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Determination of 17α-Methyltestosterone in Muscle Tissues of Tilapia, Rainbow Trout, and Salmon Using Liquid Chromatography−Tandem Mass Spectrometry

PAK-SIN CHU,* MAYDA LOPEZ, STAN SERFLING, CHARLIE GIESEKER, AND RENATE REIMSCHUESSEL

Center for Veterinary Medicine, U.S. Food and Drug Administration, 8401 Muirkirk Road, Laurel, Maryland 20708

An analytical method was developed to quantitate and confirm the presence of 17α-methyltestosterone in the muscles of tilapia, rainbow trout, and salmon. The method employed two liquid−liquid partitioning steps and two solid-phase extraction columns for sample cleanup. The final extracts were analyzed on an isocratic reverse-phase liquid chromatography−tandem mass spectrometry system with atmospheric-pressure chemical ionization in the positive ion mode. The method was validated at levels from 0.40 to 1.6 ng/g, with MT-d_3 used as an internal standard. The accuracy was between 100% and 110%, and coefficients of variation of <10% were obtained for all three fish species. Muscle tissues from dosed fish were also assayed to demonstrate the effectiveness of the method for recovering the parent drug.

KEYWORDS: Methyltestosterone; tilapia; salmon; trout; LC−MS/MS; method

INTRODUCTION

The synthetic androgen 17α-methyltestosterone (MT, Figure 1) is commonly used in newly hatched tilapia fry for sex reversal (*1*−*3*). In tilapia aquaculture, an all-male population is desired because males grow faster and larger than do females. In the United States, MT is not approved for use in fish. Its use in tilapia for sex reversal, however, is permitted under a compassionate Investigational New Animal Drug Application (INAD). Under field conditions, MT is administered orally to newly hatched tilapia fry via the feed for 28 days. When the proper withdrawal time is observed, residues are expected to be diluted through growth of the fish to market size, thus posing no threats to human health at the time of consumption. Failure to observe the proper withdrawal time could cause residues to be found in edible tissues. Furthermore, potential use of MT as a growth promoter poses regulatory concerns. Analytical methods are therefore needed to monitor MT residues in fish.

A number of analytical procedures have been described for the detection and quantitation of methyltestosterone residues in tissue, urine, feed, and hair (*4*−*9*). Among them are methods based on radioimmunoassay (*5*), chemiluminescence immunoassay (*7*, *10*), gas chromatography−mass spectrometry (GC−MS), and liquid chromatography (LC). Stolker et al. (*11*) described a method for determining MT at low parts per billion in bovine urine using supercritical fluid extraction. Daeseleire et al. (*5*) developed an HPLC method combined with radioimmunoassay for the screening of MT residues in meat samples.

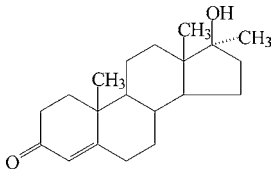

Figure 1. Structure of MT.

This method, however, is labor-intensive and requires HPLC fractionation and the handling of radioactive materials. A GC−MS method was reported by Casademont et al. (*6*) for the determination of 12 anabolic agents, including MT, in calf urine after derivatization into heptaflurobutyryl derivatives. More recent methods involving the detection of anabolic hormones in hair using GC−MS/MS have also been reported (*9*, *12*).

Despite the wealth of methods developed for urine, hair, and meat of terrestrial animals, few have been developed for aquatic species. A method was reported by Cravedi and Delous (*13*) for the determination of two MT metabolites, 5α-androstane-17α-methyl-3α-17β-diol and 5β-androstane-17α-methyl-3α-17β-diol, in trout using an immobilized enzyme reactor. The reported limit of quantitation was 1 μg/kg, and the method was able to detect MT residues up to 3 days post-dose. Goudie (*14*) described a method for determining parent MT in tilapia at 1 μg/g. This level, however, is too high for practical use in monitoring and surveillance purposes. In light of previous depletion studies on MT (*15*, *16*), a sensitivity of <1 ng/g is needed if the parent MT is selected as the marker for monitoring residues in fish. This article describes a sensitive LC−MS/MS

* To whom correspondence should be addressed. Phone: 301-210-4583. Fax: 301-210-4653. E-mail: Pak.Chu@FDA.GOV.

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method capable of determining MT in three fish species at levels below 1 ng/g, a level sensitive enough to address regulatory concerns.

MATERIALS AND METHODS

Reagents and Supplies. LC-grade water used in preparing solutions was purified in-house with a Milli-Q Plus water system. Methanol (MeOH), acetonitrile (ACN), hexane, and ethyl acetate (EtOAc) were of HPLC grade (Burdick & Jackson, Muskegon, MI). Sodium chloride (Mallinckrodt, Paris, KY) and formic acid (FA, Sigma-Aldrich, St. Louis, MO) were of reagent grade. MT was obtained from Sigma-Aldrich, and MT-d_3 was obtained from RIVM (Rijksinstituut voor Volksgezondheid en Milieu, Dutch National Institute for Public Health and Environment, Bilthoven, The Netherlands). BondElut strong anion exchange (SAX) (3 cm^3, 500 mg) solid-phase extraction (SPE) columns were obtained from Varian (Walnut Creek, CA), and florisil SPE columns were obtained from Waters Corp. (Milford, MA). A concentrated sodium chloride solution was prepared by dissolving 50 g of sodium chloride in 200 mL of water. The HPLC mobile phase consisted of ACN/0.1% aqueous FA (60/40, v/v). The preparation of 0.1% aqueous FA was as follows: One milliliter of FA was measured into a 1-L volumetric flask. Water was added to the mark. The solution was stirred and filtered through a 0.2-μm nylon filter. The aqueous and organic components of the mobile phase were either premixed or mixed by the HPLC instrument.

Standard Solutions. *Primary Stock Solution (100 μg/mL).* Approximately 10 mg of MT was accurately weighed to the nearest 0.1 mg into a 100-mL glass volumetric flask. Methanol was added to dissolve the solid. Where applicable, the concentration was corrected for purity. This stock standard solution was stored at −10 °C or below.

Intermediate Stock Solution (2 μg/mL). Two milliliters of the primary stock solution (100 μg/mL) was pipetted into a 100-mL volumetric flask, brought to volume with MeOH, and mixed well. The volume pipetted was adjusted for the concentration difference of the primary stock solution from the nominal 100 μg/mL. This solution was stored at −10 °C or below.

Final Stock Solution (0.2 μg/mL). Five milliliters of the intermediate stock solution (2 μg/mL) was pipetted into a 50-mL volumetric flask, brought to volume with MeOH/water (40/60, v/v), and mixed well. This solution was stored at −10 °C or below.

Working MT Standard Solutions. Working MT standard solutions at 0.01, 0.02, 0.04, 0.08, and 0.1 ng/μL were prepared by diluting the final stock solution (0.2 μg/mL) or its diluted solutions with MeOH/water (40/60, v/v). These solutions were stored at −10 °C or below.

Primary MT-d_3 Internal Standard Stock Solution (2 ng/μL). This standard was prepared in accordance with the manufacturer's instructions. One milliliter of ethanol was pipetted into an ampule containing 0.1 mg of MT-d_3. The ampule was vortex mixed for at least 1 min and sonicated for at least 5 min. Its contents were quantitatively transferred to a 50-mL volumetric flask, and the volume was brought to the mark with MeOH. This solution was stored at −10 °C or below.

Intermediate MT-d_3 Internal Standard Solution (0.1 ng/μL). Five milliliters of the primary MT-d_3 internal standard stock solution (2 ng/μL) was measured into a 100-mL volumetric flask and brought to the mark with MeOH/water (40/60, v/v). This standard was stored at −10 °C or below.

Calibration Standards. A four-point calibration curve of MT was prepared at the following concentrations: 0.2, 0.4, 0.8, and 2.0 ng/g equivalents (ppb) by adding 100 μL of the appropriate working MT standard solutions (0.01, 0.02, 0.04, and 0.1 ng/uL) to 50 μL of the intermediate MT-d_3 internal standard solution (0.1 ng/μL) and 50 μL of 40/60 MeOH/water. Accordingly, each calibration standard contained a fixed level (1 ng/g) of the internal standard, MT-d_3. In the preparation of these standards, the units ng/g are in reference to a 5-g sample and are based on a final volume of 200 μL for the sample extracts.

Fish Dosing. Control fish fillets were purchased from local grocery stores or obtained from our Center for Veterinary Medicine aquaculture facility. Dosing of fish was performed at our aquaculture facility. Tilapia (*Oreochromis* species, not sex reversed) were obtained from Aquasafra Inc. (Bradenton, FL) as 1-g fry and raised in a recirculating system consisting of two 500-gal tanks at a temperature of 20−24 °C to a suitable size (400−700 g) for dosing. Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Maryland Department of Natural Resources as 100-g juveniles and raised in a recirculating system consisting of two 500-gal tanks at a temperature of 14−18 °C to a suitable size (400−700 g) for dosing. Atlantic salmon (*Salmo salar*) were obtained from the University of Maine as 40-g juveniles and raised in a recirculating system consisting of four 500-gal tanks at a temperature of 14−18 °C to a suitable size (900−1300 g) for dosing. Fish were fed gel food at a dose of 30 mg of MT/kg once a day for four consecutive days. Tilapia were sacrificed at 1, 2, 3, 7, 14, 21, 28, and 35 days after the last dose. Rainbow trout were sacrificed at 1, 2, 3, 5, 7, 14, and 21 days after the last dose. Salmon were sacrificed at 1, 3, 5, 7, 14, and 21 days after the last dose. Typically, one fish was used for each collection time. Fillets with adhering skins were removed and homogenized with dry ice using blenders. After homogenization, samples were temporarily stored in a −10 °C freezer for 2−4 days to sublime the CO_2. Thereafter, they were stored at −80 °C until analysis.

Incurred Tissues for Method Validation. Two levels of incurred residues at approximately the target concentration were prepared for method validation as follows: For tilapia, incurred level 1 was prepared by mixing 30 g of the day-3 dosed tissues with 30 g of control; incurred level 2 was the day-3 dosed tissues. For rainbow trout, incurred level 1 was the day-5 dosed tissue; incurred level 2 was prepared by mixing 15 g of the day-2 dosed tissue with 45 g of control. For salmon, incurred level 1 was prepared by mixing 15 g of the day-7 dosed tissue with 45 g of control, and incurred level 2 was prepared by mixing 30 g of the day-7 dosed tissue with 30 g of control.

Extraction Procedure. Homogenized muscle tissue (5.0 ± 0.2 g) was weighed into a 50-mL polypropylene centrifuge tube. Fortification of samples was performed at this step. To each sample was added 5 mL of ACN, and the mixture was briefly vortex mixed to disperse the tissue. Samples were then placed on a multitube vortexer (VWR, Bridgeport, NJ) for 5 min and then centrifuged at 3800 rpm (∼3000*g*) for 5 min at 15 °C. After centrifugation, the supernatant (ACN) was decanted into a 15-mL glass centrifuge tube. The ACN extraction step was repeated once. Chunks of tissue were broken up with a spatula before the samples were placed into the multitube vortexer. The ACN fractions were combined and evaporated to <0.5 mL in a TurboVap LV evaporator (Zymark Corp., Hopkinton, MA) set at 50 °C.

Liquid−Liquid Partitioning. Two milliliters of concentrated NaCl solution was added to each sample, which was then subjected to liquid−liquid partitioning twice with 5 mL of 100% ethyl acetate. The ethyl acetate fractions were combined and washed with 2 mL of water. The bottom aqueous layer was discarded. The top ethyl acetate was evaporated to dryness in a TurboVap LV evaporator set at 50 °C. After evaporation, 1 mL of MeOH was added to each sample, and the mixture was vortex mixed for 15 s and sonicated for 5 min to dissolve the residue. Then, 1 mL of water was added to each sample, which was then vortex mixed. A large volume of white solid always formed as soon as water was added to the methanolic extract.

SAX Cleanup. SAX columns were conditioned sequentially with 2 mL of MeOH and 2 mL of 50% aqueous MeOH. Prior to sample loading, glass centrifuge tubes were in place for immediate collection. As soon as the sample was loaded, the eluent was captured into a 15-mL glass centrifuge tube. The flow rate was maintained at ∼2 drops/s. The sample tube was then rinsed with 2 mL of 50% aqueous MeOH. The rinse was poured into the respective SPE column. After sample elution, the SPE column was vacuum-dried. The eluent was evaporated to ∼1.8 mL in a TurboVap LV evaporator set at 50 °C. It was found to be critical that MeOH was completely evaporated prior to hexane partitioning; otherwise, MT could be lost in the aqueous layer.

Liquid−Liquid Partitioning. Each sample was partitioned three times with 3 mL of hexane. The hexane fractions were combined and further cleaned on florisil SPE columns.

Florisil SPE Cleanup. The combined hexane fractions from the previous step were loaded onto a florisil column, which had been conditioned sequentially with 4 mL of ethyl acetate and 4 mL of hexane. The sample was allowed to drip at a rate of ∼2 drops/s. The sample tube was rinsed with 2 mL of ethyl acetate/hexane (10/90, v/v). After brief vortex mixing, the rinse was added to the respective SPE column. Both the throughput and rinse were discarded. The analyte was eluted

from the column with two aliquots of 4 mL of ethyl acetate/hexane (60/40, v/v) into a centrifuge glass tube. The columns should not be vacuum-dried. The eluate was evaporated almost to dryness in a TurboVap LV evaporator set at 50 °C. To each sample tube was added 1 mL of MeOH, and the mixture was vortex mixed at high speed for 15 s. This was to prevent analyte loss on the side of the tube. The samples were then placed in the TurboVap LV evaporator again and continued with evaporation to dryness. The residue was reconstituted in 200 μL of a 40% aqueous MeOH solution. The sample was vortex mixed, sonicated for 5 min, and vortex mixed again. Each sample was filtered through a Gelman PTFE filter (13 mm, 0.2 μm) into an autosampler vial with a 300-μL insert.

Liquid Chromatographic Conditions. The LC system consisted of a Perkin-Elmer Series 200 Micro pump and a Perkin-Elmer ISS-200 autosampler equipped with a 200-μL loop (Norwalk, CT). Extracts were analyzed for MT using the following isocratic LC conditions: mobile phase, ACN/0.1% aqueous FA (60/40, v/v); flow rate, 0.2 mL/min; and run time, 15 min. The column was a C_8, Prodigy 5 μ, 150 × 2.0 mm, column with a guard column of the same packing (C_8, 4 mm × 2.0 mm i.d., Phenomenex, Torrance, CA). An optional precolumn filter (Upchurch, Oak Habour, WA) was installed between the autosampler and the guard column. The injection volume was 20 μL. A typical injection sequence was as follows: a solvent blank, the standards, the sample set, and the standards again to bracket the samples.

Mass Spectrometry. An Applied Biosystems (Foster City, CA) Sciex API 2000 triple-quadrupole mass spectrometer with an atmospheric-pressure chemical ionization (APCI) interface in the positive ion mode was used. The APCI interface operated at 5500 V and a temperature of 450 °C. The $[MT + H]^+$ ion at *m*/*z* 303 and the $[MT\text{-}d_3 + H]^+$ ion at *m*/*z* 306 were selected as the precursor ions for collision-induced dissociation (CID). The following product ions were identified for selected reaction monitoring (SRM) analysis: MT, *m*/*z* 303 → 109, 97; MT-d_3, *m*/*z* 306 → 109, 97. The dwell time for each monitored transition was 150−200 ms. The collision energies were 41 and 39 V for the MT transitions *m*/*z* 303 → 109 and *m*/*z* 303 → 97, respectively. The first quadrupole was set at unit resolution, and the third quadrupole set at low resolution. For quantitation, peak areas were computed by summing the *m*/*z* 97 and 109 product ions of the respective analytes using Analyst version 1.3 from PE-Sciex. For confirmation, the ion ratio between *m*/*z* 97 and 109 was determined.

Additional experiments were performed on a Waters Quattro Micro triple-quadrupole mass spectrometer (Milford, MA) with an electrospray-ionization (ESI) interface in the positive ion mode. The capillary was set at 0.81 kV and the cone at 32 V. The source temperature was set at 125 °C and the desolvation temperature at 350 °C. The cone gas and desolvation gas flow rates were 100 and 600 L/h, respectively. The collision energies were 27 and 23 V for the MT transitions *m*/*z* 303 → 109 and *m*/*z* 303 → 97, respectively.

External Standard Calibration Method. A four-point calibration curve of peak area versus concentration (ng/g) of MT analyte was plotted. The sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y is the peak area and X is the concentration of standard in ng/g. Correlation coefficients for each of the calibration curves were routinely >0.99. A mean detector response was used to determine recovery of MT-d_3. When unknown or incurred samples were assayed, a negative and a fortified control were always processed along with each set for quality control.

Internal Standard Calibration Method. The internal standard calibration curve was constructed by plotting the area ratio of MT/MT-d_3 versus the concentration. The sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where Y is the ratio of the MT peak area to the IS peak area and X is the concentration of standard in ng/g. Correlation coefficients for each of the calibration curves were routinely >0.99.

RESULTS

The method was validated by fortifying control muscle tissues of tilapia, rainbow trout, and salmon with MT at levels from 0.4 to 1.6 ng/g. A fixed level of MT-d_3 (1 ng/g) was added to each sample as an internal standard. The results were calculated using both external and internal standard calibration methods. Results calculated with the external standard calibration method are presented in Tables 1−3 for tilapia, rainbow trout, and salmon, respectively. The average recoveries of MT and MT-d_3 were 84% and 79% from tilapia, 78% and 76% from salmon, 78% and 75% from rainbow trout, respectively. Typical chromatograms of control, fortified control, and incurred fish for the three species are shown in Figures 2−4. In accordance with our method development guideline, the method was tested on tissues derived from treated animals. The MT levels found in muscles of dosed fish over 35 days are reported in Table 4. In tilapia and rainbow trout tissues, highest levels of MT were found at day 1 post-dose. A noticeably high level (61 ng/g) of MT in salmon tissues at day 3 post-dose might be attributed to fish-to-fish variation. This incursion study was intended for range finding for method validation and not intended as a depletion study. For method validation, two levels of incurred residues were prepared for each fish species and assayed in accordance with the test method. Their results are listed in Table 5, with coefficients of variation (CVs) of <10%. For all three species, the parent drug was clearly present in the chromatograms of treated fish, thus demonstrating the effectiveness of the method in recovering MT from biologically incurred tissues. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as defined in the U.S. Pharmacopeia-National Formulary (*17*) and were estimated to be 0.04 and 0.09 ng/g for MT, respectively.

For comparison, results were also calculated using the internal standard calibration method. This quantitation method corrected for procedural recovery loss and compensated for mass spectrometer ionization differences due to matrix effects. As Tables 1−3 show, the method accuracies among the three species are

Table 1. Method Validation of MT for Tilapia: Fortified Samples[a]

	external standard		internal standard	
fortification level (ng/g)	recovery (%)	CV (%)	accuracy (%)	CV (%)
0.4	82	6	104	4
0.8	87	5	109	5
1.6	83	2	106	3

[a] $N = 6$.

Table 2. Method Validation of MT for Rainbow Trout: Fortified Samples[a]

	external standard		internal standard	
fortification level (ng/g)	recovery (%)	CV (%)	accuracy (%)	CV (%)
0.4	79	4	107	2
0.8	80	5	105	4
1.6	77	4	103	5

[a] $N = 6$.

Table 3. Method Validation of MT for Salmon: Fortified Samples[a]

	external standard		internal standard	
fortification level (ng/g)	recovery (%)	CV (%)	accuracy (%)	CV (%)
0.4	81	6	107	1
0.8	79	5	106	5
1.6	74	5	102	4

[a] $N = 6$.

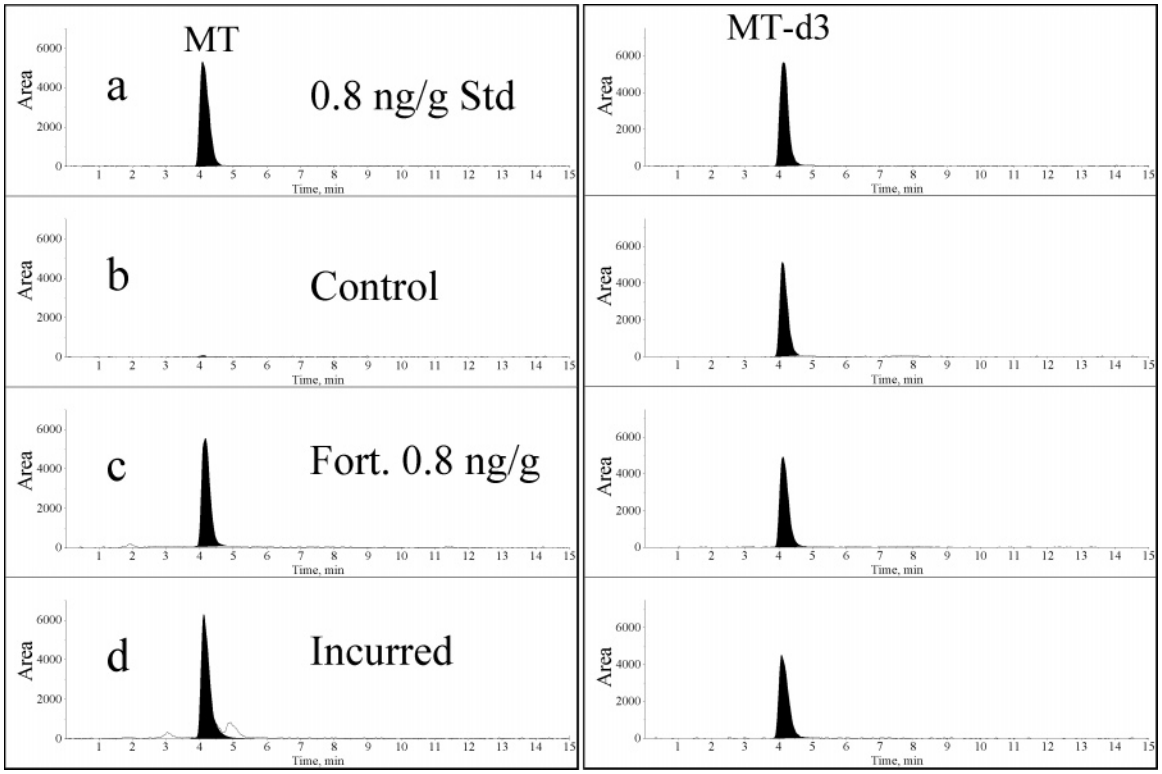

Figure 2. Reconstructed ion chromatograms of MT extracted from tilapia: (a) 0.8 ng/g standard with 1 ng/g MT-d_3, **(b)** control fortified with 1 ng/g MT-d_3, **(c)** control fortified with 0.8 ng/g MT and 1 ng/g MT-d_3, **(d)** incurred MT fortified with 1 ng/g MT-d_3.

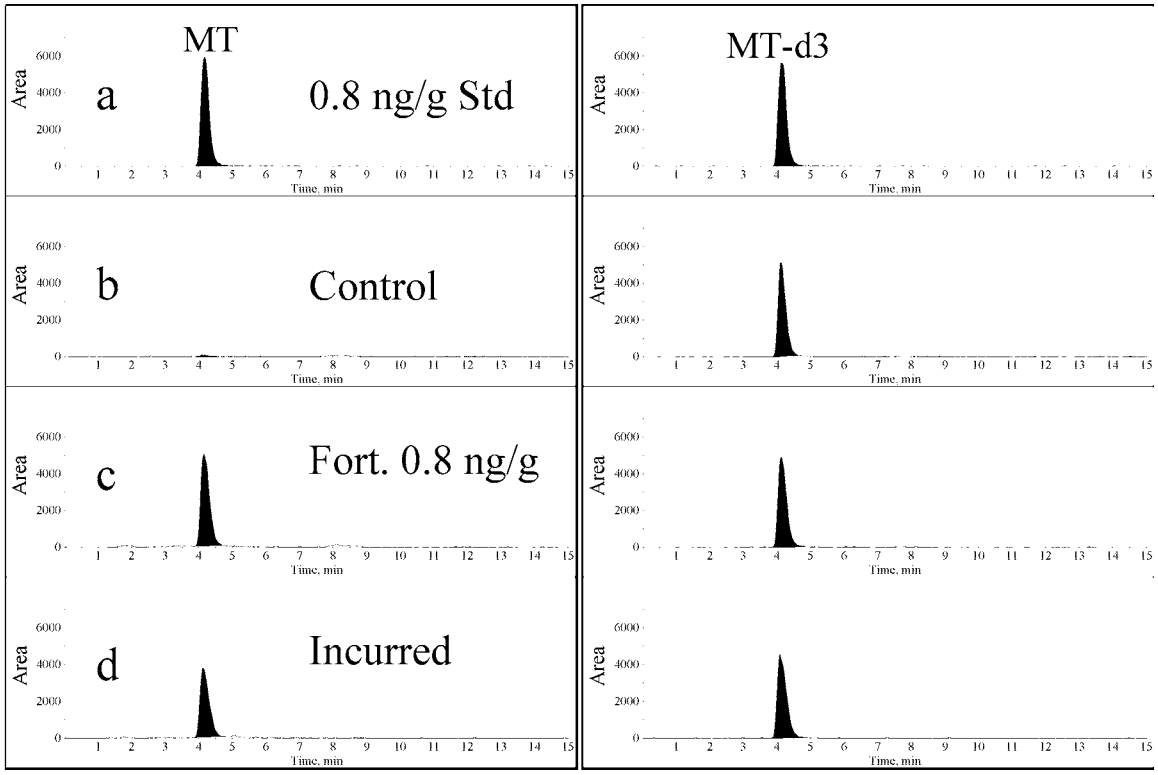

Figure 3. Reconstructed ion chromatograms of MT extracted from rainbow trout: (a) 0.8 ng/g standard with 1 ng/g MT-d_3, **(b)** control fortified with 1 ng/g MT-d_3, **(c)** control fortified with 0.8 ng/g MT and 1 ng/g MT-d_3, **(d)** incurred MT fortified with 1 ng/g MT-d_3.

between 100% and 110% with CVs of less than 10%. An accuracy of greater than 100% is linked to the slight difference (3−5%) in recovery between MT and MT-d_3.

For confirmation, the analyte retention time and its ratio between the *m*/*z* 97 and 109 ions were computed and compared to those of standards. A sample is confirmed to be positive if its retention time agrees within 2% and the ion ratio agrees arithmetically within 10% compared to standards. Each confirmation ion should have a signal-to-noise ratio of at least 3:1. With these criteria (*18*), all fortified and incurred samples of all three species were confirmed, whereas all negative controls were not.

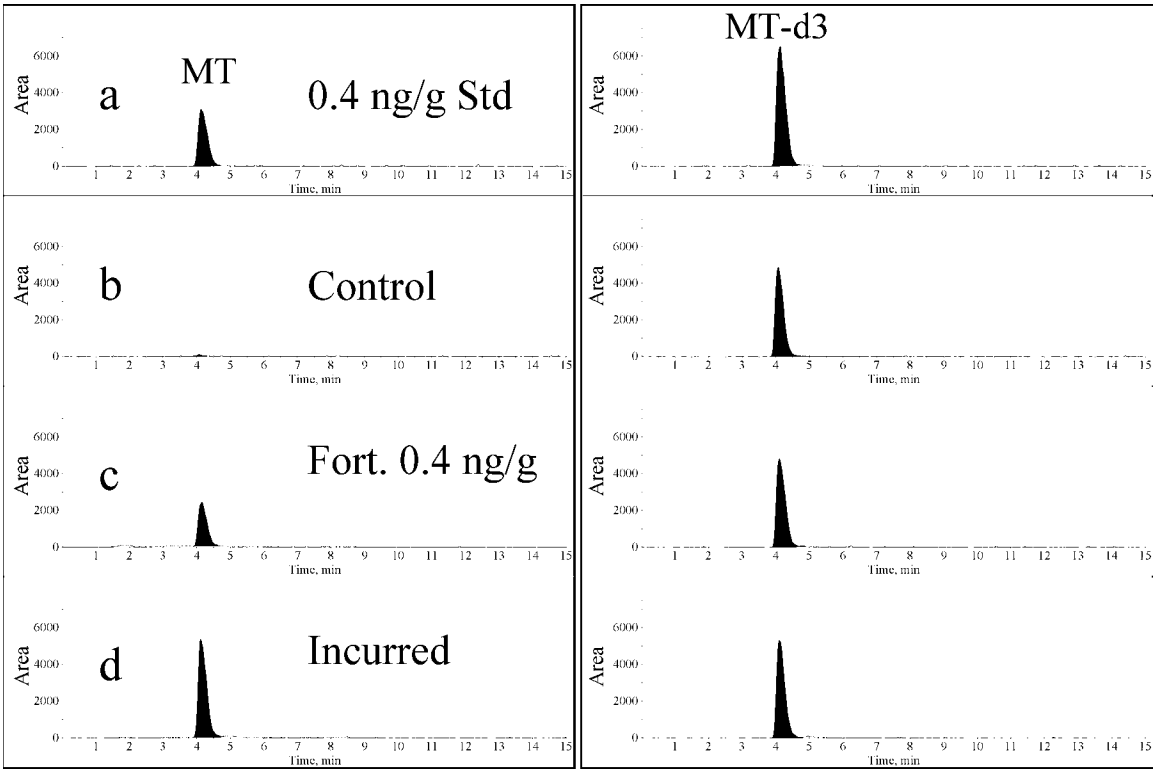

Figure 4. Reconstructed ion chromatograms of MT extracted from salmon: (a) 0.4 ng/g standard with 1 ng/g MT-d_3, **(b)** control fortified with 1 ng/g MT-d_3, **(c)** control fortified with 0.4 ng/g MT and 1 ng/g MT-d_3, **(d)** incurred MT fortified with 1 ng/g MT-d_3.

Table 4. Levels of MT (ng/g) Found in Muscles of Dosed Fish[a]

days after last dose	tilapia	rainbow trout	salmon
1	72	22	22
2	3.9	5.9	NC[b]
3	1.2	1.1	61
5	NC	0.69	3.9
7	0.19	0.47	3.4
14	0.13	0.12	0.52
21	ND[c]	0.09	ND
28	ND	NC	NC
35	ND	NC	NC

[a] $N = 1$. [b] NC = Tissues not collected. [c] ND = Levels below the LOQ.

Table 5. Method Validation of MT: Incurred Residues[a]

	external standard		internal standard	
sample	level found (ng/g)	CV (%)	level found (ng/g)	CV (%)
tilapia				
incurred level 1	0.48	5	0.63	3
incurred level 2	0.92	7	1.2	5
rainbow trout				
incurred level 1	0.51	3	0.64	1
incurred level 2	1.2	1	1.6	4
salmon				
incurred level 1	0.62	2	0.85	4
incurred level 2	1.2	4	1.7	3

[a] $N = 6$.

DISCUSSION

Among the three fish species we studied, salmon presented the greatest challenge in terms of sample cleanup and matrix interferences. Part of the problem is that the salmon fillets sold in grocery stores contained a reddish-orange substance (possibly astaxanthin) that caused significant problems with carry-over peaks and with matrix effects upon MS ionization. These led to a gradual increase in detector response across an injection sequence and nonlinear calibration curves. We reduced the problem by further sample cleanup. SPE columns such as C_{18}, silica, florisil, strong cation exchange (SCX), strong anion exchange (SAX), and their combinations were tried. Some success was achieved when the combination of a C_{18} column with a florisil SPE column was used for sample cleanup. However, this approach was not robust, as interfering peaks occasionally appeared in a small number of samples. Other cleanup procedures involving acid and base partitioning were also explored, but they were of little use in resolving the problems. After several attempts, we employed the SAX column, which was very effective in removing the reddish orange substance present in salmon. The SAX column was primarily used for retaining the unwanted reddish-orange substance instead of retaining the analytes. MT simply passed through the SAX column and was immediately collected in the eluent. A combination of SAX and florisil SPE columns was very effective for sample cleanup and markedly improved the precision and ruggedness of the method.

Method validation data were acquired on an Applied Biosystems Sciex API 2000 apparatus equipped with an APCI source. To facilitate method transfer to other laboratories and to ensure adequate method performance on other LC−MS/MS systems, sample extracts were tested on a Waters Quattro Micro system equipped with an ESI interface. With the Quattro Micro ESI interface, the MT and MT-d_3 recoveries decreased by 5−10%. This observation might be due to the dissimilarities between the two interfaces toward matrix effects. Other investigators have reported that APCI is more robust toward matrix effects than is ESI (*19*). In general, this is also our experience. Despite the recovery difference between the two interfaces, the method accuracy was not affected as long as an

internal standard calibration method was used for quantitation. Hence, the use of an internal standard is strongly recommended because it serves as a surrogate and corrects for any procedural loss and matrix effects on the response.

During sample evaporation, it is critical to use glass tubes and to employ vortex mixing and sonication. We initially used plastic tubes during evaporation, but the results were highly variable. Goudie (*14*) observed a similar phenomenon and postulated that adhesion of steroids to glass surfaces during evaporation might have caused the large coefficients of variation for her results. Therefore, we added sonication steps to our procedure whenever samples were evaporated to dryness. With this change, the precision of the method was significantly improved.

The stability of the incurred tissues and the sample extracts was investigated. Tissues derived from dosed fish were stable for at least 7 months when stored at −80 °C. Similar stability was observed for sample extracts stored at −20 °C.

In the United States, six drugs are approved for use in aquaculture, namely, chorionic gonadotropin, oxytetracycline, sulfamerazine, sulfadimethoxine, tricane methansulfonate (MS-222), and formalin. These substances were, therefore, chromatographed to determine whether they would interfere with the detection method. Other drugs potentially used for aquaculture were also included for interference testing. These drugs included β-lactams such as amoxicillin, ampicillin, cloxacillin, dicloxacillin, penicillin G, cephalexin, and oxacillin; sulfonamides and their potentiators such as sulfadiazine, sulfermerazine, sulfadimethoxine, ormetroprim, and trimethoprim; tetracyclines such as oxytetracycline, tetracycline, chlortetracycline, and doxycycline; quinolones and fluoroquinolones such as ciprofloxacin, difloxacin, enrofloxacin, sarafloxacin, danofloxacin, flumequine, and oxolinolic acid; macrolide antibiotics such as erythromycin, lincomycin, tylosin, emamectin, and ivermectin; imidazoles such as albendazole, metronidazole, fenbendazole, and ketoconazole; and others drugs such as colistin, florfenicol amine, malachite green, and leucomalachite green. None of these drugs interfered with the assay at the target concentration.

In this article, we describe a method suitable for quantitation and confirmation of MT in three fish species. The method can be used for monitoring and surveillance purposes, as well as for drug disposition studies. Eight samples can be assayed as a batch, and the procedure takes 1 day for completion.

ABBREVIATIONS USED

MT, methyltestosterone; ESI, electrospray ionization; APCI, atmospheric-pressure chemical ionization; LC−MS/MS, liquid chromatography−mass spectrometry/mass spectrometry.

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