Determination of Levamisole in Animal Tissues Using Liquid Chromatography with Ultraviolet Detection

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An efficient and sensitive liquid chromatographic method is described for the determination of the anthelminthic drug levamisole, in muscle, liver, kidney and fat of sheep, pigs and poultry, using thiabendazole as internal standard. Samples were extracted by homogenizing with chloroform, and were applied to Supelco Si solid-phase extraction columns and eluted with methanol. Chromatographic analysis was performed on a LiChrospher 60 RP−Select B column using methanol/ammonium acetate buffer 0.05 M (55/65, v/v) as mobile phase and reading at 220 nm. The quantification limit for the assay was 4 ng/g. Mean recoveries were about 84% for liver, 85% for kidney, 89% for muscle and 84% for fat. The assay has been used for statutory testing purposes.

Keywords: *Levamisole determination; anthelmintic; animal tissue; veterinary drug residues*

INTRODUCTION

Levamisole, the L-isomer of tetramisole, is an anthelminthic used for many years for the control of gastrointestinal parasites in domestic ruminants. Its fast, intense therapeutic effect and versatility (it can be administered in many ways) explain its widespread use, especially in intensive meat production.

Several papers concerning the determination of residues of this drug in milk, biological fluids, and animal tissues but not in fat have been published. The main existing techniques are high-performance liquid chromatography (HPLC) (*1*−*3*), gas chromatography (GC) with nitrogen−phosphorus detection (*4*, *5*) or mass spectrometric detection (*6*), and liquid chromatography−thermospray mass spectrometry (LC-MS) (*7*).

This paper describes a chromatographic method for assaying this active ingredient in animal tissues such as muscle, liver, kidney, and fat of sheep and pigs, and liver, kidney, and fat + skin of poultry.

The proposed method gave results similar, in terms of recoveries (about 84% for liver, 85% for kidney, 89% for muscle, and 84% for fat), to reports in the literature concerned with determination of levamisole residues in tissues; literature values ranged from 63 and 86% (*3*, *4*−*7*). The most innovative aspect of our method concerns the quantification limits achieved (4 ng/g), which are much lower than those reported for techniques based on LC with UV detection (limits of the order of 20 ng/g of matrix) (*1*−*3*). To obtain similar results, much more sensitive methods, such as GC (*4*) or LC−MS (*7*) (quantification limits of ∼5 ng/g), have hitherto been necessary.

EXPERIMENTAL PROCEDURES

Materials. Levamisole was obtained from Sigma-Aldrich S.r.l. (Gallarate, Milan, Italy) and thiabendazole used as internal standard from Merck (Darmstadt, Germany). Solvents and reagents were from Merck. Three milliliter SPE Si columns were obtained from Supelco (Bellefonte, PA), and 0.45 μm LIDA filters (Kenosha, WI) were used to filter samples before injection into the chromatograph.

Equipment. A Perkin-Elmer LC series 410 pump and an SEC-4 solvent environmental control apparatus for degassing of solvents (Norwalk, CT), connected to a Perkin-Elmer UV/VIS LC295 detector, were used for chromatographic analysis. Chromatograms were recorded with a Perkin-Elmer 1022 Plus integrator.

Analytical Method. *Extraction from Muscle, Liver, Kidney, and Fat.* Internal standard (200 μL of a methanolic solution containing 20 μg/mL of thiabendazole) was added to an exact weight of 5 g of matrix (muscle, liver, kidney, or fat) of the various animals to a concentration of 800 ng/g of matrix. Extraction was performed after the addition of 1 g of solid NaCl, 1 g of solid $NaHCO_3$, and 10 mL of chloroform and homogenization with an IKA blender for 5 min at 20500 rpm. The sample was then centrifuged at 3000*g* for 15 min. The organic solvent was separated, and a second extraction of the matrix was performed with another 20 mL of chloroform. After centrifugation, the two chloroform extracts were pooled prior to evaporation in a Rotavapor apparatus. The residue was made up with 5 mL of a chloroform/cyclohexane mixture (1:3 v/v).

The sample thus prepared was ready for solid−liquid extraction. The SPE Si columns were conditioned with 5 mL of chloroform/cyclohexane (1:3 v/v) before the sample was passed; levamisole and the internal standard were retained in the column. The columns were then washed with 5 mL of the same mixture and dried; the compounds of interest were eluted with 2 mL of methanol. The residue obtained by evaporating off the solvent was made up with 500 μL of the mobile phase. After filtration with 0.45 μm filters, the sample was injected into the chromatograph.

Quantification was performed using a calibration curve based on the ratio of levamisole peak area to internal standard area.

Chromatographic Method. Chromatographic analysis was performed with a Merck LiChrospher 60 RP-Select B (5 μm, 125 × 4 mm) column. The mobile phase was methanol/ammonium acetate buffer, 0.05 M (55:65 v/v) at 1.0 mL/min. Before use, the solvents were filtered through 0.45 μm cellulose nitrate filters. The UV detector was used at a wavelength of 220 nm. The areas of the chromatographic peaks expressed in mV/time were used to quantify the compounds.

Recovery of Levamisole from the Various Matrices. Known quantities of levamisole, corresponding to the lower and upper points of the respective calibration lines, were added

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Table 1. Mean Recoveries and Standard Deviations Obtained for the Determination of Levamisole in the Various Matrices (mean of at least five determinations at each concentration)

animal	concn[a]	muscle	liver	kidney	fat
sheep	a	89.34 ± 3.41	84.61 ± 4.12	85.49 ± 4.81	87.03 ± 3.77
	b	90.28 ± 2.68	84.57 ± 3.04	84.61 ± 3.24	88.40 ± 3.99
pigs	a	88.35 ± 3.68	83.47 ± 3.68	87.30 ± 3.77	82.67 ± 4.15
	b	89.72 ± 3.08	84.12 ± 3.22	87.37 ± 3.63	82.59 ± 3.77
poultry	a	90.52 ± 4.01	85.96 ± 4.35	84.32 ± 4.28	87.45 ± 4.80
	b	91.23 ± 3.66	84.97 ± 3.08	85.92 ± 2.67	88.22 ± 3.32

[a] The following concentrations were used for the determinations: (a) 0.020 and (b) 0.800 μg/g for liver samples and (a) 0.004 and (b) 0.800 μg/g per for the other matrices.

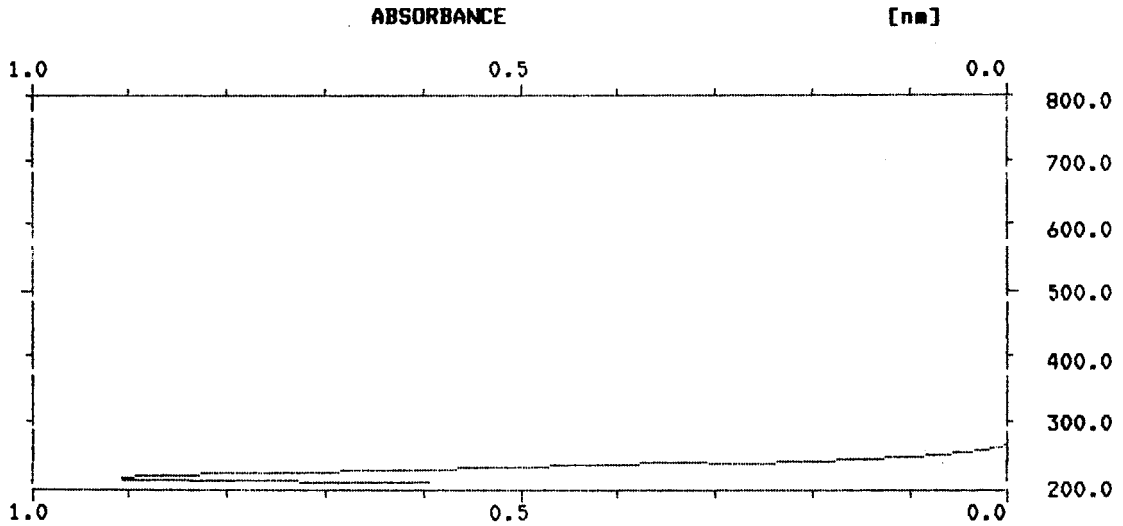

Figure 1. UV−vis spectrum of the methanol solution of levamisole in the range 0f 200−800 nm.

to five aliquots of muscle, liver, kidney, and fat from untreated animals. Five samples of each matrix were extracted as described under Experimental Procedures. Comparison was made with respect to direct injection of solutions of levamisole.

Construction of Calibration Curves. To construct the calibration curves and check the linearity of the method, five samples were prepared for each of the seven concentrations of levamisole selected. The samples were obtained by adding 200 μL of the following methanol solutions of levamisole to muscle, liver, kidney, and fat from animals not treated with this compound: 0.5, 1.25, 2.5, 5, 10, 15, and 20 μg/mL for muscle, kidney, and fat and 0.1, 0.5, 1.25, 2.5, 7.5, 15, and 20 μg/mL for liver. The samples were then extracted and analyzed as described for each method under Experimental Procedures.

The calibration curves for the different matrices were constructed so as to cover a range of content from 0.004 to 0.800 μg/g for muscle, kidney, and fat and from 0.020 to 0.800 μg/g for liver.

RESULTS AND DISCUSSION

Extraction for Determination of Levamisole. The extraction method was found to be operationally valid and to have good recovery. Recoveries of levamisole and thiabendazole were determined from the various matrices and from water and were not significantly different for the two compounds (92.87 ± 2.12 and 91.39 ± 2.37%, respectively). Thiabendazole as internal standard was selected on the basis of the similar recovery from water for the two compounds. The samples used to evaluate recovery are reported together with the method under Experimental Procedures.

Means and standard deviations (SD) for levamisole recovery are reported in Table 1. It is interesting that there were no significant differences in recovery between samples with low and high contents of the drug. Figure 1 shows a UV−vis spectrum of the levamisole.

Table 2. Results Obtained in Precision and Accuracy Determination of the Method (Mean of Five Samples for Each Concentration Used)

animal	concn[a]	muscle	liver	kidney	fat
		Mean			
sheep	a	0.0210	0.0041	0.0203	0.0212
	b	0.413	0.408	0.408	0.435
	c	0.812	0.787	0.792	0.794
pigs	a	0.0198	0.0042	0.0210	0.0186
	b	0.387	0.374	0.398	0.394
	c	0.783	0.784	0.813	0.836
poultry	a	0.0216	0.0037	0.0218	0.0193
	b	0.392	0.425	0.426	0.421
	c	0.817	0.812	0.822	0.755
		CV Percent			
sheep	a	3.56	4.35	4.22	3.59
	b	3.26	3.84	3.27	4.03
	c	3.54	3.64	3.41	2.80
pigs	a	3.84	4.08	4.09	3.48
	b	3.75	4.09	3.64	2.94
	c	3.08	3.25	2.48	3.06
poultry	a	4.01	4.67	3.86	3.60
	b	2.89	4.27	4.05	3.41
	c	2.67	3.96	3.07	3.52
		Recovery Percent			
sheep	a	105.00	102.50	99.00	106.00
	b	103.25	102.00	102.00	108.75
	c	101.50	98.37	100.50	99.25
pigs	a	99.00	105.00	105.00	103.00
	b	96.75	93.50	99.50	98.50
	c	100.37	98.00	101.62	104.50
poultry	a	108.00	92.50	109.00	96.50
	b	97.87	106.25	106.50	105.25
	c	102.12	101.50	102.75	94.37

[a] The following concentrations were used for the determinations: (a) 0.020, (b) 0.400, and (c) 0.800 μg/g for liver samples and (a) 0.004, (b) 0.400, and (c) 0.800 μg/g for the other matrices.

Figure 2 shows some examples of chromatographic profiles obtained by analysis of blank samples and

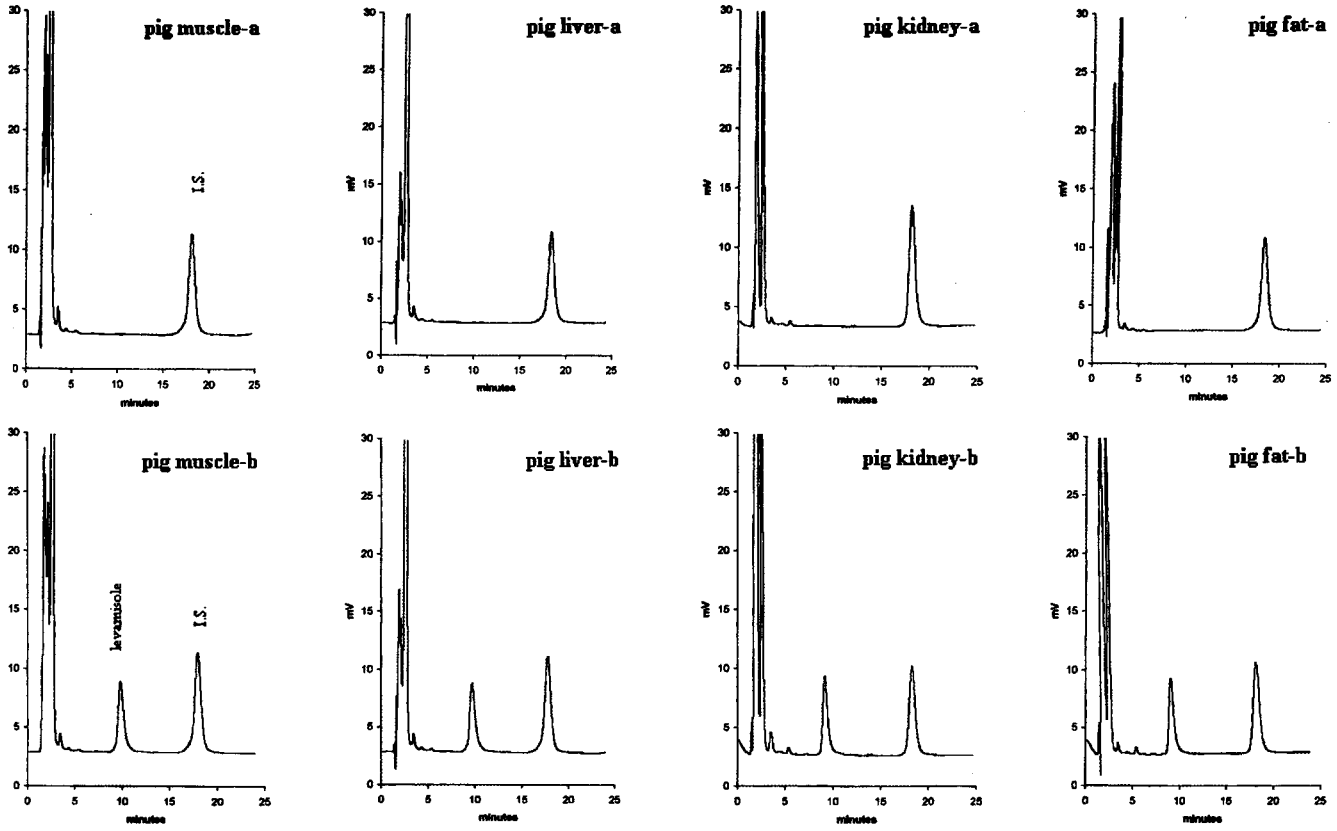

Figure 2. Examples of chromatographic profiles obtained by analysis of blank samples (a) and samples spiked with levamisole (b) (I.S. = internal standard, thiabendazole).

samples of the various matrices spiked with levamisole.

Analytical Method. For the chromatographic method we determined specificity, robustness, precision, accuracy, and linearity for the various matrices.

Specificity was determined using two samples of each matrix, both from an untreated animal but one spiked with a known quantity of solution of the substance in question and internal standard. After extraction, these samples were analyzed by chromatography as reported under Experimental Procedures. With all matrices, the chromatograms showed an absence of interfering peaks at the wavelength and retention times used for analysis.

The robustness of the method was evaluated by varying the ratios of the components of the mobile phase by 2% and the flow by 10%. In no case were changes in the specificity of the method observed, and the peak areas were not significantly different for the two compounds.

Precision was determined using 15 samples at three different concentrations of levamisole for each matrix. These samples were prepared by adding exact quantities of the three solutions of known concentration, corresponding to the lowest, an intermediate, and the highest value of the calibration line, to samples from untreated animals. After these additions, the samples were extracted and analyzed under the conditions reported under Experimental Procedures. Percentage coefficients of variation were never >10%.

Accuracy was determined with the same samples as for determination of precision. For none of the matrices were differences from the theoretical value >10%.

Results obtained in the precision and accuracy determination are shown in Table 2.

The mean calibration parameters of the curves constructed for the various matrices are shown in Table 3.

Table 3. Parameters of Calibration Lines Obtained with the Various Matrices (Mean of at Least Five Determinations)[a]

animal		muscle	liver	kidney	fat
sheep	intercept	0.0019	−0.0029	0.0003	0.0018
	slope	0.6754	0.7356	0.6257	0.7258
	R^2	0.9986	0.9987	0.9994	0.9979
pigs	intercept	0.0027	−0.0011	−0.0002	0.0014
	slope	0.6789	0.7108	0.6148	0.7004
	R^2	0.9989	0.9992	0.9989	0.9988
poultry	intercept	0.0015	−0.0027	0.0005	0.0022
	slope	0.6440	0.7189	0.6258	0.6706
	R^2	0.9991	0.9993	0.9988	0.9994
quantification limits (μg/g)		0.004	0.020	0.004	0.004

[a] The following ranges of concentrations were chosen for calibration: 0.020−0.800 μg/g for liver samples and 0.004−0.800 μg/g for the other matrices.

The quantification limit for levamisole was 4 ng/g for muscle, kidney, and fat and 20 ng/g for liver.

Figure 2 shows some examples of chromatographic resolutions obtained in the analysis of blank samples of the various matrices and samples spiked with levamisole.

The quantification limit was determined by analysis of samples with known concentration of analyte and establishing the minimum level at which the levamisole can be quantified with acceptable accuracy and precision.

The methods were found to be precise and accurate, with quantification limits for the various matrices that make them suitable for determining residue levels of levamisole in animals treated with this drug; in fact, the statutory maximum residue limits (MRLs) of levamisole, set by the European Union (*8*), are 10 ng/g in

muscle, kidney, and fat and 100 ng/g in liver of beef cattle, sheep, pigs, and poultry.

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