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Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for C_{19}-Δ^{16}-Steroids in Sera of Boar Pigs

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A monoclonal antibody was prepared against 5α-androst-16-en-3-one, the major steroid compound responsible for the urine-like odor in the adipose tissue of uncastrated male pigs, and incorporated into a sensitive competitive indirect enzyme-linked immunosorbent assay (ELISA). The limit of detection for 5α-androst-16-en-3-one in the ELISA was 0.05 ng/mL (2.5 pg/assay). Cross-reactivities in the ELISA, measured as concentration (ng/mL) required to inhibit 50% binding of antibody to a 5α-androst-16-en-3-one–ovalbumin conjugate solid phase, were 3.2, 7.2, and 1.3 for 5α-androst-16-en-3-one, 5α-androst-16-en-3-α-ol, and 5α-androst-16-en-3β-ol, respectively. The latter two compounds, which also contribute to boar odor, thus exhibited 44% and 246% cross-reactivity relative to the parent compound. The assay was used directly for detection of the boar odor steroids in plasma and serum after appropriate dilutions without extraction. This rapid and sensitive monoclonal antibody-based ELISA should be readily applicable for the routine screening of pigs for boar odor on the assumption that a relationship exists between the concentration of the responsible odorous compounds in the blood and fatty tissues.

Meat from boars emits a strong characteristic perspiration-like or urine-like odor upon heating or cooking that is called boar odor or boar taint (Malfors and Lundstrom, 1983). Patterson (1968a), utilizing gas chromatography–mass spectrometry, isolated and identified 5α-androst-16-en-3-one as the compound responsible for the urine-like odor in the adipose tissue of the boar. Other researchers (Berry and Sink, 1971; Berry et al., 1971; Thompson et al., 1972; Thompson and Pearson, 1977) have confirmed this finding and identified two other C_{19}-Δ^{16}-steroids (5α-androst-16-en-3-α-ol and 5α-androst-16-en-3β-ol) as contributors to boar odor.

The C_{19}-Δ^{16}-steroids are synthesized in the Leydig cells of the testes and are delivered to the blood where they are taken up by the adipose tissue and stored in the fat as a result of their lipophilic characteristics (Brooks and Pearson, 1986). Concentrations of these steroids can reach the microgram per gram level in fat, and at these concentrations they are perceptible to the human senses (Brooks et al., 1986). The compound 5α-androst-16-en-3-one is thermally stable and is not greatly affected during processing of boar meat. However, heating in open containers may lead to a moderate decrease in the concentration due to the volatility of the C_{19}-Δ^{16}-steroids (Claus et al., 1985).

Although immunological procedures have recently been developed for blocking C_{19}-Δ^{16}-steroid production (Shenoy et al., 1982; Williamson and Patterson, 1982; Williamson et al., 1985; Brooks et al., 1986), the inhibition of these compounds is reported to vary from 50 to 90% (Brooks et al., 1986). The early methods (Patterson, 1968b; Berry and Sink, 1971; Thompson et al., 1972; Kauffman et al., 1976) for quantitating C_{19}-Δ^{16}-steroids involved extraction, steam or vacuum distillation techniques, and labor-intensive thin-layer chromatography for purification of the samples prior to gas chromatography–mass spectrometry (GC–MS) analysis. Other methods not only involve tedious techniques but also incorporate the use of expensive radioisotopes as internal standards in the gas chromatographic analysis (Claus and Hoffman, 1971).

Radioimmunoassays for 5α-androst-16-en-3-one utilizing rabbit antibodies have been described by Andresen (1974) and Claus (1974) but involve extraction of the steroid before estimation. While radioimmunoassay methods are faster and more sensitive than GC–MS techniques, they have the disadvantages of using costly and potentially hazardous radioactive materials. Furthermore, the specificity and affinity of polyclonal antibodies vary from animal to animal. Hybridoma technology provides the potential to produce high-affinity specific antibodies to steroids that can be used for diagnostic screening of the pigs for boar taint. Here we describe the development of a specific monoclonal antibody against 5α-androst-16-en-3-one and its utilization in a diagnostic enzyme-linked immunosorbent assay (ELISA) to measure the concentration of the C_{19}-Δ^{16}-steroids in the plasma or serum of sexually mature boars. The approach eliminates the need for tedious purification steps and instrumental analysis and facilitates rapid, efficient, and sensitive assay with large throughput of samples.

MATERIALS AND METHODS

Materials. All inorganic chemicals and organic solvents were of reagent grade. All steroids were purchased from Steroids, Inc. (Wilton, NH). Bovine serum albumin (BSA) (fraction V), ovalbumin (OA) (crude and fraction VII), polyethylene sorbitan monolaurate (Tween-20), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, dimethylformamide (DMF), poly(ethylene glycol) (PEG) (MW 1450), hypoxanthine, aminopterin, thymidine, and pristane were purchased from Sigma Chemical Co. (St. Louis, MO). Tetrahydrofuran was purchased from Aldrich Chemical Co. (Milwaukee, WI), while Freund's complete and incomplete adjuvants were obtained from Difco Laboratory (Detroit, MI). Goat anti-mouse IgG conjugated to horseradish peroxidase was obtained from Cooper Biomedical (Malvern, PA); Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin solution (pen/strep) (100 000

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U/mL), NCTC supplemental medium, fetal bovine serum (FBS), and sodium pyruvate were from Gibco Laboratories (Grand Island, NY). Microculture plates (96- and 24-well plates) were purchased from Costar (Cambridge, MA) and microtiter plates (Immunolon-2 Removawells) from Dynatech Laboratories. The immunoglobulin subclass identification kit was purchased from Boehringer Mannheim Biochemical (Indianapolis, IN). The myeloma cell line P3/NS 1/1-Ag4-1 (NS-1) (ATCC TIB 18) was purchased from The American Type Culture Collection (Rockville, MD). Mice (BALB/c) were purchased from Charles River Laboratories (Wilmington, MA).

Preparation of Immunogen. The *O*-(carboxymethyl)oxime of 5α-androst-16-en-3-one was prepared by the method of Erlanger et al. (1957) and separated by preparative thin-layer chromatography using hexane–ethyl acetate (8:2, v/v) as the solvent system. The 5α-androst-16-en-3-one oxime was conjugated to bovine serum albumin (BSA) for use as an immunogen and also to ovalbumin (OA) for use as a solid-phase antigen in the competitive indirect enzyme-linked immunosorbent assay by the modified activated ester method of Kitagawa et al. (1981). The number of moles of steroid conjugated per mole of BSA or OA was 23 and 29, respectively, as estimated by the method of Erlanger et al. (1959). The final solution of 5α-androst-16-en-3-one oxime–protein conjugate was adjusted to 1 mg/mL and stored at −80 °C for subsequent use.

Immunization. Four groups of 6–8-week-old female BALB/c mice (five each) received subcutaneous or intraperitoneal injections of 5α-androst-16-en-3-one oxime–BSA (30 or 50 μg of protein/mouse), which was emulsified with one volume of saline and 2 volumes of Freund's complete adjuvant. At 2-week intervals, two booster injections were given exactly as above, except that incomplete Freund's adjuvant was used. One week after the last injection, serum was obtained from the retrobulbar plexus of each mouse to determine titer value and antibody specificity. Three days before removal of the spleen for fusion, a subcutaneous injection of the conjugate in saline solution was given to the mouse whose antiserum showed the maximum inhibition by competitive ELISA.

Enzyme-Linked Immunosorbent Assay. An indirect ELISA (Dixon et al., 1987) was used to determine titer, sensitivity, and specificity of anti-5α-androst-16-en-3-one antibodies produced during immunization in mice sera or in culture following fusion and cloning. For titer determination assay, microtiter plates were coated overnight (4 °C) with steroid oxime–OA (5 μg/mL) in 0.01 M carbonate buffer (pH 9.6). The plates were washed four times with 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 0.05% (v/v) Tween-20 (PBS–Tween). To each well was added 300 μL of 1% (w/v) ovalbumin (crude) in PBS for 30 min to block the unbound sites of solid phase and to minimize nonspecific binding. After washing four times in PBS–Tween, 50 μL of the serially diluted mouse serum or hybridoma culture supernatant was added to each well and the resultant mixture incubated for 1 h at 37 °C. Wells were washed four times. Bound antibody was then determined after the addition of anti-mouse IgG peroxidase conjugate (100 μL/mL of 1:500 in 1% OA–PBS) and the resultant mixture incubated for 30 min. After the wells were washed eight times in PBS–Tween, bound peroxidase was determined with ABTS substrate, with the absorbance being read at 405 nm as described by Casale et al. (1988).

The competitive assay was essentially identical with titer determination, except that, after ovalbumin blocking and washing, 50 μL of 5α-androst-16-en-3-one standard (or plasma or boar serum) was added with 50 μL of the appropriate dilution of antisera or 50 μL of crude hybridoma culture. Standard 5α-androst-16-en-3-one and its related analogues were prepared fresh daily in PBS buffer from a stock solution containing 1 mg/mL in absolute ethanol.

Fusion, Cloning, and Production of Antibody. Mouse spleen cells (2×10^8) were fused with NS-1 myeloma cells (2×10^7) with PEG as the fusion reagent according to the protocol of Galfre and Milstein (1981). Following fusion, the cells were suspended in Dulbecco's modified medium containing 20% fetal bovine serum (20% FBS–DMEM) supplemented with 1% NCTC, 10 mM sodium pyruvate, and pen/strep solution, then distributed into the wells of microculture plates, and incubated at 37 °C in a humid atmosphere of 7% CO_2 in air. After 24 h, half of the supernatant fraction from each well was removed and an equal volume of selective growth medium containing hypoxanthine, aminopterin, and thymidine (HAT) in 20% FBS–DMEM was added. This was repeated every 3 days. After approximately 2 weeks, the HAT medium was eliminated by gradual replacement with HT medium (the same composition of HAT medium but without aminopterin). Supernatants from hybridoma cultures were tested for the presence of the specific antibody as described earlier. Contents of wells containing specific antibodies were transferred into six wells of another 96-well plate and fed with HT medium. Positive wells were expanded into 24-well plates and then into 50- and 250-mL tissue culture flasks. Hybrid lines were cloned by limiting dilution (Goding, 1980) in 20% macrophage-conditioned medium (Sugasawara et al., 1985). Subclones yielding antibody that showed at least 90% inhibition in CI-ELISA were stored in fetal bovine serum–dimethyl sulfoxide (9:1) under liquid nitrogen.

Table I. Titers and Percent Inhibition of Mouse Antibody Binding to 5-Androstenone Solid Phase after Three Subcutaneous and Intraperitoneal Immunization by 30 and 50 μg/Mouse 5α-Androst-16-en-3-one Oxime–BSA As Determined by Competitive Indirect ELISA

mouse gp	dose	immunizn[a]	mean titer	mean % inhibn
1	30	ip	1540 (100–3200)[b]	16 (5–35)[b]
2	50	ip	2880 (1600–3200)	32 (15–55)
3	30	sc	3200 (1600–6400)	48 (18–66)
4	50	sc	6400 (3200–12 800)	59 (12–86)

[a] Immunization: ip = intraperitoneal; sc = subcutaneous. [b] Range.

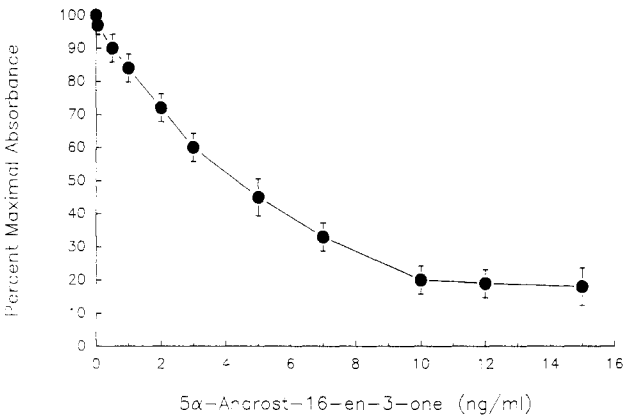

Figure 1. ELISA standard curve for 5α-androst-16-en-3-one.

Mass Production of Monoclonal Antibodies. BALB/c female and male mice were primed by intraperitoneal injection of 0.5 mL of pristane (Galfre and Milstein, 1983). After 10 days, each mouse received 5×10^6 cells suspended in 0.5 mL of 20% FBS–DMEM. Abdominal fluid was collected and pooled, and the antibody was purified by precipitation with 50% saturated ammonium sulfate (Hebert et al., 1973).

Estimation of 5-Androstenone in Plasma and Serum of Boars. The 5α-androst-16-en-3-one content in serum or plasma of boars was estimated by competitive indirect ELISA directly without any extraction or chromatographic procedures. Plasma or serum was diluted 100-fold in PBS in order to enable estimation in the linear part of the standard curve and to eliminate interference by serum components.

RESULTS AND DISCUSSION

Hybridoma Production. One week following the third injection, approximately half the immunized mice in each group exhibited specific antibodies to 5α-androst-16-en-3-one (Table I). Subcutaneous injection of 50 μg of antigen/mouse not only resulted in the highest end point titer (12 800) but also was more efficient in eliciting sensitive antibodies than that induced by intraperitoneal injection or by low dose (30 μg/mouse) antigen injection sub-

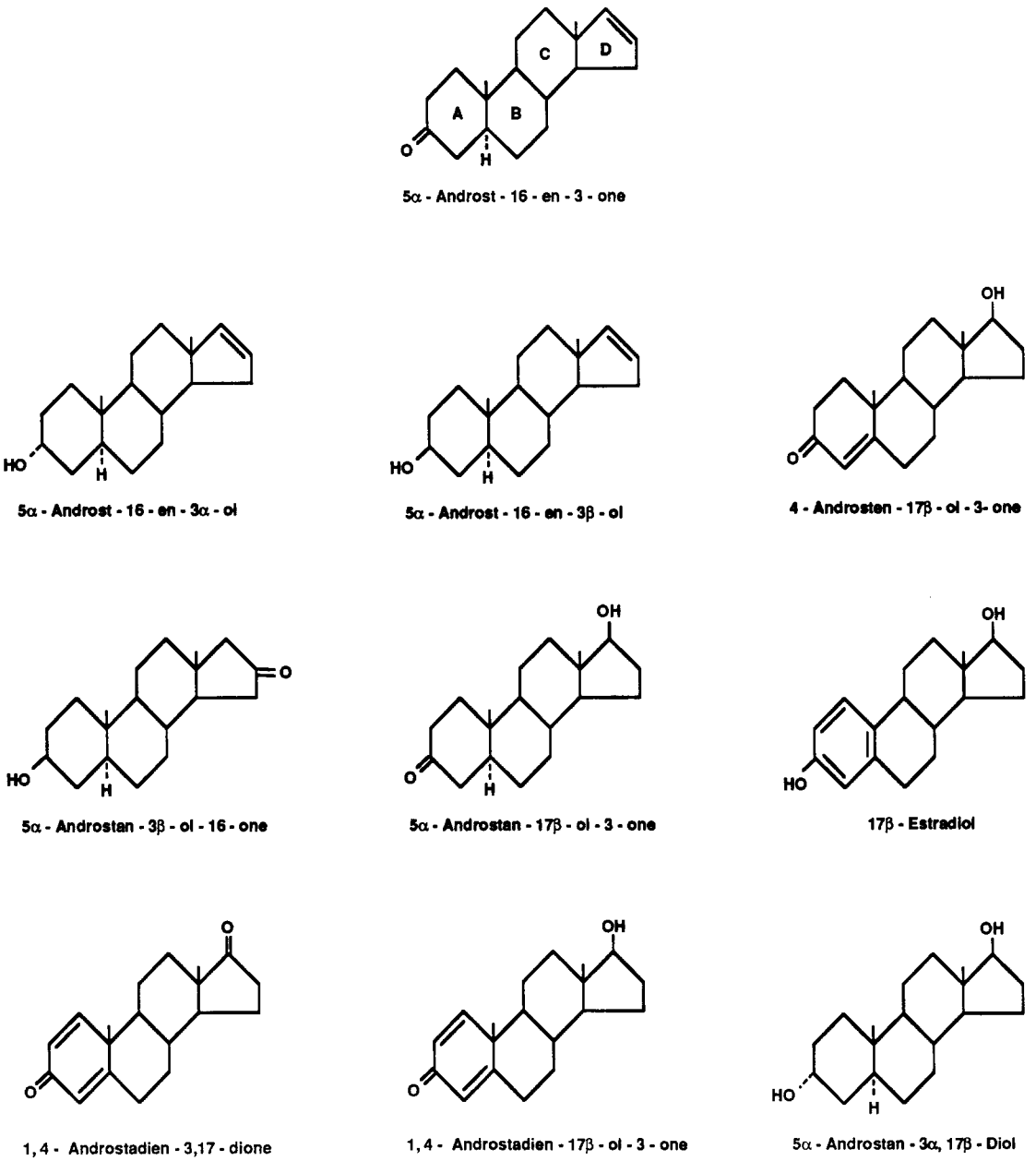

Figure 2. Structures of 5α-androst-16-en-3-one and related analogues.

cutaneously. The spleen from the mouse in the group that yielded antiserum most susceptible to inhibition by 5α-androst-16-en-3-one was used for fusion with myeloma cells.

Fusion efficiency (the number of wells with growing colonies/total number of wells seeded) was greater than 91% (711/780). Of these, 24 wells exhibited strong inhibition (70–88%) of antibody binding when the supernatant of the wells was incubated with 1 μg/mL of 5α-androst-16-en-3-one and evaluated by ELISA. Upon further cloning, three subclones, with the maximal sensitivity and desirable specificity for 5α-androst-16-en-3-one, were used to inject mice for mass production of antibody in ascites fluid. The isotype of all three subclones immunoglobulins was IgG_1, with κ light chain. Monoclonal antibody secreted by the cell line designated as F10 was used for the remainder of the study.

ELISA. Performance of the monoclonal antibody for 5α-androst-16-en-3-one was determined in the indirect ELISA using 5α-androst-16-en-3-one oxime–OA as the solid-phase antigen. Figure 1 depicts a standard ELISA curve for 5α-androst-16-en-3-one based on seven replications. The sensitivity range for the assay was from 0.05 to 10 ng/mL (or 2.5–200 pg/assay).

Specificity of the monoclonal antibody was assessed by evaluating the reactivity for other related steroids relative to 5α-androst-16-en-3-one (Figure 2), based on the amount of each steroid required to inhibit 50% of binding of the antibody to the solid phase in indirect ELISA (Table I). The antibody cross-reacted with other C_{19}-Δ^{16}-steroids having identical structures in the A, B, or D rings to 5α-androst-16-en-3-one. For example, 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol exhibited 44 and 246% cross-reactivity, respectively, with the antibody as compared to the parent compound (5α-androst-16-en-3-one). This suggested that although the monoclonal antibody was prepared against the steroid conjugated at the C3 position, it discriminated between the α- or β-orientation of the hydroxyl group at the C3-position in A ring. Data on relative levels of the various compounds in plasma are not available, although values for adipose tissue are published elsewhere (Berry et al., 1971; Thompson and Pearson, 1977). We have found previously similar discrimination between the C6′ α- and β-hydroxyl groups

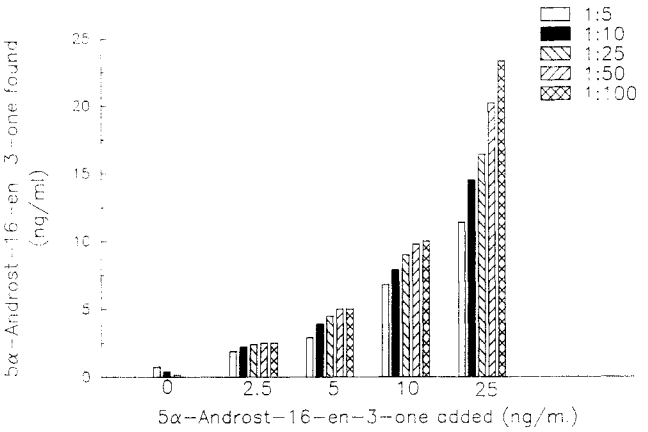

Figure 3. Effect of dilution on recovery of 5α-androst-16-en-3-one from porcine plasma.

Table II. Specificity of Monoclonal Antibodies for 5α-Androst-16-en-3-one in Competitive Indirect ELISA

steroid	50% inhibn, ng/mL	% cross-reactivity[a]
5α-androst-16-en-3-one	3.2	100
5α-androst-16-en-3β-ol	1.3	246
5α-androst-16-en-3α-ol	7.2	44
5α-androstan-17β-ol-3-one	25.0	12.8
5α-androstan-3β-ol-16-one	32.0	10.0
4-androsten-17β-ol-3-one	90.0	3.6
1,4-androstadien-17β-ol-3-one	115.0	2.8
5α-androstane-3α,17β-diol	550.0	0.58
1,4-androstadiene-3,17-dione	>1000.0	<0.32
17β-estradiol	>1000.0	<0.32

[a] Cross-reactivity defined as (nanograms per milliliter of 5-androstenone required for 50% inhibition/nanograms per milliliter of 5α-androst-16-en-3-one analogue required for 50% inhibition) × 100.

in isomers by a monoclonal antibody that was prepared from zearalenone 6′-(carboxymethyl)oxime (Dixon et al., 1987). The antibody to 5α-androst-16-en-3-one had much less reactivity than those steroids with structures that differed from 5α-androst-16-en-3-one in the A or D rings. The presence of a double bond at the C_{16}–C_{17} position in the D ring was also critical for determining the cross-reactivity.

Application to Porcine Serum or Plasma. Plasma and serum of a female pig (gilt) were spiked with 5α-androst-16-en-3-one, and recovery was estimated by competitive indirect ELISA (Figure 3). At low dilutions (1:5 to 1:25) of plasma, the recovery of added 5α-androst-16-en-3-one was incomplete (Figure 3), possibly due to the interference by binding to other plasma components in the assay. However, estimation of 5α-androst-16-en-3-one in gilt plasma diluted 1:50 or 1:100 resulted in 96–100% recovery of the spiked steroid. Since the maximum inhibition of antibody binding in ELISA (2.5–200 pg/assay) was far below the amount of 5α-androst-16-en-3-one typically present in boar blood (Andresen, 1974), appropriate dilution of the plasma or serum should not only eliminate the interference of other compounds in the assay but also enable determination of concentration within the linear portion of the standard curve.

To evaluate the applicability of the monoclonal antibody-based ELISA to screening of boar odor, 5α-androst-16-en-3-one in sera or plasma (1:100 dilution) of barrows (castrated pigs), gilts, and boars was quantitatively determined (Table II). Boar odor steroids were undetectable (<5 ng/mL) in the plasma of gilts, whereas the plasma and serum samples from barrows contained 13.8 and 17.1 ng/mL, respectively. For six boar samples, the value ranged from 20.9 to 210 ng/mL of plasma or 26.3 to 266 ng/mL of serum, the mean values being 97.6 and 110.8 ng/mL, respectively. The mean coefficients of variation for the boars and barrows were 9.9% and 9.3% for plasma and serum, respectively.

Table III. Determination of 5α-Androst-16-en-3-one in Plasma and Serum of Boars by Competitive Indirect ELISA

		plasma		serum	
pig sample	no. of assays	mean ± SD, ng/mL	CV,[a] %	mean ± SD, ng/mL	CV, %
1[b]	7	210 ± 13.9	6.6	266 ± 21.4	8
2[b]	4	20.9 ± 1.6	7.7	26.3 ± 0.6	2.0
3[b]	4	64.8 ± 11	16.9	70.8 ± 7	9.8
4[b]	3	88.3 ± 5.4	6.1	94 ± 6	6.4
5[b]	4	102 ± 8	7.8	106.8 ± 5.4	5.0
6[b]	7	99.3 ± 10.1	10.2	100.7 ± 20.7	20.6
7[c]	7	17.8 ± 2.2	12.4	23 ± 2.6	11.3
8[c]	7	9.8 ± 1.1	11.2	11.2 ± 1.3	11.6
9[d]	5	<5		<5	
10[d]	5	<5		<5	

[a] Coefficient of variation. The results of triplicate determinations of 5α-androst-16-en-3-one in the same plasma and serum on separate days. Means of the coefficient of variation of plasma and serum samples were 9.9 and 9.3%, respectively. [b] Uncastrated boar pigs. [c] Castrated male pig. [d] Female (gilt) pig.

The values for 5α-androst-16-en-3-one in boar blood obtained by ELISA in this study were about 65% higher than those reported by Andresen (1974) using a radioimmunoassay. The difference might be explained by the fact that in the latter study determination of 5α-androst-16-en-3-one involved organic solvent extraction from plasma with a recovery of only 35%, whereas the recovery of the steroid in our assay was over 85%.

Summary. Boar odor is a very serious quality defect in swine. USDA regulations specify that carcasses with "strong odor" must be condemned. In view of the fact that about 65–70% of all sexually mature boars produce carcasses with boar taint (Williams et al., 1963), the Canadian government meat inspection regulations require condemnation of all boar and stag carcasses. In West Germany, legal regulations prohibit marketing of fresh meat from boars at a carcass weight of 40 kg and above. The monoclonal antibody based ELISA procedure described herein should find use as a means of screening pigs for boar odor in slaughterhouses due to its simplicity, rapidity, sensitivity, minimal cost of reagents, and ease of sample preparation. With use of a 96-well microtiter plate, 36 samples can be screened for the taint odor steroid in less than $2^1/_2$ h, allowing high sample throughput in the routine determinations of 5α-androst-16-en-3-one in live pigs or pork carcasses. In preliminary work we have also adapted the ELISA to detection of boar odor in adipose tissue (Ashgar et al., 1988). However, research is still needed to more fully characterize the relationship between the concentration of the C_{19}-Δ^{16}-steroids in the sera and adipose tissue. Such studies are currently under way in our laboratory.

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Registry No. 5α-Androst-16-en-3-one, 18339-16-7; 5α-androst-16-en-3α-ol, 1153-51-1; 5α-androst-16-en-3β-ol, 7148-51-8.

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