Bulletin UASVM, Veterinary Medicine 67(1)/2010
ISSN 1843-5270; Electronic ISSN 1843-5378

Analysis of Mycotoxins from Biological Fluids in Bovids

Violeta-Elena SIMION[1), M d lina GEORGESCU[2)], C t lin NEGREANU[2)],
Adriana AMFIM[1)], Elena MITRANESCU[3)]

[1)] Faculty of Veterinary Medicine, SPIRU HARET University, 47 Ma ina de Pâine Street, Bucharest, Romania, ushmv_simion.violeta@spiruharet.ro
[2)] Sanitary Veterinarian Direction and for the Safety of Foods, 16 E Ilioara Street, Bucharest, Romania, geomadalina@yahoo.com
[3)] Faculty of Veterinary Medicine, University of Agronomical Sciences and Veterinary Medicine Bucharest, 105 Splaiul Independentei, Romania, mitranescuelena@gmail.com

Abstract. The consumption of feeds contaminated with mycotoxins (particularly aflatoxins, ochratoxin A, zearalenone) by the animals causes various hepatic and renal; disorders, severe immunodeficiency, reproductive disorders and the presence of these mycotoxins or their metabolites in various fluids (such as aflatoxin M_1, metabolite of aflatoxin B_1). Various studies have determined the presence of aflatoxin M_1 and of zearalenone in milk, of aflatoxin B_1 in the hepatic tissue and in the serum, of ochratoxin A in the liver, of zearalenone in the bile; the presence of these mycotoxins in different organs depends on the selective place of metabolisation of these mycotoxins. The research aimed to determine the aflatoxins (AFB_1, AFB_2 and AFM_1), ochratoxin (OTA) and zearalenone (ZEA) in some biological fluids (milk, ruminal fluid, urine and bile) collected from bovids (cows and sheep), using ELISA and CSS techniques. The results have shown the presence of mycotoxin metabolites in all the analysed matrices, with values between 0 (below the detection limit) and 2.23 ppb for total AF in the ruminal fluid; 25.06 ppb for OTA in the ruminal fluid; 42 ppt for AFM_1 in milk and absence of mycotoxins AFB_1, AFB_2 and ZEA in the samples of urine and bile.

Keywords: mycotoxins, biological fluids, bovids

INTRODUCTION

Presently, the kinetics of transformations and the risk associated to the ingestion and absorption of aflatoxins is well established, both in animals and in humans. The main route for aflatoxin conjugation is glucuronoconjugation, the complex resulting from this process being eliminated through the bile. The liver is the target organism for aflatoxins. At this level, mycotoxin metabolisation takes place under the action of the microsomal enzymes. The reaction products are eliminated from the organism through the excretion products (faeces and urine) and through milk, unmodified and as metabolites.

In vitro studies have shown that over 90% of ZEA is degraded in the rumen by the ruminal microsymbionts, with the production of -zearalenol (Kiessling *et al.*, 1984), compound which is four times more estrogenic than the parental toxin, and with the production of -zearalenol, toxic compound for the endometrial cells (Tiemann *et al.*, 2003). The highest rate of transformation of ZEA in metabolism products was noticed for the first time at incubation (Moschini *et al.*, 1999), the protozoa playing an important role in this transformation (Kiessling *et al.*, 1984). ZEA is transformed, in lower amounts, in zeranol (-zearalanol) too, through a process of hydrogenation of the -zearalenol, the end product being detected in the rumen and in the bile too (Kennedy *et al.*, 1998). ZEA can also be metabolised in the rumen and liver, with the formation of zearalenol, compound which is not toxic for the

ruminal bacteria which degrade the mycotoxins. This supports the almost full metabolisation of this mycotoxin within the dairy cows' organism.

OTA is absorbed passively, unionized, from the digestive tract, particularly from the small intestine, at a pH of 7.04. After OTA gets into the organism, it binds to plasma albumins and starts to get metabolised quite rapidly, function of the animal species. OTA displays the highest affinity for the liver and kidneys. At the hepatic level, OTA is metabolised by hydroxylation into 4-hydroxyochratoxin A, metabolite eliminated via the renal pathway (Krogh, 1992; Ruhland *et al.*, 1996). Another important metabolite, OT- , forms in the digestive tract, where it is subsequently absorbed, and eliminated via the digestive and renal pathways. Other metabolites that are formed in the organism follow an entero-hepatic circuit after which they are eliminated through the faeces or urine (Curtui, 1998).

An important aspect of OTA metabolisation in the organism is its renal; absorption in the proximal tubules (2/3), in the distal tubules and in the collecting duct (1/3). This phenomenon takes place because of the disturbed pH homeostasis of the nephron cell walls, which affects the trans-epithelial acid-alkali transportation and determines urine acidification. Urine acidification favours OTA reabsorption, which makes the mycotoxin build up in the organism by decreasing its rate of removal (Gekle *and* Silbernagl, 1996).

The purpose of the research was to determine the aflatoxin, ochratoxin and zearalenone from some biological fluids collected from bovids (cows and sheep).

MATERIALS AND METHODS

The laboratory techniques used to determine these mycotoxins are different, from immunoenzyme screening to liquid chromatography or liquid chromatography coupled with mass spectrometry. The immunoenzymatic technique (ELISA) is usually used in Romania to detect the mycotoxins from biological fluids. The principle of this method is to couple the antigen with the antibody in the presence of the substrate coupled with an enzyme. A peculiarity for zearalenone detection is that when the mycotoxin is detected in a specific concentration, that sample will also be analysed for zeranolol presence because of the crossed reactions that may develop between these two substance with a very similar chemical structure.

Because the maximal admitted limits for mycotoxins in the biological fluids are regulated by European regulations only for milk (maxim 50ng/kg in fresh milk, according to EC 1881/2006), the mycotoxin (aflatoxins B_1, ochratoxin A, zearalenone) level must be monitored constantly in other biological matrices as well.

The purpose of the research was to analyse by ELISA aflatoxin M_1 (AFM_1), aflatoxin B_1 (AFB_1) and zearalenone (ZEA) in milk (48 samples), urine (3 samples), ruminal fluid (10 samples) and bile (11 samples) collected from bovids in various counties and localities throughout Romania.

The biological fluids were collected in sterile containers, observing the hygiene rules and the acting regulations (SR RN ISO 707/2002 for milk). The samples were transported under refrigeration and the mycotoxicological assay was performed within four hours from sampling.

RESULTS AND DISCUSSIONS

For total AF, AFB_1 and OTA determination in the ruminal fluid, samples were collected from 10 cows whose forages were documented for the presence of mycotoxins. Table 1 shows the results of the analysis.

Tab. 1.

Total AF, AFB_1 and OTA levels in the ruminal fluid

No.	Sample	total AF ppb		AFB_1 ppb		OTA ppb	
1	ruminal fluid	2.17					
2	ruminal fluid	2.23					
3	ruminal fluid	2.21					
4	ruminal fluid			0.98			
5	ruminal fluid			0.99			
6	ruminal fluid			0.98			
7	ruminal fluid (diluted with distilled water)					25.06	
8	ruminal fluid (non diluted)					23.91	
9	ruminal fluid (diluted with buffer solution)					34.61*	
10	ruminal fluid (non diluted)					24.98	
Limits of variation		2.17-2.23		0.98-0.99		23.91-25.06	
Positive analyses. no./%		3	100.0	3	100.0	4	100.0
Total analyses. no./%		3	100.0	3	100.0	4	100.0

* result not relevant

The analysis of Table 1 results shows that:

- total AF were determined in 100% cases (3 samples), with variation limits between 2.1 – 2.2 ppb in the three analysed samples;

- AFB_1 was determined in 100% cases (3 samples), with variation limits between 0.98 – 0.99 ppb in the three analysed samples.

The presence of AF and its metabolites in the ruminal fluid was reported in many scientific papers. Contradictory results were reported in relation with AFB_1 biodegradation in the rumen. Some researchers observed a significant decrease of the initial AFB_1 concentration when it was incubated with ruminal fluid, while others didn't report this phenomenon (Kiessling *et al.*, 1984). Generally, the ruminal degradability of AFB_1 is minor and the toxicity of its metabolites is similar with that of the parent molecule.

Auerbach *et al.* (1998b) notices that the addition of 9.5 ng AFB_1/ml ruminal fluid didn't alter *in vitro* alfalfa digestion and didn't influence the VFA production, while in another study, the addition of 1 µg AFB_1/ml decreased the rumen capacity to produce volatile fatty acids. *In vivo* studies have also reported the presence of AFM_1 in the ruminal content, which takes us to the conclusion that the AFM_1 produced in the liver may get back to the rumen via the rumen-hepatic route (Auerbach *et al.*, 1998a).

AF affects the ruminal function by reducing the ruminal motility, the capacity of fibre digestion, the capacity of VFA production and the proteolysis capacity (Cook *et al.*, 1986). The ruminal fluid and particularly the bacterial population from the rumen of the cows and sheep don't have the capacity to convert AF into other metabolites, except for AFM_1 metabolite which was detected in large amounts in the milk.

OTA was determined in 100% cases (4 samples), with variation limits between 23.91 (non diluted sample) and 25.06 (the sample diluted with distilled water) ppb in three of the four analysed samples; the value of 34.61 ppb in one sample (sample diluted with buffer

solution) was not considered relevant, possibly because of the influence of the solution on the composition of the fluid.

In vitro study that after the ruminal fluid was incubated, 60% of the ingested OTA was degraded to OTA- . Protozoa are judged to play a major role in OTA degradation to OTA- . (Diaz, 2005). Müller *et al*., (1995) evaluated by *in vitro* studies that the adult cows are able to degrade 33 to 72 mg OTA/day, and the sheep, 3 to 7 mg OTA/day.

The food composition influences the structure of the ruminal microsymbionts and implicitly the capacity to degrade OTA. When the mycotoxin is found in large amounts in the diet, the microbial capacity of detoxification is low and ochratoxicosis symptoms appear. OTA metabolism on the rumen is much lower when a higher amount of concentrated feeds is ingested than when the diet consists mainly of fibrous feeds (Xiao *et al.,* 1991a, 1991b).

The research also analysed 48 milk samples collected from bovids reared in 18 counties and localities (Bac u, Boto ani, Br ila, Bucure ti, Buz u, C l ra i, Constan a, Covasna, Dâmbovi a, Giurgiu, Ialomi a, Ilfov, Olt, Pantelimon, Suceava, Vaslui and Vrancea). The samples were analysed by ELISA for AFM_1 (with LOD 5 ppt). The results show the presence of the mycotoxin in 16.66% of the cases (8 samples), with limits of variation between 0 - 42 ppt, while in 41.66% of the cases (40 samples) AFM_1 was not detected by analysis.

Other research has shown that after the oral administration of AFB_1, the metabolites are rapidly found in the urine and milk, while small amounts can be detected in the faeces,, which supports the fast absorption of AFB_1 in the digestive tractul and its hepatic metabolism.

In the dairy cows, of the total 4.52% AF detected in the organism, 1.55% was traced in urine, 2.79% in the faeces and 0.18% in the milk, as AFM_1 which means 0.35% of the given dose. In the lactating ewes, of the 8.1% of the ingested amount, 6.4% was detected in the urine, 1.6% in the faeces, and 0.1% in the milk. After 6 days from administration, AF was no longer detected in the milk, after 8 days in the urine and after 9 days in the faeces (250).

The presence of mycotoxins AFB_1 (ELISA, LOD 0.5 ppb), AF B2 (CSS 1 ppb) and ZEA (ELISA, LOD 0.05 ppb) in urine was ascertained on a sample of urine collected from a bovid from Br ila County, which was analysed by ELISA for ZEA, on a sample of urine from a bovid from Boto ani County, which was analysed by ELISA for AFB_1 and by CSS for AFB2, and on a sample of urine from a bovid from Buz u County, which was analysed by ELISA for ZEA. The results were negative for all analyses, none of these mycotoxins being detected using the specified methods.

The studies of distribution and metabolism of C^{14} marked AFB_1 in the organism of animals, have shown both the elimination of significant amounts of the mycotoxin during the first 24 hours and the build up of residual amounts in various organs (muscle, stomach, liver, heart, etc.), build up which depends on the amount of ingested mycotoxin. The lipophilic mycotoxins with a molecular mass lower than that of AFB_1 are absorbed in the digestive tract through passive diffusion.

After the administration of a total dose of 300 mg AF (0.5 mg AF/kg) we evaluated the metabolism and level of AF distribution in the body of the dairy cows and lactating ewes. AF identification in the excretion products using CSS revealed that 85% of the total ingested AF was excreted in the cows and 90% in the sheep within 48 hours from ingestion.

AFB_1 metabolism runs in two stages. The first stage comprises mainly the transformation of AFB_1 into AFB_1 – 8,9-epoxide, while the second stage consist in the conjugation of the epoxide with glutation, a major pathways of detoxification, followed by the formation of the glucuronoconjugated and sulphurconjugated compounds (Guerre *et al*., 1996). The biotransformations from the first stage occur mainly by substrate oxidation and

less by substrate reduction. For AF and for a larger number of xenobiotics, the main enzymatic system involved in these transformations is that of cytochrome P450. This biotransformation route runs mainly in the hepatocytes, but it also takes place in a number of different cell structures (Guerre *et al*., 1996).

We have also conducted mycotoxicological analyses on the incidence and amount of mycotoxins in the bile. Thus, we analysed by ELISA for ZEA (LOD 0.275 ppb), 10 samples of bile collected from bovids and a bile sample of bovid for AFB_1 (ELISA, LOD 0.5 ppb) and AF B2 (CSS, 1 ppb). The results were negative for all analyses, none of these mycotoxins being detected using the specified methods.

About 90% of the blood AFB_1 is in the plasma, the fraction associated to the proteins being particularly bound to albumins. AF are oxidized in the liver with formation of very reactive molecules, able to bind nucleic acids or functional proteins. This hepatic bioactivation has an outstanding importance for animal health because of the reactive metabolites that form in situ, at tissue level. AF metabolisation in the liver is done under the action of the microsomal enzymes, the most active one being P450.

An experimental study which administered ZEA for 7 weeks (385-1925 ppb ZEA), didn't show any changes in the milk yield or the presence of ZEA residues in the milk, urine, serum or tissues. and -zearalenol are derivatives of ZEA which are removed from the animal organism through faeces and urine, and less (amounts in the range of μg/l) through the milk. Of them, -zearalenol is considered the metabolite with the strongest estrogenic activity. Another ZEA metabolite, zeranol, has a strongly anabolysing effect. Both ZEA and its metabolites are reabsorbed at the intestinal level, going through the entero-hepatic cycle (Chinchila *et al*., 1998; Kennedy *et al.*, 1995; Kennedy *et al*., 1998).

CONCLUSIONS

The analysis of the ruminal fluid samples by ELISA showed that the total AF were determined in 100% cases (3 samples), with variation limits between 2.1 – 2.2 ppb in the three analysed samples; AFB_1 was determined in 100% cases (3 samples), with variation limits between 0.98 – 0.99 ppb in the three analysed samples; - OTA was determined in 100% cases (4 samples), with variation limits between 23.91 (non diluted sample) – 25.06 (the sample diluted with distilled water).

The analysis of the 48 milk samples collected from bovids reared in 18 counties and localities by ELISA for AFM_1 showed the presence of the mycotoxin in 16.66% of the cases (8 samples), with limits of variation between 0 - 42 ppt, while in 41.66% of the cases (40 samples) AFM_1 was not detected by analysis.

The analysis by ELISA and CSS for ZEA and AFB_1 of 10 samples of bile collected from bovids, the results were negative for all analyses, none of these mycotoxins being detected using the specified methods.

The presence of metabolites from aflatoxins, ochratoxin and zearalenone in various biological fluids collected from bovids and the absence of maximal admitted loads requires a continuous monitoring to assess their concentration in various biological matrices.

REFERENCES

1. Auerbach, H., E.Oldenburg, F. Weissbach (1998b). Incidence of Penicillium roqueforti and roquefortine C in silages. J. Sci. Food Agr. 76:565-572.

2. Auerbach H., R.F.M. Maas, H.J.M. Op Den Camp, A. Pol, J. Fink-Gremmels (1998a). Biodegradation of aflatoxin B1 by bovine rumen microorganisms in vitro and its effects on rumen fermentation, Revue Méd. Vét. 149, 573.
3. Chichila T.M., Silvestre D., Covey T.R., Henion J.D. (1988). Distribution of zeranol in bovine tissues determined by selected ion monitoring capillary gas chromatography/mass spectrometry, J. Anal. Toxicol., Nov-Dec; 12(6):310-8.
4. Cook W.O., J.L. Richard, G.D. Osweiler, D.W. Trampel (1986). Clinical and pathological chamges in acute bovine aflatoxicoses: Rumen motility and tissue and fluid concentrations of aflatoxin B1 and M1, Am. J. Vet. Res., 47:1817.
5. Curtui V. Ghe., 1998 – Implica iile poluan ilor micotici din nutre uri asupra s n t ii i produc iei puilor broiler, Tez de doctorat, FMV, USAMV a Banatului Timi oara.
6. Diaz D.E. (2005). The mycotoxin blue book, Nottingham University Press.
7. Gekle M., S. Silbernagl (1996). Renal toxicodynamics of ochratoxin A: a pathophysiological approach, Kidney Blood Press Res., 19(5):225-35.
8. Guerre P., P. Galtier, V. Burgat (1996). Le métabolisme : un factor de susceptibilité à la toxicité des aflatoxines, Revue Méd. Vét., 147, 12 : 879-92.
9. Kiessling, K., H. Pettersson, K. Sandholm, M. Olsen (1984). Metabolism of aflatoxin, ochratoxin, zearalenone and three trichothecenes by intact rumen fluid, rumen protozoa and rumen bacteria. Appl. Environ. Microbiol. 47:1070.
10. Kennedy D.G., Hewitt S.A., McEvoy J.D., Currie J.W., Cannavan A., Blanchflower W.J., Elliot C.T. (1998). Zeranol is formed from Fusarium sp. toxins in cattle in vivo, Food Addit. Contam. 15 (1998) 393-400.
11. Kennedy D.G., S.A. Hewitt, J.D.G. McEvoy, J.W. Currie, A. Cannavan, W.J. Blanchflower, C.T. Elliott (1995). Possible naturally occurring zeranol in bovine bile in Northern Ireland, J. Vet. Med. Series B, 42:509-12.
12. Krogh P. (1992). Role of ochratoxin in disease causation, Food Chem. Toxicol., 30(3):213-24.
13. Moschini M., F. Masoero, T. Bertuzzi (1999). In vitro rumen metabolism of zearalenone. In: Recent Progress in Animal Production Science, Piacenza, Italy, pp.363-5.
14. Müller H.M., K. Müller, H. Steingass (1995). Einfluss der fütterung und anderer faktoren auf den umsatz von ochratoxin A in pansenflüssigkeit in vitro und in vivo, Ph.D. Thesis, Hohenheim University, Stuttgart, Germany.
15. Ruhland M., G. Engelhardt, W. Schafer, P.R. Wallnofer (1996). Transformation of the mycotoxin ochratoxin A in plants: 1. Isolation and identification of metabolites formed in cell suspension cultures of wheat and maize, Nat. Toxins, 4(6):254-60.
16. Tiemann U., T. Viergutz, L. Jonas, F. Schneider (2003). Influence of the mycotoxins - and - zearalenol and deoxynivalenol on the cell cycle of cultured porcine endometrial cells, Reprod. Toxicol. 5506:1-10.
17. Xiao H., R.R. Marquardt, A.A. Frohlich, G.D. Phillips, T.G. Vitti (1991a, 1991b). Effect of a hay and a grain diet on the bioavailability of ochratoxin A in the rumen of sheep, J. Anim. Sci., 69:3715-23; 69:3706-14.