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Note

Use of thin-layer chromatography for the testing of avermectins produced by *Streptomyces avermitilis* strains

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Avermectins are macrocyclic disaccharide antiparasitic agents having anthelmintic and insecticidal properties and are produced[1] by fermentation of *Streptomyces avermitilis*. Their structure was described[2] as sixteen-membered lactones containing an α-L-oleandrosyl-α-L-oleandrosyl disaccharide attached to the lactone ring through the allylic C_{13} hydroxy group. The complex contains four closely related major components, A_1a, A_2a, B_1a and B_2a, in varying proportions and four minor components, A_1b, A_2b, B_1b and B_2b, each of which is a lower homologue of the corresponding major component.

Avermectins do not show any antibiotic activity and therefore instead of microbiological methods for their determination a biological test based on the movement paralysis of the free-living nematode *Caenorhabditis elegans* is commonly used[3,4]. The test is simple, but for a higher accuracy of determination of individual major components in an avermectin complex it is desirable to combine it with a chromatographic assay. The avermectin complex produced by investigated microorganism strains can be tested by means of thin-layer chromatography (TLC) of extracts of the cultivating medium. The advantages of this technique are its simplicity and rapidity and the possibility of simultaneously determining a larger number of samples.

However, the separation of the major compounds under the TLC conditions described so far[1,2,5–7] is not sufficient to make a densitometric determination possible. The purpose of this investigation was to achieve an effective TLC separation of the major components A_1a, A_2a, B_1a and B_2a of avermectins, permitting their densitometric determination.

EXPERIMENTAL

Preparation of samples

The samples for TLC were prepared by extracting (5 min) the homogeneous

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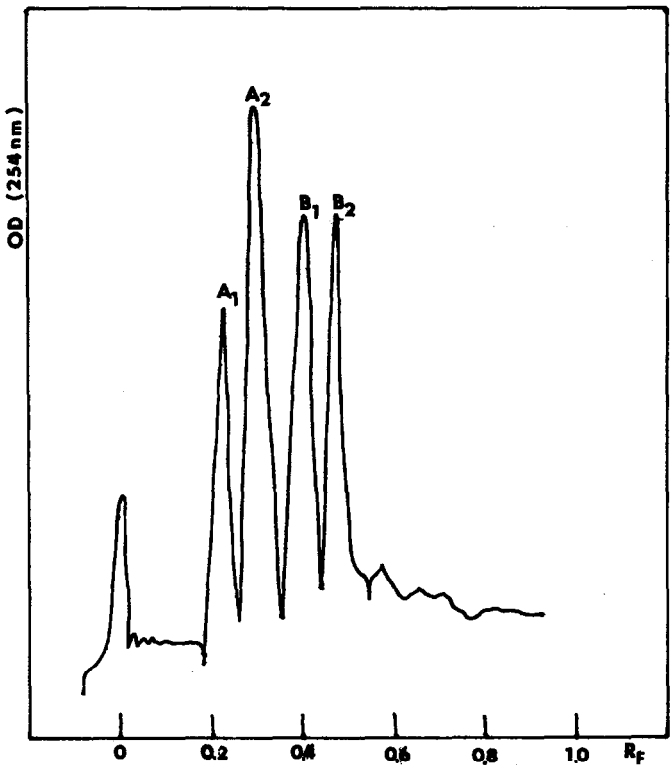

Fig. 1. TLC of an extract from the cultivation medium of *Streptomyces avermitilis* producing avermectins. Solvent system: hexane–acetone–methanol (14:6:0.05). R_F values of avermectins: A_1 = 0.41; A_2 = 0.36; B_1 = 0.26; B_2 = 0.20. Detection at 254 nm.

cultivation medium (5 ml) with a volume of chloroform so as to give a final avermectin concentration in the organic layer of *ca.* 1 mg/ml. The chloroform layer was separated by centrifugation (10 min, 5000 *g*).

Thin-layer chromatography

The samples (5–10 μl of organic layer) were applied to TLC plates (LK6F, 20 × 20 cm; Whatman, Maidstone, U.K.) with a Linomat III instrument (Camag, Muttenz, Switzerland). The plates were developed for 30–40 min with different solvent systems. The best results were obtained with hexane–acetone–methanol (14:6:0.05) or hexane–isopropyl alcohol–methanol (14:4:0.05). For quantitative evaluation, the plates were measured at 254 nm by means of a TLC Scanner II densitometer (Camag) with Hewlett-Packard HP 3390 A integrator. Typical chromatograms are shown in Figs. 1 and 2. The concentrations of individual types of avermectin were determined from a calibration graph (Fig. 3). Ivermectin [22,23-dihydrogen avermectins (Merk, St. Louis, MO, U.S.A.) (modified B_1a and B_1b avermectins giving one peak only on TLC plates), similarly to a main component A_1 which is a mixture of A_1a and A_1b compounds; see below] was used as a standard.

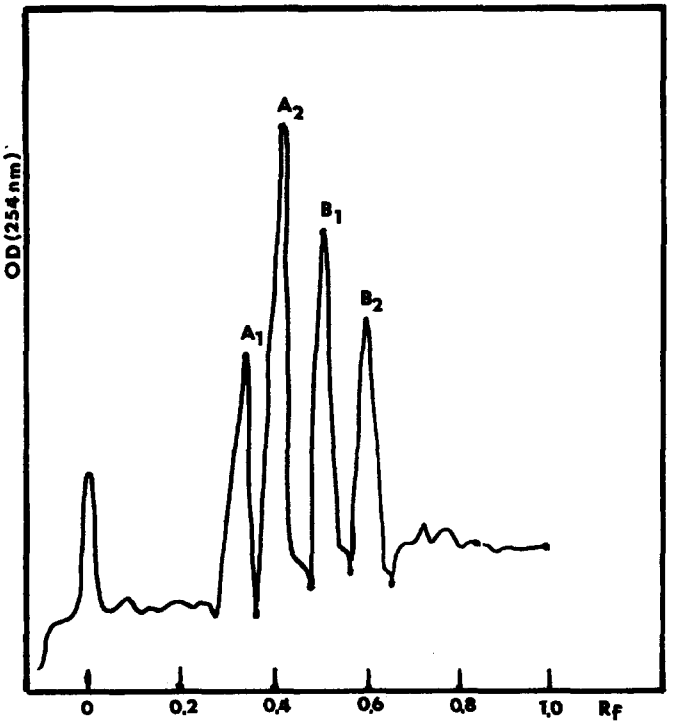

Fig. 2. TLC of an extract from the cultivation medium of *Streptomyces avermitilis* producing avermectins. Solvent system: hexane–isopropyl alcohol–methanol (14:4:0.05). R_F values of avermectins: A_1 = 0.53; A_2 = 0.45; B_1 = 0.38; B_2 = 0.30. Detection at 254 nm.

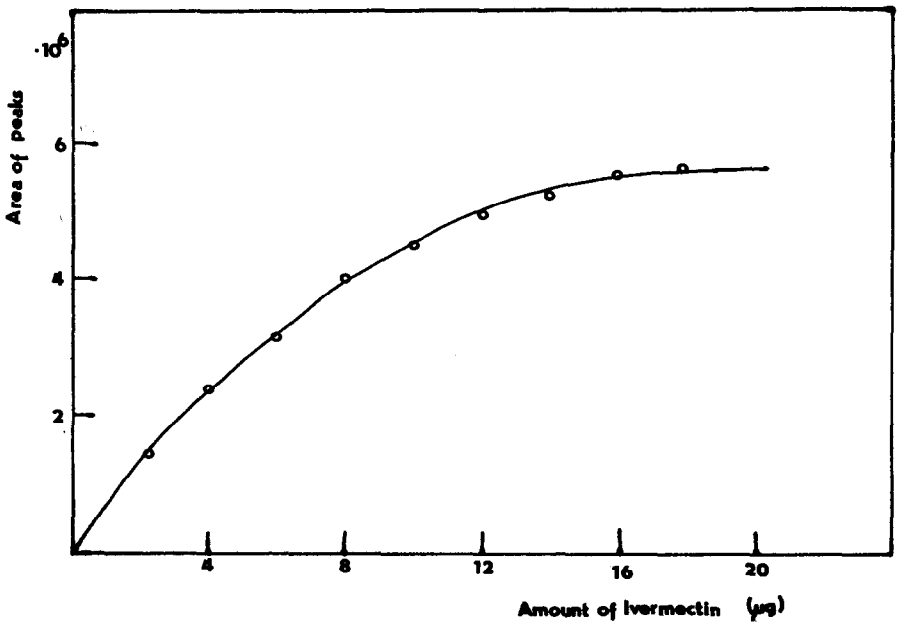

Fig. 3. Dependence of the peak area (measured densitometrically on TLC plates) on the amount of ivermectin.

RESULTS

Different solvent systems were tested for the determination of individual avermectins in cultivation media by TLC: chloroform–ethyl acetate–methanol–methylene chloride (9:9:1:2) (according to ref. 1), ethyl acetate–ethanol (95:5), methylene chloride–methanol (95:5 and 98:2), diethyl ether–light petroleum (30:70 and 180:20), hexane–ethyl acetate (85:5) (according to ref. 6), hexane–ethyl acetate (2:1), methylene chloride–tetrahydrofuran–ethanol (90:9.7:0.3 and 89.7:10:0.3) (according to ref. 7), and some other combinations of these solvents. The best results were obtained with the systems hexane–acetone–methanol (14:6:0.05) and hexane–isopropyl alcohol–methanol (14:4:0.05). Under these conditions, the mixture of avermectins was separated into four main components, A_1, A_2, B_1 and B_2, each containing both the major and the minor parts (*e.g.*, component A_1 included compounds designated A_1a and A_1b). From a comparison of Figs. 1 and 2 it is evident that the second solvent system gives a better separation of the components, with higher R_F values. The determination of individual avermectins in the cultivation medium based on measurement of corresponding peak areas using the calibration graph (Fig. 3) showed an inter-assay deviation of ± 10% under standard conditions. The shapes of the peaks of the individual avermectins and the ivermectin standard were very similar over the whole sample concentration range applied (the percentage deviation may be affected by possible differences in the molar absorptivities for the individual avermectins and ivermectin).

The TLC technique described here permits an effective screening of *Streptomyces avermitilis* strains from the viewpoint of the qualitative and quantitative composition of the avermectins produced.

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