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Thin-layer chromatographic detection of ivermectin in cattle serum

W.G. Taylor*, T.J. Danielson, R.L. Orcutt

Agriculture and Agri-Food Canada Research Centre, P.O. Box 3000 Main, Lethbridge, Alb. T1J4B1, Canada

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Abstract

A study was conducted on the detection of ivermectin (22,23-dihydroavermectin B_1) in cattle serum by thin-layer chromatography (TLC) after derivatization of this parasiticide by the known reaction with trifluoroacetic anhydride–1-methylimidazole and visual examination of the chromatograms under long-wavelength ultraviolet light. By derivatization of reference samples of ivermectin in acetonitrile, approximately 0.1 ng of a highly fluorescent material, tentatively identified as 2,5,6-tetradehydro-5,7-dideoxy-22,23-dihydroavermectin B_1, could be detected on silica-gel thin layer plates. Extraction of fortified serum samples with methyl *tert.*-butyl ether followed by derivatization, hydrolysis, partitioning between water–hexane and TLC gave a limit of detection of 1–2 ng/ml. With these simple techniques ivermectin could be detected in cattle serum for 3–4 weeks after subcutaneous treatment of Hereford heifers.

1. Introduction

Ivermectin, a macrocyclic lactone disaccharide derived from avermectin B_1, is currently used extensively to control internal and external parasites of beef cattle and other livestock. This parasiticide is effective at low doses (0.2 mg/kg of body weight) when administered by subcutaneous injection to cattle. The injection vehicle, a mixture of glycerol formal and propylene glycol, promotes prolonged but low ivermectin blood levels by slowing the absorption process from the injection site [1]. Typically, maximum ivermectin concentrations of 30–45 ng per millilitre of cattle serum or plasma have been reported at 1–2 days after treatment, gradually declining to very low levels (<1 ng/ml) by approximately 28 days posttreatment [1–3]. The terminal elimination half-life has been estimated at 8.3 days [1]. In Canada, a withdrawal time of 35 days is currently required before treated cattle are slaughtered for human consumption. Residues in cattle liver, the target tissue for residue monitoring by regulatory agencies [4], tend to be higher than in the blood [5]. Ivermectin is also administered to cattle by a topical pour-on formulation [6].

Several sensitive procedures based on high-performance liquid chromatography (HPLC) have been developed for the determination of ivermectin in serum and plasma. These methods employed UV detection [7–10] or, after derivatization, fluorescence detection [11–14]. The reported detection limits with serum or plasma

* Corresponding author.

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samples of 1–5 ml ranged from 1–5 ng/ml by photometry to 0.01–0.5 ng/ml by fluorescence.

Tolan et al. [11] described the chemistry involved in the derivatization of avermectins with acetic anhydride and pyridine (basic catalyst) to produce the fluorescent derivatives on which their HPLC assay was based. With ivermectin, the hydroxyl groups at positions 4″, 5 and 7 were first acetylated and then base-catalyzed elimination reactions occurred in the cyclohexene ring to yield a fluorescent aromatic derivative. These authors noted that the major derivative from ivermectin (4″-O-acetyl-2,5,6-tetradehydro-5,7-dideoxy-22,23-dihydroavermectin B_{1a}) gave a highly fluorescent spot on a silica-gel TLC plate. Recently, an improved derivatization procedure for ivermectin that utilized trifluoroacetic anhydride and 1-methylimidazole has been reported [13].

The objective of the present study was to investigate the utility of TLC for the detection of ivermectin in cattle serum. Using common laboratory equipment in combination with straightforward extraction, derivatization and cleanup techniques, a fluorescent derivative of ivermectin could be visually detected under a long-wavelength UV lamp at levels of 1–2 ng/ml. This detection limit on ordinary silica-gel plates was equal to or better than that reported by HPLC with UV detection and allowed the detection of ivermectin residues in the serum of two cattle for 25 and 32 days after treatment.

After this study was completed, Abjean and Gaugain [15] reported on the detection of a fluorescent ivermectin derivative at low ng levels using silica-gel plates and a TLC scanner equipped with a mercury lamp. The authors claimed that ivermectin could be determined in liver and fat samples at a concentration of 5 ng/g.

2. Experimental

2.1. Reagents

1-Methylimidazole (>99%) and ammonia (2 *M* solution in methanol) were purchased from Aldrich (Milwaukee, WI, USA). Trifluoroacetic anhydride (>99%) was obtained from Pierce (Rockford, IL, USA). Acetonitrile, methyl *tert.*-butyl ether, hexane, acetone and methanol were OmniSolv glass-distilled grade (supplied by BDH, Edmonton, Alb., Canada). Distilled-in-glass decane was obtained from Caledon Laboratories (Edmonton, Alb., Canada). Water was distilled and further purified by a Barnstead NANOpure II system.

A sample of ivermectin (lot L-640,471-000W109) was supplied as a white solid (≥80% 22,23-dihydroavermectin B_{1a}) by Merck (Rahway, NJ, USA). A stock solution of ivermectin (2 mg/ml) was prepared in acetonitrile and stored at −15°C. Working solutions were appropriately prepared by dilution with acetonitrile to give 1, 2, 4, 10, 20 or 100 ng of ivermectin in 25 μl solution.

2.2. Serum samples

Two Hereford heifers were maintained in a controlled-environment room as previously described for other experimental cattle [16]. The animals weighed 292 kg and 266 kg at the time of subcutaneous treatment (0.2 mg/kg) with a nonaqueous, injectable formulation of ivermectin (IVOMEC, supplied by MSDAGVET, a division of Merck Frosst Canada, Kirkland, Que., Canada). Posttreatment bleeding times were at 6, 24, 48, 72 and 96 h, and subsequently on day 7, 8, 9, 10, 11, 14, 16, 18, 21, 23, 25, 32 and 39. Blood was collected from a jugular vein into red-stoppered tubes (Vacutainer no. 6430, Becton Dickinson Canada, Mississauga, Ont., Canada) and allowed to stand at room temperature for 2 h. Serum was obtained by centrifuging at 900 *g* for 25 min and stored at −80°C in polypropylene tubes for analysis.

2.3. Serum extraction

Cattle serum was thawed, vortex-mixed for 10 s (VWR vortex-mixer) and aliquots (1 ml) were transferred to test tubes (100 × 13 mm) equipped with PTFE-lined screw caps.

Drug-free serum samples collected before

treatment were spiked with working solutions of ivermectin, initially at a concentration of 100 ng/ml, then routinely at concentrations of 1 and 4 ng/ml. These samples, as well as serum blanks and serum samples from an ivermectin-treated animal, were vortex-mixed and extracted twice with 2 ml of methyl *tert.*-butyl ether using a reciprocal shaker (Ames aliquot mixer) for 5 min followed by a 1–2 min centrifugation (250 *g*). The top organic layer was transferred with a disposable pipette to a test tube and the combined extracts were evaporated at 40°C under nitrogen with a Meyer N-EVAP apparatus (Organomation Associates, Berlin, MA, USA). The residue was dissolved in acetonitrile (200 μl) with the aid of a Branson ultrasonic bath (5 min).

2.4. Derivatizations

After cooling the 200-μl solution with a crushed ice bath, 100 μl of 1-methylimidazole–acetonitrile (1:1, v/v) were added. The tube was capped and the mixture was vortex-mixed at room temperature (21–23°C) and recooled. Trifluoroacetic anhydride–acetonitrile (1:1, v/v) was added (150 μl) followed by vortex-mixing and allowing the mixture to come to room temperature. A 2 *M* solution of ammonia in methanol was added (250 μl). After 15 min at room temperature, the mixture was concentrated to approximately 100 μl by evaporating for 20 min under nitrogen at 40°C (N-EVAP). Water (750 μl) was added and the mixture was vortex-mixed. The mixture was extracted with hexane (2 × 700 μl) using the reciprocal shaker (5 min) and low-speed centrifugation to separate the layers. The combined hexane extracts (in a tapered 1.5-ml polypropylene tube) were centrifuged for 1 min at 16 000 *g* (Eppendorf Model 5415) and transferred to a clean polypropylene tube, avoiding the bead of water at the bottom. The hexane was evaporated under nitrogen and the residue was redissolved in hexane (50 μl). The solution was vortex-mixed before TLC.

Reference samples were derivatized as outlined above by adding working solutions of ivermectin (1–100 ng) to 200 μl of ice-cold acetonitrile.

2.5. Thin-layer chromatography

Precoated silica-gel 60 F_{254} plastic sheets of 0.2 mm thickness (Merck, Darmstadt, Germany) were cut to size (4 × 8 cm). Solutions (usually 25 μl) were applied to the plate with a pipette (Clay Adams Accu-fill 90 micropet). The spot was approximately 0.25 cm in diameter. Plates were developed at room temperature in small glass tanks (4.5 × 5 × 9 cm) with a mixture of hexane–acetone–decane–methanol (59:30:10:1, v/v). The solvent front migrated 6–7 cm from the origin. Plates were transferred to an adjacent dark room and viewed under a long-wavelength UV lamp (Blak-Ray B-100A, Ultraviolet Products, San Gabriel, CA, USA) equipped with a 100-W General Electric mercury light (HR100PSP44-4). The fluorescent ivermectin derivative appeared as a white spot, R_F 0.43. Solutions of the derivative could be stored in the dark at −15°C for 3 months with no apparent loss of fluorescence. Low levels of the derivative ($\leq$1 ng applied to a plate) faded quite rapidly (within 1 min) during visualization under UV light. Higher amounts ($\geq$2 ng) were readily visible for 5 min.

In experiments on the detection of ivermectin in serum of treated cattle, six samples were extracted, derivatized and examined by TLC each day. One plate was used to chromatograph a sample from a treated animal, a blank (from drug-free serum) and a spiked serum sample (4 ng/ml; 2 ng ivermectin on the plate). A second plate was used to analyze a duplicate serum sample representing the same posttreatment time point, another serum blank and a spiked serum sample (1 ng/ml; 0.5 ng on the plate). The chromatograms were read after a 5–10 s exposure under the lamp.

Precoated silica-gel 60 HPTLC plates (10 × 10 cm, Merck) with and without fluorescent indicator were also evaluated in a derivatization experiment with reference solutions and spiked serum samples. Using the solvent mixture indicated above and a Camag HPTLC 28510 de-

veloper, the sensitivity to the fluorescent derivative was unaffected by the presence or absence of the UV indicator in the silica gel. Sensitivity with this apparatus was approximately the same as that observed by ascending development with the 4 × 8 cm silica-gel 60 plates.

3. Results and discussion

Reference samples of ivermectin in cold acetonitrile were initially derivatized with trifluoroacetic anhydride and 1-methylimidazole (catalyst) according to the procedure of De Montigny et al. [13]. The crude reaction products were examined by TLC using hexane-based solvent mixtures that were previously employed for the separation of avermectins in extracts of fermentation media [17]. Mixtures of hexane and acetone containing methanol gave two fluorescent white spots that gradually faded under the UV light. Addition of decane to the developing solvent increased the persistence of these fluorescent spots. Other fluorescence enhancers have been evaluated by Abjean and Gaugain [15]. With hexane–acetone–decane–methanol (59:30:10:1, v/v), the R_F values were 0.43 and 0.54 (Fig. 1).

Fig. 1. Thin-layer chromatogram illustrating the separation of fluorescent spots on silica gel following derivatization of a reference sample of ivermectin with trifluoroacetic anhydride and 1-methylimidazole. After development with hexane–acetone–decane–methanol (59:30:10:1, v/v), fluorescence was detected under long-wavelength UV light. The aromatic derivatives of ivermectin appeared as white spots (shaded on the chromatogram) whereas reagent-related background, if detectable, was visibly blue (dotted circles).

In theory, these components could represent a homologous mixture of fluorescent derivatives because ivermectin is composed of ≥80% 22,23-dihydroavermectin B_{1a} (*sec.*-butyl group at C-25) and ≤20% 22,23-dihydroavermectin B_{1b} (isopropyl group at C-25). Indeed, under similar derivatization conditions, the homologous mixture of fluorescent derivatives can be separated by HPLC [13,18]. It has also been shown that the fluorogenic 4″-trifluoroacetyl ester derivative of ivermectin was hydrolytically unstable [14]. We subsequently concluded that the homologues remained unresolved on the TLC plates and that the component of R_F 0.54 was the 4″-trifluoroacetyl ester whereas the component of R_F 0.43 was the fluorescent 4″-alcohol (Fig. 2).

Additional derivatizations of reference samples showed that the relative intensity of these two spots varied from day to day. This was true whether the crude derivatization mixture was spotted directly or whether the acetonitrile was evaporated after derivatization, the residue dissolved in water and a hexane extract applied to the plate. Sommer et al. [19] recently suggested that methanol promoted hydrolysis of the 4″-trifluoroacetyl group and added this solvent to the derivatization mixture prior to HPLC analysis. In our experiences, conversion of the 4″-ester to the 4″-alcohol was usually promoted by evaporating the hexane and redissolving the derivatized samples in methanol but the procedure was not always successful.

Since reproducibility and sensitivity should be improved if the derivatization reaction gave a single fluorescent spot, an efficient procedure was needed to hydrolyze the 4″-trifluoroacetyl group. In this regard, Ericson et al. [20] have shown by mass spectrometry that the 4″-trifluoroacetyl group of doramectin, an avermectin analogue, can be selectively removed by the use of ammonia in methanol following derivatization with trifluoroacetic anhydride and triethylamine. HPLC procedures for the determination of abamectin (avermectin B_1) in fruits and vegetables [21,22] used similar reagents (ammonium hydroxide and methanol) after derivatization with 1-methylimidazole and trifluoroacetic anhydride.

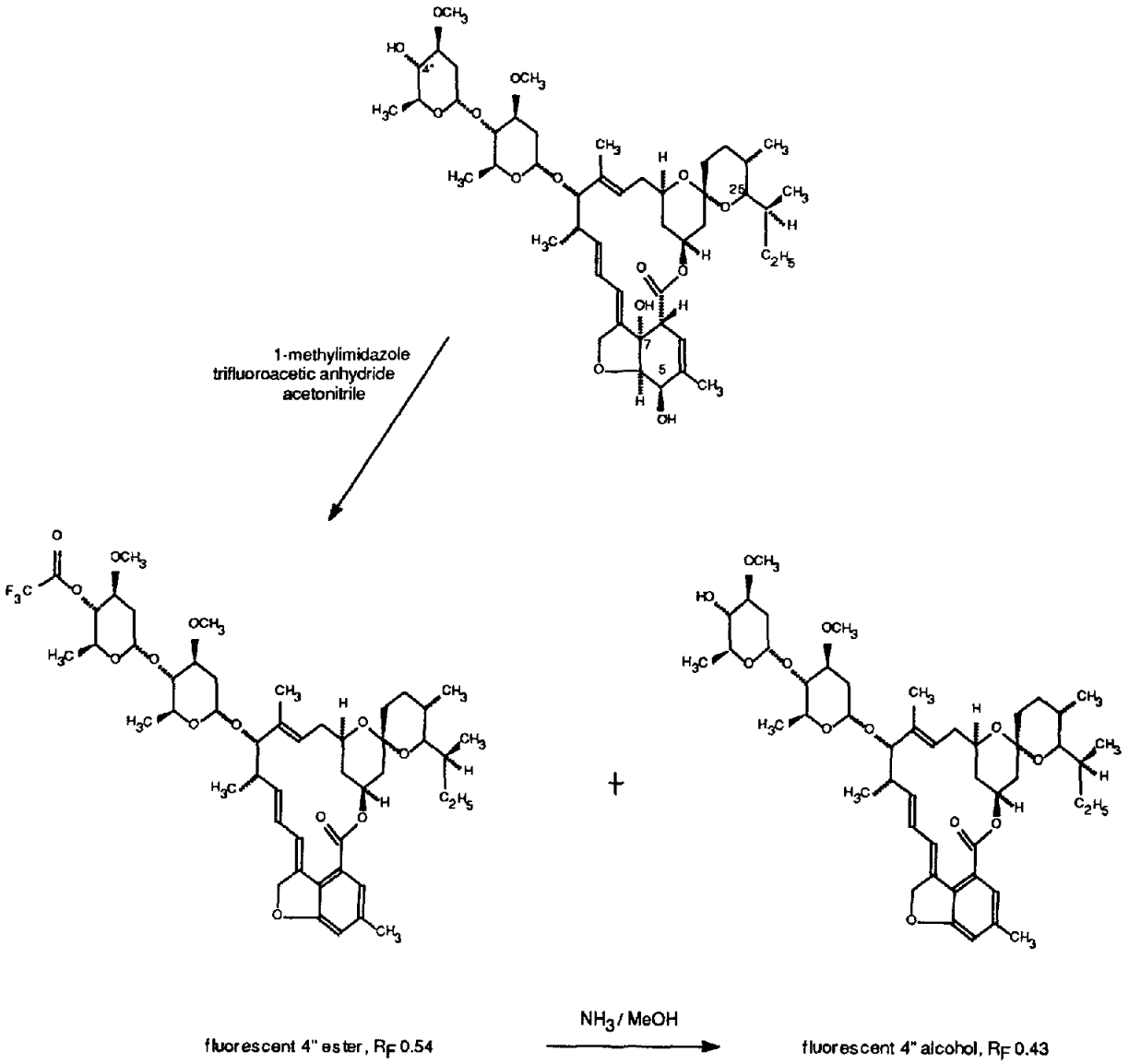

Fig. 2. Derivatization and deblocking reactions studied in the TLC detection of fluorescent ivermectin derivatives. The structure of the major homologue of ivermectin is illustrated at the top.

We found that the 4"-trifluoroacetyl group was readily hydrolyzed using the former procedure [20]. Isolation of the fluorescent derivative proceeded in the same manner as in experiments conducted without ammonia. TLC of concentrated hexane extracts showed the spot of R_F 0.43 (alcohol) without a trace of the ester component.

It should be emphasized that both the ester and alcohol derivatives were highly fluorescent when viewed under long-wavelength UV light. Derivatizations conducted without ammonia treatment showed both components when reactions were performed with as little as 4 ng of ivermectin and applying one-half of the final hexane extract to the plate (2 ng on-plate sen-

sitivity). In derivatization experiments with the ammonia treatment, the on-plate sensitivity of the alcohol corresponded to 0.1 ng of ivermectin.

Experiments with drug-free serum were initially conducted by spiking serum samples with ivermectin at a concentration of 100 ng/ml. This was followed by extraction with methyl *tert.*-butyl ether, a solvent previously utilized in the solid-phase extraction of this drug [23]. The residue was derivatized and treated with ammonia. TLC on one-quarter of the final hexane extract, corresponding to 25 ng of ivermectin, clearly revealed the fluorescent alcohol derivative. Serum blanks prepared in an identical manner appeared to be free of interfering fluorescent spots.

Since these were promising results, the same techniques were applied on different days to serum samples that were spiked at lower ivermectin concentrations. In 37 experiments in which one-half of the final hexane extract was applied to a plate, the fluorescent spot of the alcohol was always visible at ivermectin concentrations of 4 ng/ml. The spot persisted for 1–2 min. In 29 of the 37 experiments, the fluorescent spot corresponding to 1 ng/ml (0.5 ng on-plate sensitivity) was visible. At this low serum concentration, however, the spot only remained visible under the UV lamp for 15–30 s and, in eight experiments, the spikes of 1 ng/ml could not be differentiated from the serum blanks. That problem did not occur with extracts from serum samples spiked at concentrations of 2 ng/ml or higher.

This procedure was applied to detect ivermectin residues in the serum of cattle following injection of a commercial product at the recommended dose. Ivermectin was detected in the first blood sample, taken 6 h after dosing. By visually comparing the intensity of fluorescence on the TLC plates, levels of ivermectin in serum appeared to peak at 2–4 days posttreatment (animal 1) and at 3–7 days posttreatment (animal 2). The fluorescent TLC spot of the alcohol remained visible in all serum samples until at least 25 days posttreatment. Thereafter, blood was collected at weekly intervals and ivermectin could not be detected in serum samples collected at 32 days posttreatment in animal 1 and at 39 days in animal 2. These observations agreed with blood concentration–time profiles that have been defined for cattle by HPLC [1–3].

The qualitative TLC techniques described here provide a new approach to detect ivermectin in cattle serum down to concentrations of 1–2 ng/ml. Several technicians have implemented these techniques in our laboratory and encountered very few problems. Reagent-related background on the TLC plates can become significant, especially if the bottle containing 1-methylimidazole, an air-sensitive reagent, loses its inert atmosphere from repeated puncturing of the liner. The ammonia solution should also be replaced at regular intervals. If interferences are encountered near the fluorescent spot of the alcohol, the samples can be derivatized without the use of ammonia and subsequently visualized by means of the fluorescent spot of the ester, albeit with variable and reduced sensitivity.

These simple techniques will be useful to check for residues of this antiparasitic agent, for example to screen blood samples to show that recommended withdrawal times have been met before cattle are slaughtered.

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