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A recombinant Fab fragment-based electrochemical immunosensor for the determination of testosterone in bovine urine

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Abstract

This work describes the development of an electrochemical, recombinant Fab fragment-based immunosensor for the detection of testosterone in bovine urine. The sensor comprised of a testosterone conjugate on the surface of screen-printed electrodes, and recognition followed by an anti-testosterone Fab fragment. The use of an IgG-horseradish peroxidase conjugate determined the degree of competition. Chronoamperometry at a potential of +100 mV, was chosen to reductively measure the product of the catalysis of 3,3′,5,5′-tetramethylbenzidine catalysis. ELISA was primarily used to investigate the assay system, prior to transferring to SPEs. The final Fab-based sensor exhibited the linear range of 300–40,000 pg/ml with limit of detection of 90 ± 13 pg/ml. Furthermore, the developed Fab sensor allowed for the determination of testosterone in bovine urine directly after dilution, omitting the necessity of extraction and hydrolysis. Comparison of administrated bovine urine samples between the developed Fab sensor and GC–MS data showed quantitative or semi-quantitative results and enabled identification of suspicious samples for further extensive analysis by established analytical techniques. With simple sample preparation, low limit of detection, and good repeatability, the proposed method can offer alternative advantages as a primary screening tool for meat quality control.

Keywords: Testosterone; Screen-printed electrode; Recombinant Fab; Immunoassay; Bovine urine

1. Introduction

Testosterone is an anabolic steroid often used for illegal growth promotion in livestock farming and thereby causing potential health risks to consumers. The use of steroids for growth promoting purposes in animals is banned in the European Union for food safety reasons (Directive 88/146/EEC). The determination of residues is required in animals and in fresh meat as given by the EU Directive (86/849/EEC).

There are various analytical methods used for the detection of testosterone in biological fluids including, HPLC (Li et al., 2002; Navajas et al., 1995), GC–MS (Samuels et al., 1998; Saudan et al., 2004) and LC–MS offers a simplified, specific and sensitive alternative to GC–MS (Leinonen et al., 2004). Whilst these techniques have been used as quantitative and confirmatory methods, they do need expensive instrumentation, specialized personnel and suffer from considerable time delays between sampling and obtaining results. These disadvantages limit their routine use and restrict assaying to extensive laboratory environments.

Immunological techniques are based on the molecular recognition of antigens by antibodies to form a stable complex which are capable of detecting low level concentrations of analytes in biological matrices. Various assays have been developed to measure testosterone in plasma (Rajkowski et al., 1989), serum (Dhar and Ali, 1992), and human urine (Al-Dujaili, 2006). The cumbersome handling procedures involved, hinder their use as on-site detection systems. By comparison, immunosensors combining sensitivity of the antibody–antigen interaction with other advantages, including cost-efficient, speed of analysis and portable screening, allows for fast, decentralised measurement away from the lab environment. They are widely applied in the area of human and animal health and in the food sector (D'Orazio, 2003; Mello and Kubota, 2002). Electrochemical-based screen-printed electrodes (SPE) provide an approach to develop cost-effective devices through disposable means and have been reported to measure steroids, like progesterone in cow's milk (Pemberton

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et al., 1998; Kreuzer et al., 2004) and estradiol in human serum (Pemberton et al., 2005).

Current immunoassays capable of detecting steroid hormones are still performed using polyclonal and monoclonal antibodies. It has been suggested that the main limitation of immunosensor technology is related to the antibodies and their properties (Hock, 1997). Steroid hormones are small, rigid, hydrophobic molecules with only a few functional groups capable of specific interaction with antibodies. Furthermore, they are poorly immunogenic in mice and rats; two species from which monoclonal antibodies are usually generated, making it difficult and expensive to produce. The supply of good polyclonal antibodies of uniform quality is not easy and requires continuous immunization of many laboratory animals (Killard et al., 1995). Antibody engineering provides excellent tools to tailor the properties of antibodies in respect to affinity, specificity and performance for different applications. The possibility to express soluble, active antibody fragments, Fab and scFv, in *E. coli* (Better et al., 1988; Bird et al., 1988; Huston et al., 1988) allows cost-effective production, feasible engineering of the binding properties and fusions with peptide tags enhancing purification, detection and immobilisation. Furthermore, the antibody phage display library technology has opened completely new possibilities to produce novel binding specificities avoiding the limitations inherent in the mammalian immune response, as reviewed in Hoogenboom (2005). A number of recent publications describe the development of recombinant antibody fragments to steroid analytics by antibody engineering techniques (Hemminki et al., 1998; Valjakka et al., 2002). To our knowledge, there is little information of the application of a recombinant Fab fragment to detection of testosterone in electrochemical immunosensor area.

2. Materials and methods

2.1. Chemicals and reagents

Testosterone, 19-nortestosterone, boldenone, methyltestosterone, methylboldenone, eticholanolone, epieticholanolone, epitestosterone, 4-androsten-3β,17β-diol, 4-androsten-3α,17β-diol, 2α-, 6β-, 16α-, and 16β-hydroxytestosterone were purchased from Steraloids Inc. (RI, USA). Bovine serum albumin (BSA), testosterone 3-(*o*-carboxymethyl)oxime: BSA conjugate, anti-mouse IgG peroxide conjugate, 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) were purchased from Sigma–Aldrich (Dublin, Ireland). The inks used in screen-printing (Electrodag® B-0851, 423-SS and 451-SS) were purchase from Acheson (Plymouth, UK). All other solvents and reagents were of analytical grade and were purchased from Sigma–Aldrich (Dublin, Ireland).

The anti-testosterone Fab fragment (clone 220) was isolated from a phage display library constructed from mice immunized with testosterone 3-(*o*-carboxymethyl)oxime: BSA (Sigma–Aldrich). The phage library construction, selection and clone analysis was essentially done as described in Pulli et al. (2005). The Fab clone 220 was isolated by selecting the library for four rounds with the immunogen, testosterone-CMO-BSA, immobilised on microtiter plate wells. For large-scale, soluble production of Fab 220, the Fab expression unit with six histidine residues at the C-terminus of the heavy chain was cloned into the pKKtac expression vector (Takkinen et al., 1991). The Fab 220 was produced in *E. coli* strain RV308 (ATCC 31608) by high cell density fermentation and purified by immunoaffinity and protein G chromatography as described in Nevanen et al. (2001).

2.2. Apparatus

Ninety-six-well flat-bottomed polystyrene microtiter plates were purchased from NUNC (Dublin, Ireland). Bio-Tek Instruments (VT, USA) supplied the microplate reader (model EL 311). Incubations at elevated temperatures were carried out in a thermostat oven supplied by Heraeus Instruments (Hanau, Germany). Electrodes were prepared using a DEK (model 247) screen-printer (Dorset, UK). Chronoamperometric measurements were performed using a potentiostat (μAUTOLAB) with GPES software (ECO-CHEMIE, Netherlands).

2.3. Urine sample preparation

Urine samples of heifers injected intramuscularly with testosterone (200 mg per animal) were provided by Dr. M. Crowe (Faculty of Veterinary Medicine, National University of Ireland, Dublin). The study was performed in compliance with protocols approved by the Ethics Committee, University College Dublin, the Cruelty to Animals Act (Ireland, 1876), and the European Union Directive 86/609/EC. An aliquot (1 ml) of the urine specimen of interest was defrosted and centrifuged at $3000 \times g$ for 3 min. The supernatant was collected, aliquoted (20 μl) and frozen at −20 °C until assayed to avoid repeated freeze–thaw cycles of samples.

2.4. ELISA protocol

Ninety-six-well ELISA plates were coated with 50 μl of the appropriate dilution of testosterone–BSA conjugate in phosphate buffered saline (PBS, pH 7.4) at 37 °C for 45 min. After washing three times with washing buffer (PBS containing 0.05% (v/v) Tween 20), 200 μl per well of blocking buffer (PBS containing 1% (w/v) BSA) was added and plates were then incubated at 37 °C for 45 min or at 4 °C overnight followed by washing as described above. Thereafter, an optimised, one-step incubation protocol was used for the remaining immunoreagents. A volume of 25 μl of each different concentration (0–1000 ng/ml) of testosterone standards, 25 μl of the suitable dilution of the Fab fragment and 25 μl enzyme labelled anti-species (α-IgG–HRP) were all added at the same time into the wells and incubated at 37 °C for 15 min. After a further washing step, 100 μl per well of substrate solution containing 0.1 mg/ml TMB and 0.2% (v/v) hydrogen peroxide in 0.05 M phosphate citrate buffer (pH 5, containing 0.1 M potassium chloride) were added and the wells, left to develop colour for 30 min at room temperature and subsequently stopped by the addition of 50 μl of 2 M sulphuric acid. Absorbance was read at 450 nm. Standard-dose response curves