A Harmless Method for Determining Trenbolone Acetate Together with 17β-Trenbolone in Beef

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Abstract

An inexpensive, harmless, and quick technique of sample preparation followed by a reversed-phase high-performance liquid chromatography (HPLC) for the simultaneous quantification of trenbolone acetate and 17β-trenbolone in cattle muscle is presented. Analysis by HPLC with photo-diode array detector was performed using a short C1 column with an isocratic mobile phase. The method was validated by the analyses of spiked samples, resulting recoveries (≥ 87.9%; relative standard deviations ≤ 3.4%), analytical total time (< 20 min/sample, where, a batch of 12 samples in 2 h), and quantitation limits (≤ 1.8 ng/g). No harmful organic solvents and reagents were used at all.

Introduction

Trenbolone acetate (TBa), a synthetic steroid hormone, is licensed as growth promoter for farm beeves in the United States and Canada. TBa is administered as a subcutaneous implant. Previous residual studies for TBa in cattle have found that the compound administered is hydrolyzed to 17β-trenbolone (β-TB) in muscle (1,2).

Overuse or misuse of TBa in beef cattle is of great concern because it can result in its appearance in marketed beef. To assure the safety of beef (cattle muscle) for the consumer, the FDA, Codex, and Japan set a safe concentration for TBa of 80 ng/g (3), maximum residue limit (MRL) for TBa of 2 ng/g (4), or MRL for TBa and β-TB, respectively, of 2 ng/g (5).

Because determinations for β-TB together with TBa in beef are therefore an important specific activity to guarantee food safety, a useful analytical method for the simultaneous determining TBa and β-TB is presently required. The acceptable (or ideal) method must have the following qualities: it must be simple, economical in time and cost, and cause negligible harm to the environment and analyst.

Previous methods involving HPLC (6–9), GC–MS (10,11), or LC–MS (9) for determination of TBa and/or β-TB in cattle tissue or urine have crucial disadvantages: (*i*) all of the methods consume harmful organic solvents (such as acetone, acetonitrile, methanol, etc.) as the HPLC or LC–MS–MS mobile phases as well as harmful organic solvents (such as dichloromethane, diethyl ether, hexane, chloroform, etc.) (12) for extraction and deproteinization in sample preparation. Discharging harmful organic solvents is a severe problem on a world-wide scale (13–15). Risks associated with these solvents extend beyond direct implications for the health of humans and wildlife to affect our environment and the ecosystem in which we all reside. Additionally, incineration for disposal of waste organic solvents has steadily increased over the past 10 years, and huge amounts of money have been spent (16,17). From a standpoint of environment conservation, human health, and economy, reducing the use of organic solvents, in particular, eliminating the harmful solvents, is a very important goal. (*ii*) Most of the methods could not simultaneous detect TBa and β-TB, and they involve at least one extracting/purifying step using large amounts of organic solvent, resulting in time- and cost-consumption that do not permit the determination of large number of samples.

The harmful organic solvent-free technique described here is an environmentally benign method that enables rapid and low-cost determination of TBa and β-TB in beef muscles.

Experimental

Reagents and materials

TBa and β-TB standards and other chemicals were purchased from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Distilled water and ethanol, no direct harm (12), were of HPLC grade. Stock standard solutions of TBa and β-TB were prepared by dissolving each of the compounds in ethanol to a concentration of 100 µg/mL. Working mixed standard solutions of these compounds were prepared on by diluting the stock solution with water. The following apparatuses were used in the sample preparation: a handheld ultrasonic-homogenizer (model HOM-100, 2 mm i.d. probe, Iwaki Glass Co., Ltd., Funabashi, Japan); a micro-centrifuge (Biofuge fresco, Kendo Lab. Products, Hanau, Germany); an Ultrafree-MC Biomax (nominal molecular weight limit =

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5,000 Da, maximum sample size = 0.5 mL, Biomax high-flux polysulphone ultra-filtration membrane) (Millipore, Bedford, MA) as a centrifugal ultra-filtration unit (18). The following six types of C1 non-polar sorbent columns (3- or 5-µm dP) (4.6 mm i.d. × 50 or 75 mm length) with their guard columns (4.6 × 5 mm) for HPLC analysis were used: Column-A, CAPCELL PAK C1 UG120 (S-5) (5-µm dP, 75 mm length) (Fine Chemicals Business Dept., Shiseido Co., Ltd., Tokyo, Japan); Column-B, Daisopak SP-200-3-C1-P (3-µm dP, 75 mm length) (Daiso Co., Ltd., Osaka, Japan); Column-C, Daisopak SP-200-3-C1-P (3-µm dP, 50 mm length) (Daiso); Column-D, Develosil TMS-5 (5-µm dP, 50 mm length) (Nomura Chemical Co., Ltd., Aichi, Japan); Column-E, Develosil TMS-3 (3-µm dP, 50 mm length) (Nomura Chemical); Column-F, YMC-Pack TMS (5-µm dP, 75 mm length) (YMC Co., Kyoto, Japan) (Table I).

HPLC

The HPLC system included a model PU-980 pump and DG-980-50 degasser (both from Jasco Corp., Tokyo, Japan), as well as a model SPD-M10A VP photo-diode array (PDA) detector (Shimadzu Scientific Instruments, Kyoto, Japan). The analytical column was a Daisopak SP-200-3-C1-P (3 µm dp, 4.6 × 50 mm) column (Daiso) equipped with a guard column (4.6 × 5 mm) containing the same packing material. The isocratic mobile phase was a 27% (v/v) ethanol (in water), and the flow rate was 1.0 mL/min. PDA detector was operated at 190–380 nm: monitoring wavelength was adjusted to 353 nm, which is an average maximum for all the target compounds. The column temperature was operated at 40°C.

Procedure

The present sample preparation was as follows: an accurately weighed 0.1 g cattle muscle sample was placed in micro-centrifuge tube and homogenized with a handheld ultrasonic homogenizer for 30 s with 0.6 mL of ethanol. After being homogenized, the capped tube was centrifuged at 12,000 × g for 5 min. A 50-µL portion of supernatant liquid was placed into the Ultrafree-MC Biomax and centrifuged at 5,000 × g for 5 min, and the ultra-filtrate was injected into the HPLC system.

Result and Discussion

Sample preparation and HPLC conditions

The present procedure provided an easy-to-use, rapid, method that did not use harmful organic solvents and had a shorter operating time, resulting in high recovery and reproducibility with considerable cost savings.

Considering the packed non-polar sorbents in the columns for reversed-phase HPLC separation, the C1 (methyl-silica) sorbent is the most non-retentive sorbent when retention was based on non-polar interactions alone. To reduce the volume of elution solvent (= ethanol) required phase and provide a more rapid separation, this study tested six types of the short packed-C1 columns (see Table I: A–F). The physical/chemical specifications are listed in Table I. A mixture of ethanol and water as the isocratic mobile phase was used, and mobile phases with the ethanol concentrations of 5–35% (v/v), column temperatures of 25–50°C, HPLC flow rates ≥ 0.5 mL/min, and HPLC measuring times (retention times) ≤ 20 min were used. Because the HPLC separations were performed serially, the time per run became critical in routine residue monitoring. The short run time not only increased sample throughput for analysis but also affected the method-development time.

The six columns were compared with regard to how the analysis separated among TBa and β-TB, their interfering peaks, and the sharpness of the peaks obtained upon injection of equal amounts. The observed chromatographic separations and peak forms formed within the condition ranges examined are also presented in Table I.

Columns A and F had difficulty separating TBa and β-TB, and the interferences of resulting sample extract throughout the examined condition ranges. Neither TBa and β-TB were eluted from columns D and E. Column B provided tailing

Table I. Physical/Chemical Specifications of the C1 Columns* Used and Chromatographic TBa and β-TB Peak Forms Obtained Under the HPLC Condition Ranges Examined†

Designation	Column length (mm)	d_p (µm)	Surface area (m2/g)	Pore diameter (Å)	Carbon content (%)	HPLC target compounds	
						Separation	Peak form
A: CAPCELL PAKC1 UG120 (S-5)	75	5	300	120	4	Not separated	Broadening
B: Daisopak SP-200-3-C1-P	75	3	300	200	3	Remarkably	Tailing
C: Daisopak SP-200-3-C1-P	50	3	300	200	3	Remarkably	Symmetrical and Sharp
D: Develosil TMS-3	50	3	300	140	4.5	Not eluted	
E: Develosil TMS-5	50	5	300	140	4.5	Not eluted	
F: YMC-Pack TMS	75	5	Nd‡	120	4	Not separated	Rounded

* i.d.= 4.6 mm; † Mobile phases of ethanol concentrations 5–35 (%, v/v, in water); column temperatures 25–50°C; HPLC flow rates ≥ 0.5 mL/ min; HPLC measuring times (retention times) ≤ 20 min; ‡ No data.

Table II. Method Validation Data

	TBa	β-TB
Linearity (*r**)	0.999	0.998
Accuracy†	≥ 87.9	≥ 90.8
Precision‡	≤ 3.4	≤ 3.3
QL§	1.8	1.5

* Mean of three determinations using spiked cattle muscle samples for calibration curves; range of concentration was 2–20 ng/g; *r* is the correlation coefficient.
† Means from six replicates at three different spiked levels (0.2, 0.5, and 10 ng/g).
‡ Values in parentheses are RSDs.
§ Quanatitation limit (ng/g).

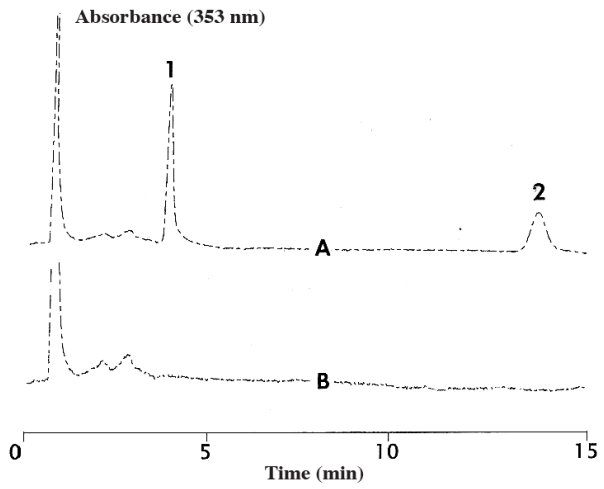

Figure 1. Chromatograms obtained from the HPLC system. A cattle muscle sample spiked with the target compounds (20 ng/g each compound) (A); a blank cattle muscle sample. Peaks: 1, β-TB (Rt = 3.9 min); 2, TBa (Rt = 13.7 min) (B).

peaks of the target compounds.

An ideal chromatogram with complete separation of TBa, β-TB, and interfering peaks, their symmetrical natures, and their short retention times was obtained by the Column-C, and an isocratic mobile phase of 27% ethanol with a flow rate of 1.0 mL/min and a column temperature of 40°C. The HPLC analysis accomplished optimum separation within 15 min and enabled also the multiple sequential injections, without the risks of interfering late-eluting peaks. The short length and 3% carbon contents in the column used here were necessary at least to obtain the described findings. There was no interference from cattle muscle extract with the elution of TBa and β-TB, respectively, as illustrated in Figure 1. In the present HPLC system, separation, identification, and quantitation were achieved in a mere 15 min/run without requiring pre-column washing following analysis.

Method validation

The present method was qualified in terms of analytical performance parameters calculated according to the FDA regulation decision in 1987 (19). Table II summarizes the main performance parameters.

Linearity and range

The spiked recovery graph was generated as practical calibration line by plotting peak areas of fortified sample extracts ranging from 2 to 20 ng/g versus their concentrations. The resulting line showed an excellent linearity for individual compound ($r \geq 0.998$, $P < 0.01$).

Linearity, accuracy, and precision quanatitation limit

The average recoveries from cattle muscle samples at three different spiking levels (2, 5, and 10 ng/g) (total n = 18) were ≥ 87.9% with relative standard deviations (RSDs) of ≤ 3.4% for TBa and ≥ 90.8% with RSDs of ≤ 3.3% for β-TB, respectively. These values are well within "acceptable criteria" for the residue analysis that the Codex setup: average recoveries of 80–110% with RSDs < 15% when the MRL for the target compound is ≥ 0.1 µg/g and when the analytical method can be performed with acceptable precision (20).

Quantitation limit

The quantitation limit (QL) should correspond to the concentration for which the signal-to-noise ratio is less than 10. The QLs in cattle muscle samples were 1.8 ng/g for TBa and 1.5 ng/g for β-TB, respectively, which were less than the TBa MRL of 2 ng/g or the TBa FDA safe concentration of 80 ng/g.

Selectivity

The photo-diode array detector chosen provides an easy way to confirm peak identity and enable the separation and identification of TBa and β-TB by their retention times and spectra. The TBa and β-TB spectra obtained from sample were practically identical to those of the standards. Because of the high absorbance of TBa and β-TB and the satisfactorily purification, UV detection (PDA using) at trace levels is fully available. It is, therefore, instructive to show purification effectiveness of the sample preparation. The present HPLC system made it unnecessary to use very expensive MS to identify the target compounds.

Robustness

Some chromatographic parameters were performed using a spiked (10 ng/g of each compound) cattle muscle sample obtained under the established procedure. Changes of ± 5% of the flow rate, the HPLC column temperature, and the ethanol concentration of the mobile phase had no effect on the peak areas, whereas the variations in the retention times were obtained with both the flow rate and the column temperature. Normal retention times for TBa and β-TB were 13.7 and 3.9 min, respectively. At + 5% the flow rate, the two retention times were decreased, ranging between 1.5 and 4.6 % and at –5%, the times were increased ranging between 6.0 to 8.1%. By changing the column temperature by + 5% (42°C), decreasing retention times obtained were 1.8–8.3%; however, no significant variations were observed with –5% (38°C).

Conclusion

The proposed technique for determination of TBa and β-TB in beef (cattle muscle) is a useful tool for the routine residue monitoring in beef and the withdrawal control of beef farm. There are four advantages: (*i*) harmless to the environment (does not use harmful organic solvents and reagents at all); (*ii*) shorter total analysis time (< 20 min/sample, where a batch of 12 samples in 2 h); (*iii*) economical (budget = 5.4 US$/sample as December 7, 2007); (*iv*) reproducible recoveries (≥ 87.9% with RSD ≤ 3.4%)

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