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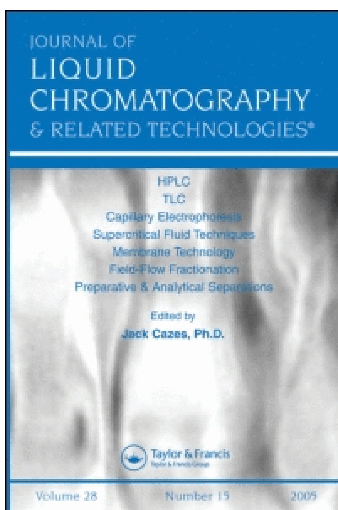

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SEMI-PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CARBON-14 LABELED AVERMECTIN B_1a FROM A MIXTURE OF AVERMECTINS

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ABSTRACT

A semi-preparative high performance liquid chromatographic method has been developed to separate carbon-14 labeled avermectin B_1a from a fermentation mixture of carbon-14 labeled avermectins, i.e., avermectins A_1a, A_1b, A_2a, A_2b, B_1a, B_1b, B_2a, and B_2b. Two HPLC systems were employed for the separation: I. A Whatman M20, Partisil 10, normal phase column and a solvent system of 10% ethanol in isooctane (v/v), and II. A Whatman M20, Partisil 10, ODS-3, reverse phase column and a solvent system of acetonitrile/methanol/water (56:18:26, v/v/v); the flow rate was 18 ml/min. Avermectin separations were monitored using ultraviolet detection (254 nm). Further analyses of avermectin B_1a were done using analytical HPLC and TLC/radioassay to check compound purity and identity.

INTRODUCTION

Avermectin B_1a is one of the major avermectins (Figure 1) produced by the actinomycetes *Streptomycetes avermetilis* (1). It

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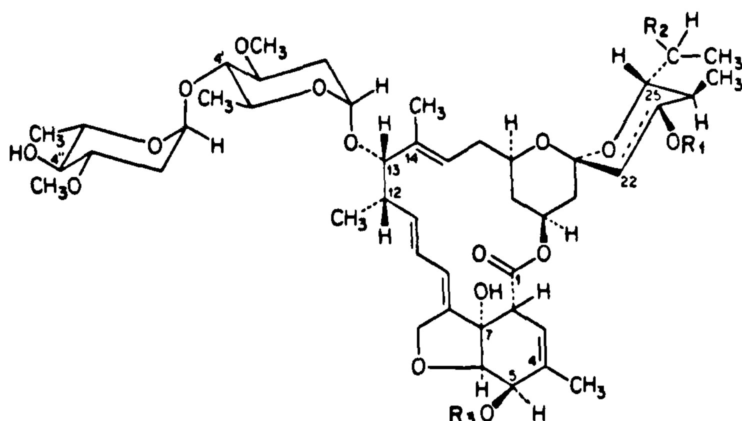

Figure 1. Structure of the Avermectins.

AVERMECTIN	R_1	R_2	R_3
A_1a		C_2H_5	CH_3
A_1b		CH_3	CH_3
A_2a	OH	C_2H_5	CH_3
A_2b	OH	CH_3	CH_3
B_1a		C_2H_5	H
B_1b		CH_3	H
B_2a	OH	C_2H_5	H
B_2b	OH	CH_3	H

Where R_1 is absent, the double bond (-----) is present.
Both sugars are o-L-oleandrose.

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is active at extremely low dosage against a wide variety of nematode and arthropod parasites, apparently by virtue of its action on the mediation of neurotransmission by γ-aminobutyric acid (2). In addition, it exhibits excellent activity in controlling different phytophagus pests of field crops and citrus (3), and red imported fire ants (4). Pure, carbon-14 avermectin B_1a is needed for various metabolism studies and environmental chemistry studies. Miller et al. (5) separated avermectin major components, A_1, A_2, B_1, and B_2, by using a partition chromatography system: Sephadex LH-20.

EXPERIMENTAL

Material

Solvents used for the semi-preparative and analytical high-performance liquid chromatographic separations of avermectins were HPLC grade.

Crude mixture of carbon-14 labeled avermectins, used to obtain pure avermectin B_1a by semi-preparative HPLC, were isolated from the fermentation broth using the procedure reported by Ku *et al.* (6).

The TLC plates were E. Merck Sil GF, 0.25 mm thick plates. Autoradiography was achieved on Kodak ARO X-ray film. Both normal phase and reverse phase semi-preparative columns were purchased from Whatman.

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Apparatus

The HPLC system consisted of two Altex model 110A pumps, a Rheodyne injector with a 4 ml sample loop, an Altex dual wavelength model 151 UV detector with a preparative flow cell (0.5 mm pathlength) and a recorder. The normal phase semi-preparative column (50 cm x 22 mm I.D.) was a Whatman M20, Partisil 10. Operating conditions were: mobile phase, ethanol-isooctane (10:90, v/v); flow rate, 18 ml/min; temperature, ambient; UV wavelength, 254 nm; chart speed, 12 cm/hr. The reverse phase semi-preparative column (50 cm x 22 mm I.D.) was a Whatman M20, Partisil 10, ODS-3. Operating conditions were: mobile phase, acetonitrile/methanol/water (56:18:26, v/v/v); flow rate, 18 ml/min; temperature, ambient; UV wavelength, 254 nm; chart speed, 12 cm/hr.

The analytical column (25 cm x 4.6 mm I.D.) was a Zorbax ODS (DuPont). Operating conditions were: mobile phase, methanol/water (85:15, v/v); flow rate, 1 ml/min; column temperature, ambient; UV wavelength, 245 nm; chart speed, 0.5 cm/min.

METHODS

Semi-Preparative HPLC Separations

A methanol solution of carbon-14 labeled avermectins was obtained from an isolate of the fermentation broth. One ml of this solution contained approximately 35 mg [^{14}C]avermectins with approximately 600 μCi of radioactivity. Three ml of the solution was injected per each run. A total of seven runs was made for the normal phase separation. The fractions containing avermectin B_1a were collected and concentrated using rotary evaporation. The separation of avermectin B_1a and B_1b was achieved by reverse phase HPLC. The pure avermectin B_1a was obtained by another normal phase separation to remove the trace contaminant, avermectin A_2. The fractions containing pure avermectin B_1a were collected and concentrated using rotary evaporation.

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Analytical HPLC/Radioassay

The purified avermectin B_1a was analyzed for chemical purity by analytical HPLC. The effluent was collected in fractions for radioassay using standard liquid scintillation counting technique to determine the radiopurity.

TLC/Radioassay

The purified avermectin B_1a was also analyzed by TLC technique. The developing solvent was hexane/isopropyl alcohol (51:9, v/v). The avermectin B_1a spot on the developed plate was visualized by both autoradiography and UV light (254 nm). The radiopurity was determined by liquid scintillation counting of the TLC plate scrapings in a liquid scintillation counter.

RESULTS AND DISCUSSION

The chromatogram of the semi-preparative normal phase separation of avermectins A_1, A_2, B_1, and B_2 is shown in Figure 2. Through TLC with radioassay, the first peak with a retention time

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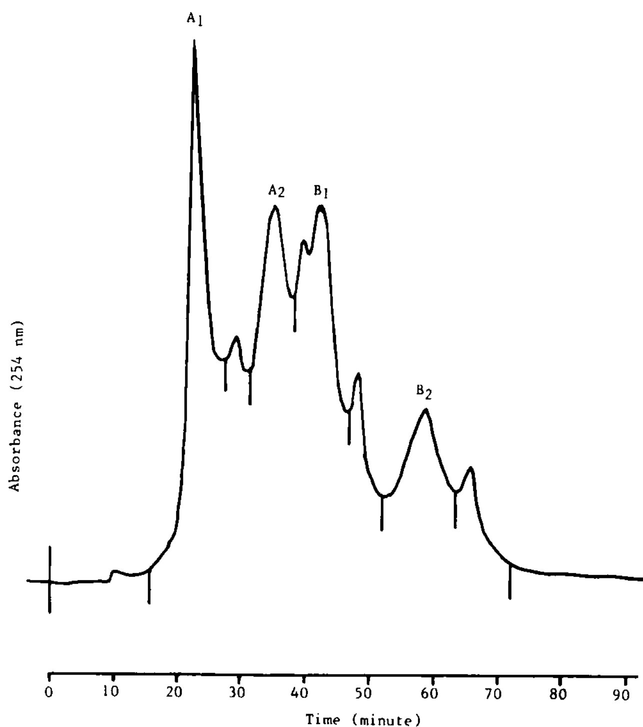

Figure 2. Semi-preparative Normal Phase HPLC Chromatogram of A_1, A_2, B_1, and B_2.

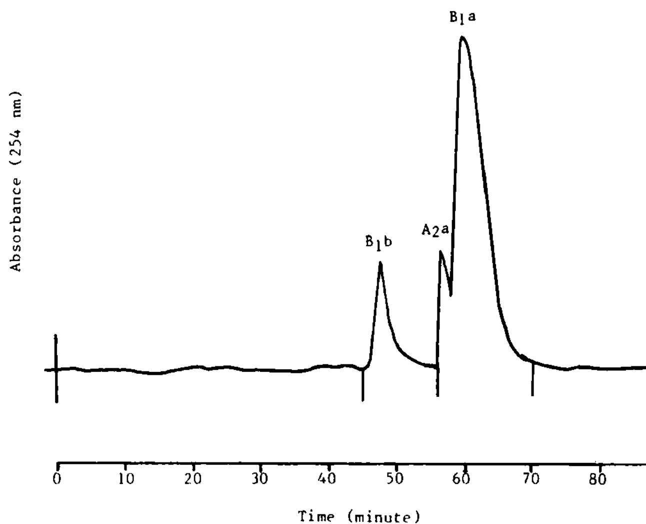

Figure 3. Semi-preparative Reverse Phase HPLC Chromatogram of Avermectins B_1a and B_1b.

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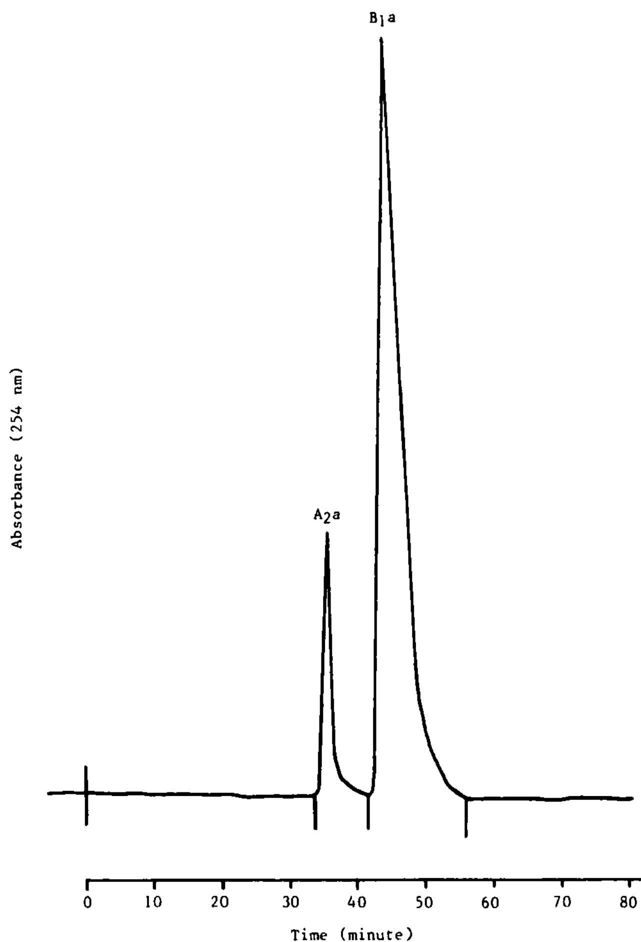
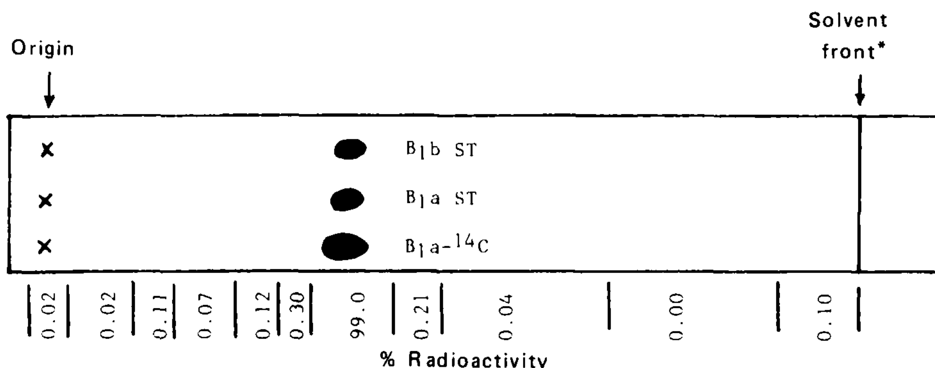


Figure 4. Semi-preparative Normal Phase HPLC Chromatogram of Avermectins B_1a and A_2a.

(R_T) of 22.5 min is identified as avermectin A_1, the peak with R_T = 35.5 min is avermectin A_2, the peak with R_T = 42.5 min is avermectin B_1a, and the peak with R_T = 59.0 min is B_2. In all four fractions, i.e., avermectin A_1, A_2, B_1, and B_2, the components a and b of the same avermectin e.g., A_1a and A_1b, were not separated



*Developed 3 times with hexane/isopropyl alcohol (51:9, v/v).

Figure 5. TLC Chromatogram of Avermectin B_1b (B_1b ST), Avermectin B_1a (B_1a ST) and Avermectin (B_1a-^{14}C) Collected from the Semi-preparative Separations. St, standards.

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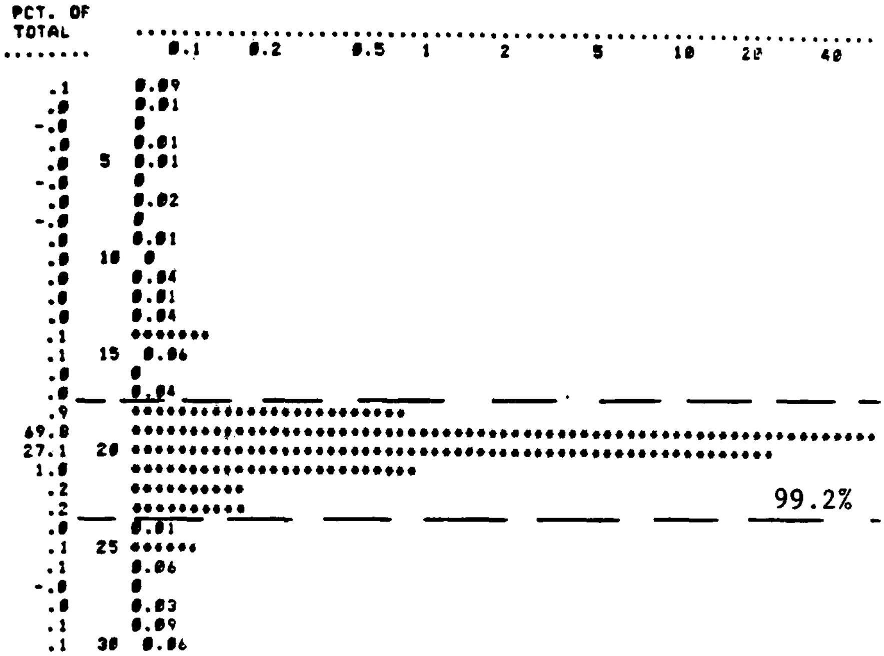
on normal phase HPLC. The absorption properties of silica gel for components a and b are nearly the same because the only difference between a and b structurally is the alkyl side chain at C-25, i.e., a is isobutyl and b is isopropyl (See Figure 1). However, when the fraction containing avermectin B_1 was concentrated and reinjected onto a semi-preparative reverse phase HPLC, a baseline separation of avermectin B_1a and B_1b was obtained. The chromatogram is shown in Figure 3. From the results of TLC and radioanalysis, the peak with R_T = 47.5 min is avermectin B_1b, the peak with 57.0 min is avermectin A_2a, and the peak with R_T = 59.5 min is avermectin B_1a.

Since avermectin B_1a contained some avermectin A_2a, these two components were collected together and concentrated, then reinjected onto the semi-preparative normal phase HPLC again to obtain a baseline separation of the two avermectins. The peaks again were analyzed by TLC and radioanalysis. The chromatogram is shown in Figure 4.

The fractions containing pure avermectin B_1a were collected and concentrated. In order to ascertain the chemical purity and radiopurity, and to check the identity of the purified avermectin B_1a, analytical HPLC and TLC/radioassay were employed. The results are presented in Figure 5 for the analyses by TLC and Figure 6 for the evaluation by analytical HPLC. The identity and



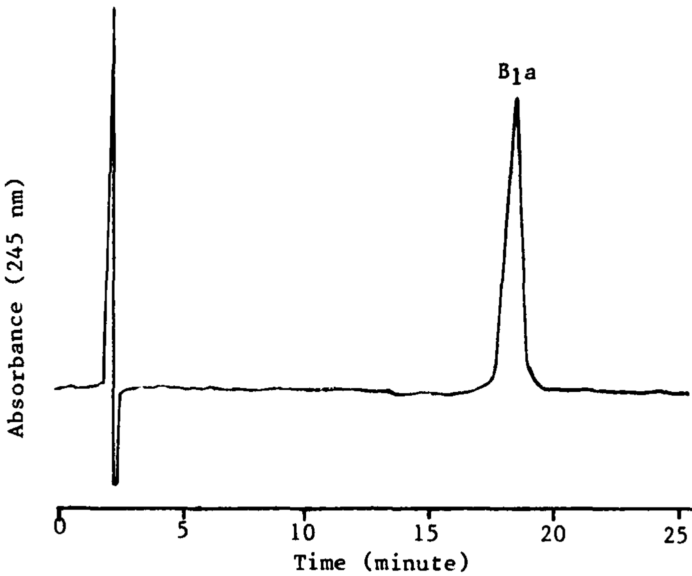


Figure 6. Analytical HPLC Chromatogram/Radio-histogram of Avermectin in B_1a.

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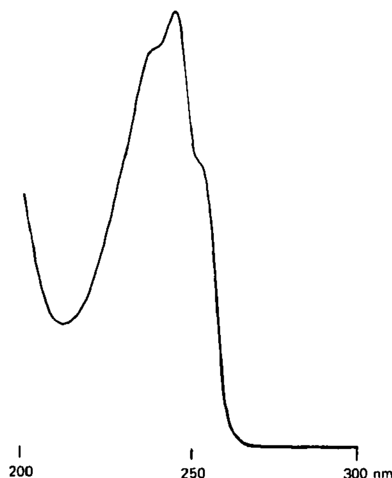

Figure 7. UV Spectrum of Avermectin B_1a-^{14}C.

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concentration of the purified avermectin B_1a were confirmed and measured by UV spectrometry (Figure 7).

This semi-preparative HPLC method offers a rapid means of obtaining [^{14}C]avermectin B_1a that are chemically and radiochemically pure (99^+%) in sufficient quantity to do metabolism and environmental chemistry research.

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