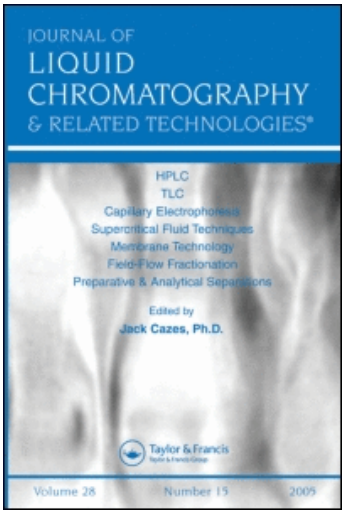

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DETERMINATION OF THE ANTIPARASITIC DRUG IVERMECTIN IN LIVER, MUSCLE AND FAT TISSUE SAMPLES FROM SWINE, CATTLE, HORSES AND SHEEP USING HPLC WITH FLUORESCENCE DETECTION

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ABSTRACT

A rapid and very effective analytical procedure for the isolation, derivatization and fluorescence HPLC determination of Ivermectin (IVM) residues in all the animal tissues and organs listed in the EEC Regulation 2901/93 has been developed and tested. First, the drug is extracted from the samples with acetonitrile. Then, the IVM extracts, mixed with water and triethylamine, are cleaned up on and eluted with terbutylmethylether from SPE C_{18} columns. Prior to the derivatization step, the IVM eluates are purified from the interfering lipophilic substances through a liquid-liquid partition step. Finally, IVM is derivatized and analyzed by HPLC

with fluorescence detection. The mean recoveries from IVM fortified samples were 76% for liver and 77% for fat tissue in a concentration range of 10-120 ng/g. The limit of determination was 2 ng/g.

This paper also reports the results of a short trial on the pharmacokinetics of the IVM depletion in guinea pigs dosed with the drug.

INTRODUCTION

Ivermectin (IVM) is a broad-spectrum antiparasitic drug of the chemical family of Avermectins, used against Nematodes and Arthropods in food-producing animals.[1] IVM is a mixture of two homologs, not less than 80% of 22,23 - dihydroavermectin B_{1a} (H_2B_{1a}) and not more than 20% of 22,23 - dihydroavermectin B_{1b} (H_2B_{1b}). H_2B_{1a}, the component in the largest amount in the mixture, is metabolized more slowly than the other drug component H_2B_{1b} and therefore is considered to be a satisfactory marker compound for measuring total residues.[2] IVM displays a prolonged persistence in the animal and its pharmacological action has been reported to be effective at very low dosage levels.[3]

With its Regulation 2901/93 the EEC[4] definitively fixed the MRL's for IVM in tissues from several food-producing animal. The animal species included were swine, cattle, horses and sheep and the tissues recommended for the analysis were liver and fat. A number of analytical methods for the determination of IVM in animal tissues has been published, by Tway et al.,[2] Markus et al.,[5] Salisbury[6] and Degroodt et al.[7] In general, all these procedures involve a liquid-liquid extraction step of the drug from the samples, followed by a clean-up step of the resulting IVM extracts, either with liquid-liquid purifications or with SPE column technique.

Recently, Schenk et al.[8] and Iosifidou et al.[9] have reported an extension of the matrix solid phase dispersion (MSPD) technique to the determination of IVM in tissues. To perform the quantification of the drug by fluorescence detection, nearly all the authors use, except Stong,[10] the same chemical derivatization step of the IVM extracts.

Before the quantification of IVM by fluorescence-HPLC, the method we report employs sequentially two very effective clean-up procedures for the IVM extracts from the tissues: an SPE column extraction step and a very fast liquid-

liquid partition step. This makes the method suitable for the routine analysis of IVM in all the tissues from all the animal species listed in the EEC Regulation 2901/93. This paper also reports the results of a short trial on the pharmacokinetics of the IVM depletion in guinea pigs dosed with the drug.

MATERIALS

Reagents and Equipment

Acetonitrile, methanol (HPLC grade), hexane, chloroform, triethylamine and terbutylmethylether were obtained from Merck. Water for LC analysis was prepared with a Nanopure Ultrapure Water System of Barnstead. 1-Methylimidazole, acetic anhydride and N,N- dimethylformamide were obtained from Sigma.

C_{18} Bakerbond SPE columns, each with 500 mg of stationary phase and a 6-mL volume were obtained from J.T. Baker (code no. 7020-06). Silica SPE columns were obtained from Waters (code no. WAT051900). The columns were connected to an SPE vacuum manifold block, obtained from Supelco Inc.

The HPLC system consisted of a Model 9010 ternary pump from Varian, equipped with a loop injector with a 100 µL loop from Rheodine Corp. A Model 9070 fluorescence detector from Varian and a Model 561 recorder from Perkin-Elmer (chart speed: 0.5 cm/min) was used for fluorimetric detection. The HPLC separation was carried on a 5 µm Supelcosil LC - 18 150 x 4.6 mm column at room temperature, with a 5 µm Supelguard LC - 18 20 x 4.6 mm guard column (both the columns from Supelco Inc.).

The operating conditions for fluorescence-HPLC were: mobile phase 95:5 v/v methanol:water, flow 1.8 mL/min, injection volume 20 µL, chart speed 5 mm/min, excitation wavelength 360 nm, emission wavelength 470 nm, absorbance units of full scale (AUFS) 0.2-0.5.

Standard and Standard Solutions

The IVM (Reference standard L-640.471 - 076P005) standard solution at a concentration of 1.39 % w/w, stored at -20° C, was a generous gift of Merck Sharp and Dohme (Research Laboratory) of Germany. IVM stock solution was prepared in methanol at a concentration of 13.9 µg/mL.

The IVM working solutions at concentrations of, respectively, 0.35, 0.69, 1.39, 2.78 and 5.56 μg/mL, were prepared in methanol on the day of use from the stock solution and were employed to fortify the samples for the recovery study, giving IVM concentrations in the 10-120 ng/g range.

Aliquots of these solutions (100 μL) were derivatized according to the procedure described below. A standard curve was prepared with each batch of samples.

EXPERIMENTAL

Extraction and Clean-up Procedure

5.00 g minced tissue sample (liver, muscle or fat) was weighed into a 50 mL polypropylene centrifuge tube; 15 mL acetonitrile was added and the mixture was homogenized with an Ultraturrax homogenizer at high speed for 3 min. The homogenizer rod and blades were rinsed into the tube with 2 mL acetonitrile and the tube was placed into an ultrasonic bath for 15 min.

After centrifugation for 5 min at 3000 rpm, the supernatant layer was poured from the tube, through a folded paper filter, into a 100 mL flat-bottomed flask. The samples were re-extracted as previously described, again with 10 mL acetonitrile, the two acetonitrilic portions were combined, and the filter was washed with 3 mL acetonitrile. 70 mL distilled water and 0.1 mL triethylamine (TEA) were then added to the flask and the resulting mixture was throughly stirred.

A C_{18} SPE column was placed on the vacuum manifold block, and a 75 mL reservoir was attached to its top. Before the extraction step, the column was activated with, respectively, 3 x 5 mL methanol, 5 mL acetonitrile and 3 x 5 mL 70:30 v/v water:acetonitrile mixture containing 0.1% TEA. The sample extract was added into the reservoir and passed through the SPE column. The eluate was discarded. The column was washed with 2 x 5 mL v/v 50:50 water:acetonitrile mixture. IVM was eluted with 7 mL terbutylmethylether from the column into a 10 mL glass centrifuge tube.

To obtain the best IVM recoveries, the eluition flow rate should be of about 2 mL/min. The tube was stocked overnight in a refrigerator at -20°C.

The cool, clear supernatant etheric layer was throughly decanted to another 10 mL centrifuge glass tube, after discarding the lower frozen water layer. Then the etheric layer was reduced to dryness under N_2 at 50 °C. 3 mL methanol and then 0.1 mL bidistilled water were added to the tube and the resulting solution was washed with 2 x 3 mL hexane, vortex-mixing after every addition. The two hexanic washings were re-extracted with 1 mL methanol and this methanolic portion was combined with the hydromethanolic layer in the tube. The mixture was reduced to dryness under N_2 at 50°C. Finally, all the traces of the solvents were exhaustively removed from the sample extract by heating the tubes for 30 min in a vacuum oven at 50°C.

Derivatization

150 µL freshly prepared derivatizing reagent (1-methylimidazole-acetic anhydride-dimethylformamide, 2+3+9 v/v) was added to the tube, then it was tightly capped, vortex-mixed for 30 sec, and heated for 60 min in an oven at 100°C. Then the tube was removed and cooled, and 1 mL chloroform was added. After vortex-mixing, the dark brown solution was transferred onto a silica SPE column, previously activated with 8 mL chloroform and placed on the vacuum manifold block. The tube was rinsed with 3 x 3 mL chloroform. The rinses were loaded onto the column and eluted into a 10 mL centrifuge glass tube.

The chloroformic eluate in each tube was reduced to dryness under N_2 at 50°C. The residue was dissolved in 0.4 mL methanol. After vortex-mixing, 20 µL derivatized IVM solution was injected onto the fluorescence-HPLC system, operating as previously described.

Trial in Guinea Pigs

Eight guinea pigs (approximately 500 g body weight) were administered a pharmaceutical preparation containing IVM, namely Ivomec®, from Merck Sharp and Dohme, i.e. 0.2 mg/kg b.w., injected subcutaneously in the retroscapular region. The animals were fed ad libitum both before and during the experiment.

Groups of two animals were sacrificed at 2, 4, 6 and 13 days after dosing and samples of liver, muscle and fat tissue were collected from each of the two animals, combined and frozen at -20 °C until analysis was performed. The samples were analyzed in duplicate according to the procedure described below, in order to study the "in vivo" IVM depletion profile and, also, to further validate the analytical procedure of IVM determination.

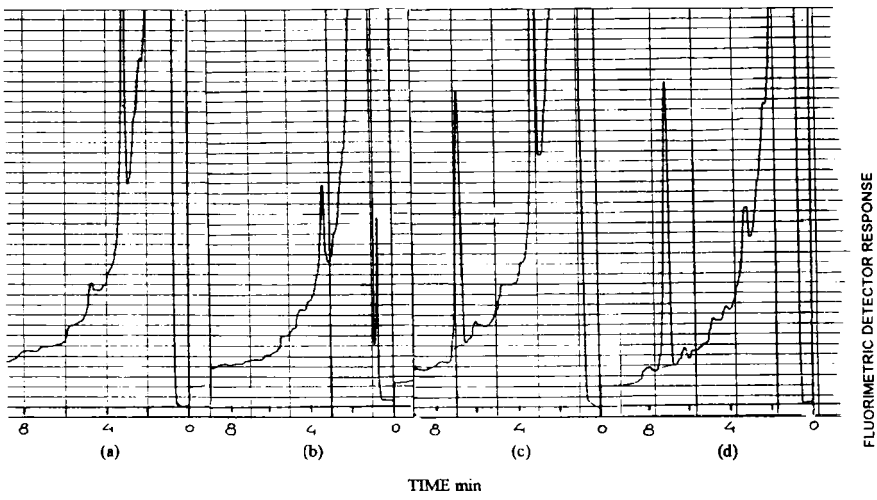

Figure 1. Representative chromatograms of IVM: - (a), (b) blank control samples of sheep liver and fat tissue respectively - (c), (d) IVM-fortified (20 ng/g) sheep liver and fat tissue respectively.
Conditions: mobile phase, methanol-water (95+5 v/v); column, 150 x 4.6 mm, C_{18}; flow rate, 1.8 mL/min; recorder sensitivity, 0.5 AUFS; chart speed, 0.5 cm/min; and injection volume, 20 µL from a final volume of 400 µL.

RESULTS AND DISCUSSION

Several papers are available in the scientific literature on the determination of IVM in different animal matrices and tissues, mostly in milk,[11] plasma,[12] muscle and liver.[6-9] However, only very few studies report reliable analytical procedures to be contemponeously applied both to liver and fat tissues,[2,5] which represent the two matrices recommended for the IVM analysis in the EEC Regulation 2901/93.

Compared to the methods of this kind published so far, this analytical procedure has a major advantage in its extremely effective clean-up procedure. Carried out with an SPE C_{18} extraction, followed by a very fast liquid - liquid partition, this method can actually yield sample extracts with a minimum of interfering substances. This improvement makes the present method suitable for the analysis of IVM, not only in derivatized liver and fat tissue extracts by fluorescence - HPLC, but also, as reported by Dickinson,[13] in non-derivatized muscle tissue extracts by HPLC - UV.

Table 1

Average IVM Recoveries of Six Replicates (% - ± Standard Deviation) From Spiked Samples of Blank control Fat and Liver Tissue of Cattle, Horse, Swine, and Sheep

Added IVM (ng/g)	Swine		Sheep		Horse		Cattle	
	Fat	Liver	Fat	Liver	Fat	Liver	Fat	Liver
10	88 ± 4	74 ± 8	70 ± 5	76 ± 4	84 ± 5	77 ± 7	---	---
20	77 ± 1	77 ± 4	79 ± 9	78 ± 3	77 ± 6	78 ± 5	71 ± 8	---
30	77 ± 3	78 ± 4	73 ± 5	73 ± 5	76 ± 9	82 ± 6	75 ± 8	---
40	71 ± 4	76 ± 7	70 ± 4	77 ± 7	76 ± 9	82 ± 9	82 ± 4	---
60	---	---	---	---	---	---	79 ± 4	73 ± 3
80	---	---	---	---	---	---	---	68 ± 7
100	---	---	---	---	---	---	---	74 ± 5
120	---	---	---	---	---	---	---	75 ± 3

The present procedure was also applied to the fat samples without performing the liquid-liquid partition step, but our results suggest that, in these conditions, the derivatization of the fat extracts was not successful. Also, the procedure was carried through omitting the final silica SPE clean-up, but, in these conditions, the derivatized extracts were not purified enough, so that their injection dramatically shortened the life of the HPLC column.

Fig. 1 shows the chromatograms of a blank sheep liver tissue extract (a), of a sheep liver tissue extract, fortified with 20 ng/g IVM (b), of a blank sheep fat tissue extract (c), and of a sheep fat tissue extract fortified with 20 ng/g IVM (d). The response of the fluorimetric detection is linear in the working range of 2-120 ng/g IVM concentrations in tissue. The limit of determination of IVM is 2 ng/g for both tissues.

The chromatographic profiles of the liver and fat tissue extracts from the other animal species listed in the EEC Regulation 2901/93 (cattle, horses and swine) are practically identical to those shown in Fig. 1. In Table 1 are listed the recovery and standard deviation percentages of IVM of liver and fat tissue samples from different animal species, fortified to give IVM concentrations of 10, 20, 30, 40, 60, 80, 100, 120 ng/g. The overall recovery percentages of IVM from fortified samples are 76% with SD 6% for liver and 77% with SD 7% for fat samples.

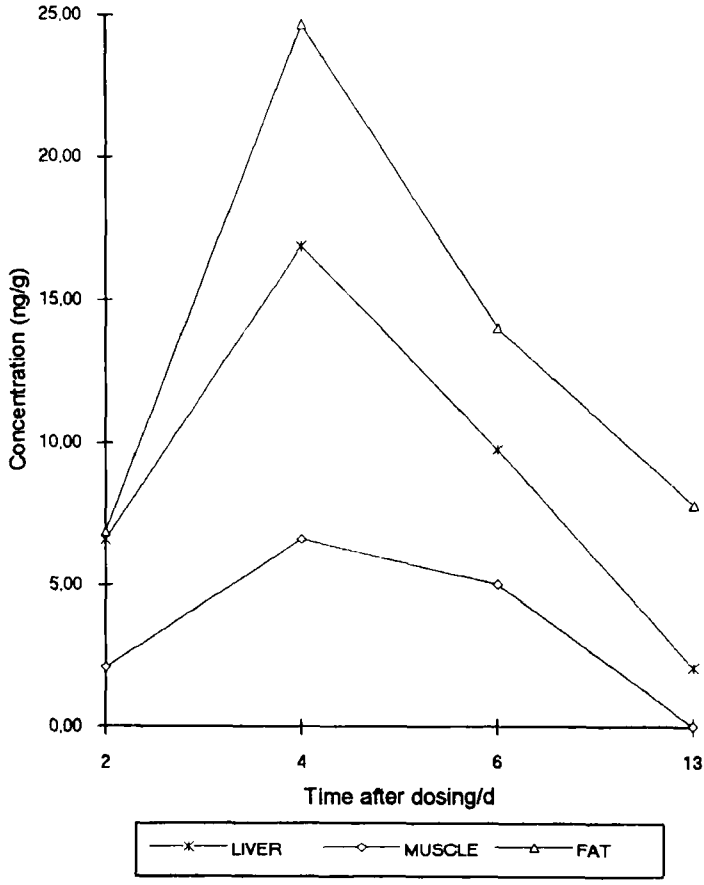

Figure 2. Residual concentration of IVM in muscle, liver and fat tissue of guinea pig following a single subcutaneously dose (0.2 mg/kg body weight). Data represent the mean for two guinea pigs at each time point.

In order to further validate this procedure, and also to study the pharmacokinetics and the depletion features of the drug in guinea pigs administered IVM, a short trial has been carried out. Its results, shown in Fig. 2, confirm the findings of other authors on the depletion of IVM in guinea pigs[14] and in pigs.[15] IVM concentrations increased to reach, after 4 days, their maximal levels of, respectively, 7, 17 and 25 ng/g in muscle, liver and fat. Then

they slowly decreased thereafter. This short study demonstrates that liver and fat tissues are the preferred matrices for IVM accumulation in guinea pigs, as in other animals.[2,15] Moreover, as the intra-assay variation for IVM determinations in duplicate is less than 5 % on all the samples from the treated animals, it can be concluded that this method is effective not only on fortified samples, but also on incurred ones.

This method provides a protocol for IVM determination on food-producing animals, which is in full agreement with the recommendations of the European Community. In fact, it completely fulfils the most important analytical requirements of EEC Regulation 2901/93, both in terms of the IVM limit of determination, and of the animal tissues and species to which IVM analysis should be extended.

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