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Development and validation of an indirect competitive enzyme-linked immunosorbent assay for monitoring organoarsenic compounds in edible chicken and pork and feed

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ABSTRACT

For the first time in this study, we used molecular modelling to design a suitable hapten (arsanilic acid, ASA) and produced a broad-specificity monoclonal antibody (mAb). This mAb exhibited the IC_{50} for ASA was 913.7 μg L^{-1} and showed the cross-reactivity to ASA (100%), carbarsone (849.2%), and nitarsone (1159.5%), respectively. Based on this mAb, an optimised indirect competitive enzyme linked immunosorbent assay (ic-ELISA) protocol was developed to monitor organoarsenic compounds (OAs) in edible chicken and pork and feed, which the detection limit for OAs in a muscle matrix ranged from 74.2 μg kg^{-1} to 143.3 μg kg^{-1} and in a feed matrix ranged from 7.4 mg kg^{-1} to 11.8 mg kg^{-1}, the recoveries were 61.3–109.6% with a coefficient of variation of less than 10.8%. These data demonstrated that the developed ic-ELISA is a reliable and useful tool for screening OAs in edible chicken and pork and feed.

1. Introduction

To increase weight gain, improve feed efficiency, and improve pigmentation, organoarsenic compounds (OAs) such as arsanilic acid (ASA), nitarsone (NPA), roxarsone (ROX), and carbarsone (CBA) have been widely used as feed additives for intensive poultry and swine farming in the USA, Canada, Australia, and China (Chapman & Johnson, 2002; Ferslew & Edds, 1979; Aghajani & Hasani Amiri, 2013; Sarmah, Meyer, & Boxall, 2006). These OAs can also be used to control disease. For example, ASA and ROX have been used to prevent and treat coccidiosis in poultry and haemorrhagic enteritis in swine (Mcdougald et al., 1992), while NPA and CBA were drugs of choice to prevent and treat histomoniasis (blackhead) in turkeys, a disease caused by the protozoan *Histomonas meleagridis* (Silbergeld & Nachman, 2008).

However, many studies have noted that these OA additives can bioaccumulate in the body of an animal (Datta, Sarkar, Sharma, & Sand, 2006; Morrison, 1969) or even cause toxic effects in livestock, such as blindness, ataxia, muscle tremors, posterior paralysis and quadriplegia, when used at higher than the recommended levels (Kerr, Narveson, & Lux, 1969; Laster, Hoerr, Bilgili, & Kincaid, 1999; Lu et al., 2014; Zhu, Wang, Liu, Qin, & Zhou, 2014). On the other hand, most of these OAs in the feed are excreted in the manure unchanged and then released into the environment by application of the contaminated litter to agricultural fields (Fisher, Yonkos, & Staver, 2015). In the soil and water environment, OAs undergo biogeochemical degradation and are transformed into stable but more toxic inorganic arsenic species such as arsenite and arsenate, which may pose a potential risk to both human health and the environment (Datta et al., 2006; Zheng, Cai, & O'Shea, 2010; Zhu et al., 2014). Therefore, maximum residue limits (MRLs) for such OAs in edible animal tissue and maximum permissible limits in feed have been established (0.5 mg kg^{-1} in muscle, 2 mg kg^{-1} in other edible tissues (particularly liver), and 100 mg kg^{-1} in feed) in the USA and China.

Although regulations exist, noncompliance remains a serious issue because of the economic benefits of OAs in food animals. For instance, the Institute for Agriculture and Trade Policy (IATP) tested for total arsenic in retail packages of raw chicken and in "fast food" chicken sandwiches and nuggets in 2004 and 2005. The results strongly suggest that the use of arsenic-containing compounds in poultry feed leads to arsenic residues in chicken marketed and eaten in the U.S., and as a result, the FDA announced that the Zoetis and Fleming Laboratories would voluntarily withdraw current ROX, ASA, and CBA approval, leaving only NPA approval in place in September 2013 (FDA, 2013). Therefore, the

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need for an effective monitoring programme for OAs to be put in place is clear to avoid the misuse of this class of additives.

Numerous methods, including spectrophotometric (Roerdink & Aldstadt, 2005), HPLC (Chen et al., 2011; Cui, Xiao, Dai, Zhao, & Wang, 2013), gas chromatography–mass spectrometry (GC–MS) (Roerdink & Aldstadt, 2004), capillary electrophoresis (CE) (Li & Hu, 2011), and liquid chromatography–mass spectrometry (LC–MS) (Liu, Zhang, Hu, & Cheng, 2013), have been previously published for the determination of OAs. Although highly sensitive and reliable, with limits of detection (LOD) that ranged from 0.092 ng mL^{-1} to 2 μg mL^{-1}, such methods often require extensive sample clean-up or derivatization as well as sophisticated laboratory equipment with highly trained operators and are unsuitable for a high sampling frequency or the rapid assessment of results. Thus, it is necessary to develop a rapid, high-throughput screening method to monitor the residues of OAs in edible animal tissue and feed.

In practice, an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has high-sensitivity, low-cost and the ability to screen numerous samples and is the most popular bioassay for the detection of drug residues in animal tissues. Until now, only one specific polyclonal antibody against ROX has been produced by Shelver and was used to develop an ELISA method with a LOD of 1.5 ng mL^{-1} for the determination of ROX in animal feed (Shelver, 2011). To the best of our knowledge, no studies have been published regarding the production of a broad-specificity antibody against OAs or the development of ic-ELISA methods for the simultaneous determination of OAs in edible animal tissue and feed.

The purpose of this study was to develop and validate an ic-ELISA method for the simultaneous determination of OAs in animal tissue and feed. Therefore, ASA was selected as a hapten and was coupled with carrier proteins to produce an immunogen to prepare a broad-specificity monoclonal antibody (mAb) against OAs. The most sensitive hapten and generic mAb were used to develop an ic-ELISA with high sensitivity and low cost for the determination of OAs in edible animal tissue and feed that did not require complicated sample preparation and clean-up. Finally, the developed ic-ELISA was compared with HPLC results in an analysis of edible chicken and swine feed samples containing ASA.

2. Materials and methods

2.1. Chemicals

Standards, including ASA, CBA, NPA and ROX, were purchased from Sigma–Aldrich (St. Louis, MO, USA). These four standards were over 97% pure. N-dimethylformamide (DMF), 3,3′,5,5′-tetramethylbenzidine (TMB), bovine serum albumin (BSA), ovalbumin (OVA), N-hydroxysuccinimide (NHS), glutaraldehyde (GA), polyethylene glycol 4000 (PEG 4000, 50%), peroxidase-labelled goat anti-mouse immunoglobulins (HRP-IgG), dimethyl sulfoxide (DMSO), hypoxanthine–aminopterin–thymidine (HAT) and hypoxanthine–thymidine (HT) medium supplements, complete and incomplete Freund's adjuvants, and RPMI-1640 culture media were also purchased from Sigma–Aldrich (St. Louis, MO, USA). Foetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were all of analytical grade.

2.2. Synthesis of antigens

2.2.1. Synthesis of ASA–BSA/$OVA_{(DIA)}$

The hapten ASA was diazotized (DIA) and coupled to the carrier protein BSA (or OVA) to prepare the antigen ASA–$BSA/OVA_{(DIA)}$ based on the modified method of Degand, Bernes-Duyckaerts, and Maghuin-Rogister (1992). Briefly, the hapten ASA (21.7 mg) was dissolved in 1 mL HCL (0.1 mol L^{-1}). A $NaNO_2$ solution (100 mg mL^{-1}, 0.6 mL) was added dropwise to this mixture with stirring in the dark at 4 °C. After 30 min, the reaction was stopped by addition of an ammonium sulphamate (100 mg mL^{-1}, 0.3 mL) solution. Then, the solution of the diazo derivative was added in 50-μL portions to 6 mL of carbonate buffered saline (CBS, pH 9.6), containing BSA (70 mg) or OVA (132 mg). During this operation, the pH was maintained at a value of 9.5 by the addition of 1 mol L^{-1} NaOH. The reaction mixture was left for 6 h at 4 °C and then dialysed against PBS (0.01 mol L^{-1}, pH 7.4) for 72 h. The dialysed solutions were stored at −20 °C until use.

2.2.2. Synthesis of ASA–BSA/$OVA_{(GA)}$

The hapten ASA was conjugated to BSA/OVA via a linker GA following the modified procedure of Peng et al. (2012) to prepare ASA–$BSA/OVA_{(GA)}$. Briefly, 21.7 mg of the hapten ASA was dissolved in 1 mL of DMF. The solution was added slowly to 70 mg of BSA (or 132 mg of OVA) with 15 mL CBS (pH 9.6) with stirring. Then, 0.1 mL 25% GA was added dropwise. After incubation for 6 h at 4 °C, the solution was centrifuged and the supernatant was dialysed against PBS (0.01 mol L^{-1}, pH 7.4) for 72 h. The dialysed solutions were stored at −20 °C until use.

Verification of conjugate synthesis and estimation of the hapten/protein ratio were performed using an 8453 UV–Visible spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) and a Matrix-assisted laser desorption ionisation time-of-flight tandem mass spectrometer (MALDI-TOF-MS) (Applied Biosystems, Foster City, CA, USA). The mass spectrometer was equipped with an electrospray ionisation (ESI) source and operated in the positive mode. Liquid nitrogen was used as the nebulising gas at a flow rate of 1.5 L min^{-1}. The capillary and skimmer voltages were set at 4.5 kV and 1.6 kV, respectively. The CDL and heat block temperatures were both maintained at 200 °C. With the 8453 UV–Visible spectrophotometer, the number of ASA residues conjugated to the carrier molecules was estimated according to the ultraviolet absorbance spectra of the haptens, carrier proteins, and conjugates as follows: $[\varepsilon_{(\text{conjugation})} - \varepsilon_{(\text{protein})}]/\varepsilon_{(\text{hapten})}$, where ε is the absorbance coefficient of the analytes. Based on the MALDI-TOF-MS, the number of ASA residues conjugated to the carrier molecules was estimated according to the molecular weight of haptens, carrier proteins, and conjugates as follows: $[M_{(\text{conjugation})} - M_{(\text{protein})}]/M_{(\text{hapten})}$, where M is the molecular weight of analytes.

2.3. Preparation of monoclonal antibodies

All animal experiments in this study adhered to the Huazhong Agricultural University animal experiment centre guidelines and were approved by the Animal Ethics Committee (HZAUCH2014-018). Eight female BALB/c mice (6–8 weeks old), purchased from the Hubei Center for Disease Control and Prevention (Wuhan, China), were inoculated with the immunizing conjugates (ASA–$BSA_{(DIA)}$ and ASA–$BSA_{(GA)}$). The first dose consisted of 50 μg of conjugate that was intraperitoneally injected in an emulsion of PBS and complete Freund's adjuvant. Two subsequent injections were given at 2-week intervals that were emulsified in incomplete Freund's adjuvant. One week after the last vaccination, serum was collected from the caudal vein of each mouse, and the antiserum titre was determined by an indirect ELISA. Three days before cell fusion, the mouse that produced antiserum with a high titre was given a final soluble intraperitoneal injection of 200 μg of the conjugate without adjuvant in PBS.

Spleen cells of the immunized mice were fused with myeloma cells Sp2/0 at a ratio of 10:1 according to a previously published procedure (Zhou et al., 2014). Hybridoma from wells that showed