

Production and characterisation of monoclonal antibodies for the detection of AOZ, a tissue bound metabolite of furazolidone

M. VASS¹, L. KOTKOVA¹, I. DIBLIKOVA¹, Z. NEVORANKOVA¹, K.M. COOPER², D.G. KENNEDY³, M. FRANEK¹

¹Veterinary Research Institute, Brno, Czech Republic

²Queen's University Belfast, Department of Veterinary Science, Northern Ireland, UK

³Chemical Surveillance Branch, Veterinary Sciences Division, Department of Agriculture & Rural Development, Belfast, Northern Ireland, UK

ABSTRACT: 3-amino-2-oxazolidinone (AOZ) is a tissue bound toxic metabolite derived from the nitrofurans antibiotic, furazolidone. AOZ is detected in the derivatised form of 3-[[2-nitrophenyl] methylene] amino-2-oxazolidinone (NP AOZ). 3-[[3-carboxyphenyl]-methylene] amino-2-oxazolidinone (CP AOZ) was used as the immunising hapten for the production of monoclonal antibodies against NP AOZ. Monoclonal antibodies were produced using hybridomas from the fusion of murine myeloma cells and spleen cells isolated from BALB/c mice immunised with CP AOZ-ethylenediamine-human serum albumin (CP AOZ-ed-HSA). The antibody production in ascitic fluids from clones 3B8/2B9 and 2D11/A4 was monitored during a 16 month period. Repeated cultures of these hybridomas, followed by injection into mice and cloning did not change the assay parameters. Clone 2D11/A4 exhibited long term stability in antibody production throughout the experiment whereas clone 3B8/2B9 demonstrated variability in particular antibody yields whilst retaining assay sensitivity. Reasons for this production variability in clones are discussed. In an optimised direct ELISA format, the antibodies exhibited a 50% binding inhibition in the range of 0.52–1.15 ng/ml with NP AOZ (0.22–0.50 ng/ml, respective AOZ equivalents) and showed high specificity towards this analyte. The sensitivity of monoclonal antibodies incorporated into the ELISA is compatible with the European Union MRLP and is currently in use for routine analysis.

Keywords: furazolidone; AOZ; hybridomas; monoclonal antibodies; ELISA; immunochemical characterisation; production stability

Nitrofurans are a class of synthetic broad spectrum antibiotics. Members of the nitrofurans family were commonly employed as feed additives for use in the treatment and prevention of bacterial enteritis caused by *Escherichia coli* and salmonella in swine, poultry and cattle and also as growth stimulators. In 1995, the use of nitrofurans was completely banned in food animal production in the European Union (EU) due to concerns about carcinogenicity of their residues in edible products (Commission

Decision (EC) 1442/95). As a result of the low costs of nitrofurans and their treatment benefits, it has been proven that illegal abuse of these compounds is still taking place (EC Rapid Alert System, 2004). From thorough inspection by authorities in the EU, contamination was found in products originating from over 20 countries in 2003/4 including Indonesia, India, Taiwan, Malaysia, France, Germany, Bulgaria, Italy and Spain (EC Rapid Alert System, 2004). Nitrofurans metabolites have

Supported by the European Commission, Project QLK1-CT1999-00142 "FoodBRAND" and, in part by the Ministry of Agriculture of the Czech Republic, Project MZ-00027162201.

been found in poultry and swine products, and recently more so in aquaculture products (especially shrimp) originating from S.E. Asia.

Monitoring of illegal nitrofurantoin parent drugs (furazolidone, nitrofurantoin, furaltadone, and nitrofurazone) has not been effective in the past due to the instability and rapid metabolism of the parent drugs. The parent drugs have a short half-life which causes them to disappear rapidly from blood, however, protein bound metabolites of these drugs are detectable in animal tissue for up to 6 weeks after treatment and hence are more practical for monitoring public compliance of the EU ban. It has been concluded that when nitrofurans are treated with mild acid, specific side chains are released (i.e. furazolidone releases 3-amino-2-oxazolidinone (AOZ)). These protein-bound residues are stable in tissue matrices, even after long-term storage *in vitro*, heat processing and cooking, therefore the ingestion of such protein bound residues in meat or fish products may result in release of metabolites, such as AOZ, in the human body during digestion (Horne et al., 1996; DeAngelis et al., 1999; Hoogenboom et al., 2002), thus causing a health risk for consumers.

Analytical detection of banned nitrofurantoin drugs (furazolidone, nitrofurantoin, furaltadone, nitrofurazone) can be monitored by measuring concentrations of bound nitrofurantoin metabolites 3-amino-2-oxazolidinone (AOZ) (Figure 1), 1-amino hydantoin (AHD), 3-amino-5-methylmorpholino-2-oxazolidinone (AMZO) and semicarbazide (SEM). Prior to analysis these metabolites can be further derivatised with *o*-nitrobenzaldehyde (*o*-NBA) forming stable nitro phenyl (NP) deriva-

tives. Various instrumental reference and screening methods (such as ELISA, HPLC-MS, LC-MS/MS and LC-UV) for the determination of nitrofurantoin residues have since been developed (Soliman et al., 1990; Horne et al., 1996; McCracken and Kennedy, 1997; Leitner et al., 2001; Cooper et al., 2004b), but a growing need remains for low cost and rapid screening methods that are sensitive toward a single analyte such as AOZ, or more of the nitrofurantoin metabolites simultaneously. Competitive enzyme-linked immunosorbent assay (ELISA) based on the use of a specific antibody and peroxidase conjugate is one such a method.

The first polyclonal rabbit antibodies raised against a derivatised AOZ hapten were produced under the FoodBRAND project (QLK1-CT1999-001142) (Cooper et al., 2004a) and used in ELISA for the determination of AOZ in prawn tissue (Cooper et al., 2004b). These antibodies allowed the detection of AOZ (in the form of NP AOZ) following derivatisation with *o*-NBA. The antibodies were highly specific for NP AOZ and did not cross-react with other nitrofurantoin metabolites, their nitro-phenyl derivatives or a range of other veterinary drugs. The antibody incorporated into an immunoassay satisfied current criteria for monitoring of veterinary drug residues. Although monoclonal and polyclonal antibodies may have similar analytical characteristics, the principal advantage of monoclonal antibodies is that they are highly standard reagents and can be produced in virtually unlimited quantities (Howard and Bethell, 2000). Monoclonal antibodies represent homogeneous antibody entities that have identical affinity and specificity to an antigen. Thus, mono-

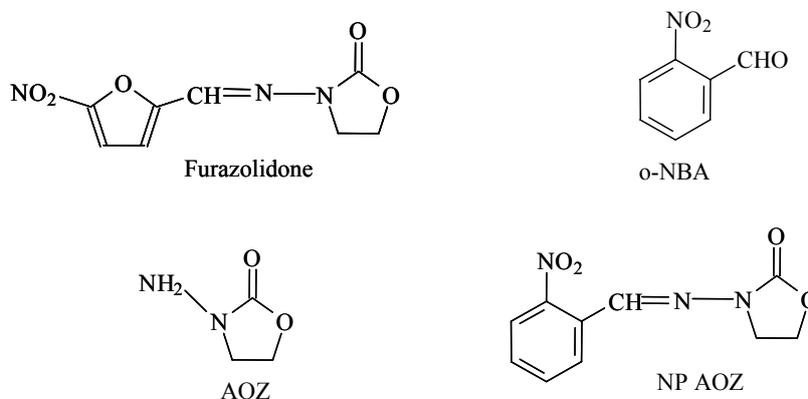


Figure 1. Chemical structure of the nitrofurantoin parent drug (furazolidone), metabolised residue AOZ, derivatising agent *o*-nitro benzaldehyde (*o*-NBA) and target analyte NP AOZ

clonal antibodies can be utilised in various immunochemical techniques including those having higher consumption of antibody reagent.

In previous years, this laboratory has successfully produced monoclonal antibodies against herbicides, 2,4-dichlorophenoxyacetic acid (Franek et al., 1994), atrazine (Deng et al., 1999), estrogenic disruptor nonylphenol (Zeravik et al., 2004), sulphonylureas and triazines (Kolar et al., 2002) and utilised them in environmental and food analysis. The immunogen based on the 3-carboxyphenyl-AOZ (CP AOZ) hapten, previously used for the preparation of rabbit antibodies (Cooper et al., 2004a), was utilised for the production of monoclonal antibodies in this work. Details of this monoclonal antibody production are reported in this paper. However, the aim of this work was not only to develop hybridomas producing monoclonal antibodies against NPAOZ, but also to characterise the antibody species in terms of cross reactivity and assay sensitivity, to study the influence of repeated cultures and cloning of hybridomas on the effectiveness of antibody production and quality, to examine assay parameters of individual antibodies in the ELISA and to monitor long-term hybridoma production in terms of antibody yield and immunoassay characteristics of monoclonal antibodies produced at different periods after fusion.

MATERIAL AND METHODS

Chemicals and biochemicals

3[[2-nitrophenyl)methylene]-amino]-2-oxazolidinone (NP AOZ) was provided by WITEGA laboratorien, (Berlin, Germany). 3-amino-2-oxazolidinone (AOZ), protease Type XXIV, (bacterial), bovine serum albumin (BSA), rabbit-anti-mouse IgG, polyethelene (PEG) glycol, pristane and Sephadex G-25 were purchased from Sigma-Aldrich (Heidelberg, Germany). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). Dimethyl sulfoxide (DMSO), polyoxyethylenesorbitan monolaurate (Tween-20 (T₂₀)), *N*-hydroxysuccinimide (NHS), *N,N*-dicyclohexylcarbodiimide (DCC), Freund's adjuvant (complete), horseradish peroxidase (HRP), and *N,N*-dimethylformamide (DMF) were purchased from Sigma (St. Louis, USA). Dulbecco's minimum essential medium (DMEM) was purchased from GIBCO-BRL (USA) and fetal

calf serum (FCS) from ZVOS (Hustopece, Czech Republic). All other chemicals were of analytical grade. Microtitre plates (Nunc-Immuno™ Plate, Maxi-Sorp™ Surface) were supplied by NUNC (Roskilde, Denmark). Preparation of 3-[[3-carboxyphenyl)methylene]amino]-2-oxazolidinone (CP AOZ) and CP AOZ-ed-HSA (CP AOZ – ethylene diamine – human serum albumin) has been described previously (Cooper et al., 2004a).

Instruments

EL808 Ultra microplate reader with the software KC4™ (Kineticcalc for windows, v3.1) was used for the measurement of absorbance levels and the processing of ELISA results. Auto strip washer, ELX50, was used for the washing of microplates. All instruments were supplied by Bio-tek Instruments Inc, Vermont, USA.

Buffers and solutions

The following buffers and solutions were used: Coating buffer solution (0.05 M, pH 8.6), containing 1.5 g Na₂CO₃ and 2.93 g NaHCO₃ in 1 l of deionised water. Phosphate buffered saline (PBS) concentrate containing 8.5 g NaCl, 2.686 g Na₂HPO₄(12H₂O) and 0.34 g NaH₂PO₄(2H₂O) in 1 l deionised water and a dilute 1 : 10 (PBS concentrate : deionised water) PBS solution was used for the standards preparation. PBS-BSA (0.5% w/v BSA) consisted of 2.5 g BSA dissolved in 50 ml of PBS buffer concentrate. Washing buffer was 100 ml of PBS (T₂₀) concentrate (0.1w/v) diluted to 1 l with deionised water. Substrate buffer was 0.1 mol/l sodium acetate, pH 5.5 (adjusted by addition of 1 mol/l citric acid). Substrate solution for HRP was prepared by addition of 1 ml of substrate buffer, 200 µl of 1% (w/v) solution of TMB in DMSO and 20 µl of 6% (w/v) H₂O₂ to 20 ml MilliQ-water. Stopping reagent was 2M H₂SO₄.

Standards

Stock solutions of NPAOZ and cross-reactants were prepared in methanol at a concentration of 10 000 ng/ml. Standard concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 10 ng/ml were prepared by dilution with PBS buffer.

Synthesis of the Peroxidase conjugate

CP AOZ (5 μmol , 1.17 mg), NHS ester (5 μmol , 0.58 mg) and DCC (5 μmol , 1.03 mg) were dissolved in 60 μl of DMF. The mixture was stirred gently at room temperature overnight (18 hrs), then centrifuged (2 500 rpm for 10 min). 27.2 μl of the supernatant was added drop wise to 3.4 mg of HRP in 0.13 mol/l NaHCO_3 (300 μl) in order to obtain a hapten : HRP molar reaction ratio of 10 : 1. The conjugation mixture was then stirred at room temperature for 5 hours. The tracer was purified on Sephadex G-25, using 0.01 mol/l of NaHCO_3 as the eluent. A purified fraction of the conjugate CP AOZ-HRP (Figure 2) was obtained, diluted with the same volume of glycerol and stored at -20°C until use.

ELISA procedure

Direct ELISA. Media and antisera were tested as follows: 200 μl of rabbit-anti-mouse IgG in coating buffer (1 : 10 000) was pipetted into a microtitre plate and incubated at 4°C overnight. Plates were washed 3 times using 0.3 ml/well of washing buffer solution. 200 μl of medium containing antibody or 200 μl of serum diluted with PBS was pipetted into wells. The plate was then incubated at 4°C for 1 hour then washed again using the above procedure. 100 μl of both standard and tracer (CP AOZ HRP conjugate) solution were then added to each well. Unbound standard was removed by washing (as above) after a 1hr incubation period at 4°C . 200 μl of developing substrate solution was then added to each well and the enzymatic reaction was stopped after 15 min incubation at room tempera-

ture by addition of 100 μl /well of stopping solution. Absorbance values were measured at 450 nm.

Ascites fluids were tested as follows: Ascitic fluid from mice was diluted with coating buffer and 200 μl of this solution was pipetted into wells of a microtitre plate and incubated at 4°C overnight. Plates were washed 3 times using 0.3 ml/well of washing buffer. 100 μl of standard and peroxidase conjugate were then added to wells. Unbound compounds were removed by washing (as above) after 1hr incubation at 4°C . 200 μl of substrate solution was then added to each well and the enzymatic reaction was stopped after 15 min incubation at room temperature, by addition of 100 μl /well of stopping solution. Absorbance values were measured at 450 nm.

Production of clones and monoclonal antibodies

Four BALB/c mice (aged 10 to 15 weeks) were immunised 3 times with 75 μg of CP AOZ-ed-HSA in Freund's adjuvant, injected intraperitoneally, at 2 week intervals. Four and eleven week periods elapsed between the 3rd – 4th and 4th – 5th immunisations respectively. Blood samples were taken from tail vena 10 days after the 5th immunisation and tested for antibody production by direct ELISA. The two mice exhibiting the highest antibody titres and sensitivity to NPAOZ were sacrificed after the last injection and their spleens removed for use in hybridoma production. Spleen cells from the mice and myeloma cells were fused at a ratio of 1 : 10 in 1 ml of 50% PEG 1500 containing DMEM without serum. After cell fusion, the cells were selected using selection medium containing 20%

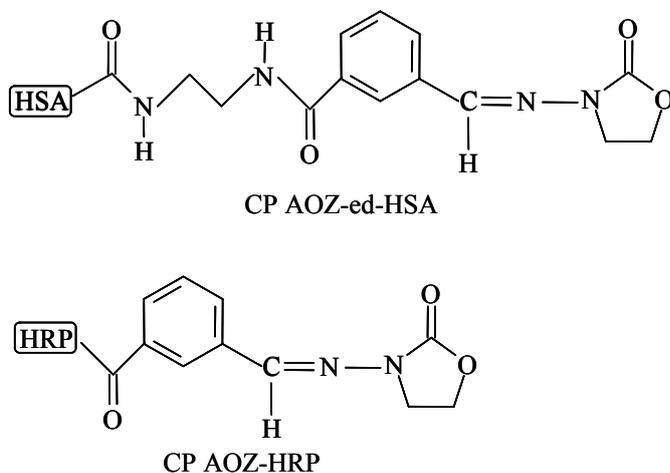


Figure 2. Hapten structures used in immunogen and peroxidase conjugate (tracer)

FCS and hypoxantin-azaserin solution in microtitre polystyrene plates. After 8 days, the growing hybridoma cells were screened for antibody production. Hybridoma cells exhibiting the best assay parameters in ELISA-positive wells were cultured in DMEM and then cloned using the method of limited dilution. The positive clones were used for antibody production by ascites growth. Priming of the mice was carried out by injecting 0.5 ml of pristane into the peritoneal cavity. Seven days after priming, hybridoma cells were resuspended in sterile PBS to a final concentration of 2×10^6 cells per ml and 0.5 ml of the suspension was injected into each mouse intraperitoneally. Mice were sacrificed 7–10 days after inoculation. Ascites fluids were collected, centrifuged and stored at -20°C until use.

Sensitivity and cross-reactivity evaluation

To determine IC_{50} values, ELISA standard curves were fitted into a four-parameter logistic curve using Kineticalc for windows, v. 3.1, according to the following formula:

$$y = \{(A1-A2)/[1+(x/x_0)^p]\} + A2$$

where: A1, A2 = the upper (theoretical B_0) and lower (theoretical non-specific binding) asymptotes of the curve, respectively

p = the slope at inflection point of the sigmoid

x_0 = the concentration of NP AOZ resulting in 50% inhibition of tracer binding

Therefore, the mean of each absorbance standard was normalised against the mean absorbance of the zero standard (B/B_0), and the midpoint of each displacement standard curve was then calculated in order to determine the IC_{50} .

Cross-reactivity (CR) for a given substance was calculated from standard curves as IC_{50} of NP AOZ divided by the IC_{50} of the respective cross-reactant multiplied by 100.

RESULTS AND DISCUSSION

Immunogen and analyte

Hapten molecules, such as the nitrofurans and their metabolites, must be covalently attached to

a carrier protein prior to immunisation in order to evoke a specific immune response. In previous work, an effort was made to produce separate antibodies that were sensitive to AOZ, AHD and AMOZ. Derivatives of these metabolites containing aliphatic carboxylic side chains were bound to carrier proteins and applied to experimental animals, but attempts to prepare functional antibodies against these metabolites were not successful (Diblikova et al., 2005). All the rabbit antibodies formed exhibited binding affinity towards the corresponding hapten-protein conjugate but no binding inhibition was evident in the presence of free, underivatized metabolite (AOZ). Functional antibodies for the detection of NP AOZ were prepared only when the AOZ hapten structure was enlarged with 3-carboxybenzaldehyde and conjugated to carrier proteins (Cooper et al., 2004a). These antibodies exhibited high sensitivity and specificity towards NP AOZ, which therefore became the target analyte for assay development. The AOZ moiety in derivatised form was situated in the immuno-dominant position by linkage via a carboxy-phenyl group to a protein carrier. High specificity toward NP AOZ was also expected using this carboxy-phenyl AOZ (CPAOZ) immunogen in monoclonal antibody production. Figure 2 shows the CP AOZ-protein conjugates used in this work as immunogens for the preparation of antibodies and a peroxidase tracer for use in a competitive ELISA.

Monoclonal antibody production

Due to the low predictability of results in hybridoma technology and in order to ensure sufficient material for repeated cell fusions, it is usually necessary to use a large number of experimental mice for immunisations. Due to limited availability of immunogen, only four experimental mice were used for immunisation with CP AOZ-ed-HSA. Lengthening the period between the 4th and 5th booster immunisations from 2 to 11 weeks aimed to increase the affinity of mice antibodies to NP AOZ prior to fusion. The clonal selection theory suggests that antigen-driven selection will result in an increase in antibody affinity as the concentration of circulating antigen diminishes over time. Thus, longer periods between booster injections will enable fuller clearance of circulating antigen, stimulating production of higher affinity antibodies (Cooper et al., 2004a).

The second cell fusion carried out in November 2002 provided an excellent collection of antibody producing hybridomas. Of a total of 616 cell colonies, 28 exhibited rapid colour development in microwells, indicating strong antibody binding. Four of these cells were cloned by the method of limiting dilution and antibodies were given codes according to the origin of the hybridoma. In the initial screening stages, monoclonal antibodies secreted by clone 3B8/2B9 and clone 2D11/A4 in November 2002, proved to be most responsive to the target analyte NP AOZ and were used further for immuno-analytical characterisation. Antibodies produced by these clones were used for comparison

with antibodies produced throughout the course of the project. These original clones were cultured and injected into mice on four separate occasions throughout the course of the study. After collection of ascites fluid from mice, additional antibodies were produced and characterised as described in sections 3.3–3.5.

Antibody sensitivity

In order to determine antibody sensitivity produced by the clones within the period June 2003 to April 2004, binding and competitive properties

Table 1. Assay sensitivity of monoclonal antibodies for NP AOZ in an optimised ELISA

Clone number*	Ascites fluid dilution	Tracer dilution	Absorbance at zero (450 nm)	Sensitivity (IC ₅₀ , ng/ml)
November 2002				
3B8/2B9	1 : 2 000	1 : 60 000	1.25	0.70
2D11/A4	1 : 5 000	1 : 40 000	1.25	0.55
June 2003				
1B1 (Ab3)	1 : 5 000	1 : 20 000	1.27	0.55
2E9 (Ab7)	1 : 500	1 : 20 000	1.12	1.01
3B8/2B9 (Ab11)	1 : 500	1 : 20 000	0.50 (max.)	1.05
3B8/2D2 (Ab12)	1 : 10 000	1 : 20 000	1.11	0.52
3B8/12H9 (Ab14)	1 : 500	1 : 20 000	1.16	0.61
2D11/A4 (Ab15)	1 : 5 000	1 : 20 000	1.08	0.74
February 2004				
3B8/2B9 (41)	1 : 500	1 : 20 000	0.57 (max.)	1.15
2D11/A4 (42.2)	1 : 5 000	1 : 40 000	1.17	0.64
2D11/A4/B2 (46.1)	1 : 2 000	1 : 20 000	1.23	0.68
2D11/A4/B2 (46.2)	1 : 500	1 : 20 000	1.10	0.78
2D11/A4/H12 (47.1)	1 : 500	1 : 20 000	1.16	0.52
2D11/A4/H12 (47.2)	1 : 10 000	1 : 20 000	1.30	0.76
April 2004				
3B8/2B9 (3.3)	1 : 1 000	1 : 10 000	1.17	0.58
2D11/A4 (3.1)	1 : 1 000	1 : 20 000	1.24	0.79
2D11/A4 (3.2)	1 : 500	1 : 20 000	1.23	0.75
2D11/A4 (3.3)	1 : 500	1 : 10 000	1.21	0.85

*The table shows four time periods of antibody production and each row details the characterisation of a respective clone/antibody. The information in brackets indicates the assigned antibody number. Lettering in bold indicates re-injection of the main subjects (produced November 2002) into mice at various time periods. Subject names separated by a slash indicate different generations within the cloning procedure

of the antibodies obtained were examined in direct ELISA systems. Table 1 summarises results of the characterisation of monoclonal antibodies originating from hybridomas produced and cloned at different intervals after fusion. IC_{50} values, which lie within the region of the ELISA standard curve with the highest assay sensitivity, were used as an indicator of the sensitivity towards NPAOZ for individual antibodies. The antibodies obtained from original clones 3B8/2B9 and 2D11/A4 within the first weeks after fusion (November 2002) showed high sensitivity (IC_{50} = 0.70 and 0.55 ng/ml, respectively) in an optimised direct ELISA system and were used as comparative materials for the following experiments. The results obtained within this period demonstrate considerable variation in optimised immuno-reagent dilutions while assay sensitivity remained relatively constant. Of the 17 antibodies that underwent hybridoma fusion and cloning in June 2003, only six exhibited good binding ability and sensitivity in the optimised assay. When compared to the main subjects produced in November 2002, antibodies 1B1 (Ab3), 3B8/2B9 (Ab12) and 2D11/A4 (Ab15) exhibited comparable characteristics, whereas a 10-fold decline in ascites dilution was observed in three other antibodies (Abs 7, 11 and 14). In February 2004, clones 3B8/2B9 and 2D11/A4 were re-injected into mice and re-cloned in an attempt to improve antibody binding and competitive characteristics. All seven clones originating from 3B8/2B9 (41) produced low binding affinity antibodies and hence are not detailed in this report. 2D11/A4 (42.2), however, maintained its titre strength showing a slight drop in assay sensitivity, changing from 0.55 to 0.64 ng/ml.

Antibody generations derived from the cloning of 2D11/A4 (42.2), were comparable in sensitivity to the original although they exhibited a decline in titre values (except for clone 2D11/A4/H12 (47.2)). Small differences in assay sensitivity may be caused by the fact that we were not able to reach the same "absorbance at the zero calibration point" of approximately 1.2 absorbance units. Additionally, a low absorbance at zero was produced by antibodies 3B8/2B9 (Ab11) and 3B8/2B9 (Ab41) because it was the maximum attainable absorbance. These particular clones showed poor ascites fluid dilutions but high sensitivity and hence affinity was retained, leaving the antibody concentration produced by individual mice as the possible variable in these cases.

It can be concluded that the hybridoma clones produced in various time periods retained their pro-

duction capabilities and produced antibodies with similar sensitivity in optimised ELISA systems.

Antibody specificity

The monoclonal antibodies exhibiting good binding and assay sensitivity towards NP AOZ were tested for cross reactivity with parent drugs (furazolidone, nitrofurantoin, furaltadone and nitrofurazone), free nitrofurans metabolites (AOZ, AMOZ, AHD and SEM) and their respective o-NBA derivatised metabolites (Tables 2–4). A general trend in cross reactivity was seen throughout the duration of the experiment. Tables 2 to 4 show that the antibodies exhibited low or negligible cross reactivity with all tested metabolites and parent drugs except furazolidone. Cross-reactivity with furazolidone was 6.6–36.7% and the respective IC_{50} values ranged from 15.5 to 27.1 ng/ml. Furazolidone is however, unlikely to occur as real assay interference in practice because furazolidone is known to be metabolised rapidly *in vivo* (McCracken et al., 1995). Additionally, IC_{50} values one order of magnitude higher were observed for nitrofurazone (156.4–302.9 ng/ml) and negligible dose responses to parent drugs nitrofurantoin and furaltadone were seen. One can conclude that all the antibodies exhibited high specificity toward target NP AOZ, having sufficient sensitivity for screening analysis and therefore can be used as specific reagents for the detection of AOZ in food matrices.

Production stability of clones

To investigate the stability of antibody production, clones 3B8/2B9 and 2D11/A4 were studied over a long term period (November 2002–April 2004) and a comparison chart of the production yield based on antibody titre level was constructed. The two clones were applied to BALB/c mice at various periods throughout the duration of the experiment and the titre of the antibodies produced was examined (Table 5). The antibody titre is defined as the dilution of ascitic fluid at which absorbance at zero in a direct ELISA is approximately 1.2. By examining the antibody titre values it can be seen that clone 2D11/A4 retained long-term stability (and viability) throughout the 16 month period after fusion, whereas 3B8/2B9 showed significant variability in antibody production.

Table 2. Cross reactivity among NP AOZ and related compounds in ELISA using monoclonal antibodies produced in November 2002

Compound	3B8/2B9 (2002)		2D11/A4 (2002)	
	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)
NP AOZ	100.0	1.7	100.0	3.0
Furazolidone	16.9	9.8	16.9	17.5
AOZ	< 0.1	5 781	< 0.1	7 082
NP AHD	1.1	154.8	1.1	289.6
Nitrofurantoin	< 0.01	> 10 000	< 0.01	> 10 000
AHD	< 0.1	6 593	< 0.1	8 621
NP AMOZ	< 0.01	>10 000	< 0.01	> 10 000
Furaltadone	< 0.1	> 10 000	< 0.1	6 641
AMOZ	< 0.1	6 434	< 0.1	8 915
NP SEM	0.2	837.5	0.4	820.9
Nitrofurazone	1.3	128.7	1.8	166.7
SEM	< 0.1	7 074	< 0.01	7 289

The results of clone 3B8/2B9 demonstrate that production stability of the clone was not retained as the titre values fluctuated from December 2002 (1 : 40 000) to April 2004 (1 : 200). This could be due to various factors, e.g. contamination within the original sample, culture conditions or the instability of a hybridoma. It is also possible that a group

of cells producing antibodies derived from one well may appear as one single cell line hence interfering in antibody selection. Presence and over growth of non-producing cells in producing cell wells and mutual cell adherence influencing the cloning procedure should also be taken into account (Brichta and Franek, 2003). Although it does not seem to be

Table 3. Cross reactivity among NP AOZ and related compounds in ELISA using monoclonal antibodies produced in June 2003

Compound	1B1 (Ab3)		2E9 (Ab7)		3B8/2D2 (Ab12)		3B8/12H9 (Ab14)		2D11/A4 (Ab15)	
	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)
NP AOZ	100.0	1.8	100.0	1.9	100.0	5.7	100.0	2.4	100.0	2.6
Furazolidone	6.6	27.0	7.5	25.5	36.7	15.5	11.8	20.0	9.8	27.1
AOZ	< 0.1	4 133	< 0.01	> 10 000	0.1	6 042	< 0.1	> 10 000	< 0.1	7 903
NP AHD	0.4	448.7	0.5	377.1	2.8	200.5	1.0	230.7	0.6	469.6
Nitrofurantoin	< 0.01	> 10 000	< 0.1	> 10 000	< 0.1	> 10 000	< 0.1	> 10 000	< 0.01	> 10 000
AHD	0.1	2 011	< 0.1	> 10 000	0.1	7 549	–	–	< 0.1	4 697
NP AMOZ	< 0.1	6 451	< 0.1	4 783	0.1	5 993	< 0.01	> 10 000	< 0.01	> 10 000
Furaltadone	< 0.1	> 10 000	< 0.1	7 241	0.1	6 355	< 0.1	6 534	< 0.1	7 857
AMOZ	< 0.01	> 10 000	0.1	3 790	–	–	< 0.1	8 905	< 0.01	>10 000
NP SEM	0.1	1 408	0.1	1 215	0.4	1 607	0.9	2 954	0.2	1 481
Nitrofurazone	0.6	302.9	0.8	252.9	3.6	156.4	1.2	196.6	1.1	250.9
SEM	< 0.1	6 018	0.1	6 549	0.1	8 887	–	–	< 0.1	4 884

Table 4. Cross reactivity among NP AOZ and related compounds in ELISA using monoclonal antibodies produced in February 2004

Compound	2D11/A4 (42.2)		2D11/A4/B2 (46.1)		2D11/A4/H12 (47.1)		2D11/A4/H12 (47.2)	
	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)	CR (%)	IC ₅₀ (ng/ml)
NP AOZ	100.0	2.8	100.0	3.7	100.0	2.2	100.0	4.0
Furazolidone	14.4	19.7	12.3	29.9	6.9	32.2	15.2	26.2
AOZ	0.1	6 295	0.1	6 005	0.1	4 274	< 0.1	8 669
NP AHD	0.7	403.6	0.9	424.9	< 0.01	>10 000	0.6	619.5
Nitrofurantoin	< 0.01	> 10 000	< 0.1	> 10 000	< 0.01	>10 000	0.1	7 374
AHD	< 0.01	> 10 000	< 0.1	> 10 000	0.4	502.8	< 0.01	> 10 000
NP AMOZ	0.1	5 905	< 0.1	9 031	0.04	5 963	0.1	5 344
Furaltadone	0.1	5 970	< 0.1	8 421	< 0.01	> 10 000	0.1	6 077
AMOZ	1.6	179.7	< 0.01	> 10 000	0.1	2 804	< 0.1	6 911
NP SEM	0.2	1 414	0.3	1 427	0.2	1 186	0.3	1 432
Nitrofurazone	1.7	171.2	1.1	345.6	0.8	276.3	1.9	214.7
SEM	–	< 0.01	–	< 0.01	–	< 0.01	–	–

Table 5. Production of monoclonal antibodies at different periods of time after fusion

Antibody	December 2002	June 2003	February 2004	April 2004 (1)	April 2004 (2)	April 2004 (3)
3B8/2B9	1 : 40 000*	1 : 500	1 : 2 000	1 : 20 000	1 : 200	1 : 10 000
2D11/A4	1 : 20 000	1 : 10 000	1 : 10 000	1 : 10 000	1 : 10 000	1 : 5 000

*Titre values of monoclonal antibodies at different periods of time after fusion at the constant peroxidase tracer dilution of 1 : 10 000

the case, another factor resulting in fluctuation of titre values could be chromosome loss in hybridomas. Chromosome loss does not only occur within 7 days post-fusion, it can occur at any time in the culture of hybridomas. Hybridoma cells are not “typical” cells and hence are less stable and prone to stress (Howard and Bethell, 2000) therefore a slight disturbance of optimal growth conditions may interfere in achieving desired results.

In order to verify results from December 2002 to February 2004, the same hybridoma cell lines were injected into 6 experimental mice in April 2004. Looking at the titre levels of the three 3B8/2B9 antibodies in particular, it is clearly evident that individual mice differed significantly in production yield capabilities, as injections of the same clone show dramatic change in the titre levels of the resulting antibodies in ascitic fluid produced by each mouse (Table 5). Interpretation of these

findings in molecular or cellular terms is difficult and other laboratories have experienced similar problems (Brichta and Franek, 2003; Chen et al., 2003). Pasqualini and Arap (2003) stated that although hybridomas can be immortal, they may depend on a feeder cell layer and/or may be genetically unstable. Other possible causes have been discussed above.

Although fluctuations in titre levels were observed in 3B8/2B9 within the studied time period, the 2D11/A4 clones maintained a stable titre level throughout the duration of the experiment demonstrating that antibody production stability was retained.

CONCLUSIONS

To increase antibody affinity prior to fusion, the period before the last booster immunisation of the

mice was lengthened. A series of monoclonal antibodies against the derivatised metabolite, NPAOZ, have been produced by hybridomas originating from one fusion experiment. The production of the most sensitive antibodies in ascitic fluids from clones 3B8/2B9 and 2D11/A4 was followed by measuring antibody titres during a 16 month period. Although both the clones provided highly sensitive antibodies, they differed in production efficiency throughout the experiment. Clone 2D11/A4 exhibited good stability in respect to antibody titre, whereas clone 3B8/2B9 showed dramatic variability in antibody yields whilst retaining assay sensitivity. To interpret the differences in production efficiency, various factors within the cloning procedure, chromosome changes and individual mice variability were considered. Additionally, repeated injection of the original hybridomas clones into mice did not change the assay parameters. In an optimised direct ELISA format, the antibodies exhibited a 50% binding inhibition in the range of 0.52–1.15 ng/ml for the target analyte NP AOZ, whilst the cross-reactivity pattern remained fairly constant in the time period of observation.

The sensitivity of the presented monoclonal antibodies in ELISA is approximately four times lower than the most sensitive rabbit polyclonal antibody (R670) prepared in the previous stage of this project (Cooper et al., 2004a). However, this sensitivity is still compatible with the Minimum Required Performance Limit (MRPL) for nitrofurans metabolites, which is set by European Commission at 1 µg/kg tissue (Commission Decision 2003/181/EC). The validated ELISA test based on monoclonal antibody 3B8/2B9 is currently in use for the routine screening analysis of AOZ in food samples (Diblikova et al., 2005).

REFERENCES

- Brichta J., Franek M. (2003): Identification of monoclonal antibodies against 2,4-D herbicide by ELISA and DNA sequencing. *Journal of Agricultural and Food Chemistry*, 51, 6091–6097.
- Chen B., Bautista R., Yu K., Zapata G.A., Mulkerrin M.G., Chamow S.M. (2003): Influence of histidine on the stability and physical properties of a fully human antibody in aqueous and solid forms. *Pharmaceutical Research*, 20, 1952–1960.
- Commission Decision 2003/181/EC, 2003, 13/03, Off. J. Eur. Commun., L71, 17–18.
- Commission Decision (EC) 1442/95, 1995, 26/06, Off. J. Eur. Commun., L143, 26–30.
- Cooper K.M., Caddell A., Elliott C.T., Kennedy D.G. (2004a): Production and characterisation of polyclonal antibodies to a derivative of AOZ, a metabolite of the nitrofurans furazolidone. *Analytica Chimica Acta*, 520, 79–86.
- Cooper K.M., Elliott C.T., Kennedy D.G. (2004b): Detection of 3-amino-2-oxazolidone (AOZ), a tissue-bound metabolite of the nitrofurans furazolidone, in prawn tissue by enzyme immunoassay. *Food Additives and Contaminants*, 21, 841–848.
- De Angelis I., Rossi L., Pedersen J.Z., Vignoli A.L., Vincentini O., Hoogenboom L.A.P., Polman T.H.G., Stamatii A., Zucco F. (1999): Metabolism of furazolidone: alternative pathways and modes of toxicity in different cell lines. *Xenobiotica*, 29, 1157–1169.
- Deng A., Franek M., Kolar V. (1999): Determination of atrazine in soil samples by ELISA using polyclonal and monoclonal antibodies. *Food and Agricultural Immunology*, 11, 135–144.
- Diblikova I., Cooper K.M., Kennedy D.G., Kotkova L., Franek M. (2005): Monoclonal antibody-based ELISA for the quantification of nitrofurans metabolite 3-amino-2-oxazolidinone in tissues using a simplified sample preparation. *Analytica Chimica Acta*, 540, 285–292.
- European Commission Rapid Alert System for Food and Feed (2004): http://europa.eu.int/comm/food/food/rapidalert/index_en.htm, updated weekly.
- Franek M., Kolar V., Granatova M., Nevorankova Z. (1994): Monoclonal ELISA for 2,4-dichlorophenoxyacetic acid: characterization of antibodies and assay optimization. *Journal of Agricultural and Food Chemistry*, 42, 1369–1374.
- Hoogenboom L.A.P., van Bruchem G.D., Sonne K., Enninga I.C., van Rhijn J.A., Heskamp H., Huvenneers-Oosprong M.B.M., van der Hoeven J.C.M., Kuiper H.A. (2002): Absorption of a mutagenic metabolite released from protein-bound residues of furazolidone. *Environmental Toxicology and Pharmacology*, 11, 273–287.
- Horne E., Cadogan A., O'Keefe M., Hoodenboom L.A.P. (1996): Analysis of protein-bound metabolites of furazolidone and furaltadone in pig liver by high-performance liquid chromatography mass spectroscopy. *Analyst*, 121, 1463–1468.
- Howard G.C., Bethell D.R. (2000): *Basic Methods in Antibody Production and Characterization*. CRC press LLC, USA, 63–70.
- Kolar V., Deng A., Franek M. (2002): Production and characterization of generic antibodies against s-triazine and sulfonylurea herbicides. *Food and Agricultural Immunology*, 14, 91–105.

- Leitner A., Zollner P., Linder W. (2001): Determination of the metabolites of nitrofurantoin antibiotics in animal tissue by high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 939, 49–58.
- McCracken R.J., Kennedy D.G. (1997): Determination of the furazolidone metabolite, 3-amino-2-oxazolidinone, in porcine tissues using liquid chromatography-thermospray mass spectrometry and the occurrence of residues in pigs produced in Northern Ireland. *Journal of Chromatography B*, 691, 87–94.
- McCracken R.J., Blanchflower W.J., Rowan C., McCoy M.A., Kennedy D.G. (1995): Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectrometry and a study of the pharmacokinetics and stability of its residues. *Analyst*, 120, 2347–2351.
- Pasqualini R., Arap W. (2003): Hybridoma-free generation of monoclonal antibodies. *Proceedings of the National Academy of Sciences USA*, 101, 257–259.
- Soliman M.M., Long A.R., Barker S.A. (1990): Method for the Isolation and Liquid Chromatographic Determination of Furazolidone in Chicken Muscle-Tissue. *Journal of Liquid Chromatography*, 13, 3327–3337.
- Zeravik J., Skryjova K., Nevorankova Z., Franek M. (2004): Development of direct ELISA for the determination of 4-Nonylphenol and octylphenol. *Analytical Chemistry*, 76, 1021–1027.

Received: 05–05–17

Accepted after corrections: 05–06–06

Corresponding Author

Dr. Milan Franek, DrSc., Veterinary Research Institute, Hudcova 70, 621 32, Brno, Czech Republic
Tel. +420 533 331 901, fax +420 541 211 229, e-mail: franek@vri.cz
