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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH STOP-FLOW ULTRAVIOLET SPECTRAL CHARACTERIZATION OF LOLITREM NEUROTOXINS FROM PERENNIAL RYEGRASS

REX T. GALLAGHER* and ALLAN D. HAWKES
Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton (New Zealand)
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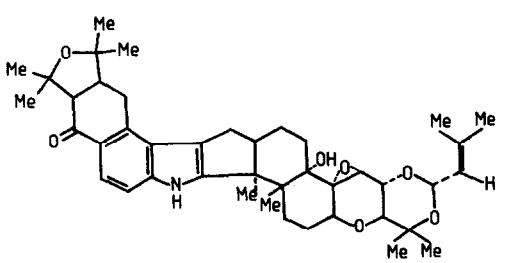
SUMMARY

The lolitrem neurotoxins, potent tremorgenic toxins isolated from perennial ryegrass, were examined using high-performance liquid chromatography with stop-flow UV spectral characterization. Comparison with some known indoles and indolic tremorgenic mycotoxins, together with chemically reduced lolitrem B, the major lolitrem neurotoxin, established the central indole chromophore of the lolitrems. The stop-flow UV spectral characterization was useful for identification of lolitrem B in ryegrass plant and seed extracts.

INTRODUCTION

The lolitrems, remarkable tremorgenic neurotoxins isolated from perennial ryegrass (*Lolium perenne* L.), induce a characteristic sustained tremor when dosed to laboratory mice[1–3]. The structure of lolitrem B (1), the major lolitrem neurotoxin, has



(1)

recently been reported[4]. These lipid-soluble lolitrem neurotoxins are the prime suspect causative agents of ryegrass staggers, a dramatic nervous disorder of sheep, cattle, horses and deer grazing ryegrass-dominant pastures[1–9]. A feature of the ryegrass staggers disorder, which is characterized by severe incoordination and hyper-

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sensitivity to external stimuli, is the consistent lack of observable specific lesions in severely affected animals and the eventual complete recovery and return to normality of such intoxicated animals[2,5,6,10]. The pronounced hydrophobic nature and medium molecular weight range (lolitrem B has molecular weight 685) of the lolitrems, together with the possession of an indole nucleus, are believed to be important properties contributing to their unique actions[1,8].

During our studies of the structure of lolitrem B, we investigated the nuclear chromophore in the lolitrems using high-performance liquid chromatography (HPLC) with stop-flow UV spectral characterization. The present paper describes our investigations with this technique, and serves to illustrate further the utility of this HPLC spectroscopic technique[16,17]. In our work, for comparative purposes, a range of indoles and tremorgenic mycotoxins[5,8,10–13] was examined. These included indole, tetrahydrocarbazole, aflatrem, janthitrem B, paspaline, paxilline, penitrem A and verruculogen. It was established that the spectra so obtained were equivalent to spectra obtained in conventioanl UV spectrophotometers. Using the above technique, we concluded that lolitrem B (and lolitrem C) contained an indole nucleus. Further, peaks in the HPLC analysis of ryegrass plant extracts[1,14] and seed extracts[2] suspected of being lolitrem B, were able to be conclusively identified. Thus, lolitrem B was found to be present in all parts of certain lines of ryegrass plants, including the leaf, stem, roots and seed[14].

EXPERIMENTAL

Apparatus

A Perkin-Elmer liquid chromatograph system was used. The components of the system were: a Series 4 liquid chromatograph with a Rheodyne 7125-075 injector; an LC-85B spectrophotometric detector equipped with a 8-μl flow cell and an LC autocontrol unit to allow spectral scanning; an ASV-1 automatic switching valve plumbed in between the LC pump outlet and the Rheodyne injector; a Model 3600 data station-chromatographics intelligent terminal; a chromatography interface module (A/D converter), to feed the analogue output from the LC-85B detector into the data station; a Model 660 graphics thermal printer-plotter. Also, the analogue output from the LC-85B detector was directed to an ancillary recorder, a Beckman 10-inch chart recorder, for plotting spectral scans.

The HPLC column used was a DuPont Zorbax silica (25 cm × 4.6 mm I.D.) column, particle size 5–6 μm. Column temperature was room temperature, 25 ± 4°C.

The plumbing configuration of the LC system is shown in Fig. 1. This system allowed stop-flow spectroscopy to be performed on selected peaks of interest in the column eluate. The function of the switching valve was to stop the mobile phase flow to the column and, at the same time, vent the stored pressure in the pump and connecting lines. This enabled "peaks of interest" to be trapped in the UV detector flow-cell, for the purpose of spectral scanning.

Conventional UV spectra of compounds in this investigation were determined with either a Beckman Model 25 double-beam spectrophotometer or an Aminco Model DW-2a UV–VIS spectrophotometer.

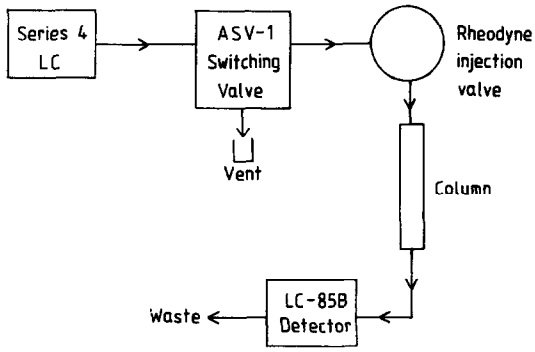


Fig. 1. Plumbing configuration of LC system.

Reagents and reference compounds

Solvents used were Ajax Unichrom HPLC grade dichloromethane and acetonitrile, and were distilled under vacuum in a Buchi rotary evaporator before use. Solvents were automatically degassed with helium in the Series 4 Liquid Chromatograph, during HPLC runs.

Anthracene, indole and tetrahydrocarbazole were purchased from Aldrich, and were purified by crystallization from ethanol; the lolitrems and tremorgenic mycotoxins were pure compounds available from the authors' research programme.

Standard solutions of the lolitrems, indoles and tremorgenic mycotoxins were made up in the HPLC mobile solvent mixture, and stored in the dark at −5°C.

Procedure for stop-flow UV spectral scanning

The general procedure for trapping a "peak of interest" in the UV detector flow-cell, and carrying out a UV spectral scan of the trapped compound, is illustrated by the following example of the UV spectral scan determination for lolitrem B. Pure lolitrem B was subjected to HPLC on silica gel, to establish suitable chromatographic conditions to give the compound as a sharp peak in the column eluate, well free of the injection "solvent front" and resolved from any minor trace impurities. With the isocratic mobile phase system dichloromethane–acetonitrile (70:30), at a flow-rate of 2.0 ml/min, and with the UV detector monochromator set at 268 nm, lolitrem B eluted from the column as a sharp peak with a retention time of 2.7 min. A "blank run" or "calibration run" was then carried out in which an analyte-free, carrier-solvent-only injection was made. At 2.6 min after injection, the automatic switching valve was activated. This turned off the HPLC pump, isolated the front end of the column, and opened the vent valve to dissipate the stored pressure in the pump and the connecting line. At 2.7 min, all solvent movement in the system had ceased, and under these conditions there was negligible diffusion of the liquid trapped in the flow cell. The LC autocontrol unit was then used in the "calibrate" mode, to scan the UV detector flow-cell from 200 nm to 400 nm. This allowed the background absorption spectrum of the solvent trapped in the UV detector flow-cell to be automatically stored in the electronic memory in the LC autocontrol unit, as the scan was carried out. The automatic switching valve was then reversed; this restarted the HPLC pump, opened the valve to the column, and closed the vent valve, thus recommencing normal

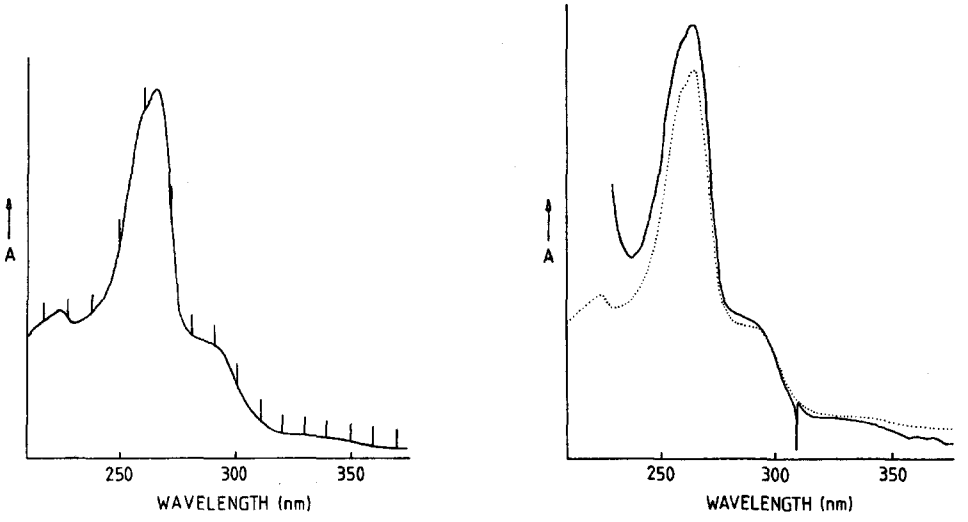


Fig. 2. UV spectrum of lolitrem B by HPLC stop-flow UV spectral scanning.

Fig. 3. UV spectra of lolitrem B determined by HPLC stop-flow spectral scanning (·····) and by conventional UV spectroscopy (———).

isocratic HPLC conditions as above. Lolitrem B (in carrier solvent) was then injected onto the column, with the UV detector monitor set at 268 nm as above. At 2.6 min after injection, the lolitrem B peak was starting to appear in the UV detector flow-cell, as expected, and thus the automatic switching valve was again activated. This trapped the lolitrem B peak in the detector flow-cell. At 2.7 min, the LC autocontrol was used in the "scan mode" to scan the detector flow-cell from 200 to 400 nm. In this mode, automatic subtraction of the solvent background absorption spectrum (stored in the electronic memory during the "calibrate" mode run above) occurred, to give the UV spectrum of lolitrem B, shown in Fig. 2. Reversal of the automatic switching valve then recommenced normal isocratic HPLC conditions as before.

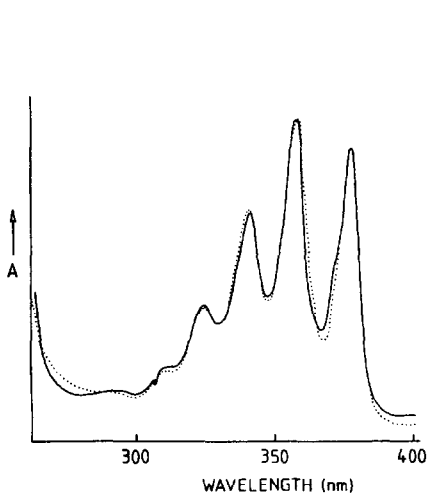
RESULTS AND DISCUSSION

The UV spectrum of lolitrem B obtained under stop-flow HPLC spectral scanning conditions with the HPLC system described in the text, is shown in Fig. 2. The spectrum was determined in the solvent dichloromethane–acetonitrile (70:30), which was the mobile phase used. The spectrum shows the presence of automatically generated calibration "pips" spaced at 10-nm intervals; these pips are useful for wavelength calibration of spectral features. A switch on the UV detector autocontrol unit allows inhibition of the calibration pips, if desired. This allows presentation of a conventional-style, "clean" spectrum, as shown in Fig. 3.

The spectrum of lolitrem B obtained as above was compared with the spectrum obtained using normal UV cuvettes and the mobile phase above as solvent, in a conventional UV-VIS spectrophotometer. The use of the ancillary chart recorder in the LC system, additional to the chromatogram recorder, facilitated the direct com-

parison of spectra of compounds generated by these two modes. With appropriate selection of recorder chart speed, scan rate and sensitivity settings, the two spectra (Fig. 3) of lolitrem B were directly comparable. Thus the shape of both spectral curves is substantially the same, with each possessing absorption maxima at identical wavelengths, at 265 nm and 290 nm, as well as an inflection point and a trough at *ca.* 282 nm and *ca.* 235 nm, respectively. Differences, not unexpected, are apparent between the two spectra in Fig. 3, especially at the lower wavelengths. The UV transmission of dichloromethane begins to decline at 250 nm, falls rapidly at 240 nm, until it is greatly reduced at 230 nm and eventually cuts out at *ca.* 225 nm[15]. Thus the spectral comparison of compounds in solvent systems containing dichloromethane, as in the present case, becomes qualitative only and eventually invalid as the wavelength falls towards 230 nm and below. This effect can be largely overcome if the HPLC solvent system can be chosen to consist only of highly UV-transparent solvents, such as acetonitrile, methanol, hexane, and water. Even so, as the far-UV portion of the spectral region is approached, differential oxygen absorption and variations in the monochromators of different instruments become more important and spectral differences are usually more evident[17]. In our investigations, the above effects did not present a problem.

The general validity of the HPLC stop-flow method[16,17] for obtaining UV spectra of compounds comparable with those obtained with the conventional spectrophotometer method, was readily verified by the determination of the UV spectra of a range of compounds by both methods. In our investigations, anthracene provided a dramatic illustration of the close comparability of the spectra attainable by the HPLC stop-flow method. Fig. 4 shows the spectra obtained for anthracene by the two methods. The characteristic complex UV-spectral fine structure of anthracene[18] is shown in both spectra, which are almost perfectly superimposable over the critical spectral region from 270 to 400 nm.



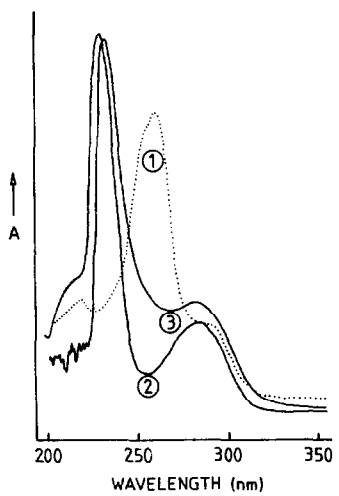


Fig. 4. UV spectra of anthracene determined by HPLC stop-flow spectral scanning (·····) and by conventional UV spectroscopy (———).

Fig. 5. UV spectra of lolitrem B, tetrahydrocarbazole, and aflatrem. Curves: 1 = lolitrem B; 2 = tetrahydrocarbazole; 3 = aflatrem.

From consideration of the UV spectrum of lolitrem B as determined above, from the biological neurotoxicity of lolitrem B[1], from its molecular formula $C_{42}H_{55}NO_7$, as determined by high resolution mass spectroscopy[1], and from the neutral nature of the molecule[1], it was speculated that the lolitrem B molecule could contain an indole nucleus as its central chromophoric moiety. Thus, a range of indoles, including tremorgenic mycotoxins of known chemical structure, were examined by the stop-flow HPLC UV spectral scanning technique. Direct comparison of the resultant spectra strongly suggested that lolitrem B could indeed contain an indole chromophore. Fig. 5 shows the UV spectra of lolitrem B, and two representative substituted indoles, tetrahydrocarbazole[19] and aflatrem[12], superimposed on the same chart. The critical diagnostic region at 280–300 nm shows for lolitrem B a partly-obscured, but undoubtedly present, somewhat flat maxima so characteristic of substituted indoles[15], as present in the spectra of tetrahydrocarbazole and aflatrem. The UV spectrum of lolitrem B is of course dominated by the intense peak with its maximum at 265 nm, which almost "overshadows" and partly obscures but does not hide the above tell-tale characteristic indolic absorption. In contrast, for tetrahydrocarbazole and aflatrem (as shown in Fig. 5), and also for paspaline, paxilline, penitrem A and verruculogen (not shown) a characteristic intense benzenoid absorption peak is located at *ca.* 230 nm, with a deep trough being present at *ca.* 250 nm. This peak is uniquely displaced in the case of lolitrem B, to higher wavelength at 265 nm. This suggested the presence of a carbonyl group in conjugation with the aromatic ring of lolitrem B. This supposition was elegantly verified by sodium borohydride reduction of lolitrem B, which gave a mixture of two isomeric alcohols[4]. The UV spectra of these alcohols revealed classic indolic spectra. Fig. 6 shows the UV spectra of the major isomeric alcohol from the lolitrem B reduction, and tetrahydrocarbazole. The central indole chromophore of lolitrem B was thus established.

From the above investigations, and from a study of the literature on the UV spectra of indoles, it was apparent that the intense maximum at 268 nm in the UV spectrum of lolitrem B was different from most other indoles and tremorgenic my-

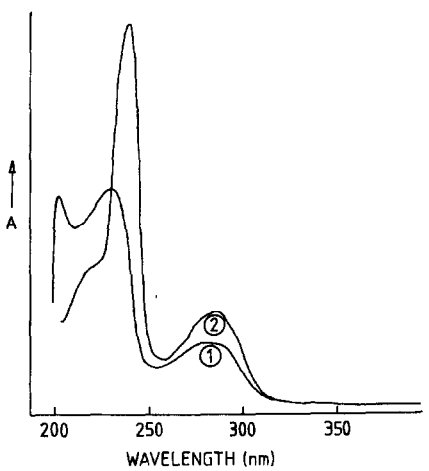


Fig. 6. Comparison of UV spectra of reduced lolitrem B and tetrahydrocarbazole. Curves: 1 = reduced lolitrem B; 2 = tetrahydrocarbazole.

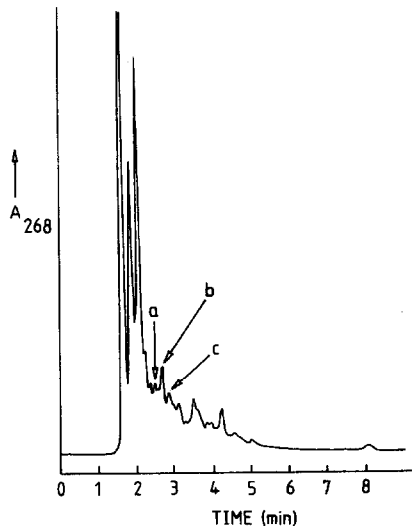


Fig. 7. HPLC of perennial ryegrass (*Lolium perenne* L.) extract, showing suspected lolitrem B peak. Stop-flow UV spectral scanning suggested identity of peak b as lolitrem B (see text).

cotoxins, and that the UV spectrum of lolitrem B was unique and characteristic of the compound. Use was made of this fact in detecting the presence of lolitrem B in ryegrass plant and seed extracts. Fig. 7 shows a typical chromatogram of a toxic ryegrass extract[1,3,14], in which a peak (labelled b) occurs at the correct retention time (2.7 min) for lolitrem B under the HPLC conditions used. However, the chromatogram contained numerous peaks, some lying on either side of the suspect lolitrem B peak. To aid positive identification of the suspect peak, stop-flow UV scans were carried out on the peak, and on peaks a and c which were peaks on either side of peak b. Peak b gave a spectrum substantially superimposable on that of authentic lolitrem B over the critical range 240–300 nm; by comparison, neither peak a nor peak c gave spectra comparable with that of lolitrem B. A range of ryegrass extracts was examined by this technique, and it was demonstrated that extracts from toxic ryegrass (*i.e.* neurotoxic to mice, and/or implicated in outbreaks of ryegrass staggers) consistently contained significant levels of lolitrem B, whereas non-toxic ryegrass contained low or negligible levels.

Our investigations clearly showed that this HPLC technique could be used for quantitative analysis of lolitrem B, since the UV peak height of lolitrem B was found to be proportional to the amount of lolitrem B. However, attempts to develop a routine quantitative analysis for lolitrem B by this technique were not pursued, as during the course of our investigations of lolitrem B a rapid, more sensitive method was discovered and subsequently developed[20].

Lolitrem C, isolated from ryegrass seed[2], had a UV spectrum obtained by the HPLC stop-flow method that was completely superimposable on that of lolitrem B. This established with high probability that lolitrem C possesses the same chromophoric moiety as lolitrem B.

It is noteworthy that the tremorgenic mycotoxin janthitrem B[13] gave a UV spectrum that showed an intense absorption peak at 265 nm, like that of lolitrem B

TABLE I

RETENTION TIMES, ABSORPTION WAVELENGTHS AND MOLECULAR WEIGHTS OF LOLITREMS, INDOLES, AND TREMORGENIC MYCOTOXINS

Compound	*HPLC** t_R *(min)*	*UV*** λ_{max} *(nm)*	*MW*
Lolitrem B	2.70	265, 290	685
Lolitrem C	2.90	265, 290	687
Indole	1.82		117
Tetrahydrocarbazole	1.75	231, 284	171
Paspaline	2.65	234, 284	421
Aflatrem	1.90	232, 280	501
Janthitrem B	5.28	266, 331	585
Paxilline	3.05	234, 284	435
Penitrem A	4.65	238, 299	633
Verruculogen	3.30	230, 278, 297	511

* Solvent, dichloromethane–acetonitrile (70:30); flow-rate, 2 ml/min; t_R = retention time.
** Solvent, dichloromethane–acetonitrile (70:30).

and lolitrem C. However, the indolic absorption of janthitrem B is dramatically shifted upfield to 329 nm, uniquely different from the lolitrems.

Finally, Table I lists useful data on the HPLC retention times, λ_{max} values of UV spectra, and molecular weights of the compounds used in this investigation. It is apparent that HPLC on silica gel, with stop-flow UV spectral scanning, is a useful technique for separating and identifying tremorgenic mycotoxins and related indolic neurotoxins.

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