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DETERMINATION OF LEVAMISOLE IN PLASMA AND ANIMAL TISSUES BY GAS CHROMATOGRAPHY WITH THERMIONIC SPECIFIC DETECTION

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SUMMARY

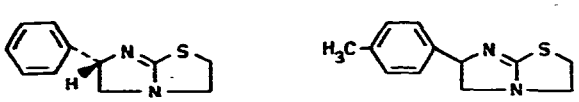
A rapid and sensitive method has been developed for the determination of the anthelmintic levamisole in plasma and tissues from man and animals. The procedure involves the extraction of the drug and its internal standard from the biological material at alkaline pH, back-extraction into sulphuric acid and re-extraction into the organic phase (heptane—isoamyl alcohol). Several extraction steps can be omitted, however, whenever the gas chromatographic background permits and some operations can be simplified using Clin Elut™ extraction tubes.

The analyses were carried out by gas chromatography using a nitrogen-selective thermionic specific detector. The detection limit was 5 ng, contained in 1 ml of plasma or in 1 g of the various tissues, and recoveries were sufficiently high (79—86%).

The method was applied to human plasma samples in a comprehensive bioavailability study of levamisole in healthy volunteers, and to plasma and tissues in a residue trial in cattle. The effect of the blood collection technique on the plasma levels was also studied and pointed to decreased plasma concentrations when Vacutainer® tubes were used.

INTRODUCTION

Levamisole, (*S*)-(—)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1b]thiazole (I, Fig. 1), is widely used as a potent broad-spectrum anthelmintic agent [1—3].



I II

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

More recently it has also been shown to possess a marked influence on the immunological responses of animals and man, particularly in those individuals whose immune defences have for some reason become impaired [4, 5].

A gas—liquid chromatographic (GLC) method was described for determining levamisole residues in milk using an alkali flame ionization detector and external standard calibration [6]. Animal tissue concentrations have been determined by a polarographic method [7].

The present paper describes a rapid and reliable method for the determination of levamisole in plasma and animal tissues by GLC using a thermionic specific detector. The method was used to obtain more detailed information about the pharmacokinetics of the drug in man and animals.

EXPERIMENTAL

Reagents

Levamisole hydrochloride (R 12 564) and the internal standard (R 8493) or (+)-2,3,5,6-tetrahydro-6-(4-methylphenyl)imidazo[2,1b]thiazole hydrochloride (II, Fig. 1) were originally synthesized in our research laboratories and were of analytical grade. Spectrophotometric grade *n*-heptane and methanol were used (Uvasol, E. Merck, Darmstadt, G.F.R.); all other solvents and chemicals were of analytical grade. The Clin Elut™ tubes were of analytical purity (CE 1003. Analytichem International, Lawndale, CA, U.S.A.).

Standard solutions

Using the hydrochloride salts of I and II, stock solutions were prepared corresponding to 1 mg of the free base per ml of methanol. The levamisole stock solution was diluted to concentrations ranging from 0.05 to 30 μg/ml of methanol.

To spike the samples with the internal standard, the stock solution of II was further diluted to 5 μg/ml for the plasma samples and to 1 μg/ml for the various tissue samples.

Extraction procedure

Plasma. Plasma samples (1 ml) were pipetted into 15-ml glass centrifuge tubes and spiked with 0.5 μg of the internal standard. The solution was made alkaline with 2 ml of 1 *M* sodium hydroxide and 4 ml of a heptane—isoamyl alcohol mixture (95:5, v/v) were added. The tubes were carefully rotated for 10 min (35 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 *g*). The upper organic layer was transferred to a second centrifuge tube and the plasma was extracted again with 4 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were then extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation. The remaining acidic phase was made alkaline with 0.15 ml of concentrated ammonia (pH 10) and extracted twice with 2-ml aliquots of the heptane—isoamyl alcohol. The combined organic layers were finally evaporated to dryness under a stream of nitrogen in a water bath at 60°C.

Tissue samples. Aliquots of 1 g of the various tissues (muscle, liver, subcutaneous fat and kidney), ground by means of a Waring commercial blender,

were weighed into 15-ml glass centrifuge tubes and 0.1 μg of the internal standard was added. After the addition of 2 ml of 1 *M* sodium hydroxide, the samples were further homogenized with an Ultra-turrax TP 18/10 homogenizer and extracted as described above.

Use of the disposable extraction columns

Plasma samples of 1 ml, made alkaline and spiked with the internal standard as described above, were poured into the Clin Elut™ columns. After 2 min, 4 ml of the extraction solvent were added and the organic phase was allowed to drip into 5-ml test tubes. This step was repeated then with one more 2-ml aliquot of extraction solvent and the combined eluents were evaporated to dryness.

Calibration procedure

Using the levamisole standard solutions, samples of blank control plasma (1 ml) were spiked with levamisole at concentrations ranging from 0.01 to 3 μg/ml, and with the internal standard at a fixed concentration of 0.5 μg/ml.

The calibration tissue samples (rat muscle, 1 g) corresponded to 0.005—1 μg/g and the amount of internal standard was 0.1 μg per sample. These samples were then taken through the extraction procedures described previously.

Apparatus

All the chromatographic analyses were performed on a Varian Model 3700 gas chromatograph equipped with a Varian Model 8000 automatic sample injector and a thermionic specific detector containing an electrically heated ceramic-alkali bead. The glass column (200 × 0.2 cm) was packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column temperature was 255°C and the injector and detector temperatures were 290°C and 340°C, respectively. Nitrogen was used as a carrier gas at a flow-rate of 30 ml/min. The detector was operated at a bias voltage of —4 V and the bead heating current was adjusted at 3 A, corresponding to a bead temperature of about 800°C. To optimize the detector for the specific detection of nitrogen compounds, the detector bead was in the path of a gas stream comprising hydrogen and air at flow-rates of 4.5 and 175 ml/min, respectively.

Area integrations, calculations and plotting of the chromatograms as well as the control of the autosampler functions were carried out by a Spectra-Physics Model 4000 data system.

Gas chromatography

The extraction residues from plasma samples were reconstituted with 100 μl of methanol by vigorous vortexing, centrifuged for a few seconds, transferred to 200-μl microvolume vials and placed in the autosampler. Sample volumes of 2 μl were then automatically injected into the gas chromatograph. Tissue extraction residues were dissolved in 50 μl of methanol and aliquots of 2 μl were manually injected.

Calculations

Results were calculated by determining the peak area ratios of levamisole related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of the calibration samples.

RESULTS

The recovery of the extraction procedure, obtained after the analysis of 1 μg of levamisole added to 1 ml of control plasma or to 1 g of blank tissue, amounted to 86 ± 4% (mean ± S.D., $n = 5$) for the plasma extraction procedure using Clin Elut™ columns, and to 79 ± 5% (mean ± S.D., $n = 5$) for the tissue extraction procedure; the detection limits were 10 ng/ml of plasma and 5 ng/g of tissue. The precision was calculated as the coefficient of variation (C.V.) of the peak area ratios obtained after ten identical injections of one sample (plasma, 100 ng of levamisole per ml) and was 2.4%. The reproducibility was determined after 10 analyses of the same sample (plasma, 100 ng of levamisole per ml) resulting in a day-to-day variation of 8.9 (C.V., %). The retention times of levamisole and the internal standard were 3.4 and 4.4 min, respectively.

The concentration ranges for levamisole and the internal standard as well as the correlation coefficient and the mathematical expression of the standard curves for levamisole in both plasma and animal tissue are summarized in Table I.

TABLE I

CONCENTRATION RANGES AND MATHEMATICAL EXPRESSIONS OF THE LEVAMISOLE STANDARD CURVES

Biological material	Internal standard (ng/sample)	Levamisole range (ng/sample)	Regression equation $y = ax + b$*		Correlation coefficient	
			a	b	r	n
Plasma (1 ml)	500	10—3000	2.33	—0.01	0.9993	5
Animal tissue (1 g)	100	5—1000	13.23	—0.13	0.9998	5

*y = peak area ratio (levamisole/internal standard); x = ng of levamisole per sample.

DISCUSSION

Initially, a rapid and sensitive method was sought for the determination of levamisole in plasma samples from clinical and bioavailability studies in man.

In most plasma samples, obtained after blood collection in polyethylene tubes containing heparin as the anticoagulant, the gas chromatographic background permitted the omission of the back-extraction steps described in the Experimental section. The procedure could even be further simplified using the Clin Elut™ disposable extraction columns, reducing time-consuming

shaking, centrifuging and phase-transfer steps. In addition, the use of the autosampler allowed the analysis of the samples overnight.

Problems appeared, however, with the extraction of plasma samples whenever Vacutainer® tubes (Model 3200 QS, Becton-Dickinson & Co., Rutherford, NJ, U.S.A.) were used to collect the blood specimens. Interfering peaks were observed and this resulted in the necessity of back-extraction with sulphuric acid and re-extraction into the organic extraction solvent after alkalinization. These purification steps too could be simplified using a second Clin Elut™ tube. However, in comparison with the procedure using single tubes,

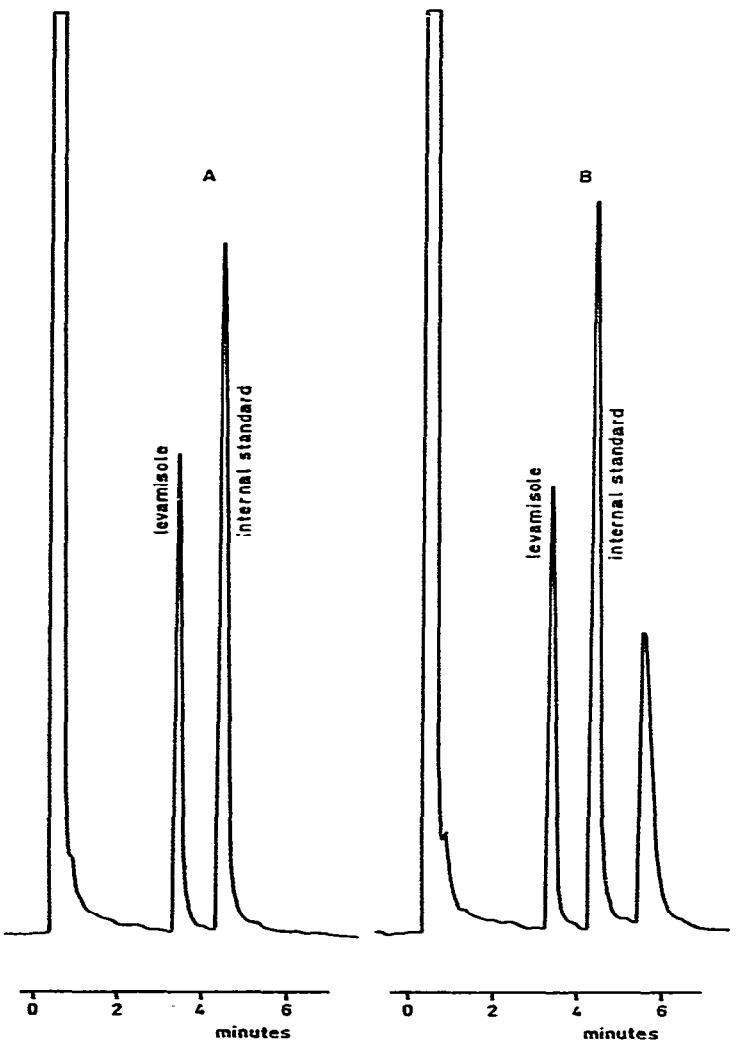


Fig. 2. Chromatograms of plasma extracts from a volunteer, 2 h after oral administration of levamisole, (A) after blood collection in a polyethylene tube, and (B) after collection in a Vacutainer® tube. The extractions were carried out using Clin Elut™ colums. Blood collection in the Vacutainer® tube required the use of an additional purification step using a second Clin Elut™ column. Chromatographic conditions were as indicated in the text.

the total recovery for both I and II declined about 5%, and non-interfering extraneous peaks were still observed, as can be seen in Fig. 2, which shows chromatograms from extracts of the same blood sample collected in a polyethylene tube containing heparin and in a Vacutainer® tube containing EDTA as the anticoagulant. An even more important draw-back of the use of Vacutainer® tubes was the resulting decrease of levamisole plasma concentrations in comparison to other blood collection techniques. Table II shows the levamisole plasma levels in two volunteers after oral administration of 100 mg of the drug. Blood samples were collected from the antecubital vein with Plastipak-BD syringes and transferred to Vacutainer® tubes with rubber stoppers and also to polyethylene tubes with polyethylene stoppers. All samples were then carefully rotated for 5 min (10 rpm); the plasma was then separated after centrifugation and both series of plasma samples were analyzed as described in the Experimental section. In all measurable plasma samples, exposure to the lubricated rubber stoppers lowered the levamisole concentration and resulted in a relative loss of 19.9 ± 8.3% (mean ± S.D.), indicating a highly significant difference ($p < 0.001$ by a paired t-test). A similar phenomenon has previously been reported for propranolol [8], lidocaine [9] and tricyclic antidepressants [10], in which cases it was attributed to the displacement of the drugs from the plasma proteins by stopper constituents and diffusion thereafter into the erythrocytes [11]. The use of Vacutainer® tubes should therefore be avoided whenever levamisole plasma levels are to be determined.

TABLE II

LEVAMISOLE PLASMA LEVELS IN TWO VOLUNTEERS AFTER ORAL ADMINISTRATION OF 100 mg OF THE DRUG

Values are expressed in μg/ml of plasma.

Time after dosage	Subject 1		Subject 2	
(h)	A*	B**	A*	B**
0	≤0.010	≤0.010	≤0.010	≤0.010
1	0.019	0.015	≤0.010	≤0.010
2	0.307	0.249	0.126	0.107
4	0.411	0.261	0.246	0.198
6	0.164	0.133	0.095	0.086

*Blood collected in 10-ml polyethylene tubes with polyethylene stoppers.
**Blood collected in Vacutainer® tubes.

The autosampler can not be used if low tissue residues of I must be detected, since the smaller volumes of methanol used to reconstitute the residues would be consumed in flushing the injection system. If the reconstituted residues are injected by hand, 5 ng of levamisole could be easily detected in 1 g of tissue as well as in 1-ml plasma samples.

The application of the method to the assay of several hundred plasma and tissue specimens demonstrated its suitability; interferences were not

observed and the gas chromatographic column proved to be extremely stable under the conditions used.

APPLICATIONS

The method described has been used in a bioavailability and dose proportionality study of levamisole in human volunteers. Plasma levels from a male volunteer receiving an oral dose of 150 mg of levamisole in three 50-mg tablets are shown in Fig. 3.

The method has also proved to be valuable in tissue residue trials in cattle after topical and oral dosage of the drug. Residues were measured in muscle, liver, subcutaneous fat and kidney at several times after administration.

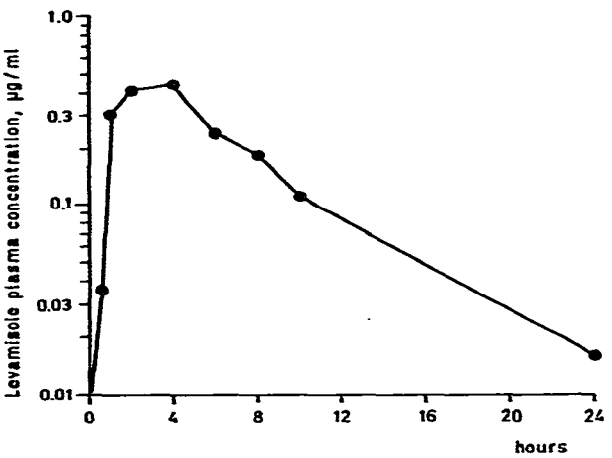


Fig. 3. Plasma concentration profile of levamisole in a human subject following oral administration of 150 mg of the drug in a tablet formulation.

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