HPLC FLUORESCENCE DETERMINATION OF AVERMECTIN B1 IN POND WATER AND SEDIMENT

Method No. 8001

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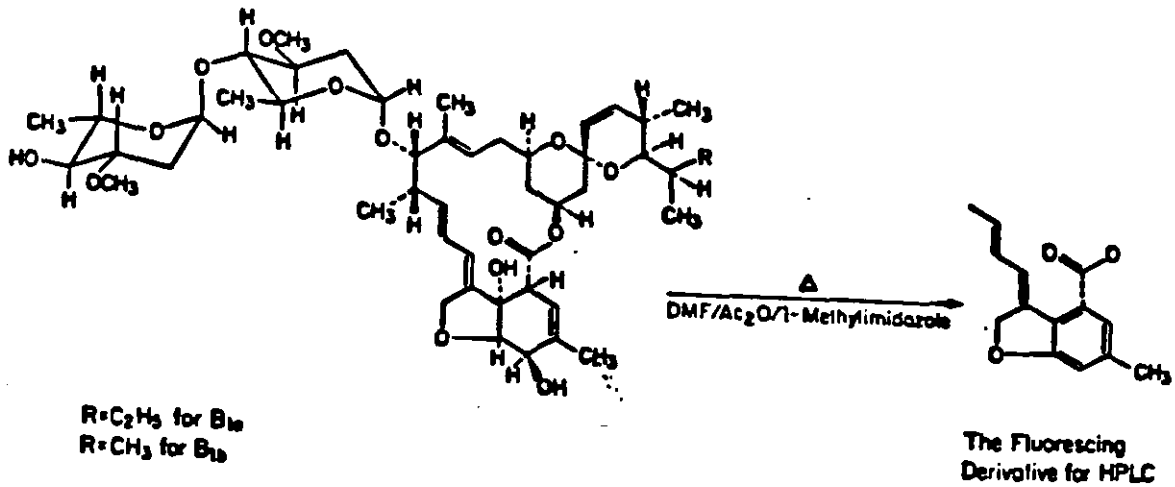
Principle

Residues of avermectin B1 in sediment are extracted with methanol. The sediment methanol extract is made aqueous (10% methanol in water). Similarly, methanol is added to the pond water, to give a 10% solution. Both the sediment extract 10% methanol aqueous solution and the pond water 10% methanol solution are passed through a C-8 column which retains the avermectin B1. Avermectin B1 is then eluted from the column with a small amount of methanol. The fluorescent derivative is formed by reaction with N,N dimethylformamide/ acetic anhydride/1-methylimidazole reagent for 1 hour at 95 C. The reaction mixture is dissolved in chloroform and passed through a silica gel column for separation of the derivatized residue from derivatization reagents. The eluant is taken to dryness and dissolved in methanol. The derivatized residue is determined by reversed-phase liquid chromatography with fluorescence detection. Background fluorescence in the samples is minimized by the use of column switching.

Structure of avermectin B1 and the derivatization reaction



General Apparatus

(a) Shaker- Burrell Wrist-Action Shaker Model 75-775-12

(b) Centrifuge- IEC Tabletop Model HN-S II.

(c) Sonicator- L&R Transistor/Ultrasonic model T-21.

(d) Vacuum manifold- Baker 10 SPE System, J.T. baker Co.

(e) Glass vials- 25ml,70mm x 28mm, Arthur Thomas Co.

(f) Luer type needles- Pipeting needles, 16 gauge x 4 inches (blunt), No. 8957-6-46 -Arthur Thomas Co.

HPLC Apparatus

(a) Pumps- Beckman model 112 or 114M.

(b) Injector- Waters Wisp model 710B.

(c) In-line filter- SSI high pressure 0.5 micron pre-filter.

(d) Pre-column- Whatman Inc. 70mm x 21mm column containing 25-37 micron Co-Pell ODS packing.

(e) Column 1-Regis"Little Champ",3 micron C-18,50mm x 4.6mm.

(f) Column 2-Rainin"Short-One",3 micron C-18,100mm x 4.6mm.

(g) Column heater- YSI Thermistemp temperature controller, YSI series 400 probe, Thermolyne/Briskheat silicone rubber heating tape 1.3 x 244 cm.

(h) Column switching valve-Autochrom model 401 with Rheodyne model 7000 valve and Autochrom solenoid interface.

(i) System controller- Nelson Analytical model 4415.

(j) Fluorescence detector- Kratos Instruments model FS950

(k) Recorder/integrator- Nelson Analytical 4415 Data System

Preparation of standard solutions

(a) To prepare a 500 ng/ml stock standard of avermectin B1, weigh 26.5 mg of the glycerol formal solution (.945% avermectin B1a w/w) into a glass weigh boat. Rinse the contents into a 500 ml volumetric flask and make up to volume with methanol.

(b) To prepare 25, 50, 75, 100, and 125 ng/ml standards, use a 2 ml pipet, graduated 1/10 (to deliver), to transfer 0.25, 0.50, 0.75, 1.00, and 1.25 ml respectively of the 500 ng/ml standard to 15 ml silanized centrifuge tubes. Take to dryness at 50 C. Derivatize and perform all subsequent operations for injection on the HPLC.

(c) To prepare a 50 ng/ml stock standard of avermectin B1, pipet 10 ml of the 500 ng/ml stock standard into a 100 ml volumetric flask. Make up to volume with methanol.

(d) To prepare 1, 3, 5, 7, 10 ng/ml working standards use a 1.0 ml pipet, graduated 1/100 (to deliver), to transfer 0.1, 0.3, 0.5, 0.7, and 1.0 ml respectively of the 50 ng/ml standard to silanized 15 ml centrifuge tubes. Take to dryness at 50 C. Derivatize and perform all subsequent operations for injection on the HPLC.

Reagents

(a) Avermectin B1 (MK-0936) analytical standard- Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065.

(b) Solvents- methanol, 2,2,4 trimethylpentane (isooctane), methylene chloride, acetone- J.T. Baker HPLC grade; chloroform- Burdick and Jackson; t-butanol- Fisher Scientific; Sylon CT- Supelco Inc.; ultrapure water- Milli-Q water purification system.

(c) Inorganics- anhydrous sodium sulfate- Fisher Scientific; 6 ml Bond Elut column containing 0.5 g C-8 - Baker Chemical Co.; SEP-PAK silica cartridges- Waters Associates.

(e) Derivatization reagents- acetic anhydride, N,N-dimethylformamide- MCB Reagents; 1-methylimidazole- Aldrich. To prepare derivatization reagent add 0.6 ml acetic anhydride to 1.8 ml N,N-dimethylformamide in a 15 ml centrifuge tube. To this mixture add 0.4 ml 1-methylimidazole. Vortex to mix. Reagent must be prepared just prior to derivatization.

Preparation of silanized centrifuge tubes

Soak clean 15 ml graduated centrifuge tubes in methylene chloride for 4 hours. Remove methylene chloride. Soak in a detergent solution (Arthur Thomas Co. cleaning compound No. 3298 or equivalent) for 4 hours. Rinse with hot water, followed by distilled water, then acetone. Dry until free of moisture or solvent.

Fill the tubes completely with Sylon-CT, silylate for 30 minutes. Remove the Sylon-CT from tubes, rinse with toluene 5 times, followed by methanol 2 times. Fill the tubes completely with methanol, soak for 30 minutes. Rinse with acetone, dry tubes until free of solvent.

Fill tubes completely with warm detergent solution, soak overnight. Rinse tubes 10 times with distilled water, followed by acetone. Dry until free of moisture or solvent. Place tubes in a 120 C oven for 1 hour just prior to use.

Between uses soak overnight in methylene chloride, rinse with distilled water, soak in detergent solution for at least 4 hours, rinse with distilled water then acetone. Dry tubes until free of moisture or solvent. Place tubes in a 120 C oven for 1 hour just prior to use.

Extraction of sediment

Allow the soil sediment collection tubes to partially thaw. Push out sample of sediment and pond water, and decant off pond water. Air dry sediment over night. Grind 50 grams of air dried sediment with a morter and pestal. Sift through a US standard #18 sieve. Weigh out 20 g of sieved sediment and transfer to a 500 ml erlenmeyer flask. Add 75 ml methanol. Stopper and shake for 1 hr on a Burrell shaker at setting 3. Slurry the sediment-methanol mixture and pour into a 63 mm porcelain funnel containing a Whatman no. 50 filter paper prerinsed with 3 ml methanol, and fitted with a rubber stopper to a 250 ml filter flask. Remove the solvent under vacuum. Rinse the 500 ml erlenmeyer flask with 2 x 5 ml methanol. Slurry to remove the remaining sediment and add to the funnel while under vacuum. Quantitatively transfer the contents of the filter flask to a 100 ml graduated cylinder with 2 x 5 ml methanol. Adjust the volume to 100ml with methanol. Mix. Transfer 50 ml to a 500 ml separatory funnel containing 450 ml distilled water. Quantitatively transfer the remaining 50 ml to a suitable container for repeat analysis if necessary.

C-8 column clean-up/enrichment chromatography

For the pond water samples (approximately 1 liter), measure 450 ml into a 500 ml sepatatory funnel. Retain the remaining sample for repeat analysis if necessary. Add 50 ml methanol to the separatory funnel, mix. For both pond water 10% methanol solution and the 10% methanol soil extract aqueous solution, place a 6 ml Baker column containing 0.5 grams of C-8 column packing, fitted with a 15 ml reservoir, in a luer type needle fitted to a 500 ml filter flask with a rubber stopper. Drain the 10% methanol solution into the reservoir as to maintain a constant flow through the column when a vacuum of approximately 125 mm Hg is applied to the filter flask. Discard the eluant. Attach the C-8 column and reservoir to the Baker 10 SPE system. Elute the column with 15 ml methanol (use 2 x 5 ml to rinse the separatory funnel) into a 25 ml vial (70 mm x 25 mm, Arthur Thomas Co., No. 9710L17) by applying a vacuum of approximately 125 mm Hg. Reduce the methanol eluant to 2-3 ml with nitrogen at 50 C. Quantitatively transfer the methanol concentrate to a 15 ml silanized centrifuge with 3 x 3 ml of methanol by passing the concentrate and rinses through a small glass funnel containing 2.5 grams anhydrous sodium sulfate held in place with a small amount of silanized glass wool.

Derivatization

Take the sample to dryness with nitrogen at 50 C. Add 0.1 ml of the derivatization reagent to the silanized centrifuge tube containing the residue, vortex and sonicate. Standards should be derivatized along with unknowns. Centrifuge briefly, tape the stopper, and put unknowns and standards together in a 95 C oil bath for 1 hour. (If after 1 hour the residue is not black the reaction was not quantitative. The remaining half of the sample can be used to repeat the derivatization with the next set of standards.) Allow tubes to cool, add 1 ml of chloroform, vortex and sonicate. Quantitatively transfer the sample using chloroform to a 5 ml syringe fitted with a silica-gel Sep-Pak, prewashed with 5 ml chloroform. Install the plunger and place the sample on the Sep-Pak. Collect the eluant in a 15 ml centrifuge tube. Wash the silanized centrifuge tube with 3 x 1 ml chloroform into the syringe and through the Sep-Pak. Elute the Sep-Pak with chloroform to give a final eluant volume of 13 ml. Take the sample to dryness with nitrogen at 50 C (65 C may be necessary to remove the last few fractions of a milliliter of chloroform remaining). For the standards, dissolve the residue in 5 ml methanol. Dissolve samples in a suitable volume of methanol based on expected concentration. Vortex and sonicate. Centrifuge briefly prior to HPLC analysis.

Reversed-Phase Liquid Chromatography

Operating conditions

Mobile phase-7% ultrapure water in methanol (v/v); flow rate-1.5 ml/min; injection volume-40 microliters; detector parameters: excitation lamp-mercury vapor; excitation filter-365 nm; emission filter-418 nm; sensitivity (PMT gain)-570 to 780 V; Range-0.02 microamps; time constant-6; column temperature- 32 C.

Column switching parameters (see diagram): For 0 to 2 minutes following injection the eluant from column 1 goes to waste. At 2 minutes the valve switches. Column 1 is connected to column 2 for further separation and detection. The timing of the valve switch is controlled by the Nelson Analytical 4416 Data System. A Beckman 421 system controller or other similar device with timed contact closures or TTL logic may also be used.

Note: These conditions are for the HPLC equipment and columns described above. With other equipment or columns minor changes in operating conditions may be required to obtain equivalent performance. Timing for the valve switch is determined by connecting column 1 directly to the detector. For some samples acceptable chromatography can be obtained without column switching, however column switching minimizes the high "background fluorescence" observed at instrument settings and sample dilutions needed for detection and quantitation near 150 pg injected.

Determination

A Nelson Analytical 4416 Data System is used for HPLC data reduction. Retention times for the fluorescent derivatives of avermectin B1a and avermectin B1b are approximately 10 minutes and 8.5 minutes respectively. As avermectin B1b is at most 20% (usually (10%) of the active ingredient and residue levels are generally less than 1.0 ng/g for sediment and 0.1 ng/ml for pond water (below which results are reported as none detected), it is identified but not quantitated.

An analysis set is comprised of 5 standards run before and after no more than 12 samples. The concentration of avermectin B1a in unknowns is determined from the linear regression of the standards' peak height vs. concentration in ng/ml. Both sets of standards are used to determine the regression coefficients-slope and intercept, by the method of least squares. Occasionally it has been observed that the peak height for one standard is much lower that expected. Because it is known that this observation can be attributed to low derivatization reaction yield, a single errant standard may be discarded in determining the regression coefficients. The concentration of avermectin B1a in a residue sample is determined as follows:

$$C=(PK\ HT-I)/S \qquad UNK=(CxFV)/SS$$

Where: C=concentration of avermectin B1a in ng/ml in the final volume used for HPLC analysis, PK HT=peak height of avermectin B1a, I=intercept, S=slope, FV= final volume used for HPLC analysis, SW=sample size (grams or milliliters), UNK=concentration of avermectin B1a in ng/g sediment or ng/ml pond water.

VII. NOTE TO THE ANALYST

Avermectin B1 has a very low water solubility, approximately 5 ppb. In addition, there are indications that this compound will form a monolayer at phase boundaries [liquid-air, liquid-liquid, or liquid-solid (glass or plastic) interface]. Because of these properties, loss of avermectin B1 due to adsorption to glassware may be critical when working in the low ppb range. Care should be taken not to take samples to dryness wherever possible. When samples are taken to dryness, sonication upon dissolution is crucial.

Diagram of HPLC System With Column Switching