Abstract: Most published methods for quantifying neurotoxic alkaloids such as lolitrem B from perennial ryegrass rely on separation using normal phase HPLC followed by fluorescence detection. We report here a method based on reversed-phase HPLC using a C18 column followed by electrospray ionisation liquid chromatography/mass spectrometry (ESI LC-MS). Lolitrem B was easily quantified with this method at very low levels of abundance (limit of detection = 1 pg injected on column). Extraction of dried ryegrass foliage with various solvents showed dichloromethane to be most efficient at recovering lolitrem B, but acetonitrile recovered 75% of the amount extracted by dichloromethane and has numerous advantages over dichloromethane as an extraction solvent. This method should prove to be very useful for quantitating lolitrem B in perennial ryegrass samples because it offers great sensitivity and selectivity and relies on commonly used chromatographic columns and solvents.
An improved method for recovering and quantifying neurotoxic alkaloids from endophyte-infected ryegrass

P.A. Weston\textsuperscript{A}, J.C. Quinn\textsuperscript{B} and L.A. Weston\textsuperscript{C}

\textsuperscript{A}Charles Sturt University and E.H. Graham Centre for Agricultural Innovation, Wagga Wagga, NSW 2678: pweston@csu.edu.au

\textsuperscript{B}Charles Sturt University and E.H. Graham Centre for Agricultural Innovation, Wagga Wagga, NSW 2678: jquinn@csu.edu.au

\textsuperscript{C}E.H. Graham Centre for Agricultural Innovation, Wagga Wagga, NSW 2678: leweston@csu.edu.au

Abstract: Most published methods for quantifying neurotoxic alkaloids such as lolitrem B from perennial ryegrass rely on separation using normal phase HPLC followed by fluorescence detection. We report here a method based on reversed-phase HPLC using a C\textsubscript{18} column followed by electrospray ionisation liquid chromatography/mass spectrometry (ESI LC-MS). Lolitrem B was easily quantified with this method at very low levels of abundance (limit of detection = 1 pg injected on column). Extraction of dried ryegrass foliage with various solvents showed dichloromethane to be most efficient at recovering lolitrem B, but acetonitrile recovered 75% of the amount extracted by dichloromethane and has numerous advantages over dichloromethane as an extraction solvent. This method should prove to be very useful for quantitating lolitrem B in perennial ryegrass samples because it offers great sensitivity and selectivity and relies on commonly used chromatographic columns and solvents.

Key words: analytical chemistry, livestock production, ryegrass staggers

Introduction

Endophytic fungi produce a variety of compounds which confer advantageous properties to the plants they inhabit including drought tolerance and resistance to insects and weeds (Schardl \textit{et al.} 2007). However, these compounds can also have a devastating effect on livestock grazing on them. One such example is the potent endophytic neurotoxin lolitrem B which is found in the forage species \textit{Lolium perenne} (perennial ryegrass)(Gallagher \textit{et al.} 1981). Lolitrems, a family of closely related ergopeptine alkaloids produced by \textit{L. perenne} infested with the endophyte \textit{Neotyphodium lolii}, cause tremors in livestock and result in decreased production and increased mortality (Parton and Chambers 2001). The lolitrems are active at very low levels (ca. 5 ppm)(Gallagher \textit{et al.} 1985), so being able to quantify these compounds in perennial ryegrass tissues and seed is of importance to the livestock industry.

To date, most reports quantititating lolitrems in ryegrass have relied on the original method reported by Gallagher \textit{et al.} (1985). This method requires the use of normal phase HPLC, halogenated solvents, and fluorescence detection. Since then, significant advances have been made in analytical techniques for separation of compounds in mixtures, particularly the widespread use of reversed phase columns, and use of ESI LC/MS (liquid chromatography coupled with mass spectrometry using electrospray ionisation to introduce analytes into the mass analyser). These advances have the potential to increase the sensitivity of methods for quantitating lolitrems from ryegrass while decreasing reliance on halogenated solvents, which are potent carcinogens.

The objectives of this study were to apply reversed phase HPLC coupled with ESI LC/MS to quantify lolitrem B in perennial ryegrass samples, and to compare extraction efficiency of lolitrem B using a variety of organic solvents. The overall goal was to develop a method for detecting lolitrem B at very low levels using commonly available analytical chemistry techniques.

Methods

Sample preparation

Dried samples of perennial ryegrass were weighed (100 mg) and extracted with 2 mL of solvent. The following solvents were compared: acetonitrile, dichloromethane, hexane, methanol and dichloromethane:methanol (4:1). Samples were vortexed for 30 sec, then gently shaken on an orbital shaker for 24 h and centrifuged at 8,000 rpm for 5 min. The supernatant was drawn off with a pipette and filtered through a 0.22 \textmu m filter (dichloromethane extracts could not be filtered because the filter membrane and housing was not compatible with halogenated solvents). Solvent was removed under a stream of nitrogen.
while warming the samples on a heat block; after weighing the samples, they were dissolved in dichloromethane (2 mL) and subjected to clean-up on silica gel solid phase extraction (SPE) cartridges (Agilent SimpliQ, 3 mL). SPE cartridges were preconditioned with methanol followed by dichloromethane, and samples were eluted with dichloromethane (3 mL, to waste) followed by 4:1 dichloromethane:methanol (2 mL).

**Quantitation of lolitrem B**

Samples were analysed using an Agilent 1200 HPLC coupled with an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray source. HPLC solvents were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Sample were eluted with a solvent gradient from 50:50 A:B, increasing to 100% B over 8 min at a flow rate of 0.4 mL/min. After reaching 100% B, the column was held at 100% B for 2 min and returned to 50:50 A:B for 3 min before the next injection. The column was C18 (Zorbax Eclipse XDB-C18, 4.6 x 50 mm, Agilent). Samples were introduced into the mass analyser via electrospray ionisation using a drying gas flow of 10 L/min and drying temperature of 350°C. Preliminary analyses were run in full scan mode with a fragmentation voltage of 135V, and quantitation was conducted using multiple reaction mode, filtering on a precursor ion of m/z 686 and a product ion of m/z 629, based on optimisation results (fragmentation voltage = 180V, collision energy = 35). Purified lolitrem B was purchased from AgResearch Ltd., NZ.

**Results and discussion**

Lolitrem B was readily detected using the chromatographic and mass spectroscopic techniques described above. In addition to lolitrem B, lolitrem F was often detected in samples (Figure 1). Lolitrem F is a stereoisomer of lolitrem B, differing only in the orientation of the hydrogen atom on carbon 35 (Munday-Finch et al. 1996). Despite this subtle difference, these two molecules were eluted with complete baseline separation, differing by 10-15 sec in retention time. Dilution series analysis of lolitrem B revealed an apparent limit of detection (LOD) of ca. $10^3$ fg (1 pg) (Fig. 2). This is significantly more sensitive than previous studies utilising fluorescence detection, which has a LOD of 0.5 ng (Gallagher et al. 1985). Based on our sample size of 100 mg of dried tissue, our detection limit of lolitrem B in dried perennial ryegrass foliage is equivalent to 10 ng/kg, which is also a dramatic improvement over detection relying on fluorescence detection, reported by Moyano et al. (2009) as 0.05 mg/kg (50 ug/kg). This represents a 5000-fold increase in sensitivity.

Another significant advantage of ESI LC/MS is the exceptional selectivity when operating in multiple reaction mode. In this mode, ionised molecules entering the system are first filtered to exclude all except those with a molecular weight of the compound of interest, and then only characteristic product ions resulting from fragmentation of the parent molecule are quantitated. Combined with knowledge of the retention time of the compound eluting from HPLC, it is a virtual certainty that ions detected at specified points in time can result only from the presence of the parent molecule in a mixture. This selectivity contributes to the high signal-to-noise ratio, making it possible to quantify molecules at extremely low levels.

Comparison of extraction of lolitrem B with a range of solvents revealed that dichloromethane was the most efficient solvent (Table 1). Acetonitrile and hexane extracted about 75% of the amount extracted by dichloromethane, but the least efficient solvent for extracting lolitrem B was 4:1 dichloromethane:methanol, which extracted only 30% of the lolitrem B recovered by dichloromethane. Dichloromethane also extracted much more material (a wider range of compounds other than lolitrem B from grass samples) compared to hexane and acetonitrile (Table 1). This is in contrast to extraction of the ergot alkaloid ergovaline from tall fescue seed; in this case, methanol provided highest extraction efficiency of ergopeptide-related alkaloids (Garner et al. 1993). This difference is no doubt explained by the high lipophobicity of lolitrem B and the relatively more polar nature of the ergopeptide alkaloids.

Acetonitrile may therefore be the solvent of choice when quantifying lolitrem B in perennial ryegrass samples because it extracted a high percentage of lolitrem B (ca. 75%) without significant amounts of irrelevant and potentially interfering compounds. Acetonitrile has other advantages over dichloromethane: 1) it is compatible with plastics commonly used in disposable sample filtration units, 2) it is fully miscible with solvents used for reversed-phase HPLC (e.g. water and acetonitrile), 3) it is less toxic than halogenated solvents such as dichloromethane and 4) wastes do not require the same degree of special handling as halogenated solvents.
The extraction procedure described above could potentially be simplified for routine extraction of lolitrem B from perennial ryegrass samples. Owing to the lower limit of detection, smaller quantities of plant material could likely be used, and vortexing samples with solvent might obviate the need for an orbital shaker.

**Conclusions**

This novel method for quantitating lolitrem B in perennial ryegrass offers significant advantages over previously published methods that are widely cited in the literature. HPLC methods that are currently used in analytical laboratories (e.g. reversed phase separation using acetonitrile and water in the mobile phase) are well suited to analysis of lolitrem B, and the use of ESI LC-MS offers extremely high sensitivity and selectivity (Callahan et al. 2009). Further refinements to the extraction procedure are likely to result in a greatly simplified and rapid method for analysing this environmentally important molecule.

**Acknowledgments**

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**References**


**Table 1.** Relative recovery of lolitrem B and total weight of material extracted from a perennial ryegrass sample extracted with various solvents. Recoveries are expressed as a percentage of the amount of lolitrem B extracted by dichloromethane.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Relative recovery of Lolitrem B (%)</th>
<th>Total weight of extract (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>100.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Hexane</td>
<td>77.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>73.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Methanol</td>
<td>52.0</td>
<td>22.3</td>
</tr>
<tr>
<td>Dichloromethane plus methanol (4:1)</td>
<td>30.5</td>
<td>16.2</td>
</tr>
</tbody>
</table>
Figure 1. Total ion chromatogram of authentic standard of lolitrem B as separated via reversed-phase HPLC and detected via ESI LC-MS in multiple reaction mode (precursor ion=686 \textit{m/z}, product ion=629 \textit{m/z}). The small peak preceding the lolitrem B peak (the large peak at RT=10 min) is lolitrem F.

Figure 2. Dose/response of lolitrem B as detected by ESI LC-MS in multiple reaction mode. Below $10^3$ (1 pg) of on-column injection, lolitrem B was not accurately quantitated. In the range of $10^3$ to $10^7$ g of compound injected, the detector response was well correlated with quantity injected ($r^2 = 0.9896$).