Journal of Chromatography, 445 (1988) 385–392
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 500

ASSAY FOR TRENBOLONE AND ITS METABOLITE 17α-TRENBOLONE IN BOVINE URINE BASED ON IMMUNOAFFINITY CHROMATOGRAPHIC CLEAN-UP AND OFF-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–THIN-LAYER CHROMATOGRAPHY

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(First received December 14th, 1987; revised manuscript received March 18th, 1988)

SUMMARY

An high-performance liquid chromatography (HPLC)–thin-layer chromatography (TLC) method was developed to detect the illegal use of the xenobiotic growth promotor Trenbolone acetate® (TBA). Very effective clean-up of bovine urine was achieved by immunoaffinity chromatography (IAC). The active form of TBA, the steroid 17β-Trenbolone (17β-TB), as well as its major metabolite 17α-Trenbolone (17α-TB), were assayed simultaneously with HPLC and on-line UV detection. The fraction containing 17α-TB and 17β-TB (TB-fraction) was collected, and for confirmation 17β- and 17α-TB were subsequently separated and identified by TLC. The limit of detection by on-line HPLC-UV (350 nm) was 1–2 μg TB/l. Off-line TLC detection was even more sensitive, 0.5 μg 17β- or 17α-TB/l. The assay was validated by investigating urine samples from veal calves implanted with TBA. The presence of 17β- and 17α-TB was clearly demonstrated. A survey of the illegal use of TBA in cattle was performed by applying the assay to urine obtained at slaughter. No residues of TBA or its metabolites were found in any of the 144 random samples from the Dutch public health surveillance programme.

INTRODUCTION

Trenbolone acetate® (TBA) is one of the most important xenobiotic anabolic steroids often used as growth promotors for fattening veal calves and cattle. No toxic effects have so far been found in humans[1] and its use, for example, in some countries of the European Community (EC) is common practice. However, in other EC countries, such as The Netherlands, the use of all growth promoters, including TBA is totally banned and their use will be banned in all EC countries starting from 1988[2]. To screen for the illegal use of this compound, a method for detecting residues in bovine urine is required. Such a method will be used for the large-scale detection of trace amounts of the anabolic agent, including its active form 17β-Trenbolone

0021-9673/88/$03.50

(17β-TB) and its major metabolite 17α-Trenbolone (17α-TB), in urine from veal calves and cattle[3].

For screening purposes, radioimmunoassay (RIA) is usually used[4] and this type of method was applied by us[5] previously. With such an assay a relatively large number of samples can be investigated with a rather high specificity and a low detection limit. Because of the restricted availability of the essential radioactive reagents and in order to reduce their use in general, we developed a suitable alternative method based on high-performance liquid chromatography (HPLC) with UV detection[6] which was especially suitable for urine from veal calves.

For samples from cattle, an additional purification is necessary. Immunoaffinity chromatography (IAC) is a very powerful technique when small amounts of a compound have to be isolated from a complex matrix[7,8]. In this paper we present an IA–HPLC method for the determination of TB in 1-ml samples of urine at a level of 1 μg/l (1 ppb) suitable for both calves and adult cattle. A very effective column clean-up was achieved with IAC after enzymatic hydrolysis of the urinary trenbolone glucuronides to obtain free TB. To increase the sensitivity, the chromatographic system was chosen in such a way that 17β-TB and 17α-TB were not separated. The results of the HPLC analyses were confirmed by thin-layer chromatography (TLC), separating 17β- and 17α-TB completely and detecting the two epimers by *in situ* fluorescent reaction[9,10].

MATERIALS AND METHODS*

The antiserum used (batch H 148181) was a polyclonal antiserum raised in a rabbit by immunization with a TB hemisuccinate (HS)–bovine serum albumin conjugate (TB-17HS–BSA)[11]. This immunogen, the standards 17β-TB and 17α-TB and radiolabelled [^{3}H]17β-TB were kindly donated by Roussel-Uclaf, France and were checked for purity by HPLC and/or TLC if applicable. Protein A-Sepharose® and tresyl chloride-activated Sepharose® were from Pharmacia. To hydrolyse the samples, suc d'*Helix Pomatia* (Industrie Biologique Francaise) was used. Prior to use the crude enzyme preparation was purified by gel filtration chromatography (PD-10, Pharmacia)[6].

The urine samples used in this study originated from a model experiment in which male veal calves aged 13 weeks were implanted with preparations containing 20 mg 17β-estradiol and 140 mg TBA (animal no. 19) or 36 mg zeranol® and 140 mg TBA (animals 11 and 26). Animal 7 served as a control. The experiment (no. 61.08) was performed in may 1982 at the Institute for Animal Nutrition Research (ILOB) at Wageningen, The Netherlands. Urine samples were stored at −20°C during the 4 years between sampling and analysis.

The HPLC system consisted of a pump (Model 6000, easy prime, Waters), a sample injector (WISP 710B, Waters), an UV detector (spectroflow 773, Kratos) and a fraction collector (Redirac, LKB). The desired selectivity was obtained using a

* Reference to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Protection, to the exclusion of others which may also be suitable.

chromatographic column (150 mm × 4.6 mm I.D.), obtained from Chrompack, which was packed with LiChrosorb Diol, 5 μm (Merck) using a column packing instrument (Shandon). The eluent was isooctane–ethanol (95:5, v/v) at a flow-rate of 2.0 ml/min.

Preparation of the immunosorbent

The immunoglobulin G (IgG) fraction was isolated from the antiserum (H 148181) with Protein A coupled to Sepharose and the protein content of this fraction was estimated using the method of Lowry *et al.*[12]. Subsequently the protein content was adjusted to 2 mg IgG/ml, either by dilution in 0.1 mol/l sodium hydrogencarbonate or by ultrafiltration (Amicon MC5, exclusion limit 5000 daltons). The antibodies were coupled to tresyl chloride-activated Sepharose according to the supplier's instructions. The immunoaffinity matrix obtained was stored at 4°C in 0.5 mol/l phosphate-buffered saline, pH 7.0, prior to and between analyses.

Procedure

To 1.0 ml urine a few drops of 0.5 mol/l acetic acid were added to adjust the pH to 5.2. To this solution, 0.375 ml of the purified enzyme (equivalent to 0.25 ml of the crude preparation) were added and the mixture was incubated for 1 h at 37°C. The incubate was then cooled to room temperature and applied to the immunosorbent column (open column made of glass, 10 cm × 0.8 cm) containing 1 ml gel, previously washed with 5 ml water. The column was washed three times with water, using portions of 1, 1 and 5 ml respectively. The absorbed TB was eluted with 5 ml ethanol–water (40:60, v/v). The volume of the TB fraction was reduced to approximately 0.8 ml under a stream of nitrogen. Water was added to adjust the volume of the eluate to 1 ml. In order to make possible injection of the sample on the diol HPLC column, the aqueous sample was extracted with a single 6-ml portion of diethyl ether. After removing the ether completely under a stream of nitrogen the residue of the extract was dissolved in 0.2 ml HPLC eluent. Of this solution, 0.18 ml were injected onto the HPLC column. The UV absorbance at 350 nm was monitored and the fraction eluting around the retention time of TB, starting 30 s before and ending 30 s after, was collected. This HPLC fraction was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.05 ml chloroform. A 0.015-ml aliquot of the chloroform solution was applied to each of three HPTLC plates (5 cm × 5 cm), coded A, BI, BII. On plate BI, 0.5 ng 17β-TB and on plate BII, 0.5 ng 17α-TB were superimposed on the extract spot. The plates were developed simultaneously in *n*-hexane–ethyl acetate–acetone (50:40:10, v/v/v), after which they were dried under a stream of cold air. Next the plates were dipped for 10–20 s in a fluorescence reagent mixture containing 100 ml ethanol (96%, v/v), 100 ml diethyl ether and 4 ml concentrated sulphuric acid. After drying the plates for 5 min under a stream of cold air, they were developed in the second direction in *n*-hexane–ethyl acetate–dichloromethane (20:40:40, v/v/v). Both TB epimers were observed as green fluorescent spots when the plates were viewed above an UV lamp (Transilluminator, 366 nm).

RESULTS AND DISCUSSION

The protein content of the IgG fraction obtained after chromatography on

Protein A-sepharose was 2.1 mg/ml. This material was tested against the original antiserum, in its freeze-dried form (batch H 156777)[11]. Serial dilutions were made and the binding of freshly HPLC-purified [^{3}H]17β-TB was monitored. The corresponding dilution curves are shown in Fig. 1. The maximum binding of the tritiated 17β-TB was 84%, for both the freeze-dried antiserum and the IgG fraction. At 50% binding there was a slight deviation. For the freeze-dried serum this point on the dilution curve corresponded with a dilution of 1:11 600 (v/v), and for the IgG fraction with a dilution of 1:9200 (v/v). The recovery of the binding activity after IgG isolation was estimated to be 80%.

Characterization of the gel

Columns were filled with 1 ml of the immunoaffinity matrix and increasing amounts of either 17α-TB or 17β-TB, starting at 1 ng and increasing to 50 ng, were applied. The recovery of both TB epimers was high (>90%) and constant over the range from 1 to approximately 30 ng. If higher amounts were applied to the columns a significant reduction in the recovery was observed. Since amounts of 1–30 ng are well within the range of interest as far as urine samples are concerned, no changes in the amount of gel were necessary.

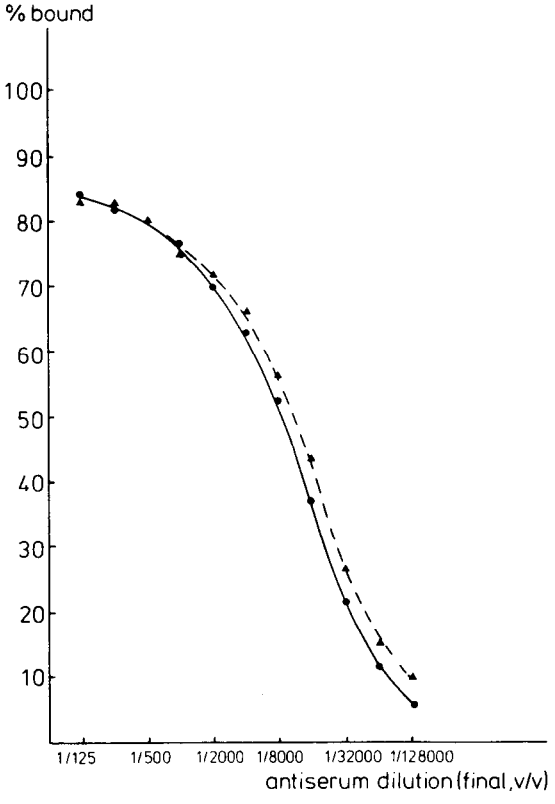

Fig. 1. Antibody dilution curves for the original serum (H 156777, ▲) and the corresponding IgG fraction obtained by Protein A chromatography (H 148181, ●).

Each 1-ml aliquot of gel was used repeatedly. After using the gel ten times, including the analysis of urine samples, no reduction in binding capacity was observed. As a quality-control procedure, we pooled the contents of all columns, mixed the resulting gel gently but thoroughly and divided it again over the number of columns used. The capacity was tested in triplicate each time. The same pool of gel has been in continuous use for over 2 years.

Detection of TB in urine from veal calves implanted with TBA

Urine samples from veal calves implanted with TBA were investigated with the procedure described. All TB HPLC fractions were checked by TLC. Urine samples from the control animal (no. 7) did not show any TB. Not one compound was observable in the extract with HPLC-UV and subsequent TLC.

Fig. 2 shows the chromatograms of the samples obtained at day 0 till 35. A single response was observed at the retention time of 17β- and 17α-TB (6.1 min). The results are summarized in Table I. The presence of TB was clearly demonstrated up to 48

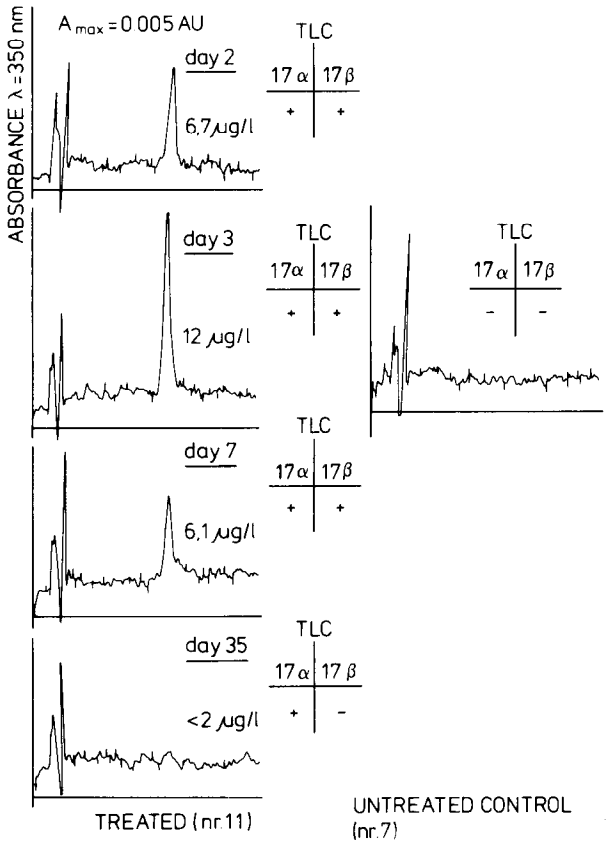

Fig. 2. HPLC chromatograms obtained for urine samples from veal calves implanted with TBA. The right side shows the chromatogram for a blank sample. Results of TLC investigations are also shown. For chromatographic details see the text.

TABLE I

RESULTS OBTAINED FOR SAMPLES OF URINE FROM VEAL CALVES

Animals 11, 19 and 26 were implanted with 140 mg TBA; animal 7 was a control. Identification by TLC.

Animal 7				*Animal 11*				*Animal 19*				*Animal 26*			
*Day**	*HPLC total TB (ppb)***	*TLC* α-*TB* +/−***	β-TB +/−	*Day*	*HPLC total TB (ppb)*	*TLC* α-*TB* +/−	β-*TB* +/−	*Day*	*HPLC total TB (ppb)*	*TLC* α-*TB* +/−	β-*TB* +/−	*Day*	*HPLC total TB (ppb)*	*TLC* α-*TB* +/−	β-*TB* +/−
3	<2	−	−	2	6.7	+	+	3	<2	+	+	3	8.5	+	+
5	<2	−	−	3	12	+	+	6	14	+	+	5	11	+	+
7	<2	−	−	7	6.1	+	+	7	6.6	+	+	7	3.6	+	+
35	<2	−	−	35	<2	+	−	35	<2	+	−	35	<2	+	−
48	<2	−	−	48	<2	+	−	48	<2	+	−	48	§	§	§

* Number of days after implantation.
** Results expressed as μg TB per liter urine.
*** +, Fluorescent TB spot visible; −, no spot visible.
§ No sample available.

days after treatment. It is concluded that detection by TLC is more sensitive than by HPLC. Samples showing a positive (> 2 μg TB/l) response by HPLC all contained 17β- and 17α-TB. At day 35 or day 48 after treatment, no TB was detected by HPLC. However, TLC investigation of the HPLC TB fraction still showed the presence of 17α-TB. In general, if a positive response was detected by HPLC, the sample contained both 17β- and 17α-TB. When no response was obtained by HPLC–UV, and the sample was from a treated animal, even 48 days after treatment 17α-TB was detected by TLC. The observed urinary residue excretion is expected from the known metabolism of TBA in veal calves[13].

In 1986 a total of 144 samples of urine taken at random from slaughtered cattle were analysed using the method described, including TLC confirmation. None of the samples showed any residues indicating the use of TBA.

HPLC has been shown to be a potent technique in residue analysis, amongst others for anabolic agents[6,14–17]. Obtaining suitable separation and detection techniques is always an important part of the development of a new method. Even more important is the sample clean-up, especially for residues at ppb levels in biological matrices. A variety of materials and techniques are available at present. However, their specificity is limited, especially when the results have to be used for forensic purposes. A combination of two or even more clean-up steps is usually necessary. In contrast, IAC is based on the rather specific interaction of the analyte and an antibody raised against it. When such an antibody is available, IAC surpasses many other techniques as far as the sample clean-up is concerned. For efficient control of the illegal use of TBA in bovines, TB residues down to a level of 0.5–1 ppb in urine are detected with an high specificity based on immunochemical solid-phase column clean-up and HPLC retention time(s), together with confirmation by two-dimensional TLC with *in situ* fluorescence reaction[9]. In routine use, a per serial throughput of 20 samples per technician a day is achievable. Meanwhile, our pilot experiments also showed the suitability of the reported IAC method for the identification of 17β-TB residues at the 0.1 ppb level in meat using in combination of IAC and gas chromatography with mass selective detection.

ACKNOWLEDGEMENTS

This investigation was performed as part of project 368301 on behalf of and for the account of the Dutch Veterinary Chief Inspectorate of Public Health. Labelled and unlabelled 3-ketotriene steroids and immunogen were gifts from Drs. J. C. Bouffault and A. Jouquey (Roussel-Uclaf, France). The authors are indebted to Ir. P. L. M. Berende (Dutch State Institute for Quality Control of Agricultural Products, Wageningen) for the urine samples from implanted veal calves and to Dr. N. F. Cunningham (MAFF, Central Veterinary Laboratory, Weybridge, U.K.) for his critical comment on the manuscript.

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