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Determination of trenbolone and zeranol in bovine muscle and liver by liquid chromatography–electrospray mass spectrometry

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Abstract

A sensitive and selective method using liquid chromatography (LC)–electrospray mass spectrometry for the determination of growth promoters, trenbolone and zeranol, in bovine muscle and liver has been developed. The LC separation was performed on a Zorbax XDB-C18 column (150×2.1 mm I.D.) using 0.005% acetic acid–acetonitrile (60:40, v/v) as the mobile phase at a flow-rate of 0.2 ml/min. The positive ionization produced typical $(M+H)^+$ molecular ions of α-trenbolone and β-trenbolone. On the other hand, the negative mode produced $(M-H)^-$ ion of zeranol. The calibration graphs for α-trenbolone, β-trenbolone and zeranol were rectilinear from 2.5 pg to 1.0 ng with selected ion monitoring. The drugs were extracted with 0.2% metaphosphoric acid–acetonitrile (6:4, v/v), and the extracts were cleaned up on a OASIS HLB (60 mg) cartridge. The recoveries of the hormones from bovine muscle fortified at 2 ng/g were 82.3–85.1%, and detection limits were 0.5 ng/g for each drug.

Keywords: Food analysis; Trenbolone; Zeranol; Growth hormones

1. Introduction

Hormonal anabolic agents such as zeranol (ZER) and trenbolone acetate (TBA)(Fig. 1) are widely used for the growth promotion in animal breeding [1,2]. ZER (α-zearalanol) is a natural mycooestrogen derived from zearalenone, produced by different species of *Fusarium* molds [3]. On the other hand, TBA is a synthetic androgen having anabolic activity several fold above that of testosterone. These synthetic hormones are not used in Japan. However, 60% or more of the beef domestically consumed are imported from the United States, Australia, etc. Therefore, after the safety of ZER and TBA was evaluated, and the maximum residue limits (MRLs)

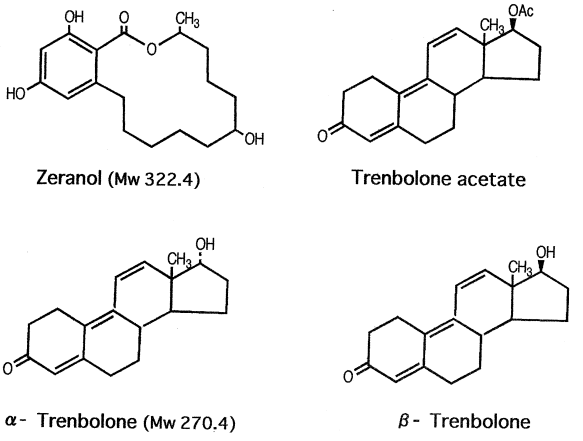

Fig. 1. Structures of zeranol, trenbolone acetate, α-trenbolane and β-trenbolone.

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for the residues of these hormones were established in Japan in 1995. The established MRLs are the same as the Codex Alimentarius, which is an international standard [4].

TBA and ZER are administered by subcutaneous implantation in the ear. TBA upon entering the circulatory system is rapidly hydrolyzed to its active free form, 17β-trenbolone (β-TBOH). In cattle, the 17α-trenbolone (α-TBOH) is the major metabolite occurring in the liver while the β-TBOH is the major metabolite occurring in the muscle [5]. The FAO/WHO Codex Committee recommended the MRLs for TBA and ZER in muscle and liver. The MRLs for ZER in bovine muscle and liver are 2 μg/kg and 10 μg/kg, respectively [4]. The Committee recommended MRLs for β-TBOH in the muscle and α-TBOH in the liver at 2 μg/kg and 10 μg/kg respectively. These MRLs are not likely to be exceeded with good practice when using these hormones.The incorrect use of these hormones may leave residues in edible tissues. In other words, the use of anabolic agents in animals involves possible health risks if harmful residues remain in the meat products intended for human consumption [6]. Therefore, monitoring of these residues is necessary to ensure that they are not present at levels that may pose health risks to the public. Analytical methodologies have been developed and established to identify and quantify these hormones.

As the official method [7], HPLC–UV was established for the measurement. However, as the MRLs of ZER and TBA are quite low, an extremely complicated cleanup procedure is adopted to remove all impurities. This paper describes a simple, rapid and reliable LC–MS method for the determination of ZER and TBA (α-TBOH and β-TBOH) at 0.5 ng/g in bovine muscle and liver.

2. Experimental

2.1. Materials and reagents

ZER, α-trenbolone and β-trenbolone were obtained from Hayashi (Osaka, Japan).

OASIS HLB (60 mg) cartridges were purchased from Waters (Milford, MA, USA). The cartridges were conditioned by washing with 5 ml of methanol and then 10 ml of water before use. Hyflo Super-Cel was obtained from Johns-Manville (Denver, CO, USA). Other chemicals were of analytical-reagent or HPLC grade. Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA) prior to use.

2.2. Preparation of standard solutions

Stock standard solutions of zeranol, α-trenbolone and β-trenbolone were prepared by dissolving 50 mg of each compound in 50 ml of methanol. The standard solutions were kept at 5°C in amber glass vessels and were stable for up to six months. Working standard solutions were prepared by diluting the stock solution with 40% acetonitrile. The working standard solutions were stored in the refrigerator and were stable for up to ten days.

2.3. Apparatus

Experiments were carried out using a Hewlett-Packard (Palo Alto, CA, USA) Model HP 1100 Series LC–MSD system with a electrospray interface (ESI). The separation was performed on a Zorbax Eclipse XDB-C18 column (5 μm, 150×2.1 mm I.D., Hewlett-Packard) with 0.005% acetic acid–acetonitrile (60:40) as the mobile phase at a flow-rate of 0.2 ml/min. The LC–MS operating conditions are summarized in Tables 1 and 2. The other instruments used were a Model N-1 rotary evaporator (Tokyo Rikakikai, Tokyo, Japan) and a Model NS-50 Physcotron homogenizer (Niti-on, Chiba, Japan).

2.4. Calibration graphs

Standards at concentrations of 0.5, 1, 2, 5, 20, 50 and 200 ng/ml of ZER, α-TBOH and β-TBOH were prepared from the standard stock solutions. A 5 μl volume of these solutions was injected into the column. Calibration graphs were obtained by measurement of the peak areas on the m/z 321, m/z 271 and m/z 271 selected ion monitoring (SIM) chromatograms for the amount of zeranol, α-TBOH and β-TBOH, respectively.

Table 1
Operating Conditions of LC–MS for trenbolone (TBOH) and zeranol (ZER)

MS Conditions		HPLC Conditions	
Ionization mode	TBOH: ESI, Positive Mode	Column	Zorbax XDB-C18 (150×2.1 mm)
	ZER: ESI, Negative mode	Eluent	0.005% acetic acid–acetonitrile (60:40)
Fragmentor	TBOH: 100 V	Flow rate	0.2 ml/min
	ZER: 120 V	Oven temp.	40°C
Nebulizer	N_2 (40 p.s.i., 1 p.s.i.=6894.76 Pa)	Injections	5 μl
Drying gas	N_2 (10 l/min, 350°C)		
V-cap	4500 V		
SIM ion	TBOH: *m/z* 271.1		
	ZER: *m/z* 321.2		

2.5. Sample preparation

The sample preparation was done as follows based on previous papers [8,9]. A 5 g sample was homogenized at high speed for 2 min with 100 ml of 0.2% metaphosphoric acid–acetonitrile (6:4, v/v) used as the deproteinizing extractant. The homogenate was filtered through ca. 2 mm of Hyflo Super-Cel coated on a suction funnel. For a liver sample, several grams of Hyflo Super-Cel were added to the homogenized solution before the filtration. After a slight mixing, the obtained mixture was filtered. The filtrate was evaporated under reduced pressure at 45°C to approximately 30 ml volume. The flask contents were passed through an OASIS HLB cartridge. After washing with 5 ml of 20% acetonitrile, the cartridge was then eluted with 5 ml of acetonitrile. The eluate was evaporated to dryness under reduced pressure at 45°C, and the residue was dissolved in 1 ml of 40% acetonitrile. A 5 μl sample of the solution was then injected into the LC–MS system.

3. Results and discussion

3.1. Chromatographic conditions

For the interface, ESI was selected, which is excellent for the manipulation and suitable for ionizing polar and non-polar compounds [10]. ZER is a weak acidic compound, which has a phenol hydroxyl group, and it was found that the negative mode was suitable for its ionizing. On the other hand, the positive mode was found suitable for α-TBOH and β-TBOH. The negative mode was then

Table 2
Effect of methanol or acetonitrile additive to metaphosphoric acid extraction solvent on the recovery of zeranol, α-trenbolone and β-trenbolone from bovine muscle[a]

Extracting solvent		Recovery (%)		
		Zeranol	α-Trenbolone	β-Trenbolone
0.2% MPA[b]		14.2	39.3	36.5
0.2% MPA–methanol	(8:2)	49.7	60.5	56.5
	(7:3)	65.3	77.9	74.4
	(6:4)	80.5	82.5	80.1
	(5:5)	88.7	84.5	86.5
0.2% MPA–acetonitrile	(8:2)	59.3	59.5	61.9
	(7:3)	82.3	81.3	79.3
	(6:4)	88.2	84.5	83.5
	(5:5)	85.8	84.3	81.3

[a] Samples were spiked with 50 ng/g of each drug. Mean result of three replicates. These recoveries were measured by HPLC–UV analysis.
[b] Metaphosphoric acid.

adopted for the measurement of ZER, and the positive mode was adopted for the analysis of α-TBOH and β-TBOH. The authors [11] previously reported that the sensitivity improved by adding a small amount of acetic acid to the mobile phase in the analysis of bisphenol A using LC–MS. Therefore, the influence of the concentration of acetic acid on the detectability was then examined. As shown in Fig. 2, adding a small amount of acetic acid to the mobile phase of α-TBOH, β-TBOH and ZER resulted in a better sensitivity and a better detectability, but as the concentration increased, the detection sensitivity decreased. Based on these results, 0.005% acetic acid–acetonitrile (60:40) was used in the mobile phase in consideration of the retention time, the peak shape, the ionic strength, etc. The pseudo-

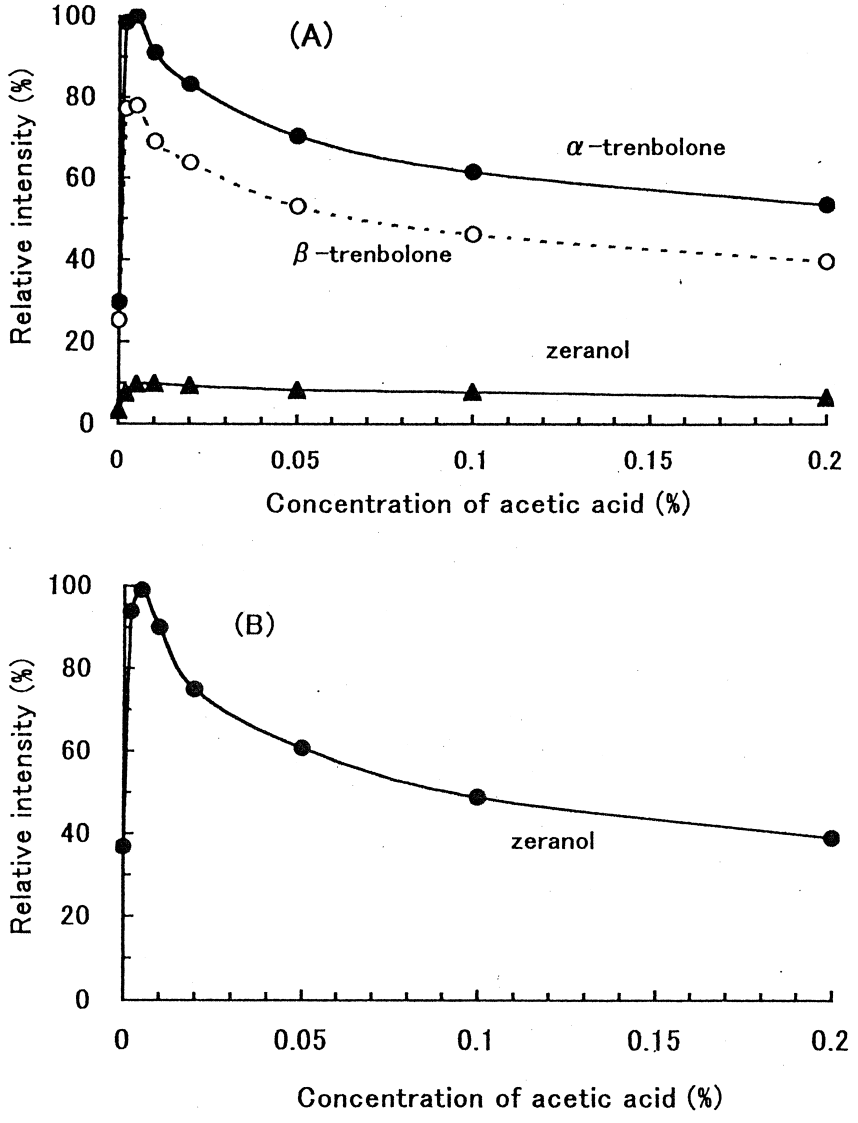

Fig. 2. Effect of the concentration of acetic acid–acetonitrile (60:40) mobile phase on the intensity of (A) the $(M+H)^+$ ions of trenbolone and zeranol and (B) the $(M-H)^-$ ion of zeranol.

molecular ions of ZER ($[M-H]^-=m/z$ 321), α-TBOH and β-TBOH ($[M+H]^+=m/z$ 271) observed under this condition were excellent.

3.2. Influence of fragmentor voltage

ESI is a mild ionization method, while it lacks structural information, can easily obtain molecular weight information on any compound. A method was then used in which the structural information (fragment ion) was obtained after changing the collision conditions with nitrogen molecule in the ion source by changing the voltage at the capillary exit (fragmentor voltage) [12]. As shown in Fig. 3, it was

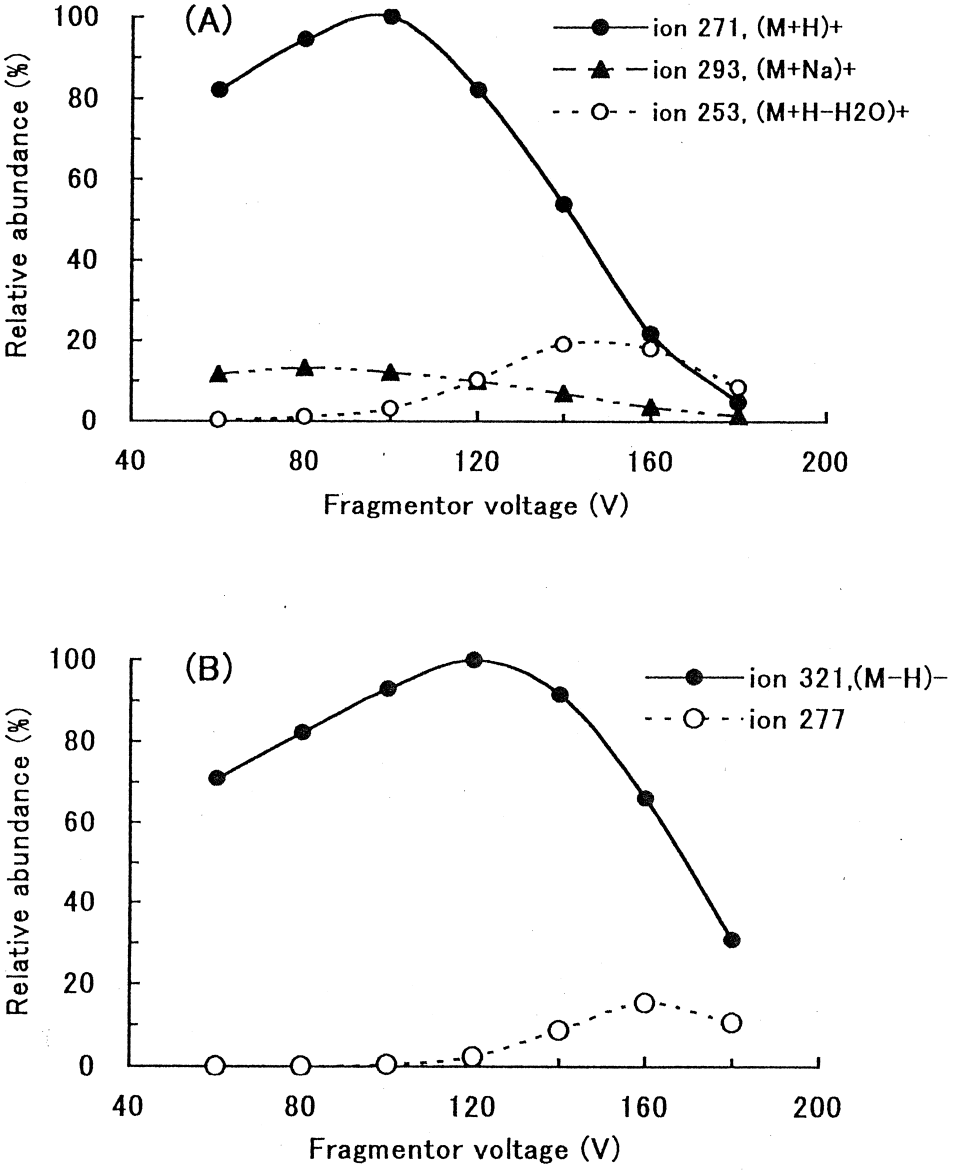

Fig. 3. Effect of fragmentor voltage on the relative abundance of (A) $(M+H)^+$, $(M+Na)^+$ and product ion of α-trenbolone and (B) $(M-H)^-$ and product ion of zeranol.

possible to change the type of ion generated by changing both the fragmentor voltages of ZER, α-TBOH and β-TBOH. The fragmentor voltages were set at 100 V and 120 V, then pseudomolecular ions of α-TBOH and β-TBOH, and ZER were efficiently generated.

In addition, the best measurement conditions for the nebulizer pressure, drying gas pressure, temperature, capillary voltage, etc., were examined, and the conditions shown in Table 1 was established. The main ions of α-TBOH, β-TBOH and ZER, which had been obtained under these conditions, were presumed to be pseudomolecular ions, and also pseudomolecular ions from which water or CO_2 was eliminated. Moreover, for the determination of quality, a fragmentor voltage was adopted, so that fragment ions were efficiently generated (Figs. 4 and 5). The detectability limit of α-TBOH, β-TBOH and ZER, which had been obtained under these conditions, was 0.5 ng/ml (2.5 pg as the absolute amount), when the monitor ions were presumed to be pseudomolecular ions in the SIM mode.

3.3. Analytical accuracy of SIM

The calibration curves of α-TBOH, β-TBOH and ZER were drawn based on SIM, which chose $[M+H]^+$ (m/z 271) or $[M-H]^-$ (m/z 321). The calibration curves of each ion showed excellent linearity within the range of 2.5 to 1000 pg. Moreover, the five replicate measurements taken for each injection rate (10, 50 and 100 pg) to obtain the relative

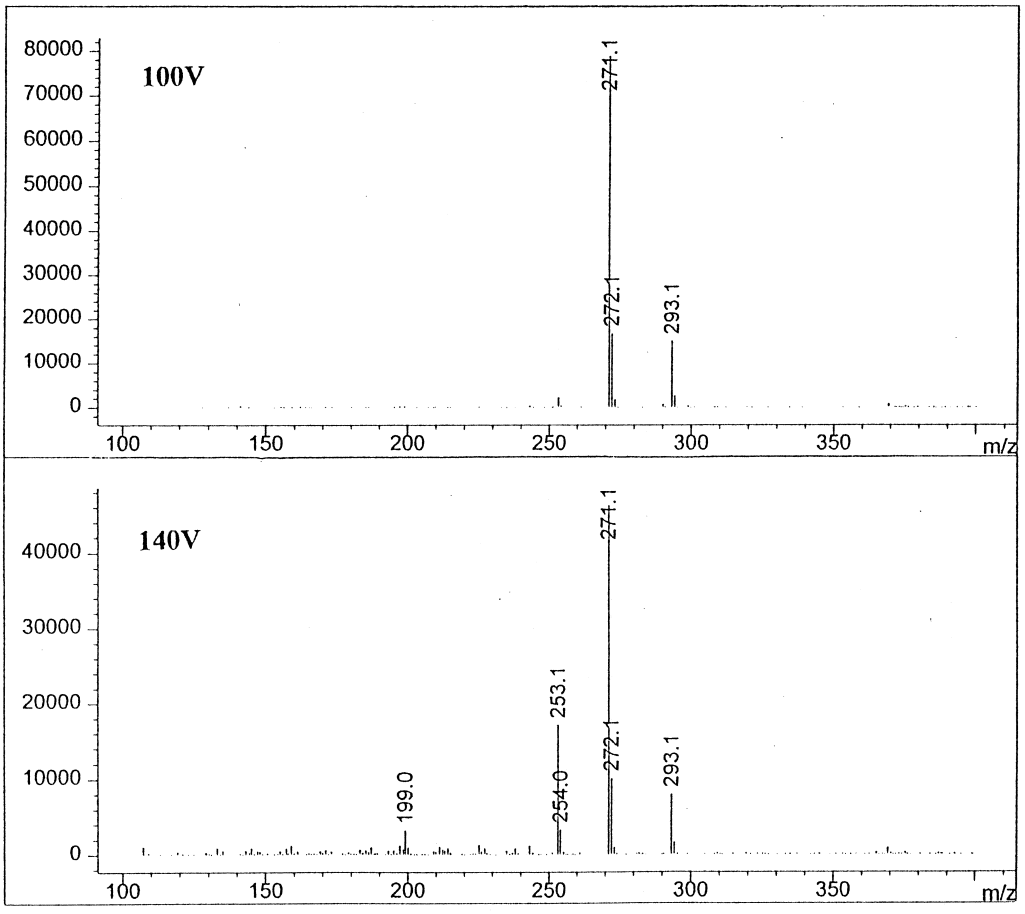

Fig. 4. LC–ESI-MS spectra of α-trenbolone at fragmentor voltages of 100 and 140 V.

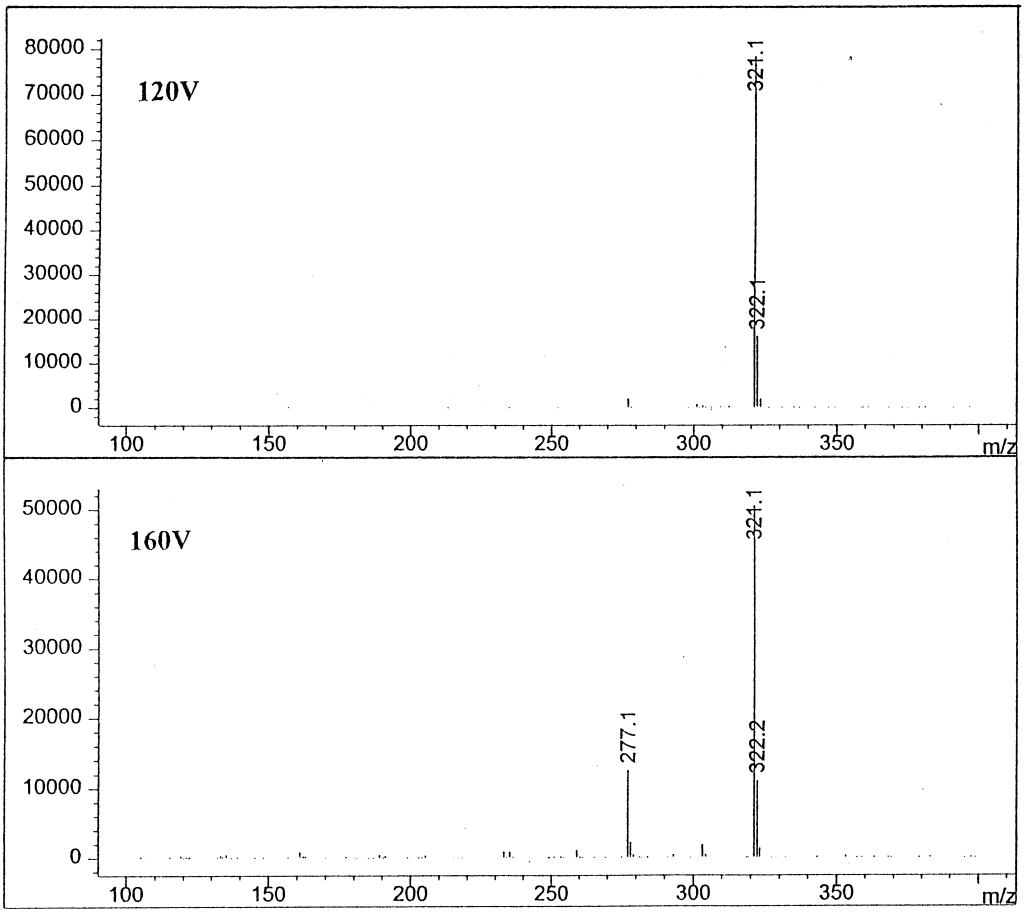

Fig. 5. LC–ESI-MS spectra of zeranol at fragmentor voltages of 120 and 160 V.

standard deviations (RSDs) in the peak area were found satisfactory within 2% for each injection rate.

3.4. Sample preparation

Several cleanup methods had been developed for the analysis of ZER and TBA [13–17]. Recently, a pretreatment method was reported in which the solid phase extraction (SPE) was combined with immunoaffinity [18]. This method is quite excellent for the clean-up effect, but the manipulation is rather complicated. In Japan, the HPLC–UV method has been established as the official method for the analysis of ZER and TBA (α-TBOH and β-TBOH). However, due to the low MRLs of ZER, α-TBOH and β-TBOH, an extremely complicated pretreatment method (in which a liquid–liquid partition, an anion-exchange resin cartridge, and a Sephadex column are combined) was adopted [7].

Recently, the SPE method has been extensively applied for the cleanup of animal tissue samples prior to being quantitatively analyzed for trace amounts of veterinary drugs [18]. The most widely used solid-stationary phase for extraction and cleanup is octadecylsilane bonded to silica gel, called C_{18} or ODS. As the LC–MS measurement technique has an excellent selectivity, we then decided to use a reversed-phase system cartridge, which is also excellent for preparing samples. Several reversed-phase cartridges were examined and all of them showed sufficient recovery rates. The polymer type OASIS HLB was adopted this time.

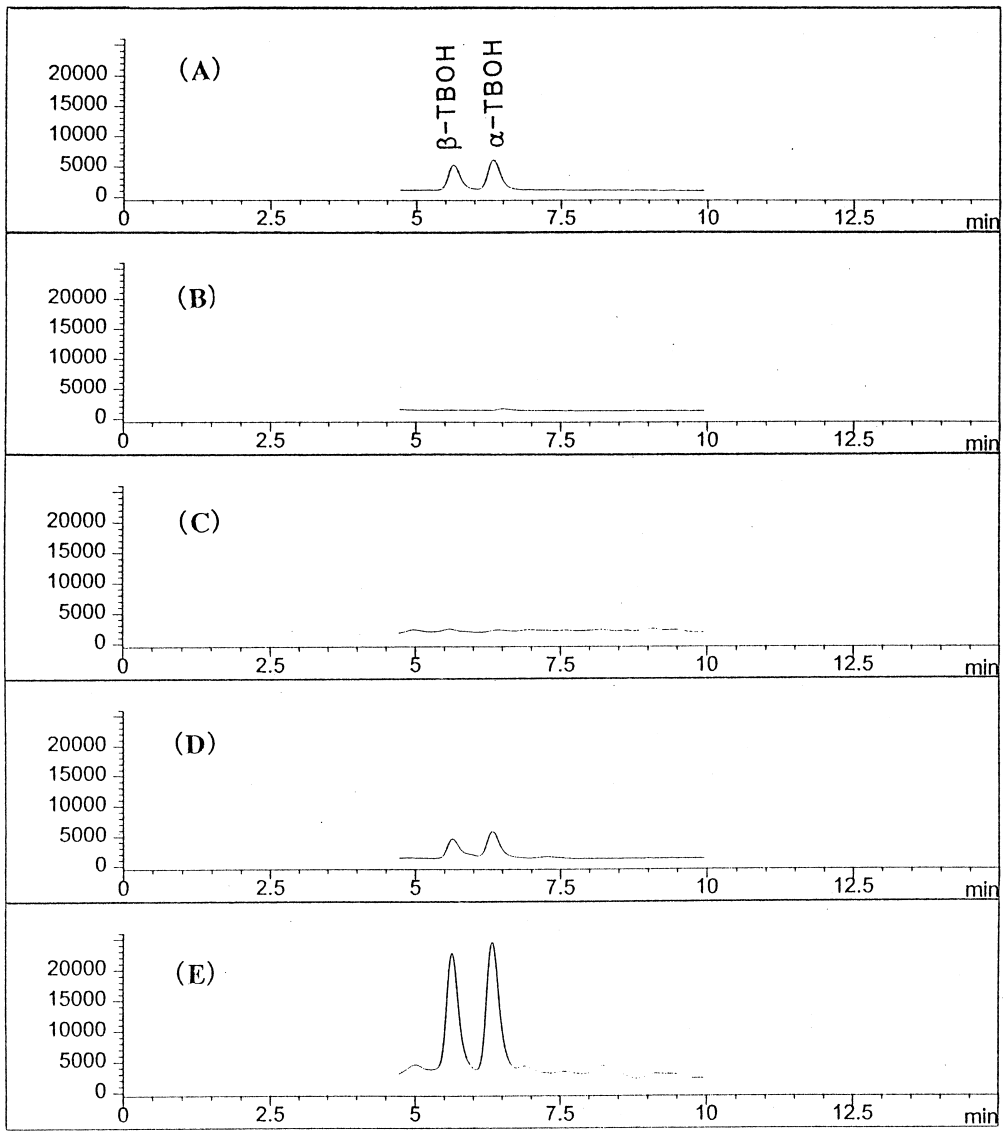

Fig. 6. LC–ESI-MS-SIM chromatograms of (A) standard mixture of α-and β-trenbolone (10 pg), (B) bovine muscle extract, (C) bovine liver extract, (D) extract of bovine muscle fortified at 2 ng/g and (E) extract of bovine liver fortified at 10 ng/g.

Next, the extracting solvents were examined. We have previously reported that the content of methanol or acetonitrile in the extracting solvent (0.2% metaphosphoric acid (MPA)–organic solvent system) affected the recovery of the veterinary drugs [6,7]. Table 2 shows the results of the recovery experiments for bovine muscle tissues fortified with 50 ng/g of each drug. An increase in the methanol content resulted in improved recovery. For the LC–MS measurement, the decrease (about 30%) in the strength of the pseudomolecular ions of these hormones was seen due to a matrix effect for the MPA–methanol system. On the other hand, the same influence was hardly seen in the MPA–acetonitrile system. Therefore, we used the MPA–acetonitrile system for the sample extraction. An increase in the acetonitrile content also resulted in improved recovery. Acetonitrile at 40% added to 0.2% MPA

provided good recoveries while minimizing elution of the contaminants.

Using this method, a good recovery was obtained without any interference from any coexisting substances as shown in Figs. 6 and 7.

3.5. Recovery

Linear calibration graphs (r^2>0.997) were obtained from 2.5 to 1000 pg (equivalent to 0.1–40 ng/g) for the hormones. Table 3 summarizes the recoveries of the drugs from samples of bovine muscle and liver fortified with 2 and 10 ng/g, respectively. Greater than 70% overall mean recoveries and within 10% RSD were obtained with both samples. The detection limits of the method were 0.5 ng/g for the hormones (signal-to-noise ratio >3) in both samples.

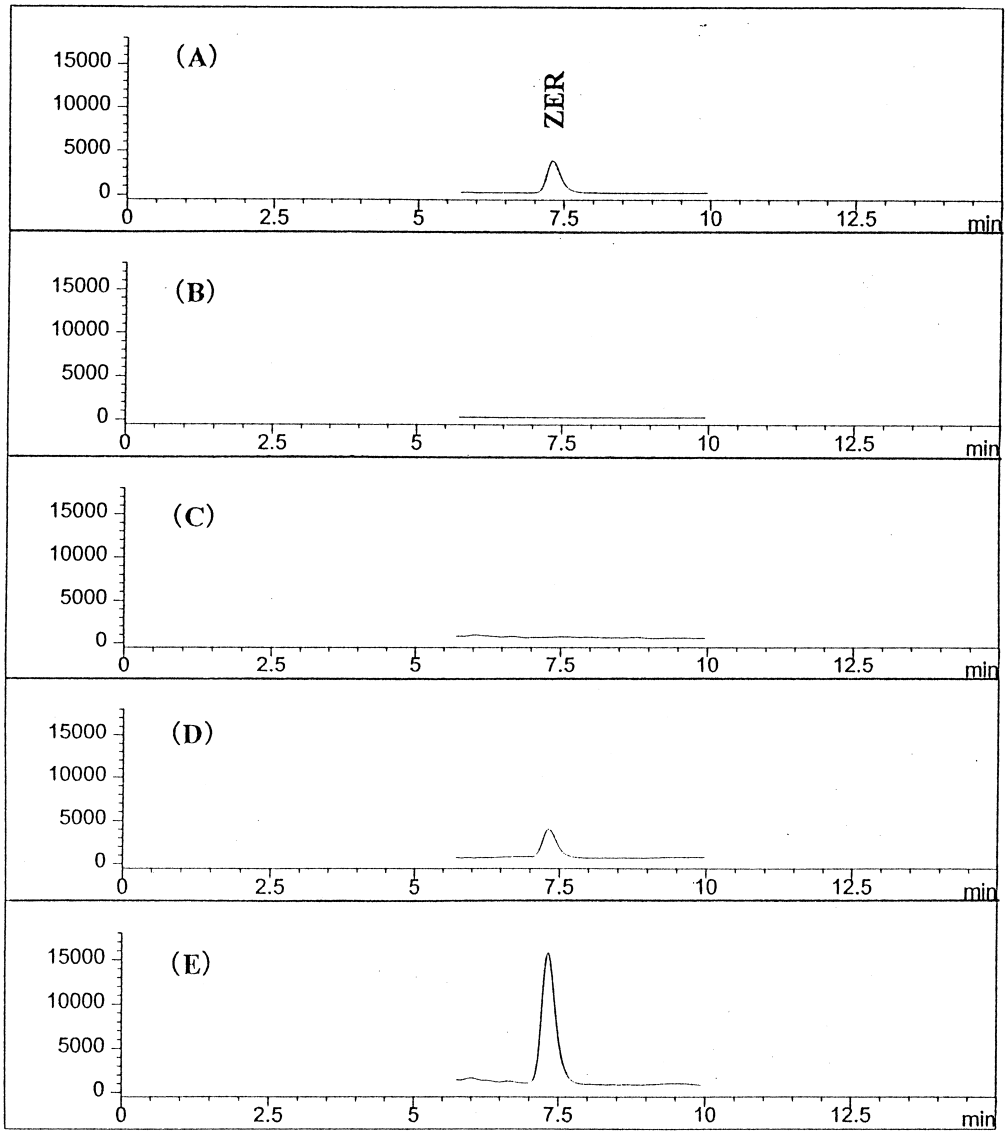

Fig. 7. LC–ESI-MS-SIM chromatograms of (A) standard mixture of zeranol (10 pg), (B) bovine muscle extract, (C) bovine liver extract, (D) extract of bovine muscle fortified at 2 ng/g and (E) extract of bovine liver fortified at 10 ng/g.

Table 3
Recoveries of zeranol, α-trenbolone and β-trenbolone from bovine muscle and liver

Sample	Added (ng/g)	Recovery (mean±SD, n=5) (%)		
		Zeranol	α-Trenbolone	β-Trenbolone
Bovine muscle	2	85.1±4.6	82.4±5.1	82.3±5.7
Bovine liver	10	78.9±4.7	76.3±7.3	79.1±6.9

HPLC–UV is generally used for the residual analysis of veterinary drugs, but it is necessary to use an extremely complicated pretreatment in order to use HPLC–UV in the analysis of ZER and TBA, both of which have low MRLs. However, as LC–MS is quite excellent in selectivity and detection sensitivity, it became possible to use a simple pretreatment method. It is now expected that LC–MS will be applied as a qualitative and quantitative analysis method for toxic chemical substances in foodstuffs, including pharmaceutical drugs for animals.

4. Conclusions

The method described for the determination of ZER and TBA in bovine muscle and liver samples yielded good recoveries and precision. In addition, the detection limits of the method were 0.5 ng/g for these hormones in bovine tissues, and the time required for the analysis of one sample was less than 1.5 h. A combination liquid chromatography–mass spectrometric method is available to reliably measure the recommended MRLs in bovine liver and muscle. Therefore, we recommend this proposed method for the routine analysis of the residual ZER and TBA in bovine tissues.

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