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Quantitative determination of several toxicological important mycotoxins in pig plasma using multi-mycotoxin and analyte-specific high performance liquid chromatography–tandem mass spectrometric methods

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ABSTRACT

A sensitive and reliable multi-mycotoxin method was developed for the identification and quantification of several toxicological important mycotoxins such as deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZON), zearalanone (ZAN), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1) in pig plasma using liquid chromatography combined with heated electrospray ionization triple quadrupole tandem mass spectrometry (LC–h-ESI-MS/MS). Sample clean-up consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in water/methanol (85/15, v/v). Each plasma sample was analyzed twice, i.e. once in the ESI+ and ESI− mode, respectively. This method can be used for the assessment of animal exposure to mycotoxins and in the diagnosis of mycotoxicoses. For the performance of toxicokinetic studies with individual mycotoxins, highly sensitive analyte-specific LC–MS/MS methods were developed.

The multi-mycotoxin and analyte-specific methods were in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9974–0.9999 and 2.4–15.5%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification for the multi-mycotoxin and analyte-specific methods ranged from 2 to 10 ng/mL and 0.5 to 5 ng/mL, respectively, whereas limits of detection fell between 0.01–0.52 ng/mL and <0.01–0.15 ng/mL, respectively.

1. Introduction

Mycotoxins are secondary metabolites produced by different fungal species. Over 100 mycotoxins have been identified, although only a few of them present a significant toxic effect and are of major concern. These include deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZON), ochratoxin A (OTA), aflatoxin B1 (AFB1) and fumonisin B1 (FB1) [1]. The contamination of feed with mycotoxins is a continuing feed safety issue leading to economic losses in animal production [2], especially for pigs as they are known to be one of the most susceptible species for the stated mycotoxins.

Diagnosing mycotoxicosis in animals and assessing animal exposure to mycotoxins is usually performed by analysis of commodities and feed. However, several disadvantages are present when estimating mycotoxin intake as such. In particular, no measurement of exposure at the individual level is possible, the variability of feed contamination is high and it does not provide a good dose response and specificity for the target mycotoxin. This can be overcome by measuring specific biomarkers or target compounds in body fluids, e.g. plasma or urine. Measuring the target compound in blood is also the most appropriate way for other purposes, including toxicokinetic studies with mycotoxins and evaluation of the efficacy of mycotoxin reduction strategies. Adding mycotoxin detoxifying agents to feed contaminated with mycotoxins is commonly used and seems to be the most promising way of counteracting the harmful effects of mycotoxins in animals [3]. Manufacturers have to prove the efficacy of these products, which is generally based on in vitro adsorption studies. However, the European Food Safety Authority (EFSA) has recently proposed guidelines for efficacy testing of mycotoxin detoxifying agents [4]. In their guidelines, it is stated that in vivo efficacy trials should be performed based on absorption, distribution, metabolisation and excretion (ADME) studies. Devreese et al. [5] has recently developed in vivo models for efficacy

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testing of mycotoxin detoxifying agents based on these guidelines.

It is obvious that for assessing animal exposure to mycotoxins (screening) on the one hand and the investigation of mycotoxin toxicokinetics on the other hand, the availability of sensitive and specific validated analytical methods is mandatory. In recent decades, high-performance liquid chromatography (HPLC) has become the most important method for the analysis of mycotoxins in food, feed and other matrices. The mass spectrometer is a reliable detector for HPLC and it is an important analytical tool for routine analysis of mycotoxins in complex matrices because of unambiguous identification and accurate quantification [1]. A huge amount of literature on high-performance liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis of several mycotoxins in food and feed is available [6,7], however, analytical methods for detection of mycotoxins in biological matrices, such as blood, are scarce. In addition, most methods are analyte-specific, which means that they allow the detection and/or quantification of only a limited number of mycotoxins at the same time. Until now, no multi-mycotoxin method for the analysis of plasma has been reported.

Therefore, the goal of this study was to develop such methods for the simultaneous determination and quantification of several toxicological important mycotoxins in animal plasma. The final methods should be applicable in two areas of expertise, i.e. screening for animal mycotoxin exposure and evaluation of the toxicokinetics of individual mycotoxins for the efficacy testing of mycotoxin detoxifying agents. Since a large amount of samples had to be analyzed, special attention has been paid to the development of a high-throughput sample preparation procedure and LC–MS/MS analysis, but not at the expense of reliability and sensitivity.

2. Materials and methods

2.1. Chemicals, products and reagents

The analytical standards of ZON, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, HT-2 and DOM-1 were obtained from Sigma-Aldrich (Bornem, Belgium). Standards of DON, T-2, OTA, FB1 and AFB1 were obtained from Fermentek (Jerusalem, Israel). Internal standards (IS) ($^{13}C_{15}$-DON, $^{13}C_{24}$-T-2, $^{13}C_{18}$-ZON, $^{13}C_{20}$-OTA, $^{13}C_{34}$-FB1 and $^{13}C_{17}$-AFB1) were purchased from Biopure (Tulln, Austria). All standards were stored at ≤−15 °C. Water, methanol and acetonitrile (ACN) were of LC–MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate, ethyl acetate and glacial acetic acid were of analytical grade and were obtained from VWR (Leuven, Belgium). Millex®-GV-PVDF filter units (0.22 μm) were obtained from Millipore (Brussels, Belgium).

2.2. Preparation of standard solutions

Standard stock solutions of DON, T-2, HT-2, ZON, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, T-2, OTA, FB1 and AFB1 were prepared in acetonitrile (analyte concentration: 1 mg/mL). Following standards were purchased as solutions: DOM-1 (50 μg/mL ACN), $^{13}C_{17}$-AFB1 (10 μg/mL ACN), $^{13}C_{15}$-DON, $^{13}C_{24}$-T-2, $^{13}C_{18}$-ZON, $^{13}C_{20}$-OTA and $^{13}C_{34}$-FB1 (all 25 μg/mL ACN). The stock solutions were stored at ≤−15 °C.

Working solutions of 100 μg/mL were prepared in ACN/water (50/50, v/v). Serial dilutions of separate working solutions were prepared to achieve analyte concentrations of 10, 1, 0.1 and 0.01 μg/mL. A combined working solution of 10 μg/mL of all analytes (except IS) was prepared by transferring 10 μL of each stock solution of 1 mg/mL and 200 μL of the stock solution of DOM-1 (50 μg/ml) to an eppendorf cup, followed by further dilution with ACN/water (50/50, v/v) up to a final volume of 1 mL. By appropriate dilution of this solution with ACN/water (50/50, v/v), combined working solutions of 1, 0.1 and 0.01 μg/ml were obtained. For the internal standards, individual and combined working solutions of 1 μg/mL were prepared in ACN/water (50/50, v/v). All working solutions were stored at 2–8 °C.

2.3. Biological samples

Blank plasma samples were obtained from pigs (19.4 ± 1.8 kg BW) that received blank feed for an acclimatization period of one week. The blank plasma samples were used for the preparation of matrix-matched calibrator and quality control (QC) samples.

After the acclimatization period, a bolus toxicokinetic study was performed. Six pigs received a single oral bolus of the following mycotoxins (Fermentek, Jerusalem, Israel): DON, T-2, ZON, OTA and AFB1 (all 0.05 mg/kg BW). Blood samples were drawn before (0 min) and at 15, 30 and 45 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h post-administration. Blood samples were taken in heparinized tubes and centrifugated (2851 × *g*, 10 min, 4 °C). Aliquots (250 μL) of plasma samples were stored at ≤−15 °C until analysis.

This animal experiment was approved by the Ethical Committee of Ghent University (Case number EC 2012/08).

2.4. Sample pretreatment

To 250 μL of plasma were added 12.5 μL of the combined IS working solution and 750 μL of ACN, followed by a vortex-mixing (15 s) and centrifugation step (8517 × *g*, 10 min, 4 °C). Next, the supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (45 ± 5 °C). The dry residue was reconstituted in 200 μL of water/methanol (85/15, v/v). After vortex mixing (15 s), the sample was passed through a Millex® GV-PVDF filter (0.22 μm) and transferred into an autosampler vial. An aliquot (2.5–10 μL) was injected onto the LC–MS/MS instrument.

2.5. Liquid chromatography

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler with temperature-controlled tray and column oven, type Autosampler Plus, from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil Gold column (50 mm × 2.1 mm i.d., dp: 1.9 μm) in combination with a guard column of the same type (10 mm × 2.1 mm i.d., dp: 3 μm), both from ThermoFisher Scientific. The temperatures of the column oven and autosampler tray were set a 45 and 5 °C respectively.

For multi-mycotoxin screening analysis, two gradient elution programs were performed depending on the MS/MS detection mode, i.e. positive or negative electrospray ionization (ESI). The flow rate was set at 300 μL/min. An overview of the gradient programs is given in Table 1.

For the toxicokinetic studies, separate LC–MS/MS methods were run for each type of mycotoxin and its metabolites, i.e. DON and DOM-1, T-2 and HT-2, ZON and metabolites, FB1, OTA and AFB1. Instrument parameters (mobile phase, gradient programs and MS/MS conditions) were optimized in such a way that optimal chromatographic separation and sensitivity could be reached for all analytes of interest. An overview of the HPLC conditions for the analysis of the individual mycotoxins (and their metabolites) is shown in Table 1.