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Analytica Chimica Acta 520 (2004) 79–86
ANALYTICA CHIMICA ACTA
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Production and characterisation of polyclonal antibodies to a derivative of 3-amino-2-oxazolidinone, a metabolite of the nitrofuran furazolidone

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Received 24 November 2003; received in revised form 12 May 2004; accepted 14 May 2004

Abstract

Polyclonal antibodies were produced to detect 3-amino-2-oxazolidinone (AOZ), a stable metabolite of the nitrofuran antibiotic furazolidone, following derivatisation with *o*-nitrobenzaldehyde. A carboxyphenyl derivative of AOZ was prepared, purified and conjugated to immunogenic carrier protein. Six antisera were produced from the immunisation of seven rabbits using various immunogen doses and time-scales. IC_{50} values, as determined by competitive enzyme-linked immunosorbent assay (ELISA) suggested that reducing immunogen dose from 0.3 to 0.05 mg, while lengthening rest periods between booster immunisations from 2 to 8 weeks, increased the sensitivity of the antibodies to 3-{[(2-nitrophenyl)methylene]amino}-2-oxazolidinone (NPAOZ) from 3.8 to 0.3 $\mu g\,l^{-1}$. An IC_{50} of 0.065 $\mu g\,l^{-1}$ (AOZ in the form of NPAOZ) was achieved with antiserum R670 by altering ELISA conditions. This antibody was highly specific for NPAOZ and did not cross-react with various nitrofuran metabolites, their nitrophenyl derivatives or a range of veterinary drugs. Antibody R670 is suitable for incorporation into an immunoassay for AOZ with sufficient sensitivity to satisfy current criteria for monitoring of veterinary drug residues. This is the first publication of an antibody for detection of a nitrofuran metabolite.

Keywords: Nitrofuran metabolites; AOZ; Furazolidone; Antibodies; Maturation; Cross-reactivity

1. Introduction

Furazolidone [*N*-(5-nitro-2-furfurylidene-3-amino)-2-oxazolidinone] belongs to the class of 5-nitrofuran antibiotics and has been widely and effectively used for the prevention and treatment of gastrointestinal infections caused by *Escherichia coli* and *Salmonella* spp. and as a growth promoter in cattle, pigs and poultry. However, the European Union (EU) was unable to assign a maximum residue limit for furazolidone because of the potential carcinogenic effects of its residues on human health [1]. As a consequence, the administration of furazolidone to food-producing animals was prohibited by placing it in Annexe IV of Council Regulation 2377/90 [2] in 1995 [3]. EU Member States are required to monitor compliance with the prohibition on the use of banned substances through their annual national residues control plans [4]. However, monitoring residues of the parent drug furazolidone is unsuitable because of the rapid excretion of the nitrofurans and their marked instability in vitro and in vivo [5,6]. It is possible, nevertheless, to monitor tissue-bound metabolites of furazolidone containing the 3-amino-2-oxazolidinone (AOZ) moiety, which is released upon mild acid hydrolysis. AOZ (Fig. 1) has been shown to persist in pig tissues for at least six weeks after drug withdrawal [7] in contrast to the disappearance of furazolidone from tissue within 12 h of withdrawal [6]. Furthermore, AOZ residues are stable in tissue, even after long-term storage [8].

Unlicensed preparations of furazolidone have been seized previously by customs officials within the EU, suggesting there may be a black market for this antibiotic. Furthermore, during 2002 a number of European laboratories reported finding tissue-bound residues of nitrofurans in poultry and aquaculture products imported from a range of countries in S.E. Asia and Brazil [9–11]. The most common nitrofuran-related residue detected in these products was

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doi:10.1016/j.aca.2004.05.074

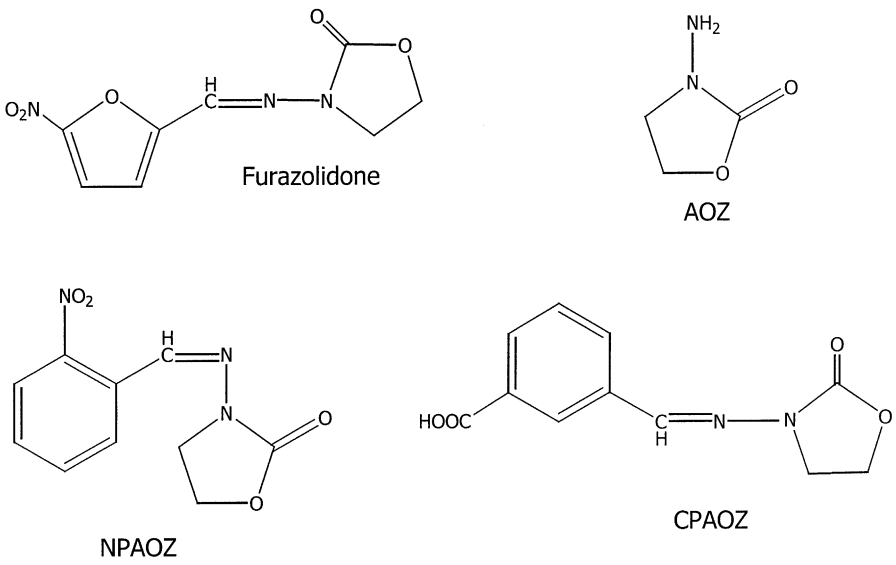

Fig. 1. Chemical structures of the banned nitrofuran furazolidone, its marker residue AOZ, the target derivative NPAOZ and the immunogen hapten CPAOZ.

AOZ. These findings resulted in the publication of two European Commission Decisions [12,13]. These required Member States to test all consignments of aquaculture products from Vietnam and Thailand and all consignments of poultry from Thailand for the presence of tissue-bound nitrofuran residues. Only those consignments shown to be free of nitrofuran residues would be permitted to enter the EU. In late 2002, poultry imports from Brazil were placed under similar restrictions [14]. During the early months of 2003 tissue-bound residues of furazolidone and, predominantly, another nitrofuran, furaltadone, were also reported in poultry meat produced within the EU (Portugal) [15], pork meat purchased within three EU countries (Portugal, Italy and Greece) [16] and egg powder produced in India [17]. Furthermore, later in 2003 the European Commission issued notifications to Member States via its Rapid Alert System for Food and Feed concerning findings of AOZ in fish (tilapia and milkfish) from Taiwan, crayfish and salted hog casings from China, prawns from Bangladesh, catfish from Thailand, egg powder from Mexico and poultry meat products from Romania and Bulgaria [18]. In Northern Ireland, exploratory testing of prawn shipments from various countries detected nitrofuran residues in samples originating in Thailand, Vietnam, China, India, Malaysia and Ecuador. Of these positive samples, 68% contained AOZ and 30% contained semicarbazide (SEM), indicating the use of the nitrofuran drugs furazolidone and nitrofurazone, which are known ingredients of the commercially available antibiotic preparations Furazan Gold and Furazone Green. Residues of nitrofuran antibiotics in meat of various species are clearly of topical importance and use of these drugs, in particular, furazolidone, is more widespread than previously thought.

Various methods have been published for the determination of AOZ by HPLC–UV [19–21], LC–MS [8] and LC–MS–MS [22]. However, with the advent of testing third Country imports and meat produced in the EU, there is now an urgent need for rapid, high capacity screening methods for tissue-bound residues of the nitrofurans. Currently, there are no immunoassay-based screening tests for any of the tissue-bound nitrofuran residues. Furthermore, no one has yet described the production of suitable antisera for incorporation into an immunoassay test. “FoodBRAND”, an EU-funded Research and Development project (QLK1-CT1999-00142), set out to (a) develop immunoassay and HPLC screening tests for residues of the nitrofurans, (b) develop LC–MS–MS based confirmatory tests for residues of the nitrofurans, (c) prepare deuterated nitrofuran internal standards for mass spectrometry, (d) conduct a pan-European survey of the presence of nitrofuran residues in retail pig meat, and (e) disseminate FoodBRAND and other findings to the European Consumer and the EU Community and National Reference Laboratory networks. Further project details can be found at the project website http://www.afsni.ac.uk/foodbrand.

This paper describes the production of polyclonal antibodies capable of detecting AOZ following derivatisation with *o*-nitrobenzaldehyde. These antibodies are suitable for incorporation into an enzyme-linked immunosorbent assay (ELISA) screening test, which would enhance the ability of Member States to fulfil European Commission Decisions 2002/250/EC and 2002/251/EC, Council Directive 96/23/EC and promote the safety of the European consumer.

2. Materials and methods

2.1. Materials

All chemicals, unless otherwise stated, were supplied by Sigma–Aldrich (Gillingham, UK). 3-Amino-2-oxazolidinone was obtained from the Sigma–Aldrich Library of Rare Chemicals (Gillingham, UK). 3–{[(2-Nitrophenyl)methyl-

ene]amino}2-oxazolidinone (NPAOZ) was a generous gift from Dr. Robert McCracken (DARD, Belfast, UK) while scavenger resin 3-(4-(hydrazinosulfonyl)phenyl)propionyl AM was obtained from CN Biosciences (Nottingham, UK). Horseradish peroxidase was obtained from Roche Diagnostics (Lewes, UK) and isobutyl chloroformate from Fluka (Gillingham, UK). Immunoassay Stabiliser blocking solution was supplied by Advanced Biotechnologies (Columbia, MD, USA) and Falcon microtitre plates by Becton-Dickinson (Franklin Lakes, NJ, USA). Trimethylbenzidine substrate solution was obtained from Chemicon (Temecula, CA, USA). LC–MS analyses were performed on a VG Platform LC–MS system (Micromass, Manchester, UK) with a Merck–Hitachi AS-2000A autosampler and L6000/L6200A pumps. System control and data handling were managed by MassLynx software (Micromass). LC–MS sample separation employed a PLRP-S column (Polymer Labs., UK) containing poly(styrene–divinylbenzene) copolymer and a mobile phase of 35% acetonitrile and 65% 0.1 M ammonium acetate. Buffer A was a 1 mM aqueous solution of sodium acetate. Buffer B was 0.1 M sodium phosphate buffer pH 7.2 in 0.15 M sodium chloride solution (saline) containing 0.05% polyoxyethylenesorbitan monolaurate (Tween 20).

2.2. Synthesis of AOZ derivative

A two molar excess of AOZ over 3-carboxybenzaldehyde (CBA) was refluxed overnight in dry pyridine over Type 3A molecular sieve. Thin-layer chromatography of the reaction mixture demonstrated formation of a product (elution in 5% methanol in chloroform; CBA R_F = 0.11 and product R_F = 0.03 visualised under UV light, AOZ R_F = 0.27 visualised by ninhydrin staining). LC–MS analysis demonstrated that over 99% of the CBA was derivatised. Residual unreacted CBA was removed by hydrazino scavenger resin treatment. Scavenging was carried out in dry pyridine containing 5% acetic acid at room temperature for three days, gently stirring an estimated twenty molar excess of resin over residual CBA. After removal of scavenger resin by filtration through porosity five sintered glass, the pyridine solution was diluted with water to approximately 7% pyridine, acidified with concentrated hydrochloric acid to pH 1–2, and extracted into 3 ml × 25 ml ethyl acetate. Residual water was removed from the ethyl acetate extract by shaking with anhydrous sodium sulphate. A pale yellow powder was recovered after evaporation of the ethyl acetate under a flow of nitrogen at 45 °C. LC–MS analysis demonstrated that this powder was CPAOZ, 3-{[(3-carboxyphenyl)methylene]amino}-2-oxazolidinone (Fig. 1), and was free from unreacted AOZ and CBA.

2.3. Preparation of CPAOZ conjugates

CPAOZ was conjugated to an immunogenic carrier protein human serum albumin (HSA) via an acid anhydride reaction scheme under basic conditions provided by *N*-methylmorpholine. The carboxylic acid on the hapten (Fig. 1) was activated with isobutylchloroformate to produce a mixed anhydride which then reacted with amine groups on HSA, forming an amide bond.

CPAOZ (4.1 mg) was dissolved in 0.4 ml dry *N*,*N*-dimethylformamide (DMF). This was equivalent to an approximately two molar excess over the amine groups found in 10 mg of HSA which contains 60 mol of amines per mol of protein [23]. *N*-Methylmorpholine (15 μl) was added. CPAOZ was then activated with isobutyl chloroformate (10 μl; approximately four molar excess over CPAOZ), stirring for 15 min at room temperature. Activated CPAOZ was added very slowly while stirring to 10 mg HSA dissolved in 1 ml of buffer A, then stirred for 2 h at room temperature. CPAOZ–HSA conjugate was purified by exhaustive dialysis against saline (4 × 4 l).

Cationised HSA was also prepared by conjugation of ethylenediamine to HSA via peptide linkages under weakly acidic conditions catalysed by ethyl(dimethylaminopropyl) carbodiimide hydrochloride for 3 h. Excess ethylenediamine was removed by Sephadex G25 gel filtration before the cationised protein solution was lyophilised and stored at 4 °C.

An immunogen containing the ethylenediamine spacer was prepared by conjugation of CPAOZ (4.1 mg) to cationised HSA (10 mg) via the acid anhydride scheme described above.

Protein concentrations of the immunogen solutions were estimated by the method of Lowry et al. [24] using HSA or cationised HSA calibration curves as appropriate. The incorporation rate of CPAOZ into protein was then estimated by the method of Habeeb [25] using the colourimetric reaction of trinitrobenzenesulphonic acid with free (unreacted) amine groups on the conjugated protein. The absorbance at 340 nm of the conjugated protein is expressed as a percentage of the absorbance at 340 nm of unconjugated protein at the same protein concentration as read from a linear HSA or cationised HSA calibration curve, to give the hapten incorporation rate.

CPAOZ (5.4 mg) was also conjugated to 10 mg horseradish peroxidase (HRP) via the acid anhydride scheme described above. As HRP possesses only two amine groups which are free to participate in conjugation reactions [26], this was equivalent to an approximately 50 molar excess over available amines. Peroxidase conjugate was stored at −20 °C as aliquots of a 50:50 glycerol mixture to minimise freeze–thaw damage.

2.4. Immunisation of rabbits

CPAOZ–HSA or CPAOZ-ethylenediamine–HSA immunogen, in 2 ml sterile saline, was added slowly while vortexing to 2 ml Freund's adjuvant. Immunogen emulsions were prepared by repeatedly passing this mixture through a narrow bored (1 mm) cylindrical Pyrex block, drilled

Table 1
Comparison of immunisation schemes and IC_{50} results of seven CPAOZ polyclonal antisera

Antibody	Immunogen	Scheme	Immunogen dose (mg)	Weeks between doses	Time elapsed (months)	IC_{50} ($\mu g\,l^{-1}$) for AOZ as NPAOZ
R659[a]	CPAOZ–HSA	A	0.3	2	1.5	3.8
R660	CPAOZ–HSA	A	0.3	2	1.5	No response
R661	CPAOZ-ed–HSA	A	0.3	2	1.5	4.0
R662	CPAOZ-ed–HSA	A	0.3	2	1.5	24.9
R676	CPAOZ–HSA	B	0.1	4	5	8.2
R677[a]	CPAOZ–HSA	B	0.1	4	5	0.5
R670[a]	CPAOZ–HSA	C	0.05	8	6	0.3

[a] Standard curves for the most sensitive antibody in each scheme are illustrated in Fig. 2.

at each end to accommodate two syringes. For the first inoculation, complete adjuvant containing heat-killed *Mycobactehum tuberculosis* was used. Subsequent injections used incomplete adjuvant.

Three schemes were used for immunising a total of seven rabbits (Table 1). Scheme A (two CPAOZ–HSA and two CPAOZ-ethylenediamine–HSA rabbits) employed 0.3 mg immunogen per injection, with booster injections every 2 weeks. Scheme B (two CPAOZ–HSA rabbits) employed 0.1 mg immunogen with booster injections every 4 weeks. Scheme C (one CPAOZ–HSA rabbit) employed 0.05 mg immunogen with booster injections every 8 weeks. Test serum samples were taken 10 days after each immunisation.

2.5. Determination of antibody titre

Test bleeds taken regularly from immunised rabbits were assessed for antibody content in an ELISA chequerboard titration using a direct competitive assay format. Test antiserum at four serial dilutions in buffer A was immobilised (100 μl per well) in 96-well Falcon microtitre plates overnight at room temperature. After removal of excess antiserum, four serial dilutions of CPAOZ–HRP conjugate in 50 μl assay buffer ($2\,g\,l^{-1}$ bovine serum albumin in buffer A) were applied to the plate in a 4 × 4 chequerboard formation. Negative controls (50 μl of buffer A) and positive controls (50 ng NPAOZ standard in 50 μl of buffer A) were applied along with the peroxidase conjugate dilutions to demonstrate displacement of the conjugate from binding to immobilised antibody. Plates were incubated for 2 h at 37 °C on a plate shaker then washed seven times with ELISA wash buffer (0.1% Tween 20 in saline). Substrate solution (100 μl trimethylbenzidine) was added and incubated for 15 min at 37 °C on a plate shaker. Reaction was halted by addition of 25 μl of 2.5 M sulphuric acid and absorbance was read at 450 nm on a Bio-Tek Instruments EL-312e plate reader with online Multicalc data reduction software (EG & G Wallac, Turku, Finland).

2.6. Assessment of antibody sensitivity

The competitive ELISA format described above was used to determine the sensitivity to free NPAOZ (Fig. 1) of test bleeds of the polyclonal antisera. Although the antisera were raised against a carboxyphenyl-AOZ hapten, sensitivity to nitrophenyl-AOZ was assessed with a view to the development of an ELISA for AOZ in tissue samples. Derivatisation of tissue with *o*-nitrobenzaldehyde, yielding NPAOZ from a sample containing tissue-bound residues of furazolidone, is well characterised and routinely used in mass spectrometric and HPLC determinations of AOZ [8,19]. Consequently, all sensitivity and specificity data in this paper are calculated on the basis of underivatised AOZ (M_r 102), a more relevant measurement than the derivatised form NPAOZ (M_r 236).

Optimum antiserum and peroxidase conjugate dilutions were determined previously by chequerboard titration. NPAOZ standards were prepared in the range 0–92.5 $\mu g\,l^{-1}$ (equivalent to 0–40 $\mu g\,l^{-1}$ of AOZ) in buffer B. Standard (50 μl) was added to 50 μl of peroxidase conjugate (in buffer B containing $2\,g\,l^{-1}$ BSA) in triplicate microtitre plate wells which had previously been blocked with Immunoassay Stabiliser solution (100 μl per well, 2 h at 37 °C) following antibody immobilisation. The mean absorbance of each standard was normalised against the mean absorbance of the zero standard (B/B_o). The midpoint of each displacement standard curve was calculated in order to determine its IC_{50}, that is, the standard concentration in $\mu g\,l^{-1}$ at the midpoint of the curve.

The antibody most sensitive to NPAOZ was selected, a final bleed taken from the appropriate rabbit, and sensitivity to NPAOZ defined further by performing ELISA analysis in buffer A on unblocked plates.

2.7. Assessment of antibody specificity

The cross-reactivity of the most sensitive NPAOZ antibody to various other veterinary drugs and metabolites was estimated using the procedure outlined by McCaughey et al. [27], determined by measuring their IC_{50} values in the competitive ELISA described above using the midpoint of the NPAOZ standard curve in all cases. Cross-reactivity values were calculated as follows:

$$\text{Cross-reactivity} = \frac{\text{IC}_{50}\text{ of NPAOZ}}{\text{IC}_{50}\text{ of competitor}} \times 100$$

3. Results

3.1. Preparation of CPAOZ conjugates

In the CPAOZ–HSA immunogen, approximately 42% of the amine groups on HSA were conjugated to hapten, equating to 25 mol of CPAOZ per mol of HSA (assuming all 60 amine groups in HSA are available for conjugation), as measured by the method of Habeeb [25]. Available amines in cationised (ethylenediamine) HSA were estimated by comparison with native HSA assayed in the same run. Cationised HSA contained approximately 25% more amines than native HSA, that is approximately 75 mol of amine groups per mol cationised HSA. Incorporation of hapten into cationised HSA was approximately 38%, equating to 28 mol of CPAOZ per mol of protein.

Incorporation of hapten into HRP is limited by the availability of only two amine groups for binding in each HRP molecule [26]. Being within the margin of error of the Habeeb method, incorporation into HRP cannot be accurately estimated but is likely to be less than 1 mol of CPAOZ per mol of HRP.

3.2. Antibody sensitivity

Comparisons of the immunisation schemes and IC_{50} values in the presence of NPAOZ for the seven polyclonal antisera are shown in Table 1. NPAOZ standard curves obtained using the most sensitive antiserum in each immunisation scheme (R659, R677 and R670) are illustrated in Fig. 2. These curves are based on interim test bleeds taken at different periods during immunisation. Table 1 and Fig. 2 suggest that reducing the dose of CPAOZ–HSA immunogen from 0.3 to 0.05 mg, while lengthening rest periods between booster immunisations from 2 to 8 weeks increased the sensitivity of the antibodies to NPAOZ from 3.8 to 0.3 $\mu g\,l^{-1}$ (IC_{50} calculated as AOZ).

R670, the most sensitive antibody for NPAOZ, was selected for further characterisation with regards to sensitivity and specificity and a final bleed was taken 28 weeks after first immunisation. Subsequent characterisation was performed using assay buffer A and unblocked plates. Fig. 3 illustrates a typical R670 NPAOZ standard curve, with an AOZ IC_{50} of 0.065 $\mu g\,l^{-1}$.

3.3. Antibody specificity

The cross-reactivity of antibody R670 with NPAOZ, other nitrofuran metabolites, their nitrophenyl derivatives, and various other veterinary drugs was determined by competitive ELISA as described above. Table 2 presents results as percentage cross-reactivity with respect to the target compound, NPAOZ. The data demonstrate that R670 was highly specific for NPAOZ, with negligible cross-reactivity with other metabolites, their derivatives or the derivatising agent *o*-NBA. The only significant cross-reactivity was with furazolidone (35.2%), the parent nitrofuran that yields AOZ as a metabolite. Furthermore, R670 exhibited negligible cross-reactivity with a range of nitroimidazole, tetracycline and sulphonamide drugs.

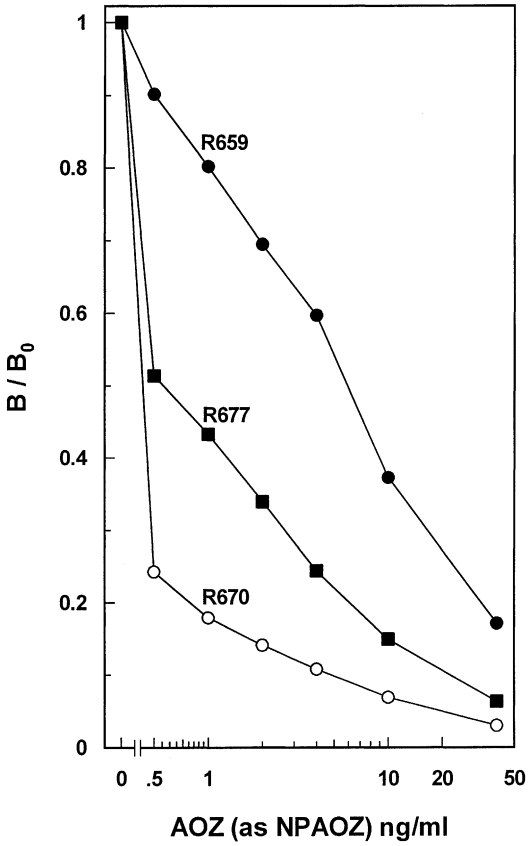

Fig. 2. Standard curves for the most sensitive antibodies in three immunisation schemes. B/B_0 is the normalised response relative to the zero standard.

Table 2
Cross-reactivity of antibody R670 with various drugs and marker residues

Competitor	Cross-reactivity (%)
(NP)AOZ	100
(NP)AHD, (NP)AMOZ, (NP)SEM	<0.01
AOZ	0.13
AMOZ, AHD, SEM	<0.01
o-NBA, CBA	<0.01
Furazolidone	35.2
Nitrofurazone	0.23
Nitrofurantoin, furaltadone, nifursol	<0.01
Dimetridazole, hydroxydimetridazole, ipronidazole, ronidazole, metronidazole	<0.01
Sulphamethazine, sulphadiazine	<0.01
Oxytetracycline, chlortetracycline	<0.01

In the case of the four nitrophenyl derivatives of the nitrofuran metabolites, concentrations were calculated on the basis of underivatised metabolites.

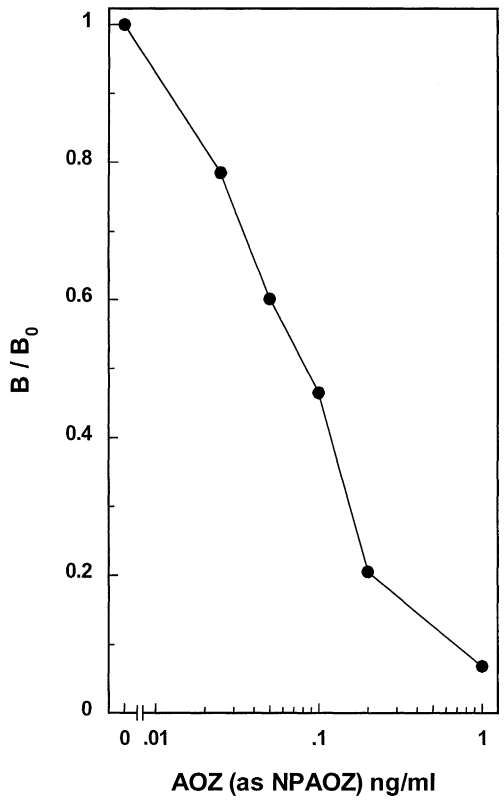

Fig. 3. Optimised ELISA standard curve for R670 AOZ antibody. B/B_0 is the normalised response relative to the zero standard.

4. Discussion

There is an urgent need for the development of immunochemical screening methods for detection of metabolites of the nitrofuran antibiotics. These drugs are prohibited for use in food-producing animals within the EU and all foods imported into the EU must be free of residues of the nitrofurans or their metabolites. However, 2002 and 2003 saw numerous reports of nitrofuran metabolites being detected in foods destined for the European consumer. These included prawns produced in Thailand, Vietnam, India, China, Ecuador and Bangladesh; poultry produced in Brazil, Thailand and Portugal; egg powder produced in India and pork purchased in Portugal, Italy and Greece. This heightened awareness of the prevalence of nitrofurans in food has been driven largely by European inspection laboratories and consumers groups involved in the FoodBRAND project co-ordinated by the authors of this paper.

Thousands of food samples imported into the EU are currently being subjected to nitrofuran analysis using expensive and time-consuming mass spectrometric technology. There is clearly a need for rapid and inexpensive screening techniques to process the increased volume of samples arising from European Commission Decisions and testing schemes initiated within EU Member States. In a survey of European National Reference Laboratories carried out by FoodBRAND in 1998, 55% of NRLs stated they wanted immunoassay test kits for nitrofuran metabolites for screening purposes. In light of recent revelations that nitrofurans are being found in a wide range of meat products, it is likely that this figure may be higher. However, there are currently no immunoassay-based screening tests available for any of the tissue-bound nitrofuran residues. Furthermore, no one has yet described the production of suitable antisera for incorporation into any such immunoassay test.

4.1. CPAOZ conjugates

We have now described the production of the first antibody capable of detecting a derivative of AOZ, a tissue-bound metabolite of the nitrofuran furazolidone. The antibody was raised against a carboxyphenyl derivative of AOZ. The use of this derivative served two purposes. Firstly, derivatisation introduced a carboxylic acid moiety into the hapten, which was a convenient functional group for conjugation to carrier protein (initial studies demonstrated that the hydrazide group in AOZ is not readily conjugated directly to protein). Secondly, by using CBA, we had also hoped that this structural element might have increased the chances of obtaining a generic antibody capable of cross-reaction with other similarly derivatised nitrofuran metabolites. This will be discussed below.

Determination of hapten incorporation into an immunogen conjugate by the method of Habeeb [25] is an indirect estimation, in that it measures a reduction in the number of available (reactive) amine groups following a conjugation reaction. Nevertheless it is a useful indicator of the success of a conjugation reaction (control analyses in the absence of hapten, and preliminary studies using inappropriate conjugation solvents such as pyridine, demonstrated that the presence of CPAOZ hapten was necessary to produce a reduction in the number of available amine groups). It was shown that CPAOZ successfully reacted with 42% of available amines in HSA, equating to 25 mol CPAOZ per mol HSA. Introduction of ethylenediamine spacers into HSA marginally increased incorporation to 28 mol/mol HSA, but, as shown in Table 1, did not improve on the sensitivity of antibodies raised against CPAOZ–HSA immunogens. The immunogenicity of the CPAOZ derivative was demonstrated clearly by six of the seven immunised rabbits producing detectable antibodies to NPAOZ (Table 1).

4.2. Antibody sensitivity

The use of three immunisation schemes allows a tentative comparison of the effects of size of immunogen dose and length of rest period between booster immunisations. Individual animals can vary greatly in the properties of their immune response to a given immunogen so a large number of animals would need to be immunised under different schemes to demonstrate conclusively the effects of these factors. However, some suggestions can be made from the six rabbits which produced antibodies to CPAOZ. When the

most sensitive antibodies from each immunisation scheme are compared (Table 1 and Fig. 2), it appears that reducing the dose of CPAOZ–HSA immunogen from 0.3 to 0.05 mg, while lengthening rest periods between booster immunisations from 2 to 8 weeks increased the sensitivity of the antibodies to NPAOZ from 3.8 to 0.3 $\mu g\,l^{-1}$ (IC_{50} calculated as AOZ).

These findings are in keeping with the well documented phenomenon of antibody affinity maturation [28,29], first described by Siskind and Benacerraf [30]. The clonal selection theory suggests that, following immunisation, the host lymphocytes possessing the highest affinity receptors will bind the greatest amount of antigen and will consequently be triggered to produce high-affinity antibodies. Such selection of high affinity lymphocytes will be most marked when the concentration of antigen is limited. Thus lower antigen concentrations (immunogen dose) may produce higher sensitivity antibodies, as demonstrated by the reduction of immunogen dose from 0.3 to 0.05 mg and the consequent increase in antibody sensitivity from 3.8 to 0.3 $\mu g\,l^{-1}$. Furthermore, as the concentration of circulating antigen diminishes over time, this antigen-driven selection will continue to increase antibody affinity [31]. Longer periods between booster injections will allow fuller clearance of circulating antigen, stimulating production of the highest affinity antibodies. Extended rest periods also allow the circulating level of antibody to drop sufficiently to prevent rapid clearance of newly injected antigen [29]. The current paper also supports this mechanism-the most sensitive NPAOZ antibody was obtained from the rabbit with the longest rest periods (8 weeks).

In addition to selection of high affinity lymphocytes, repeated immunisation induces somatic hypermutation within antigen-stimulated B cells further enhancing the affinity of the resulting antibodies [28]. Thus an extended period of immunisation may yield higher sensitivity antibodies as demonstrated by Rose et al. [32] in the production of antibodies to ceftiofur sodium. This is supported by the current paper wherein immunisation over 6 months yielded antibodies more sensitive to NPAOZ than immunisation over 6 weeks.

4.3. Antibody specificity

Increasing sensitivity during antibody affinity maturation may be accompanied by increasing specificity for the antigen. High affinity lymphocyte clones specific for the antigen come to dominate the circulating population at the expense of other less specific clones which may have cross-reacted with structurally related compounds. The cross-reactivity profile of an antiserum may therefore change over subsequent test bleeds [33]. The current paper also supports this mechanism. A short immunisation period (6 weeks, R659) yielded a NPAOZ antibody exhibiting 24% cross-reactivity with NPAHD (data not shown), a closely related nitrophenyl derivative of the metabolite (1-aminohydantoin) of the nitrofuran nitrofurantoin. A longer immunisation period (6 months, R670) yielded an antibody with the desired higher sensitivity but which exhibited less than 0.01% cross-reactivity with NPAHD. Thus the aim of raising a generic antibody capable of recognising NPAHD and other nitrofuran metabolite derivatives was not successful in this instance but was counterbalanced by the production of an antibody with high sensitivity for NPAOZ.

This most sensitive antibody (R670) demonstrates remarkable specificity for NPAOZ—its only significant cross-reactant is furazolidone, the parent compound of AOZ. However, furazolidone is known to be metabolised rapidly in vivo [6], and as a consequence such cross-reactivity is unlikely to occur in practice.

Currently, screening of samples for nitrofuran residues is carried out using liquid chromatography–tandem mass spectrometry. Such technology is capable of confirming the presence of AOZ in meat samples at around 0.3 $\mu g\,kg^{-1}$. The European Commission has now established a minimum required performance limit (MRPL) of 1.0 $\mu g\,kg^{-1}$ for the nitrofuran metabolites [34]. Antibody R670 exhibits an IC_{50} of less than 0.1 $\mu g\,l^{-1}$ in the absence of sample matrix. This antibody is therefore suitable for incorporation into an immunoassay test with sufficient sensitivity to meet the new EU MRPL and the demands of existing confirmatory analyses.

Acknowledgements

The authors acknowledge the financial support of the European Union for the project QLK1-CT1999-00142 “FoodBRAND” of which this work is a part.

References

[1] J.E.M. Van Koten-Vermeulen, M.F.A. Wouters, F.X.R. Van Leeuwen, Report of the 40th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), World Health Organisation, Geneva, 1993, p. 85.
[2] Council Regulation (EEC) 2377/90, Off. J. Eur. Commun. No. L224 (1990) 1.
[3] Commission Regulation (EC) 1442/95, Off. J. Eur. Commun., No. L143 (1995) 26.
[4] Council Directive (EEC) 96/23, Off. J. Eur. Commun., No. L125 (1996) 10.
[5] J.F.M. Nouws, J. Laurensen, Vet. Q. 12 (1990) 56.
[6] R.J. McCracken, W.J. Blanchflower, C. Rowan, M.A. McCoy, D.G. Kennedy, Analyst 120 (1995) 2347.
[7] R.J. McCracken, M.A. McCoy, D.G. Kennedy, Food Addit. Contam. 14 (1997) 287.
[8] R.J. McCracken, D.G. Kennedy, J. Chromatogr. B 691 (1997) 87.
[9] Food Standards Agency UK web site, news archive, http://www.foodstandards.gov.uk/news/newsarchive/51418, 15 March 2002.
[10] Food Standards Agency UK web site, press release, http://www.foodstandards.gov.uk/news/pressreleases/nitrofuran, 11 April 2002.
[11] Food Standards Agency UK web site, news archive, http://www.foodstandards.gov.uk/news/newsarchive/95293, 23 October 2002.

[12] Commission Decision (EC) 2002/250, Off. J. Eur. Commun., No. L84 (2002) 75.
[13] Commission Decision (EC) 2002/251, Off. J. Eur. Commun., No. L84 (2002) 77.
[14] Commission Decision (EC) 2002/794, Off. J. Eur. Commun., No. L276 (2002) 66.
[15] Food Standards Agency UK web site, press release, http://www.foodstandards.gov.uk/news/pressreleases/portugesechickens, 4 March 2003.
[16] Test Aschats magazine, 466, Test Achats, Brussels, June 2003, p. 8.
[17] AFSCA Belgium web site, press release, http://www.pressreleases.be/script_FR/newsdetail.asp?language=FR&ID=7532, 3 April 2003.
[18] European Commission Rapid Alert System for Food and Feed web site, http://europa.eu.int/comm/food/fs/sfp/ras_index_en.html.
[19] L.A.P. Hoogenboom, M.C.J. Berghmans, T.H.G. Polman, R. Parker, I.C. Shaw, Food Addit. Contam. 9 (1992) 623.
[20] A. Conneely, A. Nugent, M. O'Keeffe, Analyst 127 (2002) 705.
[21] A. Conneely, A. Nugent, M. O'Keeffe, P.P.J. Mulder, J.A. van Rhijn, L. Kovacsics, A. Fodor, R.J. McCracken, D.G. Kennedy, Anal. Chim. Acta 483 (2003) 91.
[22] A. Leitner, P. Zollner, W. Lindner, J. Chromatogr. A 939 (2001) 49.
[23] B. Meloun, L. Morávek, V. Kostka, FEBS Lett. 58 (1975) 134.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[25] A.F.S.A. Habeeb, Anal. Biochem. 14 (1966) 328.
[26] G.T. Hermanson, Bioconjugate Techniques, Academic Press, London, 1995.
[27] W.J. McCaughey, C.T. Elliott, S.R.H. Crooks, Food Addit. Contam. 7 (1990) 259.
[28] C. Berek, C. Milstein, Immunol. Rev. 96 (1987) 23.
[29] E. Harlow, D. Lane, Antibodies: a Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1988.
[30] G.W. Siskind, B. Benacerraf, Adv. Immunol. 10 (1969) 1.
[31] S.L. Kaattari, H.L. Zhang, I.W. Khor, I.M. Kaattari, D.A. Shapiro, Dev. Comp. Immunol. 26 (2002) 191.
[32] B.G. Rose, C. Kamps-Holtzapple, L.H. Stanker, Bioconjug. Chem. 6 (1995) 529.
[33] S. Pullen, B. Hock, Anal. Lett. 28 (1995) 781.
[34] Commission Decision (EC) 2003/181, Off. J. Eur. Commun. L71 (2003) 17.