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Quantitation of levamisole in plasma using high performance liquid chromatography

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SUMMARY

A rapid and sensitive procedure is described for the quantitation of levamisole in plasma using high-performance liquid chromatography (HPLC). The procedure involves sample preparation using a reverse-phase C18 cartridge prior to chromatography and quantitation using peak area ratios (UV absorbance detection, 225 nm) of levamisole to the internal standard, quinine.

The limit of detection was 21 ng/ml and the limit of quantification was 72 ng/ml, both contained in 1 ml of plasma. The recoveries were sufficiently high (73.1%) and overall coefficient of variation of the procedure was 0.25 %. This procedure has been used to determine levamisole levels in human and cattle plasma. A comparison of using two C18 columns (Nova-pak®, Puresil®) was also studied and discussed.

INTRODUCTION

Levamisole, (S)-(–)-2,3,5,6-tetrahydro-6-phenyl imidazo [2,1b] thiazole (I, Fig. 1) is widely used as an anthelmintic agent in veterinary and human health (1–3). More recently, due to its immunostimulant properties, levamisole has been used to treat diseases with immunity insufficiency (4,5) and for his anticancer activities (6).

Several analytical assays have been described for quantitation of levamisole in plasma, in animal tissue, and in milk (Table I). The polarographic determination and gas-liquid chromatography (GLC) have the advantage of giving good reproducibility and a low detection limit (7–10). Gas-liquid chromatography allows the use of an internal standard to realize the assay. A high performance liquid chromatography method for quantitation of levamisole in biological fluids has been described (11) but it does not use an internal standard and the mobile phase has a low stability.

In this paper, we report a rapid and reproducible

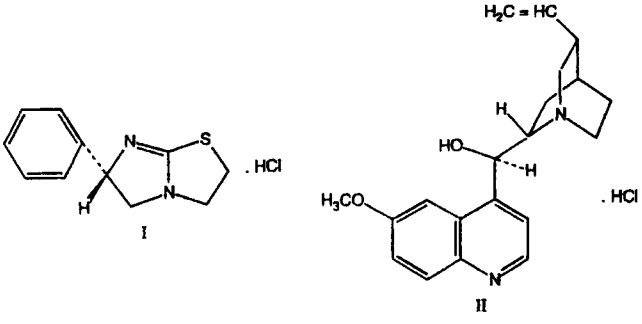

Fig. 1 : Chemical structures of levamisole hydrochloride (I) and quinine hydrochloride, the internal standard (II).

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paired-ion chromatography technique for quantitation of levamisole in plasma or for pharmacokinetic studies (limit of detection, 21 ng/ml using UV detection). Comparison of the results obtained using the described method with two C18 columns (Nova-pak®, Puresil®) is also presented.

MATERIALS AND METHODS

Materials and reagents

Levamisole hydrochloride, quinine hydrochloride and bovine plasma were obtained from Sigma Chemical Co. (St Louis, MO, USA). Levamisole was used in the preparation of spiked plasma standards. Quinine hydrochloride was used as the internal standard. 1-Pentane sulfonic acid sodium salt was obtained from Janssen Chimica (Belgium). Human plasma was purchased from Biological Specialty Corporation (Lansdale, PA, USA).

Potassium dihydrogenphosphate and phosphoric acid were obtained from Merck (Darmstadt, Germany). Acetonitrile was glass distilled, certified HPLC grade and obtained from UCB Belgium. All HPLC solvents were filtered on 0.45 µm Millipore filters and then deaerated under reduced pressure prior to use. Reverse-phase C18 sample cartridges (Analytichem Bond Elut LRC®, Varian, Sunnyvale, CA, USA) were of analytical purity and used in sample preparation.

Collection of plasma samples

5 ml blood samples were withdrawn before administration of levamisole and other blood samples were collected at 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after oral administration to human volunteer and after transcutaneous administration to cattle. Blood samples were centrifuged and plasma was frozen at –20°C until analysed.

HPLC assay

The quantitative determination of levamisole was realized with an HPLC-Waters delivery system (pump M45, injector U6K, detector M481, integrator baseline 810, sensitivity at 0.01 AUFS).

The analytical columns (C18 Nova-pak® : 3.9 mm i.d x 150 mm, 4 mm particle size and a porosity of 60 Å; C18 Puresil® : 4.6 mm x 150 mm, 5 mm particle size and a porosity of 120 Å) were used for in vitro analytical determinations. C18 Puresil® was used for in vivo quantitative determinations. The mobile phase consisted of 15% (v/v) acetonitrile and 85% (v/v) 0.05 M potassium dihydrogenphosphate. The paired-ion

Table I : Different analytical methods proposed for the assay of levamisole.

Analytical method	*Column and detector*	*Limit of detection*	*Internal standard*	*Biological fluid*	*References*
Gas-liquid chromatography	3% OV-17 80/100 mesh; AFID	4 ng/ml	2,3,5,6-tetrahydro-6-(4-methylphenyl) imidazo [2,1b] thiazole hydrochloride	Plasma	(7)
Gas-liquid chromatography	3% OV-17 80/10 mesh; AFID	5 ng/ml	2,3,5,6-tetrahydro-6-(4-methylphenyl) imidazo [2,1b] thiazole hydrochloride	Plasma, ruminal fluid	(8).
Gas-liquid chromatography	5% Apiezon L 10% KOH Anakrom ABS 110–120 mesh; AFID	10 ng/ml	—	Milk	(9)
Polarography		5 ng/g 1 ng/ml	—	Animal tissue, milk	(10)
High-performance liquid chromatography	C18; UV 220 nm	20–50 ng/ml	—	Plasma, ruminal fluid	(11)

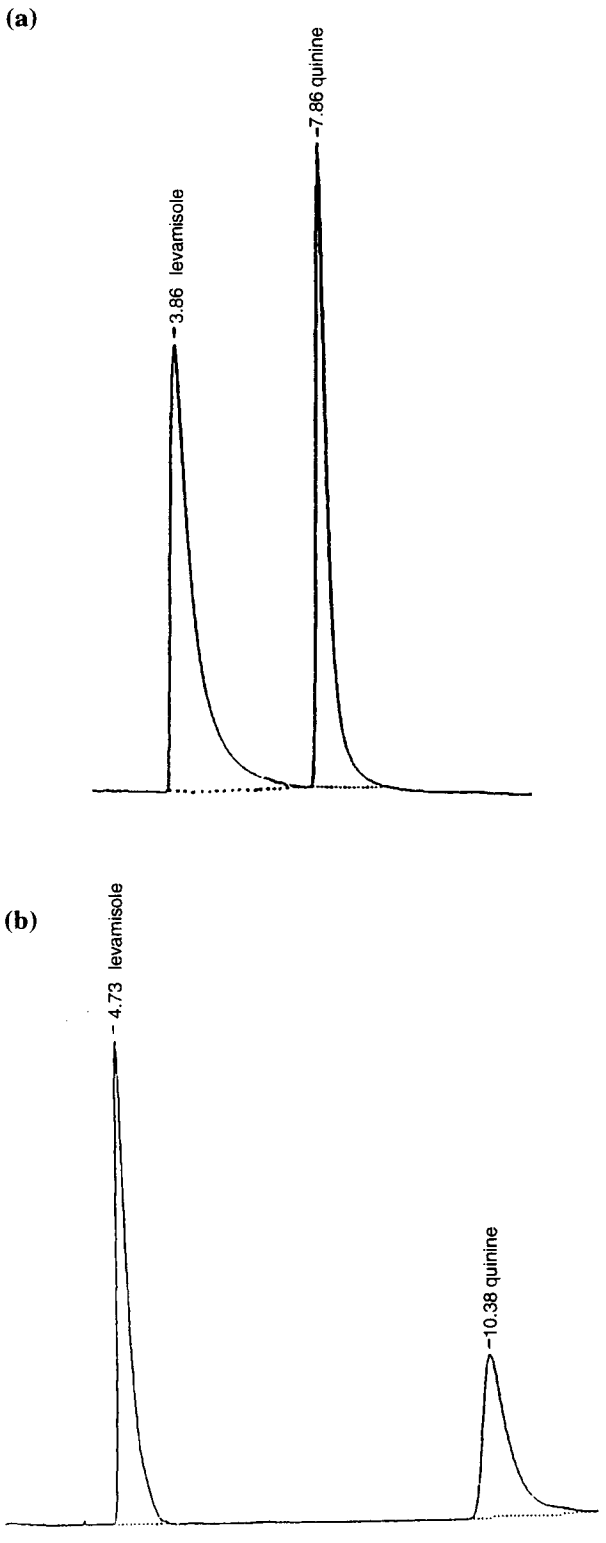

Fig. 2 : (**a**) HPLC chromatogram of 10 µg/ml of levamisole and 1 µg/ml of quinine (column Puresil®). (**b**) HPLC chromatogram of 10 µg/ml of levamisole and 1 µg/ml of quinine (column Nova-pak®).

agent (1-pentane sulfonic acid sodium salt) was used at 5 mM in the aqueous phase. The pH of the mobile phase was adjusted to 3 with phosphoric acid. The flow rate was maintained at 1.0 ml/min.

Methods

Deproteinization and elimination of polar serum constituents was accomplished using C18 reverse phase sample preparation cartridges. The cartridges were conditioned by washing with 2.0 ml of acetonitrile followed by 5.0 ml of water. A 1.0 ml aliquot of plasma sample or plasma standard was placed in a 2.0 ml capped plastic tube. Quinine hydrochloride, 100 µl of a 10 µg/ml solution, was then added to each sample to serve as an internal standard. An alkaline pH was achieved by the addition of 0.125 ml of 0.033 M NH_3 to each sample. The samples were mixed by vortexing and each was added to a reverse-phase cartridge. Each cartridge was then washed with 2.0 ml of water to eliminate the water soluble substances followed by 3.0 ml of acetonitrile to elute levamisole and quinine. The eluant was collected in a disposable glass tube.

The sample was evaporated to dryness using a gentle nitrogen stream at 50°C, and the residue reconstituted with 100 µl of acetonitrile and mixed by vortexing. A 25 µl aliquot was injected into the HPLC system.

Levamisole hydrochloride plasma standards were prepared by spiking human and cattle plasma with 100 µl of levamisole hydrochloride stock solution to give final concentrations ranging from 0.01–10 µg/ml. Levamisole was quantitated by comparison of the peak area ratio of the drug to the internal standard using a calibration curve. The peak area ratios were plotted against concentrations of levamisole and analysed by linear regression to generate calibration curves. The limit of detection of the procedure was determined by assaying spiked plasma samples at concentrations ranging from 10 ng/ml to 1 µg/ml. The limit of detection was then defined as the concentration at which the signal-to-noise ratio was 3. The variability was assessed by performing replicate analyses (n = 5) using spiked plasma samples containing 10 µg/ml of levamisole. Aqueous solutions containing known amounts of levamisole (0.01 µg/ml to 10 µg/ml) were compared with spiked plasma standards undergoing analysis to calculate the percent recovery. Statistical analyses to determine means, standard deviations, correlation coefficients, coefficients of variation and linear regressions were performed using a Baseline 810 integrator (Waters Associates).

Table II : Determination of the chromatographic parameters for assay of levamisole with two C18 columns (Nova-pak® and Puresil®).

Chromatographic parameters	*Nova-pak®* *Mean (n = 5)*	*SD (n = 5)*	*CV %*	*Puresil®* *Mean (n = 5)*	*SD (n = 5)*	*CV %*
Theoretical efficiency $N = 16\left(\frac{t_1}{w_1}\right)^2$	155	19	12.26	429	53.39	12.44
Factor of capacity K'1 $K'1 = \frac{t_1 - t_0}{t_0}$	3.36	0.02	0.59	2.60	0.00	0.00
Factor of capacity K'2 $K'2 = \frac{t_2 - t_0}{t_0}$	7.73	0.03	0.39	7.47	0.01	0.13
Factor of selectivity $\alpha = \frac{K'_2}{K'_1}$	2.30	0.01	0.43	2.88	0.00	0.00
Factor of resolution $R = 2\frac{t_2 - t_1}{w_1 + w_2}$	2.61	0.11	4.21	5.56	0.01	0.18

RESULTS AND DISCUSSION

Representative chromatograms from assayed samples of levamisole (10 µg/ml) and quinine (1 µg/ml) in an aqueous solution with a C18 Nova-pak® column and a C18 Puresil® column under the same analytical conditions are shown in Figure 2. Retention times for levamisole and quinine are respectively 3.8 and 7.8 min for the C18 Nova-pak® column and 4.7 and 10.3 min for the C18 Puresil® column. The correlation coefficient of the standard curve was 0.999 for both C18 columns. The average recovery of levamisole obtained from the sample preparations over the range of concentrations used in the standard curve was 73.1% (SD 0.25%). The recovery of quinine was 87.2% (SD 0.36%). Using the described procedure with a C18 Puresil® column, 21 ng/ml concentrations of levamisole can be detected while maintaining a signal-to-noise ratio of 3. The limit of quantification in these analytical conditions is 72 ng/ml. However, the limit of detection and quantification are respectively 56 ng/ml and 186 ng/ml with a C18 Nova-pak® column. Both C18 columns (Puresil® and Nova-pak®) gave a good separation of the two compounds and levamisole did not interfere with the internal standard peak. However, the levamisole peak showed a considerable tailing with a C18 Nova-pak® column (Fig. 2). This fact explains the difference of limits of detection and quantification between the two C18 columns. Table II lists the results for different chromatographic parameters, for both columns. The significant advantages of the present procedure over the previously published HPLC assay (11) were the use of an internal standard which facilitates accurate and precise levamisole quantitation and the use of a mobile phase with good sta-

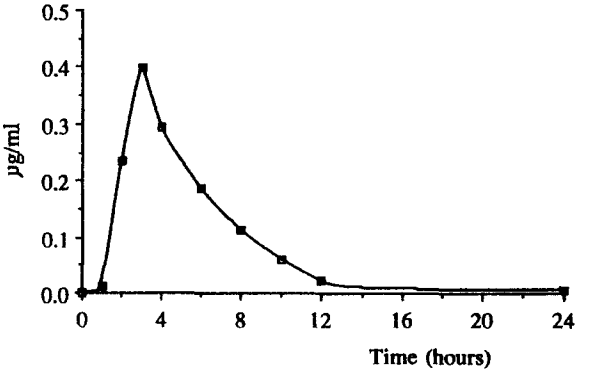

Fig. 3 : Plasma concentration profile of levamisole in a human subject following oral administration of 150 mg of the drug (Ergamisol®, Janssen Pharmaceutica).

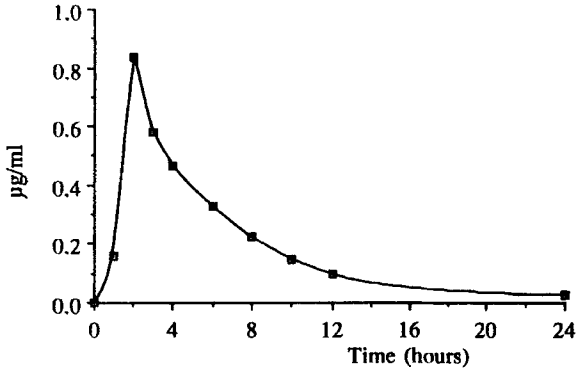

Fig. 4 : Plasma concentration profile of levamisole in two cows after transcutaneous administration of 10 mg/kg of levamisole (L-Ripercol pour-on®, Janssen Pharmaceutica).

bility. The previous HPLC method did not use internal standardization although it involved extraction and concentration procedures (11). Finally, several chromatographic parameters such as selectivity, capacity, resolution and theoretical efficiency for the two C18 chromatographic columns are compared under the same chromatographic conditions.

APPLICATIONS

This procedure has been used to successfully analyse human and cattle plasma. Plasma levels from an 82 kg male volunteer receiving an oral dose of 150 mg levamisole in three 50 mg tablets (Ergamisol®, Janssen Pharmaceutica) are shown in Figure 3. This method was also applied to the determination of the pharmacokinetic profile of levamisole from two cows weighing respectively 282 kg and 324 kg after transcutaneous administration (10 mg/kg) of a solution (L-Ripercol pour-on®, Janssen Pharmaceutica) containing 200 mg/ml of levamisole. These plasma levels are shown in Figure 4.

In summary, an HPLC method by paired-ion using UV detection has been found to be rapid, reproducible and sensitive. Moreover this procedure allowed the use of an internal standard. This analytical assay has been able to determine the pharmacokinetic profile after administration of levamisole to a human subject and to cattle.

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