Monoclonal Antibodies for Immunoassay of Avermectins

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We have developed a panel of mouse monoclonal antibodies (MAbs) that recognize ivermectin and abamectin, the major avermectins used in veterinary and agricultural formulations. Conjugates of ivermectin 4″-hemisuccinate on three different proteins were used to raise the antibodies and develop a quantitative competition enzyme immunoassay (EIA) for avermectins. The five MAbs that gave the lowest detection limits for ivermectin and abamectin were used to optimize the EIA. Several types of EIA plates, plate coating conditions, blocking agents, incubation times and temperatures, and enzyme-conjugated detecting antibodies were compared to determine acceptable assay conditions. One of the MAbs, C4D6, bound ivermectin in buffers containing up to 30% (v/v) of organic solvent. The lower limit of detection for standards in this EIA was approximately 0.5 ppb for ivermectin and 1 ppb for abamectin, with half-maximal inhibition (I_{50}) around 3 ppb for ivermectin and 7 ppb for abamectin. The MAbs and EIA appear to be usable for quantifying avermectin residues in agricultural and environmental matrices.

INTRODUCTION

Avermectins are naturally occurring antibiotics secreted by the actinomycete *Streptomyces avermitilis*. They are active against helminths and arthropods (Chabala et al., 1980), but have no significant antibacterial or antifungal activity (Burg et al., 1979). The use of avermectins is rapidly increasing in livestock pest management, veterinary medicine, parasitology, and traditional chemical control of insect pests on orchard and field crops. As the number of new applications grows, so does the need for simple, precise, and inexpensive means of detecting avermectin residues.

Current methods for detecting avermectins and their analogues include partition chromatography and high-performance liquid chromatography (Miller et al., 1979; Pivichny et al., 1987; Oehler and Miller, 1989; Tolan et al., 1980; Tway et al., 1981). These instrumental methods are accurate but expensive and time-consuming, requiring lengthy sample extraction and cleanup procedures. Biological assays for avermectin potency are logistically difficult to conduct, are only semiquantitative, and may be sensitive to solvents and other components of the sample material. By contrast, immunoassays generally are faster and much less expensive and require less sample preparation than instrumental methods, and they have been successfully developed for other compounds that are difficult to analyze by instrumental methods (Jung et al., 1989). Since no immunological assays for avermectins or structurally related compounds have been reported, we undertook this project.

We report here the production of a panel of monoclonal antibodies (MAbs) to ivermectin and the development of a sensitive and reproducible enzyme immunoassay (EIA) for avermectins. The MAbs recognize ivermectin and abamectin, the major avermectins used in veterinary and agricultural applications. The classical competition EIA using these MAbs has a detection limit similar to that of the presently used instrumental analysis methods. The EIA should be compatible with the established procedures for recovery of avermectins from biological and environmental samples.

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MATERIALS AND METHODS

Ivermectin (22,23-dihydroavermectin B_1) and abamectin (avermectin B_1) were provided by Merck, Sharpe, & Dohme, Inc. (Three Bridges, NJ). Reference solutions were prepared in methanol and standardized by spectrophotometry, using the following extinction coefficients: for ivermectin, ϵ_M = 27 100, 30 100, and 19 300 at λ = 238, 245, and 254 nm, respectively (Chabala et al., 1980; Merck Index 10, 1983, p 753); for abamectin, ϵ_M = 29 120, 31 850, and 20 510 at λ = 237, 243, and 252 nm, respectively (Merck Index 10, 1983, p 128). The reference solutions were stored at 4 °C in Teflon vials (Pierce Chemical Co.). Ivermectin–protein conjugates were stored in glass vials in phosphate-buffered saline (PBS; 0.01 M KH_2PO_4–K_2HPO_4, pH 7.4–0.15 M NaCl) at 4 °C. Unless otherwise specified, the EIAs were performed in Immulon 2 plates (Dynatech); samples of other EIA plates were generously provided by manufacturers or distributors. Enzyme–antibody conjugates were obtained from Sigma Chemical Co. or Fisher Scientific. For cell fusion, polyethylene glycol 4000, gas chromatography grade, was purchased from EM Science (Cherry Hill, NJ). All other chemicals used for the work reported here were of analytical reagent grade or better. Cell culture media and additives were purchased from GIBCO Laboratories (Grand Island, NY), and fetal bovine serum was from Intergen, Inc. (Kankakee, IL). Swiss Webster mice were purchased from Simonsen Laboratories (Gilroy, CA), and Biozzi and B10.Q mice were from stock bred in the University of California, Berkeley, Hybridoma Facility mouse colony.

Hapten Synthesis. Ivermectin hapten syntheses were based on variations of the chemistry described by Mrozik et al. (1982) for preparation of avermectin acyl derivatives. Nuclear magnetic resonance (NMR) assignments were facilitated by comparison to data given in the same paper for avermectin B_{1a}

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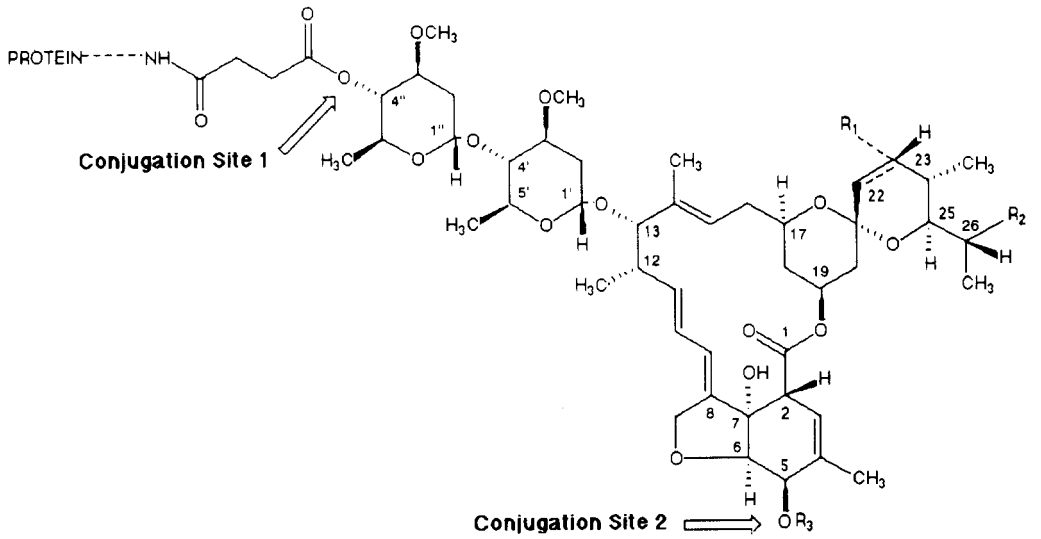

Figure 1. Structure of ivermectin, showing the sites that were derivatized to form the hapten–protein conjugates used as immunizing and screening antigens. The 4″-hemisuccinate (site 1) or the hemisuccinate formed through the oxygen on carbon 5 (site 2) was conjugated to carrier proteins as described under Materials and Methods.

derivatives. Ivermectin is an approximately 80:20 mixture of the 22,23-dihydroavermectins B_{1a} and B_{1b}. This mixture was used throughout this study and can be observed in the hapten mass spectra data. NMR spectra were obtained on a Nicolet NT-300 (300 MHz) instrument. Chloroform-*d* ($CDCl_3$) was used as the solvent, and chemical shifts are reported downfield from tetramethylsilane. Fast atom bombardment mass spectral data (FABMS) were obtained on a VG70-HF spectrometer using a dithiothreitol (DTT)/dithioerythritol (DTE) matrix, and 8-kV Xe atom bombardment. All products were purified by flash chromatography (Still, 1978).

Ivermectin was conjugated at the two positions indicated in Figure 1. $Iver_1$ (4″-*O*-succinoylivermectin) was prepared in a three-step sequence involving (a) protection of the 5-hydroxy group as the *tert*-butyldimethylsilyl ether, (b) succinylation of the 4″-hydroxy group using succinic anhydride and 4-(dimethylamino)pyridine (DMAP), and (c) deprotection of the 5-hydroxy group. The use of DMAP is essential to effecting succinylation of the 4″-hydroxy group without employing a longer indirect process such as that described by Mrozik et al. (1982) for preparation of 4″-*O*-succinoylavermectin B_{1a}. In contrast, $iver_2$ was prepared by direct succinylation of ivermectin using pyridine instead of DMAP. Additional support for the hapten structures was obtained by conversion to the corresponding methyl esters using ethereal diazomethane.

4″-*O*-Succinoylivermectin: R_f 0.4 (1% HOAc/5% iPrOH/CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$) δ 5.9–5.7 (m, 3 H), 5.45–5.3 (m, 2 H), 5.0 (br d, 1 H), 4.78 (d, 1 H, J = 3 Hz), 4.69 (t, 1 H, J = 9.4 Hz, C4″-*H*), 4.68 (br s, 2 H, C8-CH_2), 4.3 (br d, 1 H, J = 6 Hz, C5-*H*), 3.97 (d, 1 H, J = 6.3 Hz, C6-*H*), 3.95 (br s, 1 H), 3.89–3.82 (m, 2 H), 3.7–3.58 (m, 4 H), 3.43 (s, 3 H, C3′-OCH_3), 3.36 (s, 3 H, C3″-OCH_3), 3.23 (t, 1 H, J = 8.9 Hz, C4′-*H*), 2.75–2.65 (m, 4 H, COCH_2CH_2CO), 2.4–2.2 (m, 4 H), 1.98 (dd, 1 H, J = 5, 12 Hz), 1.88 (br s, 3 H, C4-CH_3), 1.50 (br s), 1.25 (d, 3 H, J = 6 Hz, CH_3), 1.17 (d, 3 H, J = 6.8 Hz, CH_3), 1.14 (d, 3 H, J = 6.3 Hz, CH_3), 0.93 (t, 3 H, J = 7.3 Hz, CH_3), 0.86 (d, 3 H, J = 6.7 Hz, CH_3), 0.79 (br d, 3 H, J = 6 Hz, CH_3).

FABMS: negative ions, m/z [relative intensity; assignment] 1127.3 [17.2; M (succinoyl 22,23-dihydroavermectin B_{1a} component) + DTT – H], 973.4 [100; M (succinoyl-22,23-dihydroavermectin B_{1a} component) – H], 959.4 [15.5; M (succinoyl-22,23-dihydroavermectin B_{1b} component) – H], 873.5 [12.9; M (succinoyl-22,23-dihydroavermectin B_{1a} component) – succinate – H], 567.3 (46.8), 433.3 (25.4), 405.1 (44.0).

5-*O*-Succinoylivermectin: R_f 0.4 (1% HOAc/5% iPrOH/CH_2Cl_2); ^1H NMR (300 MHz, $CDCl_3$) δ 5.9–5.65 (m, 3 H), 5.54 (br s, 1 H, C5-*H*), 5.45–5.25 (m, 2 H), 4.99 (br d), 4.77 (d, J = 3 Hz), 4.64 (dd, 1 H, J = 1.9, 14.1 Hz, C8-CH*H*′), 4.58 (dd, 1 H, J = 1.5, 14.1 Hz, C8-CH*H*′), 4.05 (d, J = 6.3 Hz), 3.94 (br s), 3.88–3.58 (m, 2 H), 3.43 (s, 3 H, C3′-OCH_3), 3.42 (s, 3 H, C3″-OCH_3), 3.23 (t, 1 H, J = 8.9 Hz, C4′-*H*), 3.17 (t, 1 H, J = 9.2 Hz, C4″-*H*), 2.73 (m, 4 H, COCH_2CH_2CO), 2.4–2.2 (m, 4 H), 1.75 (br s, 3 H, C4-CH_3), 1.50 (br s), 1.28 and 1.26 (overlapping d, 3 H each, J = 6.3 and 6.0 Hz, respectively, C5′- and C5″-CH_3), 1.16 (d, 3 H, J = 7.1 Hz, CH_3), 0.93 (t, 3 H, J = 7.3 Hz, CH_3), 0.86 (d, 3 H, J = 6.5 Hz, CH_3), 0.8 (br m).

FABMS: positive ions, m/z [relative intensity; assignment] 1151.6 [15; M (succinoyl-22,23-dihydroavermectin B_{1a} component) + DTT + Na^+], 1013.5 [17; M (succinoyl-22,23-dihydroavermectin B_{1a} component) + K^+], 997.5 [30; M (succinoyl-22,23-dihydroavermectin B_{1a} component) + Na^+]; negative ions, m/z [relative intensity; assignment] 1127.7 [40; M (succinoyl-22,23-dihydroavermectin B_{1a} component) + DTT – H], 973.8 [50; M (succinoyl-22,23-dihydroavermectin B_{1a} component) – H].

Conjugate Preparation. The ivermectin hapten, calculated to be a 20–30-fold molar excess over the carrier protein, was preactivated to form an *N*-hydroxysuccinimide ester before coupling to the protein carrier. This procedure was typically carried out in a minimal volume (0.2–0.3 mL) of dimethylformamide with a 2-fold molar excess (calculated over hapten) of *N*-hydroxysuccinimide (NHS) and a 2-fold molar excess (calculated over hapten) of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide. The activation reaction was stirred at room temperature overnight. The protein carriers were bovine serum albumin (BSA), conalbumin (Con), or keyhold limpet hemocyanin (KLH). Typically 10–20 mg of protein was dissolved in 0.1 M borate (pH 9.4) to a concentration of 4–5 mg/mL, and dimethylformamide was added to a concentration of 9–12% (v/v). The preactivated hapten solution was added directly to the protein solution dropwise over 1 h at room temperature with stirring. The coupling solution routinely became turbid with a finely suspended solid by the end of the hapten addition. Final DMF concentration in the coupling solution ranged from 12 to 16% (v/v). The coupling solution was stirred for an additional hour at room temperature and then at 5 °C overnight. The sample was transferred to cellulose dialysis tubing and dialyzed versus 2-L volumes of phosphate-buffered saline (pH 7.2) for a minimum of 2 days and three dialysate changes. Any precipitate remaining after dialysis was removed by centrifugation. The dialyzed supernatant was further purified on a Sephadex G-100 (40–120-μm beads) column (1.7 × 48 cm) equilibrated with PBS. Ivermectin–protein conjugates were stored in glass vials in phosphate-buffered saline (pH 7.4) at 4 °C, and aliquots were centrifuged for 3 min at 1000*g* to remove traces of aggregated material prior to use.

Table I lists the hapten density of the ivermectin conjugates used for immunization and EIA screening. The bracketed letters refer to the pool fraction collected after Sephadex gel filtration. Hapten content was determined by UV–visible

Table I. Ivermectin Conjugates Used for Antibody Production and EIA[a]

conjugate	molecules of iver per molecule carrier	moles of iver per 100 amino acids	I_{50}, ppb	slope factor
site 1 conjugates				
$iver_1$–BSA [A]	17	2.9	1.7 ± 0.3	1.95 ± 0.03
$iver_1$–BSA [B]	21	3.5	3.9 ± 0.5	1.61 ± 0.14
$iver_1$–BSA [C]	12	2.0	3.3 ± 0.5	1.32 ± 0.16
$iver_1$–BSA [D]	5	0.8	4.1 ± 0.6	1.46 ± 0.15
$iver_1$–con-albumin [E]	15	1.9	6.2 ± 0.5	1.28 ± 0.02
$iver_1$–con-albumin [F]	7	0.9	4.9 ± 0.4	1.08 ± 0.01
$iver_1$–con-albumin [H]	16	2.0	3.4 ± 0.1	1.54 ± 0.02
$iver_1$–KLH [G]	12	0.4	2.6 ± 0.4	1.61 ± 0.17
site 2 conjugates				
$iver_2$–BSA [A]	30	5.0	2.9 ± 0.2	1.58 ± 0.09
$iver_2$–con-albumin [C]	31	4.0	1.8 ± 0.4	4.49 ± 0.09

[a] Hapten density and protein content were determined as described under Materials and Methods, and the I_{50} values and slope factors were determined by competition EIA with MAb C4D6.

spectrophotometry, and protein was measured by the method of Lowry et al. (1954). The number of ivermectin molecules per 100 amino acids was computed by using molecular weights of 65 400, 86 180, and 360 000 for BSA, conalbumin, and KLH, respectively, and an average molecular weight of 110 per amino acid residue.

Immunization. Pairs of Swiss Webster, Biozzi, and B10.Q mice were immunized four times with mixtures consisting of 25 μg of a single ivermectin–protein conjugate ($iver_1$–BSA [A], $iver_1$–Con [E], or $iver_1$–KLH [G]) and 50 μg of Ribi adjuvant (MPL + TDM emulsion, Ribi Immunochem Research, Hamilton, MT) in 0.1 mL of physiological saline, delivered intradermally in three sites on the back. Injections were given on days 1, 7, 21, and 45, and ivermectin antibody titers were determined by direct EIA of tail vein blood samples taken on days 27 and 52. Competition EIA was used to determine the ability of free ivermectin to inhibit the binding of a limiting amount of mouse antibody to an ivermectin conjugate. In all cases, the conjugates used in the EIAs had carrier proteins different from that of the immunizing conjugate.

Three mice (two Swiss Webster and one B10.Q) that developed the best serum responses (highest titer in the direct EIA, lowest detectable dose, and lowest I_{50} for ivermectin in the competition EIA) were given final "booster" doses of conjugate 4 and 3 days prior to the preparation of hybridomas. The Swiss Webster mice received 25 μg of their immunizing conjugate in 0.05 mL of saline via the tail vein, and the B10.Q mouse received two intradermal doses consisting of 25 μg of immunizing conjugate and 50 μg of Ribi adjuvant.

Media and Cell Culture. The "complete medium" for hybridoma culture was Iscove's Modified Dulbecco's Medium (IMDM) (Iscove, 1984), supplemented with 20% (v/v) fetal calf serum, 10 μg/mL kanamycin sulfate, 5×10^{-5} M β-mercaptoethanol, and 1 μg/mL of 1/3 iron-saturated transferrin (human type III, Sigma). Hybridomas were selected in complete medium supplemented with Iscove's lipid emulsion, J774A.1 macrophage conditioned medium (Sugasawara et al., 1985), 10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3×10^{-5} M thymidine (HAT). Myelomas were adapted to grow in medium with hypoxanthine and thymidine prior to fusion, and aminopterin was added to the hybridomas 24 h after fusion. The hybridomas were adapted to grow in complete IMDM without emulsion, macrophage medium, or HAT after they were expanded to 24-well culture plates. Cultures were grown at 37 °C in 5% CO_2 and saturating humidity. Hybridomas were frozen at 10^7 cells/mL in medium consisting of complete IMDM/FBS/DMSO (6:3:1) by using a Cryomed 910 programmable cell freezer, and the cultures were stored in liquid nitrogen.

Hybridoma Production. Splenocytes from the responding mice were fused with log-phase P3X63AG8.653 myelomas, at a ratio of 2.5 myelomas per splenocyte, essentially as described by Fazekas de St. Groth and Scheidegger (1980). The fusing agent was 50% (w/v) polyethylene glycol 4000 in water containing 5% (v/v) dimethyl sulfoxide. A total of 9888 cultures (103 96-well plates) were seeded at 3.5×10^4 splenocytes per well, a density at which colonies that developed were better than 95% likely to be monoclonal. Twelve to 21 days later, culture supernates were sampled from colonies and tested for ivermectin-specific antibodies by direct EIA.

Ivermectin-specific hybridomas were expanded to 24-well dishes, and supernates from these cultures were again screened by direct EIA on wells coated with the hapten conjugate or the unconjugated carrier protein. Ivermectin-specific antibodies that did not react with unconjugated carrier were tested for inhibition by free ivermectin in a competition EIA. Selected cell lines were subcloned by limiting dilution and expanded to produce approximately 1 L of culture medium containing sufficient antibody for several hundred thousand assays. Immunoglobulin subclass was determined by EIA using a commercial kit (Southern Biotechnology Associates, Birmingham, AL).

Enzyme Immunoassays. The EIAs were based on procedures and utilized buffers described by Voller et al. (1976). Conjugates were adsorbed to EIA plates in "coating buffer" (0.015 M Na_2CO_3–0.035 M $NaHCO_3$–0.003 M NaN_3, pH 9.6). Antibodies and antibody–analyte mixtures were diluted in PBS–Tween (0.01 M KH_2PO_4–K_2HPO_4, pH 7.4–0.15 M NaCl–0.02% NaN_3–0.05% Tween 20). For the experiment using peroxidase–antibody conjugates, the NaN_3 was omitted from the PBS–Tween. The substrate solutions for color development were as follows: for alkaline phosphatase conjugated goat anti-mouse Ig, and for Extravidin avidin–alkaline phosphatase conjugate used with biotinylated goat anti-mouse Ig, *p*-nitrophenyl phosphate (Sigma 104 substrate tablets) 1 mg/mL, in 10% (w/v) diethanolamine hydrochloride, pH 9.8–0.4 mM $MgCl_2$–3 mM NaN_3; for horseradish peroxidase, *o*-phenylenediamine N hydrochloride, 0.4 mg/mL, in 0.02 M citric acid–Na_2HPO_4, pH 5–0.009% (v/v) H_2O_2 (Goding, 1986); for urease, bromocresol purple, 0.08 mg/mL, in 0.2 mM ethylenediaminetetraacetic acid–NaOH, pH 4.8, containing urea, 1 mg/mL (Chandler et al., 1982).

Individual EIA plates were tightly covered during all incubation steps to prevent evaporation. Between incubation steps, the plates were washed with PBS–Tween from a squeeze bottle three times and dried by rapping on lint-free towels. "Blocking solutions" to prevent adventitious binding were prepared by dissolving the indicated material in PBS–Tween and filtering through a 0.45-μm Millipore filter to remove aggregates before use.

Direct EIA. A "direct EIA" was used to detect antibodies to ivermectin. Wells were coated overnight at 4 °C with conjugate containing 500 ng of carrier protein in 0.1 mL of coating buffer. The wells were washed, blocked for 30 min at room temperature with PBS–Tween–1% BSA, and incubated for 2 h at room temperature with 0.1 mL of the antibody-containing serum or cell culture fluid diluted with PBS–Tween. The wells were washed again and incubated for 2 h at room temperature with 0.1 mL of alkaline phosphatase conjugated goat anti-mouse antibody (1:1000 in PBS–Tween). After a final wash, 0.1 mL of substrate solution was added to the wells, color development was monitored on a Multiskan EIA reader (Flow Laboratories) interfaced with a Macintosh computer, and the rates of the reaction (ΔA_{405}/min × 10^3) were calculated by linear regression.

Competition EIA. A competition EIA was used to quantitate the avermectins and to compare assay conditions and recognition by different antibodies. Subsaturating (limiting) amounts of ivermectin–protein conjugate and antibody were determined by direct EIA. Immulon 2 wells were coated overnight at 4 °C with a limiting amount of conjugate ($iver_1$–Con [E], unless stated otherwise), usually equivalent to 25 or 50 ng of carrier protein in 0.1 mL of coating buffer. For one experiment, Dynatech Immulon C plates, which are treated to covalently bind the coating antigen, were coated with antigen in PBS at pH 7.5 and blocked per the manufacturer's instructions. Standards (0.1 ppb–1 ppm) or unknowns were mixed

with the limiting dilution of antiserum or hybridoma culture fluid (MAb C4D6, unless stated otherwise) in PBS–Tween containing 10% (v/v) acetonitrile or other organic solvent, as indicated, and incubated overnight at room temperature in tightly sealed polypropylene tubes. The coated wells were washed as described for the direct EIA, and aliquots (0.1 mL) of the antibody–analyte mixture were applied. After 2 h at room temperature, the wells were washed, and the remainder of the assay performed as described for the direct EIA. Color development was inversely proportional to the amount of avermectin in the sample.

Data Analysis. Dose–response curves (generally 11 dilutions in triplicate from a spectrophotometrically standardized stock solution) were fitted by iterative regression to the four-parameter logistic equation (Canellas and Karu, 1981) using Passage II (Passage Software, Inc., Fort Collins, CO) on a Macintosh computer. The characteristics of the dose–response curve were expressed as the EIA rate at the limiting low dose (LLD) of 0.1 ppb avermectin, I_{50} (the concentration that half maximally inhibited the EIA), and the slope of the best-fit curve at the I_{50}. The slope ($\Delta_{response}/\Delta_{dose}$) is one of the four parameters that define the dose–response curve, and it is one indicator of the sensitivity of the assay with a particular MAb or coating antigen. The data were expressed as the mean ± standard error. Significant differences in the data were determined by repeated measure analysis of variance (ANOVA) and Fisher's protected least-squares difference (LSD) (Little and Hills, 1978; Snedecor and Cochran, 1980). P values that are included in statements about significance indicate the likelihood that the result could have occurred by chance. For example, $P < 0.01$ indicates less than 1% likelihood that the result is *not* significant.

RESULTS AND DISCUSSION

Synthesis of Haptens and Conjugates. Ivermectin hapten syntheses were based on variations of the chemistry described by Mrozik et al. (1982) for the preparation of avermectin acyl derivatives. Hapten 1 (4″-*O*-succinoylivermectin) was prepared in a three-step protection–succinylation–deprotection sequence that utilized DMAP [(dimethylamino)pyridine] to effect the succinylation of the 4″-hydroxy group. Hapten 2 (5-*O*-succinoylivermectin) was prepared by direct succinylation. The conjugation procedure employed an *N*-hydroxysuccinimide activated ester "bridge" to improve coupling efficiency and reduce the degradative effects usually observed with the direct EDC method (Weetall, 1976).

Responses of Mice to Ivermectin Conjugates. To survey the widest range of ivermectin antibody expression, we immunized pairs of three different mouse strains with six different ivermectin–protein conjugates. The ivermectin-specific antibody titers of sera taken 28 days after initial immunization differed by more than 10-fold, with the B10.Q mice and some Swiss Websters raising the best responses. The Biozzi mice developed the poorest titers to all of the immunizing conjugates. Sera from 16 of the 18 mice had antibodies that were inhibited by ivermectin in the competition EIA, with I_{50} values that differed over 10-fold. The mean I_{50} values of the mouse sera taken on day 52 were between 20 and 50 ppb. The two Swiss Webster mice and one B10.Q mouse that had the highest titer in the direct EIA and the lowest I_{50} in the competition EIA were used to prepare the hybridomas. Pairs of mice immunized and boosted with each of the iver$_2$ conjugates failed to raise a significant response to ivermectin.

Properties of the Hybridomas. Hybridoma culture fluids were screened on avermectin conjugated to a protein other than that of the immunizing conjugate. Of the 1686 hybridoma colonies that developed, 485 culture fluids reacted with ivermectin hapten, and 33 of these were inhibited by ivermectin in the competition assay. Fourteen of these MAbs had I_{50} values for ivermectin below 50 ppb, which was the most sensitive I_{50} value we observed with any of the mouse sera. The five cell lines with the lowest I_{50} values were subcloned, and the specificity and other properties of these MAbs were investigated further. Although the five MAbs were of the same immunoglobulin subclass, their I_{50} values for ivermectin and abamectin (Table II), ivermectin and abamectin monosaccharides, and other avermectins were sufficiently different to indicate that all five MAbs had unique recognition patterns (Karu et al., 1990). MAbs from six subclones of each of these cell lines gave results identical with those observed with their parent line.

Table II. Antibody Subclass and Sensitivity of Competition EIA for Ivermectin and Abamectin[a]

monoclonal antibody	Ig subclass	ivermectin		abamectin	
		I_{50}, ppb	slope	I_{50}, ppb	slope
C4D6	$IgG_1\kappa$	3–14	0.9–1.0	28–59	1.0–1.1
C5D6	$IgG_1\kappa$	6–20	1.2–1.7	33–72	1.5–1.8
B2A2	$IgG_1\kappa$	8–10	0.9–1.2	7–12	2.2–3.1
C1A3	$IgG_1\kappa$	10–31	1.0–1.2	10–13	1.2–1.8
B11C2	$IgG_1\kappa$	12–15	1.3–1.4	11–13	0.9–2.3

[a] These data are the range of dose–responses during 3 months of competition EIA experiments.

Optimization of the Competition EIA. *Plates.* Eight brands of EIA plates from six manufacturers were tested for their utility in the competition assay using PBS–Tween with 5% methanol or PBS–Tween with 10% acetonitrile–25 mM H_3PO_4 as diluent for the precompetition incubation step. Beckman EPB, Corning E-Z Wash, Falcon Pro-Bind, Nunc Immuno 2, Costar 3590, and Dynatech Immulon 2 and Immulon 4 plates all gave responses with acceptable precision. EIA rates at the limiting low dose of 0.1 ppb of ivermectin were between 10 and 19.5, and the I_{50} values ranged from 2.4 to 3.6 ppb. I_{50} values were slightly higher (3.1–4.7 ppb) in PBS–Tween with acetonitrile–H_3PO_4 than in PBS–Tween with methanol, but otherwise the results were identical. The EIA using Immulon C plates, to which the ivermectin conjugate was presumed to be covalently bound, had low rates, scatter in individual data points, and sharp breaks at both ends of the standard curves, making them difficult to fit. We did not attempt to improve the results with Immulon C plates, nor did we explore other methods of covalently attaching coating antigens to these plates.

Plate Coating Conditions. The rate of color development, I_{50} value, and slopes of the competition EIA dose–response curves were compared for Immulon 2 plates coated with iver$_1$–Con [E] for various times at different temperatures. MAb C4D6 was used for these experiments. The results were nearly identical, whether plates were coated at room temperature (22 °C) for 2 or 3 h or overnight at 4 °C. Plates coated for 30 min or overnight at room temperature gave significantly lower EIA rates ($P < 0.01$), but did not have significantly different I_{50} values ($P < 0.01$). Plates coated at 37 °C for various lengths of time gave significantly lower ($P < 0.01$) EIA rates and greater variance in the dose–response compared to plates coated at room temperature. The most reproducible results were obtained by coating for 3 h at room temperature or overnight (approximately 14 h) at 4 °C. The poorer dose–response curves obtained with plates coated at 37 °C or overnight at room temperature may have been due to degradation or denaturation of the conjugate, but we did not investigate this further.

Storage of Conjugates and Coated Plates. During 3 months of competition EIAs with iver$_1$–Con [E] as coating antigen and MAb C4D6 as probe, we found that the mean

Table III. Ivermectin Competition EIA with MAb C4D6, with Coating Antigens Stored Refrigerated or Frozen for 72 Days

coating antigen	storage temp, °C	rate at LLD[a]	I_{50}, ppb
$iver_1$–conalbumin [E]	4	17.1 ± 0.3	4.7 ± 0.6
	−70	21.0 ± 1.8	6.5 ± 2.5
$iver_2$–BSA [A]	4	9.3 ± 0.6	1.6 ± 0.01
	−70	13.3 ± 1.0	1.5 ± 0.20

[a] LLD, limiting low dose; 0.1 ppb in this experiment.

I_{50} values for spectrophotometrically standardized ivermectin stocks rose by approximately 6 ppb. The ivermectin–protein conjugates were stored under refrigeration (approximately 4 °C). Although the refrigerated conjugates did not show visible aggregation or deterioration, their aging may have reduced the sensitivity and uniformity of the assay. In an effort to limit these effects, we investigated the quality of conjugates that were stored at −70 °C.

In Table III, competition EIAs were conducted on plates coated with $iver_1$–Con [E] and $iver_2$–BSA [A] conjugates that had been stored at −70 °C for 72 days. The EIA rates were significantly higher ($P < 0.01$) on plates coated with the frozen conjugates than on plates coated with the refrigerated conjugates, but the I_{50} values were not significantly different. The two conjugates in Table III could be stored frozen without losing their potency, although use of frozen conjugates increased the variance of the I_{50} measurements. This experiment was not done with ivermectin–KLH conjugates, because KLH has a tendency to form insoluble precipitates upon freezing and thawing.

Experiments were conducted to determine whether the EIA could be run with Immulon 2 plates that were stored frozen after the coating step. Plates were coated overnight at 4 °C and stored at −20 °C under three conditions. For group A, the wells were coated and then emptied, and the plates were stored dry. Group B plates were coated and stored at −20 °C with the coating solution remaining in the wells. Group C plates were coated, the wells were rinsed with coating buffer, and the plates were stored with coating buffer in the wells. Plates from these groups were removed from storage at various intervals from 1 to 30 days, and their performance in competition EIAs was compared with that of plates coated overnight at 4 °C.

The I_{50} and slope values for plates from groups A–C were not significantly different ($P < 0.05$) from one another. The I_{50} values of the "freshly prepared" plates increased by approximately 2.5 ppb over the 30-day test period, while I_{50} values for plates stored at −20 °C increased by less than 1.5 ppb. Furthermore, the between-assay differences in I_{50} values were significantly less ($P < 0.05$) than for plates coated the night before use. Thus, plates stored at −20 °C for periods up to 1 month after coating gave better between-assay uniformity (less day-to-day plate variation) than plates coated as needed, the day before use.

Comparison of Ivermectin Conjugates. Table I lists the estimated hapten densities for various ivermectin conjugates, as well as the I_{50} and slope values of the competition EIA dose–responses with MAb C4D6. The direct EIA response at limiting low dose was significantly higher ($P < 0.01$) on plates coated with the $iver_1$ conjugates than on plates coated with the $iver_2$ conjugates, but the I_{50} and slope of the competition EIA dose–response curves were better on plates coated with the $iver_2$ conjugates.

Optimal amounts of coating antigen for EIA are customarily determined empirically and expressed in terms of the amount of carrier protein, which is easier to quantify than the amount of conjugated hapten. In Table I the competition EIA was run on plates coated with conjugate on the basis of equal amounts of carrier protein. The I_{50} value for $iver_2$–Con [C] was lower and the slope of the dose–response curve was greater than for the $iver_1$ conjugates. This conjugate gave the most sensitive EIA. However, the EIA rates at the limiting low dose of 0.1 ppb (not shown) were significantly greater ($P < 0.01$) on plates coated with the $iver_1$ conjugates than on plates coated with the $iver_2$ conjugates. Thus, use of the $iver_1$ conjugates minimized assay development time and error in monitoring the rate of color development.

To more accurately compare the quality of the coating antigens in Table I, we performed competition EIAs with coating calculated from the amount of hapten per unit of carrier molecule. $Iver_1$–KLH [G], with a hapten density of 0.4 mol of ivermectin/100 amino acids of KLH, required more protein to present the same amount of ivermectin than $iver_1$–Con [E] with 1.9 mol of ivermectin/100 amino acids of conalbumin. When plates were coated to present a saturating amount of ivermectin hapten per well, the responses of the EIAs, including the rates at limiting low amounts of free ivermectin, were not significantly different ($P < 0.01$). Thus, these two conjugates were equally good coating antigens when the coating was based on the amount of ivermectin hapten rather than on the amount of carrier protein. However, the hapten density of the KLH conjugates was too low to be practical for routine plate coating, because such large amounts of the conjugate were required to present usable amounts of the ivermectin in the EIA wells.

Blocking Agents. Blocking coated plates with additional protein before the competition step had no appreciable effect on the accuracy and reproducibility with which ivermectin or abamectin standards were measured. The EIA rate and I_{50} values were similar whether or not the assay included a blocking step, but unblocked plates had a higher background value; i.e., EIA rates at the high-dose asymptote (>50 ppb) were significantly higher ($P < 0.05$) on unblocked plates than they were on plates blocked with BSA. Blocking for 30 min with 0.1 or 1% BSA or calf serum did not significantly change the I_{50} values or the expected between-plate variation of the EIA. Blocking with 0.1–10% of nonfat dry milk reduced the EIA rate and raised the background. Eliminating the blocking step may reduce reagent costs and assay time by 30–45 min, but blocking with BSA or calf serum may prove to be necessary with some sample matrices.

Organic Solvent Tolerance. Avermectins have limited solubility in aqueous solutions, and extraction and recovery of avermectin residues from various sample matrices require organic solvents. Consequently, we tested the ability of the MAbs to react in solutions containing water-miscible organic solvents that could be used to recover avermectin residues. All five MAbs reacted with ivermectin in PBS–Tween containing 5% methanol. The curves in Figure 2 demonstrate that MAb C4D6.1 was able to bind ivermectin in PBS–Tween containing 5, 10, or 20% (v/v) acetonitrile, acidified acetonitrile, or dimethylformamide with no significant differences ($P < 0.05$) in the dose–response. MAb C4D6 reacted with ivermectin in PBS–Tween containing up to 30% methanol or acetonitrile without significant reductions in the EIA rate or I_{50} for ivermectin. PBS–Tween with 5% dimethyl sulfoxide or 10% tetrahydrofuran gave dose–response curves nearly identical with those obtained with 5% acetonitrile and 5% methanol (data not shown). At a concentration of 20%, dimethyl sulfoxide only slightly reduced the EIA rate but did not change the slope of the curve. Reaction of C4D6

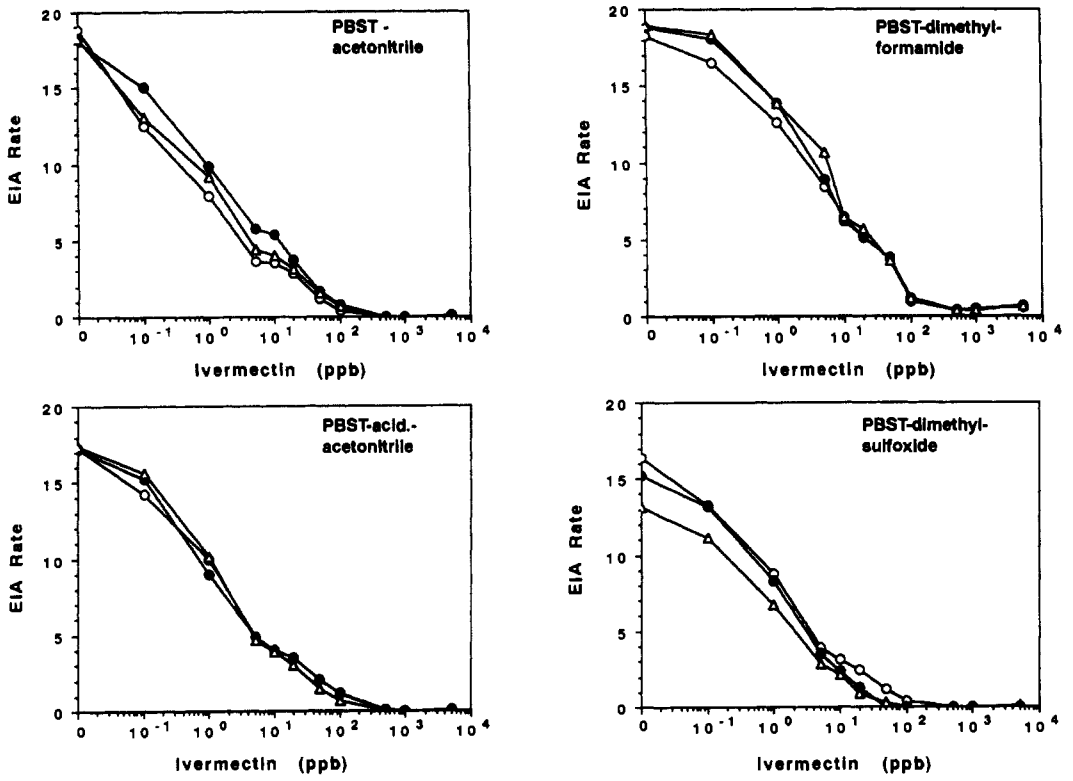

Figure 2. Ivermectin competition EIA dose–responses with organic solvents in the diluent used for the precompetition incubation step. Ivermectin standards were diluted in PBS–Tween containing the indicated solvent, and each was incubated overnight at 22 °C in sealed polypropylene tubes with an equal volume of MAb C4D6 culture supernate diluted 1:100 in the same PBS–Tween solvent. Aliquots (0.1 mL) of these mixtures were added to triplicate Immulon 2 plates coated overnight with $iver_1$–Con [E] (25 ng of protein per well), and the remainder of the competition EIA was performed as described under Materials and Methods, using only PBS–Tween as diluent. Symbols represent PBS–Tween containing (○) 5, (●) 10, or (△) 20% (v/v) of the indicated solvent in the precompetition step. The units of the EIA rate are thousandths of an OD unit per minute (ΔA_{405}/min × 10^3).

with ivermectin in PBS–Tween with more than 5% tetrahydrofuran gave dose–response curves with increased scatter at the low doses (<1.0 ppb of ivermectin). This range of organic solvent tolerance should facilitate the development of protocols for EIA of residues recovered from liquid- or solid-phase extraction procedures.

Incubation Conditions for Competition. The competition EIA required three incubation periods. In the first ("precompetition") incubation step, the MAb and standard or unknown analyte were mixed; in the second ("competition") step, the MAb–analyte mixture was added to the coated EIA plates, and in the last step, enzyme anti-mouse conjugate was added to the plates.

It proved advantageous to give the analyte a "head start" at binding to the MAb in the precompetition incubation step, to obtain the greatest within-assay reproducibility. If the avermectin and MAb were added directly to the coated plate, the dose–response curves were shallow and inhibition was not complete. To obtain a repeatable dose–response and complete inhibition at limiting high doses, it was necessary to incubate the avermectin and MAb for 1–2 h at room temperature before the mixture was added to the coated plates. Binding of the analyte by the MAb appeared to reach saturation in about 2 h, since overnight incubation only slightly improved the inhibition and did not significantly affect the I_{50} ($P < 0.01$). Following the overnight incubation at 22 °C the MAb–ivermectin mixture was applied to the EIA plate for various lengths of time (Figure 3). The I_{50} values were not significantly different ($P < 0.01$) for incubations of 30 min and 1 and 2 h, but a 4-h incubation resulted in a significantly lower ($P < 0.01$) I_{50}, i.e., a more sensitive assay. However, for routine use the improvement did not appear to be worth the increased assay time. Conducting the competition step at 37 °C increased the error at most dosages.

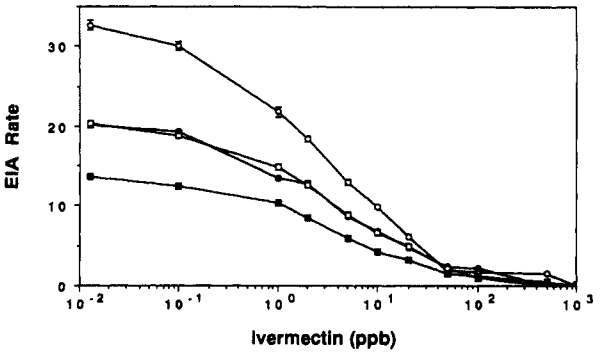

Figure 3. Effect of duration of the competition incubation step. MAb C4D6 (1:100) and ivermectin standards were diluted in PBS–Tween–10% acetonitrile and incubated overnight (approximately 14 h, 22 °C). Aliquots (0.1 mL) were added to wells coated as described in Figure 2 and incubated at 22 °C for (■) 30 min, (□) 1 h, (●) 2 h, and (○) 4 h. The remainder of the competition EIA was conducted as described under Materials and Methods. Data are the mean ± standard error of triplicates. The units of the EIA rate are thousandths of an OD unit per minute (ΔA_{405}/min × 10^3).

Antibody–Enzyme Conjugate. Four types of commercially available enzyme conjugates with goat anti-mouse Ig were tested in the competition assay. All four proved to be usable for quantitative EIA of avermectins, but Table IV shows that there were significant differences ($P < 0.01$) in the sensitivity of the EIA using the different conjugates. The lowest I_{50} value was obtained with alkaline phosphatase, but the slope of the dose–response curve was less steep with this conjugate than with the urease or avidin–alkaline phosphatase conjugates. These differences

Table IV. Ivermectin Competition EIA Using Four Different Detecting Antibody–Enzyme Conjugates[a]

enzyme–anti-mouse conjugate	I_{50}, ppb	slope
alkaline phosphatase	3.7 ± 0.4	0.77 ± 0.06
urease	6.0 ± 1.4	0.91 ± 0.03
biotin (followed by avidin–alkaline phosphatase)	10.2 ± 0.5	1.18 ± 0.04
horseradish peroxidase	12.3 ± 0.4	0.76 ± 0.03

[a] Following the competition step using MAb C4D6, plates were washed and incubated for 2 h at 22 °C either with goat anti-mouse Ig (whole molecule) conjugates of alkaline phosphatase, urease, or peroxidase or with biotinylated goat anti-mouse Ig at the manufacturer's recommended working dilution (1:1000 to 1:2000). In the latter case, the plate was rinsed and incubated for an additional 30 min with avidin–alkaline phosphatase conjugate. Rates of color development were read at 405, 595, and 492 nm for phosphatase, urease, and peroxidase conjugates, respectively.

included between-plate variation and may also have been due to differences in the binding activity of the goat anti-mouse antibodies used to manufacture these particular lots of conjugates.

The rate of color development in the EIA was also dependent on the dilution and length of incubation with the goat anti-mouse alkaline phosphatase conjugate. Increasing the incubation time with this detecting antibody conjugate, or the concentration of conjugate, dramatically increased the EIA rate at the limiting low dose. However, neither the I_{50} nor the slope was significantly altered ($P < 0.01$) by increasing the incubation time or concentration of the conjugate, and good results could be obtained even if the amount of detecting antibody–enzyme conjugate was not sufficient to saturate the MAb bound after the competition step. As in the competition step, incubation of the plates with the detecting antibody–enzyme conjugate at 37 °C increased the scatter of replicate points in the assay.

CONCLUSIONS

The monoclonal antibodies and EIA we describe here are usable for quick and accurate detection of avermectins. The unique structure of the avermectins suggests that the EIA should be specific for this antibiotic, its analogues, and some of its metabolites. MAb C4D6 gave the most sensitive detection of ivermectin, the analogue most commonly used in medical and veterinary applications, while MAbs B11C2, C1A3, and B2A2 were about equally sensitive for detection of abamectin, which is used in agricultural formulations. The EIA had a threshold and range of detection for ivermectin and abamectin comparable to that of the instrumental analysis methods in current use (Oehler and Miller, 1989; Tolan et al., 1980; Tway et al., 1981).

MAbs have several well-documented advantages over antisera for small-molecule immunoassay. MAbs have invariant affinity and characteristic specificity and are available in potentially unlimited quantities as long as the hybridoma lines are maintained. All of the most sensitive avermectin MAbs proved to be IgG_1 immunoglobulins, which are relatively easy to purify by affinity chromatography. The competition EIA uses limiting amounts of antibody and requires only about 0.5 μL of the unpurified hybridoma culture supernate per well.

Development of an effective EIA for the avermectins depended on maximizing the selectivity as well as the affinity of the antibodies. The critical factors we attempted to optimize were hapten design, the immunization process, and screening of large numbers of MAbs. Because hapten design involves modification of the target analyte, it is usually a compromise between feasible chemistry and minimal alteration of the analyte. The most accessible functional groups for linker attachment on ivermectin are the 5- and 4″-hydroxyls (Mrozik et al., 1982). In addition, we expected that attachment to the 4″ moiety would cause the least alteration, because it is on a pendant sugar and is not part of the aglycon. This may have been why the site 1 (4″) conjugates were the better immunogens. We cannot explain why the ivermectin site 2 conjugates were virtually nonimmunogenic, but they proved to be useful as coating antigens in the competition EIA. This may have been due to their higher hapten density, the different orientation of the hapten, or a combination of these two factors.

The avermectin family of antibiotics has only been known for a few years, but these compounds have been shown to be enormously beneficial and virtually nontoxic to humans in a wide variety of applications. Their rapidly increasing use, however, will lead to greater exposure of consumers to avermectin residues and metabolites and of agricultural workers to concentrates and aerosols, as well as greater concern for the environmental consequences of their use. Widespread employment may accelerate the development of resistant pest strains, and increased human exposure or consumption may promote development of resistant parasitic organisms. Agricultural use may affect populations of beneficial organisms used for biological control and pollinators (such as bees), and field drainage could disperse avermectins in concentrations sufficient to affect fish. A quantitative EIA offers a highly cost- and time-effective alternative to the analytical methods and bioassays presently available for detection of avermectins and their metabolites. The EIA is not likely to be influenced by the same matrix effects as the instrumental methods. Although we have only tested our MAbs in the microplate EIA, their properties indicate that they should be compatible with other immunoassay formats. Efforts are under way in our laboratories to interface the competition EIA with compatible avermectin residue recovery methods and to develop protocols to quantitate avermectins in meat and plant tissue.

NOMENCLATURE AND ABBREVIATIONS

Subscripts $iver_1$ and $iver_2$ refer to the attachment position of the succinyl moiety on the ivermectin molecule. Bracketed letters refer to the pool fraction collected from the gel filtration column during synthesis. Conjugates used for immunization and immunoassay screening are denoted by a combination of these abbreviations (e.g., $iver_1$–Con [E] for ivermectin site 1 hemisuccinate, conjugated to conalbumin, pool E) in Table I. Avermectin is the generic name for a family of macrocyclic lactone antibiotics secreted by the soil microorganism *S. avermitilis*. Avermectins are 16-membered lactones that contain an α-L-oleandrosyl-α-L-oleandrosyl disaccharide attached to the lactone ring through the C_{13}-hydroxy group (Mrozik et al., 1982). Ivermectin is the nonproprietary name for the mixture of approximately 80% 22,23-dihydroavermectin B_{1a} ($C_{48}H_{74}O_{14}$) and 20% 22,23-dihydroavermectin B_{1b} ($C_{47}H_{72}O_{14}$) (Chabala et al., 1980). Ivermectin is primarily found in formulations for veterinary use, e.g., MK-933, Eqvalan, and Ivomec (Merck). Abamectin is a mixture of approximately 80% avermectin B_{1a} ($C_{48}H_{72}O_{14}$) and 20% avermectin B_{1b} ($C_{47}H_{70}O_{14}$). Abamectin is mainly used in agricultural insecticide and miticide formulations such as Avid.

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