Influence of the Hapten Conjugation Site on the Characteristics of Antibodies Generated against Metabolites of Clostebol Acetate

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4-chloro-androst-4-ene-3,17-dione (CLAD) and 4-chlorotestosterone (clostebol, β-CLT or CLT) were made immunogenic by coupling to protein carriers via the 3 and 17 positions, respectively. These immunogens were used to elicit polyclonal and monoclonal antibodies to CLAD and to clostebol. The antibodies were characterized in an enzyme immunoassay for sensitivity and specificity. Polyclonal antisera generated through position 17 reacted preferentially with 4-chlorotestosterone-17-acetate (clostebol acetate, CLTA), 4-chloro-epitestosterone (epi-clostebol, 17α-clostebol, 17α-CLT), and clostebol, whereas polyclonal antisera generated through the 3 position almost did not react with these derivatives. Interestingly, the monoclonal antibody generated through the 3 position recognized (35%) epi-clostebol. These results suggest that polyclonal antisera generated through the 17 position have a broad specificity profile and can be used to analyze by immunoassay methods urinary metabolites of clostebol acetate and thereby detect the illegal use of clostebol acetate in livestock farming.

Keywords: *Immunogen synthesis; conjugation site, metabolites, clostebol acetate, 4-chlorandrost-4-ene-3,17-dione, cross-reactivity*

INTRODUCTION

Clostebol acetate is an anabolic steroid which is often used for illegal fattening purposes in livestock farming. To safeguard public health, the use of anabolic steroids as growth-promoting agents in cattle has been prohibited under Directive 86/469 of the European Commission (EC, 1986).

In Belgium, routine screening of illegal use is organized by the Institute of Veterinary Inspection, which depends on the Ministry of Public Health. Notwithstanding the total ban, illegal use in Belgium is rife. Analyses in our laboratory (Van Oosthuyze et al., 1994) showed that the contribution of clostebol acetate to the positive injection sites increased from 52% in 1990 to 76% in 1993.

In previous studies, the excretion and biotransformation of clostebol acetate in humans (Debruyckere et al., 1992, 1994; Van Puymbroeck et al., 1996) and in cattle (Hendricks et al., 1994; Le Bizec et al., 1993, 1996, 1998; André et al., 1994; Leyssens et al., 1994) after intramuscular injection or oral intake were studied, and a wide range of urinary metabolites (Figure 1) were identified using HPTLC and GC-MS.

When analyzing urine samples of treated animals, in general no traces of the parent drug clostebol acetate were detected. Comparing the work of several authors, there is no doubt about the identity of the different metabolites, though their relative concentrations do not always correspond.

The importance and abundance of certain metabolites also depend on the method of administration of the parent drug, the age, and the sex of the animals. In general, it is possible to distinguish two groups of major metabolites (Walshe et al., 1998): after intramuscular administration the main metabolites are epi-clostebol, 4-chloro-4-androst-4-en-3α-ol-17one, and CLAD, while after oral intake in addition to these there is also a higher abundance of 4-chloro-4-androsten-3α,17β-diol and 4-chloroandrostane-3β-ol-17-one.

About 95% of these appear in urine as sulfate conjugates. Only 4-chloro-4-androst-4-en-3α-ol-17-one has been found for 20% to 25% as glucuronide conjugate.

On the basis of this knowledge, the abuse of clostebol acetate can be evidenced by the presence of certain unique metabolites in urine. The parent drug and its metabolites have a typical 4-chlorine group which gives them a higher anabolic and a less androgenic activity compared to non-chlorinated steroids. Since there is no knowledge of the existence of endogenous chlorinated steroids in cattle, the presence of one of those chlorinated metabolites in urine can be used as an indication of illegal use. This explains why the 4-chloro group is an important feature for recognition by the antibodies.

The main objective of this work was to generate a suitable antiserum for immunochemical screening of the metabolites of clostebol acetate (e.g., CLAD, epi-clostebol, etc.) in urine of food-producing animals.

Since the site of attachment of clostebol to protein carriers elicits formation of antibodies with enhanced specificity toward selected parts of the steroid molecule, we utilized two different hapten-conjugation methods, one through the 3 position and the other through the 17 position. In this report we describe the characteristics of monoclonal and polyclonal antibodies (CLAD antise-

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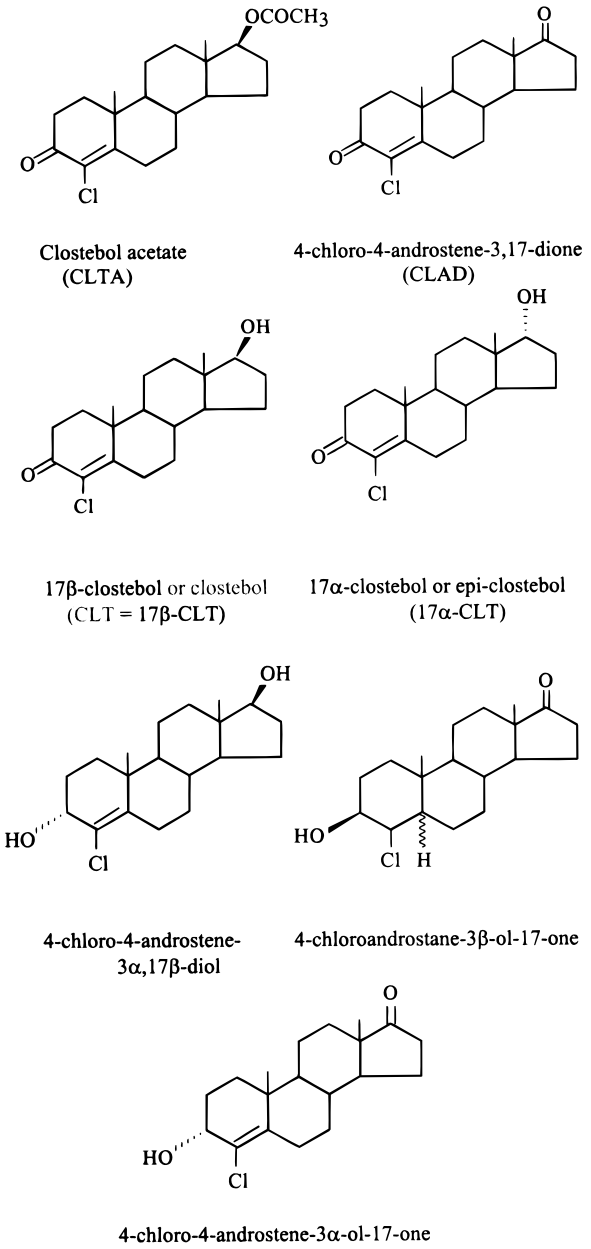

Figure 1. Structures of clostebol acetate and its main metabolites.

rum) generated through position 3 and of polyclonal antibodies (clostebol antiserum) elicited through position 17 of clostebol. Using CLAD as the reference standard for cross-reaction studies, our data indicate that the polyclonal antibodies elicited through the 17 position react preferentially with clostebol acetate, epi-clostebol, and clostebol. On the other hand, the monoclonal and polyclonal antibodies generated through position 3 do not recognize clostebol acetate; they cross-react only to a low extent with clostebol, while only the monoclonal antibodies do recognize epi-clostebol relatively well and 4-chloro-androstene-3α-ol-17-one to a low extent.

Until now only one paper (Walshe et al., 1998) has reported an enzyme-linked immunosorbent assay (ELISA) for the screening of clostebol metabolites, using a commercial kit for the analysis of urine samples. This paper is the first to describe the actual development of antibodies, their characteristics, and their application in ELISA. Our data indicate that with regard to cross-reactivity with clostebol metabolites, one of our antisera scores better than the commercial kit.

MATERIALS AND METHODS

Reagents. Steroids, bovine serum albumin (BSA), goat anti-rabbit IgG (whole molecule; containing 3.6 mg of specific antibody per mL), horseradish peroxidase (HRP), ProClin 300, Tween 20, casein, antifoam A emulsion, and tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (Bornem, Belgium). Fluoxymesterone and 17α-19-nortestosterone were purchased from Steraloids (Wilton, NH). Organic solvents were of analytical reagent grade. 'HiPerSolv for HPLC' water, methanol, and acetonitrile were from BDH Laboratory Supplies (Poole, England). *Helix pomatia* juice was purchased from Boehringer Mannheim (Germany). Clostebol was obtained from Alltech (Applied Science Laboratories, State College, PA), and CLAD was a gift from Dr. L. Leyssens (Dr. Willems-Instituut, Diepenbeek, Belgium). Epi-clostebol was generously donated by RIVM (The Netherlands). The metabolites 4-chloro-androstene-3α-ol-17-one and 4-chloro-androstene-3α,17β-diol were kindly donated by Dr. G. Pieraccini (CISM, Florence, Italy). Rabbit anti mouse IgG was obtained from DAKO (Glostrup, Denmark).

Solutions. (a) Assay buffer: Phosphate-buffered saline (PBS), pH 7.5 (0.2 g of KH_2PO_4, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$, 8 g of NaCl, 0.2 g of KCl/L of water) containing 0.05% Tween 20; 0.1% BSA. (b) Coating buffer (Tijssen, 1985): 50 mM carbonate buffer, pH 9.6 (1.59 g of Na_2CO_3; 2.93 g of $NaHCO_3$/L of water). (c) Blocking solution: PBS containing 2% casein. (d) Wash solution: PBS with 0.005% Tween 20, 0.1% casein, and 0.004% Antifoam A Emulsion. (e) Substrate solutions A and B: prepared as described by Dürsch and Meyer (1992) with little modification. Solution A (pH 5) contained 1.00 g of urea hydrogen peroxide, 18.00 g of $Na_2HPO_4 \cdot 2H_2O$, 10.30 g of citric acid$\cdot H_2O$ per liter water. Solution B (pH 2.4) contained 500 mg of TMB, 40 mL of dimethyl sulfoxide, 10.30 g of citric acid$\cdot H_2O$, and 960 mL of water. Both solutions were stored separately and protected from light at 4 °C. Before the assay, equal volumes of A and B were mixed. To all buffers and solutions, 0.05% ProClin was added as a preservative.

Disposables and Equipment. Flat-bottomed microtiter plates (96 wells, Nunc Maxisorp) were obtained from Life Technologies (Merelbeke, Belgium). Optical activities were measured with a Micro Plate Reader, model MPR-A4, from Eurogenetics (Tessenderlo, Belgium).

PD-10 columns (Sephadex G-25 M) and the Mab-Trap GII kit for total IgG isolation was purchased from Pharmacia Biotech (Roosendaal, The Netherlands). The UltraFree-15 centrifugal filter device (Biomax−50 NMWL) was purchased from Millipore (Brussels, Belgium). The commercial ELISA kit was supplied by Laboratoire d'Hormonologie (Marloie, Belgium).

Preparation of 3-Carboxymethyl-oxime-CLAD. The required 3-carboxylmethyl-oxime-CLAD (CMO−CLAD) was prepared in three steps. In the first step, clostebol acetate (220 mg) was dissolved in absolute ethanol (2 mL). Carboxymethoxylamine hemihydrochloride (263.2 mg) and pyridine (144 μL) were added, and the reaction was stirred overnight at room temperature. Examination of the reaction mixture by TLC in the solvent system chloroform:methanol:acetic acid (94.7:5:0.3) showed a single product of $R_f = 0.46$.

In the second step, the reaction mixture containing the product 3-carboxymethyl-oxime-clostebol acetate was hydrolyzed with acid (2 N HCl, 2 mL) for 3 h at 80 °C. The reaction mixture was then cooled, and the ethanol was evaporated. The formed precipitate was filtered and washed with water until neutral.

In the third step of the synthesis, the crude products were oxidized with Jones reagent. For this purpose, the crude products (250 mg) were dissolved in acetone (20 mL) and cooled to 0 °C. Jones reagent (prepared by adding 26.72 g of CrO_3 to 23 mL of concentrated H_2SO_4 and diluted to 100 mL with double distilled water) (290 μL) was then added dropwise. The

reaction was continued for 5 min and stopped by the addition of water (10 mL). The acetone in the reaction mixture was evaporated under nitrogen, and the reaction mixture was extracted into chloroform. The organic layer was washed, dried, and evaporated. The residue was chromatographed on silica gel 60. Elution with chloroform:methanol (95:5) gave the desired product 3-carboxymethyl-oxime-CLAD in a very low yield. This product was rechromatographed on silica gel 60 using chloroform:methanol (95:5) as a solvent system. 3-Carboxymethyl-oxime-CLAD (CMO−CLAD) showed one spot of $R_f = 0.13$ on TLC in the solvent system chloroform:methanol: acetic acid (94.7:5:0.3).

The ^{1}H NMR analysis of this compound in dimethyl sulfoxide showed the following signals: δ 0.994 (3-H, singlet, C-18 CH_3, 1.268 (3-H, singlet, C-19 CH_3) and 4.76 (2-H, singlet, O−CH_2−COOH).

Conjugation of 3-Carboxymethyl-oxime-CLAD to Macromolecules. CMO−CLAD was conjugated to BSA and HRP via a two-step reaction. In the first step of the synthesis, the CMO-CLAD (3.15 mg) was dissolved in dry dioxane (200 μL), and *N*-hydroxysuccinimide (1.7 mg) and carbodiimide (3.5 mg) were added to the reaction mixture. After an overnight reaction at room temperature, urea was formed and the supernatant analyzed by TLC in a solvent system of chloroform:methanol: acetic acid (74.5:25:0.5) for the presence of the active *N*-hydroxysuccinimide ester derivative of CMO−CLAD. A compound with an R_f of 0.95 was obtained. The *N*-hydroxysuccinimide active ester of CMO−CLAD in dioxane (200 μL) was then used in the next step without further purification. BSA (10 mg) was dissolved in 1 mL of 0.13 M $NaHCO_3$ (pH 8.5) followed by dropwise addition of the active ester (200 μL). The reaction mixture was stirred for 2.5 h at room temperature, dialyzed overnight against phosphate-buffered saline (PBS), pH 7.4 at 4 °C, and stored at −20 °C until use.

The reaction scheme is shown in Figure 2. UV analysis according to the method of Erlanger (Erlanger et al., 1957) indicated that the conjugate contained 32.8 mol of CLAD per mole of BSA.

For the preparation of a conjugate of HRP to CMO-CLAD, HRP (4.8 mg) was dissolved in 1 mL of 0.13 M $NaHCO_3$ (pH 8.5) and 50 μL of the *N*-hydroxysuccinimide ester derivative of CMO−CLAD in dioxane was added. The reaction mixture was stirred for 3 h at room temperature, dialyzed twice against PBS, pH 8, followed by PBS, pH 7.2. UV-analysis (Erlanger et al., 1957) indicated that the HRP conjugate contained 2 mol of CLAD per mole of HRP. It was stored at −20 °C until use.

Preparation of Clostebol-17-hemisuccinate. Clostebol acetate (20 mg) was dissolved in a 20 mL mixture of acetone:1 N HCl (9:1, v/v) and left to react for 15 h at 75 °C under reflux and continuous stirring. The reaction mixture was then made alkaline with 1 M Na_2CO_3 and extracted with diethyl ether. By examination on HPTLC (silica gel 60 with F_{254}) in the solvent system hexane:ethyl acetate (2:1; v/v), no clostebol acetate ($R_f = 0.57$) was left; it had been almost completely hydrolyzed into clostebol ($R_f = 0.18$).

Clostebol (100 mg) was redissolved in pyridine (3 mL) with an excess of succinic acid anhydride (1 g) and stirred at room temperature for 5 days. After extraction with diethyl ether, it was purified over a silica gel G 60 column and eluted with dichloromethane and 10% acetonitrile. Examination of the reaction product by HPTLC (silica gel plate with F254 indicator; mobile phase = dichloromethane with 10% acetonitrile) showed a spot with $R_f = 0.41$. The structures of clostebol and clostebol-17-hemisuccinate were confirmed by ^{1}H NMR analysis. Comparison of the spectra clearly revealed the presence of the succinylic protons (CH_2CH_2) as a complex multiplet (δ: 2.55−2.75).

Preparation of Macromolecular Conjugates of Clostebol Hemisuccinate. *(i) Clostebol-17-hemisuccinate-BSA.* The clostebol-17-hemisuccinate (CLT-HS, 9.8 mg) was dissolved in 0.5 mL of dimethylformamide (DMF). A solution of *N*-hydroxysuccinimide (6 mg) in 0.5 mL of DMF was added, followed by a solution of dicyclohexylcarbodiimide (13.4 mg) in 1 mL of DMF, while the mixture was cooled on ice. Afterward, the reaction mixture was left overnight at room temperature under continuous stirring. Subsequently, the mixture was added dropwise to a solution of BSA in 0.1 M phosphate buffer (pH 8) and left overnight stirring at room temperature. The pH was checked at regular intervals and adjusted when necessary. Finally, the solution was diluted with 0.05 M $NaHCO_3$ (pH 8) up to a final percentage of 10% DMF and dialyzed, concentrated, and lyophilized.

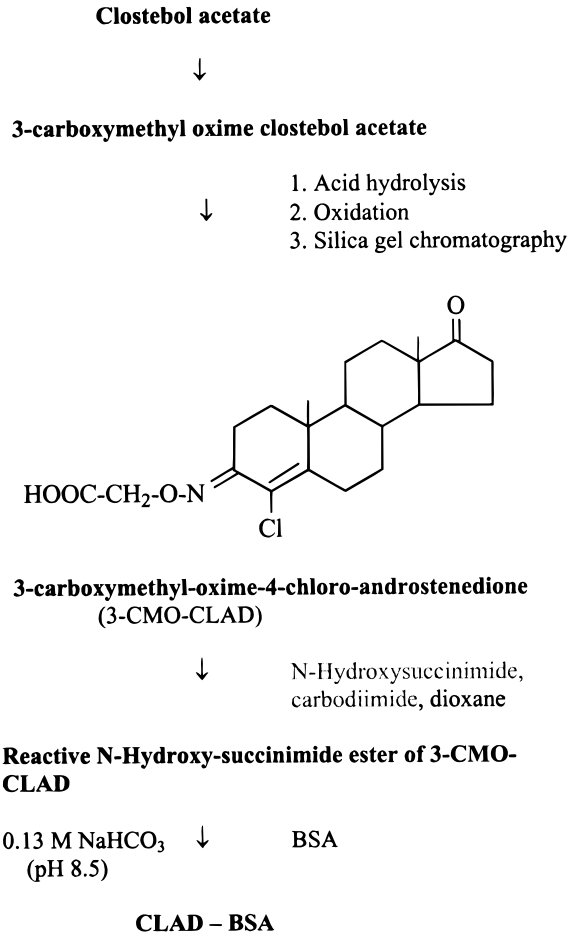

Figure 2. Scheme of synthesis of 3-CMO-CLAD-BSA.

The hapten/carrier ratio was determined spectrophotometrically according to the method of Erlanger (Erlanger et al., 1957); results showed a molar ratio of 8.59 mol of clostebol-hemisuccinate per mole of BSA.

(ii) Clostebol-17-hemisuccinate-HRP. For conjugation of clostebol-hemisuccinate to HRP, the same reaction procedure was followed as that for conjugation with BSA, except that the reaction mixture with HRP was left stirring at 4 °C. Finally the solution was dialyzed, preconcentrated by ultracentrifugation, and lyophilized. The molar hapten/carrier ratio was determined by UV (Erlanger et al., 1957). Analysis showed a molar ratio of CLT-HS/HRP of 1.8.

Preparation of Monoclonal Antibodies. The CLAD−BSA conjugate was used as to immunize female CD2 mice (age: 2 months; 50 μg/animal). Subsequently, three booster injections were given using the CLAD−BSA conjugate in incomplete Freund's adjuvant. After 2 months of immunization, the antibody titer was checked using rabbit anti-mouse IgG coated plates and CLAD-HRP as a label. Three months after the initial immunization, the spleen cells of the mouse with high titer of antibodies to CLAD were fused with a mouse myeloma cell line (the non-Ig producer mouse myeloma cell line NSO/1 was kindly provided by Dr. C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, UK) using the hybridoma technique of Köhler and Milstein (1976) with some modifications introduced (Kohen and Lichter, 1986). The culture supernatants of growing hybridomas were screened for antibody activity using rabbit anti-mouse IgG coated plates and

CLAD-HRP as a label. Suitable hybridomas were selected, cloned, and propagated in vitro as culture supernatant or in vivo in pristane primed mice as ascites. Purified immunoglobulins were isolated from ascites by chromatography on Sepharose-Protein A as described previously (Strasburger and Kohen, 1990).

Generation of Polyclonal Antibodies. Rabbits were immunized by intradermic multisite injection. First a stock solution of the immunogen (1 mg mL^{-1}) in NaCl 0.9% was prepared. For the first injection, 250 μL of stock solution was mixed with 250 μL of NaCl (0.9%) and 500 μL of Freund's complete adjuvant (total injection volume: 1 mL). Boosts were given every 28 days with a mixture of 150 μL of stock solution, 350 μL of NaCl (0.9%), and 500 μL of incomplete Freund's adjuvant. Every 10 days after injection, blood samples were collected and the sera were stored at −20 °C until use. The various sera were then tested for titer, sensitivity, and specificity using ELISA techniques described below.

Isolation of Total IgG from the Anti-Clostebol Antiserum. The immunoglobulin G (IgG) fraction of the antiserum was isolated with the aid of recombinant protein G (Mab-Trap GII kit) according to the procedure described by the manufacturer and then applied to a PD-10 column in order to exchange the buffer into PBS and to remove remaining salts. Before storage at −20 °C, the solution was preconcentrated using an UltraFree device for ultrafiltration. The titer of the IgG solution was determined with a checkerbord titration in combination with clostebol-17-hemisuccinate-HRP.

Development of a Competitive ELISA with Double-Antibody Technique. A microtiter plate was coated with GAR (100 μL per well) at a concentration of 5 μg mL^{-1} in coating buffer and left for overnight incubation at 4 °C.

After decanting the solution, the plate was blocked with 2% casein in PBS (300 μL per well) for 30 min at room temperature on a shaker. The plate was washed four times, and reagents were added in the following order: 50 μL of standard solutions (0.02−0.05−0.1−0.2−0.5−1−2−5 ng mL^{-1}) of CLAD in assay buffer or sample solutions, 25 μL of HRP label (CLAD−HRP or CLT-HS−HRP), and 25 μL of antiserum (respectively, CLAD antiserum or clostebol antiserum). After incubation for 2 h at room temperature, the plate was washed four times. Substrate solution was added (100 μL per well), and the plate was incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 100 μL of 1 M H_2SO_4 to each well. The absorbance was read at 450 nm.

Antisera Titer Determination. In the double-antibody, solid-phase ELISA binding studies between the antisera and hapten-HRP label were used to determine their optimal dilution. The optimal dilutions were those that resulted in an absorbance of 1.0 at 450 nm, following incubation of the substrate for 30 min at room temperature.

Antisera Sensitivity Determination. Calibration curves for CLAD (standards in assay buffer) were determined with the two polyclonal antisera according to the procedure described above.

The corresponding B/Bo 50% value and the detection limit, defined as the concentration of CLAD that corresponds with the response of 'mean Bo − 3 SD', were determined.

Antisera Specificity Determination. Cross-reactivity studies were carried out with various structurally related compounds (relative to CLAD at 100%) at different concentrations.

For testing the monoclonal antibodies, a rabbit anti-mouse IgG was used as second antibody in the ELISA instead of the GAR used for the polyclonal antisera.

The percentage cross-reactivity was determined as the ratio of the amount of cross-reacting material that yields 50% inhibition of binding with the amount of standard giving the same inhibition.

RESULTS AND DISCUSSION

Synthesis of Immunogens. It has been reported that by judicious choice of the site of conjugation it is possible to elicit the formation of antibodies with enhanced specificity toward selected parts of the steroid molecule (Bauminger et al., 1974; Kohen et al., 1975). Accordingly, in this work we investigated the influence of the site of conjugation to clostebol and to CLAD on the specificity of antibodies generated. Being the most important metabolite, CLAD was chosen as hapten for conjugation at position 3 This first mode of conjugation was intended to preserve the substituents of ring D, more specific the 17-side chain, as prime determinants in immuno recognition. The second conjugation through the 17-position, using clostebol—with its free hydroxyl group—as a hapten, is a convenient way of coupling while leaving the A ring fully exposed for antibody recognition.

Table 1. Characteristics of Polyclonal Antisera in ELISA

	Clostebol antiserum	CLAD antiserum
FD (PAb)	1:500 000	1:100 000
FD (IgG)	1:1 500 000	
FD (HRP-conjugate)	CLT-17-HS-HRP 1:400 000	CLAD-3-CMO-HRP 1:25 000
incubation time	1 h	2 h
coloring time	30 min	30 min
ED 50 (ppb)	0.09	0.36
Bo (mean)	0.987 (n = 16)	1.232 (n = 14)
standard deviation	0.054	0.041
detection limit (ppb) (in assay buffer)	0.03	0.08

Since clostebol was not immediately available as a reference standard, clostebol acetate had to be hydrolyzed as a starting material for the required synthesis. Basic hydrolysis of clostebol acetate led mainly to decomposition products. No further attempts were made using alkaline hydrolysis. Using previously described methods for acid hydrolysis (Leyssens et al., 1994; Le Bizec et al., 1996), unsatisfactory results were obtained. On the other hand, modification of the method of Le Bizec (Le Bizec et al., 1996) resulted in a more efficient hydrolysis of clostebol acetate in acidic conditions.

Development of a Competitive ELISA. Differences in characteristics (titer, sensitivity, and specificity) were examined by use of a direct competitive ELISA with a second-antibody technique.

The use of second antibodies gives better reproducibility and sensitivity and a much smaller amount of the precious antiserum is consumed. It has been shown that pH 9.6 is optimal for the passive adsorption of antibodies to microtiter plates (Butler et al., 1993). According to Tijssen (1985), the optimal concentration of antigens and antibodies for coating is usually between 1 and 10 μg mL^{-1}.

Two coating concentrations, 5 and 10 μg mL^{-1}, were compared. Both gave similar results in the ELISA. Therefore, the microtiter plates were coated with secondary antibodies at a concentration of 5 μg mL^{-1} overnight at 4 °C.

Antiserum Titer and Sensitivity. *(i) Monoclonal Antibodies to CLAD.* From the fusion experiment, 2000 hybridomas were screened, and one clone (#14H2) secreting monoclonal antibodies to CLAD with high binding activity to CLAD-HRP, specificity (Table 2), and sensitivity was selected. This clone belonged to the IgG1 class and was propagated in vitro and in vivo. Using colorimetry as an end point, a sensitivity of 0.8 ng of CLAD mL^{-1} was obtained.

(ii) Polyclonal Antibodies. Usable polyclonal antisera were obtained with both conjugates. In general, the

Table 2. Cross-Reactivity with Structural Analogues

	% Cross-reactivity		
	Clostebol antiserum	CLAD antiserum	Monoclonal antiserum
CLAD	100	100	100
clostebol acetate	65	0.01	<0.01
epi-clostebol	50	0.4	35
clostebol	39	2	1
4-chloroandrostene-3α-ol-17-one	0.7		6
4-chloro-androstene-3α,17β-diol	0.5	0.2	0.05
testosterone	0.7	1	<0.01
epi-testosterone	0.5	0.2	0.2
testosterone-17-sulfate	0.9		0
epi-testosterone-sulfate	0.2		0
testosterone-17-glucuronide	0.2	<0.01	0
19-nortestosterone	0.2	0.3	<0.01
epi-19-nortestosterone			<0.01
17α-Me-testosterone	0.9	0.1	<0.01
Progesterone	0.8	0.1	<0.01
α-estradiol	<0.001	<0.01	<0.01
β-estradiol	0.02	<0.01	<0.01
Androsterone	<0.01		<0.001
Androstene-3,17-dione	1		0.9
19-norandrostene-3,17-dione	0.07	7	<0.03
Dehydroepiandrosterone	<0.001		<0.001
Dehydroepiandrosterone-sulfate	<0.001		0
5α-androstane-3,17-dione			<0.01
5β-androstane-3,17-dione			<0.01
5α-androstane-3β-ol-17-one			<0.01
5α-androstane-3α-ol-17-one			<0.01
Boldenone			<0.01
Me-boldenone			<0.01
Fluoxymesterone			<0.01
Stanozolol			<0.01

titers of the antisera produced against the clostebol-17-hemisuccinate immunogen (clostebol antiserum) were higher than those obtained against the 3-CMO−CLAD immunogen (CLAD antiserum).

For the CLAD antiserum, all of the following ELISA tests were performed with the antiserum in a final dilution of 1:100 000 in combination with a CLAD-HRP dilution of 1:25 000.

The clostebol antiserum with the highest titer could be used in a final dilution of 1:500 000 in combination with the CLT−17-HS−HRP conjugate in a final dilution of 1:400 000. The IgG fraction could be used in a final dilution of 1:1 500 000 in combination with the HRP conjugate in a final dilution of 1:400 000, resulting in a similar calibration curve as with the crude antiserum.

Figure 3 represents the dose response curves obtained for CLAD using the polyclonal CLAD and clostebol antisera. They are characterized by an ED_{50} value of, respectively, 0.36 and 0.09 ng mL^{-1}, and the detection limits, defined as 'Bo − 3SD', are, respectively, 0.08 and 0.03 ng mL^{-1} (Table 1).

Cross-Reactivity of the Antisera. The specificity of the three antisera is shown in Table 2.

The monoclonal antibody generated through the 3 position of CLAD recognized epi-clostebol relatively well (35%) and 4-chloro-androstene-3α-ol-17-one only to a small extent. Little cross-reactivity with clostebol and clostebol acetate was observed. There was also no cross-reaction with endogenous hormones as testosterone, estradiol, and progesterone, neither with other structural analogues nor anabolics (e.g., boldenone, fluoxymesterone, stanozolol). Moreover, the polyclonal antiserum generated through the same position gave rise to antibodies that were specifically directed toward the 17 position and did not recognize epi-clostebol, clostebol, or clostebol acetate well. On the other hand, polyclonal antibodies generated through the 17 position of clostebol are directed more specifically against the free 4-Cl function of the CLT-17-HS hapten and thus show more cross-reactivity toward the metabolites of clostebol.

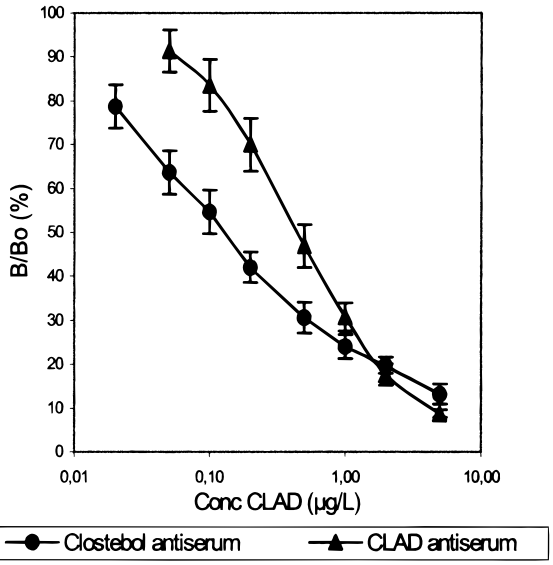

Figure 3. Sigmoïdal calibration curves of CLAD with the polyclonal antisera.

The fact that the clostebol antiserum cross-reacts to a different extent with clostebolacetate, clostebol, and epi-clostebol demonstrates that the 17 position used for conjugation is not completely deprived of its antigenic determinant. A possible explanation for why the cross-reactivity with clostebol acetate is higher than for clostebol could be the structural similarity between the hemisuccinate bridge of the immunogen and the acetate group on clostebol acetate.

The CLAD antiserum only reacts weakly with the metabolites and shows a high specificity toward CLAD. Using the 3-conjugation site, possible steric hindrance could occur, so the 4-Cl function is not recognizable as a strong antigenic determinant for the generated antibodies.

If we finally compare the cross-reactivity of our antibodies with those of the antibodies used in the commercial ELISA kit, we can see some differences. The principal cross-reactivities of these antibodies as mentioned in the information from the manufacturer are CLAD (100%), clostebol (28%), clostebol acetate (120%). The cross-reactivity for epi-clostebol, as determined at our laboratory, was found to be 8%. The high result for clostebol acetate is, however, of little importance since no parent drug occurs in urine; the cross-reactivity for epi-clostebol, on the other hand, is much more important since it represents one of the major metabolites in urine. In this view we can state that antibodies with the highest cross-reactivities toward CLAD, clostebol, and epi-clostebol are most promising when it comes to screening of urinary metabolites and avoiding false negative results.

CONCLUSIONS

The results presented in this paper confirm previous studies (Bauminger et al., 1974; Kohen et al., 1975) that the site of attachment of the steroid molecule to the macromolecular carrier molecule considerably affects the specificity of the generated antibodies. When the steroid molecule was attached to the carrier through position 17 and the 4-Cl function was left free for

recognition, we obtained antibodies that discriminate more efficiently between clostebol metabolites and endogenous hormones than do those produced through conjugation at position 3, leaving a 17-keto function free. This latter conjugation gave rise to highly specific antibodies for metabolites with a 17-keto function (or α-OH in case of the monoclonals) but not for metabolites with a β-OH function or an acetate group.

The relative merit in ELISA of the two types of antisera will also depend on the relative concentration of potentially cross-reacting (endogenous) steroids in the biological fluid of interest and the relative ease with which these can be eliminated during the initial extraction and purification steps. However, in general, it can be concluded that the polyclonal antibodies of the clostebol antiserum have a great potential for preliminary screening of clostebol acetate and its metabolites in urine.

Further studies of their application for analysis of urine samples of food-producing animals are being conducted at the moment.

ABBREVIATIONS USED

CLAD, 4-chloro-androstenedione; CLTA, clostebol acetate; CLT, clostebol; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FD, final dilution; GAR, goat anti-rabbit; HRP, horseradish peroxidase; OD, optical density; PBS, phosphate-buffered saline; Bo, the value of the OD (in the ELISA) obtained when the tracer is incubated alone with the antibodies; B, the value of the OD (in the ELISA) obtained when the tracer is incubated with the antibodies and the antigen (CLAD); SD, standard deviation; DMF, dimethylformamide; HS, hemisuccinate; CMO, carboxymethyl-oxime.

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