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Electrochemical immunosensors for the detection of 19-nortestosterone and methyltestosterone in bovine urine

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Abstract

Concerns over the use of steroid hormones as growth promoters, have prompted the EU to prohibit their use in food producing animals. Subsequently, rigorous screening procedures have been implemented in all member states to detect the illegal administration of such compounds. In the presented work, disposable immunosensors for the detection of 19-nortestosterone and methyltestosterone in bovine urine have been developed using screen printed electrodes (SPEs). Competitive immunoassays were developed, initially by enzyme linked immunosorbent assay (ELISA), and subsequently transferred to an electrochemical immunosensor format. Horseradish peroxidase (HRP) was the enzyme label of choice and chronoamperometric detection was carried out using a tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) substrate system, at +100 mV. Assays comprised of an indirect format, with immobilised antigen-protein conjugate. The EC_{50} values obtained for nortestosterone immunosensor in urine were 936 pg/ml, with an LODs of 10.5 pg/ml. The competitive assay of methyltestosterone in urine yielded an EC_{50} of 274 pg/ml with an LOD of 14.8 pg/ml. Notable cross-reactivities of anti-nortestosterone were observed with boldenone, testosterone and α-testosterone. Cross-reactivity of anti-methyltestosterone was most significant with methylboldenone. Precision, accuracy and stability studies were satisfactory for both immunosensors.

Keywords: Nortestosterone; Methyltestosterone; Immunosensor; Bovine urine; Screen printed electrode

1. Introduction

Steroid hormones are involved in the physiological regulation of growth and development [1]. In the 1950s, the recognition of the growth promoting properties of these hormones led to their introduction as a tool to increase meat production [2]. This has led to attempts to increase the weight gain and feed conversion efficiency of animals reared for meat by supplementing their normal (endogenous) hormones with extra amounts, of either these steroid hormones or synthetic equivalents. The effect of these anabolic steroids is to increase lean tissue growth. Fat deposition is reduced and since fat is so energy dense, food conversion efficiency is increased. By these criteria alone, a healthier product is produced at less cost [3].

Concern over the use of hormone implants first surfaced during the late 1970s and early 1980s, when a number of incidents linked hormone residues in meat with health problems in humans [4,5]. This prompted the EU to introduce a number of directives culminating in a ban on the use of growth promoting hormones within the EU, except for therapeutic or other veterinary purposes. The importation or intra EU trade in meat from animals treated with growth promoting hormones was also banned [6]. Member states carry out the control of residues in accordance with council directive 96/23/EC [7]. These directives also specify the maximum residue limit (MRL) for each hormone residue. Two such steroids used as growth promoters in meat producing animals are 19-nortestosterone and methyltestosterone.

19-Nortestosterone (17β-hydroxyestr-4-en-3-one, 17β-19-nortestosterone, also named nandrolone) is a C_{18} anabolic steroid hormone, which differs from testosterone in that it does not possess a C-19 methyl group [8]. 19-Nortestosterone has been used as a growth promoting agent to accelerate weight gain and improve feeding efficiency in animals [9]. The structure of nortestosterone and its 17-epimer is shown in Fig. 1. The metabolism of nortestosterone strongly follows that of testosterone; the main metabolites have been confirmed as 3α-hydroxy-5α-estran-17-one (19-norandrosterone or 19-NA) and 3α-hydroxy-5β-estran-17-one (19-noretiocholanolone or

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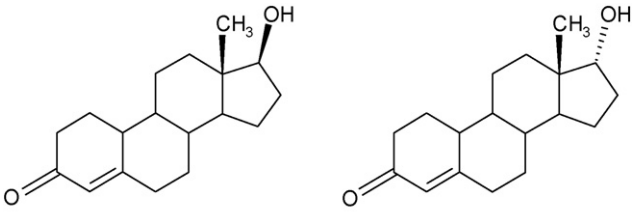

Fig. 1. Structures of 17β- and 17α-nortestosterone.

19-NE). The metabolism of 19-nortestosterone in bovines was reported by Samuels et al., which showed the presence of 17α-nortestosterone, four isomers of estrane-3,17-diol, estranetriol and 19-NE in the urine, independent of the route of administration [10].

Until 1984, the presence of nortestosterone in the urine of both humans and animals was considered to be proof of illegal administration [11]. At that time, it was proven that 19-nortestosterone was endogenous in the urine of stallions [12,13]. Studies in pigs, cows and sheep followed [14–16]. It was shown that α-nortestosterone is present in urine of pregnant cows at least 4 months before partus, 17-β-nortestosterone was not found. α-Nortestosterone was detected in the urine of pregnant sheep analysed by GC/MS, the β-epimer was not present in urine [17].

17α-Methyltestosterone (17β-hydroxy-17α-methyl-4-androstan-3-one) is a synthetic androgen, differing from testosterone by the presence of a methyl group at the C-17 position [18–20]. Methyltestosterone has been used to treat patients with androgen deficiency and infertility; it has significantly higher activity than testosterone. It is also one of the anabolic steroids illegally used as a growth promoter in cattle [18]. The structure of methyltestosterone is shown in Fig. 2. The metabolism of methyltestosterone in humans was investigated in 1962 by Rongone and Segaloff who identified 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol as the main metabolites [8]. Further studies by GC/MS found the main metabolites in human urine were the glucuronides of 17α-methyl-5α-androstan-3α,17β-diol and 17α-methyl-5β-androstan-3α,17β-diol [21]. The study of methyltestosterone metabolism has been performed in rabbits, heifers, bulls and cows [22,23].

The most popular techniques used for steroids hormones detection are GC/MS, LC/MS and various immunoassays. Bile samples from female cattle were analysed for 17β-nortestosterone and 17α-nortestosterone using GC/MS/MS [24]. 17β-Nortestosterone was not detected, 17α-nortestosterone was detected from around 120 days of gestation. Studies on the bile of male cattle concluded that 19α-nortestosterone was not endogenous in male cattle [25].

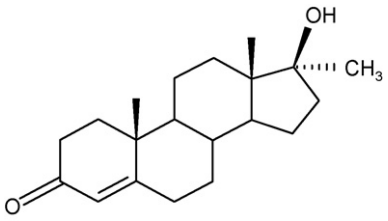

Fig. 2. Structure of methyltestosterone.

There has been much debate on the possible endogenous nature of 19-nortestosterone in humans. Studies have found links between strenuous physical activity and positive detection [26]. Le Bizec et al. studied the effect of consumption of boar meat on an athlete's urine. The group found that following consumption of boar meat the urine of the athlete was found to contain 19-NA, 19-NE and nortestosterone, after physical activity [27]. GC/MS was used as a detection method, with a LOD of 0.02 ng/ml [28]. Structural modifications of nortestosterone, such as esterification of the 17β-position, allow the compound to be administered via intramuscular injection [29–32].

Competitive EIA methods for determination of a number of anabolic residues including nortestosterone have been developed [33,34]. Concentrations of nortestosterone, which reduced the signal by 50% was reported as 8 pg/well. Roda et al. developed a competitive chemiluminescent (CL) enzyme immunoassay for analysis of 19-nortestosterone in bovine urine [35]. Van Look et al. developed a direct competitive immunoassay for the detection of nortestosterone in urine [36]. Van Puymbroeck et al. reported on the use of faeces as a matrix for the determination of anabolic steroids in cattle [37]. Casademont et al. reported on a method to determine 12 anabolic hormones, including 17α-methyltestosterone [38]. The LOD was 0.5 ng/ml and corrected recoveries from urine was quite low at 45% (±14.9%).

A number of chemiluminescent procedures have been used to detect methyltestosterone [39–42]. Van Peteghem et al. developed a chemiluminescence immunoassay for the detection of methyltestosterone residues in muscle tissue [43]. The antiserum was raised in a rabbit against methyltestosterone-3-carboxymethyloxime:bovine serum albumin (BSA). The LOD of the assay was between 7 and 21 pg, with a limit of quantification (LOQ) in muscle tissue of 0.125 ppb. Competitive EIA for anabolic androgens, including methyltestosterone, have been developed [33]. The concentration of methyltestosterone, which reduced the signal by 50% was reported as 6 pg/well. An RIA in combination with HPLC was developed to detect 17α-methyltestosterone in muscle tissue [44]. HPLC with UV spectrum diode array detection was used to analyse 117 samples of intramuscular application sites in slaughtered cattle. Of these samples, six tested positive for methyltestosterone [45].

The work presented describes the development of immunosensors to detect the presence of nortestosterone and methyltestosterone in bovine urine [46]. The format for both sensors is an indirect system, using immobilised testosterone–BSA conjugate, an antibody and a HRP-labelled anti-species. The electrochemical technique uses 3,3′,5,5′-tetramethylbenzidine (TMB) hydrogen peroxide (H_2O_2) substrate mixture. At present, there are no chronoamperometric immunosensors available for the detection of either analyte in this matrix. The minimum required performance limit (MRPL) for these sensors is 1 ppb. Limits detected by the immunosensor were considerably lower at <20 ppb bovine urine, a dilution step was used to minimize matrix effects. There are advantages to using these systems