Analysis of Deoxynivalenol and De-epoxy-deoxynivalenol in Animal Tissues by Liquid Chromatography after Clean-up with an Immunoaffinity Column

H. Valenta*, S. Dänicke, S. Döll

Institute of Animal Nutrition, Federal Agricultural Research Centre (FAL), Bundesallee 50, D-38116 Braunschweig, Germany
*, Corresponding Author

Abstract

A method for the determination of deoxynivalenol (DON) and its metabolite de-epoxy-deoxynivalenol (de-epoxy-DON) in blood serum, urine, faeces/digesta and bile fluid is described. Liquid samples (urine and bile after incubation with ß-glucuronidase) were extracted with ethyl acetate on a ChemElut column. Freeze dried faeces and digesta were extracted with a mixture of acetonitrile and water. For clean-up, serum and urine extracts could be directly applied to an immunoaffinity column (IAC). A pre-treatment prior to the IAC clean-up was necessary in the case of bile and faeces/digesta. DON and de-epoxy-DON were determined by high performance liquid chromatography (HPLC) with diode-array detection (DAD).
Because of the clean extracts, low detection limits in the range of 4 ng/ml (serum) to 20 ng/g (dried faeces/digesta) were achieved. The recovery of DON and de-epoxy-DON was in the range of 75-89% and 64-85%, respectively. DON was found in blood serum, urine, bile, digesta and faeces of pigs from several feeding studies with DON contaminated feed; de-epoxy-DON could be detected only in digesta, faeces and urine.

Keywords: mycotoxin, deoxynivalenol, de-epoxy-deoxynivalenol, method, animal tissues, HPLC

Introduction

Deoxynivalenol is one of the most important mycotoxins in middle Europe, because of its widespread occurrence in cereals and feedstuffs, and its toxic effects on livestock. Studies on the carry-over of DON showed that significant residues are not to be expected in edible tissues under practical feeding conditions (1). De-epoxy-DON is the only currently known metabolite and is far less toxic than DON.

For studies on pharmacokinetics and metabolism of DON in livestock, it was necessary to establish a sensitive method for the determination of DON and de-epoxy-DON in animal tissues, fluids and excreta. Gas chromatographic (GC) methods with mass spectrometry (MS) or electron capture (EC) detection were described in the literature (2). Recently, DON and de-epoxy-DON were analysed in the urine of swine by HPLC-MS (3). These methods require expensive instrumentation and/or derivatisation which are rather problematic. In this paper an HPLC-DAD method with an IAC clean-up is presented.

Materials and Methods

Toxin standards

The DON standard was obtained from Sigma. The de-epoxy-DON standard was a kindly gift from Dr. Meyer, Department of Animal Hygiene at the Technical University of Munich.

Incubation of urine and bile

To 2.0 ml urine (1.0 ml bile) 2.5 ml (3.5 ml) phosphate buffer, pH 6.8, was added and, in case of urine, pH was controlled and adjusted if necessary. After addition of 8,000 U (4,000 U) ß-glucuronidase (Sigma), the mixture was incubated overnight at room temperature.

Extraction

A portion of 1.5 ml serum, diluted with 1.0 ml phosphate buffer, and the incubated urine/bile samples, respectively, were applied on a disposable ChemElut column (Varian, 3 or 5 ml). The column was eluted with 35 ml (50 ml in case of 5 ml column) ethyl acetate and the eluate was evaporated to dryness.

Five g freeze-dried faeces/digesta were mixed with 24 ml water and left standing overnight. After addition of 126 ml acetonitrile, the mixture was shaken for 1 hour and filtrated.

Additional clean-up step for bile and faeces/digesta (4,5)

Bile residue was dissolved in 20 ml acetonitrile-water (84+16, v+v). This mixture and 30 ml of the extraction mixture of faeces/digesta, respectively, were defatted with equal amounts of petroleum ether and further cleaned up by shaking with 2 g (bile) or 3 g (faeces/digesta) of a mixture of charcoal, alumina and celite (7+5+3, w+w+w) for 10 minutes. After filtration, an aliquot of 10 ml (bile) and 15 ml (faeces/digesta), respectively, was evaporated to dryness with the addition of ethanol.

Clean up with IAC (4,5)

The extracts were dissolved in 1.5 - 4.0 ml of 5% PEG (polyethylene glycol)-solution, filtrated with membrane filter (0.45 µm) and 1 ml (according to an aliquot of 1 ml serum, 0.5 ml urine, 0.25 ml bile and 0.25 g faeces/digesta, respectively) was applied on an IAC (DONtest HPLC™, VICAM). The column was washed 3 times with 2 ml water and the toxins were eluted with four 1 ml portions of methanol. The eluate was evaporated to dryness and re-dissolved in 0,2 ml mobile phase B.

HPLC

Column:	Synergi Hydro RP, 4 µm, 250x4,6 mm (Phenomenex)
Guard column:	SecurityGuard C18, 5 µm, 4x3 mm (Phenomenex)
Mobile phase A:	acetonitrile
Mobile phase B:	acetonitrile - water for HPLC (13+87, v+v)
Gradient B:	0-15 min: 100%; 18-33 min: 5%; 36-53 min: 100%
Flow rate:	1 ml/min
Injection volume:	50 µl
Column temp.:	40 °C
Detector:	DAD, 190-300 nm, quantification at 218 nm

Results and Discussion

The clean up by IAC was performed according to a method for DON determination in feed from the VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten) (4) as described in (5). The immunoaffinity columns also proved suitable for analysis of the metabolite de-epoxy-DON. However, the columns were shown to be very sensitive to impurities in the extracts. Good results were obtained with extraction of liquid samples with ethyl acetate on a ChemElut column. Serum and urine extracts could be loaded on the IAC without further clean up. Bile and faeces/digesta extracts were purified according to the VDLUFA method (4,5) prior to IAC clean up and only equivalents of 0.25 g of a sample could be given to the column in order to obtain a good recovery.

Because of the IAC clean up, very clear chromatograms were obtained (see figure 1 and 2). Retention times of DON and de-epoxy-DON were approximately 6.5 and 11 minutes, respectively. A gradient was run after elution of de-epoxy-DON in order to clean the HPLC column.

Because only a limited amount of the de-epoxy-DON standard was available, the metabolite was quantified according to the calibration curve for DON. The detection limit for both toxins was approximately 4 ng/ml, 8 ng/ml, 16 ng/ml and 20 ng/g for serum, urine, bile and freeze-dried faeces/digesta, respectively. The recovery of DON/de-epoxy-DON was 89/85% in serum, 82/81% in urine, 83/64% in faeces and digesta and 75/70% in bile.

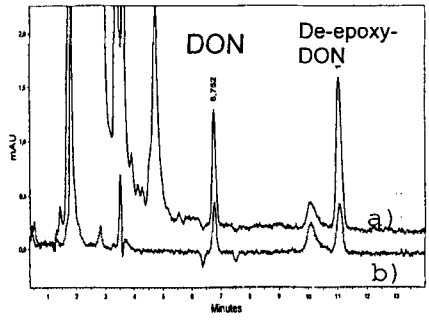

Figure 1:
Chromatograms of a) naturally contaminated digesta of swine with 145 ng/g DON and 274 ng/g de-epoxy-DON; b) standard solution with 79 ng/ml DON and 125 ng/ml de-epoxy-DON

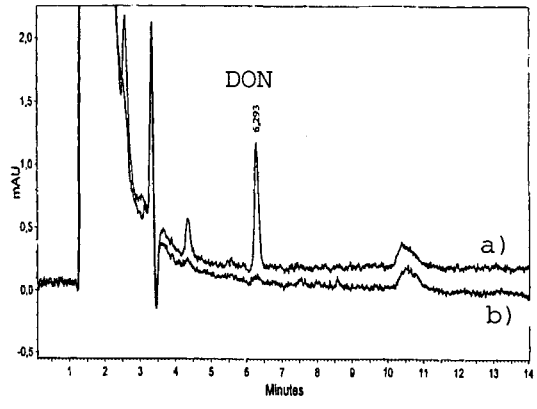

Figure 2:
Chromatograms of a) naturally contaminated serum of swine with 33 ng/ml DON; b) non contaminated serum of swine

Janes and Schuster described a similar method for determination of DON in blood, bile, urine and excrements of swine using IAC clean up and HPLC-UV detection, but they employed preparative HPLC for clean up prior to IAC and the analysis did not include de-epoxy-DON (6).

The present method was used for analysis of animal fluids and/or excreta from several feeding studies with pigs, bulls and ducks with DON contaminated feed. DON was found in blood serum, urine, bile, digesta and faeces of pigs, de-epoxy-DON could be detected only in digesta, faeces and urine (7,8). When naturally contaminated urine samples were analysed with incubation by ß-glucuronidase, higher concentrations of DON and de-epoxy-DON were measured than without incubation. This shows that in urine DON and its metabolite are present partly in conjugated form as glucuronides. No change in the concentration of both toxins in serum and faeces/digesta was measured after incubation. In the case of bile, the results were not clear.

For confirmation, extracts of some serum, urine and faeces samples were additionally analysed by HPLC/MS at the Institute of Nutrition at the Veterinary University in Vienna. In all samples, the presence of DON and de-epoxy-DON could be confirmed and concentrations measured by HPLC/DAD and HPLC/MS were of the same magnitude (7).

In conclusion, the method described is a useful alternative to GC/MS or LC/MS methods for determination of DON and de-epoxy-DON in animal tissues, fluids and excreta. The sensitive and quick analysis of serum and urine is especially appropriate for monitoring purposes.

References

1 Dänicke, S., Valenta, H., Ueberschär, K.-H. (2000) in Dänicke, S., Oldenburg, E. (eds.) Risikofaktoren für die Fusariumtoxinbildung in Futtermitteln und Vermeidungsstrategien bei der Futtermittelerzeugung und Fütterung. Landbauforschung Völkenrode, Sonderheft 216: 108-139.

2 Dahlem, A.M., Swanson, S.P., Cote, L.-M., Yoshizawa, T., Buck, W.B. (1986) Quantitation of deoxynivalenol and its metabolite in bovine urine and feces by gas chromatography with electron-capture detection. J. Chromatogr. 378: 226-231.

3 Razzazi, E., Böhm, J., Kettner, B., Hochsteiner, W., Kahlbacher, H. (2002) Metabolisierung von Deoxynivalenol beim Schwein: Bestimmung von DON und DOM-1 im Urin vom Schwein. Mycotoxin Res. 18A: 84-88.

4 VDLUFA-Methodenbuch III – 5. Ergänzung 200X, VDLUFA-Verlag, Darmstadt

5 Valenta, H., Dänicke, S., Wolff, J. (2002) Vergleich einer HPLC- und einer ELISA-Methode zur Bestimmung von Deoxynivalenol in Mühlenstäuben, Kleien und Getreide. 114. VDLUFA-Kongress, 16.-20.9.2002, Leipzig, VDLUFA-Schriftenreihe, in Druck.

6 Janes, W., Schuster, M. (2001) Determination of Deoxynivalenol in Blood, Bile, Urine and Excrement Samples from Swine Using Immunoaffinity Chromatography and LC-UV-Detection. Mycotoxin Res. 17: 88-95.

7 Dänicke, S., Goyarts, T., Valenta, H., Razzazi, E. and Böhm, J. (2003) On the effects of increasing deoxynivalenol (DON) concentrations in pig feed on growth performance and utilization of nutrients and on DON metabolism. *submitted*

8 Döll, S., Dänicke, S., Ueberschär, K.-H., Valenta, H., Schnurrbusch, U., Ganter, M., Klobasa, F., Flachowsky, G. (2003) Effects of graded levels of *Fusarium* toxin contaminated maize in diets for female weaned piglets. Arch, Anim. Nutr., in press.

Presented at the 25th Mykotoxin Workshop in Giessen, Germany, May 19-21, 2003