SZENT ISTVÁN EGYETEM

ÁLLATORVOS-TUDOMÁNYI DOKTORI ISKOLA

Development of sensitive immundiagnostics for determination of toxic residues (mycotoxins, drugs) in biological fluids and animal feeds

PhD. Thesis

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1. Introduction and outline of the subject

Complex food-control comprises not only the analysis of natural food components (carbohydrates, proteins, fats, vitamins, colorants, flavours) but also the determination of harmful compounds that may be dangerous for human and animal health. The list of contaminants is quite long, including pesticide residues, antibiotics, drug residues, hormones and mycotoxins.

As a result of the numerous food scandals in many EU countries that have occurred recently, such as hormones, PCBs and dioxins, BSE, consumers are becoming dissatisfied with the current production process. The food and feed safety became immediately the first political matter in EU countries and Brussels planned to set up a Food-Control-Authority in the near future. The main task of this new Committee would be to make stronger legislation and to control the whole process of the food-chain, beginning from the farm – up to the table of consumers. These changes refer to Hungary as well, as Hungary will join to the EU in the near future.

For detection of the harmful residues in foods, acceptable, sensitive and reproducible methods are required such as enzyme-immunoassay (ELISA) based on antigen-antibody reaction. The ELISA combines the sensitivity of an enzyme reaction with the specificity of a good quality antibody (polyclonal or monoclonal).

In the Diagnostic Laboratory of ABC, Gödöllő, a long-term research program has been started 12 years ago for development of a series of monoclonal antibody based ELISA tests along with assay technology for determination of *mycotoxins and drug residues* in foods and feeds. The aim of our work was to develop simple test-kits for screening these harmful components and the cost per test should be less than the that of the imported diagnostic kits. In Hungary, the

Diagnostic Laboratory of ABC Gödöllő is the only one which is dealing with development, production and distribution of such reagent kits. The whole process of development was performed at ABC, Gödöllő.
The mycotoxin research is partly sponsored by the Hungarian Academy of Science and Ministry of Agriculture and Landscape Development.

1.2 Objectives of the research

- Production of monoclonal antibodies against ***ochratoxin A (OA) and fumonisin B_1 (FB_1)*** and developing direct, competitive ELISA tests and reagent kits for quantitative determination of these mycotoxins in cereal samples.

- The ochratoxin A test was modified and used for quantitative determination of OA concentration in human and swine serum, in seminal plasma of boars and in different tissues of broiler chickens (liver, kidney, thigh and breast muscles). The test was validated for these matrices. As collaborating partner in the experiment, we wanted to investigate the distribution of OA in the different poultry tissues after long-term feeding of chicken with OA doses approximating the range of natural contamination in feed.

- Production of monoclonal antibodies against ***gentamicin and sulphadiazine (SD)*** and development of direct, competitive ELISA test for quantitative determination of these drug residues in biological fluids (milk and sera). Validation of the tests.

2. Materials and Methods

2.1 Production of monoclonal antibodies and their characterisation

Using the benefits of hybridoma technology for raising antibodies with appropriate affinity and specificity, **monoclonal antibodies** of high affinity were produced against **ochratoxin A, fumonisin B_1, gentamicin and sulphadiazine.** The antibodies were characterised with the following data: Cross-reactivity (%) with some related compounds, 50% displacement value (IC_{50}), subclass and affinity constant.

2.2 Preparation of ochratoxin A-HRP conjugate by mixed anhydride method

Ochratoxin-A (2.48 μmol) was dissolved in 100 μl of dry DMF and cooled to –15 ^{o}C. To this solution 1.5 μl Tri-n-butilamine was added and cooled again to –15 ^{o}C. After 15 min. incubation, 1 μl of i-butil-chlorophormate was added, stirred and cooled again to –15 ^{o}C and incubated for 15 min. HRPO (RZ:3.0, 0.05 μmol) was dissolved in 250 μl distilled water + 250 μl of DMF and cooled to –15 ^{o}C . The pH of the peroxidase solution was raised to 9.0 with some drops of 0.1 mol/l of $NaHCO_3$. The activated ochratoxin-A solution was added quickly, but drop by drop to HRPO solution and stirred, the mixture was incubated for 1 hour at –15 ^{o}C, than 2 hours at + 4 ^{o}C. The pH of the mixture should be 9.0-10.0 during the incubation period. The solution was dialysed for 3 days against PBS buffer. The estimated Mol $_{OA}$/ Mol $_{HRPO}$ = 50. The working dilution of this conjugate was assessed in direct ELISA (Mw of HRPO: 40000, Mw of OA 403).

2.3 Preparation of Fumonisin B_1, Gentamicin and Sulphadiazine–HRP conjugates

Eight mg of HRPO (0.2 μmol) was dissolved in 2 ml distilled water, then 0.2 ml of 0.1 M sodium periodate was added and stirred for 20 min at RT, then dialysed overnight in 1 mM sodium acetate buffer (pH: 4.4). The pH of the activated HRPO was adjusted to 9-10 with 0.1 M NaOH, mixed with 2 mg of FB_1 (2.77 μmol) dissolved in 0.5 ml distilled water and the mixture was stirred for 3 hours at RT. The reaction was stopped with 0.2 ml of a sodium borohydride solution (4 mg/ml) and incubated overnight at 4 ^{o}C. Finally the conjugate was dialysed against 0.1 M phosphate-buffered saline (PBS) and stored at - 20 ^{o}C. (Mol $_{FB1}$/ Mol $_{HRPO} \cong 14$).
The preparation of GE-HRP and SD-HRP conjugates were similar to the above method but with different molecular rates. (Mol $_{GE}$ /Mol $_{HRPO} = 5.6$; Mol $_{SD}$ /Mol $_{HRPO} \cong 400$).

2.4 Direct competitive ELISA (dc-ELISA) for detection of OA in cereals, sera, seminal plasma, animal tissues and FB_1 in cereals

Microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with anti-mouse Ig rabbit IgG globulin, then 120 μl of diluted anti-OA ascites fluid or anti-FB_1 ascites fluid was pipetted to each well and incubated for 18 hours at RT. After washing with distilled water, plates were dried and stored in a foil bag for up to several months at 4 ^{o}C. Fifty-microliter OA or FB_1 standard solution or extracted samples were co-incubated in wells with 50 μl of diluted OA-HRPO or FB_1-HRPO conjugate in PBS-Tween 20 (0.1 %) for 1 h at RT. Plates were washed five times with distilled water and 150 μl of tetramethylbenzidine-H_2O_2 (TMB/H_2O_2) substrate per well added and incubated for 15 min at RT. The colour reaction

(blue colour) was stopped with 50 µl of 6N sulphuric acid (yellow colour) and the OD_{450} was measured by an automated microplate reader (Labsystems Multiscan PLUS, Finland).The developed colour (absorbance at 450 nm) is inversely proportional to the mycotoxin concentration in the sample.

2.5 Direct competitive ELISA (dc-ELISA) for detection of GE or SD in biological fluids

Microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with 120 µl of diluted anti-GE ascites fluid or anti-SD ascites fluid and incubated for 48 hours at RT or 18 hours at 37 ^{o}C. After washing with distilled water, plates were dried and stored in a foil bag for up to several months at 4 ^{o}C. Twenty µl of GE or SD standard solution or samples were co-incubated in wells with 100 µl of diluted GE-HRP or SD-HRP conjugate in PBS-Tween 20 (0.1 %) for 1 h at RT. After four washing steps, the peroxidase activity was measured as before.

Plotting the standard curves:

The standard curve of OA, FB_1, GE or SD was constructed by plotting $\log_{10}$ concentration (*x-axis)* against B/B_o (*y-axis)*:
B/B_o = (OD of standard or sample)/(OD of blank [no analyte added]), where optical density (OD) is the mean value.

2.6 Preparation of cereal samples for OA determination

Cereal samples (maize, barley, soya, mixed concentrates etc.) were triturated in a grinder and 2 g were transferred into a 50-ml Erlenmeyer flask. Thereafter 10 ml dichloromethane and 5 ml 1 M citric acid were added to the flask and the sample was vortex mixed for 5 minutes, sealed with parafilm and agitated

for 2 hours at RT (about 22 ^{O}C) on a horizontal shaker. The whole suspension was then transferred to a centrifuge tube and centrifuged for 30 min at 3000 x g. Three phases, aqueous (upper), - sample cake (middle) and dichloromethane (lower) were obtained after centrifugation. The upper aqueous phase was discarded, the sample cake was cut through and 2 ml of the dichloromethane phase was transferred to another conical tube. Two milliliters of 1% sodium bicarbonate buffer was added to this, sealed with parafilm and shaken for 30 min during which OA was transferred into the aqueous solution. The mixture was centrifuged (20 min at 3000 x g) in order to obtain a clear buffer solution. Four hundred and ninety microliters of the upper buffer solution was pipetted into a test tube and 10 µl of 1 N HCl added. The sample was mixed thoroughly and 50 µl of this solution was used directly in the ELISA. After four washing steps, the measure of peroxidase activity and the calculation of mycotoxin concentration was done according to point 2.4.

2.7. Preparation of human, pig, chicken serum and seminal plasma of boars for OA determination

Two milliliter of serum or seminal plasma, citric acid (2.5 ml 1 M) and 4 ml dichloromethane were added to centrifuge tube, vortexed for 5 minutes, sealed with parafilm and agitated for 1 hours at RT (about 22 ^{O}C) on a horizontal shaker, and finally centrifuged for 20 min at 3000 x g. The further part of the process was the same as under point 2.6.

2.8 Preparation of tissue sample for OA determination

Tissue samples (muscle, kidney, liver) of chickens were homogenised in a grinder and 5 g of each sample was transferred to a centrifuge tube. Seven milliliters of citric acid

and 10 ml dichloromethane were added, vortexed for 5 min, agitated on a horizontal shaker for 1 h at room temperature.The further part of the process was the same as point 2.6.

2.9 Preparation of cereal samples for FB_1 determination

To 5 g of finely ground cereals (wheat, maize, rye) 20 ml extraction solvent (composed of 50 parts acetonitrile, 39 parts water, 10 parts 0.5% KCl, 1 part 6% H_2SO_4) were added and shaken for 2 hours at RT. The extracts were centrifuged for 30 min at 4500 rpm and the supernatants diluted 1:5 with PBS-Tween 20 (0.1 %), shaked well then centrifuged again. From the clear supernatant 50 µl was used directly in the ELISA. The FB_1 concentration in sample extracts were assessed by using the calibration curve and are expressed in nanograms per gram by multiplying the nanogram-per-milliliter value by 20.

2.10. Preparation of cattle milk samples for GE and SD determination by ELISA

Raw milk samples were de-fatted by centrifugation (3000 x g) and 20 µl of samples were tested directly by ELISA according to point 2.5..

2.11. Preparation of porcine serum for determination of GE and SD by ELISA

Serum samples of swine were diluted 1:10 with dilution buffer of PBS/Tween 20, containing 0.1 % 8-Anilinonaphtalene-1-sulfonic acid (ANS) and 20 µl was tested further according to point 2.5.

3. Results

3.1. Characterisation of the optimised ELISA test for OA determination in cereals

The measuring range of the OA test is 0.5-10 ng/g (without sample dilution), but 10-100 ng/g (with sample dilution), the detection limit for cereals is 0.5 ng/g. The reproducibility data measured by within-assay and inter-assay coefficients of variation (CV) were less then 10%. The precision of the test was characterised by recovery data (%): - this value was 90-110 % in maize, barley, soya, goose-feed and piglet-feed.

The specificity of the test: - ochratoxin A 100 %, ochratoxin B 9.3 %. (No cross reaction with ochratoxin-α, coumarin, 4-hydroxy-coumarin, D,L - phenylalanine).

For easier application of the ELISA test in practice, reagent kit-package has been set up. The branded name of this commercialised kit is **TOXIKLON Ochratoxin A.** With this kit 40 cereal samples can be determined in duplicate.

3.2. Application of TOXIKLON A kit for determination of OA content of human sera

First we had to modify the protocol of the sample preparation (see Materials and Methods **2.7),** using almost half volume of dichloromethane and citric acid. The OA test was validated with a pool of negative (0.2-0.3 ng/ml of OA) and a pool of positive (2-3 ng/ml of OA) human sera. A total of 355 serum samples from random internal medicine patients was collected at the Szent János Hospital (Budapest). The OA concentration of 355 sera samples varied from <0.2 to 10 ng/ml OA but 75% of the samples contained 0.2-1.0 ng/ml. This amount reflects a tolerable daily intake (TDI) value of toxin. However, in some cases (6.8%), more than 1.0 ng/ml OA was measured, which is probably a result of elevated intake of OA, which may even exceed the “virtually safe dose”. Our data indicate that, like in

many other countries, OA is present in food or feed products available in Hungary, and in order to save the health of consumers, their regular control is desirable.

3.3. Detection of OA in serum and seminal plasma

Literature data are available on nephrotoxic effect of OA, but no reports were found in literature dealing with toxin concentration in seminal plasma after OA challenge despite the fact that high mycotoxin exposure is suspected to contribute to fertility problems in pig breeding units. The aim of the experiment was to investigate whether or not OA can be detected in seminal plasma after feeding the toxin in five and 10 times of the human tolerable daily intake (TDI) with breeding boars. The estimated human TDI value is 16 ng/kg/day. As collaborating partner in this work, the earlier optimized OA test was used to measure the OA concentration. Before this, the OA test had to validated for seminal plasma and pig sera as well. Our results showed that shortly after the toxin intake, OA appeared in serum and seminal plasma with different OA values but the shape of dose-response curves were similar. It seems, that the elevated OA content of sperm might have the potential to effect sperm production and semen quality of boars.

3.4 Detection of OA in tissue of chickens

As collaborating partner in this experiment, we wanted to investigate the OA content in poultry tissues and organs after long-term administration of OA doses approximating the range of natural contamination in feed. For this experiment the TOXIKLON ochratoxin-A test was used for measuring the OA content in different tissue samples. To test the OA in tissue, the sample preparation and clean-up had to be modified and first the test had to be validated for the different tissue samples

(Materials and Methods **2.8).** During the experimental period (28 day), the highest toxin concentration was found in the liver on day 7 and the OA concentration was still detectable even on day 28. Compared with the residual toxin amount in the liver, a considerably lower concentration of OA was detected in the kidney with ELISA. On the second week the OA concentration decreased markedly and was not longer detectable on day 28. In the thigh and breast muscle OA was identified at low level on day 14 by ELISA, and a slight increasing tendency was found on day 28. OA residues were not detected in any organs or tissues of control animals. According to this experiment, residues of OA were found in serum and in all tissues (kidney, liver and muscle), indicating wide tissue distribution.

3.5 Development of ELISA test for determination of Fumonisin B_1 in cereals

The measuring range of the optimised ELISA test in cereals is 10-500 ng/g, with detection limit of 7.6 ng/g FB_1.

The reproducibility data measured by within-assay and inter-assay coefficients of variation (CV) were less then 10%. The precision of the test was characterised by recovery data (%): - this value was 61-84 % in maize-, rye- and wheat flours.

The specificity of the test: FB_1 100 %, FB_2 91.8 %, FB_3 209 %, HFB_1 0 %.

To compare the immunoassay method with an established procedure, 11 naturally infected maize samples were extracted and their fumonisin content were analysed by HPLC. In most of the samples good correlation was obtained by two methods, at some samples higher ELISA values than HPLC were measured. We believe that the higher ELISA values at these samples are partly due to greater sensitivity of ELISA and partly to the high reactivity of our monoclonal antibody to other fumonisins. This cross-reactivity is particularly

advantageous since it enables simultaneous detection of all three fumonisins in doubtful or suspicious samples. In our case total fumonisins were measured rather than FB_1 alone.

3.6. Development of ELISA test for determination of gentamicin (GE) in biological fluids (milk, serum)

A monoclonal antibody based direct, competitive ELISA test was developed for quantitative screening of GE in milk and swine sera. The sample preparation is very simple: de-fatted raw milk directly (without dilution), the sera after dilution (1:10) can be assayed by our GE test, the volume of the test material is 20 µl. The measuring range 1.5-100 ng/ml in serum and that of 0.1-10 ng/ml in milk. The detection limit of GE in serum is 1.2 ng/ml and that of milk is 0.03 ng/ml. According to the EU regulations the acceptable GE content in biological fluids and meat is 100 µg/kg. Our ELISA test fits to this requirement. One of the most important data of the validation is the precision measured by the recovery (%) of GE from artificially infected swine serum and cattle milk. The added GE versus detected GE resulted a very good correlation with a correlation coefficient (r) of 0.999.

3.7 Development of ELISA test for determination of sulphadiazine in biological fluids

Among the most important sulphonamides, first we started the work with the sulphadiazine (SD). Our plan was to develop a sensitive ELISA for detection of SD in swine sera and cattle milk according to the EU directives.
A direct competitive ELISA based on monoclonal anti-SD was developed and used for quantitative detection of SD in serum and milk. The set up of the test was similar to GE ELISA. The measuring range was 25-500 ng/ml in serum and 2.5-50 ng/ml

in milk. The detection limit of SD in serum is 10 ng/ml and that of the milk is 1.5 ng/ml. According to the EU regulations the acceptable SD content in biological fluids and meat is 100 µg/kg. Our ELISA test fits to this requirement. According to the validation data the added and measured SD resulted a very close correlation.

4. Discussion and conclusions

- Specific, high sensitivity and high affinity monoclonal antibody against OA and (with the modification of conjugation method) peroxidase enzyme labelled OA have been prepared. Using of these basic reagents, direct, competitive ELISA test has been developed. This test was used in the practice for quantitative and/or qualitative determination of OA concentration in corn-based foods and feeds (wheat, maize, barley, rye, mixed feeds). A new extraction method using *dichloromethane/citric acid* mixture was developed. This cleanup procedure proved to be as effective for OA extraction as protocols using strong acids. For easier application of this test in practice a reagent-kit package has been set up. The branded name of this commercialised kit is **TOXIKLON ochratoxin A.** The reagent kit is sufficient for quantitative analysis of 40 cereal samples or for qualitative determination of 46 samples in duplicate.
- With modification of the cleanup procedure of OA the TOXIKLON ochratoxin A reagent kit was used for quantitative determination of OA content in different sample matrices (human, swine, chicken serum, swine seminal plasma, chicken tissues).
- According to some researchers high affinity antibody can be obtained with the use of longer cross-linker (about 16 atoms) between hapten and carrier protein. In contrast to

the literature, for the preparation of monoclonal antibody against FB_1 the FB_1-GA-KLH immunogen using shorter cross-linker reagent as glutaraldehyde *elicited high-titer, high affinity antibody.* Our antibody recognised the main fumonisin molecules (FB_1 and FB_2) equally well and FB_3 much better, *similar antibody has not been published yet* in the literature. The developed ELISA test has been commercialised, the branded name is **TOXIKLON FB_1.**

- At present, with the OA and FB_1 kits, we have five reagent kits in practice. These TOXIKLON kits can be used for rapid, quantitative screening of mycotoxins in foods and feeds. Some selected samples containing mycotoxins around the regulatory limit or above have been confirmed by accepted analytical methods (ie. HPLC, GC-MS).
- Monoclonal antibody based *ELISA tests* for simple, rapid and quantitative determination of GE in milk and serum have been developed. The sample preparation of milk was very simple, it could be used *directly* in the test. The non-specific binding effect of serum could be compensated by dilution of serum (1:10) with a special salt in dilution buffer (0.1 % ANS-PBS-0.05% Tween 20).
  Our plan is to apply this test for detection of GE in meat.
- Among the sulphonamids, first the sulphadiazin (SD) was selected. A monoclonal antibody against SD was produced by –azo- coupling of SD-BSA. The SD-HRP conjugate was produced by periodate method. Using this so called heterogeneous system, direct competitive ELISA tests for SD determination in milk and sera have been developed. The sensitivity of the test was increased by the change the volume rate of sample/conjugate (1:5). Our plan is partly to develop the SD-ELISA test for determination of SD in meat as well. Our other plan is to synthesise a sulphathiazole derivative (TS) and prepare TS-BSA immunogen. With this special immunogen a versatile

antibody against several sulphonamid components could be prepared.

- Our work for development of ELISA tests was qualified by obtaining ISO 9001:2001 certification for “production of specific monoclonal antibodies, development and manufacture of “ELISA” diagnostic tests for measurement of mycotoxins and antibiotics”.

5. List of original publications

Barna-Vetró, I.; Solti, L.; Téren, J.; Gyöngyösi, Á.; Szabó, E. and Wölfling, A. (1996): Sensitive ELISA test for determination of Ochratoxin-A. J. Agric. Food Chem. **44,** 4071-4074.
IF: 1.732

Solti, L., Salamon, F., **Barna-Vetró,** I., Gyöngyösi, Á., Szabó, E., Wölfling, A. (1997): Ochratoxin-A content of human sera determined by a sensitive ELISA. J. Anal. Toxicol. **21,** 44-48
IF: 2.168

Barna-Vetró I., Szabó E., Fazekas B. és Solti L. (1999): ELISA-teszt a fumonizin B1 toxin mérésére. Növényvédelem **35,** 609-617.

Solti, L., Pécsi, T**., Barna-Vetró**, I., Szász Jr., F., Biró, K., Szabó, E. (1999): Analysis of serum and plasma after feeding ochratoxin A with breeding boars. Anim. Reproduct. Sci. **56,** 123-132. IF: 0.813

Barna-Vetró I., Szabó E., Fazekas B., Solti L. (2000): Development of a sensitive ELISA for the determination of Fumonisin B1 in cereals. J. Agric. Food Chem. **48**, 2821-2825.
IF: 1.56

Barna-Vetró, I., Balázs, E., Solti, L. (2000): Immunodiagnostics for food and feed safety. Am. Lab. **32,** 64-66.
IF: 0.593

Biró, K.; Solti, L.; **Barna-Vetró**, I.; Bagó, Gy.; Glávits, R.; Szabó, E. and Fink-Gremmels, J. (2002): Tissue distribution of ochratoxin A as determined by HPLC and ELISA and histopathological effects in chickens. Avian Pathol. **31,** 141-148.
IF: 1.655

6. Glossary

ANS - 8-anilinonaphtalene-1-sulphonic acid
BSA- bovine serum albumin
CV - variation coefficient
DMF - dimetil-formamid
ELISA - Enzyme-Linked –Immunosorbent Assay
FBs - fumonisins
GE - gentamicin
HRP - horse radish peroxidase
HPLC - high-performance -liquid chromatography
Ig - immunoglobulin
IgG - immunoglobulin with IgG subclass
IC_{50} - concentration measured at 50 % displacement value
KLH - Keyhole-limpet hemocyanin
KCl - potassium chloride
OA - ochratoxin-A
SD - sulphadiazine
TDI - tolerable daily intake

Antigen - a large, chemically complex molecule or hapten-carrier conjugate that can induce an immune response, resulting in the formation of specific antibodies.

Antibody- a protein produced in response to an antigen that binds a specific antigen or hapten and form a complex.

Clone- a group of homogeneous cells that are the progeny of a single cell.

Hapten - a small molecule that cannot induce antibody production unless it is covalently bound to a carrier molecule. A hapten can react with the specific antibodies produced in response to the hapten-carrier conjugate.

Monoclonal antibody- a homogeneous antibody population derived from one specific antibody-producing cell.