

# SCREENING OF TRENBOLONE-17 $\beta$ IN MILK SAMPLES AFTER APPLICATION OF TRENBOLONE ACETATE TO A CULL COW

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## Abstract

An extraction method to detect the illegal application of trenbolone acetate in cull cows by HPLC/EIA analysis of milk samples was developed.

One cull cow was treated once with 200 mg of trenbolone acetate 6 weeks before slaughter. Milk samples were taken during regular milking and analysed for trenbolone-17 $\beta$  and its metabolites trenbolone-17 $\alpha$  and trendione.

The sample preparation consisted of enzymatic proteolysis and hydrolysis of steroid glucuronides and sulfates and was followed by liquid-liquid extraction and solid-phase clean-up. To detect each of the three metabolites of trenbolone, fractionizing by high-performance liquid chromatography was essential. The final quantification was carried out with a specific enzyme immunoassay.

Only trenbolone-17 $\beta$  was detected. Its concentrations reached 15 pg/mL after treatment and slowly decreased to 4 pg/mL within the following 6 weeks.

The results suggest that it is possible to detect illegal trenbolone treatment in lactating cull cows' milk.

## Introduction

The xenobiotic steroid hormone trenbolone is known for its strong anabolic activity, which is attributed to its androgenic and anti-glucocorticoid effects (Danhaive and Rousseau, 1986). In many countries outside the EU trenbolone acetate is licensed as growth promoter for steers and heifers. In addition, its tremendous effectiveness in dry and lactating cull cows (38 % increased weight gain accompanied by lower fat deposition) was reported (Galbraith, 1980; Béranger and Malterre, 1968). As there are only data available about the kinetics of excreted radioactivity through milk following implantation of tritiated trenbolone acetate (less than 1 % of the implanted dose) (Pottier et al. 1975), the present investigation aimed to detect extractable and immunoreactive residues in milk with the help of HPLC/EIA.

## Materials and methods

### *Animal experiment and sampling*

One healthy lactating Brown Swiss cull cow - raised at our own experimental farm - was treated with a single dose of Finaplix-H (Hoechst Roussel Vet, Sommerville, NJ, USA), containing 200 mg of trenbolone acetate. According to the manufacturers instructions the implant was administered to the middle third of the back side of the ear. Within the following six weeks until slaughter the cow had free access to straw, hay and maize silage. During regular milking, samples were taken and stored at  $-20^{\circ}\text{C}$  until analysis.

The whole milk production of the cow was discarded during the experimental period.

### *Preparation of the samples (proteolysis, hydrolysis)*

3 mL of whole milk were incubated at  $55^{\circ}\text{C}$  in a shaking waterbath (model 1083, GFL, Germany) for 2 hours with 3 mL of 0.5 M Tris-HCl pH 8.0 containing 1 mM  $\text{CaCl}_2$  and 50  $\mu\text{g}/\text{mL}$  Proteinase K. The enzyme was deactivated at  $70^{\circ}\text{C}$  for 30 min and the samples were equilibrated to room temperature. Subsequently 3 mL of 0.5 M sodium acetate pH 4.8 with 5  $\mu\text{L}/\text{mL}$   $\beta$ -glucuronidase/arylsulfatase from *Helix pomatia* were added. The hydrolysis was performed for 2 hours at  $37^{\circ}\text{C}$  (shaking waterbath, see above).

### *Liquid-liquid extraction*

To extract the steroids the samples were shaken thoroughly with 6 mL of methyl tert-butyl ether each. After freezing for at least 30 min at  $-60^{\circ}\text{C}$  the organic phase was decanted and the extraction procedure was repeated. The ether phases were combined, evaporated in a shaking waterbath ( $60 - 70^{\circ}\text{C}$ ) (model 1083, GFL, Germany) and redissolved in 1,25 mL of methanol, frozen for at least 30 min at  $-60^{\circ}\text{C}$  and centrifuged for 15 min at  $-10^{\circ}\text{C}$  and 2000 x g. The methanol was decanted and diluted with 5 mL of water.

### *Solid-phase clean-up*

Then the extracts were applied to octadecyl-silicagel-cartridges (Bakerbond SPE  $\text{C}_{18}$  Product No. 7020-01, J. T. Baker Inc., Philipsburg, NJ, USA): Washing with 2 x 1 mL of methanol, was followed by equilibrating with 2 x 1 mL of 20 mM Tris-HCl pH 8.5/methanol, 80 : 20 (v : v). After adding the milk extracts, the columns were washed with 2 x 1 mL of 20 mM Tris-HCl pH 8.5/methanol, 80 : 20 (v : v) and 2 x 1 mL of 40 % methanol. Finally the steroids were extracted with 2 x 0.75 mL of 80 % methanol. The solvent was evaporated to dryness at reduced pressure in a centrifugal evaporator (Univapo 100 H, Uni Equip, Martinsried, Germany) and the residue was redissolved in 300  $\mu\text{L}$  of acetonitrile/20 mM Tris-acetate pH 7.2, 80 : 20 (v : v).

### *High-performance liquid chromatography (HPLC)*

250  $\mu$ L of the extracts were applied to a RP18-column (Prontosil 120-5-C18 5.0  $\mu$ m, Bischoff, Leonberg, Germany) by an autosampler (LC 507e, Beckman, München, Germany) and eluted with acetonitrile/20 mM Tris-acetate pH 7.2 38/62 at 25 °C (column thermostat Jetstream Plus, Beckman, München, Germany) and a flow rate of 1 mL/min (pump LC 125, Beckman, München, Germany). Fractions were collected around the retention times of trenbolone-17 $\beta$ , trenbolone-17 $\alpha$  and trendione (fraction collector FRAC-100, Pharmacia, Uppsala, Sweden), evaporated and redissolved in 40 % methanol.

### *Enzyme immunoassay (EIA)*

The trenbolone-17 $\beta$ -contents in 20  $\mu$ L of each fraction were analysed in duplicate by an specific enzyme immunoassay published earlier (Meyer and Hoffmann 1987). Calibration curves of the EIA were prepared in 40 % methanol. The working intervall ranged from 0.16 pg (80 % rel. binding) to 2.0 pg (20 % rel. binding) trenbolone-17 $\beta$ /20  $\mu$ L.

The antibody showed the following cross-reactivities: trenbolone-17 $\beta$  100 %, trenbolone-17 $\alpha$  68 % and trendione 123 % (calculation based on the values of 50 % rel. binding). Therefore trenbolone-17 $\beta$ , trenbolone-17 $\alpha$  and trendione could be parallely detected within the same test.

To determine accuracy and precision of the extraction procedure, 3 mL aliquots of a milk pool from untreated animals of our own herd were fortified with 20, 40 or 81 pg/mL (n=3) trenbolone-17 $\beta$  in the form of trenbolone-17 $\beta$ -glucuronide and analysed according to the standard protocol. The mean recovery was 19 %, the coefficient of variation 11 % (see table 1). The detection limit was 0.5 pg trenbolone-17 $\beta$ /mL whole milk.

Table 1: Results of the validation

|      | recovery (%) of trenbolone-17 $\beta$ |          |          |
|------|---------------------------------------|----------|----------|
|      | 20 pg/mL                              | 40 pg/mL | 81 pg/mL |
|      | 19.02                                 | 19.68    | 19.16    |
|      | 20.11                                 | 19.60    | 19.70    |
|      | 17.02                                 | 14.49    | 16.10    |
| mean | 18.72                                 | 17.92    | 18.32    |
| SD   | 1.57                                  | 2.97     | 1.94     |
| CV   | 8.37                                  | 16.59    | 10.60    |

## Results and discussion

The aim of our efforts was to develop a highly efficient procedure to detect trenbolone residues in milk excluding false negative results.

Whole and skimmed milk samples were screened by EIA. Since the measured values after defatting (data not shown) were even less than those of whole milk samples, we supposed that trenbolone residues are adsorbed by lipid membranes of the milk fat micelles, and chose whole milk samples for HPLC/EIA analysis.

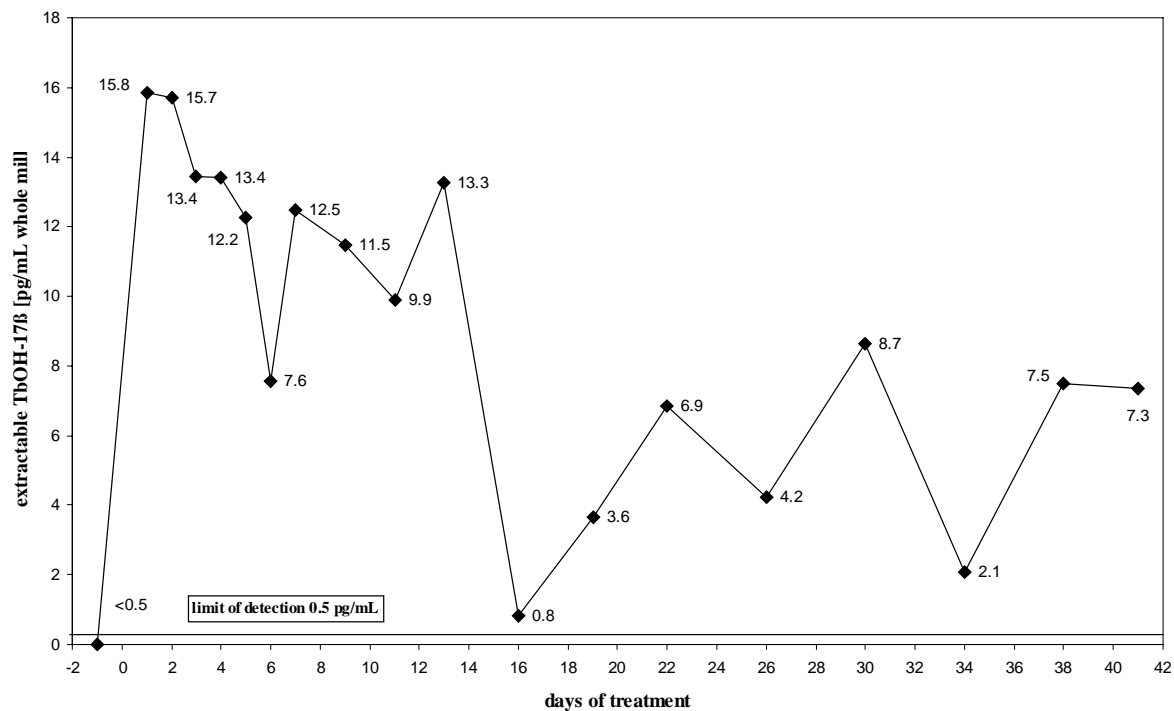
Hydrolysis with *Helix pomatia* juice was carried out to comprise even the hydrophilic conjugated metabolites of trenbolone, which should predominate in milk as in other hydrous excreta like bile or urine.

Proteolysis turned out to be necessary, because casein often coagulated during shaking with ether. On the other hand we know that trenbolone binds to proteins (Ryan and Hoffmann, 1978). The poor recoveries near 20 % in fortified samples suggest that we could not release bound residues after destroying the protein structures with Proteinase K.

Figure 1 shows the results of the 19 milk samples` analysis. Only the contents of extractable and immunoreactive trenbolone-17 $\beta$  lay above the detection limit of 0.5 pg/mL. During the first thirteen days they reached 15 pg/mL, while they decreased to about 4 pg/mL in the following days of the treatment. Linear regression yields a theoretical half life of 34 days. Taking into account that the JECFA has determined an ADI of 0.7  $\mu$ g, at first sight the residues in milk seem to have no toxicological relevance by themselves, but they constitute an possible incremental value of additional exposure.

As the detection limit of trenbolone-17 $\beta$  was 0.5 pg/mL whole milk, treatment could be detected in all samples, whereas the sample before the implantation was beyond the detection limit. In spite of the poor recoveries, the method is suitable to control illegal treatment of cull cows, which might be necessary considering its tremendous effectiveness and therefore high potential of abuse. As trenbolone is a xenobiotic compound, qualitative detection is sufficient for a screening method.

Figure 1: Kinetics of trenbolone-17 $\beta$ -excretion in whole milk after implantation of 200 mg of trenbolone acetate



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