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Note

Rapid Extrelut column method for determination of levamisole in milk using high-performance liquid chromatography

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Levamisole (Fig. 1) is a broad-spectrum anthelmintic drug used in cattle, sheep and pigs. Methods have been developed for levamisole quantification in biological fluids, based on polarographic [1], gas—liquid chromatographic (GLC) [2, 3] and high-performance liquid chromatographic (HPLC) [4, 5] procedures. A sensitive GLC method has been reported for the determination of levamisole in milk [6, 7]. However, this method is too time-consuming for determining residues of levamisole in a large number of milk samples.

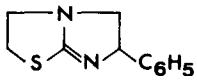

Fig. 1. Chemical structure of levamisole.

The purpose of this study was to develop a rapid, simple, and sensitive method for the determination of levamisole in milk. The method described is based on clean-up on an Extrelut column and quantitation by a HPLC procedure.

EXPERIMENTAL

Chemicals and reagents

Dichloromethane and hexane, both for pesticide residue analysis, were obtained from E. Merck (Darmstadt, F.R.G.), and levamisole hydrochloride from American Cyanamid (Princeton, NJ, U.S.A.). Levoripercol® vet (88.4 mg levamisole hydrochloride per ml) was purchased from AB Leo (Helsingborg, Sweden). All other chemicals were of analytical reagent grade.

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Standards

A stock solution of levamisole (1 mg/ml) in methanol was prepared using the hydrochloride salt. For HPLC calibration, the stock solution was diluted with methanol—0.05 *M* ammonium carbonate solution (65:35, v/v) to obtain standard solutions (1.0, 2.5, 5.0, 10, 20 and 40 μg/ml). For recovery experiments, various standard solutions of levamisole in methanol were prepared. The stock solution and all standard solutions were stored at about 4°C.

Apparatus

The liquid chromatograph consisted of a ConstaMetric III pump module with a SpectroMonitor III variable-wavelength ultraviolet (UV) detector (LDC, Riviera Beach, FL, U.S.A.), and a Valco N60 injector (Valco, Houston, TX, U.S.A.) with a 22-μl loop. The column (250 × 4.6 mm I.D.) was packed with TSK-GEL LS 410 ODS SIL (C_{18} chemically bonded silica gel, particle size 5 μm) (Toyo Soda, Tokyo, Japan).

Chromatographic conditions

The mobile phase was methanol—0.05 *M* ammonium carbonate solution (65:35, v/v), and the flow-rate was 1.0 ml/min. The wavelength of the UV detector was set at 220 nm. The system was operated at room temperature.

Extraction procedure

To 50 ml of milk was added 0.3—0.4 ml of 3 *M* hydrochloric acid to adjust the pH to about 4.6. The suspension was mixed with 15 ml of methanol and heated in a stoppered flask on a water-bath (50°C) for 15 min. The sample was then filtered through a folded filter paper. Of the filtrate 20 ml were applied to the top of an Extrelut 20 prepacked column (E. Merck), and after 15 min the column was washed with 100 ml of hexane. Then the column was eluted with 100 ml of dichloromethane. The eluate was evaporated to dryness in a rotary evaporator, and the residue was dissolved in 1.0 ml of methanol—0.05 *M* ammonium carbonate solution (65:35, v/v). A 22-μl aliquot was injected onto the column.

Calibration curve

The calibration curve was constructed by plotting the peak areas versus the concentrations. The concentrations of unknowns were calculated from peak area ratios by interpolation of the calibration curve.

Recovery study

To measure recovery, various concentrations of levamisole were added to milk and the samples were extracted as described above. The percentage recovery was determined by comparing the peak areas of levamisole extracted from samples with peak areas obtained by direct injection of standard solutions.

RESULTS AND DISCUSSION

The earlier reported GLC method for the determination of levamisole in milk is based on several extraction steps and is rather time-consuming [6, 7]. The use of Extrelut for clean-up, after precipitation of the proteins, was found to be a rapid and simple method for extraction of levamisole in milk samples. The HPLC system used is similar to that earlier reported by Marriner et al. [4] for the determination of levamisole in plasma and gastrointestinal fluids. However, Marriner et al. also use a time-consuming clean-up procedure, which was not suitable for our purpose. Representative chromatograms of milk samples are shown in Fig. 2. In the chromatogram obtained from a blank milk sample (Fig. 2A), no peaks are present which might interfere with the determination of levamisole. Fig. 2B shows a chromatogram of an extract of milk spiked with 0.4 μg/ml levamisole. The retention time of levamisole was about 6—8 min.

The retention time varied slightly from day to day owing to instability of the mobile phase and changes of temperature in the laboratory. The retention time of levamisole was always shifted when a fresh mobile phase was used.

To prevent incorrect identification of levamisole, we constructed a new calibration curve every day. The calibration curve was linear, $y = 2.51x + 0.25$ ($n = 6$) over the range 1.0—40.0 μg/ml, with a correlation coefficient of 0.999. The average recovery of milk samples spiked with 0.2—2.0 μg/ml levamisole (Table I) corresponded to 78 ± 7.1% (mean ± S.D., $n = 11$). The limit for determination of levamisole was found to be between 0.02 and 0.05 μg/ml.

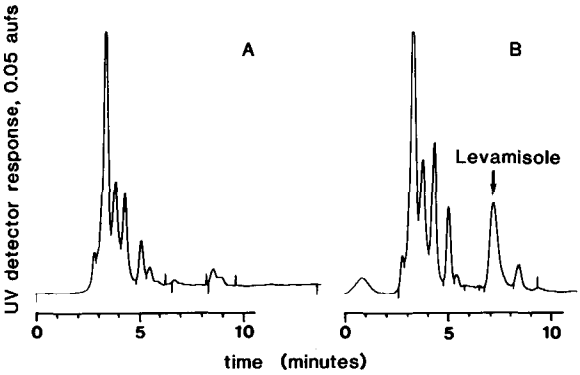

Fig. 2. Chromatograms of an extract of (A) blank milk, (B) milk spiked with levamisole (0.4 μg/ml).

TABLE I

RECOVERY OF LEVAMISOLE FROM MILK

Amount added (μg/ml)	No. of determinations	Recovery (%, mean ± S.D.)	Coefficient of variation (%)
0.2	3	76 ± 10.8	14.2
0.4	4	80 ± 5.9	7.4
2.0	4	77 ± 6.4	8.3
Mean		78 ± 7.1	9.1

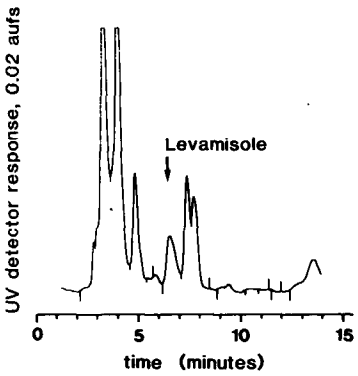

Fig. 3. Chromatogram of an extract of milk from a cow 24 h after administration of 7.1 mg of levamisole hydrochloride per kg body weight.

TABLE II

LEVAMISOLE RESIDUES IN MILK FROM TWO COWS AFTER ADMINISTRATION OF LEVAMISOLE HYDROCHLORIDE (7.1 mg/kg BODY WEIGHT)

Time after treatment (h)	Levamisole conc. (μg/ml)	
	Cow 1*	Cow 2**
0	ND***	ND
0.25	0.02	0.02
8	0.76	0.93
16	0.26	0.15
24	0.06	0.03
48	ND	ND
72	ND	ND
96	ND	ND

*Cow weight = 539 kg.
**Cow weight = 460 kg.
***ND = not detected = less than 0.02 μg/ml.

For routine analysis of a large number of milk samples, the recovery, precision and limit of determination of the described method are satisfactory. The addition of a suitable internal standard prior to extraction would permit a better precision of the method if required. (±)-2,3,5,6-Tetrahydro-6(4-methylphenyl)imidazo[2,1-*b*]thiazole hydrochloride (R-8493) has previously been used as internal standard in two GLC methods [2, 3].

The method has been applied to study levamisole residues in the milk of cows injected with this drug. Levamisole hydrochloride (Levoripercol vet) was administered subcutaneously at a dose of 7.1 mg/kg body weight, the usual therapeutic dose. Milk samples were collected in duplicate before the injection and at intervals up to 96 h after treatment. Fig. 3 shows a chromatogram of an extract of milk from a cow 24 h after administration of 7.1 mg of levamisole hydrochloride per kg body weight. The maximum level of levamisole was found 8 h after the administration and the levamisole level was less than 0.02 μg/ml at 48 h (Table II). In Sweden the recommended withdrawal period for

levamisole is two days for milk. Thus the present method is suitable for the rapid determination of levamisole residues in milk samples to check that this recommendation is followed.

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