

DETERMINATION OF LEVAMISOLE BY HPLC IN PLASMA SAMPLES IN THE PRESENCE OF HEPARIN AND PENTOBARBITAL

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ABSTRACT

A method which allows for the isolation of levamisole in plasma samples of rabbit which contained pentobarbital and heparin as well, used as an anaesthetic and an anticoagulant respectively, is described in this paper. The subsequent quantification was carried out using a ion-pair high-performance liquid chromatographic method in a reversed and isocratic phase at room temperature, using a UV detection at 225 nm. In our conditions the retention time for the levamisole was about 2.15 minutes and the average percentage of recuperation was that of 73.62 %.

INTRODUCTION

The levamisole *l*-(2,3,5,6-tetrahydro-6-phenylimidazo(2,1-b)thiazole) used initially as an anthelmintic in many animal species has also an important use as an immunomodulator in human medicine (1-5). A series of papers, in which methods for the determination of levamisole in biologic fluids, plasma being among them, by HPLC (6-9) and by GC (10), have been published over the last few years.

Owing to the experimental conditions in which we carried out our work, the determination of levamisole by direct application of the methods

described for HPLC was not satisfactory. We tried to determine levamisole in rabbit plasma which had been obtained from blood samples incoagulated with heparin. On the other hand, there were variable quantities of pentobarbital in the plasma samples, a compound previously used to anaesthetize rabbits with the aim of extracting blood from the carotid artery at short spaces of time.

The method was developed with the aim of avoiding the interference of pentobarbital and heparin, whose retention times were similar to those of levamisole, using a different extraction and purification procedure. On the other hand, the variations introduced into the chromatographic method allowed us to noticeably reduce the retention time of the compound with the consequent saving of time and mobile phase.

MATERIAL AND METHODS

Reagents

Levamisole HCl (Sigma); sodium pentobarbital (Barcia); sodium heparin 5 % solution (Leo); LC-water obtained in a Milli-Q (Millipore); ethylether, HPLC grade (Scharlau); n-hexane, HPLC grade (Scharlau); methanol, HPLC grade (Scharlau); Pic B7, low UV (Waters); sodium hydroxide, analytical grade (Merck).

Apparatus

The HPLC system employed consisted of: one pump (model SP8800, Spectra-Physic); a variable wavelength UV-VIS detector (model Uvikon 730SLC, Kontron); a computing integrator (model SP4290, Spectra-Physic); a sample injector fitted with a 20 μ l loop (model Rheodyne 7125, Rheodyne); a C₁₈ 10 μ m reversed-phase column (μ Bondapack C₁₈ 300x3.9 mm, Waters).

A slow rotatory mixer (Selecta) was used in extraction procedures.

Chromatographic conditions

A mobile phase of 0.2 % acetic acid in water: methanol (35:65, v/v), with Pic B7-low UV 0.005 M and adjusted to pH = 4.0 with acetic acid was used. The flow was 1.8 ml/min and the column effluents were monitored at 225 nm. The system was carried out at room temperature and under those conditions, normal retention times were 2.15 min, 2.38 min and 3.08 min for levamisole, heparin and pentobarbital respectively.

Preparations of standards

A levamisole stock solution (1mg/ml) in LC-water was prepared using hydrochloride salt, for HPLC calibration, the stock solution was diluted with

mobile phase in order to obtain seven standard solutions being 1, 2, 5, 10, 20, 50 and 100 μ g/ml and were injected with 20 μ l into the column.

For recovery experiments seven plasma standards were prepared by spiking a mixture of 1 ml of a drug-free pooled plasma rabbit and 0.9 ml of LC-water with a 100 μ l aliquot of each one standard solutions in water at appropriate concentrations.

Extraction procedure

0.5 ml of NaOH 10 N was added to each plasma standard solution and was then shaken on a slow rotatory mixer for five minutes. The mixture was extracted with 5 ml of ethylether:n-hexane (80:20, v/v) by rotomixing for 10 minutes. After centrifugation for 5 minutes at 2000 rpm, the organic layer was taken by aspirating and was transferred to a glass tube and then evaporated to dryness under a stream of nitrogen at 37 °C. The residue was redissolved in 100 μ l of a HPLC mobile phase and two portions were withdrawn to rinse and load the 20 μ l loop for injection.

Quantification

Four injections of each standard solution were made up. The peak height of standard solutions were analyzed by linear regression with respect to their concentration.

RESULTS AND DISCUSSION

Calibration curves with corresponding least-square regression equations and correlation coefficients (r) over the ranges studied are shown in Figure 1. It was necessary to consider two increasing concentration ranges (1 to 10 μ g/ml and 10 to 100 μ g/ml) in order to obtain better accuracy and precision. This case is also reported by Kouassi *et al.*(10).

The levamisole chromatograms in the model solution (A); that of plasma with heparin and pentobarbital (B) and that of plasma with levamisole, heparin and pentobarbital after extraction using our method (C) are shown in Figure 2. The efficiency of the extraction process can be observed in this figure which has allowed for the elimination of interferences of heparin and pentobarbital.

The minimum calculable concentration in the standard solutions is placed at 0.08 μ g/ml (equivalent to 0.8 μ g/ml of levamisole in plasma). It is still possible to detect lower concentrations but their quantification is not to be considered reliable. However the minimum calculable concentration in

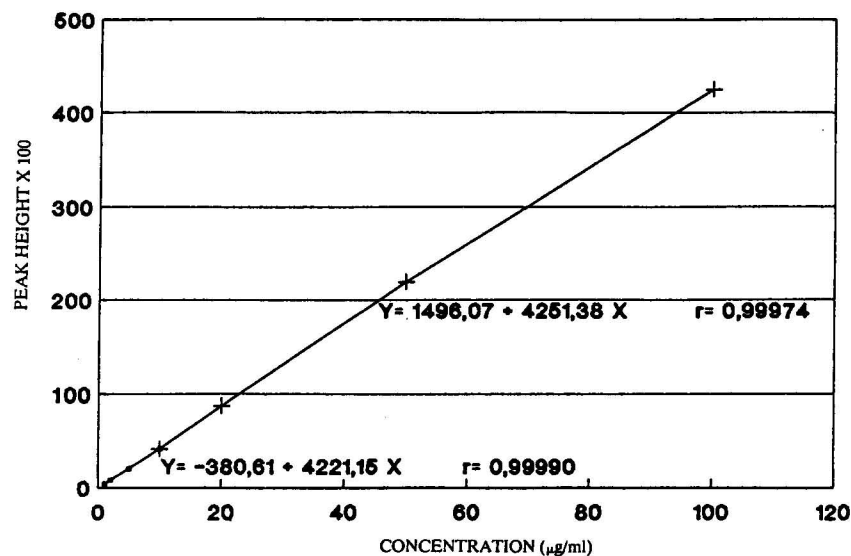


FIGURE 1.- Calibration curves for the assay of levamisole with their linear regression equations and correlation coefficients (r). Concentration range: (\bullet) 1-10 $\mu\text{g/ml}$; ($+$) 10-100 $\mu\text{g/ml}$

TABLE 1

Interday Precision and Accuracy of Methods for the Assay of Levamisole in the Standard Solution

Levamisole concentration standard solution ($\mu\text{g/ml}$)	Number of samples	Concentration found Mean \pm SD ($\mu\text{g/ml}$)	Coefficient of Variation (%)
1	32	1.09 \pm 0.10	9.11
2	8	1.89 \pm 0.05	2.55
5	11	4.82 \pm 0.27	5.70
10	9	9.46 \pm 0.43	4.58
20	12	19.11 \pm 1.03	5.41
50	7	49.29 \pm 2.84	5.77
100	9	102.67 \pm 5.92	5.77

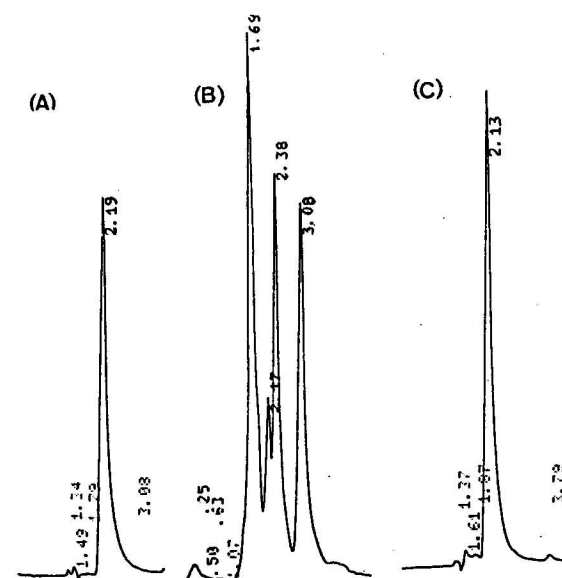


FIGURE 2.- Chromatograms of: (A) Levamisole in standard solution (0.1 μg , 0.032 AUFS); (B) Plasma rabbit spiked with Levamisole, Pentobarbital and Heparin and partially extracted using an inaccurate method and (C) Plasma rabbit spiked with levamisole (0.4 μg), Pentobarbital and Heparin extracted using our method (0.064 AUFS). For conditions see text.

plasma could increase in our case disposing of a greater volume of plasma, injecting a greater volume of solution or redissolving it to a lesser volume of mobile phase.

Data which allow for the evaluation of interday precision and accuracy of the valuation method of levamisole in the standard solutions are shown in Table 1.

The average percentage of levamisole recovery from the standard solutions was 73.62 % as can be seen in Table 2. Data which allow for the calculation the interday precision of the method in the standard solutions in plasma can also be seen in this table.

This method allowed us the determination of levamisole in plasma samples and has been applied satisfactorily to the pharmacokinetic studies of this substance in rabbits (in press)

TABLE 2

Interday Precision and Accuracy for the Assay of Levamisole in Plasma and Recoveries from Plasma

Levamisole concentration spiked plasma (μg/ml)	Number of Samples	Concentration found (μg/ml)	Mean ± SD	Coefficient of variation (%)	Recovery (%)
0.1	5	0.089	0.081 ± 0.009	10.872	88.7
		0.086			85.6
		0.067			66.8
		0.079			78.7
		0.086			85.9
0.2	5	0.152	0.149 ± 0.025	16.816	76.0
		0.155			77.5
		0.187			93.3
		0.131			65.3
		0.122			61.0
0.5	6	0.461	0.388 ± 0.056	14.495	93.3
		0.353			70.6
		0.329			65.8
		0.352			70.3
		0.378			75.5
1	7	0.454	0.648 ± 0.097	14.995	90.8
		0.663			66.3
		0.514			51.4
		0.818			81.8
		0.613			61.3
2	6	0.627	1.435 ± 0.192	13.384	62.7
		0.712			71.2
		0.587			58.7
		1.399			70.0
		1.277			63.8
5	7	1.364	3.529 ± 0.336	9.526	68.2
		1.240			62.0
		1.600			80.0
		1.731			86.6
		2.948			59.0
10	6	3.333	7.812 ± 0.491	6.290	66.7
		3.508			70.2
		3.419			68.4
		3.821			76.4
		3.745			74.9
	3.925	78.5			
		71.3			
		74.7			
		77.4			
		78.4			
		82.0			
		84.9			

Mean ± SD = 73.62 ± 10.08

Mean \pm SD = 73.62 \pm 10.08

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