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Note

Improved high-performance liquid chromatographic method for quantitation of ivermectin in whole blood, serum or muscle tissue

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Ivermectin is a macrocyclic lactone disaccharide that is a potent antiparasitic drug. It has been used to treat parasitic disease, especially helminthic diseases in animals, for several years and is now being used to treat humans for onchocerciasis and Bancroftian filariasis. The drug is effective in very low dosage (200 μg/kg total body weight) and therefore requires a very sensitive analytical procedure.

The most commonly used analytical procedure for determining ivermectin levels in both plasma and tissue is based on a method developed for avermectins, a family of closely related drugs of which ivermectin is a member [1]. This method uses a Florisil[a] column for isolating ivermectin, a derivatization reaction and fluorescence–high-performance liquid chromatographic (HPLC) quantitation of this derivative. A detection limit of 0.2 ng/ml is reported for 5-ml plasma samples. Recently, two additional fluorescence methods have been reported [2,3]. A more rapid derivatization reaction was used to apply this method to tissue analysis [4]. A more direct method uses the same reversed-phase HPLC system with ultraviolet (UV) detection and an isomer as internal

[a]Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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standard [5]. A normal-phase liquid chromatographic method for ivermectin in cattle blood or plasma with a sensitivity of 4–5 ng/ml in 5-ml samples has also been reported [6]. A very rapid reversed-phase method for determining ivermectin concentrations in serum using UV detection has recently been described [7]. This method is very simple, requiring two cartridges (one C_{18} and one silica) to isolate and purify the sample sufficiently for quantitation but requires a 5-ml serum sample to achieve a sensitivity of 2 ng/ml.

The method herein reported uses a similar mobile phase, column and detector but, due to an improved clean-up procedure, achieves the same sensitivity with one tenth the sample size and only one cartridge, making the analysis less expensive. This method also uses an internal standard that can be prepared in any laboratory from ivermectin standard. With minor modifications the procedure works well with homogenized muscle tissue but not with liver tissue. A study of the stability of whole blood samples containing ivermectin at three levels (10, 25 and 100 ng/ml) during storage in both a refrigerator and freezer is reported.

EXPERIMENTAL

Apparatus and reagents

A Varian 5060 liquid chromatograph equipped with a Rheodyne Model 7125 injector with a 50-μl sample loop, an LDC/Milton Roy Spectromonitor 3000 variable-wavelength detector set at 245 nm and a Hewlett Packard 5880A terminal as integrator was used.

The analytical column used was a Beckman Ultrasphere XL ODS, 3 mm particle size, 7.0 cm $\times$ 4.6 mm I.D., heated to 56°C with a Rainin temperature control unit Model III.

Burdick and Jackson (Muskegon, MI, U.S.A.) high-purity solvents for HPLC were used.

The mobile phase was acetonitrile–methanol–water (49:33:18) used at a flow-rate of 1.0 ml/min. At 56°C, the retention time of ivermectin is 5.6 min and that of the internal standard is 13.4 min.

C_{18} Bond Elut clean-up cartridges, 1 ml size for serum and whole blood, 3 ml size for tissue samples (Analytichem International, Harbor City, CA, U.S.A.), were prepared to receive samples by washing with 2 ml of methyl *tert.*-butyl ether, 2 ml of acetonitrile and 2 ml of 50% acetonitrile–water for the 1-ml cartridge or double these volumes for the 3-ml cartridge.

A Vertis 45 homogenizer was used to homogenize tissue samples.

Preparation of internal standard.

An acetonitrile solution (1 ml) containing 278 μg of ivermectin was added to a 15-ml glass centrifuge tube. The solvent was evaporated and 0.2 ml of 1-methylimidazole, 0.3 ml of acetic anhydride and 0.9 ml of dimethylformamide

were added to the tube. The tube was shaken to mix well, then placed in a water bath at 60 °C for 15 min, during which the solution turned brown. Acetonitrile (4 ml) was added, and the resulting solution passed through a silica cartridge to remove impurities. The solution was evaporated to approximately 0.5 ml, then diluted to 20 ml with acetonitrile. This solution was used as the internal standard without further purification. Liquid chromatographic analysis of this solution showed no trace of ivermectin, and the few impurities present do not interfere with ivermectin. A 20-μl volume of this solution was added to each 0.5-ml sample of whole blood or serum, and 50 μl were added to each gram of sample of muscle tissue.

Preparation of whole blood and serum standards

A stock solution of 0.278 mg/ml ivermectin in acetonitrile or methanol was prepared. This solution was diluted 1:50 with acetonitrile to produce a solution containing 5.56 μg/ml. A final dilution of 1:10 was required to produce the working standards (0.556 μg/ml). Individual 0.5-ml whole blood or serum standards were prepared by adding 9, 22.5, 45 or 90 μl, using a Rainin Pipetman 100, of the 0.556 μg/ml solution to the blood or serum in a 1.8-ml polypropylene microcentrifuge tube to produce 10, 25, 50 and 100 ng/ml standards, respectively, to be analyzed along with samples. A blank blood or serum sample was also analyzed with each set of standards and samples.

Extraction and clean-up of whole blood and serum samples

The 0.5-ml samples were measured into 1.8-ml polypropylene microcentrifuge tubes. A 20-μl volume of internal standard solution, 50 μl of 0.2 *M* zinc sulfate solution and 0.5 ml of acetonitrile were added to each sample and standard, and each tube was thoroughly mixed using a vortex mixer. The tubes were centrifuged for 5 min and the supernatant was transferred to a 1-ml C_{18} Bond Elut cartridge. The cartridge was washed with 2 ml of acetonitrile–water (1:1) and then eluted with 2 ml of methyl *tert.*-butyl ether. The methyl *tert.*-butyl ether solution was evaporated and the residue dissolved in 150 μl of mobile phase. Injections of 50 μl were made using this solution. The height of the ivermectin and internal standard peaks were measured and the ratio was determined. The known concentrations and the peak-height ratios were used in a linear least-square program to calculate the concentration of unknown samples.

Recovery of ivermectin from whole blood

Into each of six microcentrifuge tubes 90 μl of a solution containing 0.556 μg/ml ivermectin were added. A 0.5-ml volume of whole human blood was added to three of the tubes. To each of the six tubes, 20 μl of internal standard solution were added. The tubes containing blood were then processed as in the section on extraction and clean-up. The other tubes were dried under nitrogen and

reconstituted with 150 μl of mobile phase, the same volume used for blood samples after extraction. Full-loop (50-μl) injections were made from all six tubes. The peak-height value for ivermectin that had been extracted from blood was compared with that value which was never in blood to calculate a percentage recovery. The same comparison was made for the internal standard.

Storage stability of ivermectin in whole blood

A freshly prepared solution containing 0.556 μg/ml ivermectin was used to fortify individual 0.5-ml samples of whole blood in microcentrifuge tubes. Fourteen tubes at each of three levels were prepared by adding 9, 22.5 or 90 μl of the ivermectin solution to produce samples of 10, 25 or 100 ng/ml. A duplicate set was analyzed immediately and the remainder stored, half in the refrigerator at 5°C and half in the freezer at −15°C. Samples were removed and analyzed along with freshly prepared standards at intervals during the two-month storage stability study.

Preparation of muscle and liver tissue samples

Samples of bovine muscle tissue were blotted dry and cut into approximately 1-g strips. These strips were weighed to the nearest 0.1 mg and placed in the homogenizer jar; the appropriate amount of ivermectin solution (0.556 ng/μl in methanol) was added to produce concentrations of 10, 25 or 100 ng/ml. Internal standard was added to each sample at the level of 50 μl/g. The volume of both the ivermectin and internal standard solutions was measured to the nearest 0.1 μl if less than 100 μl or to nearest microliter if greater than 100 μl. A 6-ml volume of acetonitrile–water (1:1) was added to the jar and the tissue was homogenized. This thick mixture was transferred to a glass centrifuge tube using a disposable Pasteur pipet. The homogenizer blades and jar were rinsed with an additional 2 ml of acetonitrile–water (1:1) and this rinse was added to the centrifuge tube.

Extraction and clean-up of tissue samples

To the homogenized tissue in acetonitrile–water (1:1) were added 200 ml of 0.2 *M* zinc sulfate. The tube was inverted several times to mix and then centrifuged to precipitate the protein. The supernatant was transferred to a 3-ml C_{18} Bond Elut cartridge (previously washed as described above) and washed with an additional 4 ml of acetonitrile–water (1:1). The ivermectin was eluted with 4 ml of methyl *tert.*-butyl ether. The solvent was evaporated and the residue dissolved in 150 μl of mobile phase. Injections of 50 μl were made from this solution.

RESULTS AND DISCUSSION

Manually measured peak-height ratios were used for calculations rather than peak-area ratios because more consistent results were obtained. This is appar-

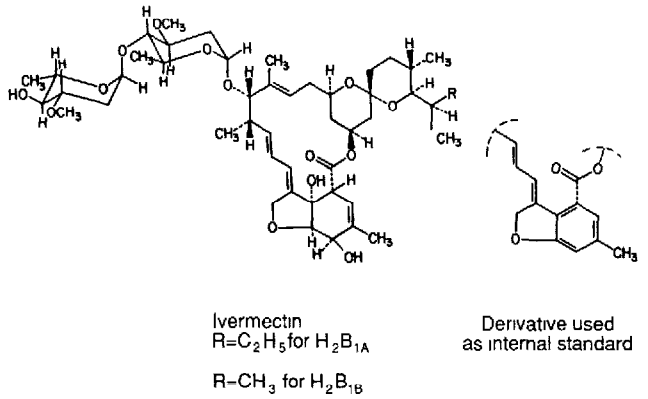

Fig. 1. Structures of ivermectin and the dehydro derivative used as internal standard.

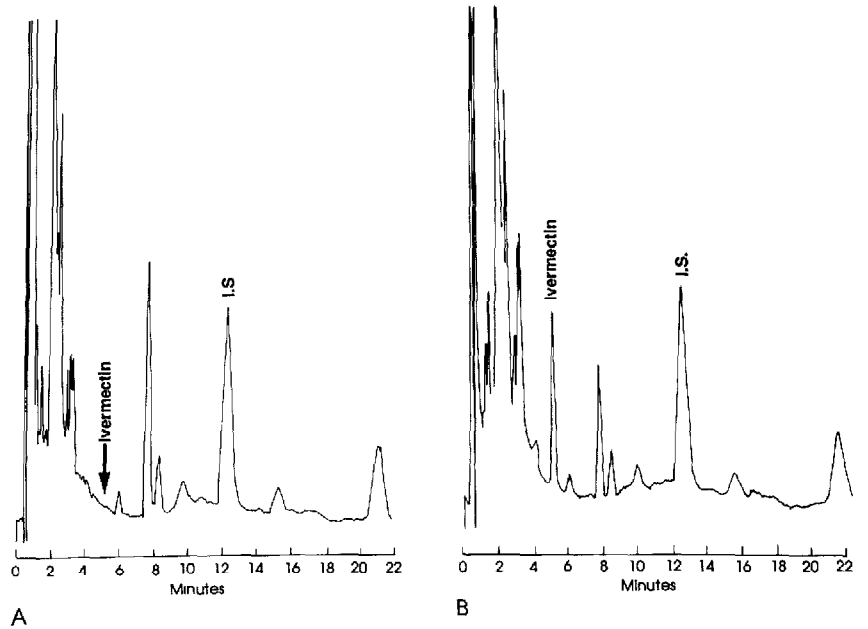

Fig. 2 Chromatograms of ivermectin and internal standard (I.S.) in whole blood. (A) Blank whole blood. (B) Whole blood sample containing 100 ng/ml ivermectin. The retention time of ivermectin is 5 2 min and of the internal standard 12.3 min.

ently due to the fact that the ivermectin peak occurs in a region of the chromatogram where the baseline is not yet level due to a previous peak and the close proximity of small impurities (see Fig. 2). Under these conditions integrators can be inconsistent in the manner in which small peaks are integrated. The chromatographic conditions do not separate the minor component of the drug, ivermectin B (Fig. 1), from a major metabolite of ivermectin and there-

TABLE I

IVERMECTIN RECOVERY AFTER EXTRACTION FROM WHOLE BLOOD

Values are peak heights (cm) of a 50-μl injection (full loop).

	Ivermectin (100 ng/ml)		Internal standard	
	Extracted	Not extracted	Extracted	Not extracted
	41.2	53.2	65.8	77.7
	45.8	58.5	49 5	76 5
	40.5	54.2	55.0	73.0
Mean ± S.D	42.5 ± 2.9	55.3 ± 2.8	56.8 ± 8.3	75.7 ± 2.4
Recovery (%)	76.8		75.0	

TABLE II

PRECISION DATA FOR IVERMECTIN ANALYSIS IN WHOLE BLOOD SAMPLES

Nominal concentration (ng/ml)	Ivermectin/internal standard peak-height ratio		
	Individual values	Mean	Deviation (%)
Intra-day precision			
10	0.06839	0.07519	8.4
	0.08081		
	0.07636		
100	0.8126	0.7731	4.5
	0.7484		
	0.7584		
Inter-day precision			
10	0.06992	0.06376	10.3
	0.06453		
	0.05682		
100	0.6957	0.7375	5.2
	0.7463		
	0.7706		

fore cannot be used to quantify this component. All ivermectin levels are based only on the major component, ivermectin A.

The dehydro derivative of ivermectin (Fig. 1) is easily prepared and serves very well as an internal standard. It has a retention time that gives good separation from all interferences, as shown in Fig. 2, and the percentage recovery is essentially the same as that of ivermectin (Table I). Using this internal standard with whole blood-fortified standards, coefficients of determination (r^2) of 0.998 are routinely obtained. Standard curves are equally good using serum instead of whole blood. Intra-day and inter-day precision are both good,

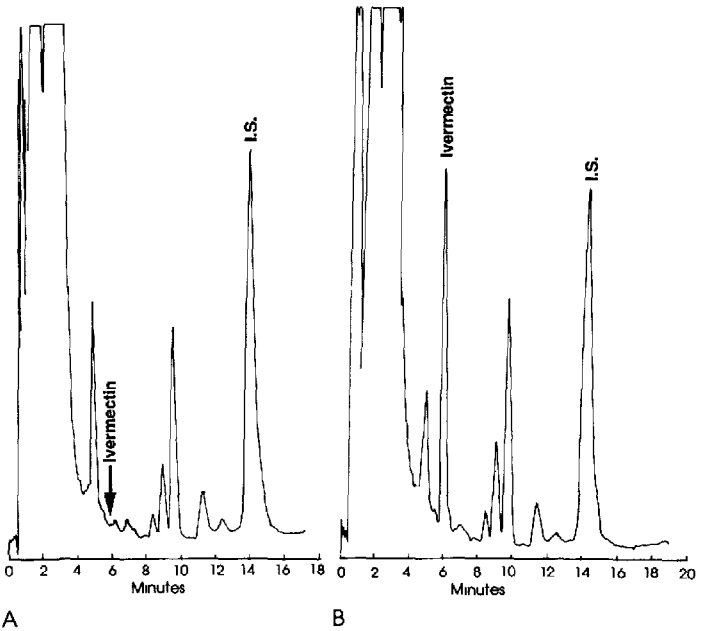

Fig. 3 Chromatograms of ivermectin and internal standard (I.S.) in homogenized bovine muscle tissue. (A) Blank muscle tissue. (B) Muscle tissue containing 100 ng/ml ivermectin.

as shown in Table II. The limit of determination is 2 ng/ml for whole blood and serum.

Whole blood samples containing ivermectin at three different levels proved stable over the two-month storage period both at refrigerator (5°C) and freezer (−15°C) temperatures. As expected, more variation was found in the 10 ng/ml samples than at both higher levels, but no significant loss on storage is apparent at any level. The data show random scatter, most of which is within the range of inter-day precision shown in Table II.

This analytical method was successfully applied, with minor changes, to 1-g samples of fortified muscle tissue. The chromatograms (Fig. 3) differ from those of extracts from whole blood in that more early eluting material is present, but no interference occurs with the ivermectin or the internal standard peaks. This method is not useful with liver tissue due to large amount of material that coelutes with ivermectin, completely obscuring the peak. The limit of determination is 2 ng/g for muscle tissue.

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