CHROM. 25 015

Liquid chromatography–thermospray mass spectrometric assay for trenbolone in bovine bile and faeces

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ABSTRACT

A liquid chromatography–thermospray mass spectrometric assay was developed and validated to confirm the presence of illegal residues of the synthetic androgenic growth promoter, trenbolone acetate, in cattle. The assay was specific for 17α-trenbolone, the major bovine metabolite of trenbolone acetate. Methods were developed for the determination of 17α-trenbolone in both bile and faeces, the most appropriate matrices for the control of trenbolone acetate abuse. The clean-up procedure developed relied on enzymatic hydrolysis, followed by sequential liquid–liquid and liquid–solid extraction. The extracts were then subjected to immunoaffinity chromatography. 17α-Trenbolone was detected by selected ion monitoring at m/z 271 using positive ion thermospray ionisation. The limit of detection was approximately 0.5 ng/g in faeces and 0.5 ng/ml in bile.

INTRODUCTION

17β-Trenbolone (17β-TBOH) is a synthetic androgen which has important anabolic activity. In the form of trenbolone acetate (17β-TBOAc) it has been used as a solid implant to promote growth, usually in combination with 17β-estradiol, in steers, heifers and veal calves [1]. Although the use of 17β-TBOAc has been banned in the European Community [2], it is still legally used in many other parts of the world.

Previous studies on the metabolism of 17β-TBOAc in cattle [3–5] have demonstrated that it is rapidly hydrolysed to the active compound 17β-TBOH. Free trenbolone can bind to both testosterone and estrogen receptors, altering the rates of protein synthesis and degradation. The net result of these interactions in an increase in

skeletal muscle mass [6]. The 17β-TBOH undergoes oxidation to trendione leading to reduction to epi-TBOH (17α-TBOH) which, in the form of a glucuronide or sulphate conjugate, is the major biliary metabolite of trenbolone [5].

To control the illegal use of 17β-TBOAc, analytical methods must be sensitive and specific for the determination of 17α-TBOH in biological samples. Bile is the sample of choice in animals presented for slaughter in abattoirs, since it is in bile that the highest levels of 17α-TBOH have been found [5]. However, in follow-up investigations on farms, faeces are frequently the only samples which are readily available.

A number of analytical methods for the determination of trenbolone in urine and tissue have been described which employ HPLC [7], enzyme immunoassay [8], GC–MS [9] and LC–MS–MS [10]. Other workers have reported the use of immunoaffinity chromatography as one of the clean-up steps in the analysis of 17α-TBOH

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in urine and tissue [11]. However, none of these assays have described the quantitative determination of 17α-TBOH in either bile or faeces.

We wish to report a procedure which uses immunoaffinity chromatography coupled with liquid chromatography–thermospray mass spectrometry (LC–MS) for the specific detection of trenbolone residues in bile and faeces. LC–MS offers several advantages to the determination of polar drugs in biological matrices. Firstly, derivatization is not necessary. Secondly, the sensitivity of LC–MS is highly compound dependent enhancing the response from a sensitive analyte over that obtained from matrix effects.

EXPERIMENTAL

Chemicals and equipment

Acetonitrile, diethyl ether and methanol were HPLC grade, other reagents were analytical-reagent grade. β-Glucuronidase/aryl sulphatase (type H2 from *Helix pomatia* was purchased from Sigma (Poole, UK). This was used without dilution for the enzymatic hydrolysis of glucuronide conjugates in faeces and bile samples. [³H]17β-TBOH was prepared by Amersham International (Amersham, UK). 17α-TBOH was obtained from RIVM (Bilthoven, Netherlands). A stock standard (1 mg/ml) in methanol was prepared. The stock standard was stored at 4°C and was stable for several months. Working standards were prepared daily as described below by dilution of the stock standard in mobile phase.

Immunoaffinity columns

The antiserum used was a polyclonal antiserum (titre 1:60 000 by radioimmunoassay) which was produced in this laboratory (by W.J. McC.) by immunising a New Zealand White rabbit with a conjugate of 17β-TBOH hemisuccinyl ester and bovine serum albumin. The specificity of the antisera was investigated by measuring the crossreactivity of other, structurally related compounds. Crossreactivity was defined as 100 times the amount of 17β-TBOH required to displace 50% of a tracer quantity of [³H]17β-TBOH from a fixed amount of antibody divided by the amount of competitor required to

produce the same displacement. The antibodies showed 85% crossreactivity with 17α-TBOH and 15% crossreactivity with 19-nortestosterone. The antibody exhibited < 0.01% crossreactivity with a wide range of other compounds including testosterone, 17α- and 17β-oestradiol, progesterone, zeranol, hexoestrol, diethylstilboestrol, cortisol, corticosterone and hydroxyprogesterone. The immunoglobulin G (IgG) fraction was isolated from the antiserum by precipitation with caprylic acid [12]. The protein content of the IgG fraction was estimated using the method of Lowry *et al.* [13], and was then adjusted to 2 mg/ml with 0.1 mol/l sodium hydrogencarbonate buffer pH 8.3 containing 0.5 mol/l sodium chloride. The antibodies were coupled to cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the suppliers' instructions to give a matrix containing 1 mg IgG per ml of swollen gel. Aliquots of the immunoaffinity matrix (1 ml) were placed in open glass columns (10 × 0.7 cm) in isotonic phosphate-buffered saline (PBS) and washed with 5 ml of the same solvent. Columns were stored at 4°C in the presence of PBS containing merthiolate (50 μg/ml). Before use, the columns were washed with 0.1 mol/l sodium acetate buffer, pH 4.0, containing 0.5 mol/l sodium chloride (5 ml) followed by 1.0 mol/l sodium carbonate (5 ml). They were then rinsed with PBS (5 ml), and the samples were applied. After
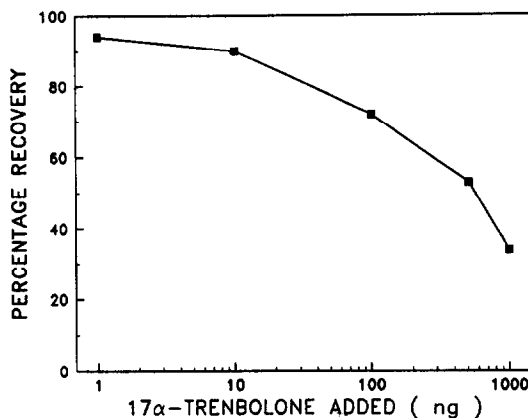

Fig. 1. Capacity of a typical immunoaffinity column for 17α-trenbolone.

use, the columns were rinsed with ethanol–water (70:30, v/v) (10 ml), water (5 ml) and stored with PBS–merthiolate as described above.

The immunoaffinity columns are stable, when stored as described above, for at least 4 months. During a 4-month period a column was spiked on 12 separate occasions with 10 ng 17α-TBOH. The recovery of 17α-TBOH did not alter during this period, being 73.7% on the first occasion and 71.1% on the last.

The capacity of the immunoaffinity columns was measured by applying a trace quantity of [^{3}H]17β-TBOH, along with varying amounts of non-radioactive 17β-TBOH, to a column and irrigating it as described below. The capacity of the columns varied somewhat from batch to batch. Fig. 1 shows a typical result obtained for one preparation.

Biological samples

Known trenbolone-free samples of bile and faeces were collected from animals born and fattened on a farm owned and maintained by the Department of Agriculture for Northern Ireland. Incurred material was obtained by implanting 15 pellets, containing a total of 300 mg 17β-TBA into the base of the left ear of a six-month old calf. Faeces and bile samples were taken 6 weeks after implantation.

Extraction procedure

The procedures developed to hydrolyse the samples and to perform the liquid–liquid extraction differed for bile and faeces. These are described separately. Thereafter all samples were treated identically.

Hydrolysis and liquid–liquid extraction: faeces. A series of negative and fortified samples (10 g each) were prepared by adding between 0 and 50 ng of 17α-TBOH to a known negative faeces sample. These, along with a series of test samples (10 g each) were incubated with β-glucuronidase (100 μl) and 0.2 mol/l sodium phosphate buffer, pH 5.5 (25 ml) either overnight at ambient temperature or for 4 h at 37°C.

Methanol (60 ml) was added to the hydrolysates which were then shaken on a reciprocating mixer for 1 h. The sample was centrifuged at 2000 g for 10 min at 4°C and an aliquot of the supernatant (50 ml) removed. The methanol was evaporated under vacuum in a rotary evaporator at 40°C and 0.1 mol/l glycine–sodium hydroxide buffer, pH 12.5, containing 0.1 mol/l sodium chloride (10 ml) added. The extract was then shaken vigorously for 1 min each with two portions (10 ml) of tert.-butyl methyl ether (t-BME) and then centrifuged at 2000 × g for 10 min at 4°C to enhance separation of the layers. The pooled t-BME extracts were then taken to dryness under a stream of nitrogen at 40°C.

Hydrolysis and liquid–liquid extraction: bile. A series of negative and fortified samples (5 ml each) were prepared by adding between 0 and 100 ng of 17α-TBOH to a known negative bile sample. These, along with a series of test samples (5 ml each) were incubated with β-glucuronidase (50 μl) and 0.2 mol/l sodium phosphate buffer, pH 7.0 (12.5 ml) either overnight at ambient temperature or for 4 h at 37°C.

The hydrolysates were loaded onto Extrelut 20 columns (Merck, Poole, UK) and allowed to stand for 20 min. The columns were irrigated with diethyl ether (2 × 20 ml) and the eluates were collected in round-bottomed flasks. It was found beneficial to allow the column to run dry between each addition of diethyl ether. The diethyl ether was removed under vacuum at 30°C.

Liquid–solid extraction: faeces and bile. The residues in the flasks were dissolved in diethyl ether (3 ml) with the aid of ultrasonication and aliquots of petroleum spirit (3 ml, 40–60°C) were added to each. These solutions were then loaded onto a Bond-Elut CN cartridge (Analytichem International, Harbor City, CA, USA) which had been preconditioned by washing with chloroform (4 ml) followed by petroleum spirit (5 ml). The flasks were then washed with aliquots (1 ml) of petroleum spirit–diethyl ether (50:50, v/v). These washings were also applied to the Bond-Elut cartridges. The cartridges were then washed with petroleum spirit (3 ml), and petroleum spirit–chloroform (50:50, v/v) (4 ml). The cartridges were then irrigated with chloroform (4 ml) to elute the 17α-TBOH. The solvent was concentrated to dryness under a gentle stream of nitrogen at 40°C and dissolved in methanol (500

μl) with the aid of ultrasonication. Finally, isotonic PBS (2 ml) was added with vortexing.

Immunoaffinity chromatography: faeces and bile. The sample solutions derived from the liquid–solid extraction were then applied to the immunoaffinity columns, which were then washed with water (3 ml). The adsorbed 17α-TBOH was eluted with ethanol–water (70:30, v/v) (3 ml). The eluate was concentrated to dryness under a gentle stream of nitrogen at 70°C. The columns were washed and stored as described above.

LC–MS system

The LC–MS system consisted of a Merck–Hitachi (BDH, Dagenham, UK) Model L-6000 pump, a Rheodyne (Rheodyne, Cotati, CA, USA) Model 7125 injector fitted with a 50-μl sample loop and a LiChrosorb (Merck, Poole, UK) RP-18, endcapped, 125 × 4 mm reversed-phase cartridge with holder. The mobile phase consisted of acetonitrile–0.1 mol/l ammonium acetate (45:55, v/v). It was degassed and filtered under vacuum through a 0.45-μm filter using a solvent filtration system (Millipore–Waters, Harrow, UK). The mobile phase was pumped at a rate of 1.0 ml/min. This system was directly coupled to a Vestec (Houston, TX, USA) Model 201A thermospray LC–MS system complete with a Technivent workstation.

The instrument was operated in two modes, the positive ion pure thermospray mode and the negative ion chemical ionisation (CI) mode using filament-assisted ionisation with a electron beam current of 250 μA. The electron multiplier voltage was 1600 V. The temperatures of the source block, tip heater and lens assembly were 260, 260 and 135°C, respectively. The vaporizer probe was operated at about 15°C below the take-off point. This was optimized for each probe used, but was typically in the region of 190°C. The instrument was operated either in the full scan mode to collect spectra, or in the selected ion monitoring mode (SIM) for maximum sensitivity. For the former, a dwell time of 3 ms was used, and for the latter the dwell time was 100 ms. In each case the sweep window was set at 0.5 u. The tuning parameters were checked daily according to the manufacturers instructions

using a 50 mg/l solution of polyethylene glycol 300 in acetonitrile–water (50:50, v/v), containing 0.1 mol/l ammonium acetate.

LC–MS confirmation: faeces and bile

The residues from the immunoaffinity chromatography step were dissolved in acetonitrile (100 μl) with the aid of ultrasonication (10 min). Water (100 μl) was added with vortexing. Aliquots (50 μl) were then injected onto the LC–MS system. Both identification and quantification were carried out by SIM of ions characteristic of 17α-TBOH in the positive and negative ionisation modes. The positive mode was operated in pure thermospray and the [M + 1] ion (m/z 271) monitored. This was the normal mode of operation for trenbolone analyses. However, negative mode, filament-assisted ionization was also sensitive when the [M] ion (m/z 270) was monitored. Calibration curves were constructed using least-squares linear regression analysis. For quantification of 17α-TBOH, the area of either the [M + 1] or the [M] peak was compared to a range of calibration standards equivalent to 0, 0.4, 2, 4, 10, 20, 30 and 40 ng/ml 17α-TBOH.

RESULTS AND DISCUSSION

LC–MS analysis of 17α-TBOH

Full-scan spectra were easily achievable using 100 ng 17α-TBOH. In the negative ion mode,
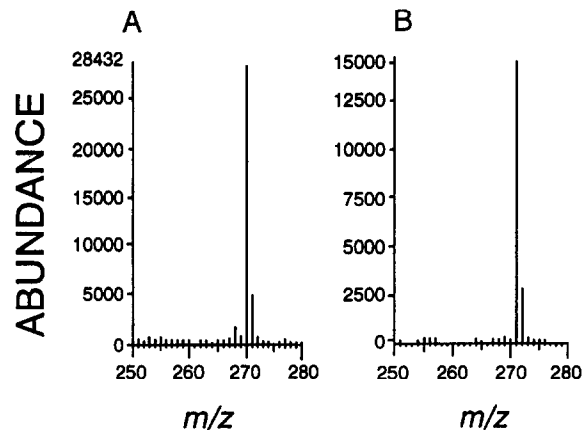

Fig. 2. Full scan spectra of 17α-trenbolone using either negative ion filament-assisted ionisation (A) or positive ion thermospray ionisation (B).

filament-assisted ionisation gave as base peak, the molecular ion at m/z 270 (Fig. 2A), while in the positive ion mode, in pure thermospray, the base peak was the protonated molecular ion at m/z 271 (Fig. 2B). No observable fragmentation was found in either mode. On SIM of m/z 270 or m/z 271, 500 pg 17α-TBOH injected onto the column were readily detectable.

The signals obtained in both modes were consistent during a run but were variable from day to day. One explanation may be the alteration of the spray characteristics of the probe as a result, perhaps, of contamination. This did not present a problem if standards were carried through with each set of samples. This could have been overcome by the use of an internal standard. However, we did not have access to deuterated trenbolone. Another possibility would have been to have employed 19-nortestosterone (19-NT). However, this was found not to be practical for two reasons. Firstly, the response produced by 19-NT on LC–MS is only approximately 10% of that produced by 17α-TBOH. Consequently, high concentrations of 19-NT would have had to be carried through the assay. Secondly, the crossreactivity of our antibody with 19-NT was only 15%, which would also have required the use of high concentrations of 19-NT. These two factors would thus have substantially reduced the capacity of the immunoaffinity columns for 17α-TBOH.

Assay development and validation

To enhance the specificity of the procedure for the isolation of 17α-TBOH from the complex matrices of bile and faeces, immunoaffinity chromatography was combined with the orthodox procedures of solvent extraction and solid-phase extraction.

In the case of faeces, adjustment of the pH to 12.5 ensured that unwanted acidic compounds were ionised and extracted into the aqueous phase while 17α-TBOH remained in the neutral form and was retained in the t-BME fraction. The use of a Bond-Elut CN cartridge in the normal phase mode, separated 17α-TBOH from any non-polar matrix components. The cleanliness of the sample after the final immunoaffinity chromatography step removed any variability caused by matrix components altering the efficiency of the thermospray ionisation mechanism.

Although 17α-TBOH produced a sensitive signal in both the positive and negative modes, positive ion thermospray was the method chosen for use throughout this study. The calibration graphs relating the peak areas of m/z 271 with the concentration of 17α-TBOH in prepared standards were linear in the range 0–40 ng/ml. The regression characteristics were, typically, $y = 209x + 33.6$, (correlation coefficient 0.9998). The accuracy and precision of this method were evaluated by analysis of fortified bile and faeces samples at concentrations of 5 and 20 ng/ml bile, and 2 and 5 ng/g faeces. Table I shows that the R.S.D. of the assay is between 4 and 8.5% for bile and between 10 and 11% for faeces. The recovery of 17α-TBOH from fortified bile or faeces ranged from 60–75%.

Sample analysis

This assay has been used to provide evidence of the illegal use in cattle of 17β-TBA-containing

TABLE I

RECOVERY OF 17α-TRENBOLONE FROM FORTIFIED SAMPLES ($n = 5$) OF BILE AND FAECES

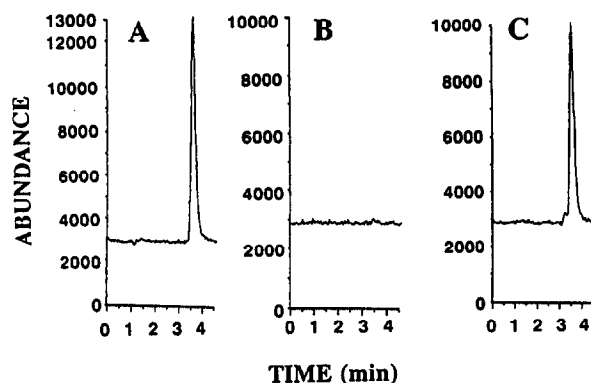| Matrix	17α-TBOH added (mean ± S.D.) (ng/ml or ng/g)	17α-TBOH recovered (mean ± S.D.) (ng/ml or ng/g)	Recovery (%)	R.S.D. (%)
Bile	5.00	3.12 ± 0.26	62.4	8.3
Bile	20.00	14.50 ± 0.60	72.5	4.1
Faeces	2.00	1.50 ± 0.16	75.0	10.7
Faeces	5.00	3.10 ± 0.30	62.0	9.7

Fig. 3. Selected ion monitoring (m/z 271) for the detection of 17α-trenbolone in a standard solution containing the equivalent of 3.6 ng 17α-TBOH per g (A), a known negative faeces sample (B) and an incurred faeces sample (C) using positive ion thermospray LC–MS.

implant pellets to promote growth. Typical SIM chromatograms of standard, negative and incurred faeces samples are shown in Fig. 3. There were no co-eluting components in the negative faeces samples which could have caused interference in the assay. This shows the power of immunoaffinity chromatography as a clean-up procedure in the analysis of faeces, a particularly dirty matrix. The 17α-TBOH concentration in the faeces sample shown in Fig. 3 was 2.5 ng/g. The SIM chromatograms obtained for bile were similar to those of faeces (data not shown). In bile the limit of detection of the assay was approximately 0.5 ng/ml, and in faeces 0.5 ng/g, based on a signal to noise ratio of 3:1.

The Commission of the European Communities (EC) has produced criteria to determine the suitability of various analytical techniques for the chemical confirmation of veterinary drug residues [14]. However, the EC criteria have not, as yet, drafted specific criteria for the suitability of LC–MS. An alternative approach was adopted by an EC panel of experts which undertook a desk-top evaluation of the components of an analytical method [15]. They assigned partial selectivity indices to each step of a procedure and concluded that a confirmatory assay should have a minimum selectivity index of 7. The described assay consists of a liquid–liquid extraction (score 0), a liquid–solid extraction (score 2), immunoaffinity chromatography (score

3) and detection of a pseudomolecular ion (score 4). Thus, the described assay has a selectivity index of 9, making it suitable for use as a confirmatory assay. We believe that this is probably an underestimate of the selectivity of LC–MS because of the very marked compound-dependent sensitivity of this technique. For example, as mentioned above, 19-NT gives very poor responses on LC–MS. Thus, although the described assay does not measure 4 diagnostic ions, the approach advocated by the EC for low-resolution GC–MS [14], the selectivity achieved by the combination of immunoaffinity chromatography and LC–MS makes this method eminently suitable for the confirmation of 17α-TBOH residues in bile and faeces.

In conclusion, the described assay is reproducible and is sufficiently sensitive to be useful as a method for the independent chemical confirmation of trenbolone in bile and faeces, which are of most value in controlling the illegal use of this compound. Bile samples are preferred from dead animals, because bile is a cleaner matrix and because analysis is faster and more economical, with up to 10 samples being processed in one working day. The equivalent figure for faeces is 5 samples in 2 working days. The described assay has now been used in this laboratory for over 2 years to confirm the presence of 17α-TBOH in bile and faeces taken from cattle in Northern Ireland, following screening by enzyme immunoassay.

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