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IDENTIFICATION AND QUANTITATION OF TRENBOLONE IN BOVINE TISSUE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Identification and quantitation of trace amounts of trenbolone in bovine tissue by capillary gas chromatography–mass spectrometry–selected-ion monitoring (GC–MS–SIM) has been developed. Three-phase liquid–liquid extraction using a mixture of water–acetonitrile–dichloromethane–hexane was utilized for the sample extraction from tissue. Target compounds were extracted from the tissue into the acetonitrile layer. The residue from this extraction was then subjected to solid-phase extraction by C_{18} and silica gel disposable cartridges using methanol–water and benzene–acetone as eluents. To overcome extensive matrix interferences, preparative reversed-phase high-performance liquid chromatographic separation was used with an octadecyl-bonded column using methanol–water as mobile phase for sample clean-up prior to GC–MS analysis. A structural analogue of trenbolone, 19-nortestosterone, was chosen as the internal standard for quantitation by GC–MS. The sample was co-injected with N,O-bis(trimethylsilyl)trifluoroacetamide–1-(trimethylsilyl)imidazole (95:5, v/v) for flash heater derivatization. Identification and quantitation were simultaneously carried out by SIM of characteristic ions of the trimethylsilyl derivatives of trenbolone and 19-nortestosterone. The limit of detection for trenbolone and epitrenbolone was 0.5 ppb in muscle and liver tissue. A comparison of sensitivity and specificity between GC–MS under electron ionization in addition to positive- and negative-ion chemical ionization conditions using methane reagent gas is also discussed.

INTRODUCTION

Trenbolone (TBOH; 17β-hydroxyestra-4,9,11-trien-3-one) is a synthetic anabolic steroid which has been exploited for improving the feed conversion rate and carcass characteristics of cattle intended for human consumption. Previous studies on the metabolism of TBOH residues in the cow [1–3] have shown that after the administration of trenbolone acetate (TBA), TBOH is the major metabolite in muscle and fat, while the epimer of trenbolone, 17α-hydroxyestra-4,9,11-trien-3-one (epi-TBOH), is the major metabolite in liver and kidney [4].

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To successfully control the illegal use of hazardous anabolics, analytical methods must be sensitive and specific enough for the simultaneous detection of trace amounts of anabolics in different biological matrices. Only a few procedures for determining TBOH have appeared in the literature. Thin-layer chromatography (TLC) was first used to determine TBOH by Oehrle et al. [5]. Radioimmunoassay (RIA) procedures for the detection of these compounds in bovine tissue and plasma were described by Hoffmann and Oettel [6] and Heitzman et al. [7]. Holder et al. [8] reported high-performance liquid chromatography (HPLC) and gas chromatography–electron-capture detection (GC–ECD) for the determination of TBA and TBOH in animal chow. Jansen et al. [9] used HPLC as the screening technique for the determination of TBOH and its metabolites in bovine urine. These techniques have been limited either by poor specificity or sensitivity. Gas chromatography–mass spectrometry–selected-ion monitoring (GC–MS–SIM) is the most sensitive and reliable residue analysis technique available for the detection and confirmation of low ppb (0.5–5.0 ppb) levels of anabolic agents. A qualitative and quantitative GC–MS–SIM determination for TBOH and its metabolites at trace levels in bovine tissue is reported herein.

EXPERIMENTAL

Chemicals and equipment

Trenbolone and its tritiated analogues were obtained from Roussel-UCLAF (France). 19-Nortestosterone was purchased from Sigma (St. Louis, MO, U.S.A.). Glucuronidase (type H-2 from *Helix pomatia*) was also obtained from Sigma for enzymatic hydrolysis of the conjugates in tissue samples and was used without dilution. Ethyl acetate, benzene, and acetone (analytical grade) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Acetonitrile and methanol (HPLC grade), C_{18} and silica solid-phase extraction cartridges were obtained from J.T. Baker (Philipsburg, NJ, U.S.A.). The cartridges were used on a twelve-port vacuum manifold available from Supelco, (Bellefonte, PA, U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1-(trimethylsilyl)imidazole (TMSI) for sample derivatization and dichlorodimethylsilane for glassware deactivation were purchased from Pierce (Rockford, IL, U.S.A.).

A Beckman Model 660 (Irvine, CA, U.S.A.) liquid scintillation counter was used for radioisotope tracer studies. The HPLC system used consisted of a Waters (Milford, MA, U.S.A.) dual-pump system with a Model 660 solvent programmer. A Kratos Model 783 UV detector set at 350 nm and equipped with an 12-μl flow cell (Ramsey, NJ, U.S.A.) was used for HPLC screening and preparative work. A Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector equipped with a 20-μl loop was used for sample introduction. A Hewlett-Packard (Avondale, PA, U.S.A.) Model 5840 gas chromatograph directly interfaced to an HP 5985B mass spectrometer equipped with chemical ionization (CI) and electron ionization (EI) modes was used for the evaluation of the optimum GC–MS ionization mode. A capacitance Baratron (Burlington, MA, U.S.A.) was used to measure the CI ion source pressure. The column used for this system was an HP 100% methylsilicone fused-silica capillary column (25 m$\times$0.32 mm I.D.). A Carlo Erba (Milan, Italy)

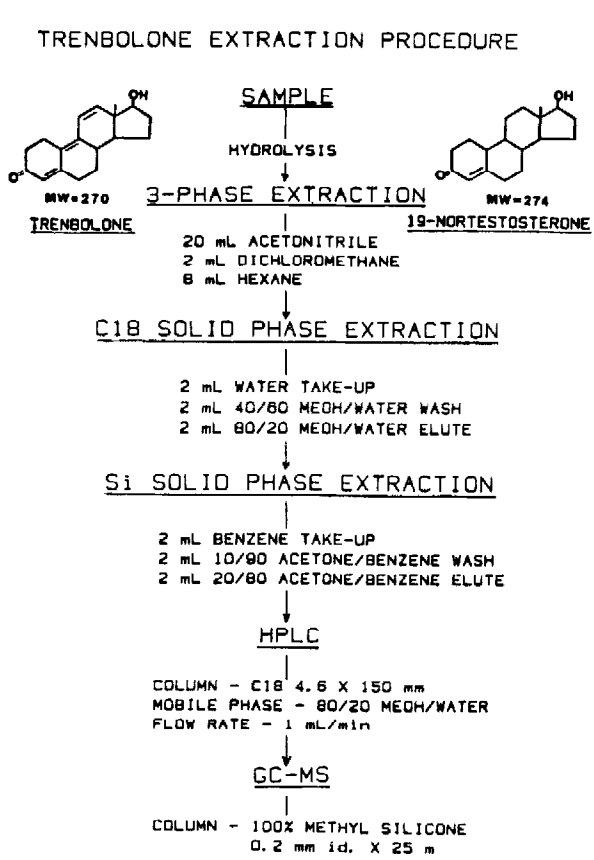

Fig. 1. Extraction procedure for isolating trenbolone and 19-nortestosterone from bovine liver and muscle.

Mega series Model 5160 gas chromatograph equipped with J & W (Folsom, CA, U.S.A.) 100% methylsilicone fused-silica capillary column (30 m × 0.2 mm I.D.) directly interfaced to an HP 5970 mass-selective detector was used for routine GC–MS tissue analysis under EI conditions. Sample introduction was performed in the splitless mode on a Grob-type capillary GC injector for both GC–MS systems.

Extraction procedure

The overall procedure can be divided into five major steps (Fig. 1): (a) enzymatic hydrolysis of the tissue, (b) liquid–liquid extraction of the tissue homogenate, (c) liquid–solid extraction by C_{18} and silica cartridges, (d) purification by HPLC after the extraction and (e) confirmation and quantitation by capillary GC–MS. Radioactive isotopes were used for the recovery determination of TBOH and 19-nortestosterone in bovine liver and muscle tissue.

Hydrolysis. A suitable amount of standard (2.5–200 ng) was spiked into 5 g of homogenized bovine tissue (liver or muscle 0.5–40 ppb). The tissue samples were then hydrolyzed enzymatically overnight at 37°C and extracted with 20 ml of

acetonitrile. The pH of the supernatant was then adjusted with 6 *M* sodium hydroxide to a value of 13.

Liquid–liquid extraction. For liquid–liquid extraction, hexane (8 ml) and dichloromethane (2 ml) were added to the aqueous acetonitrile extract to generate a three-phase liquid–liquid system. The middle, acetonitrile, layer of the three-phase extract was collected. An additional 5 ml of acetonitrile was added to the remaining hexane and aqueous layer. This was also collected and added to the previous acetonitrile layer. Because of the remaining dichloromethane, the acetonitrile layer can be washed with 5 ml water and separated. The final acetonitrile extract was then concentrated to dryness under a gentle stream of nitrogen at 60 °C.

Liquid–solid extraction. After the class separation by liquid–liquid extraction, the residue was dissolved in 2 ml water with the aid of ultrasonication. This aqueous sample was then applied to a pre-conditioned C_{18} cartridge (after a 3-ml methanol wash followed by conditioning with 3 ml distilled water), washed with 2 ml of methanol–water (40:60) and eluted with 2 ml of methanol–water (80:20). The eluate was concentrated to dryness under a gentle stream of nitrogen at 60 °C, then 500 µl acetonitrile–methanol (1:5) were added to precipitate lipid-type compounds. After discarding the resulting precipitate the acetonitrile–methanol solution was concentrated to dryness under nitrogen and the residue dissolved in benzene (2 ml) with the aid of ultrasonication and applied to a silica cartridge. The silica cartridge was then washed with 1.5 ml of acetone–benzene (10:90) eluted with 2 ml of acetone–benzene (20:80) and concentrated to dryness under nitrogen at 60 °C.

HPLC purification. After solid-phase extraction, the samples were further purified by C_{18} reversed-phase HPLC under isocratic conditions (methanol–water, 58:42). The column used was a C_{18} Perkin-Elmer cartridge column (15 cm × 4.6 mm I.D.). Targeted compounds and internal standard were preparatively collected with minimal losses. The collected fraction (1 ml) was then concentrated to dryness under nitrogen at 60 °C.

GC–MS confirmation. After preparative HPLC purification of the sample extract, the sample residues were dissolved in 10 µl ethyl acetate and subjected to GC–MS analysis. Injection was done in the splitless mode with an injector temperature of 275 °C. The oven temperature was programmed from 100 to 220 °C at a rate of 15 °C/min followed by a program of 5 °C/min from 220 to 260 °C. The sample was co-injected with BSTFA–TMSI (95:5, v/v). Both 25 m × 0.25 mm and 30 m × 0.2 mm methylsilicone fused-silica capillary columns were used. A structural analogue of TBOH, 19-nortestosterone, was used as the internal standard at a level of 3–5 ppb for quantitation by GC–MS.

Both identification and quantitation were carried out by SIM of characteristic ions of the trimethylsilyl (TMS) derivatives of TBOH and 19-nortestosterone. The molecular ion (*m/z* 342) and three significant fragment ions (*m/z* 211, *m/z* 224, *m/z* 237) were chosen for trenbolone. The molecular ion (*m/z* 346) and one fragment ion (*m/z* 256) were chosen for the internal standard, 19-nortestosterone. Both the Hewlett-Packard 5985B and mass-selective detector GC–MS systems were used for these studies.

GC–MS quantitation. Reagent blanks involved the use of all reagents, solvents and apparatus of the procedure using distilled water instead of tissue as the sample. The controls consisted of blank tissues spiked with the internal standard, 19-nortestosterone. Linear calibration curves were constructed using regression analysis and the least-squares method for fitting the calibration line. For quantitation of TBOH, the ratio of the peak area for the sum of the molecular ion of 19-nortestosterone (m/z 346) and one fragment ion (m/z 256) and the sum of the peak areas of the ions monitored for TBOH was used as the independent variable, with concentration as the dependent variable. Calibration standards were prepared by spiking control tissue samples (5 g per sample) at the 0, 1, 2 and 4 ppb levels for TBOH and epi-TBOH and at a level of 3.0 ppb for the internal standard.

Trenbolone administration. TBA (200 mg) pellets were implanted in each ear of three one-week-old calves in an effort to obtain elevated muscle and liver residues for an eventual validation study. The animals were sacrificed four weeks after implantation and the tissues stored frozen for later analysis.

RESULTS AND DISCUSSION

Three-phase liquid–liquid extraction has proven to be a preferred extraction method for diethylstilbestrol (DES) and zeranol from tissue [10,11]. This same approach also provides excellent recovery (85%) of radioactive TBOH from fortified bovine liver and muscle. By maintaining the sample at pH 13 with sodium hydroxide before the three-phase extraction, most of the undesired acidic compounds will be ionized and extracted into the aqueous layer, while the target compound and internal standard remain in their neutral form and are retained in the acetonitrile layer. Polar and ionic compounds will be extracted into the aqueous layer; low-polarity compounds will be extracted into the hexane layer and the compounds of intermediate polarity, such as DES, zeranol, melengestrol, dexamethasone and trenbolone, will be extracted into the acetonitrile layer which is the layer of interest. The overall recoveries of TBOH, epi-TBOH and 19-nortestosterone from bovine tissue extracts (liver and muscle) were 53% by radioactive tracer studies.

Due to significant matrix interference a combination of different solid-phase extraction methodologies was necessary. By applying the respective partition (reversed-phase) and adsorption (normal-phase) character of C_{18} and silica, respectively, a two-step solid-phase extraction procedure was exploited to achieve sample clean-up. Unfortunately, because of the relatively low molecular mass of the analytes, significant background interference from the extract resulted in a limit of detection (LOD) at this step of only about 5 ppb by capillary GC–MS–SIM. This problem was solved by further purification with preparative HPLC prior to capillary GC–MS analysis at the low ppb level (0.5–3 ppb) to provide more reliable GC–MS quantitation. The HPLC separation at 350 nm detection can also be utilized as a screening step (Fig. 2). With the unique UV absorbance of TBOH at 350 nm, 0.5 ppb of the analytes in bovine liver and muscle

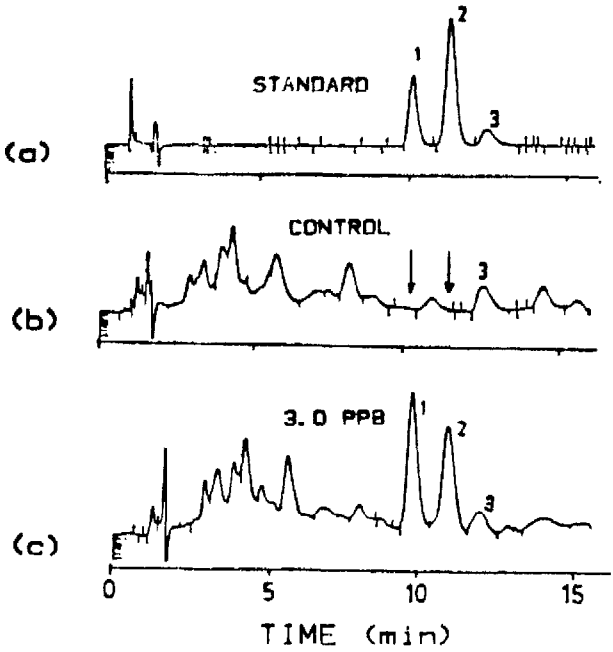

Fig. 2. HPLC profiles of bovine liver extracts. (a) Peaks: 1 = trenbolone (2 ng); 2 = epi-trenbolone (2 ng); 3 = 19-nortestosterone (2 μg); (b) liver extract control; (c) 3.0 ppb of trenbolone and epi-trenbolone in bovine liver with 19-nortestosterone (2 μg) as internal standard. The column used was a 5-μm particle C_{18}, 15 cm × 4.6 mm I.D. Perkin-Elmer cartridge column with a mobile phase of methanol–water (58:42) maintained at a flow-rate of 1 ml/min. The UV detector was set at 350 nm.

extract can be detected. At 350 nm minimal interference is expected from other xenobiotic anabolics or endogeneous androgens [12].

TMS-derivatized TBOH appeared to be more sensitive than that of the parent drug by capillary GC–MS analysis under EI conditions. The sample was co-injected with BSTFA–TMSI (95:5, v/v) in ethyl acetate to obtain quantitatively derivatized 19-nortestosterone and TBOH. This flash heater derivatization has been routinely used for this purpose with satisfactory results.

EI, positive-ion chemical ionization ((PICI) and negative-ion chemical ionization (NICI) modes were evaluated for sensitivity and selectivity for the detection of TBOH. Fig. 3 shows the EI, PICI and NICI mass spectra of TBOH utilizing the HP 5985B GC–MS system. Methane was used as the reagent gas at 0.356 Torr for the PICI and NICI modes. These results show advantages and disadvantages for each ionization mode. The NICI mode provided approximately ten times better sensitivity than the EI and PICI modes, but the lack of fragmentation under NICI conditions limits its selectivity for SIM. Monitoring only the parent ion would cause ambiguity for actual sample confirmation because of interference ions. The sensitivity of the EI and PICI mode was comparable, but the lack of useful fragment ions from PICI makes EI the ionization mode of choice. Thus, the EI mode with TMS derivatization was selected for optimum conditions for GC–MS confirmation and quantitation.

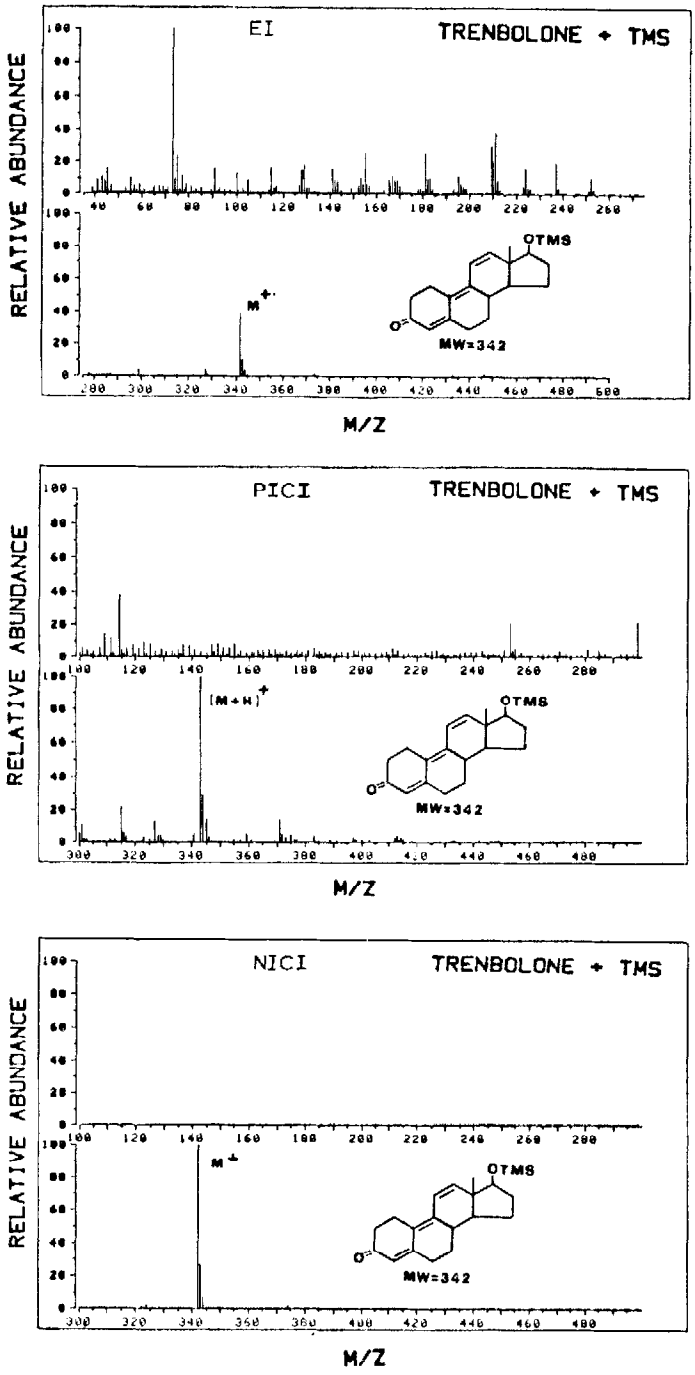

Fig. 3. Electron ionization (EI), positive-ion chemical ionization (PICI), negative-ion chemical ionization (NICI) full-scan GC–MS spectra of TMS-derivatized trenbolone. The reagent gas for PICI and NICI was methane maintained at a source pressure of 0.356 Torr.

TABLE I

EXACT MASS AND ELEMENTAL COMPOSITION FOR SELECTED IONS OF TMS-DERIVATIZED TRENBOLONE AND 19-NORTESTOSTERONE

Exact mass measurements were made by peak matching on an AEI/MS 902 system at a static resolution of 10 000 (10% valley definition) in the electron ionization mode with sample introduction by direct probe.

Trenbolone-TMS		19-Nortestosterone-TMS	
Mass assignment	Mass composition	Mass assignment	Mass composition
210.1018	$C_{15}H_{14}O$	256.1821	$C_{18}H_{24}O$
224.1184	$C_{16}H_{16}O$	346.2309	$C_{21}H_{34}O_{21}Si$
237.1282	$C_{17}H_{17}O$		
342.1989	$C_{21}H_{30}O_{21}Si$		

Capillary GC–MS–SIM met the criteria necessary for the identification and quantitation of trenbolone. These criteria include detection of the selected ions with correct relative abundance ratios ($\pm 20\%$) and with a reproducible GC retention time (± 0.01 min). For this study, the selected masses included *m/z* 210.10, 224.10, 237.10 and 342.15 for the identification of TBOH and epi-TBOH. The *m/z* 256.15 and 346.25 ions were chosen for the internal standard, 19-nortestosterone. The exact mass assignment and elemental composition for these ions

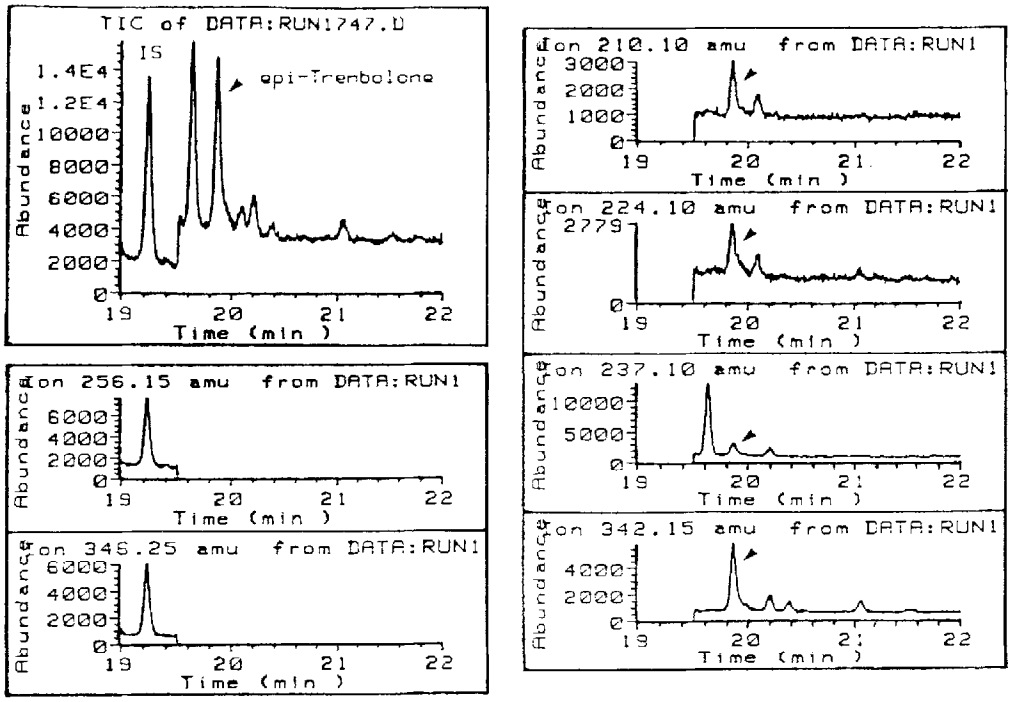

Fig. 4. Electron ionization GC–MS–SIM profiles of 19-nortestosterone-TMS (19.3 min), epi-trenbolone-TMS (19.9 min) and trenbolone-TMS (21.6 min). See text for GC–MS conditions.

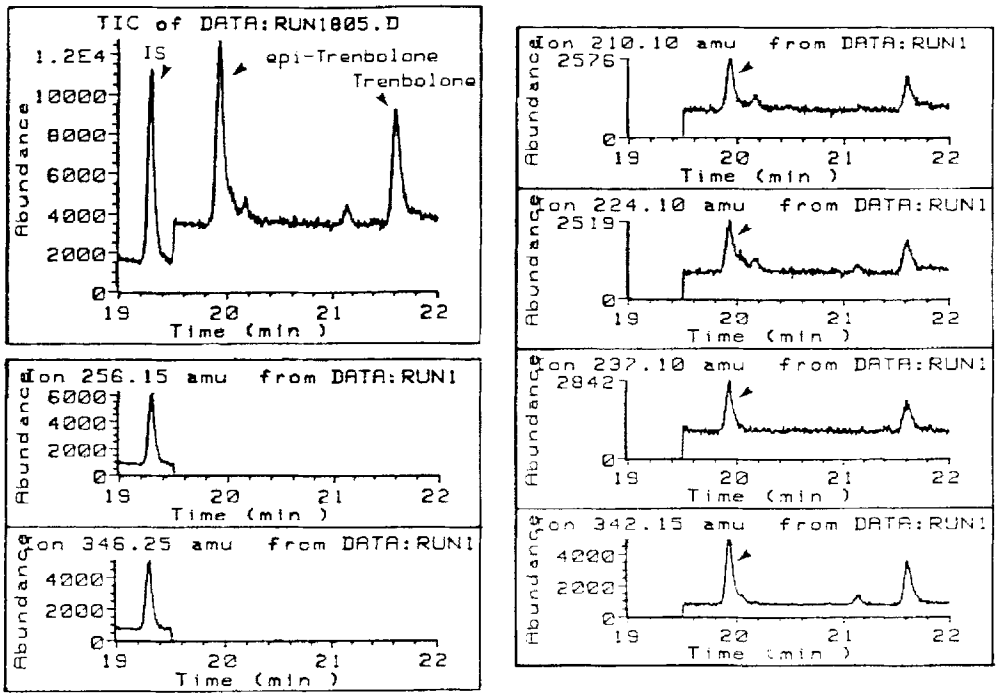

Fig. 5. Electron ionization GC–MS–SIM profiles of diluted bovine liver extract collected thirty days after 400 mg trenbolone acetate implantation in the ears of a young calf. GC–MS conditions were the same as in Fig. 4.

were obtained by high-resolution MS and are listed in Table I. Since the mass spectra of TBOH and epi-TBOH are identical, identification of these two isomers was confirmed by retention times and their corresponding selected ions (Fig. 4). Fig. 5 shows the selected-ion current chromatograms from a diluted liver extract thirty days after TBA implantation. The implantation was done in the ears of a calf with a dose of 400 mg TBA. By comparing the ion chromatograms of the standard (Fig. 4) and that of the liver extract (Fig. 5), the epi-TBOH can be clearly identified by its retention time and characteristic ions (m/z 210.10, 224.10, 237.10, 342.15). The chromatogram in Fig. 5 also shows that there is no detectable TBOH in the liver extract. This result confirms that epi-TBOH is the major metabolite of TBA in liver.

The choice of 19-nortestosterone as internal standard in this work allowed reliable GC–MS–SIM quantitative determination of both TBOH and epi-TBOH in liver and muscle. The practical limit of quantitation (LOQ) for each of these compounds was 1 ppb in both liver and muscle following preparative HPLC sample purification. Without preparative HPLC the GC–MS–SIM LOQ was 5 ppb in liver and 2 ppb in muscle.

When the incurred liver and muscle tissues were analyzed the levels for epi-TBOH in the liver were very high (110–130 ppb) and those in the muscle were about 2 ppb. The incurred liver samples from two different calves were diluted therefore with control liver (5 parts control and 1 part incurred liver) to produce

TABLE II

epi-TBOH CONTENT FOR INCURRED BOVINE MUSCLE AND DILUTED LIVER

Calf No.	Content (ppb)	Mean ± S.D. (ppb)
1	1.84	1.82 ± 0.14
	1.67	
	1.95	
3	1.93	1.99 ± 0.06
	1.99	
	2.06	

a liver homogenate containing about 2 ppb of epi-TBOH. The homogeneity of the diluted incurred liver was evaluated by GC–MS–SIM. This was done by analysing three randomly selected 5-g aliquots of diluted liver tissue homogenate plus a control liver tissue. A coefficient of variation of 14% between the samples of each individual liver was obtained. Triplicate quantitative determinations of homogenized liver aliquots from each calf produced relative standard deviations of 3.1 and 7.8%, respectively, thus demonstrating uniform distribution of the analyte in the diluted liver tissue.

The accuracy and precision of this method were evaluated by analysis of fortified liver and muscle tissues in addition to participation in a blind validation study adminstered by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS, Washington, DC, U.S.A.). This study consisted of five sets of tissues: three liver and two muscle. There were blind samples in each set which had been fortified with 0, 1, 2 and 4 ppb TBOH and epi-TBOH in addition to a blind incurred sample. Quantitative determinations using the procedure described herein were made from calibration curves prepared daily ($y = 0.702x + 0.056$; $r = 0.998$). The results of this validation study provided the correct quantitative identification by the analyst (S.H.H.) of the corresponding blind 0, 1, 2, 4 ppb and incurred levels of TBOH and epi-TBOH. In addition, triplicate analyses of two different incurred calf liver samples for epi-TBOH provided the results shown in Table II. These determinations were made on different days and represent typical precision and accuracy obtained from the procedure.

CONCLUSION

A capillary GC–MS–SIM analytical method is described for the qualitative and quantitative determination of TBOH and epi-TBOH in bovine muscle and liver. The major advantages of this method over TLC, HPLC and RIA methods are the sensitivity, selectivity and specificity. In addition, this method simulatneously combines screening and confirmation with the capability for quantitative determination of the target compounds.

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