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Short communication

Determination of α- and β-trenbolone in bovine muscle and liver by liquid chromatography with fluorescence detection

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Abstract

A sensitive and rapid high-performance liquid chromatographic screening method is described for the determination of anabolic steroid trenbolone in bovine muscle and liver. Trenbolone was analyzed as α- and β-trenbolone. Samples were extracted with ethyl acetate and cleaned up on a silica solid-phase extraction (SPE) cartridge. Liver samples were cleaned up on a multifunctional SPE cartridge before using a silica SPE cartridge. Analysis of α- and β-trenbolone was performed by reversed-phase high-performance liquid chromatography (HPLC) with a fluorescence detector. The detection limits for this method were estimated to be 0.2 and 1.0 ng/g in bovine muscle and liver, respectively. The mean recoveries spiked in muscle at 2 ng/g and in liver at 10 ng/g were over 80%.

Keywords: Trenbolone

1. Introduction

Trenbolone acetate (17β-acetoxyestra-4,9,11-trien-3-one) is a synthetic anabolic steroid used for increasing cattle weight as veterinary medicine. This compound has been known to be metabolized to β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) in muscle and α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) in liver [1]. Their maximum residue limits (MRLs) of FAO/WHO are 2 ng/g in muscle and 10 ng/g in liver, and the EU bans the use of trenbolone in food-producing animals. The analytical method requires a lower detection limit and higher sensitivity.

Various methods have been reported for determining trenbolone in animal tissues. Lagana et al. [2] developed a multi-residue analysis of anabolic steroids in animal tissue using UV-HPLC. Daeseleire [3] reported a multi-residue analysis by gas chromatography–mass spectrometry (GC–MS) after derivatization. Stubbings et al. [4] used a column switching HPLC system with gel permeation chromatography (GPC) clean-up for the determination of trenbolone. Miyazaki et al. [5] reported a multi-residue analysis with photodiode array detector (PDA)-HPLC for anabolic steroids in beef and milk. Din et al. [6] used supercritical fluid extraction (SFE) to extract trenbolone from beef. Stubbings et al. [7] described immunoaffinity chromatography clean-up method for the determination of trenbolone in animal tissue. Sawaya et al. [8] examined the accuracy and precision of an enzyme-linked immunosorbent assay (ELISA) for trenbolone acetate.

Some methods require derivatization, complicated apparatus and potentially hazardous solvents such as

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dichloromethane or benzene for extraction. Furthermore, the detection has been done by UV absorption in these HPLC methods. ELISA has been used as a screening method, however, it cannot determine α- and β-trenbolone individually due to the cross-reactivity.

We found that α- and β-trenbolone had enough fluorescence and it was about 5 times as sensitive as UV absorption. In this paper, a simple and rapid HPLC screening method is described for the determination of α- and β-trenbolone in bovine muscle and liver with fluorescence detection.

2. Experimental

2.1. Reagents and materials

All solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan) and Milli-Q water was used. α- and β-trenbolone were purchased from Hayashi Pure Chemical Industries (Osaka, Japan). Stock solutions were prepared in methanol (250 μg/ml). ISOLUTE MULTIMODE cartridges (500 mg, IST, Hengoed, UK) were pre-conditioned with 5 ml of ethyl acetate. Sep-Pak Vac RC silica cartridges (500 mg, Waters, Milford, MA, USA) were pre-conditioned with 10 ml of acetone–hexane (10:90). Samples of bovine muscle and liver were purchased at local markets in Kobe, Japan.

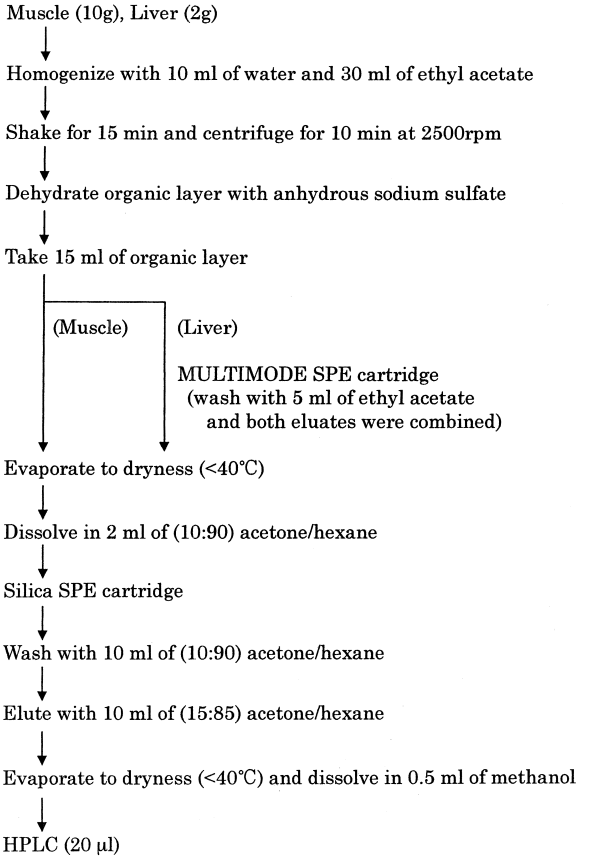
2.2. HPLC conditions

An HPLC system consisted of Shimadzu LC-10 system with a fluorescence detector RF-10AXL (Kyoto, Japan). An analytical column STR ODS-II (150×4.6 mm I.D.) was purchased from Shinwa Chemical Industries (Kyoto, Japan) and stored at 40°C. The mobile phase was methanol–water (60:40, v/v), and the flow-rate was 1.2 ml/min. The peaks were monitored at the excitation and emission wavelengths of 364 nm and 460 nm. The injection volume was 20 μl.

2.3. Extraction and clean-up

A 10 g sample of muscle or a 2 g sample of liver were placed in 50 ml centrifuge tubes. For recovery studies, the samples were spiked at 2 or 10 ng/g in muscle and 10 or 50 ng/g in liver by adding 100 μl of diluted standard solutions. Ten ml of water was added and the contents were homogenized for 30 s with an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany). Thirty millilitres of ethyl acetate was added and shaken for 15 min on a reciprocal shaker, then centrifuged at 2500 rpm for 10 min. The ethyl acetate layer was dehydrated with anhydrous sodium sulfate, then 15 ml of the extract was taken for further analysis.

For the muscle sample, the extract was evaporated to dryness on a rotary evaporator (<40°C). The residue was dissolved in 2 ml of acetone–hexane (10:90), and added to the Sep-pak silica cartridge. The cartridge was washed with 10 ml of acetone–hexane (10:90) and eluted with 10 ml of acetone–hexane (15:85). The eluate was collected and evapo-



Scheme 1. Procedure for analysis of α- and β-trenbolone.

rated to dryness, then the residue was dissolved in 0.5 ml of methanol.

For the liver sample, a MULTIMODE clean-up step was added prior to the sample evaporation. Fifteen millilitres of the extract was passed through MULTIMODE cartridge and the cartridge was washed with 5 ml of ethyl acetate. Both eluates were combined and evaporated to dryness, and then silica clean-up was done (Scheme 1).

3. Results and discussion

Methanol [2], diethyl ether [3], acetonitrile [4] and ethyl acetate [9,10] have been used as extraction solvents of steroids from animal tissue. We examined these solvents and found ethyl acetate gave cleaner extracts. Methanol and acetonitrile gave many interferences and required a re-extraction step with dichloromethane, which is hazardous and should be avoided in order to preserving the environment. Diethyl ether needed a lot of anhydrous sodium sulfate to be completely dehydrated and easily volatilizes at ambient temperature, leading to varying analytical data.

We further assessed the extraction step by using a mixture of ethyl acetate and hexane with different ratios to obtain cleaner extracts with higher recovery. The higher the ratio of ethyl acetate, the higher the recovery of α- and β-trenbolone. A mixture of (1:1) ethyl acetate–hexane was found to give the highest level of interferences. Therefore we used only ethyl acetate for extraction.

Saturated NaCl solution and 0.2 *M* NaOH solution were examined for homogenization of the samples instead of water. The alkaline and the salt solution made the tissue sample gel and the organic layer could not be taken. Thus we used water for homogenization and ethyl acetate for extraction.

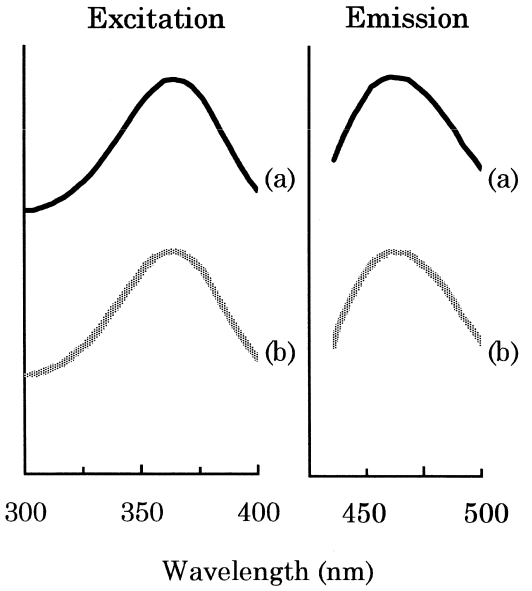


Fig. 1. Excitation and emission spectra of (a) α- and (b) β-trenbolone.

As the extract from muscle sample was found to contain much fat, hexane–acetonitrile partition and SPE (silica and florisil) clean-up were compared for their fat removal efficiency and recovery. The silica cartridge was found to give a cleaner chromatogram and higher recovery by washing out the fat with (10:90) acetone–hexane. However, the clean-up step was found to be insufficient for the liver extract. Therefore a MULTIMODE SPE cartridge which contains non-polar, strong cation and anion-exchange functional groups was added prior to the silica cartridge clean-up. The procedure for the sample preparation is shown in Scheme 1.

Table 1
Within-day and day-to-day recoveries of α- and β-trenbolone from bovine muscle and liver

Sample	Added	*n*	% Recovery (mean±SD)			
			Within-day		Day-to-day	
			α-Trenbolone	β-Trenbolone	α-Trenbolone	β-Trenbolone
Muscle	2 ng/g	5	84.3±2.3	90.5±3.3	85.1±3.8	92.7±5.0
	10 ng/g	5	82.8±2.8	83.1±2.7	91.3±4.3	92.6±3.9
Liver	10 ng/g	5	80.1±7.2	85.6±8.7	79.1±6.8	82.0±7.1
	50 ng/g	5	89.9±2.9	89.3±3.1	88.3±5.1	86.7±5.9

Although α- and β-trenbolone have been detected by UV absorption (242 nm [2], 340–350 nm [4–7]), it didn't provide sufficient sensitivity for the determination of their residual level in meat. We examined their fluorescence and found that they have a maximum excitation wavelength at 364 nm and an emission at 460 nm. Fluorescence detection was about five times as sensitive as UV detection. Excitation and emission spectra of α- and β-trenbolone are presented in Fig. 1 and chromatograms of trenbolone detected with fluorescence and UV detector are shown in Fig. 2.

Figs. 3 and 4 show typical chromatograms of extracts from blank and spiked samples of bovine muscle and liver. The blank chromatograms of both samples show no interference at the retention time of trenbolone.

The detection limits were estimated to be 0.2 ng/g in bovine muscle and 1.0 ng/g in bovine liver. Table 1 shows the within-day and day-to-day recoveries of α- and β-trenbolone spiked in bovine muscle (2 and 10 ng/g) and liver (10 and 50 ng/g). The mean recoveries ranged from 80.1 to 90.5%, with coefficients of variation from 2.7 to 10.2% over the range spiked (2–50 ng/g) in within-day trial.

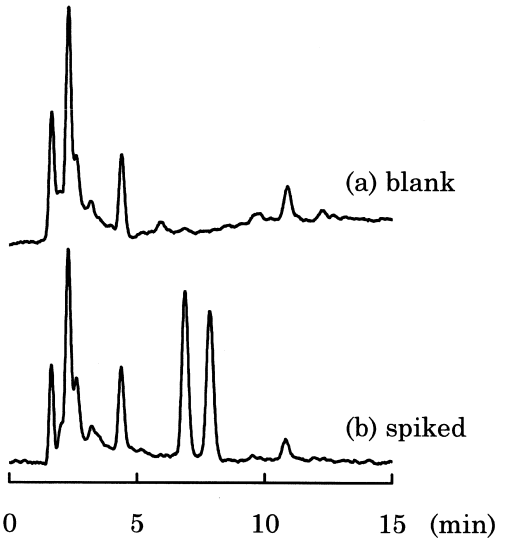


Fig. 3. Typical chromatograms of muscle extracts. (a) Blank and (b) spiked with 2 ng/g of α- and β-trenbolone.

This method was used to survey α- and β-trenbolone in commercially available meat. Ten samples of each of bovine muscle and liver were analyzed

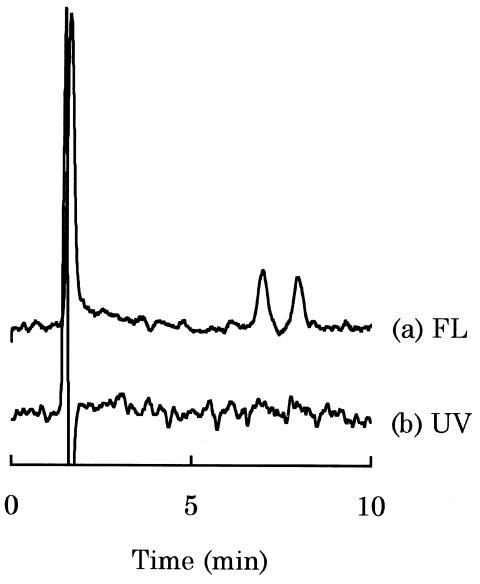


Fig. 2. Chromatograms of α- and β-trenbolone standards (40 pg each) (a) with fluorescence detector (Ex 364 nm, Em 460 nm) and (b) with UV detector (340 nm).

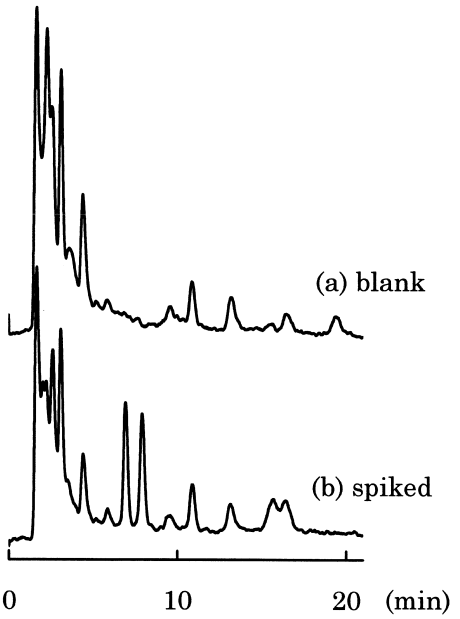


Fig. 4. Typical chromatograms of liver extracts. (a) Blank and (b) spiked with 10 ng/g of α- and β-trenbolone.

and all samples were found to be free from α- and β-trenbolone.

The method is suitable for the determination of α- and β-trenbolone in bovine muscle and liver with sensitivity and simplicity.

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