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ELISA for semicarbazide and its application for screening in food contamination

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ABSTRACT

The development of a direct competitive enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies specific for semicarbazide (SEM) is described. Molecular modelling of the hapten mimics and other key components of the assay system was conducted to explain antibody properties in relation to hapten design. The small aliphatic molecule SEM was coupled to 3-carboxybenzaldehyde to produce carboxyphenyl-SEM (CPSEM), for the generation of specific antibodies. Five rabbits produced antibodies against NPSEM (used in direct and indirect ELISA formats) exhibiting a 50% binding inhibition level (IC_{50} values) of 0.06–2.28 $\mu g\,L^{-1}$ in assay buffer for SEM. The most sensitive indirect assay based on the antibody MVK39 showed a high dynamic range providing a linear readout in the range of 0.01–0.2 $\mu g\,L^{-1}$. Antibody MVK31 (IgG) allowed specific SEM detection at an $IC_{50} = 0.14\,\mu g\,L^{-1}$ in direct ELISA and was evaluated using solvent extracted SEM-spiked porcine and baby food samples. Recovery levels determined from fortified samples (0.5, 1.0, 1.5, 5, 10 and 20 $\mu g\,kg^{-1}$) of porcine and baby food ranged from 82.9 to 105.3%, respectively, with a coefficient of variation less than 15.5%. Respective detection capability and threshold of the assay for porcine muscle, set on the basis of acceptance of no false negative results, was 0.3 and 0.11 $\mu g\,kg^{-1}$.

1. Introduction

Nitrofurazone (NFZ), as well as other nitrofuran antibiotics, were used both prophylactically and therapeutically for various bacterial and protozoan infections in fish, shrimp, bees, swine, cattle and poultry, such as gastrointestinal enteritis (caused by *Escherichia coli*, and *Salmonella* spp.), fowl cholera and coccidiosis black-heads [1,2], and also as a growth promoter. Since 1995, all nitrofurans have been prohibited for use in livestock production due to concerns about the carcinogenicity of their residues in edible tissues [3].

Monitoring of illegal administration of the NFZ parent compound is currently carried out by detection of the marker residue semicarbazide (SEM, hydrazinecarboxamide) which is the metabolite moiety derived from NFZ. Nitrofurazone is metabolised rapidly *in vivo*, forming SEM tissue bound residues in livestock. The common method of SEM analysis involves acid hydrolysis for the release of tissue bound residues in a sample, followed by derivatisation with *o*-nitrobenzaldehyde (*o*-NBA) to increase molecular mass prior to detection [2]. The formed structure is the derivatised analyte nitrophenyl semicarbazide (NPSEM). Analysis based on this approach provides data on total SEM content (bound and free) in tissue such as muscle, liver, kidney, egg, retina and prawn [4–9].

Recent research has proven that SEM formation is not only possible through the metabolism of NFZ but also through the thermal decomposition of azodicarbonamide, a blowing agent

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used in the gasket production of metal lids for certain jarred foods. SEM has been detected as a product of the decomposition reaction and its migration to the jars edible content has been confirmed [10]. This issue concerns a variety of products such as jams, honey, baby food, fruit juices, pickles, sterilised products, mayonnaise, mustard and ketchup, and due to concerns of potential harm through ingestion of contaminated food products, azodicarbonamide is banned in the EU [11]. Azodicarbonamide was also used as an additive in flour and SEM occurrence during the heating process of bread was confirmed in bakery products [12,13] as well as flour-coated poultry products [14]. SEM formation has also been observed in samples treated with hypochlorite solutions used for water disinfection or bleaching such as shrimp, chicken, soybean, carrageenan (a seaweed extract used as a food additive), starch and egg white powder [15,2].

A minimum required performance limit (MRPL) of 1 $\mu g\,kg^{-1}$ has been set for all protein bound nitrofuran metabolites (including SEM) in edible tissues of animal origin [3], however, the MRPL for SEM in other matrices has not yet been declared. The presence of SEM in jarred baby food has particularly caused great concern for infant health and has resulted in the evaluation of newly established LC–MS/MS methods [16,17]. The European Commission, Directorate General for Health and Consumer Protection (DG SANCO), organised an interlaboratory study to demonstrate the effectiveness of LC–MS/MS methods for SEM determination in baby food samples at a potential EU regulatory limit of 10 $\mu g\,kg^{-1}$ [17]. From the results, it appears that the analytical methods based on this instrumental technique can be used for regulatory purposes in the determination of SEM in baby food.

Methods such as enzyme-linked immunosorbent assay (ELISA) can provide an inexpensive, sensitive and fast screening alternative for the detection of samples containing trace amounts of low-molecular weight analytes such as nitrofurans [5,18–20]. In principle, specifically tailored antibodies are capable of detecting nitrofuran metabolites through the use of an enlarged target molecule, which can be formed by a simple derivatisation step [19,21]. Recently, the first antibodies specific for derivatised SEM were produced in two laboratories. The polyclonal antibodies developed by Cooper *et al.* [18] using 3-carboxyphenyl SEM were incorporated into a competitive direct ELISA providing a detection capability for SEM in chicken muscle at 0.25 $\mu g\,kg^{-1}$. Monoclonal antibodies raised against the 4-carboxyphenyl SEM hapten by Gao *et al.* [20] exhibited comparable sensitivity in assay buffer, however, no data on the derivatisation reaction and assay performance was reported.

Our paper presents development of two ELISA formats based on antibodies against 3-carboxyphenyl SEM applicable for samples of animal origin and baby food. The methodical approaches employed in this study for antibody development are based in part, on previous nitrofuran metabolite papers [8,19,21], and include hapten design, antibody production, assay development and evaluation. Moreover, molecular modelling of the hapten mimics, and other key components of the assay system were conducted to predict and interpret the character of the binding interaction between the analyte structure and formed antibody. Applicability of this immuno-analytical strategy as a model for other low-molecular aliphatic structures is discussed.

2. Experimental

2.1. Chemicals and reagents

3[(3-Carboxyphenyl)methylene]-hydrazinecarboxamide (CPSEM) was synthesised at Biosfor (J. Socha, Na Labisti 533 53 009, Pardubice, Czech Republic). 3[(2-Nitrophenyl)methylene]-hydrazinecarboxamide (NPSEM) was purchased from Witiga Laboratorien (Berlin, Germany). Semicarbazide (SEM) hydrochloride, nitrofurazone (NFZ), *o*-nitrobenzaldehyde (*o*-NBA), 3-carboxybenzaldehyde (3-CBA), bovine serum albumin (BSA), porcine thyroglobulin, (TG), ovalbumin (OV), *N*-hydroxysuccinimide (NHS), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant, glycerol, Sephadex G-25 and ethyl acetate were obtained from Sigma–Aldrich (Steinheim, Germany). Peroxidase Type II from horseradish (HRP), dimethyl sulphoxide (DMSO), polyethylene-sorbitan-monolaurate (Tween20), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), *N*,*N*-dimethylformamide (DMF), and (hydroxymethyl)-aminomethane were obtained from Sigma (St. Louis, USA). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). Swine immunoglobulin against rabbit immunoglobulin (SwAR)–HRP was purchased from Servaphrama a.s. (Prague, Czech Republic). Methanol, *n*-hexane and all other chemicals were of HPLC or gradient grade and purchased from Merck KGaA (Darmstadt, Germany). Affinity Pak™ Protein A Immobilization Kit used to purify the antibody and was purchased from Pierce, (IL, USA).

2.2. Instruments

A homogeniser (Ultra-Turax T18, IKA, Germany), vortex machine (MS3 basic, IKA, Germany), water bath (WB14, Memmert, Germany), centrifuge (CR 3-22, Juan, France), sample concentrator (Techne DB-3D, East Port, United Kingdom) were used for sample extraction and preparation. Maxisorp microtitre plates were supplied by NUNC (Roskilde, Denmark) and Vivapore 5 mL concentrators from Vivascience (Hannover, Germany). An automatic plate washer (ELX50, BIO-TEK Instruments, Vermont, USA) was used for washing microplates. Absorbance was measured using an EL 808 Ultra microplate reader and processed by KC4™ v3.1 software (BIO-TEK Instrumentations, Vermont, USA).

2.3. Buffers and solutions

The following buffers were used in the experiments: (1) 10 $mmol\,L^{-1}$ phosphate buffered saline containing 145 $mmol\,L^{-1}$ NaCl (pH 7.2) (PBS) for dilution of antibody and sample extracts and preparation of calibration standards; (2) 50 $mmol\,L^{-1}$ *o*-NBA in DMSO for the derivatisation of SEM; (3) 2 $mol\,L^{-1}$ NaOH in 0.1 $mol\,L^{-1}$ PBS concentrate for sample neutralisation; (4) 50 $mmol\,L^{-1}$ carbonate buffer (pH 9.6) as a coating buffer; (5) PBS with 0.5% (w/v) BSA (pH 7.2), for tracer dilution; (6) PBS with 0.1% Tween-20 as a washing