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Rapid Method for Multi-Residue Determination of Avermectins in Bovine Liver Using High-Performance Liquid Chromatography with Fluorescence Detection

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Abstract A simple method using high-performance liquid chromatography and fluorescence detection is presented for the simultaneous determination of the anti-parasitic agents avermectins (abamectin, ivermectin and doramectin) in bovine liver. Samples were extracted using acetonitrile and cleaned up on an alumina SPE column and derivatized with trifluoroacetic anhydride. The derivatives were detected using fluorescence detector set at 365 nm excitation and 475 nm emission wavelengths. As the results, the limit of quantification for three avermectins was about 1 μg/kg. Inter-assay showed the mean recoveries of avermectins in bovine liver ranged from 72% to 81%, with RSD less than 9.9%.

Keywords Avermectins · Residue · Liver · Fluorescence

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The Avermectins were originally isolated from cultures of *Streptomyces avermitilis*. They are used in the treatment of food producing animals by virtue of antiparasitic effects. They are highly effective at extremely low dose levels (0.2–0.5 μg/kg) against nematode and arthropod species in cattle, sheep, pigs and horses. They are not mutagenic or carcinogenic but may be embryotoxic in laboratory animal test. In China (Regulation No. 235, 2002) and in EU (Council Regulation EEC No. 2377/90, 1990), the Maximum Residue Limits (MRL) of avermectins in tissues ranged from 10 to 100 μg/kg depending on the species. A number of analytical methods for the determination of avermectins in animal plasma have been described mostly using high-performance liquid chromatography (HPLC) and fluorescence detection (Prieto et al. 2003; Pierre et al. 1990). In general, a chemical dehydration of the parent drug with trifluoroacetic acid and a basic catalyst (such as methylimidazole) is adapted for routine monitoring their residues in tissues. Although recently liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been described for the analysis of avermectins (David 2007), fluorescence detection is still the most commonly applied detection technique with respect to the limit of detection and quantification.

Most of the previous HPLC methods with fluorescence detection needs complicate clean-up procedures using C-18 SPE cartridges or using more than two SPE cartridges in order to avoid matrix interferences. Liver is the target tissue for avermectins residue control in cattle, however determination of nitromidazoles residue in liver is more difficult than plasma due to liver's biological complexity. Our objective was to develop a rapid analytical method for the simultaneous determination of residues of three avermectins (abamectin, ivermectin and doramectin) in bovine liver.

Materials and Methods

Acetonitrile was HPLC grade and was obtained from Merck (Darmstadt, Germany). Trifluoracetic anhydride was analytical grade and obtained from Sigma (St Louis, MO, USA). *N*-methylimidazole (99%) was purchased from ACROS (NJ, USA) and methanol was from Fisher (NJ, USA). Water was purified using a Mill-Q system (Millipore, USA). Solid phase extraction cartridges (Sep-pak aluminum B, 3CC 500 mg) were obtained from Waters (Milford, MA, USA).

Abamectin, ivermectin and doramectin (all ≥98%) were provided by Sigma (St Louis, MO, USA). Standard stock solutions of abamectin, doramectin, ivermectin (all at 1 mg/mL) were prepared in methanol. All standard stock solutions were stored at −20°C. A working standard solution (1 mg/L of abamectin, doramectin, ivermectin) was prepared from the standard stock solution, on the day of use. The working standards were taken to dryness under nitrogen, derivatized and injected onto the HPLC as described below.

Bovine liver (2 g) was homogenized on a T-25 basic mixer (IKA Germany) at 6000 rpm for 3 min and 6 mL acetonitrile was added to 2 g of homogenized liver in a tube. The tubes were stirred on an MS2 minishaker (IKA Germany) for 3 min and centrifuged for 5 min at 7,000 rpm. The acetonitrile extract was applied to an aluminum B cartridge which had previously been conditioned by irrigation with acetonitrile (4 mL). The extract was loaded at a flow rate of about 1 mL min^{-1} and the eluate was collected in a 10 mL tube. Then the cartridge was washed with 3 mL acetonitrile and the eluate was also collected in the tube. All of the eluate was evaporated to dryness with a stream of nitrogen on an Nitrogen evaporator (N-EVAP, USA) under 50°C, and then dried in an oven set at 50°C for 20 min in order to avoid the interference of water to derivatization procedure.

The dry residue was resuspended in 100 μL of a mixture of *N*-methylimidazole solution in acetonitrile (1:1, v/v) and subsequently 150 μL of a solution of trifluoroacetic anhydride in acetonitrile (1:2, v/v) was added. All the tubes were added 750 μL methanol and mixed, the solutions were transferred to autosampler vials and the vials were kept protected from sunlight until the injection. HPLC analyses were carried out within 6 h to avoid the degradation of the fluorescent derivatives.

The HPLC system consisted of a model 600 HPLC pump with a model 717 autosampler and model 474 fluorescence detector. The separation was carried out on a Novapak C18 analytical column (150 × 3.9 mm id), and equipped with μBondapak C18 guard column (Waters). The column oven temperature was 30°C, the flow rate was 1.0 mL/min, and the injection volume was 50 μL. The excitation and emission wavelengths were set at 365 and 475 nm, respectively.

Results and Discussions

The method was found to be suitable for the separation of the three avermectins (Figs. 1, 2, 3). This mobile phase gave baseline separation of the analytes, with the elution order of abamectin, doramectin and ivermectin.

Linearity of the detector response was checked from a set of eight working standards ranging in concentration from 2.5 to 200 μg/L. Calibration curves were prepared by plotting the peak area versus the analyte concentration, least-squares linear regression analysis was used to determine the slope. The curves were found to be linear over this range ($r^2 = 0.999$).The limits of detection in the HPLC system using the fluorescence detector were determined from representative blank samples. It was equal to three times signal-to-noise. The limit of quantification for the method was 1 μg/kg. Repeatability was determined by the analysis of five samples within a single run, reproducibility was determined by analyzing samples on five different days by different analysts to evaluate the run-to-run variation in the method. Accuracy was determined with the data from the repeatability assays (intra-assay variation). The extraction recovery of avermectins from bovine liver was determined at low (1 μg/kg), medium (2 μg/kg) and high (5 μg/kg) concentrations by comparing the ratios of standards in bovine liver after extraction (standard samples) with the ratios of standard spiked samples (standards in methanol, considered as 100% recovery). The specificity of the method was determined by comparing the chromatograms of drug-free bovine plasma samples with the chromatograms of bovine liver with ivermectin and bovine liver with abamectin, and bovine liver with doramectin. Intra-assay results showed the mean recovery of the analytes was between 81% and 93%. RSD were typically at less than 7.1% (Table 1). Inter-assay results showed the mean recovery of analytes was between 72% and 81% with RSD at less than 9.9% (Table 2).

The lack of interferences in the separation suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure. In this study, basic aluminum SPE cartridges were used instead of C-18 SPE cartridges commonly appeared in previous methods. It must be mentioned that the application of basic aluminum SPE cartridges at least provided two advantages, firstly, interferences from complex matrix, e.g., liver, kidney and fat were avoided. Secondly, the new method provided higher efficiency, the tedious SPE cleanup procedure was shortened from at least five steps to three steps. It should be noted that the presence of trace water in derivatization

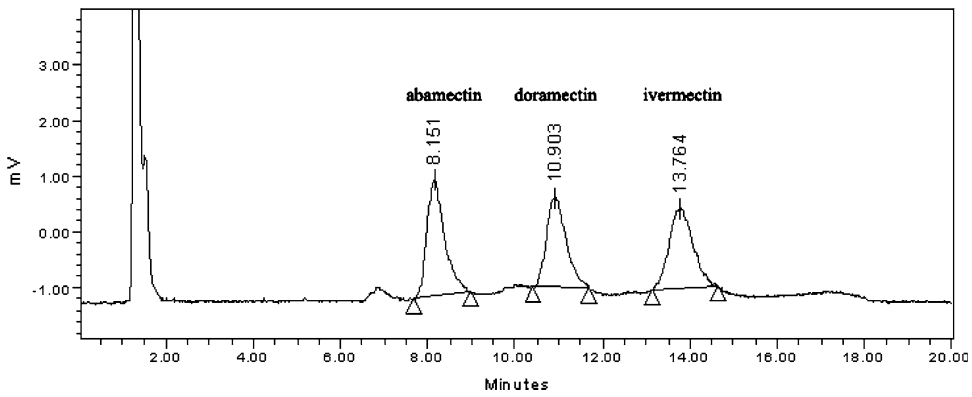

Fig. 1 Chromatograms of standards with 50 μg/kg of abamectin, doramectin and ivermectin (the three peaks are abamectin, doramectin and ivermectin in sequence)

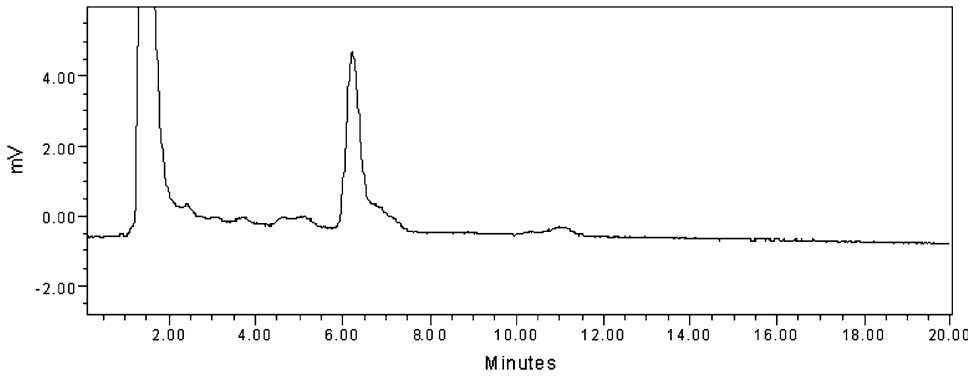

Fig. 2 Chromatograms of bovine liver extracts fortified with 0 μg/kg of abamectin, doramectin and ivermectin

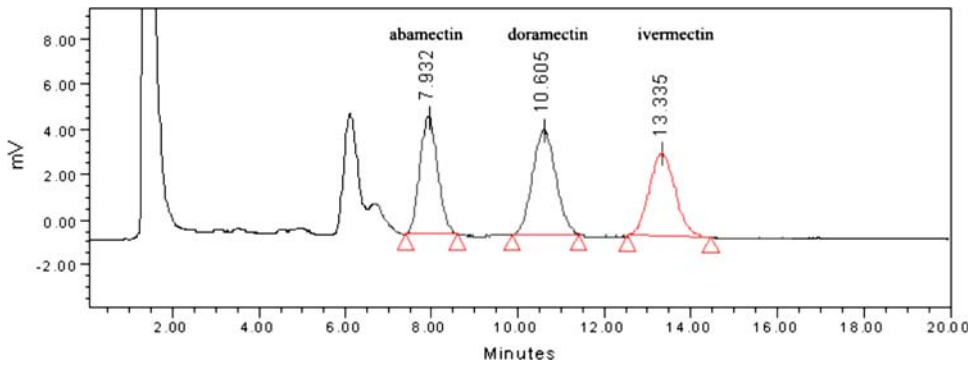

Fig. 3 Chromatograms of bovine liver extracts fortified with 50 μg/kg abamectin, doramectin and ivermectin (the three peaks are abamectin, doramectin and ivermectin in sequence)

Table 1 Intra-assay variation for the recovery of the analytes from bovine liver

Analyte	Fortification level μg/kg	Recovery (%)	
		Mean ± s (n = 5)	RSD
Abamectin	1	82 ± 5.2	6.3
	2	81 ± 4.5	5.5
	5	89 ± 4.1	4.6
Doramectin	1	86 ± 5.5	6.4
	2	85 ± 5.1	6.0
	5	90 ± 4.6	5.1
Ivermectin	1	91 ± 6.5	7.1
	2	93 ± 6.2	6.7
	5	93 ± 4.1	4.4

Table 2 Inter-assay variation for the recovery of the analytes from bovine liver

Analyte	Fortification level μg/kg	Recovery (%)	
		Mean ± s (n = 5)	RSD
Abamectin	1	75 ± 7.2	9.6
	2	76 ± 7.1	9.3
	5	81 ± 6.1	7.5
Doramectin	1	72 ± 7.1	9.9
	2	75 ± 6.1	8.1
	5	83 ± 6.0	7.2
Ivermectin	1	70 ± 6.5	9.3
	2	73 ± 6.2	8.5
	5	80 ± 5.0	6.3

should be avoided, or it might produce the hydrolysis of the trifluoroacetylated derivatives and lead to the formation of more polar products, which could be seen from the presence of interfering peaks on the chromatogram.

The limits of quantification were validated at 1, 2, and 5 μg/kg. The good and reproducible recoveries obtained for all the analytes at different fortification levels, clearly show that the method is applicable to determine avermectins residues in liver.

In the proposed method, the sample preparation and extraction procedure is simple, rapid and quantitative. The technique uses a small sample size and has a minimum number of steps which requires minimum amounts of solvents. No extensive sample clean-up procedure is required. The method described is sensitive, precise and accurate. It meets the requirements of methods used for monitoring drug residues in animal tissues (Decision 93/256/EEC) and a technician can easily perform for 30 samples a day.

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