A Method for the Determination of Trenbolone in Bovine Muscle and Liver

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ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the determination of trenbolone acetate, 17α-trenbolone and 17β-trenbolone in bovine muscle and liver. Bovine muscle sample was extracted with acetonitrile, filtered, and defatted with acetonitrile-saturated *n*-hexane. The acetonitrile extract after concentration and clean-up with Bond Elut C18 cartridge was ready for HPLC analysis. HPLC conditions were as follows, column: Inertsil ODS-3V, mobile phase: acetonitrile/methanol/H$_2$O (50:10:40, v/v), flow rate: 1 mL/min, and detecting wavelength: UV 340 nm. For bovine muscle, the recoveries were 83.8~98.9% for trenbolone acetate, 17α-trenbolone and 17β-trenbolone spiked at concentrations between 2~4 ppb, and the variation coefficients were 1.2~4.8%. For bovine liver, the recoveries were 82.6~95.7% for the three trenbolones spiked at concentrations between 10~20 ppb, and the variation coefficients were 1.9~6.7%. The detection limits for trenbolone acetate, 17α-trenbolone and 17β-trenbolone were 1, 0.5 and 0.5 ppb in bovine muscle, and 4, 2 and 2 ppb in bovine liver, respectively.

After the survey, 30 marketed bovine muscle samples showed no detection of trenbolone acetate and its metabolites.

Key words: trenbolone acetate, 17α-trenbolone, 17β-trenbolone, high performance liquid chromatography, bovine muscle, bovine liver

INTRODUCTION

Animal drugs are applied to prevent diseases, increase feed utility, promote animal growth, and multiply lean muscle quantity in animals.

Trenbolone acetate (TBA) (Figure 1), a synthetic anabolic hormone, has been permitted for application in cattle in many countries. It is usually applied in combination with 17β-estradiol as a feed supplement to promote the growth of steers, heifers and veal calves.

In cattle, TBA is rapidly hydrolysed to 17β-trenbolone (17β-TBOH) which will bind to the receptive sites of testosterone and estrogen, supporting protein metabolism, and accumulating skeletal muscle mass[1]. Twenty-four hours after application, 80% of the 17β-TBOH and its oxidative products will be converted to 17α-trenbolone (17α-TBOH) in bile metabolism, then excreted by conjugation with glucuronide or sulfate[2].

Trenbolone is not found of gene toxicity so far[3]. It is permitted by the US Food and Drug Administration for the use in promoting growth in cattles[4]. Yet in 1989, the European Economic Community (EEC) imposed a ban on the import of muscle from the US that was found with trenbolone residue. According to the Code of Federal Regulations (CFR, 1992), tolerances for TBA are 0.2 ppm (fat), 0.15 ppm (kidney), 0.1 ppm (liver), and 0.05 ppm (muscle)[5]. In Japan, the tolerance for β-TBOH is 0.002 ppm (muscle), for α-TBOH is 0.01 ppm (liver)[6]. In
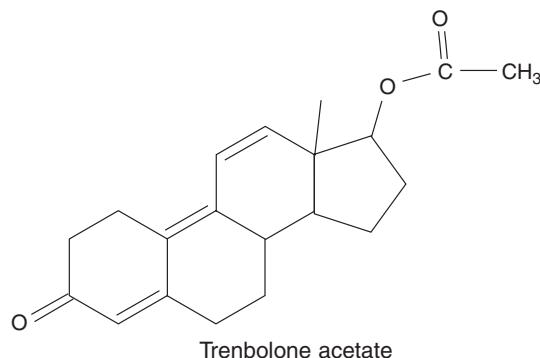

Figure 1. The chemical structure of trenbolone acetate.

Taiwan, the tolerances for TBA are 0.002 ppm (muscle) and 0.01 ppm (liver)[7].

The methods for the analysis of TBA, 17β-TBOH and 17α-TBOH include enzyme immunoassay[1,5], GC-MS[10,14,17], high performance liquid chromatography[8,9,11,13,18,19], LC-MS[12,15], and others. Considering the detection sensitivity required, high performance liquid chromatography is the method of first choice. Sensitivity can be improved by employing solvent extraction[8-11], enzyme hydrolysis[12-17] and solid phase adsorption column[1,8,9,13,16,18] for sample preparation. Therefore, the purpose of this study is to establish a schematic process of a method to reduce matrix interference and to elevate the detection sensitivity, allowing the investigation of the contents of TBA, 17β-TBOH and 17α-TBOH to ascertain the safety levels in marketed bovine muscle and liver.

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MATERIALS AND METHODS

I. *Materials*

Standards of trenbolone acetate (TBA) and 17β-trenbolone (17β-TBOH) were purchased from Sigma Chemical Co. (USA). Standard of 17α-trenbolone (17α-TBOH) was purchased from Hayashi Pure Chemical Ind. (Japan). Acetonitrile, methanol, *n*-hexane (LC grade) and anhydrous sodium sulfate (reagent grade) were purchased from Merck (Germany). Purification column (Bond Elut® C18, Varian, USA, 3 mL/500 mg, pre-treated with 10 mL of methanol and 10 mL of D.I. water), high performance liquid chromatograph (HPLC, Shimadzu LC-10AT, Shimadzu SPD-10A UV-VIS detector (Japan); Midas autoinjector (Holland)), chromatographic column (Inertsil ODS-3V, 5 μm, 4.6 × 250 mm; mobile phase: acetonitrile/methanol/D.I. water = 50/10/40, v/v; flow rate: 1 mL/min; detection wavelength: 340 nm).

II. *Preparation of Reagents*

(I) *Preparation of standard solutions*

Standards of TBA, 17β-TBOH and 17α-TBOH were separately dissolved in methanol to make stock standard solutions at a concentration of 1000 μg/mL each. The stock standard solutions were mixed at an equal ratio and diluted with methanol to make working standard mixture solutions containing 5 to 250 ηg/mL of each trenbolone.

(II) *Preparation of sample solution*

1. Extraction

Ground bovine muscle sample 10 g (bovine liver 5 g) was accurately weighed and 30 mL of acetonitrile was added, then homogenized for 5 min. The homogenate was then filtered under vacuum. The residues were extracted with 30 mL of acetonitrile one more time and then filtered. The filtrates were collected in a separatory funnel, then added 50 mL of *n*-hexane saturated with acetonitrile and shaken for 5 min to defat. The acetonitrile phase was placed in an evaporation vessel and added 10 mL of *n*-propanol (to avoid boiling), then evaporated at 40°C under vacuum.

2. Clean-up

The above concentrated residue was dissolved in 10 mL of D.I. water by ultrasonic vibration, then applied to a pretreated Bond Elut C18 cartridge. The cartridge was washed with 10 mL of methanol/D.I. water (4/6, v/v) twice (trice for bovine liver sample), and then eluted with 5 mL of methanol/D.I. water (8/2, v/v) twice. The methanol/ D.I. water (8/2) eluate was collected, evaporated to dryness, dissolved in 1 mL of methanol, filtered through a 0.45 μm nylon membrane, and analyzed with HPLC.

(III) *Preparation of standard curve*

Each of the standard mixture solutions, containing 5 to 250 ηg/mL of each trenbolone, was analyzed in triplicate using HPLC. Three standard curves were then drawn by plotting peak area against concentration of each standard.

(IV) *Identification test and quantitative test*

Twenty microliters of the sample solution and standard mixture solution were auto-injected into HPLC, respectively. The peak retention time and peak area of the sample solution were compared with those of the standard mixture solution. The concentration of TBA, 17β-TBOH and 17α-TBOH in the sample solution were calculated according to the equation shown below.

$$\text{Trenbolone concentration (ppb)} = \frac{C \times V}{W}$$

C: trenbolone concentration (ηg/mL) in sample solution extrapolated from standard curve
V: sample solution volume (mL)
W: sample weight (g)

RESULTS AND DISCUSSION

I. *Selection of HPLC Analysis Conditions*

(I) *Selection of detection wavelength*

One μg/mL solutions of TBA, 17β-TBOH and 17α-TBOH were prepared and scanned at 200-500 nm wavelength with a spectrophotometer. The maximum absorption of TBA, 17β-TBOH and 17α-TBOH were 339.5, 341.5 and 343.0 nm, respectively. Therefore, 340 nm was selected for detecting TBA, 17β-TBOH and 17α-TBOH.

(II) *Selection of mobile phase*

Referring to the mobile phase in literatures[8,9,11,13,18,19], acetonitrile/methanol/D.I. water mixtures of different ratios were used as various options. Fifty ηg/mL mixed standard solution of TBA, 17β-TBOH and 17α-TBOH was injected to HPLC and the chromatogram was compared. The best resolution was obtained when acetonitrile/methanol/D.I. water (50/10/40, v/v) was used as the mobile phase.

II. *Standard Curves*

The standard curves showed that the absorption at 340 nm of TBA, 17β-TBOH and 17α-TBOH are in good linear correlation with concentrations between 5-250 ηg/mL, with r^2 = 0.9996, 1.0000 and 1.0000, respectively.

III. *Selection of Extraction and Clean-up Conditions for Preparation of Sample Solution*

(I) *Bovine muscle*

1. Selection of extraction condition

Referring to the extraction conditions in literatures[12-17], the following solvent systems were employed for investigation in the preparation of sample solution.

(1) Extract with sodium acetate buffer solution first, then the extract was extracted with *tert*-butyl-methylether[16].

(2) Extract with sodium acetate buffer solution, adjust the pH value of the extract to 4.5-5, then extracted with ether[17].

(3) Extract with acetonitrile directly[13].

In solvent system (1), the recovery of 17α-TBOH was 44%. In solvent system (2), the recoveries of 17β-TBOH and 17α-TBOH were 24.6% and 29.5%, and the recovery of TBA was nearly zero. In solvent system (3), the recoveries were all more than 90%. Therefore, acetonitrile was used as the extraction solvent.

2. Selection of clean-up column

Sample solution, obtained from acetonitrile extraction and de-fatting with *n*-hexane, was analyzed by HPLC. The HPLC chromatogram of the concentrated sample solution showed the appearance of noise. Therefore, referring to literatures[1,8,9,13,16,18], several cartridges were tested for clean-up efficiencies.

(1) Bond Elut silica 500 mg: Sample solution (in 5 mL of methanol/D.I. water (4/6, v/v)) was loaded to a Bond Elut silica cartridge (pre-washed with 10 mL of methanol and then wetted with 10 mL of D.I. water), then eluted with methanol/D.I. water (4/6, v/v). The eluate after concentration was analyzed by HPLC. The recoveries were 91.1, 90.3 and 92.9% for TBA, 17β-TBOH and 17α-TBOH, respectively. There was still noise appearing on the HPLC chromatogram.

(2) Bond Elut CN 500 mg: Sample solution (dissolved in 10 mL of D.I. water) was loaded to a Bond Elut CN cartridge (pre-wetted with 10 mL of D.I. water), then eluted with methanol/D.I. water (8/2, v/v). The eluate after concentration was analyzed by HPLC. The recoveries were 80.9, 93.8 and 95.6% for TBA, 17β-TBOH and 17α-TBOH, respectively. The process of concentration was time-consuming because of the water present in the eluate.

(3) Bond Elut C18 500 mg: Sample solution (in 10 mL of D.I. water) was loaded to a Bond Elut C18 cartridge (pre-washed with 10 mL of methanol and then wetted with 10 mL of D.I. water). Washed with methanol/D.I. water (4/6, v/v), then eluted with methanol/D.I. water (8/2, v/v). The eluate after concentration was analyzed by HPLC. The recoveries were 83.1, 88.3 and 91.0% for TBA, 17β-TBOH and 17α-TBOH, respectively, and there was no noise on the HPLC chromatogram.

Comparing the recoveries and noise on the HPLC chromatogram, we chose the Bond Elut C18 cartridge for clean-up.

(II) *Bovine liver*

The preparation of sample solution from bovine muscle can't be applied to bovine liver for the higher matrix effect of the latter. By using Bond Elut C18 cartridge, it was found that the clean-up efficiency was satisfactory when 5 g of bovine liver sample was taken and 30 mL of methanol/D.I. water (4/6, v/v) was employed for washing the clean-up cartridge.

IV. *Recovery Test*

(I) *Bovine muscle*

Bovine muscles were spiked with TBA, 17β-TBOH and 17α-TBOH standard solution to achieve 2, 3 and 4 ppb. The recovery test was performed by using the Bond Elut C18 clean-up method established above, and a blank test was used as control. Each test was done in triplicate and the retention time and peak of sample solution were
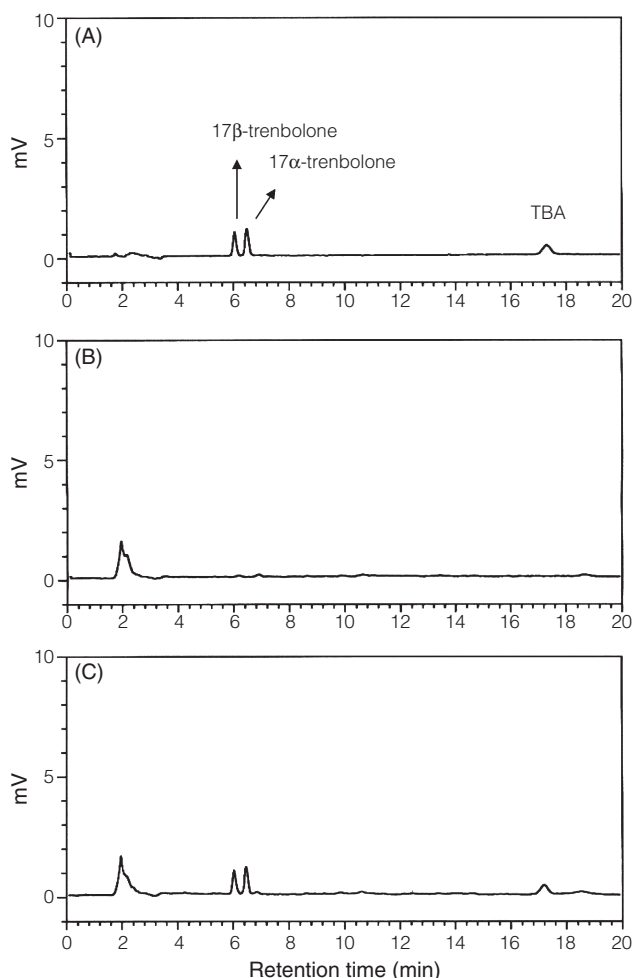

Figure 2. The high performance liguid chromatograms of trenbolone and its metabolites in bovine muscle. LC condition: column, Inertsil ODS-3V, 5 μm (4.6 i.d. × 250 mm); mobile phase, CH$_3$CN/MeOH/H$_2$O = 50/10/40; flow rate, 1 mL/min; detecting wavelength, 340 nm. (A) standard: 40 ppb, (B) sample blank, (C) sample spiked with 4 ppb each of the standards.

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Table 1. Recoveries of trenbolone and its metabolites in bovine muscle and bovine liver

Sample	Item	Recovery(%)[a]		
		2 ppb[b]	3 ppb	4 ppb
	Trenbolone acetate	86.5 (2.2)[c]	90.0 (3.4)	83.8 (1.2)
Bovine muscle	17α-trenbolone	89.3 (3.8)	93.7 (4.8)	94.5 (4.0)
	17β-trenbolone	98.9 (4.2)	97.2 (3.6)	95.0 (4.5)
		10 ppb	15 ppb	20 ppb
	Trenbolone acetate	89.8 (1.9)	94.7 (6.7)	91.3 (4.2)
Bovine liver	17α-trenbolone	93.6 (2.0)	95.7 (3.4)	92.3 (3.0)
	17β-trenbolone	88.0 (6.2)	86.4 (4.2)	82.6 (5.3)

[a]Average of triplicate analysis.　　　　　[b]Spike level.　　　　　[c]Number in parentheses represents coefficient of variation (%).
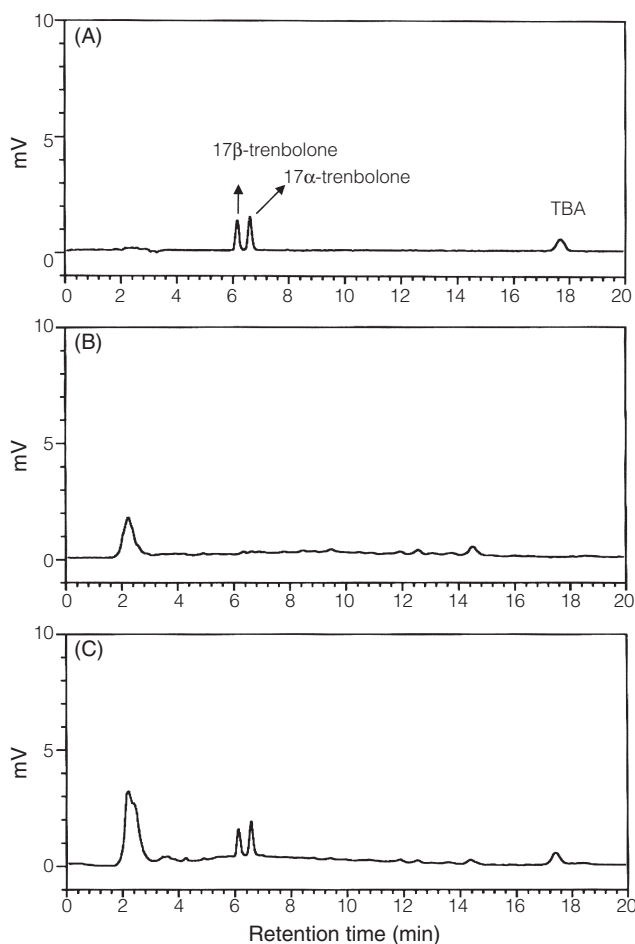

Figure 3. The high performance liguid chromatograms of trenbolone and its metabolites in bovine liver. LC condition: column, Inertsil ODS-3V, 5 μm (4.6 i.d. × 250 mm); mobile phase, $CH_3CN/MeOH/H_2O$ = 50/10/40; flow rate, 1 mL/min; detecting wavelength, 340 nm. (A) standard: 50 ppb, (B) sample blank, (C) sample spiked with 4 ppb each of the standards.

compared with the standard solution to identify and quantify, and to calculate recovery rates. The average recovery rates were 83.8-90.0%, 95.0-98.9%, and 89.3-94.5%, respectively; CV=1.2-4.8% (Table 1 and Figure 2).

(II) *Bovine liver*

Bovine livers were spiked with TBA, 17β-TBOH and 17α-TBOH standard solutions to achieve 10, 15 and 20
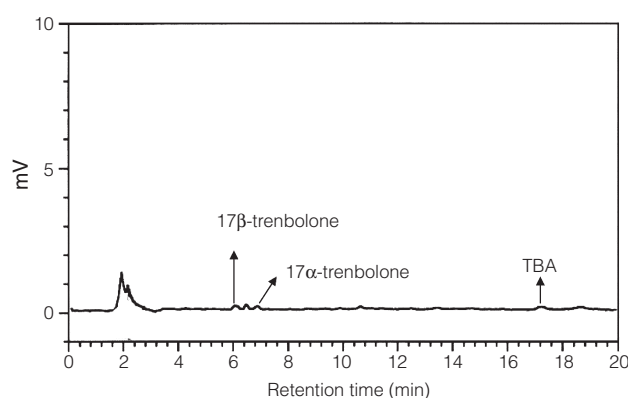

Figure 4. The high performance liguid chromatograms of trenbolone and its metabolites at detection limits in bovine muscle. LC condition: column, Inertsil ODS-3V, 5 μm (4.6 i.d. × 250 mm); mobile phase, $CH_3CN/MeOH/H_2O$ = 50/10/40; flow rate, 1 mL/min; detecting wavelength, 340 nm. Limit of quantification: TBA 1 ppb, 17β-trenbolone 0.5 ppb, 17α-trenbolone 0.5 ppb.
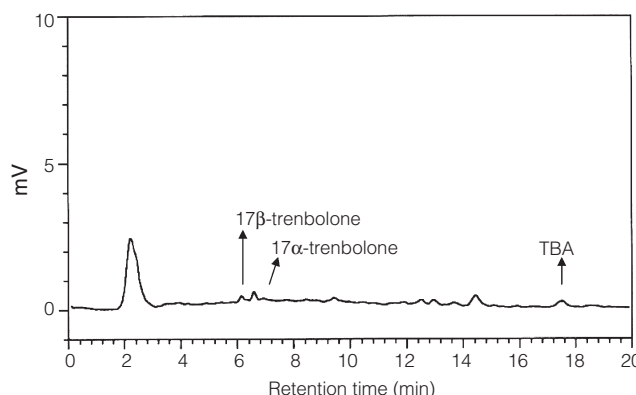

Figure 5. The high performance liguid chromatograms of trenbolone and its metabolites at detection limits in bovine muscle. LC condition: column, Inertsil ODS-3V, 5 μm (4.6 i.d. × 250 mm); mobile phase, $CH_3CN/MeOH/H_2O$ = 50/10/40; flow rate, 1 mL/min; detecting wavelength, 340 nm. Limit of quantification: TBA 4 ppb, 17β-trenbolone 2 ppb, 17α-trenbolone 2 ppb.

ppb. The recovery test was performed by using the method established above, and blank test was used as control. The average recovery rates were 89.8-94.7%, 82.6-88.0%, and 92.3-95.7%, respectively; CV = 1.9-6.7% (Table 1 and Figure 3).

The result showed both the recovery and the reproducibility were satisfactory.

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V. *Test of the Limit of Quantification*

The detection limits of TBA, 17β-TBOH and 17α-TBOH were quantified to be 1, 0.5 and 0.5 ppb in bovine muscle (Figure 4), and 4, 2 and 2 ppb in bovine liver (Figure 5), with S/N ratio above 3, respectively.

VI. *Survey of Marketed Bovine Muscle Sample*

In this study, 30 bovine muscle samples from Taipei district market were tested for TBA, 17β-TBOH and 17α-TBOH content. All of them were below the detection limit.

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