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Development and validation of a High performance liquid chromatography-Mass spectrometry method for 17α-methyltestosterone in Aquatic water systems

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Abstract: 17α-Methyltestosterone is used as feed additive to manipulate the gender of fish for aquaculture. Earlier a simple, yet specific and robust validated high performance liquid chromatographic method has been developed for the determination of 17α-methyltestosterone in fish feed. The present work describes a highly sensitive and robust Liquid Chromatography-Mass Spectrometry method for the quantitation of 17α-methyltestosterone in aquatic water systems using 17β-hydroxy-3β-methoxyandrost-5-en-7-one as internal standard. The method was validated in the concentration range of 0.2 to 25 ng of 17α-methyltestosterone on column leading to a limit of quantitation of 0.08 ppb or 0.08 mg/L in water, and has potential to increase the limit of detection and quantitation by an order of magnitude, if required.

Keywords: High-performance liquid chromatography-Mass spectrometry, Electrospray ionization, 17α-Methyltestosterone, Solid phase extraction, Method validation

INTRODUCTION

17α-Methyltestosterone (MT, I) is incorporated into fish feed and is fed to juvenile Tilapia and other fish species (e.g. hybrid striped bass, yellow perch, sunfish etc.) to manipulate gender (Beardmore *et al.*, 2001, and Gale *et al.*, 1999). Therefore, there was a need for validated methods for the analysis of MT in fish feed as well as in aquatic water systems. An FDA (USA) approved simple specific and robust validated high performance liquid chromatographic (HPLC) method for the determination of MT in fish feed has been published (Marwah *et al.*, 2005). The present work was undertaken to develop a validated liquid chromatography-mass spectrometry (LC-MS) method for the assay of MT in Aquatic water systems.

The concentration of MT in water is expected to be low and in mg/L range. Several LC-MS methods have been reported in the literature for the analysis of MT in biological matrices (Cravedi and Delous, 1991, Hauser *et al.*, 2008 and Regal *et al.*, 2010), pharmaceutical preparations (Coddington *et al.*, 2000 and Walker *et al.*, 2009) and in water (Chang *et al.*, 2008, Lagana *et al.*, 2001; Cappiello *et al.*, 2003, Sun *et al.*, 2010 and Tolgyesi *et al.*, 2010). Only a few, however, deal with analysis of MT in water. These methods do suffer with drawbacks such as sketchy details, cumbersome methodology, and/or lack of complete validation as per FDA (USA) guidelines. A nano LC-MS method developed for the analysis of steroid hormones and other organic compounds in water suffers from similar shortcomings (Cappiello *et al.*, 2003).

This paper describes a simple specific and robust LC-MS method for the determination of MT in the aquatic waters of lakes rich in clay and sand type of sediments. The method was adequately validated by following general guidelines described in Center for Veterinary Medicine (CVM), Food and Drug Administration (FDA), Bioanalytical Method validation, Guidance for Industry and established scientific norms (Shah *et al.*,2000, Rosing *et al.*, 2000, Ermer and Miller, 2005 and Swartz *et al.*, 2007) and by adhering to principles of good laboratory practice (GLP, FDA).

MATERIALS AND METHODS

17α-Methyltestosterone USP (I, Fig. 1), was supplied by Rangen Inc. (Buhl, ID, USA), and was stored at -20°C. 17β-Hydroxy-3β-methoxyandrost-5-en-7-one (II, I.S.) was synthesized in the laboratory (Marwah *et al.*, 2001). Its structure was confirmed by NMR, (^{1}H and ^{13}C) and LC-MS, and purity (>99.5%) was established by UV at 245 nm and mass spectral data in electrospray ionization (ESI)

mode. Ammonium acetate (>99.99%) and HPLC grade methanol, ethanol, acetone, and acetonitrile were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used as such. Acetic acid, HPLC grade and formic acid were purchased from Fisher (Pittsburgh, PA, USA) and Fluka (Milwaukee, WI, USA) respectively. Water, deionized and purified by Nanopure water system from Barnstead International (Dubuque, Iowa, USA) was used (18.25±0.05 MΩ-cm). Solid phase extraction (SPE) cartridges (Oasis-HLB, 6 cc) were obtained from Waters Corporation (Milford, MA, USA).

Instrumentation : The chromatographic system consisted of an Agilent's 1100 series LC-MS system (Agilent Technologies Inc. Palo Alto, CA, USA), comprised of a capillary pump (G1376A) operated in normal mode and equipped with an online degasser (G1379A), a column oven (G1316A), an autosampler (G1313A), a diode array UV detector (DAD, G1315A) and a single quadruple mass detector (G1946A). Data were acquired and processed using Agilent's ChemStation software (version A.9.0.3) or later.

Preparation of standard solutions: A series of standard solutions were prepared for the LC-MS analysis of MT in water. MT test solution, and I.S. test solution were prepared individually by dissolving an accurately weighed quantity in ethanol to give a solution of ~1.0 mg/ml. Calibration solutions were prepared by mixing appropriate volumes of MT test solution and IS test solutions and diluting with ethanol-water (1:1) to 10.0 ml in 10 ml volumetric flasks to give individually 0, 0, 2, 5, 10, 20, 50, 100, 200, 500 ng of MT and a fixed concentration (20 ng) of IS in 100 ml of 50% aq. ethanol-water. System suitability solution was prepared by serial dilution and had a concentration of about 5 ng/µL (~0.005 mg/ml) each of MT and IS in 50% aqueous ethanol.

Selection of sediments for the study: For preparing the water samples, two sediments were selected as per the protocol approved by CVM, FDA, USA (CVM File # I-011395-E-002; PMF # 5835). First sediment was collected from a marsh located on the grounds of the Lake Mills State Fish Hatchery, Lake Mills, Wisconsin, USA. This sediment had high organic carbon content (6.8%) and a fine texture (clay+silt content 50±3%). Second sediment was collected from Rock Creek, Lake Mills, Wisconsin, USA. This sediment had low organic carbon content (1.8%), and a course texture (clay+silt content 15±3%). These sediments were analyzed for their contents (Table 1). These sediments are referred to as "clay" and "sand" sediments respectively.

Preparation of water samples for the LC-MS analysis: HPLC grade water (3L) was added to a two inch thick layer of sediment in an amber glass one gallon bottle, and the bottle was manually shaken for a few minutes every day morning for one week. The bottles were allowed to stand for another one week in dark, and upper water layer was decanted carefully and frozen at -20°C till used. Clay and sand sediment were treated similarly and water layers are henceforth referred to as Clay water and Sand water respectively. These water samples were used for the preparation of calibration curves and for method validation.

Table 1. Sediment analysis of sediment used for method development.

Parameter	Sediment Type	
	Clay	Sand
pH	7.1	7.2
% Solid	33	71.1
TOC (mg/g)	85.7	9,480
Microbial Biomass (/ml)	10,000	500
Total Nitrogen (mg/Kg)	7820	516
Total Phosphorus (mg/Kg)	974	200

Extraction procedure: Calibration standard solutions were prepared for the assay of MT in Water by adding 100 µl solution of each concentration to 25 ml water matrix in 50 ml polypropylene tubes. Acetic acid (0.1 ml) was added to each sample, and samples were vortex mixed for 20s and centrifuged at 1000 g for 5 min. The water layer was applied to a preconditioned (3.0 ml acetone, 3.0 ml methanol and 3.0 ml water) solid phase extraction cartridge [Oasis-HLB, 6.0 c.c.]. The water suction was applied, if necessary, to achieve a flow rate of ~ 5 ml/min. The loaded cartridge was washed with 5% methanol (2 ml) followed by 50% methanol (4 ml) and dried under water suction (~equivalent to about 20 inches of mercury) for 2 min. Finally, MT and IS were eluted with acetone (3 ml) into a graduated polypropylene tube (15 ml). The eluted acetone was immediately evaporated under nitrogen gas at ~40°C, the residue dissolved in 0.2 ml methanol-water (1:1) and 20 µl was subjected to LC-MS analysis.

Chromatographic conditions: Chromatography was performed on a Zorbax-SB C_{18} column (3.0x150 mm, 3.5 µm, 80 Å, Agilent Technologies Inc. Palo Alto, CA, USA) protected by a Zorbax C_{18} guard column, (2.1x12.5 mm, 5 µm) at a flow rate of 0.5 ml/min and column temperature of 40.0±0.5°C. A water-90% acetonitrile gradient (70:30 (v/v) at time t=0, 10:90 at t=10, and 70:30 at t=11 min, post run time 10 min) was used as the mobile phase. Ammonium acetate (2 mM) and 0.1% formic acid were added to water as well as 90% acetonitrile. The electrospray (ESI-MS) parameters were: drying gas (N_2) 13 L/min at 350°C; nebulizer 35 psi; capillary voltage 4800V; fragmentor 125 V for MT and 110 V for IS. Analysis was carried out in selected ion monitoring (SIM) mode using m/z 303 $[M+H]^+$ as quantifying ion and m/z 285 (loss of water) as qualifying ion for MT. For IS quantifying and qualifying ions were m/z 319 $[M+H]^+$ and 287 (loss of methanol) respectively.

The dwell volume (V_D) of the system, determined

Table 2. Chromatographic parameters (Resolution (R), plate count (N, efficiency), peak width factors (w_h) and relative retention time (t_R) for pure chemical standard of MT, and for MT extracted from water spiked with 10 ng/25 ml of 17α-methyltestosterone.

Parameters	MT Chemical standard	MT Extracted from water
Plate count[a] (N)	90694 (13.6)*	107532 (13.9)*
Peak width[b] (min)	0.067 (6.0)	0.061 (6.6)
Resolution[c] (R) MT vs. IS	18.0 (5.9)	19.2 (6.2)
Relative retention time (t_R)	0.78 (0.0)	0.78 (0.1)

* Mean (%RSD); [a]Number of theoretical Plates; [b]Peak Width at half height in min; [c]Resolution factor between MT and IS.

graphically by replacing the column with a short piece (100 mm) of HPLC tubing and by running a gradient of water vs. 3% acetic acid (0-100% in 10 min) and recording the response at 220 nm using a diode array detector was found to be 0.7 ml.

RESULTS AND DISCUSSION

The first concern in developing the validated assay for MT was regarding the solubility of MT in water. The question we asked ourselves was: will MT at parts per million be soluble in water or will it precipitate out.

In order to find an answer, MT (50 mg) was added to 5 ml of HPLC grade water, and the contents were shaken for 18 hrs in a metabolic shaker at room temperature, centrifuged at 1000 g for 5 min, and 15000 g for 2 min, and a 5 µl supernatant was analyzed by LC-MS. Quantity of MT in water was calculated using an external standard method, and 5 ml water solution of MT was found to contain 2.11 ng of MT. Therefore solubility of MT in water is 0.411 mg/L or 0.4 µg/ml.

Selection of chromatographic conditions was another concern. Mass spectrum of MT showed an $[M+H]^+$ as well as a significant amount of $[M+Na]^+$ peak, and their ratio was not reproducible at different concentration resulting in unacceptable variations in calibration curve. Use of methanol in the mobile phase resulted in too much formation of sodium adduct for MT as well as IS. After several attempts it was observed that sodium adduct formation could be controlled to a large extent by using ammonium acetate and formic acid as modifiers in a water acetonitrile gradient.

Extraction recoveries: The extraction recovery of MT from water, determined by comparing areas of MT peak (m/z 303) recovered from water spiked with known amounts of MT versus area of MT peak obtained from pure chemical standard was found to be 95.13±1.83% (RSD 5.45%). The extraction recovery of IS from water, determined by comparing areas of IS peak (m/z 319) recovered from water spiked with known amounts of IS versus area of IS peak obtained from pure chemical standard, was found to be 89.01±0.76% (RSD 3.42%).

System suitability: The suitability of the LC-MS system was evaluated by analyzing in duplicate, system suitability solution (MT and I.S., 2 µL injection). The chromatograms were evaluated for peak widths at half height (W_h), column efficiency (number of theoretical plates, N, given by $N = 5.54 (t_R/W_{0.5})^2$) and signal-to-noise ratio (S/N). HPLC system is considered to be performing suitably if S/N ratio is not less than 1000, column efficiency is not less than 70,000 theoretical plates (N) calculated for MT peak, peak widths for MT and IS peak does not exceed 0.1 min, relative retention times for MT and IS are 1.0, and 0.7 respectively, and resolution factor (R) between MT and IS is not less than 15.

Specificity: Specificity is the ability to measure the analyte of interest accurately and specifically in the presence of closely related structures, impurities, degradation products, and other components that could be expected to be present in the matrix. To evaluate the specificity of the LC-MS method following experiments were carried out: i) control samples were prepared from sand water and clay water assay. The retention times of endogenous substances in water matrix were compared with retention times of MT and IS. Interference from the internal standard on the retention time of MT and vice versa was checked to rule out the presence of any interfering impurities. No endogenous substance interfered (<0.5%) at the retention time of MT. There was no interference from the MT on the retention time of internal standard and vice versa. The internal standard was well resolved from MT peak. LC-MS analysis of MT was carried out in selected ion monitoring mode, using m/z 303 $[M+H]^+$ for quantitation of MT and ion at m/z 285 $[M+H-H_2O]^+$ as a qualifier ion with a built in control value of ± 20%. The use of qualifier ion effectively ruled out interference from matrix components, degradation products and impurities. Similarly for IS, m/z 319 $[M+H]^+$ was used as quantifier ion coupled with m/z 287 $[M+H-CH_3OH]^+$ as qualifier ion; ii) the water matrix spiked with MT and IS was assayed for inter-run studies (spread over a period of ~2 months) and analyzed for resolution (R), plate count (N, efficiency), and peak width (w_h) for MT and IS. These chromatographic parameters (R, N, and w_h) were compared with same chromatographic parameters (R, N, and w_h) obtained for pure chemical samples, and were found to be comparable (Table 2). The internal standard was the major component eluting closest to MT; iii) several steroidal hormones were analyzed by the assay method and LC-MS data were scrutinized for interference if any. Estrogens and

Table 3. LC-MS analysis of various estogens and androgens, dehydroepiandrosterones, pregnanes and corticosteroids.

No.	Steroids	Retention time min	Major ions in mass spectrum (m/z)
I	MT	8.48	303; 285
II	IS	6.62	319; 287
III	Testosterone	7.97	311; 289
IV	Androstenedione[a]	8.31	286; 309
V	DHEA[b]	7.94	311; 288; 271
VI	Androstenediol[c]	7.06	273; 255
VII	7-oxo-DHEA	5.01	303; 285
VIII	7α-OH DHEA	5.01	327; 287; 269; 251
IX	7β-OH DHEA	4.19	327; 287; 269; 251
X	16α-OH DHEA	5.22	327; 305; 287; 269
XI	7β-AET[d]	3.22	329; 271; 253
XII	7α-AET[e]	3.49	329; 271; 253
XIII	7-Oxo-Diol[f]	3.92	327; 305
XIV	Estrone	7.63	271; 253
XV	Estradiol	6.81	273; 255
XVI	Estriol	3.89	311; 289; 271; 253
XVII	Progesterone	10.57	315; 297
XVIII	Pregnenolone	10.00	317; 299; 281
XIX	Cortisol	4.64	385; 362
XX	Cortisone	4.77	383; 361
XXI	Cortexolone	6.27	369; 347
XXII	Cortexone	8.01	353; 331
XXIII	Dehydrocorticosterone	5.67	367; 345
XXIV	Corticosterone	6.02	369; 347; 329
XXV	17α-OH Pregnenolone	7.55	355; 315; 297

[a]Androst-4-en-3,17-dione; [b]Dehydroepiandrosterone; [c]Androst-5-en-3β,17β-diol; [d]Androst-5-en-3β,7β,17β-triol; [e]Androst-5-en-3β,7α,17β-triol; [f]3β,17β-dihydroxy-androst-5-en-7-one.

Androgens, Dehydroepiandrosterone and its metabolites, Pregnanes and active Corticosteroids were found not to interfere in the present assay. Specifically the following hormones and steroids were studied for interference, and were found not to interfere with the present assay: Testosterone; Androst-5-en-3,17-dione; DHEA (Dehydroepiandrosterone); Androstenediol (Androst-5-en-3β,17β-diol); 7-oxo-DHEA; 7α-OH DHEA; 7β-OH DHEA; 16α-OH DHEA; 7β-AET (Androst-5-en-3β,7β,17β-triol); 7α-AET (Androst-5-en-3β,7α,17β-triol); 7-oxo-Diol (3β,17β-dihydroxyandrost-5-en-7-one); Estrone; Estradiol; Estriol; Progesterone; Pregnenolone; Cortisol; Cortisone; Cortexolone; Cortexone; Dehydrocorticosterone; Corticosterone; and 17α-OH Pregnenolone (Table 3); and iv) by subjecting MT to degradation studies at low, neutral, and high pH at elevated temperature (Marwah *et al.*, 2005). LC-MS analysis of MT samples subjected to accelerated stress study showed no interference, from degradation products.

It may be mentioned that for a compound to interfere in the present assay three requirements must be met: a) it should have same retention time; b) it should have same quantifier ion; and c) it should have same qualifier ion. It is extremely difficult for another compound to have all the three characteristic features, and therefore it is unlikely that another compound will interfere in the present assay.

Therefore, it is reasonably safe to conclude that MT water method is a highly specific method. The developed

Table 4. Intra-run accuracy and precision in the assay of 17α–methyltestosterone in aquatic water.

MT added	Intra-run (n=5)*		Inter-run (n=12)**	
(ng/25 ml)	Accuracy (% Error)	Precision (% RSD)	Accuracy (% Error)	Precision (% RSD)
2	7.35	0.42	1.70	4.92
5	-1.18	1.13	-5.62	2.52
10	3.44	0.63	2.61	2.65
20	6.53	0.81	5.25	2.78
50	4.91	0.72	3.28	2.40
100	2.06	0.65	-1.25	2.92
200	0.62	1.15	-3.30	2.97
500	5.89	2.01	7.46	5.18

* Replicate (n=5) injections of same set of samples analyzed in a single run. **Spiked samples prepared on different days and analyzed against freshly prepared calibration curve.

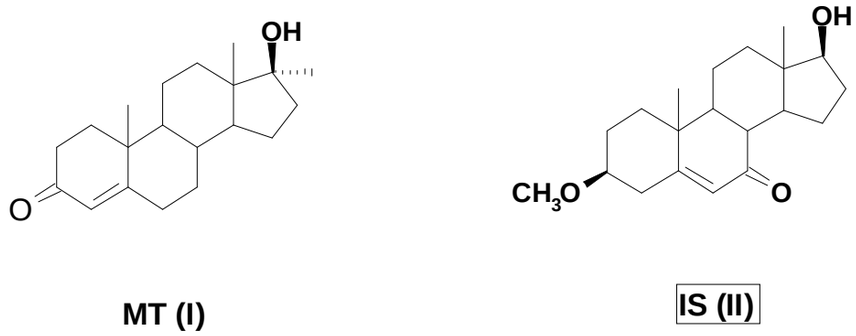

Fig. 1. *Chemical structures of 17α-Methyltestosterone (MT, I) and Internal standard (17β-hydroxy-3β-methoxyandrost-5-en-7-one, II).*

method is able to assay MT with high degree of accuracy in the presence of impurities, degradation products and matrix components.

Linearity and range: Calibration curves consisting of a blank sample (matrix sample without MT and internal standard), a zero sample (matrix sample with internal standard), and eight (2, 5, 10, 20, 50, 100, 200, and 500 ng of MT in 25 ml water) non-zero samples covering the expected range, including lower limits of quantitation (LLOQ) were plotted in the present study. Calibration curves (n=18) were generated under different conditions to ascertain precision, accuracy, ruggedness, and robustness of the method.

The range studied (2 to 500 ng MT in 25 ml water giving rise to 0.2 ng to 50 ng of MT on column) was found to be non-linear, though it was possible to plot short range linear calibration curve. However it was found that a quadratic curve with quadratic weightage gave reproducible results day after day under the same processing conditions and parameters.

For the calibration curves {$y = (-0.0235\pm0.0009)x^2 + (1.4207\pm0.0187)x + 0.0408\pm0.0019$; mean±SE, n=18} plotted for the determination of MT, the average correlation coefficient (mean±SE) was 0.9989±0.0001 (% RSD 0.05). The % RSD value for the intercept was high (~19%) but intercept contributed to only about 2% to the calculated concentration at LLOQ, and hence did not have any significant contribution to the calculated values. There was no significant difference between calibration curves plotted under different conditions (n=18, F = 0.002; $F_{critical} = 2.2$).

Extraction recoveries: The extraction recoveries of MT from water spiked with MT, were determined by comparing areas of MT peak (m/z 303) recovered from water spiked with eight different concentration of MT (0, 0, 2, 5, 10, 20, 50, 100, 200, 500 ng of MT in 25 ml of sand water matrix) and a fixed concentration of IS (20 ng/25 ml of water), processed by assay procedure versus area of MT peak (m/z 303) obtained from pure chemical standard of same concentrations. Extraction recoveries were calculated as: % Extraction Recovery = (Area MT_{water}/Area $MT_{chemical}$)x100; in which: Area MT_{water} = Area of MT in water spiked with MT, and Area $MT_{chemical}$ = Area of MT in pure chemical sample.

The extraction recovery of MT from water, determined by comparing areas of MT peak (m/z 303) recovered from water spiked with known amounts of MT versus area of MT peak obtained from pure chemical standard was found to be 95.13±1.83% (RSD 5.45%). The extraction recovery of IS from water, determined by comparing areas of IS peak (m/z 319) recovered from water spiked with known amounts of IS versus area of IS peak obtained from pure chemical standard, was found to be 89.01% (RSD 3.42%).

Accuracy and precision: Accuracy and precision of the assay were established across the range of the analytical procedure. The intra-run precision and accuracy of the method were evaluated by analyzing, during a single run, replicates of spiked samples against a separate calibration curve. Accuracy of the method was determined as percent recovery by the assay of known added amount of MT in the sample together with confidence intervals. Precision of the assay was determined as percentage relative standard deviation. Intermediate precision, resulting from within-lab variations due to random events such as differences in experimental periods, and different analysts, was studied.

The intra-run accuracy of the method was evaluated by analyzing as part of a single run, replicate sets of spiked samples prepared at eight different concentrations (0, 0, 2, 5, 10, 20, 50, 100, 200, 500, ng of MT in 25 ml of Sand Water matrix) against a separate 8-point calibration curve. Accuracy was found to be within -1.2% to +8.0% of spiked

Table 5. Stability studies of MT in water: storage at room temperature for eight hours and effect of three freeze-thaw cycles.

MT added (ng/25 ml)	MT Recovered (% RSD) Room Temp. After 8 hrs	After 3 Freeze-Thaw Cycles
10	9.67(0.66)	10.70 (0.86)
50	51.80(0.66)	51.42 (4.40)
100	101.00 (1.09)	100.63 (4.7)

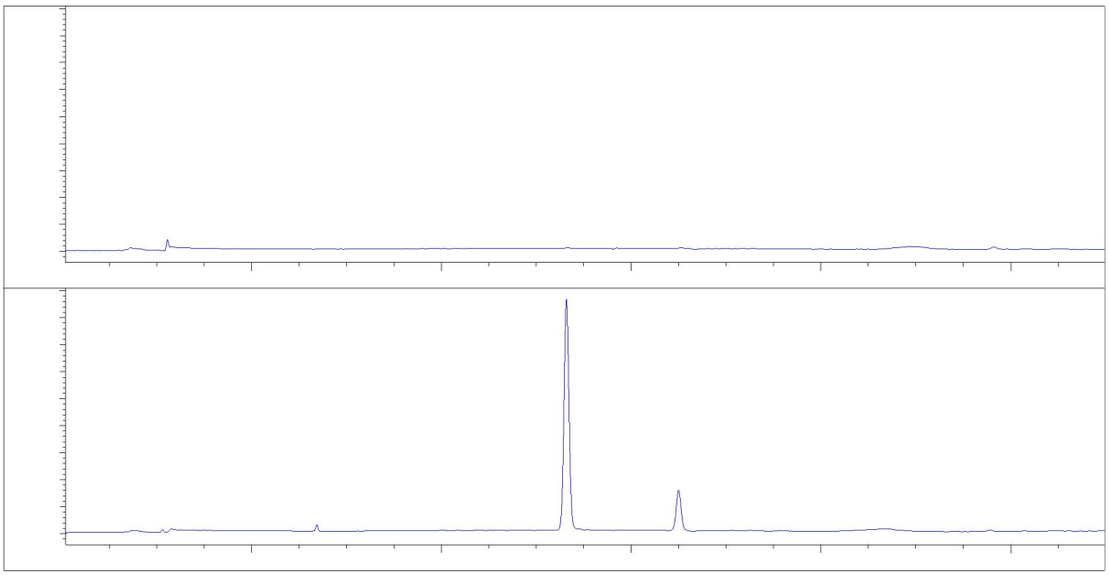

Fig. 2. *Representative chromatograms of sand water extract. (I) blank sand water matrix; (II) sand water matrix spiked with 2 ng/ 25 ml of 17α-methyltestosterone and 20 ng/25 ml of Internal standard.*

concentrations. There was no significant difference between replicates of various sets of concentrations (F=0.0005; $F_{critical}$ = 2.69).

The inter-run accuracy of the method was evaluated by analyzing replicates of spiked samples at eight different concentrations (0, 0, 2, 5, 10, 20, 50, 100, 200, 500 ng of MT in 25 ml of Sand Water matrix) against a separate 8-point calibration curve on different days (spread over a 27 day period) and during separate runs. Each run was analyzed against a separate calibration curve. Inter run accuracy was found to be within -6% to +8% of spiked concentrations and 1.7% at LLOQ. There was no significant difference between replicates of various sets of concentrations (F=0.003; $F_{critical}$ = 1.92).

The intra-run precision of the method was evaluated by analyzing as part of a single run, replicate sets of spiked samples prepared at eight different concentrations (0, 0, 2, 5, 10, 20, 50, 100, 200, 500, ng of MT in 25 ml of Sand Water matrix) against a separate 8-point calibration curve. Intra-run precision was found to be within 2% of RSD. There was no significant difference between replicates of various sets of concentrations (F=0.0002; $F_{critical}$ = 3.24).

The inter-run precision of the method was evaluated by analyzing replicates of spiked samples at eight different concentrations (0, 0, 2, 5, 10, 20, 50, 100, 200, 500 ng of MT in 25 ml of sand water matrix) against a separate 8-point calibration curve on different days (spread over a 27 day period) and during separate runs. Each run was analyzed against a separate calibration curve. Inter-run precision, expressed as the percent relative standard deviation (%RSD) was found to be within 5.2%. There was no significant difference between replicates of various sets of concentrations (F=0.003; $F_{critical}$ = 1.92) (Table 4).

Intermediate precision of the procedure was also evaluated by two different analysts. Accuracy was found to be within -3.1% to 5.3% for Analyst 1, and -6.2 to 9.6% for Analyst 2. The percentage relative standard deviation was less than 7% for both analysts. There was no significant difference between replicates of various sets of concentrations (F=0.001; $F_{critical}$ = 3.56).

Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ): The limits of detection (LOD) and quantitation (LOQ) were calculated by following the signal-to-noise approach. The chromatograms obtained by the analysis of water matrix spiked with MT were analyzed for S/N ratio using Chemstation software for data analysis. The S/N ratio was calculated for the quantifying peak (m/z 303) and also for the qualifier peak (m/z 285). LLOQ was decided based on S/N ratio of 9 or more for the qualifier peak. The LLOQ was established using five samples independent of standards and by determining the coefficient of variation and confidence interval. In the present study a range of 80 pg to 20 ng/ml of water was selected for testing curve fitting and range of the assay. A 25 ml volume of water was used for extraction giving an effective concentration of 2 ng to 500 ng of MT in the amount of water taken. Water (25 ml) extract was dissolved in 0.2 ml water, and 0.02 ml was injected into LC-MS, thus effectively giving rise to 0.2 ng injection on column for the lowest concentration studied. Therefore the limit of quantitation is 0.2 ng of MT on column or in more mundane terms, 2 ng in 25 ml water, or 0.08 mg/L or 0.08 ppb.

Limit of detection was calculated as theoretical concentrations of MT in ng/25 ml of water matrix, which will yield a S/N ratio of 3, and was found to be 0.67 ng/25 ml of water matrix leading to 0.067 ng on column injection.

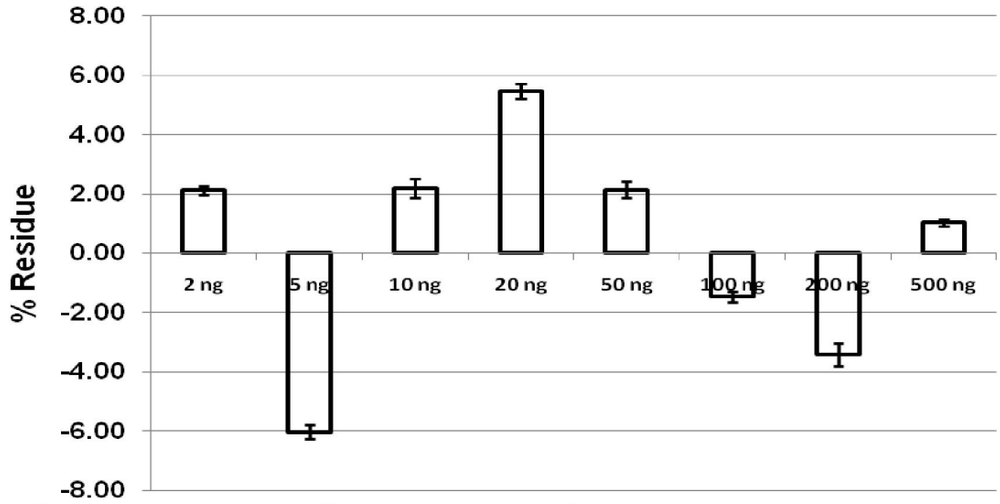

Fig. 3. *Curve Fitting ($y = ax^2 + bx + c$) for MT extracted from water. % Relative residues (n=18) for various concentration levels. Mean±SE*

The sensitivity of the method can be further improved, if required, several fold by (a) injecting larger volumes (up to 3 fold increase in injection volume was found not to affect the chromatographic performance), and (b) using larger volume of the water matrix, e.g. 100 ml or more for extraction of MT.

Robustness and ruggedness: The robustness and ruggedness of the method was evaluated by introducing small deliberate changes in extraction procedure and LC-MS conditions. Robustness was assessed early in the development of the method.

In order to test the robustness of the method deliberate small changes (~10% of the procedure values) were introduced in the extraction procedure, and samples, in triplicate, subjected to extraction procedure as usual and then analyzed by LC-MS. Areas corresponding to IS and MT were recorded, and evaluated for the effects, if any, on the results of the method. Specifically, the following variations in the extraction procedure were studied: a) concentration of acetic acid used for acidifying water matrix, b) volume of 50% methanol used for washing the solid phase cartridge, c) concentration of aqueous methanol (50±5%) used for washing the cartridge, and d) volume of acetone used to elute MT and IS from the cartridge.

The method was found to be unaffected by these small variations (~10%). No significant difference were observed in the peak areas given by MT ($F = <3$, $F_{critical} = 9.55$) and IS ($F = <5$, $F_{critical} = 9.55$). The only parameter, which was found to influence the method, was the concentration of methanol in the wash step during solid phase extraction of MT and IS. A 10% change in methanol concentration affected the elution of the internal standard but not the elution of MT. However, consistent and reproducible results were obtained when methanol concentration was maintained constant.

The method developed for the analysis of MT in environmental water is robust and rugged, and was not affected by a) the use of water from different sources or matrices, b) the use of columns from different lots, c) the use of columns of different dimensions ranging from 75 mm to 150 mm in length and 2.1 to 4.6 mm in internal diameter, and d) use of either a C_{18} or a C_8 chromatographic column.

Effect of injection volume on the analysis of MT in water: There were no significant changes in chromatographic parameters when same amount (2 ng) of MT was injected in volumes ranging from 5.0 µL to 60 µL in the present analysis. The accuracy was between -0.05% and 7.2% and a relative standard deviation was less than 5% for all the volumes injected. Peak width slightly increased from 0.0582 min to 0.0618 min (a change of 6%) but it did not affect the results of the analysis. Signal to noise ratio (S/N) significantly improved from 5 mL injection to 20 mL injection volume. Therefore a) 12 fold increase in the injection volume (from 5.0 µ L to 60.0 µL) had no significant effect on the chromatographic performance; b) the sensitivity of the method can be further improved several fold by increasing the injection volumes up to 60 µL, if desired.

Effect of change in additive concentration in the mobile phase: Reproducible results were obtained for the analysis of MT in water in the concentration range 0.2-20 ng of MT on column when additive (ammonium acetate and formic acid) concentration were changed in the mobile phase. Accuracy ranged between 2.6% to 12.2% (within ±8% for the 1st column) and standard deviation was less than 7% for all the concentration levels.

Therefore it can be reasonably concluded that this method is robust and rugged and unaffected by small variations in method parameters.

Stability studies: In the present study, the stability of

MT in samples was assessed in spiked samples at three concentration levels (0, 10, 50 and 100 ng MT in 25 ml of sand water) under different conditions and analyzed against a freshly prepared separate calibration curve.
MT was stable for eight hours at room temperature (percent recoveries 96.7-103.6%, %RSD < 1.10). MT was also stable when stored in pure water and in matrix water (Clay and Sand matrices) at-40°C or lower for extended period of time (six month). The recovery was ~109% with a % RSD of <6.0. Three freeze-thaw cycles were tolerated without any significant change in MT concentrations at three levels. No significant differences were seen among the three sets of samples analyzed after successive freeze thaw cycles. ($F = 0.0001$, $F_{critical} = 5.14$, (Table 5).

Conclusion

The analytical method for MT in water was adequately validated over the range of 80 ng/L to 20,000 ng/L in water. The described LC-MS method is highly specific, reproducible and accurate. The proposed method was found to be robust and rugged, and unaffected by small variations (~10%) in the extraction procedure and in LC-MS conditions. The method has been approved and accepted by CVM, FDA, USA. The method was subsequently used to study the degradation of MT in aquatic systems.

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