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Note

High-performance liquid chromatography of the janthitrems: fluorescent tremorgenic mycotoxins produced by *Penicillium janthinellum*

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Tremorgenic mycotoxins from *Penicillium* species and other fungi found in the pasture environment are being examined in studies on the neurological disease of sheep and cattle known as ryegrass staggers[1,2]. Recently, three tremorgenic toxins named janthitrem A, B and C (Jan A, Jan B, Jan C) were isolated from *Penicillium janthinellum* cultures, and their behaviour on silica gel thin-layer chromatography (TLC), molecular weights and molecular formulae reported[3]. The janthitrems were found to have strong UV absorption spectra and strong fluorescent spectra[3]. Methanolic solutions of Jan B, one of the major janthitrems produced, had absorption maxima at 228, 258, 265 (major) and 329 nm and a fluorescence emission maximum at 385 nm. The tremorgenic activity of cultures and TLC fractions were determined by a mouse bioassay[3].

In order to screen a range of fungal culture variants for the occurrence of these new tremorgens, and to relate the activity in mouse bioassay to the presence of these or other known tremorgens, a rapid method of identification and quantitation was required. We have developed a method of analysis for the janthitrems using reversed-phase high-performance liquid chromatography (HPLC). The use of both UV and fluorescence detectors allows differentiation of the janthitrems from other compounds of similar retention times. A fourth compound, first seen on HPLC, has been separated and isolated by preparative TLC, and is tentatively named janthitrem D (Jan D).

EXPERIMENTAL

Apparatus

The liquid chromatograph has been described[4]. The fluorescence detector was an LDC Fluoromonitor III fitted with either 370-nm bandpass excitation and a 400-nm cutoff emission filter or 254-nm line excitation and a 370-nm cutoff emission filter. It was placed in-line after the variable-wavelength UV detector.

Chromatographic conditions

The columns used were Zorbax C_8 (25 cm × 4.6 mm I.D.) (Dupont, Wilmington, DE, U.S.A.), μPorasil (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA,

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U.S.A.) and a column (25 cm × 3.1 mm I.D.) slurry-packed with Spherisorb S5 CN (Phase Separations, Queensferry, Great Britain). The solvent systems were water–methanol, hexane–ethyl acetate–methanol and hexane–isopropanol for respective columns; relevant conditions are listed in the text. The analyses were performed at room temperature, 19–22°C. Solvents were analytical grade and were purified as previously described[5].

Janthitrem samples and culture extracts

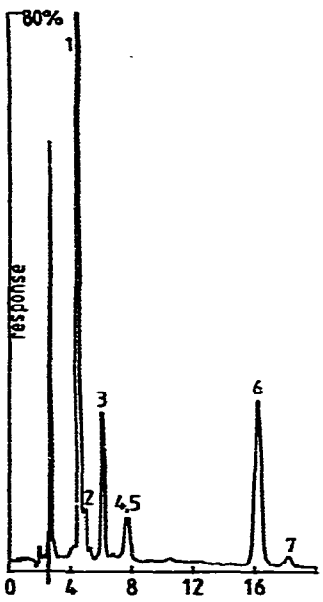
Dried mycelial mats of *P. janthinellum* cultures[2] grown on Czapek-Dox yeast extract broth[6] or potato–milk–sucrose broth[7], were mixed with chloroform–methanol (2:1) in a blender for 3 min. The resultant slurries were filtered and the filtrates dried over anhydrous sodium sulphate, filtered, and evaporated to dryness under vacuum on a rotary evaporator. The crude extracts were stored in the dark at −5°C. Pure janthitrems were separated by TLC[3] and stored dry in a similar manner. Standard solutions (10 μg/ml) of Jan B were made in methanol and ethyl acetate, and of Jan C in methanol. The vials containing these solutions were held in cardboard cylinders, and kept at 2°C between use. The Jan C standard was found to be stable for at least six months. Jan B was stable in methanol for four months, but breakdown was apparent after seven months. In ethyl acetate, considerable degradation had occurred within three months.

RESULTS AND DISCUSSION

Standard solutions of Jan B and Jan C were used to test the suitability of different chromatographic conditions. The C_8 column was found to have far greater selectivity than either the silica or the CN column. Both the silica column, using hexane–ethyl acetate–methanol (85:14.7:0.3), and the CN column, using hexane–isopropanol (90:10), showed little separation of Jan B and Jan C. The compounds could be well resolved on the C_8 column, however. An additional advantage of the C_8 column was that the janthitrems exhibited much stronger fluorescence in water–methanol than in either of the other two solvent systems.

In order to test for the presence of janthitrems in fungal cultures, extracts were analysed on the C_8 column using water–methanol (20:80). Under these conditions, the major janthitrems, Jan B and Jan C, were well separated from each other and from other common tremorgens such as penitrem A, verruculogen and fumitremorgen B (Fig. 1). To verify further the presence of the janthitrems, and to enhance sensitivity, a fluorescence detector could be used in conjunction with the UV absorbance detector. The janthitrems are the only tremorgens of those mentioned above to exhibit significant fluorescence.

In the isocratic system used to screen fungal culture extracts for janthitrems, Jan A and Jan B were eluted very close together and were only partially resolved (see Fig. 1). In addition, once several extracts had been analysed, the presence of strongly retained co-extractives on the column degraded its efficiency and Jan A merged into the tail of Jan B. This effect was aggravated by the fact that Jan B generally occurred in much greater concentrations than Jan A. Increasing the water content of the mobile phase allowed better resolution of the two peaks, and at water–methanol (36:64), near baseline separation of Jan A from Jan B was achieved at the relative



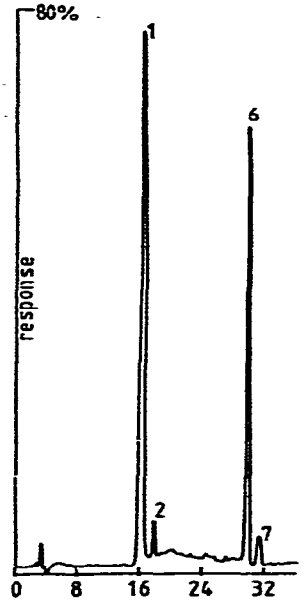


Fig. 1. HPLC of janthitrems Jan A, Jan B, Jan C and Jan D in combination with verruculogen, penitrem A and fumitremorgen B. Conditions: column, Zorbax C_8; eluent, water–methanol (20:80); flow-rate, 1 ml/min; UV detector wavelength, 265 nm; detector sensitivity, 0.04 a.u.f.s.; sample size, 20 μl. The sample was a composite mixture made by spiking a janthitrem-containing culture extract with three other tremorgens. Sample dissolved in eluent. Peaks, with approximate concentrations (μg/ml): 1 = Jan B (12); 2 = Jan A (estimate 0.5); 3 = verruculogen (24); 4 = penitrem A (20); 5 = fumitremorgen B (12); 6 = Jan C (17); 7 = Jan D (estimate 1).

Fig. 2. HPLC conditions for quantitation of janthitrems A, B, C and D in fungal culture extracts. Conditions as in Fig. 1 except for eluent, water–methanol (36:64) for 10 min followed by linear gradient over 5 min to water–methanol (20:80); UV detector wavelength, 330 nm; detector sensitivity, 0.16 a.u.f.s.; sample size, 50 μl. The sample was the extract used for Fig. 1 dissolved in water–methanol (50:50). Peaks as in Fig. 1.

concentrations commonly found in fungal culture extracts. Jan C and Jan D were retained indefinitely with this solvent, and a step gradient to water–methanol (20:80) was required to elute them in reasonable time. Quantitation of the four janthitrems in fungal culture extracts could be achieved using the conditions shown in Fig. 2.

The extract shown in Fig. 2 was used for preparative TLC[3] to isolate samples of each of the four janthitrems and to confirm their HPLC designations. The samples were recovered from TLC in order of decreasing R_F, as Jan D, Jan C, Jan A, Jan B. The isolation of Jan D has not been reported before. No molecular formula has yet been obtained for this compound, but it has been tentatively named as a janthitrem because of the characteristic UV and fluorescent properties, and because of its behaviour on HPLC and TLC. Attempts to isolate the individual janthitrems by preparative HPLC were not successful due to the low solubility of the compounds in methanol containing significant amounts of water. Further investigation of the straight-phase systems may be necessary to make preparative HPLC a viable alternative to TLC.

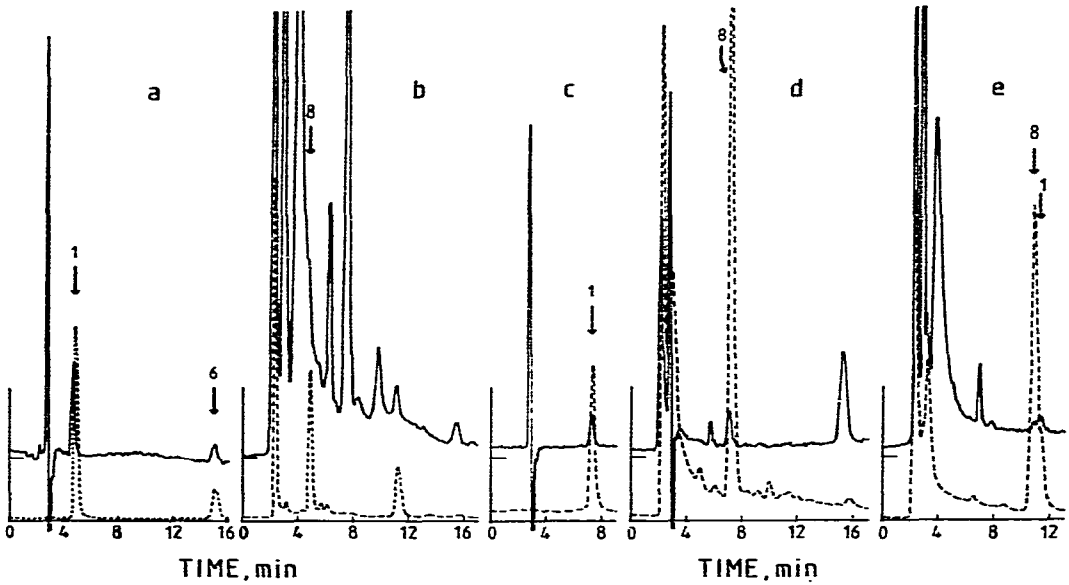


Fig. 3. Combined UV (upper trace) and fluorescent (lower trace) estimation of janthitrems in a fungal culture extract. Conditions as in Fig. 1 except for eluent, c, d, water–methanol (28:72); e, water–methanol (32:68); UV detector wavelength, a, b, e, 265 nm; c, d, 330 nm; detector sensitivity, 0.04; fluorescence excitation/emission wavelength combinations, a, b, 254/370 cutoff; c, d, e, 370/400 cutoff; fluorescence range, a, b, 500; c, d, e, 10. Samples, in methanol, were: a, 1 μg/ml Jan B plus Jan C; b, tremorgenic fungal culture extract; c, 1 μg/ml Jan B; d, as in b (first part of trace); e, 1:1 mixture of b and c (first part of trace). Peaks 1–7 as in Fig. 1; 8 = unknown fluorescent compound.

For UV detection of the janthitrems the wavelength was set at either 265 nm or 330 nm. At 330 nm the detector was more selective while at 265 nm a better indication of the presence of any co-extracted impurities could be obtained. Some strongly retained peaks, and much of the early eluting co-extractives seen at 265 nm, are not seen at 330 nm. The janthitrems also show an enhanced response at 265 nm compared to 330 nm; by 30–40% for Jan B and by 10–20% for Jan C and Jan D. On our system run at 265 nm and with water–methanol (20:80), the minimum detectable amount of Jan B was 1 ng, and of Jan C, 5 ng.

The use of a fluorescence detector allowed smaller amounts of the janthitrems to be detected. In our system, with the fluorescence detector connected in line after the UV detector, a twenty-fold increase in sensitivity over UV determination was obtained using the standard 370-nm bandpass excitation lamp and filter combination in conjunction with a 400-nm cutoff emission filter. A further 50-fold increase in sensitivity could be obtained by using a 254-nm line excitation and a 370-nm cutoff emission filter. The ratio of the absorbance response to the fluorescence response was similar for each of the four janthitrems. An added advantage of using a combination of UV and fluorescence detection was that the janthitrems could be differentiated from compounds with identical retention times but with different ratios of absorbance to fluorescence response. For example, when run in water–methanol (20:80) the culture extract shown in Fig. 3b was originally thought to contain Jan B. However, when run in water–methanol (28:72) with the UV wavelength set at 330 nm

(more specific for janthitrems) and the fluorescent excitation wavelength set at 370 nm (less sensitive for janthitrems), a simplified chromatogram results, as shown in Fig. 3d. In Fig. 3c, Jan B occurs at retention time 443 sec and has an absorbance/fluorescence ratio of *ca.* 1:4. In Fig. 3d, the extract peak occurs at retention time 428 sec and has an absorbance/fluorescence ratio of 1:16, strongly suggesting a compound other than Jan B. As Fig. 3e shows, these two compounds are partly resolved in water–methanol (32:68). Single injections show the peaks for Jan B and the unknown extract component at 678 sec and 648 sec respectively.

The monitoring system for janthitrems in fungal culture extracts described here has been used on fourteen *P. janthinellum* extracts previously tested for tremorgenic activity by mouse bioassay. Janthitrems were found in eight samples, with concentrations in the order Jan B, Jan C ≫ Jan A, Jan D. Only four of these samples, with very high levels of janthitrems, showed activity in the bioassay. The screening of fungal culture extracts for janthitrems is rapid and accurate using isocratic reversed-phase HPLC with combined variable UV–fluorescence detection. Quantitation of the individual janthitrems is possible with a gradient system or with an isocratic system set up to allow a simple step gradient.

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