Mycotoxin Res
DOI 10.1007/s12550-015-0225-7

ORIGINAL PAPER

Gliotoxin production by *Aspergillus fumigatus* strains from animal environment. Micro-analytical sample treatment combined with a LC-MS/MS method for gliotoxin determination

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Received: 17 September 2014 / Revised: 5 May 2015 / Accepted: 7 May 2015

Abstract In this study, gliotoxin production by *Aspergillus fumigatus* strains from animal environment is studied. Moreover, a rapid, easy and environment-friendly micro-analytical sample treatment procedure coupled with LC-MS/MS was applied for the determination of gliotoxin from *A. fumigatus* cultures. The ability of gliotoxin production by 143 strains was assayed in yeast extract sucrose agar, and 1 ml of chloroform was used for toxin extraction without further clean-up. Mean recoveries at two spiking levels (2500 and 7000 ng/g; n=6) were 100.3±6.6 % relative SD (RSD) and 92.4±3.8 % RSD. Repeatability and within-laboratory reproducibility for different concentration levels of gliotoxin (25 to 1000 ng/ml; n=12) ranged from 0.3 to 5.4 % RSD and from 3.9 to 12.7 % RSD, respectively. The detection limit of the analytical method was 3.5 ng/g. The ability for gliotoxin production by *A. fumigatus* revealed that 61.5 % of the strains were able to produce the toxin at levels ranging from LOQ to 3430.5 ng/g. However, all the tested samples had similar percentages of producing strains (81.8 to 86.6 %). The micro-analytical sample treatment coupled with LC-MS/MS detection is a precise and useful methodology for determining gliotoxin from fungal extracts of *A. fumigatus* and allows working both fast and safely and also reducing the effect on the environment. This toxin plays a critical role in the pathobiology of *A. fumigatus*, and its presence in animal environments could affect animal health and productivity; in addition, there are risks of contamination for rural workers during handling and storage of animal feedstuffs.

Keywords Gliotoxin · *Aspergillus fumigatus* · LC-MS/MS

Introduction

Aspergillus fumigatus, the most common cause of invasive aspergillosis in immunocompromised individuals, is a common fungus found in contaminated animal feed (Alonso et al. 2013). This fungus is able to produce tremorgenic mycotoxins and to induce neurological syndromes in farm workers who have manipulated mouldy feed (Gordon et al. 1993). One of the most abundantly secondary metabolite produced by *A. fumigatus* is gliotoxin, an epipolythiodioxopiperazine characterized by the presence of the transannular disulphide bridge responsible for its biological activity such as potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects (Nieminen et al. 2002; Upperman et al. 2003). At present, there are few reports on the ability of gliotoxin production by *A. fumigatus* isolated from animal environments. Bauer et al. (1989) isolated gliotoxin from bovine udder tissues naturally infected with *A. fumigatus*; this mycotoxin has been also associated to a case of intoxication and death in camels having consumed contaminated hay (Gareis and Wernery 1994). Many methods have been developed for gliotoxin detection and quantification from sera of cancer patients with invasive aspergillosis (Lewis et al. 2005a, b), animals tissues such as mammalian udder (Bauer et al. 1989) and feedstuff intended for domestic animals and pets (Boudra et al. 2005; Pereyra et al. 2008; Pena et al. 2010). Thin-layer

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Published online: 16 May 2015

chromatography (TLC) or high-performance liquid chromatography (HPLC) for gliotoxin analysis was used. Immunological techniques, a semi-quantitative bioassay and a semi-quantitative method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a further clean-up, were also applied (Fox et al. 2004; Grovel et al. 2006; Sulyok et al. 2007). LC-MS/MS has the unique combination of high selectivity and sensitivity that allows both qualitatively trustable and low-level assessment of mycotoxins coupled to quite easy sample treatment. Different sample treatment procedures have been applied for gliotoxin extraction from complex matrices (such as culture media) involving more than twice extraction steps and usage of big volumes of organic solvents (Richard et al. 1996; Lewis et al. 2005b; Pereyra et al. 2008). In this study, gliotoxin production by *A. fumigatus* strains from animal environment is studied. Moreover, a rapid, easy and environment-friendly micro-analytical sample treatment procedure coupled with LC-MS/MS was applied for the determination of gliotoxin from *A. fumigatus* cultures.

Materials and methods

Strains

A. fumigatus (143 strains) obtained from the National University of Río Cuarto, Córdoba, Argentina Collection Centre (RC), were assayed for their ability for gliotoxin production. They were isolated from animal feed and morphologically identified as *A. fumigatus* by the classical taxonomy according to Klich and Pitt (2002). Also, they were confirmed as *A. fumigatus* sensu stricto species by molecular characterization (Pena et al. 2014a). All strains were maintained on malt extract agar (MEA) slants at 4 °C until gliotoxin analysis.

A. fumigatus gliotoxin production

Culture

Strains were grown on yeast extract sucrose medium (YES) (sucrose 150 g/l, yeast extract 20 g/l modified with 0.5 g/l of $SO_4Mg.\ 7\ H_2O$, agar-agar 15 g/l in distilled water) at 25 °C for 7 days. Petri plates (9-cm i.d. containing 20 ml of medium) were needle-inoculated centrally by a single point from conidial suspensions in semi-solid agar at 0.2 % (Samson et al. 2004). The conidial suspensions were prepared by harvesting spores from heavy sporulating cultures (7-day-old growing colony at 25 °C) of each isolate on MEA and suspending them in the semi-solid agar to obtain 10^6 spores/ml. Three agar plugs of 6-mm diameter (6-mm profundity, approx. 85 mm^2 of surface) were removed by cutting them from edge to centre of the fungal colony using a sterile cork drill. The plugs were weighed and transferred to a 1.5-ml vial until gliotoxin extraction was done.

Sample treatment for gliotoxin extraction

Chloroform (1 ml) was added to each vial, and the sample-solvent mixture was shaken for 5 min and then centrifuged at 7500*g* for 20 min. After that, the chloroform phase was recovered and filtered through nylon filters, and 800 μl of each extract was evaporated to dryness under a gentle stream of nitrogen. Dried extracts were re-dissolved in 800 μl of mobile phase (eluent A/B 80:20 *v*/*v*; A, aqueous 1 % acetic acid/5 mmol/l ammonium acetate; B, methanol/1 % acetic acid/5 mmol/l ammonium acetate) and used for gliotoxin analysis by LC-MS/MS. Gliotoxin levels were expressed as nanogram per gram of mycelia in YES agar.

Reagents

Gliotoxin was obtained from Sigma-Aldrich Co. Organic solvents were HPLC grade from Sintorgan (Buenos Aires, Argentina). Water (HPLC grade) was obtained from a Labconco WaterPro Mobile purification system model 90901–01. Twenty-five millimetres Syringe Nylon Filters 0.22 μm were utilized for fungal extracts (MSI Micron Separation, Inc., Westborough, MA, USA).

LC-MS/MS analysis

All LC-MS/MS analyses were performed using a Waters 2695 Alliance HPLC (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2685 pump and a Waters Alliance 2695 auto-sampler, interfaced to a Quattro Ultima Platinum tandem quadrupole mass spectrometer with electrospray ionization (ESI) source. An XBridge TM C18 3.5 μm (2.1×150 mm) column with a 2.1×10-mm i.d. guard column was used (Waters).

The interface was operated in a positive-ion mode. Nebulization and desolvation gases were nitrogen-heated to 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. Nitrogen flows were adjusted to 100 and 725 l/h for cone and desolvation gases, respectively. Dwell time was 100 ms. Multiple-reaction monitoring (MRM) was used for the determination of gliotoxin. The MRM transitions and MS conditions are shown in Table 1. Data acquisition and processing were performed using Mass Lynx V.4.1© 2005 (Waters, Inc.) software. Therefore, the gliotoxin retention time, the MRM transitions of the precursor ion to two product ions (two identification criteria) and the ratio of both ion transitions were met to identify gliotoxin in fungal cultures.

The chromatographic method developed by Sulyok et al. (2007) for multitoxin analysis was applied in a different matrix. Therefore, recovery, reproducibility and repeatability

Table 1 MS conditions used for GLI production by LC-MS/MS

Compound	$[M+H]^+$	t_R (min)	Product ions (*m/z*)	CV (V)	CE (V)
GLI	327	14.97	263 (quantifier)	35	15
			245 (qualifier)	35	25

$[M+H]^+$ precursor ion, t_R retention time, *CV* cone voltage, *CE* collision energy

assays were performed for method verification in YES medium. A binary gradient of eluents A and B was used at a flow rate of 0.2 ml/min. At 0 min, the eluent was 10.5 % of B. This composition was kept for 2 min, after which a linear gradient to 97.5 % of B was performed in 12 min, and then, eluent B was kept at this composition for 3 min to clean the column. The initial conditions were stabilized over 5 min before the next injection. The column temperature was kept at 22 °C, and aliquots of 20 μl of extracts (final fungal extracts, spiked samples or spiked standards) were injected into the HPLC unit. The limit of detection (LOD) and limit of quantification (LOQ) were determined by samples and matrix-matched gliotoxin standards based on signal-to-noise ratio (S/N) of 3:1 for LOD and 10:1 for LOQ. Three agar plugs from a clean medium YES plate were extracted following the gliotoxin extraction procedure, and the re-suspended extract was further used to prepare the matrix-matched standards.

Recovery, reproducibility and repeatability assays for method verification For recovery studies, plates of YES medium were fortified at two spiking levels (2500 and 7000 ng/g) and incubated at 25 °C for 24 h. The amounts of recovered gliotoxin were determined by LC-MS/MS using matrix-matched standard calibration for quantification. Recoveries were expressed as mean value of tree replicates and relative SD (RSD) of the same replicates.

Relative standard deviations for both intra- and inter-day assays were computed to verify repeatability and reproducibility, respectively. Repeatability was estimated by assaying triplicate fungal extracts spiked with gliotoxin at levels of 25, 50, 100 and 1000 ng/ml (n=12). Samples were analyzed on the same day by the same analyst. The within-laboratory reproducibility was estimated for the same spiked fungal extracts but measured on different days.

Results and discussion

Table 2 shows the gliotoxin production by *A. fumigatus* strains from different kinds of feedstuffs and raw materials intended for different animal species. The ability for gliotoxin production by *A. fumigatus* revealed that 61.5 % of the strains were able to produce the toxin with levels ranging from LOQ to 3430.5 ng/g. In the remaining 38.5 % extracts, gliotoxin was detectable (19.6 %) or not detectable (18.9 %). The percentage of strains able to produce this toxin was similar between the tested raw materials intended for cattle and pigs. Although the number of producer strains was individually variable among the different feedstuffs, the percentage of gliotoxin producer strains was also similar to those from raw materials.

Although there were strains with strong ability to produce high levels of gliotoxin, the median values showed intermediate levels. For this reason, the strains were divided according to the gliotoxin production levels, and, as it is shown in Table 2, all the tested samples had similar percentages of producing strains 300 ng/g gliotoxin (81.8 to 86.6 %). Only pet food samples had two strains with the ability to produce more than 1000 ng/g gliotoxin (1046.74 and 1156.06 ng/g), and a corn silage sample had a strain able to produce 3430.54 ng/g gliotoxin.

At the present, there are few articles reporting *A. fumigatus* gliotoxin producer strains isolated from animal environments such as animal feedstuff and silages. Pena et al. (2010, 2014b) studied the ability for gliotoxin production by *A. fumigatus* strains isolated from feedstuffs intended for domestic animals and pets in Argentina. They found that several strains isolated from corn silage, equine feed and pet food

Table 2 Gliotoxin production by *A. fumigatus* strains after 7-day incubation in YES medium

A. fumigatus gliotoxin production							
Kind of feed	Animal species	Producer strains/Total strains		Gliotoxin level produced (ng/g)			
				Mean–median (ng/g)	<300	Between 300 and 1000	>1000
		Number of strains	Percentage (%)		Producer strains (%)		
Corn silage	Dairy cattle	16/27	59.2	340.50–36.24	86.6	13.3	6.6
Brewer's grains	Pigs	22/39	56.4	157.35–18.63	81.8	18.2	–
Feedstuffs	Pets	33/41	55.9	276.26–178.58	86.9	23.2	1.5
	Chinchillas	10/20		125.81–120.29			
	Horses	5/6		25.80–16.30			
	Poultry	1/10		12.23–12.23			

LOD, 3.5 ng/g; LOQ, 10.5 ng/g. Results not corrected by recovery

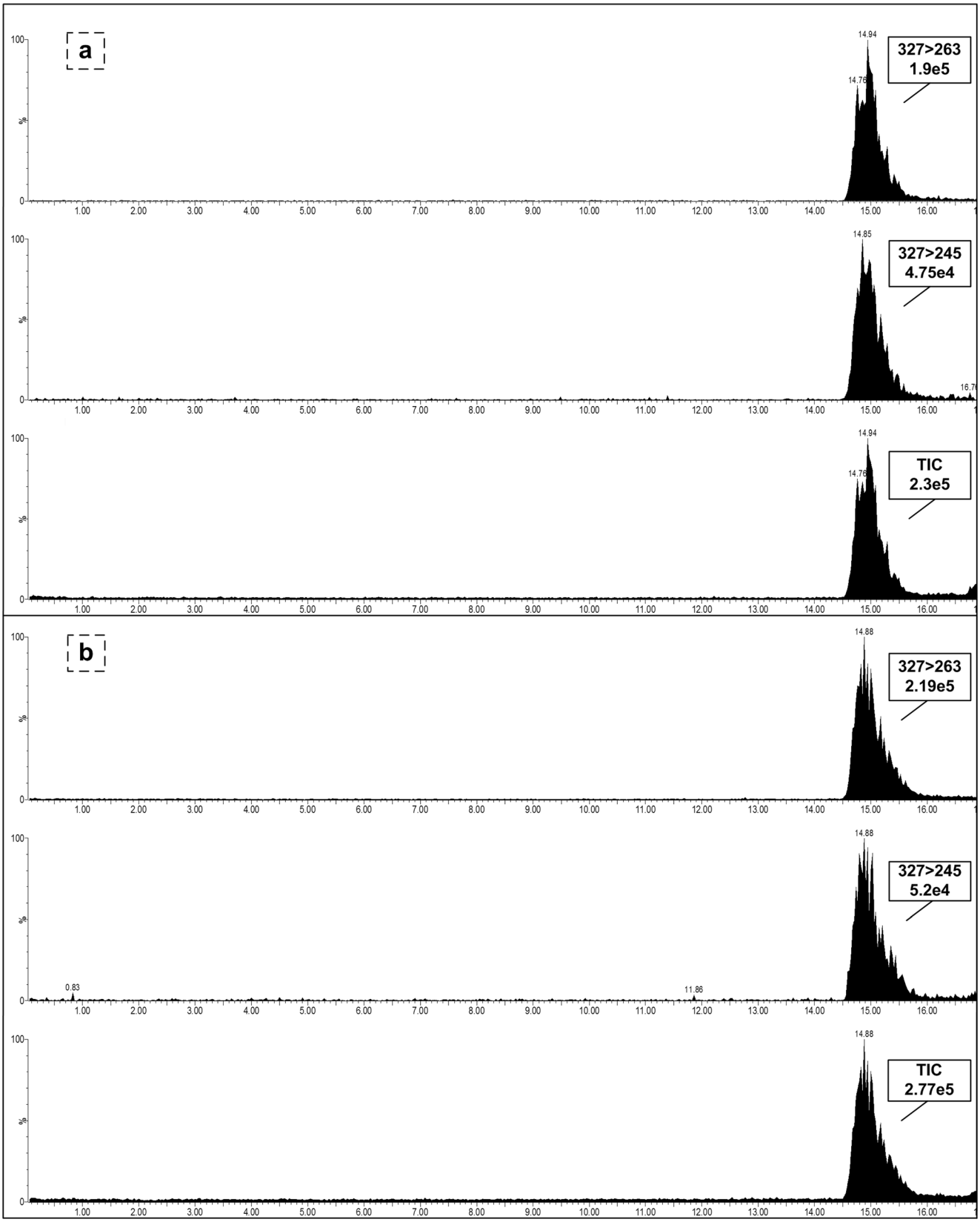

Fig. 1 LC-MS/MS chromatograms of **a** gliotoxin matrix-matched standard (1250 ng/ml) and **b** gliotoxin from fungal extracts of a pet food strain (*A. fumigatus* RC2082, 1046.74 ng/g). [*X*-axis represents retention time (min); *Y*-axis represents relative abundance of 327>263 and 327>245 product ions]. Microsoft® Office Vision®. Professional 2003 (11.8323.8405) SP 3

were able to produce the toxin at high levels, and pet food strains were again the highest producers. These results are in agreement with those found in this study since *A. fumigatus* strains isolated from pet food and corn silage were the highest producers. dos Santos et al. (2003) reported the ability for gliotoxin production by 27 *A. fumigatus* strains isolated from corn silage. However, they found that only three strains were able to produce gliotoxin. In this study, most of the tested strains isolated from the different animal feeds produced the toxin at wide ranges. Figure 1 shows chromatograms of gliotoxin in a matched-matrix standard and those detected in fungal extracts of an assayed strain isolated from pet food. Gliotoxin plays a critical role in the pathobiology of *A. fumigatus* since it modulates the immune response and induces apoptosis in different cell types (Scharf 2012). Reeves et al. (2004) studied the correlation between gliotoxin production and virulence of *A. fumigatus* on the *Galleria mellonella* larvae. They found that *A. fumigatus* strains that produced higher amounts of gliotoxin were more virulent (produced higher larvae mortality) than those who had low ability for this toxin production. Strains that are prevalent in animal environments could be involved in the development of illness in both humans and animals. Also, its presence in animal environments could affect animal health and productivity; in addition, there are risks of contamination for rural workers during handling and storage of animal feedstuffs.

In this approach, the sample preparation involved a single extraction step using a little volume of chloroform as extraction solvent without clean-up column usage. Different approaches have been performed allowing a rapid analysis of gliotoxin but involving more than one organic solvent and extraction step (Van Pamel et al. 2011) or using quite big volumes of extraction solvent (Boudra and Morgavi 2005; Pereyra et al. 2008).

In this work, the matrix-matched calibration curve, y= 5474.5x (R^2=0.9925, n=12), was linear in the range from 10 to 7000 ng/ml of gliotoxin. The instrumental limit of detection (S/N=3) was 0.25 ng. Mean recoveries at two spiking levels (2500 and 7000 ng/g, n=6) were 100.3±6.6 % RSD and 92.4±3.8 % RSD. The repeatability and within-laboratory reproducibility for different concentration levels, expressed as RSD_r and RSD_R, respectively, were found to be good according to the Commission Decision 2002/657/EC (2002) (Table 3). Furthermore, the recovery values were in agreement with the European Committee for Standardization criteria (CEN 1999). These assays allowed checking the Sulyok et al. (2007) chromatographic method and together with the suitable recoveries obtained confirmed its application to another matrix such as a culture medium. Since no ^{13}C standards were available, matrix-matched calibration was the best choice to compensate for matrix effects. Further studies on matrix effects could be conducted using culture medium extract with mycelia from non-toxicogenic strains. The developed sample treatment procedure coupled with LC-MS/MS detection is a precise and useful methodology for determining gliotoxin from *A. fumigatus* extracts. An efficient micro-extraction technique together with an easy sample treatment in order to reduce the organic solvent usage was used, allowing working both fast and safely and also reducing the effect on the environment.

Table 3 Repeatability and within-laboratory reproducibility

Gliotoxin (ng/ml)	Repeatability[a] RSD (%)	Within-laboratory reproducibility[b]
25	2.5	7.7
50	5.4	12.7
100	0.3	3.9
1000	1.6	6.5

[a] Repeatability was estimated by assaying fungal extracts spiked with gliotoxin (n=3). Samples were analyzed on the same day by the same analyst

[b] The within-laboratory reproducibility was estimated from fungal extracts spiked with gliotoxin on different days

Acknowledgments This work was supported by SECYT—Universidad Nacional de Río Cuarto and PICT—Agencia Nacional de Ciencia y Tecnología.

Conflict of interest None.

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