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Aflatoxin B_1 in wheat bran containing premix

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ABSTRACT

Premixes for medicated feedstuffs are considered to be veterinary medicinal products (VMPs) prepared in advance with a view to the subsequent manufacture of medicated feedstuffs. Medicated feedstuffs should be prepared only from market authorized premixes and premixes for medicated feedstuffs can be used only as prescribed medicines. Apart from active substances, premixes contain carriers which have a role in the homogenization of medicated feedstuffs. Wheat bran as a carrier may be a source of different, potentially harmful or toxic substances e.g. aflatoxin. In our study, 15 different batches of premixes with a wheat bran component were tested for aflatoxin B_1 (AFB_1) by means of an enzyme-linked immunosorbent assay (ELISA). The samples tested showed contamination with AFB_1 ranging from 1.5 to 35 ng/g (mean = 18.79 ng/g). However, no correlation between AFB_1 levels and the month of collection or season of production was observed. Considering the composition of the premix, the only possible source of contamination with AFB_1 was wheat bran. Contamination probably occurred before the production of the premix, probably in the field or during storage. Concentrations of active substances and citric acid (a neutralising agent for mycotoxins) were in accordance with the producer's declaration. Our discovery of AFB_1 in the tested premixes was in contravention of the definition and primary role of veterinary medicinal products. In this study, we would like to highlight the need for monitoring raw materials of biological origin for premix production. Even though carriers are not pharmacologically active substances, an efficient method for controlling potential contaminants such as mycotoxins or their toxic components should be proposed, with the aim of protecting animal health, consumers, employees and end-users in the production of veterinary medicinal products.

Key words: premix, aflatoxin B_1, wheat bran

Introduction

According to national and European legislation, premixes for medicated feedstuffs (any veterinary medicinal product prepared in advance with a view to the subsequent

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manufacture of medicated feedstuffs) must be authorised (Directive 2001/82/EC). The majority of these veterinary medicinal products (VMPs) are used to treat food-producing animals and the quality control of such products is obligatory in Croatia.

Apart from active substances, premixes contain carriers which allow the better homogenisation of medicated feedstuffs. An ideal carrier should be pharmaco-toxicologically inactive, chemically and physically inert, compatible with other ingredients, colourless and tasteless, with high fluidity or flowability and high compressibility. It should be readily available and inexpensive, characterised in detail by suppliers (i.e. in a master file), easy to store, lot-to-lot reproducible and performance consistent, accompanied by a specific dosage form (PIFFERI et al., 1999). Commercially available authorised premixes in the Republic of Croatia contain different active substances (antibiotics, sulphonamides, coccidiostatics) and different carriers. Some of them contain carriers of natural (plant) origin. The most commonly used formulations of premixes include a grain carrier or mineral compound.

The control of decomposition, specific impurities, chemical substances used during treatment, with residual limits and methods of sterilisation or decontamination of excipients, is necessary for the quality control of the VMP. The density, particle size and geometry of the premix should be similar to the feed with which it is intended to be mixed (VERMEULEN et al., 2002). Control of the purity and standard quality of carriers is also necessary. However, the quality of carriers may vary and depends on origins or storage conditions (FAZEKAS et al., 2005; SANTIN, 2005).

Some authorised premixes contain wheat bran as a carrier that may be contaminated by different substances (pesticides, herbicides, mycotoxins etc.). The most frequent contaminant of feed or raw materials, including wheat bran, is AFB_1. Aflatoxins are secondary metabolites produced by certain strains of the fungus *Aspergillus*. Well-known strains are *Aspergillus flavus*, producing AFB_1 and AFB_2, and *Aspergillus parasiticus* producing AFB_1, AFB_2, AFG_1 and AFG_2 (DIENER and DAVIS, 1966). In addition, *Aspergillus nominus* and *Aspergillus niger* have also been described as producers of aflatoxins (HORN and WICKLOW, 1983). Contamination by aflatoxins is characteristic of humid tropical areas and is more frequently associated with storage under warm conditions than field culture contamination. 68% of analysed feed samples produced in Southern Europe and 75% of feed samples from EU countries showed contamination by more than one mycotoxin (GRIESSLER et al., 2010; MONBALIU et al., 2010).

The toxicity of mycotoxins depends on the quantity, route of administration, exposure, animal species and category, genetics, age, rearing and interaction between mycotoxins (WYATT, 2005). The most prevalent and biologically active in animals and humans is aflatoxin (WILLIAMS et al., 2004; WILLIAMS et al., 2009). AFB_1 belongs to the strongest naturally occurring liver carcinogens and is one of the most important aflatoxins, in terms of occurrence and toxicity (WOGAN, 1999). Many aspects of AFB_1 toxicity both in human and animals have been investigated (KUILMAN et al., 2000; NURRED and RILEY, 2001;

OSWALD et al., 2005). Usually, animals are exposed to AFB_1 in their diets and it can cause acute, chronic and subchronic mycotoxicoses.

The aim of this study is to show that contamination of plant origin carriers with AFB_1 is possible. Our intention is to indicate the need for better quality control of raw materials or the replacement of non-standardised with standardised carriers, to protect animal and human health. We also want to point out the role of citric acid and the need for further study of its neutralising effect on AFB_1 in animals.

Materials and methods

All the analyses were performed in the accredited (ISO 17025) laboratories (Laboratory for Quality Control of Veterinary Medicines and Laboratory for Analytical Chemistry) of the Croatian Veterinary Institute.

Samples. Fifteen different batches of premixes with a wheat bran component, authorised primarily for the treatment of respiratory and digestive infections in young pigs, cattle and chickens, were tested for AFB_1. The premixes were homogenously mixed in feed for pigs (0.5%) and feed for poultry (0.15-0.5%).

Samples for analysis were obtained through the routine quality control procedure that is mandatory in Croatia. The premixes were produced during a period of 28 months (September 2007 to December 2009) and stored under recommended conditions in aluminium bags until the moment of analysis. Three samples from the same batch were mixed and a sample of the mixture taken for analysis.

Table 1. Composition of premix and proportion of wheat bran

No. of batches	Active ingredients	Wheat bran (%)
15	oxytetracycline as oxytetracycline hydrochloride, neomycin as neomycin sulphate, sulphadimidine, sulphaguanidine, citric acid	44.2

Sample preparations and analyses. The identification and assay of active substances colour, homogeneity and loss of drying was assessed according to the producer's quality control procedure. The method for determining the presence and level of citric acid has been described in European Pharmacopoiea 6th Ed. (01/2008:0455).

Representative samples were thoroughly mixed prior to extraction. Five grams of each sample was weighed, and then 25 mL of 70% methanol was added and the mixture shaken vigorously for three minutes. The extract was filtrated and 1 mL of the obtained filtrate was diluted with 1 mL of distilled water. Aliquots (50 μL) of dilutions were assayed in duplicate by ELISA. Competitive ELISA was performed as described in the instructions provided by the kit manufacturer.

A Ridascreen AFB_1 kit for ELISA was provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with capture antibodies, AFB_1 standard solutions (0, 1, 5, 10, 20 and 50 ng/mL), peroxidase-conjugated AFB_1, anti-aflatoxin antibody, substrate/chromogen, stop solution (1 N sulphuric acid) and washing buffer (contains 0.05% Tween 20). AFB_1 from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) was used for the fortification of samples. All other chemicals used in the analysis were of analytical grade. ELISA was performed by using ChemWell 2910 (Awareness Technology, USA).

Results

Validation. The estimated limit of detection (LOD) and limit of quantification (LOQ), which was calculated from the mean value of ten determinations of blank samples of different cereals, plus three- and ten-fold standard deviations, were 1.0 and 1.6 ng/g, respectively. The results of method recovery (n = 18) and repeatability (n = 54) are presented in Table 2.

Table 2. Results of method validation obtained with blank samples of different cereals spiked with AFB_1 at levels of 2, 10 and 20 ng/g

Validation parameter	No. of replicates	Spiked concentration (ng/g)	Determined concentration (ng/g)	Mean recovery R (%)	Coefficient of variation CV (%)
Recovery	6	2	1.85	92.5	7.7
	6	10	10.15	101.5	9.3
	6	20	19.34	96.7	10.2
Repeatability	18	2	1.73	86.5	9.7
	18	10	10.46	104.6	10.6
	18	20	18.86	94.3	11.5

Method validation resulted in mean recoveries ranging from 92.5% to 101.5% and repeatability ranging from 86.5% to 104.6%, with coefficients of variations (CV) of 7.7%-10.2% and 9.7%-11.5% respectively.

Qualitative analyses and occurrence of AFB_1. The quantity and identification of active substances was in accordance with the producer's specification (certificate of quality) as well as other requirements in all of the samples assessed. The quantity of AFB_1 in the premixes analysed is shown in Table 3.

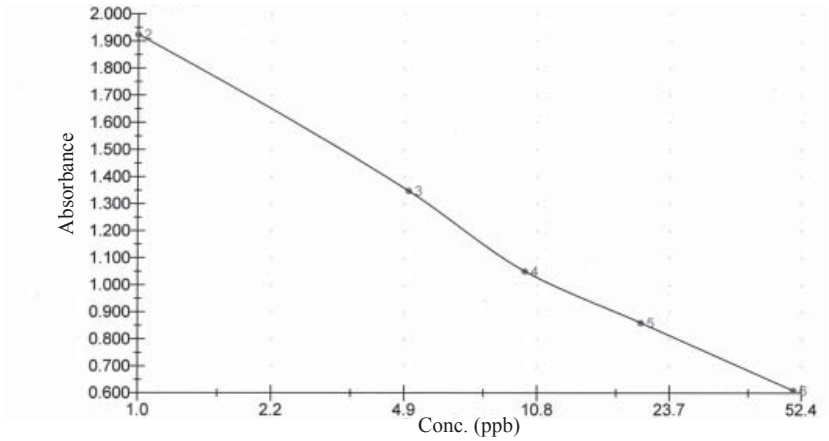

Fig. 1. ELISA standard curve for aflatoxin B1

Table 3. Concentration of AFB_1 (ng/g) in analysed premixes and descriptive statistics of results.

Month/Year	AFB_1 (ng/g)	Citric acid (g/kg)
Sept./2007	1.5	95.4
June/2008	14.1	94.6
Nov./2008	18.3	91.9
Dec./2008	11.2	94.1
Feb./2009	8.2	103.7
March/2009	19.5	97.5
May/2009	15	95.2
June/2009	30.5	96.1
Aug./2009	22	94.5
Aug./2009	16.9	98.6
Aug./2009	22	98.0
Sept./2009	27.6	97.6
Oct./2009	32.3	94.9
Oct./2009	7.8	98.8
Dec./2009	35	96.9
Mean	18.79	96.52
Standard error	2.49	0.71
Median	18.3	96.1
Standard deviation	9.67	2.76

Concentrations of citric acid were in accordance with the producer's specification in all samples analysed (90.0-110.0 g/kg).

Discussion

The safety of veterinary medicinal products is a very important part of preclinical investigation, evaluation of documentation, and the post marketing authorisation process. Animals, the environment, and people who apply or are exposed to veterinary medicinal products, should be protected from any harmful effects (WOODWARD, 2008). Apart from active substances, the safety and toxicity of excipients play an important role in medicinal products (BALDRICK, 2000; de JONG, 1999; OSTERBERG and SEE, 2003). Excipients account for most of the volume of veterinary medicinal products and must also correspond to safety requirements (PIFFERI and RESTANI, 2003). The carriers in premixes are edible materials to which drugs have been added to facilitate uniform incorporation in feedstuffs. However, the criteria which have been proposed for the quality control of different kinds of carriers in veterinary medicinal products are unsatisfactory.

The origin of raw materials and well established use of veterinary medicines are not a guarantee of quality as required by the pharmaceutical industry (PIFFERI and RESTANI, 2003). After considering our findings, we agree with LONG and CRANE (2003) that the harmonisation of veterinary medicine regulation is an ongoing process and requirements for carriers should be improved continually.

There is limited information on veterinary medicines that contain wheat bran. Wheat bran can be a source of *Aspergillus* spp. and mycotoxins. The contamination of wheat bran with aflatoxin is possible in the field, during storage, or in the manufacturing process. Requirements for wheat bran quality fall under legislation for feed, although wheat bran can also be an ingredient of veterinary medicinal products. There is no national legislation regarding the quantity of AFB_1 in wheat bran as a carrier in veterinary medicinal products. However, the maximum level of AFB_1 is 0.02 ng/g in complete feed for cattle, goats and sheep, except milk producing animals (0.005 ng/g) and (0.01 ng/g) in feedstuff for calves and lambs. The maximum content of AFB_1 in feedstuff for pigs and poultry is 0.02 ng/g, except for young animals (0.01 ng/g). The maximum concentration of aflatoxin in food in Croatia, as in the majority of European countries, must not exceed 5 ng/g.

ELZUPIR et al. (2003) reported that 63% of wheat bran samples analysed were positive for aflatoxin (average 31.19 µg/kg) in Sudan and BARA (2008) found that the concentration of aflatoxin in bran varied from 0.0 to 0.27 ppm (an average of 0.09 ppm). Rice and rice hulls also presented a risk of aflatoxin contamination (CASTELLS et al., 2007).

The harmful effects of aflatoxin on animal health have been described in many studies. Aflatoxin can cause stunted growth in lambs and provoke the selective impairment of drug-metabolizing enzymes in pig livers (FERNANDEZ et al., 1997; FERNANDEZ et al., 2000; GOWDA et al., 2007; MEISSONNIER et al., 2007). The immunosuppressant effect of many mycotoxins occurs at much lower levels of intake than the toxins affecting production parameters, such as growth rate or egg production (BONDY and PESTKA, 2000). Pigs are

very susceptible to aflatoxin, especially young pigs, and AFB_1 dietary exposure decreases cell mediated immunity, while inducing an inflammatory response (MEISSONNIER et al., 2008). Toxin ingestion can also reduce the effectiveness of vaccines (MEISSONNIER et al., 2006; MEISSONNIER et al., 2008). Even though piglets are most susceptible to AFB_1, 50 µg/kg of feed does not have a toxic effect (DILKIN et al., 2003). However, the same authors reported the negative effects on feed consumption and feed conversion in pigs that received a combination of AFB_1 (50 µg/kg) and 30 mg fumonisin B_1 (FB_1) over a period of four weeks.

Mixing AFB_1 and FB_1 was assessed by THEUMER et al. (2003) too, and it was found that such mixtures of mycotoxins caused severe changes to the immunological system in rats. BARA (2008) reported the negative effect of aflatoxin in feedstuffs on poultry production and OGUZ et al. (2002) observed that hepatic enzyme activities (AST and ALT) significantly increased in one-day old broiler chickens, after being fed (for 42 days) a diet containing 50 and 100 ng/g of AFB_1.

The immunosuppressive effects of AFB_1 in fish have also been recorded (SAHOO and MUKHERJEE, 2001). EL-SAYED and KHALIL (2009) found that sea bass were highly sensitive to AFB_1 and the consumption of sea bass reared on an AFB_1 contaminated diet could have a negative impact on human health.

Five out of twenty authorised premixes for commercial use in Croatia contain wheat bran in different proportions (from 442 to 980 g/kg). During routine control, the non-appropriate appearance of some batches of premixes was observed and we decided to analyse premix samples for the presence of AFB_1, even though the control of mycotoxins and side contaminants in premixes was not covered in the producers' documentation. In our study, the premixes tested showed a concentration of AFB_1 of between 1.5 and 35 ng/g (mean = 18.79, standard error = 2.49). Considering the composition of the premixes, the only possible source of AFB_1 contamination was wheat bran. Validation results showed that the method of sample preparation and determination of AFB_1 using ELISA as a screening method for quantitative determination was efficient and comparable with the manufacturer's data. Based on the composition of VMP, we assumed that the source of aflatoxins was wheat bran.

However, there was no correlation between AFB_1 levels and the date of the premix production. Since the premixes were packed in different packaging materials (aluminium bags, paper bags and PVC bags) we presume the packaging had no influence on the AFB_1 concentration. Contamination probably occurred before premix production, probably in the field or during the storage of wheat bran.

The concentration of AFB_1 found in premix decreases in the final composition of medicated feedstuffs, since 0.5 kg of premix is added to 100 kg of feed. Therefore the final concentration of aflatoxin is lower than 5 ng/g, i.e. within the permitted level of

concentration for young animals (0.005 ng/g). However, the presence of a certain quantity of aflatoxins or other mycotoxins may also be found in feed and the combined concentration is in that case higher. The toxic effect of AFB_1 depends not only on its total quantity, but on the animal's length of exposure, species, physiological status, age, sex etc. (HENGSTLER et al., 1999). Low levels of mycotoxins favour the development of infectious diseases through impairment of the humoral and cellular immune response and native mechanisms of resistance. In general, AFB_1 should not be present in veterinary medicinal products at any level of concentration.

In our investigation, we recorded that premixes also contain citric acid (100 g/kg). The results show small divergences within the specification indicated for standardised production. In the instructions for use, the manufacturer states that citric acid is used as an acidifier to reduce *E. coli* and anaerobic bacteria in the digestive system, not as a detoxification agent. However, citric acid has been proved to have a detoxifying effect regarding AFB_1 in broilers (GOWDA et al., 2004). Citric acid in 0.5-1% concentration is a very effective anti-fungal compound and it reduces (91-94%) aflatoxin biosynthesis by *A. parasiticus.* 1 N aqueous citric acid significantly prevented negative effects on body weight gain and transaminase activity in young ducks given feed contaminated with 110 ng/g AFB_1 (MENDEZ-ALBORES et al., 2007). Sorghum contaminated with AFB_1 can also be detoxified by citric aqueous acid during the extrusion process, without affecting the physicochemical, functional and textural properties of the extrudates (MENDEZ-ALBORES et al., 2009). Hot air ovens and sun drying reduces aflatoxin content in feed, and some chemical and herbal compounds have an anti-fungal effect, so they can also reduce aflatoxin production (GOWDA et al., 2007). The inhibition of *Aspergilus parasiticus* growth and aflatoxin production has been achieved through the use of propionic acid, ammonia, copper sulphate, benzoic acid urea, citric acid and sodium propionate acid (GOWDA et al., 2004). High dietary vitamin C enhanced protection against *Aeromonas hydrophila* infection in both healthy and AFB_1 immunocompromised fish (SAHOO and MUKHERJEE, 2001). Usually, chemical detoxification is not completely safe, due to toxic residues. Treatment with sodium bisulphite and ammoniation is successful in the inactivation of aflatoxin in peanut meals, maize and cottonseed while fungal laccase enzymes are efficacious in the degradation of AFB_1 (ALBERTS et al., 2009; MENDEZ-ALBORES et al., 2007). In our study, the concentration of citric acid was within the declared range. In accordance with previous studies carried out by many authors, we can only assume that small quantities of AFB_1 in the premixes analysed may be neutralised by citric acid in the proposed concentration in the digestive systems of animals. However, we consider that AFB_1 should not even be present in premixes and we consider that the control of raw materials for premix production, both active substances and excipients, should be clearly prescribed, according to good manufacturing practice. Non-standardised carriers in the veterinary pharmaceutical industry may be the sources of potentially harmful or toxic

substances. This contravenes the primary definition of veterinary medicinal products, in terms of their safety. Even though the carrier may not be a pharmacologically active substance, measures are necessary to minimise the undesired effects caused by some extraneous substances.

In conclusion, contamination with AFB_1 can be prevented by routine control of the plant material components of premixes. Citric acid, which was found to be a component of the samples investigated, can help to neutralise AFB_1 in premixes.

Our study was based on finished, authorised products, however, for further investigation, studies should be extended to raw materials and clinical trials (investigation of the toxic effects of low concentrations of AFB_1 as well as the neutralisation of AFB_1 with citric acid in animals). Therefore, we consider that this study highlights the absence of control regarding the raw materials for premix production, especially those of biological origin.

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SAŽETAK

Premiksi za izradu ljekovite hrane za životinje svrstani su u veterinarsko-medicinske proizvode (VMP). Ljekovita hrana mora biti propisana od veterinara i može se pripremati samo od premiksa koji imaju odobrenje za stavljanje u promet. Osim aktivnih tvari, premiksi sadržavaju i nosače koji omogućavaju njihovo bolje umješavanje u hranu za životinje. Pšenične posije kao nosač aktivnih tvari mogu biti potencijalni izvor različitih opasnih ili toksičnih tvari kao što je npr. aflatoksin. U našem istraživanju imunoenzimnim testom (ELISA) analizirano je 15 različitih proizvodnih serija premiksa za izradu ljekovite hrane za životinje na aflatoksin B_1 (AFB_1). Testirani uzorci imali su od 1,5 do 35 ng/g (M = 19,79 ng/g) AFB_1. Međutim u našem istraživanju nije ustanovljena povezanost količine AFB_1 s godišnjim dobom proizvodnje. S obzirom na sastav premiksa vjerojatno su pšenične posije izvor kontaminacije, a kontaminacija je nastala vjerojatno prije proizvodnje premiksa, odnosno prije ili tijekom skladištenja pšeničnih posija. Količina aktivnih tvari i limunske kiseline koja je potencijalni neutralizator AFB_1 odgovarala je deklaraciji proizvođača. Nalaz potencijalno štetnih tvari (AFB_1) u VMP-u u suprotnosti je s ulogom veterinarskog lijeka te se ovim istraživanjem željelo upozoriti na izostanak kontrole sirovina biološkog podrijetla u proizvodnji premiksa. Iako nosači nisu farmakološki aktivne tvari, kontrola kontaminanata kao što su mikotoksini ili druge toksične tvari trebala bi se povećati u cilju zaštite zdravlja životinja i ljudi (potrošača, osoba koje rukuju premiksima i sirovinama).

Ključne riječi: premiks, aflatoksin B_1, pšenične posije