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Journal of Chromatography B, 796 (2003) 371–377
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatographic method with ultraviolet absorbance detection for measurement of levamisole in chicken tissues, eggs and plasma

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Received 12 May 2003; received in revised form 12 August 2003; accepted 15 August 2003

Abstract

The development and validation of a high-performance liquid chromatographic and UV detection method was accomplished for quantitative determination of levamisole in chicken tissues, eggs and plasma. The chromatographic separation was achieved on Luna® 5 μm C_{18} column using a mobile phase of 0.2% acetic acid in water:methanol (50:50 (v/v)) and Pic B-7 low UV reagent and the pH was adjusted to 7.31 with ammonium hydroxide and UV wavelength was 225 nm. Limits of quantification were 0.025 μg/g for all tissues and 0.003 μg/ml for plasma. Limit of detection was 0.001 μg/g for tissues and plasma.

Keywords: Levamisole

1. Introduction

Levamisole is the laevo isomer of di-tetramisole, which is a racemic mixture. The parent compound, tetramisole, was first marketed as an anthelmintic in 1965 but it was soon noted that its anthelmintic activity resided almost entirely in the L-isomer, levamisole. Thus, it was determined that the dosage could be reduced by half using the L-isomer alone. Reducing the dosage has an advantage of decreasing the risk of toxicity with the same anthelmintic potency [1].

Levamisole is widely used as an anthelmintic in cattle, sheep, goats, swine, and poultry. It is effective against lungworms and gastrointestinal nematodes. It is also used as adjuvant therapy in the treatment of human cancer [2]. Levamisole is currently being used to treat capillaria infection in poultry [3]. Doses for the use of levamisole in poultry are available [4], although the use of levamisole in poultry is not approved by the US Food and Drug Administration. No information is available from Food Animal Residue Avoidance Databank (FARAD) or Food and Agriculture Organization (FAO) regarding the residue potential, metabolism or withdrawal times of levamisole use in poultry [5].

Although it is used in poultry industry [6], the USA tolerance level in edible tissues (0.1 μg/g) is only applicable for cattle, sheep, goats and swine [3] because there is not enough information about withdrawal in chickens. Since the withdrawal time is different in various animal species, it is difficult to predict the with-

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doi:10.1016/j.jchromb.2003.08.025

drawal time in chickens after medication with levamisole.

Various methods have been published for the determination of levamisole in biological fluids and tissues. The only analytical methods that use HPLC with UV detection to measure levamisole are for biological fluids, i.e. plasma [7–9], serum [10], and milk [11], and they resulted in early elution of levamisole and its internal standard. The analytical methods for measuring levamisole in tissues used methods other than HPLC–UV. These methods include gas chromatography (GC) with thermionic specific detection in animal plasma and tissue [12], capillary GC with mass spectrometric detection in liver [13], HPLC with thermospray-mass spectrometric detection in tissues [14], and HPLC with atmospheric pressure chemical ionization (APCI) mass spectrometry in tissues [15]. An HPLC method with photodiode array detection [16] was described for measuring of levamisole in meat, the column used was a micro Bondapak C_{18}.

In an attempt to eliminate co-elution of endogenous compounds peaks, another extraction method [12] was used with one of the chromatographic methods [15]. When the problem with co-elution of levamisole and endogenous peaks continued, it was decided to develop a new HPLC–UV analytical method. The new technique involved changing to a Luna C_{18} column and modifying the mobile phase.

This work describes a new method for the quantitative determination of levamisole in chicken tissue samples by high pressure liquid chromatography combined with UV detection. Standardization was done by using methyllevamisole as an internal standard to get precision and accuracy of the method. Published methods for multi liquid–liquid extraction steps were used for extraction of the drug from different tissue matrices. Finally, the method has been validated for quantitation of the drug at levels 0.025 μg/g for all tissues and 0.003 μg/ml for plasma.

2. Experimental

2.1. Chemicals

Levamisole hydrochloride was obtained from Sigma, St. Louis, MO, USA. Methyllevamisole hydrochloride, used as internal standard, was a generous gift from Jansen Pharmaceuticals, Beers, Belgium. All solvents for liquid–liquid extraction and mobile phase preparation (methanol, water, ethyl acetate, ethyl ether, hexane and chloroform) were HPLC grade and purchased from Fisher Scientific, Fair Lawn, NJ, USA. Chemicals used for both the extraction and mobile phase (glacial acetic acid, anhydrous sodium sulfate, sodium hydroxide, potassium hydroxide, hydrochloric acid and ammonium hydroxide) were analytical reagent grade and were purchased from Fisher Scientific. The ion-pairing compound (Pic B-7) used in the mobile phase was low UV reagent and purchased from Waters Corp., Milford, MA, USA.

2.2. Solutions

A stock solution of 1000 μg/ml levamisole was prepared in methanol. It was stored at −20 °C and replaced with a fresh stock solution every 3 months. Working solutions of levamisole were prepared by diluting stock solution with water, stored at 5 °C and replaced with fresh every month. The working solutions prepared for spiking tissues were 25, 20, 15, 10 and 5 μg/ml and for spiking plasma were 100, 50, 25, 20, 15, 10, 5, 1 and 0.5 μg/ml. Working solution of methyllevamisole at the concentration of 10 μg/ml was prepared by appropriate dilution of the stock solution with water. The storage conditions for methyllevamisole solutions were the same as for the levamisole solutions. By spiking 10 g tissues (breast muscle, thigh muscle, liver, fat, skin plus fat and egg) or 1 ml plasma with 100 μl of the 10 μg/ml working solution methyllevamisole concentrations of 0.1 μg/g of the tissues and 1 μg/ml of the plasma were obtained, and spiking each tissue or plasma with 100 μl of the respective working solution of levamisole, levamisole concentrations were 0.25, 0.2, 0.15, 0.1, and 0.05 μg/g in tissues and eggs and 10, 5, 2.5, 2, 1.5, 1, 0.5, 0.1 and 0.05 μg/ml in plasma.

2.3. Biological samples

Levamisole-free tissue samples (breast muscle, thigh muscle, liver, fat, skin plus fat and eggs) were obtained from local stores (Walmart and Kroger, Auburn, AL, USA). Plasma samples were obtained by collecting levamisole-free blood in heparinized tubes from chickens being processed at Auburn University

Poultry Farm, Auburn, AL, USA. The tissue samples were minced and homogenized using a Moulinex blender, Paris, France, and the blood samples were centrifuged for 5 min at 848 × *g* and plasma was separated. All tissue matrices and plasma were stored at −20 °C until analyzed.

2.4. Extraction procedures

2.4.1. Tissue extraction

The extraction process was done according to the method described by Heitzman [17] for measuring levamisole in animal tissues. Ten grams of tissue homogenate were transferred into a capped 50 ml polypropylene centrifuge tube and spiked with 100 μl of the working solution of the internal standard and 100 μl of the working solution of levamisole. After vortex mixing for 15 s, 5 g of anhydrous sodium sulfate was spread on the surface of the tissue and 1 ml 50% KOH was added and vortex mixed. To this mixture, 15 ml ethyl acetate was added and homogenized with Tekmar tissue-mizer (Cincinati, OH, USA) at maximum speed. The homogenized mixture was shaken with a horizontal shaker for 10 min and left motionless for another 10 min. The tubes were centrifuged at 848 × *g* for 15 min and the upper organic layer was transferred to another polypropylene tube. The tissue sample was re-extracted with another 15 ml ethyl acetate and centrifuged for 5 min at 848 × *g*. The ethyl acetate extracts were pooled and 5 ml 0.5 M HCl was added and vigorously shaken. After centrifugation, the organic layer was discarded and the acidic layer was transferred to a 12 ml polypropylene tube. The solution was made alkaline with 1 ml 50% KOH. The analyte and the internal standard were extracted with 100 μl chloroform and transferred to polypropylene micro-vial. After evaporation, the chloroform residue was dissolved in 125 μl methanol and vortex mixed and 125 μl water added giving a total volume of 250 μl. Volume injected in the HPLC system was 50 μl.

2.4.2. Plasma extraction

The extraction of levamisole from plasma was done according to a method described by García et al. [7]. Polypropylene centrifuge tube (15 ml) containing 1 ml plasma was spiked with 100 μl of the internal standard and 100 μl of the working solution of levamisole. To each tube was added 0.8 ml water and vortex mixed followed by 0.5 ml of 10N sodium hydroxide and vortex mixed followed by 5 ml of ethyl ether:*n*-hexane (80:20 (v/v)) and vigorously shaken. The mixture was centrifuged for 5 min at 848 × *g* and the organic layer was separated and dried at room temperature under a stream of nitrogen. The residue was re-dissolved in 100 μl of the mobile phase and 20 μl was injected in the chromatographic system.

2.5. Chromatography

A Waters (Milford, MA, USA) HPLC system was used. It consisted of a Model 510 pump, Model 717 autosampler, Model 746 data module, and Model 486 programmable multi-wavelength UV detector. The chromatographic condition included a Luna 5 μm C_{18} 150 mm × 4.6 mm analytical column (Phenomenex, CA, USA) and guard column packed with Perisorb RP-18 (Upchurch Scientific, Oak Harbor, Washington, USA). The mobile phase was prepared after modification of that in García et al., 1990 [7]. One liter 2% acetic acid in water:methanol (50:50 (v/v)) and one bottle of PIC B-7 low UV reagent with the pH adjusted to 7.31 with concentrated ammonium hydroxide solution. Flow rate was 1 ml/min. The UV detection was at wavelength of 225 nm. The data module attenuation level was at 64, linearity range in tissue was 0.05–0.25 μg/g and the limit of detection was 0.001 μg/g.

2.6. Validation measurements

In order to evaluate the suitability of the method for the quantitative determination of levamisole, validation studies were carried out by applying a set of parameters which are in compliance with the requirements as defined in the Rules Governing Medicinal Products in the European Community [18]. The method was validated at the tolerance level (0.1 μg/g) in cattle, sheep, goats and swine that is defined by the USDA [3], at half the tolerance level (0.05 μg/g) and at double the tolerance level (0.2 μg/g) in tissues and at 10, 5, 1, 0.5, and 0.1 μg/ml in plasma. The following parameters were examined.

The linearity of the method was evaluated by spiking blank tissue samples. For each calibration curve, different concentration levels were used, including a

zero level and concentrations of 0.25, 0.2, 0.15, 0.10 and 0.05 μg/g for tissues and 10, 5, 2.5, 2, 1.5, 1, 0.5, 0.1 and 0.05 μg/ml for plasma. Peak area ratios between levamisole and methyllevamisole were plotted against the concentration of levamisole and a linear regression was performed.

The recovery was defined as the percent of recovered amount of the drug from the spiked amount and it reflects closeness or agreement between the spiked value and the concentrations calculated in a series of experiments ($n = 6$). It was determined by analyzing six blank tissue samples spiked at the same concentration level and comparing the measured concentration to the spiked concentration. The concentrations used were 0.2, 0.1 and 0.05 μg/g for tissues and 10, 5, 1, 0.5, and 0.1 μg/ml for plasma.

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 on different days. It is expressed as the ratio between the standard deviation of the mean calculated concentrations and the mean calculated concentrations (expressed as percent). The maximum allowable tolerances for the imprecision ($R.S.D._{max}$) is calculated according to the equation $R.S.D._{max} = 2^{(1-0.5\log c)}$ with c being the spiked concentration [18]. For analyses carried out under repeatability conditions the R.S.D. (%) should not exceed two-thirds of $R.S.D._{max}$.

The specificity was evaluated by the analysis of blank tissue samples. The absence 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.

The limit of quantification (LOQ) 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 European Union Community, the LOQ has to be equal to or less than half the tolerance level (0.1 μg/g) according to the "Commission Decision No. 93/256 of the European Union Community" [18]. The limit of detection (LOD) is defined as the lowest measured content from which it is possible to indicate the presence of the analyte with reasonable statistical certainty.

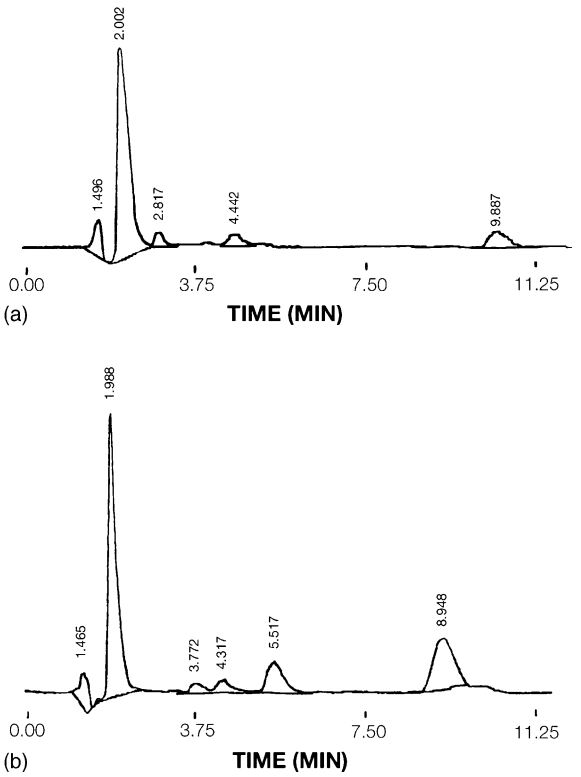

Fig. 1. Chromatograms of: (a) extracted blank breast muscle and (b) a blank breast muscle sample spiked with 0.05 μg/g of levamisole and 0.1 μg/g of methyllevamisole. The retention times for levamisole and methyllevamisole are 5.517 and 8.948 min, respectively.

Table 1
Calibration curve results of levamisole in chicken liver, breast muscle, thigh muscle, fat, skin plus fat, eggs and plasma (linear regression equation: $y = ax + b$)

Tissue	Slope a	Intercept b	r	Goodness of fit (%)[a]
Liver	8.29	−0.00895	0.993	8.6
Brest Muscle	8.29	−0.00902	0.993	7.2
Thigh Muscle	8.90	0.0764	0.997	4.6
Fat	6.37	0.113	0.980	9.0
Skin plus fat	6.53	0.0763	0.991	8.7
Egg	8.41	0.208	0.988	6.4
Plasma I (0.05–1.5)	0.689	6.22×10^{-5}	0.999	8.7
Plasma II (2–10)	0.692	0.0632	0.999	2.7

[a] Goodness of fit was calculated standard error of estimate to the mean recovered concentration expressed as percentage.

3. Results

3.1. Isolation of the compounds

Fig. 1(a) demonstrates that a blank breast muscle sample has no endogenous peaks that co-elute with the compounds of interest. Fig. 1(b) demonstrates that the extraction method used in these studies effectively extracts low levels of levamisole and methyllevamisole from spiked breast muscle tissues.

3.2. Method validation

The results of the linearity evaluation are summarized in Table 1. The correlation coefficients of the calibration curves in the various matrices were at or above 0.98. Furthermore, the goodness of fit coefficients of the calibration curves in different tissues were all below 10%, indicating the good quality of the calibration curves.

The recovery and "within-run or within-day" precision of the method were determined using six independently spiked blank tissue samples at three different spiked levels: at the tolerance level (0.1 μg/g), at half the tolerance level (0.05 μg/g) and at double the tolerance level (0.2 μg/g) in tissues and at 10, 5, 1, 0.5, and 0.1 μg/ml in plasma. The calculated concentrations were calculated according to the linear regression equation in Table 1. The results are summarized in Table 2. The precision R.S.D. (%) for all matrices was below two-thirds of the maximum R.S.D. values.

Table 2
Results of the recovery and the within-day precision evaluation of the validation samples for levamisole in chicken liver, breast muscle, thigh muscle, fat, skin plus fat, egg and plasma

Tissue	Concentration[a]	Mean concentration[b]	R.S.D.$_{max}$ (%)[c]	R.S.D. (%) within-day precision[d]	Recovery (%)[e]
Liver	0.05	0.044	25.11	4.63	86.00
	0.1	0.092	22.63	4.32	91.30
	0.2	0.180	20.39	1.81	89.00
Breast muscle	0.05	0.055	25.11	16.1	109.1
	0.1	0.100	22.63	9.81	100.1
	0.2	0.189	20.39	3.55	94.20
Thigh muscle	0.05	0.043	25.11	6.91	84.00
	0.1	0.089	22.63	2.49	88.00
	0.2	0.194	20.39	0.80	96.9
Fat	0.05	0.042	25.11	3.82	81.00
	0.1	0.110	22.63	5.61	109.0
	0.2	0.220	20.39	2.15	109.0
Skin plus fat	0.05	0.049	25.11	3.45	98.90
	0.1	0.098	22.63	1.40	97.60
	0.2	0.196	20.39	2.48	98.00
Egg	0.05	0.053	25.11	16.2	105.7
	0.1	0.095	22.63	14.1	94.70
	0.2	0.207	20.39	11.1	103.4
Plasma	0.1	0.114	22.63	7.83	110.0
	0.5	0.490	17.75	2.59	99.00
	1	1.010	16.00	3.04	100.9
	5	4.670	12.55	1.96	93.00
	10	9.720	11.31	2.79	97.1

[a] Concentration (μg/g or μg/ml) resulted from spiking blank tissue and plasma samples with levamisole ($n = 4$).
[b] Mean concentration (μg/g or μg/ml) calculated with HPLC–UV detection method ($n = 4$).
[c] R.S.D.$_{max}$ is calculated by using the equation ($\text{R.S.D.}_{max} = 2^{(1-0.5\log c)}$), with c being the spiked concentration of levamisole.
[d] R.S.D. (%) is the ratio of standard deviation of the mean calculated concentration to the mean calculated concentration in percentage [R.S.D. = (S.D./concentration) × 100].
[e] Recovery (%) = (calculated concentration/spiked concentration) × 100.

Table 3
Results of between-days precision evaluation of levamisole in chicken liver, breast muscle, thigh muscle, fat, skin plus fat, egg and plasma

Tissue	Concentration[a]	Mean concentration[b]	R.S.D.$_{max}$ (%)[c]	R.S.D. (%) between-day precision[d]	Recovery (%)[e]
Liver	0.10	0.090	22.63	6.24	89.00
Breast muscle	0.10	0.097	22.63	8.61	98.90
Thigh muscle	0.10	0.091	22.63	3.22	90.10
Fat	0.10	0.111	22.63	4.71	109.9
Skin + fat	0.10	0.105	22.63	9.18	104.8
Egg	0.10	0.100	22.63	13.4	100.3
Plasma	1.00	1.055	16.00	6.24	105.2

[a] Concentration (μg/g or μg/ml) resulted from spiking blank tissue and plasma samples with levamisole ($n = 4$).
[b] Mean concentration (μg/g or μg/ml) calculated with HPLC–UV detection method ($n = 4$).
[c] R.S.D.$_{max}$ is calculated by using the equation (R.S.D.$_{max} = 2^{(1-0.5 \log c)}$), with c being the spiked concentration of levamisole.
[d] R.S.D. (%) is the ratio of standard deviation of the mean calculated concentration to the mean calculated concentration in percent [R.S.D. = (S.D./concentration) × 100].
[e] Recovery (%) = (calculated concentration/spiked concentration) × 100.

The between-day precision was determined using independently spiked blank tissue samples at the tolerance level, and analyzed on different days. and precision (R.S.D. (%)) did not exceed two-thirds of R.S.D.$_{max}$ (Table 3).

The LOQ was established by analysing six blank tissue samples which were spiked at one-fourth of the tolerance level for levamisole (0.025 μg/g) at liver, breast muscle, thigh muscle, fat, skin plus fat and egg. The LOQ for plasma was established by analysing six blank plasma samples which were spiked at 0.003 μg/ml. The tissue and plasma samples had a recovery (%) between 81 and 110% and R.S.D. values of 9.65–11.98%, that fall within the limit of R.S.D.$_{max}$ value (27.87). The plasma samples had a recovery of 104% and R.S.D. value of 17% that falls within the limit of R.S.D.$_{max}$ value (35.5).

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 value was 0.001 μg/g for both tissues and plasma.

4. Discussion

When methods developed for HPLC with APCI mass spectrometry [15] or gas chromatography [12] were used to measure levamisole in tissues with HPLC–UV, they resulted in co-elution of levamisole–methyllevamisole with endogenous peaks. This problem was resolved by changing the column to a Luna C_{18} reversed phase column (Phenomenex) which has a large column efficiency for basic compounds (Comparison Guide to C_{18} Reversed Phase HPLC Columns, MacMod Analytical Inc., Chadds Ford, PA, USA). Levamisole is a basic compound [19]. The mobile phase described by García et al. [7] was modified by decreasing the 0.2% acetic acid in water:methanol ratio from 75:25 to 50:50 (v:v) and increasing the pH from 4 to 7.31. These changes resulted in a short retention time for the endogenous peaks and a longer retention time for levamisole–methyllevamisole. As the levamisole free base is adsorbed onto glass from solvents [20,21], therefore, polypropylene laboratory supplies were used for this work.

5. Conclusion

The method described in this study is rapid, simple, sensitive and specific. Up to 30 tissue samples or 50 plasma samples can be analyzed in a day. The assay will be particularly useful for laboratories that depend heavily on HPLC–UV methods for analysis of drugs in tissues. It can be successfully used for residue depletion studies of levamisole in animal tissues.

Acknowledgements

The authors would like to thank the Embassy of the Arab Republic of Egypt for supporting this project (GM-279).

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