JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 4351−4354 **4351**

Enzyme-Linked Immunosorbent Assay-Based Detection of Free Trenbolone in Bovine Bile

JANE FITZPATRICK,[†,‡,§] BERNADETTE MANNING,[†,||] AND RICHARD O'KENNEDY*[,†,‡]

School of Biotechnology, and National Centre for Sensor Research (NCSR), Dublin City University, Glasnevin, Dublin 9, Ireland

A rapid antibody-based detection system has been developed for the presence of free trenbolone in bovine samples. Polyclonal antibodies were produced that showed specificity toward epitopes located around the steroidal A-ring of the trenbolone molecule. These antibodies were shown to have little or no recognition for many closely related compounds. The antibodies were utilized as the specific biorecognition molecules in competitive and inhibitive enzyme-linked immunosorbent assay systems. While both assays were able to detect low nanogram concentrations of trenbolone in bovine bile, the competitive format was more sensitive (2.41 vs 17.15 ng/mL for TRAb2 and 3.31 vs 30.73 ng/mL for TRAb1). This format was also more accurate and the data produced by this assay fitted more closely to the four parameter equation used to calculate the standard curve. This was a common finding with both of the polyclonal antibodies, suggesting that this was a characteristic of the format used.

KEYWORDS: Trenbolone; ELISA; bovine bile

INTRODUCTION

Trenbolone acetate is a powerful synthetic steroidal androgen, which is used as a growth promoter in cattle. It is rapidly hydrolyzed to its metabolite 17β-trenbolone after administration to cattle (*1*). This is the active form of trenbolone acetate, and it is the main metabolite found in muscle and fat tissues of implanted cows (*2*). Trenbolone (TR) and trenbolone acetate cannot be used as growth promoters within the European Union while the U.S. FDA allows the administration of trenbolone acetate to cattle. Testing regimes are required within the EU to enforce this ban.

Many assays have been described for the detection of TR in different animal matrices, including urine, bile, muscle, liver, and faeces (*3*). These assays include radioimmunoassays (*4*), enzyme immunoassays (*3*), and immunoaffinity chromatography followed by high-performance liquid chromatography (HPLC)−thin-layer chromatography (TLC) detection (*5*). Each of these methods had its own advantages and disadvantages, but they all required lengthy sample extraction and/or cleanup before the detection of the compound can be carried out. The assay described here uses a diluted bile sample directly applied to an enzyme-linked immunosorbent assay (ELISA) system to give specific and quick detection of TR in bile with high sensitivity.

MATERIALS AND METHODS

The materials were purchased from Sigma-Aldrich Chemicals (Dublin) unless otherwise stated. All reagents were of analytical grade. Distilled water was used throughout the work. The phosphate-buffered saline (PBS) used was sourced from Oxoid and was Dulbecco's A PBS, pH 7.4. The steroids used to determine the cross-reactivity of the antibodies were generous gifts from Dr. Christopher Elliott and Dr. Steven Crooks, Veterinary Sciences Division, Northern Ireland.

Preparation and Use of Bovine Bile. Bovine bile was collected from Kepak, Co. (Dublin), centrifuged, and stored at −20 °C until required. The samples were defrosted and filtered through a 0.45 μm syringe filter before use. The standards were prepared by spiking the bile with the steroid from an ethanolic stock and performing dilutions to achieve a wide concentration range.

Derivatization of TR to TR−17-Hemisuccinate (HS). The method used was a modification of the procedure used by Jondorf in 1980 (*6*). A 100 mg amount of TR was dissolved in 3 mL of dichloromethane and 2 mL of dry pyridine. A 187 mg amount of succinic anhydride was added, and the reaction was left at room temperature for 48 h. The reaction was evaporated to dryness using a rotary vacuum evaporator with a heat gun, and the residue was dissolved in 10 mL of chloroform. The residue was washed twice with water (2 × 5 mL) to remove excess succinic anhydride, and the chloroform was then removed under vacuum. The residue was redissolved in ethanol and stored in the dark at room temperature.

Production of TR−Thyroglobulin (THY) Conjugate Using EDC/NHS Chemistry. The production of this conjugate was carried out with a modification of the carbodiimide procedure described by van Look et al. (*5*). A 5 mg of TR−HS was dissolved in 500 μL of dioxane in a glass vial. Solid NHS was added to give a final molarity of 0.1 M. A 5 mg amount of EDC was dissolved in 250 μL of distilled water and added to the steroid solution. This was incubated for 10 min with stirring. A solution containing 10 mg of THY in 700 μL of 0.05 M phosphate buffer, pH 7.8, was added to the reaction mixture. The reaction was allowed to proceed for 75 min. The resultant mixture was dialyzed for 48 h against four changes of PBS at 4 °C.

Production of TR−Bovine Serum Albumin (BSA) Conjugate Using Mixed Anhydride Chemistry. This was prepared using a modification of the mixed anhydride procedure described by Nambara

* To whom correspondence should be addressed. Tel: +353 1 7005313. Fax: +353 1 7005412. E-mail: richard.okennedy@dcu.ie.

[†] School of Biotechnology.

[‡] NCSR.

[§] Current address: National Food Centre, Teagasc, Ashtown, Dublin 15, Ireland.

[||] Current address: Schering-Plough, Ringaskiddy, Cork, Ireland.

10.1021/jf0352531 CCC: $27.50
Published on Web 06/18/2004

et al. (*7*) based on the reaction elucidated by Erlanger et al. (*8*). A 10 mg amount of TR−HS was dissolved in 200 μL of dimethylformamide (DMF). Eight microliters of tributylamine and 2.5 μL of butylchloroformate were added, and the reaction was stirred on ice for 12 min. A solution containing 20 mg of BSA in 1 mL of water, 2.5 mL of DMF, and 25 μL of 1 M NaOH was cooled to 0 °C and added to the activated hapten. The reaction was left at 4 °C for at least 20 h. The conjugate was then dialyzed overnight against 5 L of PBS to remove any unconjugated hapten.

Production of TR−Horseradish Peroxidase (HRP) Conjugate Using Mixed Anhydride Chemistry. This synthesis was carried out according to a modification of the procedure described by Meyer and Hoffmann (*3*). A 1.5 mg amount of TR−HS was dissolved in 100 μL of DMF, and 1 μL of methylmorpholine was added. This solution was cooled to −15 °C, and 1 μL of butylchloroformate was added. The solution was left to stir at −15 °C for 3 min. This mixture was added slowly to a precooled (0 °C) solution of 11 mg of HRP in 100 μL of water and 75 μL of DMF. This was stirred at −15 °C for 60 min followed by 0 °C for 120 min. One milligram of $NaHCO_3$ was then added, and the products were dialyzed overnight against PBS.

After dialysis, the spectrum of each conjugate was measured between 200 and 400 nm. If the characteristic peak of the protein was present along with the peak attributed to TR, the conjugate was used in further work.

Immunization Procedure for the Production of Rabbit Antiserum to TR−THY. Two female New Zealand White rabbits were immunized with 1 mL of TR−THY conjugate at a concentration of 0.5 mg/mL, mixed with an equal volume of Freund's Complete Adjuvant. The rabbits were then boosted with the conjugate mixed with Freund's Incomplete Adjuvant at 2, 4, and 6 weeks following initial immunization. The rabbits were sacrificed at 7 weeks, and the serum was separated and stored at −20 °C until required.

Two antibodies (designated TRAb1 and TRAb2) were isolated and purified by saturated ammonium sulfate precipitation followed by protein G affinity chromatography. Protein concentrations of the antibody solutions were determined by BCA assay (Pierce), and their purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Competitive ELISA Procedure. Nunc Maxi-sorp Immunoplates were coated overnight at 4 °C with anti-TR polyclonal antibody at the optimal concentration (4.76 μg/mL for TRAb1 and 6.59 μg/mL TRAb2) in 0.05 M carbonate buffer, pH 9.6 (100 μL/well). They were washed three times with PBST [PBS containing 0.05% (w/v) Tween 20] and three times with PBS. The plates were blocked with PBS containing 2% (w/v) skimmed milk powder for 1 h at 37 °C and washed as before. A 50 μL amount of a bile sample diluted 50:50 (v/v) with PBS (containing various concentrations of free TR) was added to the wells followed by 50 μL TR−HRP conjugate (0.5 μg/mL) in PBS. The plate was left at 37 °C for 1 h. Following a further washing step, the HRP enzymatic activity was detected using the O-Phenylenediamine Dihydrochloride (OPD) Fast Substrate system (Sigma). A 100 μL amount of OPD substrate was added to each well and left to develop in the dark for 30 min and read on a Titertek Twinreader Plus at 405 nm. Inter- and intraday assays were carried out using five replicates of each standard run on five different occasions.

Inhibition ELISA Procedure. Nunc Maxi-sorp Immunoplates were coated with TR−BSA conjugate solution in PBS (0.644 μg/mL for TRAb1 and 1.289 μg/mL vs TRAb2) and blocked as described previously. The bile samples (50 μL/well) were added, followed by 50 μL anti-TR polyclonal antibody (0.793 Mg/mL TRAb1 and 1.983 μg/mL TRAb2) and left for 1 h at 37 °C. A 100 μL amount of the secondary antibody (1/5000 dilution of commercial HRP-conjugated goat anti-rabbit IgG, Sigma) was added to each well and left for 1 h at 37 °C. After it was washed, the HRP enzyme was detected as before and inter- and intraday assays were carried out.

Both intra- and interassay investigations were carried out with five replicates. This means that each TR concentration was analyzed five times and the entire assay was carried out on five different occasions. The mean absorbance of the five replicates was used for the intraday assays while the mean normalized absorbance was used for the interassay calculations. The curves are all fitted using the four parameter

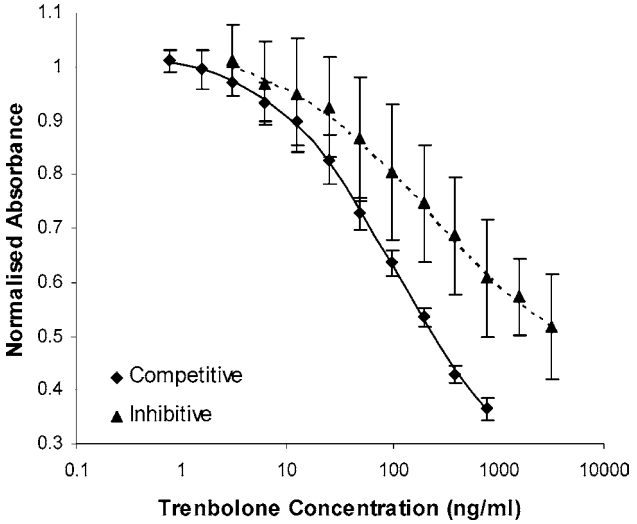

Figure 1. Interassay studies for the detection of free TR using TRAb1 in both competitive and inhibitive formats. The standard deviation for each point is shown by the vertical error bars.

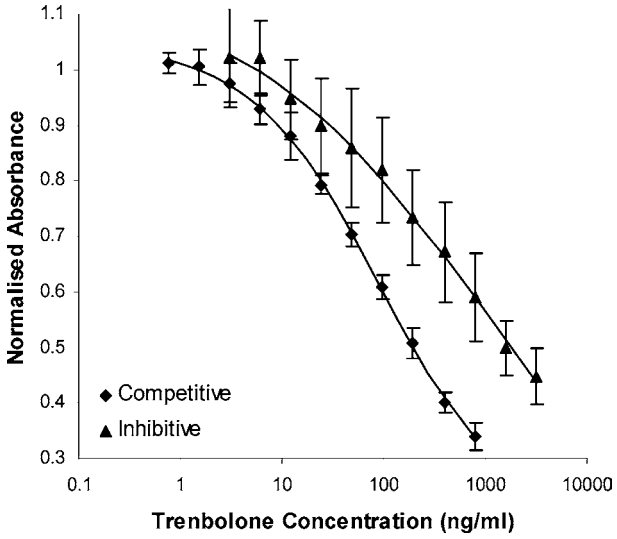

Figure 2. Interassay studies for the detection of free TR using TRAb2 in both competitive and inhibitive formats. The standard deviation for each point is shown by the vertical error bars.

equation below, and the percentage accuracy was determined from this equation.

$$y = R_{hi} - \frac{R_{hi} - R_{lo}}{1 + \left(\frac{x}{A_1}\right)^{A_2}}$$

where y is the absorbance or normalized absorbance value, R_{hi} is the absorbance value as the concentration tends to infinity, R_{lo} is the absorbance value as the concentration tends to zero, x is the concentration value, and A_1 and A_2 are fitting parameters. This model was fitted to the data using BIAevaluation 3.1 software from BIAcore.

Cross-Reactivity Studies. A TR standard curve was prepared in PBS containing 2% (v/v) ethanol. Serial dilutions of the steroid analogues to be tested were also prepared. These sets of drug containing solutions were used as the samples in a competitive assay for the detection of TR using either of the antibodies produced. The concentration that gives an absorbance value of 50% that measured for the PBS containing no steroid was determined for each steroid being tested. The concentration of TR that gives 50% signal in the assay was expressed as a percentage of the value calculated for each of the cross-reacting steroids.

Table 1. Cross-Reactivity of Anti-TR Polyclonal Antibodies TRAb1 and TRAb2

	% cross-reactivities	
compounds tested	TRAb1	TRAb2
TR	100.00	100.00
ethynyl estradiol	<0.10	<0.10
diethylstilbestrol	<0.10	<0.10
α-estradiol	<0.10	<0.10
β-estradiol	<0.50	<0.50
estrone	<0.50	<0.50
norgestrol	1.00	<0.50
19-nortestosteone	4.00	2.00
methyl testosterone	<0.50	<0.50
zeranol	<0.10	<0.10
norethisterone	1.67	0.67

RESULTS AND DISCUSSION

Antibody Specificity. The polyclonal antibodies were tested by ELISA for their ability to bind the target molecule, and their binding with related compounds was also investigated. Standard curves, consisting of doubling dilutions of a number of different compounds (ethynyl estradiol, diethylstilbestrol, α-estradiol, β-estradiol, estrone, norgestrol, 19-nortestosterone, methyl testosterone, zeranol, and norethisterone), were prepared using PBS as the diluent. Each of these were run alongside the TR standards. The percentage cross-reactivity of each compound is shown in **Table 1**. Neither antibody showed any significant recognition of the estradiols tested. This was expected as the immunogen was linked through the 17-position on the steroid allowing the immune system easy access to the A-ring of the steroid. The A-ring of this steroid is distinctly different to that of the estradiols. The compounds that are recognized by these antibodies, i.e., norgestrol, 19-nortestosterone, and norethisterone, all have similar structures about the A-ring. This implies that the antibodies are directed toward this area of the molecule. While these compounds do inhibit the binding of the antibody and the target conjugate, they do not do so at high levels. The low levels of cross-reactivity seen with these polyclonal antibodies indicate their utility for the development of specific methods for the detection of TR without significant interference from related compounds that may be present in a sample. No interference was seen in any of the blank bile samples tested.

ELISA. The polyclonal antibodies were applied to inhibitive and competitive ELISA formats, for the detection of free TR in bovine bile. The competitive assay uses a competition between free drug in a sample and labeled drug for binding to an immobilized specific antibody. The inhibitive format utilizes the inhibiting effect of free drug in a sample to prevent the binding of added specific antibody to immobilized TR conjugate on the plate surface.

After initial positive testing of the antibodies and TR conjugates, the use of bovine bile as the assay matrix was investigated. Bile was chosen as the matrix due to its ease of use, being already a liquid, and the fact that steroidal residues persist there for a longer period than other biological matrices (*9*, *10*). Bile and faeces have also been identified as the most appropriate matrices for the detection of TR (*9*). In this system, the bile is simply diluted 50:50 (v/v) with PBS before application to the ELISA. This is ample pretreatment for the detection of TR in spiked bile samples. For real samples (not available at the time), it may be necessary to deconjugate the samples prior to application to the ELISA (*3*). This frees TR molecules that have become conjugated by the animal's metabolism. However, it may only free a small amount of further TR. Previous work has estimated the amount of conjugated TR to be only 20% of the free TR (*4*). To include a time-consuming and costly deconjugation step may not be necessary if the antibodies are sufficiently specific to avoid cross-reactivity within the matrix and if the assay is adequately sensitive without the need for this additional amount of TR.

Table 2. Ranges of % Coefficient of Variation of the Standards in ELISAs for the Detection of Free TR in Bovine Bile for the Interval of 3.05–3125 ng/mL in the Inhibitive and 0.76–781 ng/mL in the Competitive ELISA

	inhibitive		competitive	
	intraassay (%)	interassay (%)	intraassay (%)	interassay (%)
TRAb1	2.30–5.38	6.51–18.72	0.59–3.20	1.98–5.65
TRAb2	0.92–4.66	6.43–13.43	1.38–3.47	1.73–7.02

Table 3. Accuracy Ranges of the Standards in ELISA for the Detection of Free TR in Bile for the Interval of 3.05–3125 ng/mL in the Inhibitive and 0.76–781 ng/mL in the Competitive ELISA

	inhibitive		competitive	
	intraassay (%)	interassay (%)	intraassay (%)	interassay (%)
TRAb1	52.36–224.14	59.87–129.23	83.84–144.81	80.52–110.24
TRAb2	65.25–132.96	55.94–121.20	66.44–127.76	87.46–133.02

The repeatability of the assay systems was tested by intra- and interday analysis. Intraday analysis consisted of each sample being repeated five times within each assay. From these data, the mean absorbance and the standard deviation for each point were determined. The testing was extended to interday analysis where the above assay ($n = 5$) was repeated on five different days. The mean absorbance values for each point were normalized with reference to the absorbance value obtained for the zero concentration point. This allows for the direct comparison of the values across the different assays. Graphs 1 and 2 show the interday standard curves obtained for the assays using bile spiked with TR as the sample.

The ranges of coefficients of variation are listed in **Table 2**. They show clearly that the competitive ELISA format is more robust and repeatable than the corresponding inhibitive assay. This implies that the antibody−drug binding reaction is more susceptible to fluctuations when the antibody is in free solution and not immobilized on a solid support. The variability in the amount of TR immobilized to the surface via the TR−BSA conjugate may also contribute to the larger well-to-well fluctuations in values seen in the inhibition assay. The binding TR to the surface via a carrier is inherently less robust and can increase variability in comparison to direct binding of TR to the surface or where antibody is bound to the surface. The competitive ELISA format also gives better accuracy for the standard curve when compared to the inhibitive assay (**Table 3**). This was calculated by the closeness of fit of the four parameter model to the data points.

The assays described here, for the detection of free TR in bovine bile, compare well to other assays for TR. The competitive assays described can quantify a sample in less than 2 h with minimal sample pretreatment. The limit of detection was taken as the average of the zero value less two standard deviations over the five intraassays. They can detect levels as low as 2.41 ng/mL in a single sample, which is close to the maximum residue limit for this steroid of 2 ng/mL in urine and plasma, and it needs only 50 μL of bile to complete the assay. If a more concentrated TR−HRP conjugate solution was used,

a larger bile sample could be incorporated into the assay, lowering the sensitivity of the assay further.

The radioimmunoassay described previously (*4*) was able to detect very low levels of TR and trenbolone acetate (40 and 70 ρg, respectively) in bovine urine and tissue. This method, however, took much longer and required the use of radioisotopes. This method also used single tubes and so was not suitable as a rapid screening method for the detection of TR residues.

The ELISA previously described (*3*) is similar to the competitive ELISA in this paper although it requires almost 2 days to complete the analysis between extraction and analysis. The antibodies can also be used to purify samples prior to their detection in a different system such as HPLC−TLC (*2*). This method uses immunoaffinity chromatography to isolate the trenbolone acetate metabolites (17α-TR and 17β-TR), which are then separated by HPLC and finally detected by TLC. This is a labor intensive and time-consuming process that has detection limits of the same order as the ELISA presented here.

The antibodies and conjugate pairs produced have been applied successfully to two similar ELISA formats, termed competitive and inhibitive. Two assays have been produced that can detect low ppb amounts of free TR in bovine bile. They have good repeatability and show acceptable accuracy as shown in both the coefficient of variation data and the accuracy ranges. These assays could be used to screen animals at the point of slaughter for residues of this illegal steroid growth promoter.

LITERATURE CITED

(1) Schiffer, B.; Daxenberger, A.; Meyer, K.; Meyer, H. H. D. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: Environmental studies. *Environ. Health Perspect.* **2001**, *109*, 1145−1151.

(2) van Ginkel, L. A.; van Blitterswijk, H.; Zoontjes, P. W.; van den Bosch, D.; Stephany, R. W. Assay for trenbolone and its metabolite 17α-trenbolone in bovine urine based on immunoaffinity chromatographic cleanup and off-line high-performance liquid chromatography-thin-layer chromatography. *J. Chromatogr.* **1988**, *445*, 385−392.

(3) Meyer, H. H. D.; Hoffmann, S. Development of a sensitive microtitration plate enzyme-immunoassay for the anabolic steroid, trenbolone. *Food Addit. Contam.* **1987**, *4* (2), 149−160.

(4) Hoffmann, B.; Oettel, G. Radioimmunoassays for free and conjugated trenbolone and for trenbolone acetate in bovine tissue and plasma samples. *Steroids* **1976**, *27*, 509−523.

(5) van Look, L. J.; Jansen, E. H. J. M.; van de Berg, R. H.; Zomer, G.; Vanoosthuyze, K. E.; van Peteghem, C. H. Development of a competitive enzyme immunoassay for 17-alpha 19-nortestosterone. *J. Chromatogr.* **1991**, *564*, 451−459.

(6) Jondorf, W. R. On the raising of antibody to the synthetic anabolic steroid trenbolone, its partial characterization and preliminary application for radioimmunoassay. *Experientia* **1980**, *36*, 394−395.

(7) Nambara, T.; Shimada, K.; Ohkubo, T.; Niwa, T. Preparation and antigenic properties of estriol-16-glucuronide and estradiol-17-glucuronide-(C-6)-BSA conjugates. *J. Steroid Biochem.* **1982**, *16*, 533−538.

(8) Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. Preparation and characterization of conjugates of bovine serum albumin with testosterone and with cortisone. *J. Biol. Chem.* **1957**, *228*, 713−727.

(9) Hewitt, S. A.; Blanchflower, W. J.; McCaughey, W. J.; Elliot, C. T.; Kennedy, D. G. Liquid chromatography-thermospray mass spectrometric assay for trenbolone in bovine bile and faeces. *J. Chromatogr.* **1993**, *639*, 185−191.

(10) McEvoy, J. D.; McVeigh, C. E.; Currie, J. W.; Kennedy, D. G.; McCaughey, W. J. Plasma, urinary and biliary residues in cattle following intramuscular injection of nortestosterone laurate. *Vet. Res. Commun.* **1998**, *22* (7), 479−491.

Received for review October 27, 2003. Revised manuscript received April 6, 2004. Accepted May 7, 2004. This work has been supported by Enterprise Ireland and the National Centre for Sensor Research.

JF0352531