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# RECENT ADVANCES IN DOPING ANALYSIS (2)

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# Detection of Trenbolone by HPLC

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## Introduction

Trenbolone (17ß-hydroxyestra-4,9,11-trien-3-one), a veterinarian preparation, is banned in most european countries for live-stock fattening [1,2]. In some kinds of sport trenbolone, received from the black market, is misused as an anabolic steroid, especially in body building. After application of trenbolone unchanged trenbolone and its main metabolite the 17-alpha isomer epitrenbolone (17α-trenbolone; Figure 1) are excreted as conjugates into human urine. The excretion of trenbolone and epitrenbolone was estimated with a ratio of 1:5 in the first 24 hours. An easy to handle enzyme linked immuno assay (ELISA) kit, which is not available in all laboratories, shows sufficient sensitivity and less crossreactions against other steroids [3]. An alternative screening procedure using high performance liquid chromatography (HPLC) for detection of trenbolone and its metabolite epitrenbolone is presented in this paper.

## Sample Preparation

To 2ml of urine 500µl of phosphate buffer (0.8m; pH 7) and 50µl of β-glucuronidase from E.coli (Boehringer, Mannheim, D) are added and the sample is hydrolyzed for 30min at 50°C. After adding 500µl of 7% aqueous potassium carbonate solution ($K_2CO_3$) and 5ml of n-pentane the sample is shaken and centrifuged. The organic layer is transferred into a new glass tube and evaporated to dryness. The solid residue is dissolved in 50µl of methanol and 25µl of the organic layer is injected onto the HPLC column. The HPLC conditions are listed in Table 1.

All chemicals are of analytical grade and solvents were destilled in our laboratory before use. The used acetonitrile is HPLC quality (Merck, Darmstadt, D).

Table 1: HPLC Parameters for the detection of trenbolone in human urine

| | |
|---|---|
| HPLC: | Hewlett Packard 1090 with automatic injection system and Diode Array Detector |
| Column: | LiChrospher RP18, 5µm, 125x4mm (HP, Böblingen/Merck, Darmstadt) + ODS Hypersil, 5µm, 20x4mm (HP) |
| Solvents: | A: Water<br>B: Acetonitile |
| Flow: | 1ml/min |
| Gradient: | 35%B → 55%B in 8 min<br>55%B → 85%B in 0.1 min (Column Flushing) |
| Stop Time: | 9.20 min |
| Post Time: | 3.00 min |
| Injectionvol.: | 25µl |
| D.A. Detector: | 350nm (Reference Wavelength 550nm) |
| Spectrum Range: | 210-500nm |
| Sampling Interval: | 640ms |

**Results and Discussion**

The analysis of trenbolone and its metabolite by gas-chromatography / mass-spectrometry in the comprehensive screening procedure for anabolic steroids [4] is connected with some problems. The derivatization with MSTFA/$NH_4I$/ethanethiol (100:0.2:0.3/v:w:w) normally used for all other steroids of this screening procedure [5], leads to unstable derivatives of 17β- and 17α-trenbolone. The instability is caused by the enolization of the 3-keto group of these steroids which results in a destruction of the molecules [6]. To avoid these disadvantages we developed a HPLC method.

HPLC is an appropriate method to screen for trenbolone and its major metabolite epitrenbolone in human urine. Figure 3 shows a HPLC-UV chromatogram of a standard solution and an urine extract containing trenbolone and epitrenbolone. The described HPLC method, based on reversed phase HPLC with on-line UV-detection at 350nm, is easy and fast to handle also in a greater batch of samples. The HPLC method further has the advantage that derivatization is not necessary.

According to the law of Lambert Beer (Formula 1) the extinction of trenbolone is very intense compared to other steroids (Table 2). The sensitivity of the analytical method is sufficient, detection limit of 1ng trenbolone shows a noise ratio of 3:1.

**Lambert Beer's law:** $E = \varepsilon * c * b$ Formula 1

E = Extinction
ε = molar extinction coefficient ($l*mol^{-1}*cm^{-1}$)
c = concentration ($mol*l^{-1}$)
b = cell length (cm)

Table 2: Comparison of the molar extinction coefficients of different steroids [7 ]

| Substance | Solvent | Wavelength | ε* |
|---|---|---|---|
| | | | [$l*mol^{-1}*cm^{-1}$] |
| Trenbolone | Ethanol | 340nm | 28.000 |
| | Ethanol | 239nm | 5.260 |
| Testosterone | Ethanol | 238nm | 16.000 |
| Cortisone | Ethanol | 238nm | 15.800 |

)* ε = molar extinction coefficient

An other main advantage for screening by HPLC for trenbolone is the UV-maximum of absorbance at 350nm (Figure 2), caused by the molecular structure of trenbolone and epitrenbolone (Figure 1). This high wavelength allows an increased detection of trenbolone as the biological background (disturbing urine matrix) with interfering substances in the lower wavelength range (200-300nm) is reduced and/or not detected.

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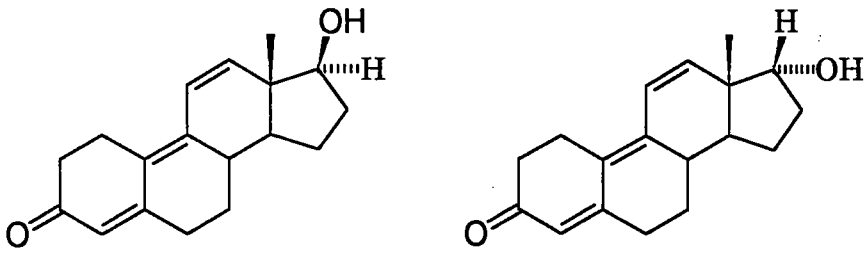


17β-trenbolone 17α-trenbolone

Figure 1: Molecular structure of 17β-trenbolone and 17α-trenbolone

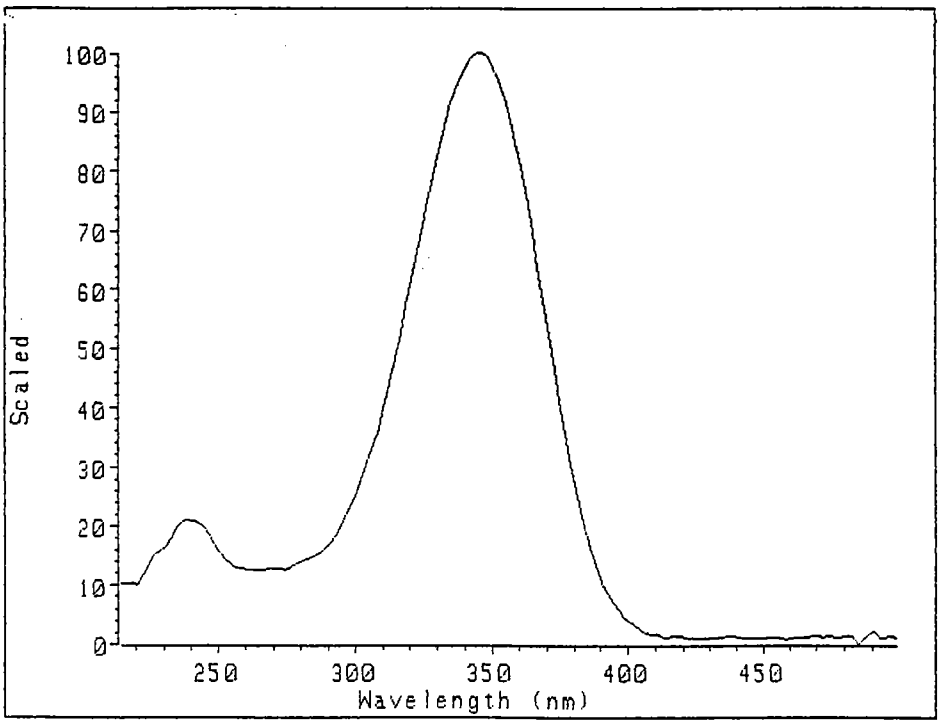


Figure 2: UV spectrum of trenbolone

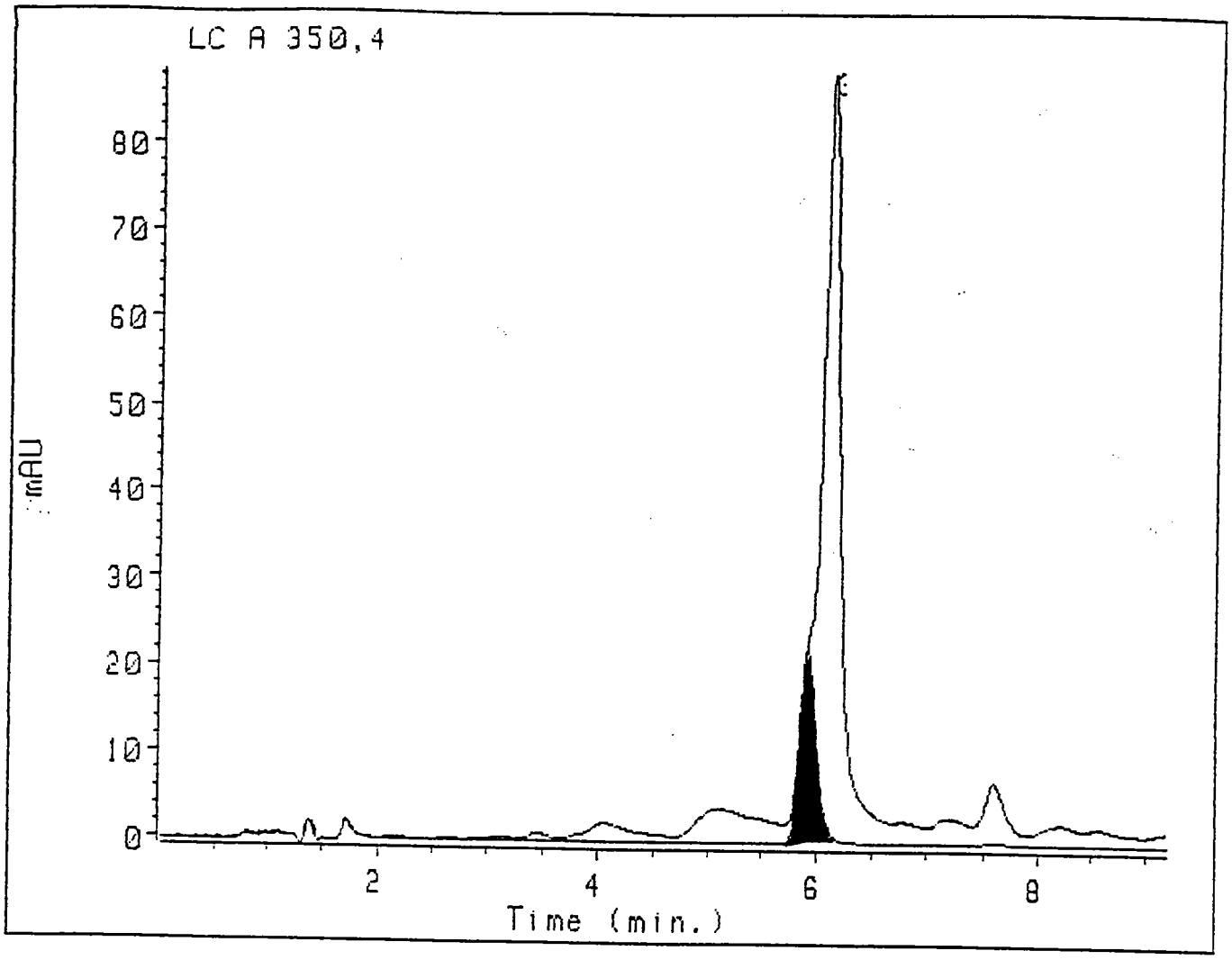


Figure 3: HPLC-UV chromatograms of a standard solution of trenbolone (shaded area) and an urine extract containing trenbolone and epitrenbolone