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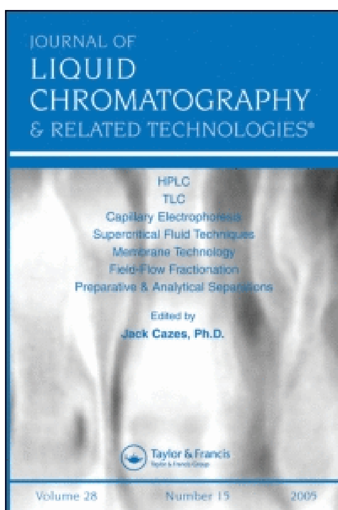

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DETERMINATION OF TRENBOLONE IN BOVINE LIVER AND MUSCLE BY HPLC AND LC/MS/MS

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ABSTRACT

A simple and sensitive detection method based on C18 reversed-phase HPLC with on-line UV-detection at 350 nm is presented for screening the xenobiotic steroid, trenbolone, and its major metabolite, epi-trenbolone, in bovine liver and muscle. On-line high performance liquid chromatography and tandem mass spectrometry (LC/MS/MS) methodology is used for confirmation of trenbolone residue in liver extracts. Trenbolone was extracted from bovine liver and muscle using three-phase liquid-liquid extraction which included a mixture of water, acetonitrile, dichloromethane, and hexane. The target compounds are extracted from tissue into the acetonitrile layer. The residue from this extraction was then subjected to solid phase extraction using C18 and silica disposable cartridges with methanol-water and benzene-acetone as eluents. Screening for trenbolone is then done by reversed-phase HPLC followed by LC/MS/MS confirmation under selected reaction monitoring (SRM) conditions. A

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structural analog of trenbolone, 19-nortestosterone, was chosen as the internal standard for quantification by HPLC. HPLC screening can be accomplished at the 0.5 ppb level in bovine liver and muscle extracts. Confirmation can be accomplished at the 1.0 ppb level by LC/MS/MS.

INTRODUCTION

Trenbolone is a synthetic anabolic steroid which has been exploited for improving the feed conversion rate and carcass characteristics of livestock intended for human consumption. The control of the illegal use of anabolics requires analytical methods which are sensitive and specific for the detection of trace amounts of these compounds in complex biological matrices. A few procedures for the determination of trenbolone in biological samples have appeared in the literature. Thin Layer Chromatography (TLC) was first used to determine trenbolone by Oehrle, et al. (5). Radio immunoassay (RIA) procedures for these compounds in bovine tissue and plasma were described by Hoffmann and Oettel (6), and Heitzman et al. (7). Bowman et al. reported HPLC and EC/GC determination of TBA and TBOH in animal chow (8). Jansen et al. used HPLC as the screening technique for the determination of trenbolone and its metabolites in bovine urine (9). These techniques have been limited either by poor specificity or sensitivity. A simple yet sensitive detection method is needed for determining the xenobiotic steroid, trenbolone, and its major metabolite, epi-trenbolone, in bovine liver and muscle tissue.

In cattle, studies of tenbolone residues (1-3) after administration of trenbolone acetate have reported that 17β-OH-trenbolone (TBOH) is the major compound in muscle and fat, while the epimer of trenbolone, 17α-OH-trenbolone (epi-TBOH), is the major metabolite in liver and kidney. However, the metabolism of trenbolone appears to be more complex. The following compounds were in fact found in bile at rather low levels: ($2,16\beta$), or (16α-hydroxy) of β-TBOH, ($1,6\beta$), (16β or 16α-hydroxy) of α-TBOH (4). Therefore, trenbolone and epi-trenbolone are the compounds of interest in this study.

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In this study, 350 nm UV wave length is used for the detection of trenbolone residue in bovine tissue extracts. At 350 nm, reduced interference is expected from other xenobiotic anabolics or endogenous androgens (10). Due to the unique absorbance of trenbolone at 350 nm, 0.5 ppb of the analyte in both bovine liver and muscle can be detected by HPLC. A structural analog of trenbolone, 19-nortestosterone was chosen as the internal standard for the quantification, but the sensitivity of 19-nortestosterone is less than that of trenbolone at 350 nm UV wavelength.

Although great strides have been made towards improving chromatographic resolution, it is not an uncommon situation to have co-eluting components during the analysis of complex biological samples. The mixture analysis capability of tandem mass spectrometry (MS/MS) should complement HPLC separation of such samples. We have, therefore, combined the attributes of HPLC and MS/MS for the rapid separation and identification of trenbolone and its metabolites in bovine tissue residues.

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EXPERIMENTAL

CHEMICALS AND EQUIPMENT

Chemicals

Trenbolone and its tritiated analogs were obtained from Roussel-UCLAF, France. 19-Nortestosterone was purchased from Sigma Chemical Co. (St. Louis, MO). 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 Company (Fair Lawn, NJ). Acetonitrile and methanol (HPLC grade), C18 and Silica solid phase extraction cartridges were obtained from J.T. Baker (Phillipsburg, NJ). The cartridges were used on a 12 port vacuum manifold available from Supelco. Inc. (Bellefonte, PA).

Equipment

A Beckman Model 660 (Irvine, CA) liquid scintillation counter was used for the radioisotop tracer studies. The high performance liquid chromatography system consisted of two Waters Model 510 pumps, driven by a Model 660 solvent programmer (Waters, Inc., Milford, MA). The UV detector was a variable wavelength Kratos Model 783 UV detector equipped with an 8 uL cell, and set at a wavelength of 350 nm. A Rheodyne (Cotati, CA) Model 7125 injector equipped with a 20 uL loop was used for sample introduction.

A Sciex TAGA 6000E triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionization (API) ion source and a heated pneumatic nebulizer (Sciex, Inc.) was used for LC/MS/MS confirmation.

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EXTRACTION PROCEDURE

The overall procedure can be divided into 5 steps: a) enzymatic hydrolysis of the tissue, b) three-phase liquid-liquid extraction of the tissue homogenate, c) liquid-solid extraction by C18 and Si cartridges, d) screening and quantification by HPLC and e) final confirmation by LC/MS/MS. Radioactive isotopes were used for the recovery determination of trenbolone from bovine liver and muscle tissue extraction.

Sample Preparation

(a) Hydrolysis: A suitable amount of standard (2.5-250 ng) was spiked into 5 g of homogenized bovine tissue (liver or muscle 0.5-50 ppb). The tissue samples were then hydrolyzed enzymatically (glucuronidase/sulfatase) overnight at 37°C and extracted with 20 mL of acetonitrile. The pH of the supernantant was then adjusted to 13 with 6N sodium hydroxide.

(b) Liquid-Liquid Extraction: Hexane (8 mL) and dichloromethane (2 mL) were added to the above aqueous acetonitrile extract to generate a

three-phase liquid-liquid system. The middle 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 combined with the previous acetonitrile layer. Because of the remaining dichloromethane in the acetonitrile layer, the latter can then be washed with water and separated. The final extract was then concentrated to dryness under a gentle stream of nitrogen at 60°C.

(c) Liquid-Solid Extraction: After the class separation by three-phase liquid-liquid extraction, the residue was dissolved in water (2 mL) with the aid of ultra sonication. This aqueous sample was then applied to a pre-conditioned 500 mg C18 cartridge (pre-washed with methanol (3 mL), and then conditioned with distilled (3 mL) water, washed with 40/60 methanol/water (2 mL), and eluted with 80/20 methanol/water (2.5 mL). The eluate was concentrated to dryness under a gentle stream of nitrogen at 60°C. Following the C18 separation, the residue was dissolved in benzene (2 mL) with the aid of ultra sonication and applied to a 500 mg silica cartridge. This cartridge was washed with 10/90 acetone/benzene (1.5 mL), eluted with 20/80 acetone/benzene (2.5 mL), and the eluate concentrated to dryness under nitrogen at 60°C.

(d) HPLC Screening and Quantification: After solid phase extraction, the internal standard was added to the sample and analysed by C18 reversed-phase HPLC under isocratic conditions (54/46 methanol/water) at 1 mL/min flow rate. The column used was a C18 Perkin-Elmer cartridge column (5 um particles, 4.6 mm id. x 15 cm) with a UV detection wavelength of 350 nm.

(e) LC/MS/MS Confirmation: For LC/MS/MS confirmation, the LC column used was a Perkin Elmer 3 um C18 cartridge (4.6 mm x 7.5 cm), and the

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mobile phase was 70/30 methanol/0.1 M NH_4OAC maintained at a flow rate of 1 mL/min, with the total eluent introduced into the ion source via the heated pneumatic nebulizer LC/MS interface. Collision induced dissociation (CID) in the central quadrupole collision cell was performed at a collision energy of 70 eV and a collision gas thickness of 2×10^{14} atoms/cm^2 (2.0×10^{-5} torr).

RESULTS AND DISCUSSION

Three-phase liquid-liquid extraction has proven to be an excellent extraction method for diethylstilbestrol (DES) and zeranol from tissue (11). The related approach described here also provides a good recovery (85%) of radioactive trenbolone from 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 will remain in its neutral form and stay in the acetonitrile layer. Polar, ionic compounds will be extracted into the aqueous layer, while low polarity compounds will be extracted into the hexane layer. Compounds of intermediate polarity, such as DES, Zeranol, dexamethasone, melengestrol and trenbolone will be extracted into the acetonitrile layer. The overall recoveries of trenbolone, epi-trenbolone and 19-nortestosterone from bovine tissue extract (liver and muscle) was approximately 53% as determined by radioactive tracer analysis of the corresponding tritiated compounds.

Due to significant matrix interference, a combination of different solid phase extraction methodology was required. By applying the partition (reversed-phase) and adsorption (normal-phase) character of C18 and Si, these adsorbants were chosen for the solid phase extraction of the sample. After C18 and Si solid phase extraction, acceptable sensitivity (1 ng of trenbolone and epi-trenbolone) and base-line separation of these compounds could be obtained at 350 nm UV wavelength.

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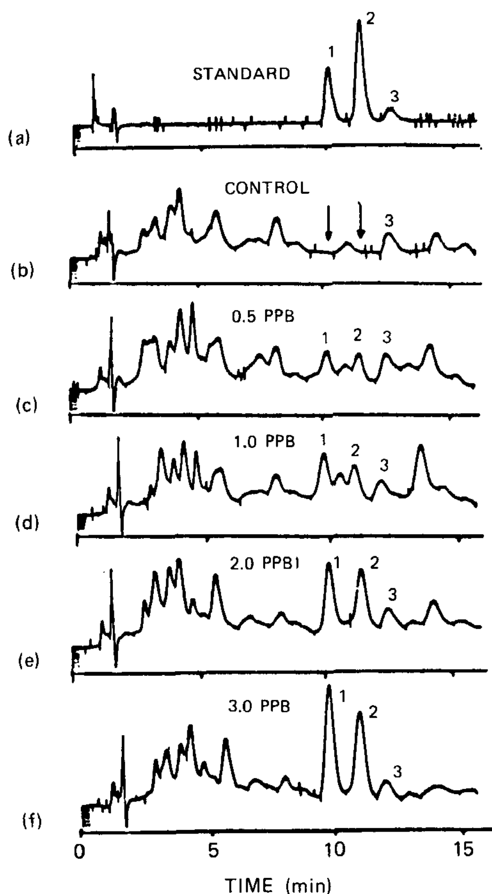

Figure 1: HPLC Chromatograms of bovine liver extract (a) Standard, ((1) Trenbolone 2 ng, (2) epi-Trenbolone 2 ng, (3) 19-nortestosterone 1 ug.), (b) Control, (c) 0.5 ppb, (d) 1.0 ppb, (e) 2.0 ppb, (f) 3.0 ppb of trenbolone and epi-trenbolone.

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Figure 1 and Figure 2 are respective HPLC chromatograms of the liver and muscle extracts. Although there is more interference in the liver extract than in the muscle, 0.5 ppb of the analyte can be readily detected in both tissues. By decreasing the methanol content of the mobile phase, base line separation of

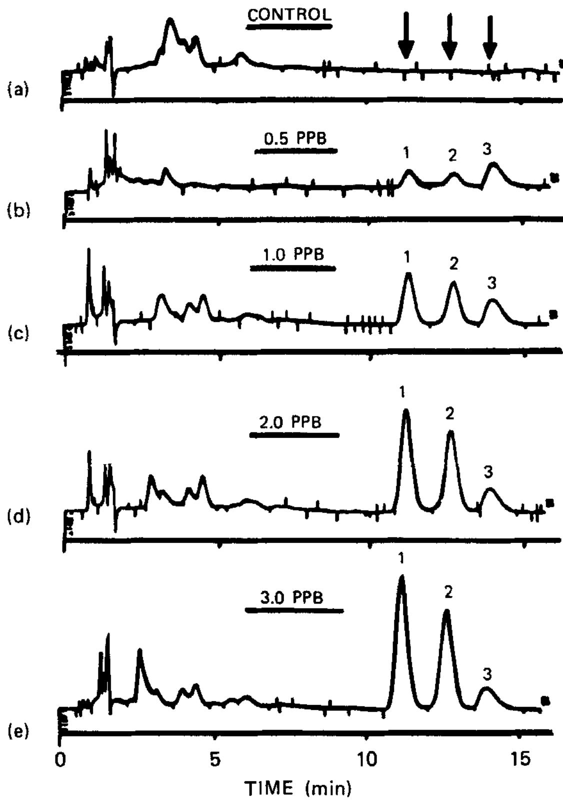

Figure 2. HPLC chromatograms of bovine muscle extract (a) Control, ((1) Trenbolone, 2 ng, (2) epi-Trenbolone, 2 ng, (3) 19-nortestosterone, 1 ug.), (b) 0.5 ppb, (c) 1.0 ppb, (d) 2.0 ppb, (e) 3.0 ppb.

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the analyte and the interference peak in the liver extract can be obtained but the analysis time is increased accordingly. Figure 3 shows the HPLC chromatogram of the liver extract from a 200 mg trenbolone acetate-implanted cow. Figure 3a shows the UV HPLC chromatogram for standard trenbolone, epi-trenbolone at the 2 ng level and 1 ug internal standard, Figure 3b is the control tissue without analytes, while Figure 3c is the tissue 15 days after implantation. The

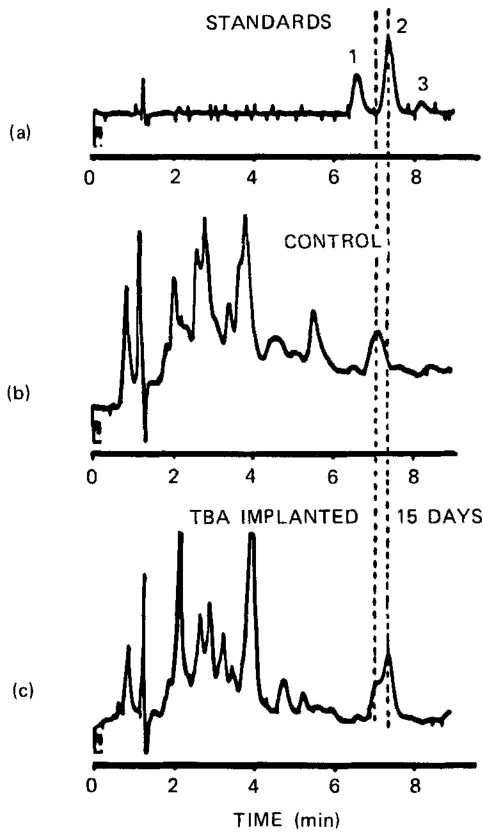

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Figure 3. HPLC chromatograms of 200 mg trenbolone acetate implanted bovine liver (a) standard compounds ((1) trenbolone, 2 ng, (2) epi-trenbolone, 2 ng, (3) 19-nortestosterone, 1 ug.), (b) control liver, (c) 15 days after implantation.

mobile phase was 58% methanol and 42% water maintained at a flow rate of 1 mL/min. The analyte can be clearly detected for screening purposes. The corresponding calibration curves of the analytes determined by HPLC in the range of 0.5 ppb to 50 ppb in liver and muscle extracts shows a linear correlation coefficients of 0.998 for the ratio of analytes to internal standard.

Most methods for on-line LC/MS produce abundant molecular ion species resulting from proton addition or abstraction processes (12-15). Structural information resulting from fragmentation of the molecular ion is generally lacking due to the mild ionization and low internal energy of the ions. However, combination of mild ionization and liquid sample introduction is well suited for the technique of MS/MS. Figure 4a shows the positive ion chemical ionization spectrum for trenbolone in the LC/MS mode using a heated pneumatic nebulizer interface. The spectrum is dominated by the $(M+1)^+$ ion due to the soft ionization process in the atmospheric pressure chemical ionization (APCI) ion source. Since the spectra of trenbolone and epi-trenbolone are identical, the identification of these two compounds is based upon the spectra and their different HPLC chromatographic retention times. In the LC/MS mode used for Figure 4a quadrupole 1 was operated in the full-scan mode beginning at m/z 50 with quadrupole 3 operating in the rf-only mode.

LC/MS/MS aprovides the opportunity to focus only ions of interest into the collision cell of the tandem mass spectrometer. Thus, the LC/MS/MS experiment allows us to select an ion within a given HPLC peak in the presence of ions characteristic of other compounds. This final mass spectrometric "separation" is followed by a daughter ion scan of the fragment ions formed by the parent ion-molecule collision in the collision cell. Figure 4b shows the full-scan daughter ion spectrum of the $(M+1)^+$ parent ion for trenbolone at a collision energy of 70 eV with argon as the collision gas. In addition to separation, increased specificity over the full mass spectrum from APCI is achieved with this full-scan daughter ion spectrum.

Figure 5 shows the LC/MS/MS selected reaction monitoring (SRM) ion chromatograms for consecutive injections of standard mixtures of dexamethasone, trenbolone, and melengestrol monitored at their parent ions, a control liver extract, followed by fortified liver extracts ranging from 1 ppb to 5 ppb level for each compound. By comparing the SRM ion chromatograms, retention times and

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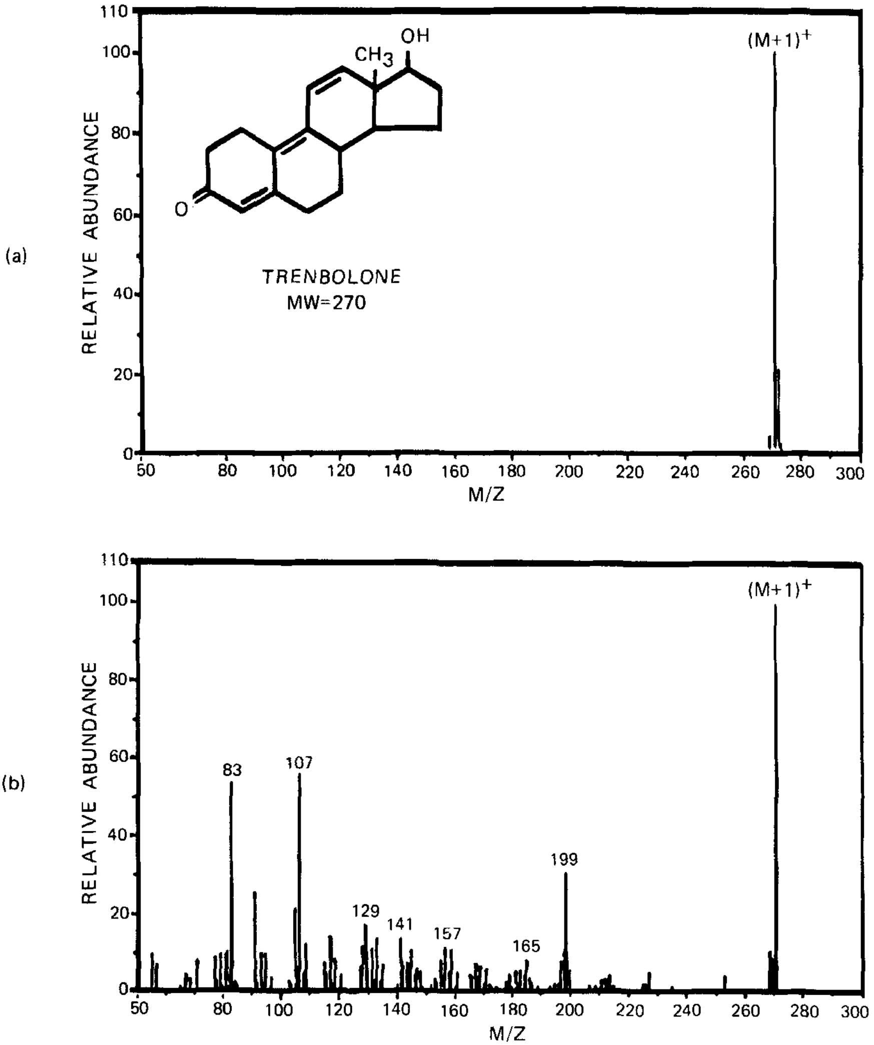

Figure 4. (a) Atmospheric pressure chemical ionization (APCI) LC/MS mass spectrum of trenbolone using a heated pneumatic nebulizer. (b) Collisionally Induced Dissociation mass spectrum of trenbolone: collision gas - argon; collision energy - 70 eV.

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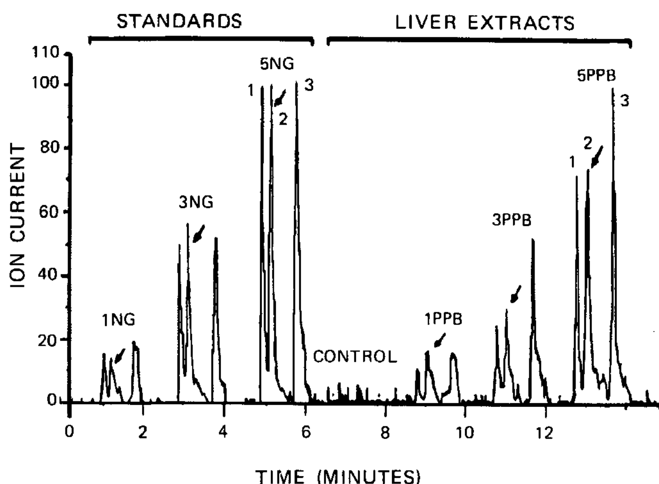

Figure 5. LC/MS/MS Selected reaction monitoring ion chromatogram of standard test solutes, Control liver extract, and liver extract containing 1 ppb to 5 ppb of analytes. Analytes: (1) Dexamethasone, (2) Trenbolone, (3) Melengestrol.

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selected ion ratios of the control and sample, the target analytes can be identified at the 1 ppb level in the liver extract.

CONCLUSIONS

An analytical method for screening and quantification of trenbolone and epi-trenbolone in bovine liver and muscle by HPLC and further confirmation by LC/MS/MS has been described. The extraction and clean-up procedure demonstrates adequate recovery (50-60%) of the analytes in fortified bovine liver and muscle samples. The limit of detection for HPLC screening and quantification of the analytes in liver and muscle is 0.5 ppb. Final confirmation by LC/MS/MS provides the specificity for the determination of the targeted analytes at 1 ppb level. It is anticipated that this method will be applicable to the determination of other anabolic agents of regulatory concern.

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