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Development and validation of an HPLC–FLD method for milbemectin quantification in dog plasma

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ABSTRACT

Milbemectin is a widely used veterinary antiparasitic agent. A high-performance liquid chromatography with fluorescent detection (HPLC–FLD) method is described for the determination of milbemectin in dog plasma. The derivative procedure included mixing 1-methylimizole [MI, MI-ACN (1:1, v/v), 100 μL], trifluoroacetic anhydride [TFAA, TFAA-ACN (1:2, v/v), 150 μL] with a subsequent incubation for 3 s at the room temperature to obtain a fluorescent derivative, which is reproducible in different blood samples and the derivatives proved to be stable for at least 80 h at room temperature. HPLC method was developed on C18 column with FLD detection at an excitation wavelength of 365 nm and emission wavelength of 475 nm, with the mobile phase consisting of methanol and water in the ratio of 98:2 (v/v). The assay lower limit of quantification was 1 ng/mL. The calibration curve was linear over concentration range of 1–200 ng/mL. The intra- and inter-day accuracy was >94% and precision expressed as % coefficient of variation was <5%. This method is specific, simple, accurate, precise and easily adaptable to measure milbemycin in blood of other animals.

1. Introduction

Among canine intestinal parasites, *Toxocara canis*, *Echinococcus granulosus*, *Ancylostoma* spp., *Giardia* spp. and *Cryptosporidium* spp., have received great attention due to their zoonotic potential [1]. Anthelmintics to those parasites had developed from metals or plant extracts to phenothiazine, piperazine, thiabendazole and levamisole, the benzimidazoles and finally the macrocyclic lactones (include avermectins and milbemycins) [2]. The macrocyclic lactones have selective toxicity to nematodicidal activity and no to their mammalian hosts.

Milbemectin (Fig. 1a and b) is a minor member of a group of analogues containing a 25-isopropyl substituent was isolated from *Streptomyces hygroscopicus* subspecies *aureolacrimosus*, which consists of a mixture of milbemycin A_3 and milbemycin A_4 at a 30:70 ratio [3,4], and the closely related chemical structure toavermectins [5–7] leads to its insecticidal activities against important pests, as has been found with the avermectins abamectin and emamectin [8–11], it was mainly used as an acaricide/insecticide for plant protection [12]. Milbemycin oxime A_3 and A_4, a related molecule, launched as a parasiticide for the control of *Dirofilaria immitis* [13] and other nematodes and arthropods. Members of pharmacology lab (NEAU, Harbin China) tested the activity of milbemectin to *Toxocara canis*, *Ancylostoma caninum* and *Dirofilaria immitis*, and it was effective both in vitro and in vivo. It is necessary to develop a detection method to study pharmacokinetics of milbemectin in animals.

The most suitable methods at present for determination of ML residues would appear to be LC fluorescence or LC–MS/MS [14,15]. Although recently a number of LC–MS/MS methods have been described for the detection of avermectins and milbemycins, the limit of detection (LOD) and quantification is not more satisfactory than the fluorescence detection which is still the most commonly applied detection technique [16–19]. Chou et al. determined milbemectin A_3 and milbemectin A_4 in bovine muscle [20] and Yoshii and coworkers developed a simultaneous analytical method for determining milbemectin in crops [21] using HPLC–FLD. To the best of our knowledge, the method has not been used in the plasma samples. The objective of the present work was to develop a HPLC–FLD method, considering the pre-column derivatization in blood and fluorescence detection, for the HPLC analysis of milbemycin in dog plasma.

2. Experimental

2.1. Chemicals and reagents

Milbemectin A_3 [10 ng/μL in acetonitrile (ACN)] and A_4 (10 ng/μL in ACN) were purchased from Wako Chemicals (Rich-

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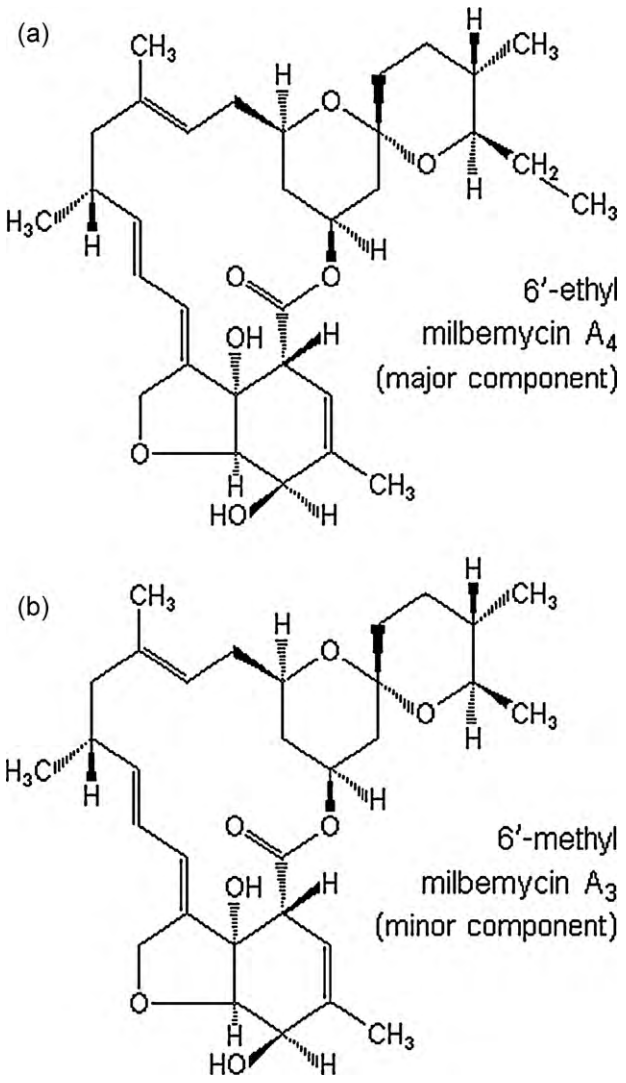

Fig. 1. Structures of milbemectin [milbemycin A_4 (a) and milbemycin A_3 (b)].

mond, VA, USA), trifluoroacetic anhydride (TFAA) (GC-grade), and N-methylimidazole of analytical-reagent grade, used in the derivatization process, were purchased from Aldrich (Sigma-Chimie, St. Quentin Fallavier, France). HPLC grade ACN and methanol were from Merk (Darmstadt, Germany). Deionized water for HPLC was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. Prepacked cartridges (Supelclean LC_{18}, 200 mg, 3 mL) for solid phase extraction were supplied by Sigma–Aldrich. For the preparation of in-house quality control and calibration samples, dog plasma was collected from normal dogs.

2.2. HPLC chromatographic conditions

Quantitative analysis of milbemectin in dog plasma was performed using an HPLC–FLD analytical system. The separation of compounds was carried out using an Agilent binary system consisting of an Agilent 1200 well plate auto-sampler fitted with a 20 μL sample loop, a quaternary pump, a column oven and a Model RF551 fluorescence detector. Chromatographic software HP ChemStation was used for data collection and processing. Separations were performed using elite C_{18} analytical column, 4 mm × 250 mm (elite, Dalian, China) packed with 5 μm particle size.

The fluorescence intensity was measured on a PerkinElmer luminescence spectrometer equipped with a xenon lamp and a Dell model 110 L, computer working with WinLab software. All the measurements were performed in a 10 mm pathlength quartz cell thermostated at 25 ± 0.5 °C, with 5 nm band-widths both emission and excitation monochromators.

Four mobile phases were tested for the elution step onto the analytical column based on acetic acid (0.2%)–methanol–ACN (8:30:62; v/v/v), 100% methanol, methanol–water (95:5, v/v), and methanol–water (98:2, v/v). The mobile phases were delivered at a constant 1.0 mL/min flow. Fluorescence detection was performed at an excitation wavelength of 365 nm and emission wavelength of 475 nm.

2.3. Extraction and cleanup

Blood samples were centrifuged for 15 min at 3000 rpm, and the supernatant was filtered through a Minisart plus syringe filter (0.2 μm pore size, Supelo) to remove remaining blood elements and high molecular weight proteins. 3 mL ACN was added to 1 mL of plasma and 1 mL of water, mixing for 20 min, and centrifuging at 2620 × *g* for 5 min, the supernatant was used.

The supernatant (≤5 mL) was manually transferred into a tube which was then placed on the appropriate rack of a Benchmate II (Hopkinton, MA, USA). Automatic sample preparation was performed as follows. Condition of the cartride: the column, positioned on the holder, was first conditioned with 3.0 mL of methanol and 3.0 mL of water (flow-rate 6 mL/min). Loading of the plasma sample: all of the supernatant was applied to the cartridge (flow-rate 3.0 mL/min). The cartridge was washed with 2 mL of water followed by 1 mL of water–methanol (75:25, v/v) at a flow-rate of 3.0 mL/min before elution, the cartridge was dried with nitrogen for 10 s (flow-rate 6.0 mL/min), then, 3.0 mL of methanol was applied to the cartridge at a flow-rate of 3.0 mL/min and the elute was collected.

2.4. Derivatization

The eluate was evaporated to dryness under a gentle stream of dry nitrogen at 50 °C in a water bath. The derivatization was started by adding 100 μL MI-ACN (1:1, v/v), then 150 μL TFAA-ACN (1:2, v/v) was added to the mixture, resulting in an exothermic reaction, coloring of the solution and the release of fumes. Exclusion of daylight for 30 s, the solution was evaporated for 10 min under a gentle stream of dry nitrogen at 50 °C in the water bath, 1 mL mobile phase was added, vortexed for 1 min, filtered through a Minisart plus syringe filter (0.2 μm pore size, Supelo) to remove the dopant, transferred into sample vials and analyzed using HPLC with fluorescence detection. The optimized procedure was tested in blood samples fortified with 1 ng/mL of milbemectin (milbemycin A_3: milbemycin A_4 = 30:70). Subsequently, the stability of the derivatised sample extracts was tested by storing for 80 h at room temperature with exclusion of daylight.

2.5. Calibration curve

Stock (1000 ng/mL) and substock (500 ng/mL) solutions of milbemectin (milbemycin A_3: milbemycin A_4 = 30:70) were prepared in ACN. A total of seven milbemectin concentrations (1, 2, 5, 10, 40, 80, 100, 150, and 200 ng/mL) in drug-free plasma were used as calibrators and three in-house quality control standards (QCs), containing 2, 10 and 150 ng/mL of milbemectin were used to estimate the accuracy and precision of the assay. All the stock and diluted stock solutions, calibrators and QC standards were stored at −80 °C until being used.