRL level and analyzed on different days.

Limit of quantification (LOQ): it is defined as the lowest concentration for which the method is validated with an accuracy and precision that fall within the ranges specified. Moreover, as recommended by the EU, the LOQ has to be at least equal to half the MRL [14].

Limit of detection (LOD): the LOD is defined as the lowest measured content from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. For mass spectrometric determinations, it is defined as: $LOD = 3 \times SD_{res}/a$, with a the slope of the calibration curve and SD_{res} the residual standard deviation (=the standard deviation of the deviation of the various calibration points of the calibration curve) [17].

Specificity: the specificity was evaluated by the analysis of blank tissue samples. The absence of the possible interference of endogenous compounds with the same retention time as levamisole and methyllevamisole, as well as the absence of the possible interference of other compounds belonging to the same class of compounds as the analyte, was demonstrated [15,17].

3. Results and discussion

3.1. Isolation of the compounds

To extract levamisole (and the internal standard) from the porcine tissue samples, two liquid–liquid extraction procedures based on procedures described

in the literature were evaluated, more precisely those of Woestenborghs et al. [2] and Wyhowski de Bukanski et al. [7]. Both methods were not suitable for HPLC coupled with conventional UV detection, which we used initially for detection of levamisole, due to remaining interfering endogenous peaks at the retention times of both levamisole and methyllevamisole (results not shown). An extra clean-up step was therefore added to the liquid–liquid extraction. Also here, different ways were followed: solid-phase extraction on an SCX column at low pH, possibly due to the basic properties of the molecule (pK_a=8.0), solid-phase extraction on a CN column [9] (CyanoPropyl, Isolute), and an extraction on an Empore® cation-exchange membrane (Varian, Zaventem, Belgium) at low pH. Based on these experiments, the liquid–liquid extraction according to Woestenborghs et al. [2] followed by a solid-phase SCX extraction, was retained for further work, since it allowed us to obtain blank tissue extracts without any interfering endogenous peaks.

3.2. Chromatography

Since the MRL of levamisole in animal tissues is set at 10 ng/g by the EU, a quantitative method must be validated at least at 5 ng/g, being half the MRL. It was not possible, on different column types and with different mobile phases, to reach such low levels with a UV detection method. Therefore, we switched to HPLC–MS detection, using a chromatographic method based on the one described by Cannavan et al. [9], using only volatile components to elute levamisole.

3.3. Mass spectrometry

The chemical structures of levamisole and methyllevamisole are shown in Fig. 1, as well as their MS and MS–MS spectra, obtained after direct infusion of a standard solution of 20 μg/ml in the APCI source. In MS mode, the most prominent ion for both compounds is the protonated molecular ion $[M-H]^+$ at m/z 205.1 and 219.1 for levamisole and methyllevamisole, respectively. The formation of only a few product ions is typical for ionization techniques such as APCI. In MS–MS mode, two prominent product ions are formed as can be seen in Fig. 1: at m/z=145.3 and 178.0 for levamisole and at m/z=159.2 and 192.0 for methyllevamisole. The product ions of both compounds show the same mass differences with respect to their protonated molecular ions, indicating that the fragmentation mechanism is the same. A third significant product ion at m/z= 202.2 is present in the methyllevamisole MS–MS spectrum.

For quantitative purposes, we preferred to operate in the MS–MS full scanning mode. Quantitation was performed on the two most abundant product ions appearing in the MS–MS spectrum of levamisole and the internal standard (at m/z=145.3 and 178.0 and at m/z=159.2 and 192.0, respectively).

Fig. 2 shows an MS–MS mass chromatogram, as well as the corresponding MS–MS spectrum, for a levamisole standard (concentration: 0.5 μg/ml), a blank kidney sample, a kidney sample spiked with levamisole at a concentration of 10 ng/g tissue (=MRL), and an incurred kidney sample (levamisole concentration: 2906 ng/g). The same data are shown in Fig. 3 for the internal standard methyllevamisole. Mass chromatograms and spectra for muscle, liver, fat and skin plus fat were similar to those shown for kidney. As can be seen from Figs. 2b and 3b, blank sample chromatograms are clean and free from endogenous interferences at the retention times of levamisole (3.4 min) and methyllevamisole (4.6 min), which demonstrates the high specificity of the LC–MS–MS technique. These chromatograms show only some noisy signals (on a very amplified scale, compared to the sample chromatograms) throughout the time window between 2 and 6 min, when the divert valve is sending the HPLC eluate to the mass spectrometer.

Moreover, for qualitative purposes, i.e. identification or confirmation of the analytes of interest, the LC–MS–MS technique has superior properties in comparison to the more traditional LC–UV or LC–DAD techniques. The identity of both levamisole and the internal standard can be confirmed, not only by their (relative) retention time, but also by their MS–MS spectrum. As can be seen from Figs. 2 and 3, high quality mass spectral data were obtained for standards (Figs. 2a and 3a), as well as for spiked calibration samples (Figs. 2c and 3c), and, more importantly, also for incurred tissue samples (Figs. 2d and 3d). In addition, the EU recommendations for

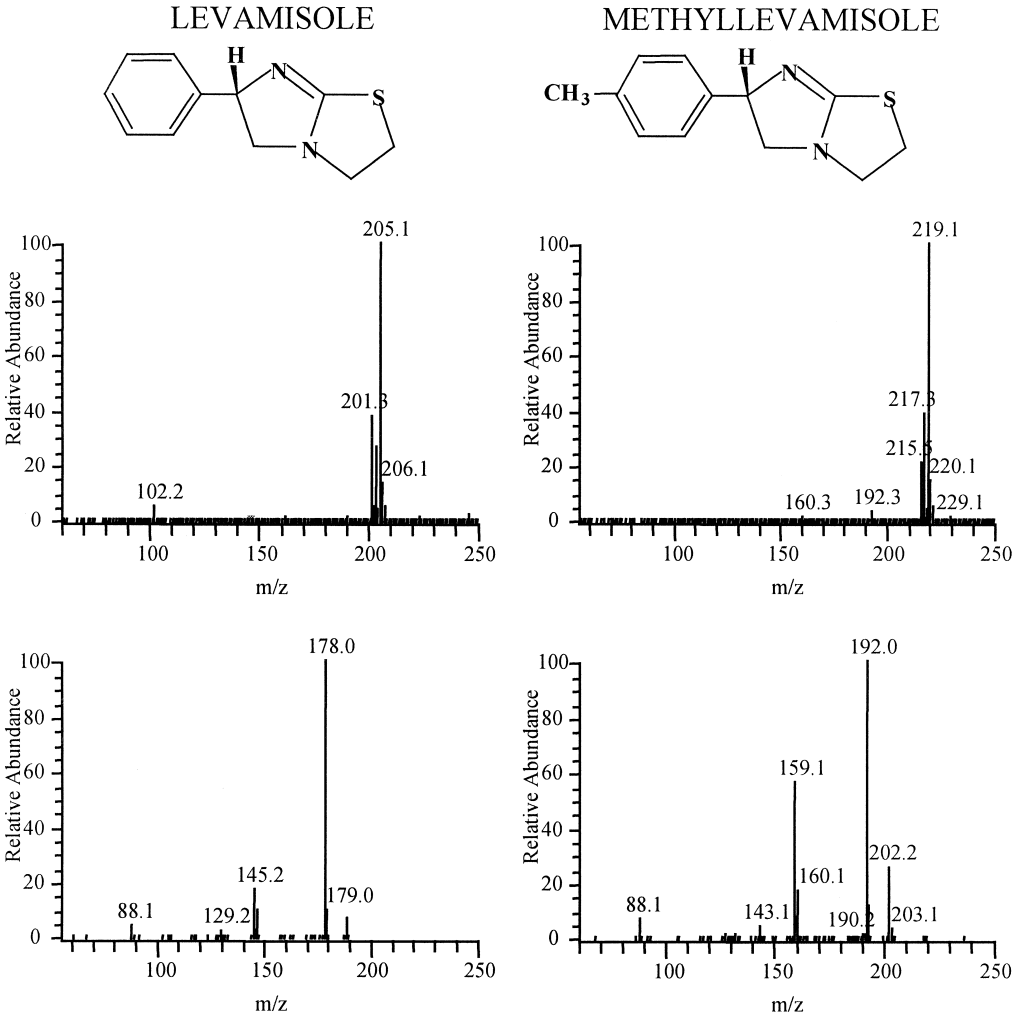

Fig. 1. Chemical structure, MS and MS–MS spectra of levamisole and methyllevamisole, obtained after direct infusion of standard solutions of 20 μg/ml of both components (APCI, positive ion mode, collision energy in MS–MS=1 V).

confirmation of the presence of an analyte in a sample by a CI mass spectrometric technique, are fulfilled: three diagnostic ions are present in the mass spectrum of methyllevamisole and the relative abundance of these ions in spiked or incurred samples, compared to the most abundant ion, has to be within a ±20% range of the relative abundance of the same ions in a standard sample (Table 1). The mass spectrum of levamisole shows only two diagnostic ions, but the criteria concerning the relative abundance of product ions (±20% range) have been fulfilled for all tissue matrices (Table 1). The identity of levamisole was also confirmed by its relative retention time (RRT) (=retention time of levamisole/retention time of methyllevamisole). The relative retention time of levamisole fell in all tissue samples (spiked and incurred) within a ±3% range, compared to a standard sample. The following RRT-values were obtained for spiked tissue samples: 0.753 for muscle, 0.746 for kidney, 0.752 for liver,

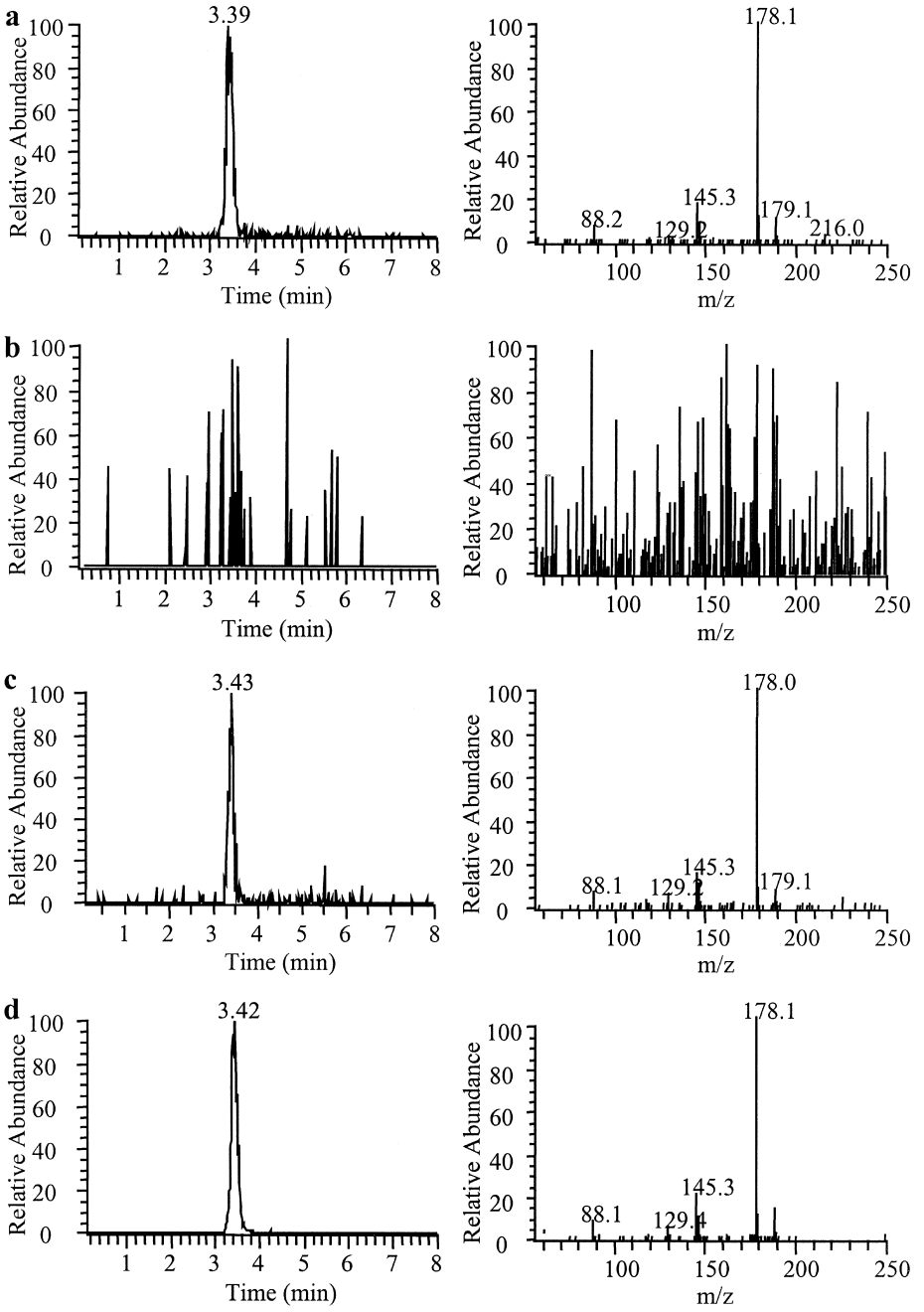

Fig. 2. Mass chromatogram and MS–MS spectrum corresponding to the MS–MS product ions of levamisole, for a standard sample at 0.5 μg/ml (a), a blank kidney sample (b), a kidney sample spiked at 10 ng levamisole/g kidney (=MRL) (c), and an incurred kidney sample (levamisole concentration: 2906 ng/g kidney; animal slaughtered 16 h after levamisole administration) (d).

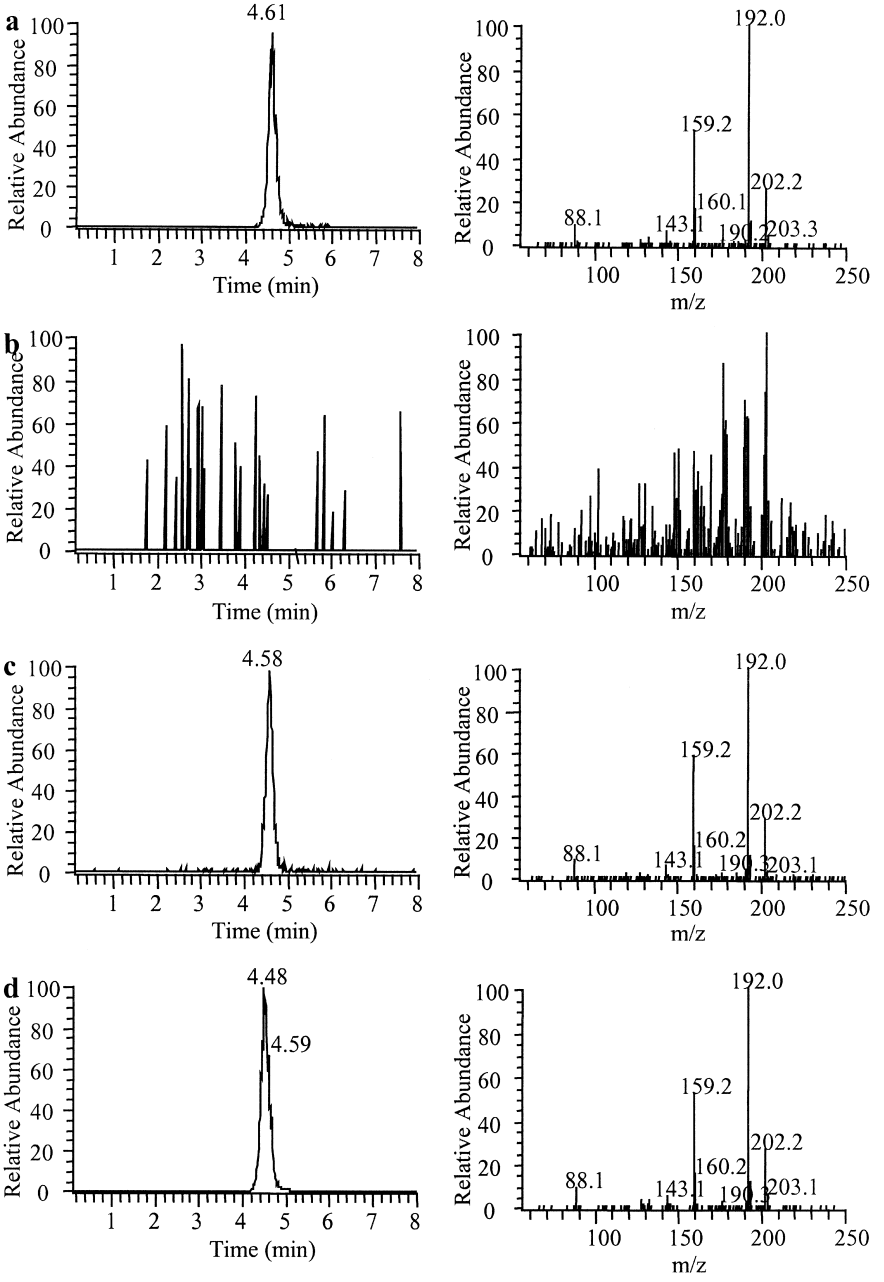

Fig. 3. Mass chromatogram and MS–MS spectrum corresponding to the MS–MS product ions of methyllevamisole, for a standard sample at 0.5 μg/ml (a), a blank kidney sample (b), a kidney sample spiked at 10 ng methyllevamisole/g kidney (c), and an incurred kidney sample (I.S.: 1000 ng/g kidney; animal slaughtered 16 h after levamisole administration) (d). All four samples correspond to the ones of Fig. 2.

Table 1
Comparison of the relative abundance of the product ions selected for quantification (at $m/z=145.3$ and 178.0 for levamisole, and at $m/z=159.2$, 192.0 (and 202.2) for methyllevamisole) for a standard sample (2 μg/ml aqueous solution), a spiked blank tissue sample (10 ng levamisole and 10 ng/g methyllevamisole/g tissue), and an incurred tissue sample (levamisole concentrations: kidney: 2906 ng/g; muscle: 626 ng/g; liver: 1855 ng/g; fat: 291 ng/g; skin plus fat: 2015 ng/g; methyllevamisole concentration in all tissues: 1000 ng/g; all tissues of animals slaughtered 16 h after administration of levamisole). The reference ion is the most abundant ion for each component and is set at 100%. For the tissue sample, the first value corresponds to the spiked blank tissue sample and the second value to the incurred sample

Compound	Product ions (m/z)	% In standard	Acceptance limits	% In muscle	% In kidney	% In liver	% In fat	% In skin plus fat
Levamisole	145.3	17	13.6–20.4	19/15	15/20	16/14	15/19	20/17
	178.0	Reference ion						
Methyllevamisole	159.2	53	42.4–63.6	56/56	55/52	58/60	54/50	52/56
	192.0	Reference ion						
	202.2	27	21.6–32.4	28/29	25/26	27/29	28/25	23/25

0.745 for fat and 0.756 for skin plus fat. The acceptance limits were 0.734 to 0.780, based on the value of 0.757 found for a standard sample.

3.4. Method validation

3.4.1. Linearity

The results of the linearity evaluation are summarized in Table 2. The correlation coefficients of the calibration curves in the various matrices were above 0.99. Furthermore, the goodness of fit coefficients g of the calibration curves in the different tissues were all below 10%, indicating the good quality of the calibration curves [16].

3.4.2. Accuracy and precision

The accuracy and 'within-run or within-day' precision of the method were determined using six independently spiked blank tissue samples at three different spike levels: at the MRL level (10 ng/g), at half the MRL (5 ng/g) and double the MRL (20 ng/g) (for liver: 100, 50 and 200 ng/g respectively), and using the calibration curves of Table 2. The results are summarized in Table 3. The accuracy fell for all matrices within the range of −30% to +10% and −20% to +10% for levamisole concentrations between 1 and 10 ng/g and >10 ng/g, respectively, showing the good accuracy of the method. The precision fell for all matrices also within the maximum RSD values. The 'between-day' precision was determined using independently spiked blank tissue samples at the MRL level, analyzed on different days. The results are summarized in Table 4. Also here, the accuracy and precision fell within the specified ranges.

3.4.3. Limit of quantification (LOQ)

The LOQ was established by analysing six blank tissue samples which were spiked at half the MRL for levamisole: at 5 ng/g for muscle, kidney, fat and skin plus fat tissues and at 50 ng/g for liver tissue. The results are summarized in Table 3.

One must remark however that for liver this might be a somewhat misleading value. It is possible to obtain for liver a calibration curve in the low range (0–25 ng/g), according to the normal acceptance criteria, indicating that also for this tissue the real LOQ might be 5 ng/g. We did, however, no valida-

Table 2
Calibration curve results of levamisole in pig muscle, kidney, liver, fat and skin plus fat (linear regression equation: $y = ax + b$)

Matrix	Slope, a (SD)[a]	Intercept, b (SD)[a]	r	Goodness of fit (%)
Muscle	0.3366 (0.0286)	0.0528 (0.0218)	0.9931	8.84
Kidney	0.3069 (0.0204)	0.0069 (0.0072)	0.9928	9.89
Fat	0.3966 (0.0071)	0.0075 (0.0159)	0.9959	6.91
Skin plus fat	0.1526 (0.0054)	0.0061 (0.0045)	0.9981	6.02
Liver	0.4458 (0.0659)	0.0018 (0.0139)	0.9932	7.50

[a] Standard deviation ($n=3$).

Table 3
Results of the accuracy and precision evaluation (within-run or within-day) of the validation samples for levamisole in pig muscle, kidney, liver, skin and skin plus fat

Matrix	Conc. (ng/g)	Mean conc. (ng/g) ($n=6$)	RSD (%) within-day precision	RSDmax (%)	Accuracy (%)
Muscle	5.0	5.06	14.9	23.7	+1.1
	10.0	9.02	20.1	21.3	−9.8
	20.0	19.69	11.4	19.2	−1.5
Kidney	5.0	4.89	12.4	See muscle	−2.3
	10.0	10.50	8.7		+5.0
	20.0	18.20	4.2		−9.0
Fat	5.0	4.39	15.1	See muscle	−12.2
	10.0	10.05	7.0		+0.5
	20.0	20.22	6.7		+1.1
Skin plus fat	5.0	5.00	8.8	See muscle	+0.1
	10.0	10.46	5.2		+4.6
	20.0	19.35	11.3		−3.2
Liver	50.0	44.4	4.7	16.7	−11.2
	100.0	101.6	4.1	15.1	+1.6
	200.0	189.9	7.7	13.6	−5.0

tion experiments at such a low level for liver, given the higher MRL value for this tissue.

3.4.4. Limit of detection (LOD)

The LOD was determined using the slope of the calibration curve and the residual standard deviation and taking into account the internal standard concentration. The calculated LOD values are all in the same range (muscle: 3.10 ng/g; kidney: 4.20 ng/g; fat: 3.10 ng/g; skin plus fat: 2.60 ng/g; liver: 3.19 ng/g), and not really much lower than the established LOQ values, except for liver. The fact that the LOD for liver is as low as for the other tissues corroborates our previous remark that also the real LOQ value of liver might be as low as for the other tissues, namely 5 ng/g.

One must remark that the above established LOQ and LOD values are likely not the lowest that can be reached with this method. Using a separate scan for levamisole and methyllevamisole at their respective retention times would increase the sensitivity of the method, since the detector is then allowed to fragmentate and to detect only one of both components during a defined time interval. We scanned simultaneously for both components over the whole run time. Working in SRM (selected reaction monitoring), monitoring in MS–MS mode only the most prominent product ions at $m/z=178.0$ and 192.0 for levamisole and methyllevamisole, respectively, is also expected to increase slightly the sensitivity of the method, without losing any confidence in identity [18]. As previously mentioned, we used a full scan of the MS–MS spectrum in the ranges 55 to 250 and 60 to 250 for levamisole and methyllevamisole, respectively.

From the above presented validation results, it is

Table 4
Results of the accuracy and precision evaluation (between-day) of samples spiked with levamisole at the MRL level in pig muscle, kidney, liver, skin and skin plus fat

Matrix	Conc. (ng/g)	Mean conc. (ng/g) ($n=4$)	RSD (%) between-day precision	RSDmax (%)	Accuracy (%)
Muscle	10.0	9.78	9.1	21.3	−2.2
Kidney	10.0	9.47	11.4	21.3	−5.3
Fat	10.0	10.44	8.8	21.3	+4.4
Skin plus fat	10.0	10.68	11.7	21.3	+6.8
Liver	100.0	104.6	10.5	15.1	+4.6

clear that the method proposed for the quantitative determination of levamisole in pig tissue samples fulfils all criteria required by the European Community. It could therefore be successfully used for residue depletion studies of levamisole in pigs.

4. Conclusion

The method described in this paper is rapid, simple, sensitive and specific. It has the advantage over some earlier published methods that it provides mass-related data which become desirable for residue analysis [14]. A simple liquid–liquid back extraction and a further solid-phase clean-up were found to be a sufficient sample preparation for this type of specific analysis. Therefore it was possible in routine to prepare up to 24 samples to be analysed within 1 working day by the same operator. It has the further advantage over some previously reported methods that it provides fully satisfactory validated quantitative data at levels as low as 5 ng/g, being half the MRL value fixed at the European level, and this for five different porcine tissue types evaluated. Therefore, it could be successfully used for residue depletion studies of levamisole in pigs, according to the European guidelines. These results will be presented elsewhere. The proposed method is in our opinion also one of the most attractive actually available to be tested on other animal species such as sheep, cattle and poultry, for which levamisole is also used.

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