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## Note

# Fast high-performance liquid chromatographic screening method for the presence of trenbolone and its major metabolite in urine of slaughter cattle

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The use of hormonal anabolics as growth promotors in lifestock fattening has been banned in many countries, The Netherlands included. Several control methods have been developed for both large-scale screening and conformation purposes. For screening, radioimmunoassay (RIA) techniques are used most frequently to indicate the presence of anabolics in excreta. We have developed RIA techniques for several compounds, such as diethylstilbestrol (DES)[1], 19-nortestosterone (NT)[2] and trenbolone (TB)[3]. A chromatographic purification step by high-performance liquid chromatography (HPLC) prior to RIA was necessary, mainly to prevent false-positive results[4]. Other screening methods have recently been applied, such as HPLC with total spectrum identification by on-line diode array detection[5]. This method, however, is restricted to samples such as application sites and formulations with high concentrations of anabolics and few interfering matrix components.

Here a fast and simple detection method based on normal-phase HPLC with on-line UV-detection at 350 nm is presented for large-scale screening of bovine urine for the xenobiotic steroid trenbolone and its major metabolite the alpha isomer.

## MATERIALS AND METHODS*

### *Apparatus*

The HPLC equipment consisted of an automatic injector (WISP, Waters Assoc.), a solvent delivery system (Model 2150, LKB), a variable-wavelength detector operated at 350 nm (Model 773, Kratos) and a printer–plotter–integrator (Model 3390A, Hewlett-Packard). The chromatographic column (150 mm × 4.6 mm I.D.), obtained from Chrompack, was packed with silica gel [5-$\mu$m Hypersil (Shandon)] using a Column Packing Instrument (Shandon). Elution conditions were 8 min isooctane–ethanol (97:3, v/v) and 2 min isooctane–ethanol (60:40, v/v) at a flow-rate of 2.0 ml/min, to elute tightly bound urine matrix components.

* 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 Hygiene, to the exclusion of others which may also be suitable.

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All solvents were of analytical grade (Merck). Anabolic standards were checked for purity by melting point, HPLC and infrared spectroscopy.

*Sample clean-up*

Bovine urine (1 ml) was hydrolysed enzymatically during 2 h at 37°C with glucuronidase/sulfatase (Suc d'Helix pomatia, IBF, France; the crude enzyme preparation was purified by gel filtration on Sephadex G-25M). After ether extraction of the urine hydrolysate, the organic layer was evaporated and the dry residue was dissolved in 0.25 ml of the HPLC mobile phase. With this procedure 40 urine samples can be processed within 4 h. Part of the extract (0.20 ml) was applied to the HPLC column. HPLC analysis takes 16 min per sample and can be performed automatically during the night[4].

## RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of standards of 17β-trenbolone and its metabolites 17α-trenbolone and triendione on a normal-phase HPLC column with detection at 350 nm. Trenbolone acetate, the active compound present in the actual anabolic formulations, was eluted at 2.25 min. At 350 nm no interference is expected from other xenobiotic anabolics or endogenous androgens[5]. From Fig. 1 it can be calculated that the absolute detection limit of the standards is *ca.* 0.3–0.4 ng, with a signal-to-noise ratio of 2.

On a silica gel column with the elution conditions described in the Experimental section, possible interfering matrix components from urine elute at retention times less than 5 min and greater than 10 min. A typical example of this chromatographic behaviour and selectivity is shown in Fig. 2. Here an aliquot of 1 ml of bovine urine, enriched with 10 ng of 17α-trenbolone, 17β-trenbolone and triendione was injected on the normal-phase column. The two trenbolone standards can clearly be identified

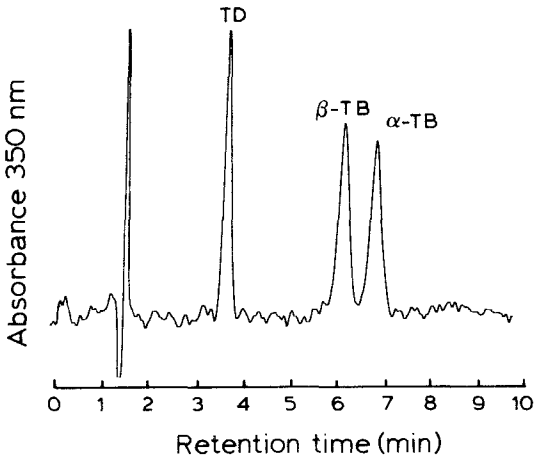


Fig. 1. A typical HPLC chromatogram of a standard mixture of 2 ng each of 17α-trenbolone (α-TB), 17β-trenbolone (β-TB) and triendione (TD) on a normal-phase Hypersil column. For Experimental conditions, see text.

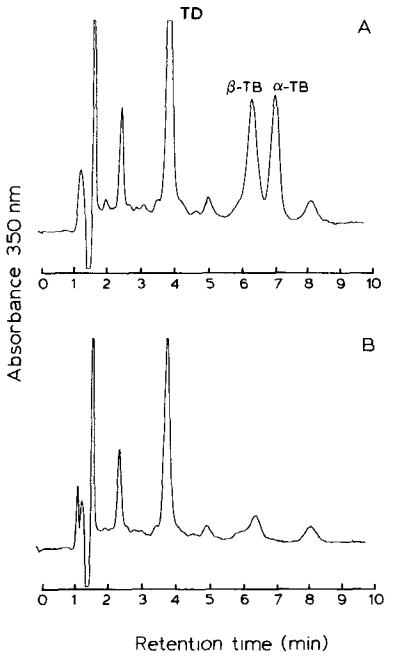


Fig. 2. HPLC chromatograms of an extract of urine sample before (B) and after (A) enrichment with 10 ng standards of 17α- and 17β-trenbolone and triendione.

from the chromatogram. No interfering peaks have as yet been observed in the chromatogram from 5 to 9 min. Only at the retention time of the minor metabolite triendione interferences are consistently found. From Fig. 2 the relative detection limit is 1–2 μg/l. This is even lower than that obtained with fluorescence detection in bovine tissue[6]. With reversed-phase HPLC in urine, low concentrations of urinary trenbolone and triendione cannot be monitored as a result of the large UV-absorption of matrix component at the retention times of these compounds[7]. Trenbolone and trenbolone acetate could be detected in human urine on a reversed-phase column[8] at the microgram per litre level only after a laborous purification procedure.

The reported detection method is a simple and fast screening method for the presence of 17β-trenbolone and its major metabolite 17α-trenbolone[9] at the ppb level in bovine urine. For forensic screening purposes a check of a large number of different samples of blank urine from practice is in progress to determinate the percentage of possible false-positive identifications caused by interfering matrix components. The results look very promising and the method is a possible suitable alternative to the thin-layer chromatographic method[10] or RIA[3] used thus far. A sensitive dual- or multi-wavelength detector will increase the specificity and consequently decrease the likelihood of false-positive identifications.

At present, purification methods are under development for monitoring other anabolics, such as stilbene derivatives and xenobiotic androgens and gestagens at 240 nm using the same procedure.

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