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Development and validation of a high performance liquid chromatography assay for 17α-methyltestosterone in fish feed

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Abstract

17α-Methyltestosterone (MT) is used to manipulate the gender of a variety of fish species. A high performance liquid chromatography (HPLC) internal standard method for the determination of 17α-methyltestosterone in fish feed using 3β-methoxy-17β-hydroxyandrost-5-en-7-one as internal standard (IS) has been developed. The method has been validated for the quantitation of MT in fish feed using 245 nm UV absorbance as the parent wavelength and 255 nm as a qualifier wavelength. The method was validated in the concentration range of 15.0–120 mg/kg of 17α-methyltestosterone in fish feed. Method was also found to be suitable for other feeds.

Keywords: High-performance liquid chromatography; Ultraviolet; Electrospray ionization mass spectrometry; 17α-Methyltestosterone; Fish feed; Solid phase extraction

1. Introduction

17α-Methyltestosterone (MT, Fig. 1) is used to manipulate the gender of a variety of fish species [1–4]. MT induced phenotypical masculinization of gynogenetic northern pike juveniles fish has been reported [1]. Adding MT to fish feed is the easiest and most convenient mode of hormone-induced sex reversal. Several high performance liquid chromatography (HPLC) and liquid chromatography–mass spectroscopy (LC–MS) methods have been reported for the analysis of MT in various biological matrices and pharmaceutical preparations [5–15], but only a few deal with analysis of MT in fish feed [5,6]. MT concentration in fish feed was determined [5] by following a multi-step extraction technique followed by HPLC analysis that is a modification of a method developed by Goudie [6] to analyze fish tissue for MT. The HPLC method suffers drawbacks such as sketchy details, cumbersome methodology, lack of proper validation, an absence of a qualifier peak (wavelength) for MT quantitation, etc.

The objective of the present work was to develop a selective and robust method for the analysis of MT that meets the accepted criteria of analytical method validation for type C medicated feeds.

This paper describes a simple, specific and robust validated HPLC method for the determination of 17α-methyltestosterone (MT) in fish feed using 3β-methoxy-17β-hydroxyandrost-5-en-7-one (**II**, Fig. 1) as the internal standard (IS). The method has been validated for the quantitation of MT in fish feed at 245 nm UV absorbance for quantitation and by using absorbance at 255 nm as a qualifier wavelength. The method was also found to be applicable to other animal feeds.

2. Experimental

2.1. Materials and reagents

17α-Methyltestosterone USP, fish feed, and fish feed with MT (0.006%, w/w, Batch A # R39D-163-2003, and

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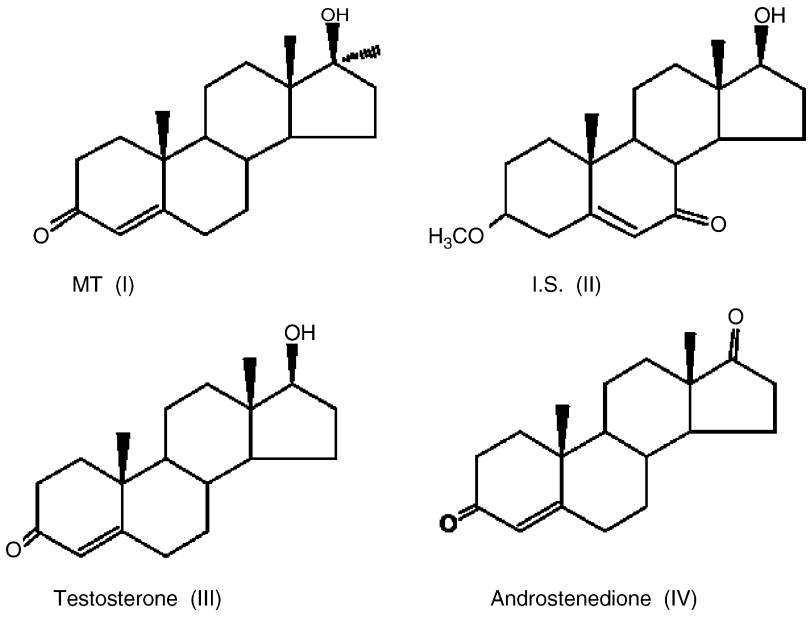


Fig. 1. Chemical structures of 17α-methyltestosterone (MT, **I**), the internal standard (17β-hydroxy-3β-methoxyandrost-5-en-7-one, **II**), testosterone (**III**) and androstenedione (androst-4-ene-3,17-dione, **IV**).

Batch B #R48 252) were supplied by Rangen Inc. (Buhl, ID, USA), and were stored at −20 °C. 17β-Hydroxy-3β-methoxyandrost-5-en-7-one (**II**, IS) was synthesized in this laboratory. 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 measurement in electrospray ionization (ESI) mode. Testosterone (**III**, Fig. 1), androst-4-ene-3,17-dione (androstenedione, **IV**, Fig. 1), HPLC grade methanol, ethanol, hexane, 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. Distilled 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, 3 cc) were obtained from Waters Corporation (Milford, MA, USA).

2.2. Instrumentation

The chromatographic system consisted of an Agilent 1100 series HPLC–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). A quaternary pump (G1311A) with on line degasser was used for the post column addition of formic acid. Data were acquired and processed using Agilent's ChemStation software (version A.8.0.3).

2.3. Preparation of standard solutions

A series of standard solutions were prepared to establish the suitability of the HPLC system for the assay of MT in fish feed. MT test solution, IS test solution, and testosterone solution were prepared individually by dissolving an accurately weighed quantity in ethanol to give a solution of ∼0.5 mg/ml. System suitability solution was prepared by diluting (×100) MT and IS test solutions in the same volumetric flask to achieve a concentration of ∼5 ng/μL each of MT and IS in 50% aqueous ethanol. Assay suitability solution was prepared by diluting (×25) MT and IS test solutions, and testosterone solution in the same flask to achieve a concentration of ∼20 ng/μL each of MT, IS and testosterone in 50% aqueous ethanol. Calibration solutions were prepared by dissolving accurately weighed amounts of MT and IS in volumetric flasks to give a concentration of 0.75, 1.50, 3.00, 4.50, and 6.00 mg/ml of MT and 3.00 mg/ml of IS, respectively.

2.4. Extraction procedure

MT was extracted from fish feed by following a combination of liquid–liquid and solid phase extraction techniques. Fish feed (5.0 g in a 50 ml polypropylene tube) was spiked with appropriate quantities of MT. After 20 min, hexane (3.0 ml) was added to each sample, and samples were vortex mixed for 20 s. Methanol (12.0 ml) and IS (0.3 mg in 0.1 ml ethanol) were added and the tubes were again vortex mixed for 2 × 20 s. Water (3.0 ml) was added to each tube, vortex mixed for 2 × 20 s, and then centrifuged at ∼1000 × *g* for 5 min. The methanol–water (2.0 ml) was pipetted out and

taken into polypropylene centrifuge tubes (15 ml), and again washed with hexane (2.0 ml). The hexane layer was discarded, and methanol–water layer was evaporated to ~0.3 ml volume at 40 °C under a slow stream of nitrogen. It was diluted with water to 3.0 ml volume and applied to a preconditioned solid phase extraction (SPE) cartridge (Oasis-HLB, 3.0 cc, preconditioned with methanol (2.0 ml) and water (2.0 ml)). The loaded cartridge was washed twice with 1.0 ml methanol–water (1:1) containing 2% acetic acid. MT and IS were eluted with methanol (2 × 1.0 ml) into a graduated polypropylene tube (15 ml). The eluted methanol was diluted with water to a final volume of 4.0 ± 0.1 ml and centrifuged at ~1000 × *g* for 5 min. About 0.5 ml of the final solution was transferred into a 2.0 ml autosampler vial, and 10 μL was injected into the HPLC for analysis.

2.5. Chromatographic conditions

Chromatography was performed on a Zorbax-SB C_{18} column (3.0 mm × 150 mm, 3.5 μm, 80 Å, Agilent Technologies Inc. Palo Alto, CA, USA) protected by a Zorbax C_{18} guard column, (2.1 mm × 12.5 mm, 5 μm) at a flow rate of 0.5 ml/min and column temperature of 40.0 ± 0.5 °C. A water–acetonitrile gradient (80:20 (v/v) at time $t = 0$, 4:96 at $t = 19$, and 80:20 at $t = 20$ min) was used as the mobile phase. A 12 min post run time was used to reequilibrate the column. MT and IS were detected at 245 nm (bandwidth 4 nm) for quantitation and 255 nm (bandwidth 4 nm) as qualifier using a diode array detector (DAD). An online mass detector was used to evaluate specificity. The MS parameters were: drying gas (N_2) 13 L/min at 350 °C; nebulizer 35 psi; capillary voltage 4500 V; fragmentor 100 V; and multiplier voltage 1700 V.

2.6. Method validation

The method has been developed and validated by following the guidelines of U.S. Department of Health and Human Services (DHHS), Food and Drug Administration (FDA) for Validation of Analytical Procedures for Type C Medicated Feeds [16].

2.6.1. Dwell volume

The dwell volume (V_D) of the system was determined graphically by replacing the column with a short piece (100 mm) of HPLC tubing and by running a gradient of water versus 3% acetic acid (0–100% in 10 min) and recording the response at 240 nm.

2.6.2. System suitability

The suitability of the HPLC system was evaluated by analyzing in duplicate system suitability solution (MT and IS, 5 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).

2.6.3. Assay suitability

The suitability of the HPLC system for the assay of MT was evaluated by analyzing in duplicate assay suitability solution (MT, testosterone and IS, 5 μL injection). The chromatograms were evaluated for resolution factor (R) and tailing factor (T_f).

2.6.4. 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. Specificity of the method was studied for the presence of interferences from impurities, matrix components and degradation products of MT. To evaluate specificity, fish feed samples were subjected to the assay procedure, and the retention times of endogenous substances in feed were compared with those of MT and IS. Interference from IS on the retention time of MT and vice versa was checked. System suitability was measured by plate count, tailing factors, and resolution between MT and IS peaks. Specificity was also evaluated by peak purity algorithm, and also by LC–MS. The mass spectrum of the pure MT was compared with mass spectrum of MT as seen in the sample extracted from the matrix.

MT was also subjected to accelerated stress study to generate likely degradation product of MT. The stress study was carried out at low, neutral, and high pH at elevated temperature (70 °C). MT (0.1 mg/ml) was taken in, (a) methanol–water (1:1), (b) methanol–water (1:1) containing 1 N hydrochloric acid, and (c) methanol–water (1:1) containing 1 N sodium hydroxide. The samples in thick walled screw capped Pyrex test tubes were heated in water bath at 70 °C for 1 h. Solvent was evaporated at 40 °C under a slow stream of nitrogen gas. The residue was reconstituted in water (2.0 ml) and subjected to SPE using Oasis-HLB, 3 cc cartridges. The cartridges were washed with water (2.0 ml) and eluted with methanol (2.0 ml). The methanol was evaporated, residue dissolved in methanol–water (1:1, 0.5 ml) and analyzed by LC–MS.

2.6.5. Linearity and range

The recommended concentration of MT in fish feed used to manipulate the gender of fish species, is 60 mg/kg of fish feed. For the assay of a drug in a medicated feed, the non-binding recommendation [USDHHS, 16] for range is typically 50–150% of the labeled concentration. In the present study a range of 25–200% of recommended concentration (60 mg/kg) was selected for testing linearity and five point (15.0, 30.0, 60.0, 90.0, and 120.0 mg/kg of MT in fish feed) calibration curves were generated under different conditions to ascertain precision, accuracy, ruggedness, and robustness of the method.

2.6.6. Extraction recoveries

The relative extraction recoveries of MT were calculated taking into account the recoveries of IS The recovery of MT

from fish feed was determined by comparing quantities of MT recovered from fish feed spiked with known amounts of MT (15.0, 30.0, 60.0, 90.0, and 120.0 mg/kg) versus quantities of MT obtained by spiking the extracted fish feed samples with said concentrations of MT. The experiments were carried in duplicate. The quantities of MT were calculated against an independently plotted calibration curve.

The absolute extraction recoveries of MT from fish feed were determined by comparing areas of MT peak recovered from fish feed spiked with known amounts of MT (15.0, 30.0, 60.0, 90.0, and 120.0 mg/kg of MT in fish feed), processed as per the procedure, versus area of MT peak obtained by spiking the extracted fish feed samples with said concentrations of MT in fish feed.

2.6.7. Accuracy and precision

Accuracy and precision of the assay were established across the range of the analytical procedure (15.0–120 mg/kg). 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.

2.6.8. Limit of detection and limit of quantitation

No experimental efforts were made to establish the limits of detection (LOD) and quantitation (LOQ), since it was not considered a requisite for this method dealing with the determination of the active gradient (MT) in a finished product. However, LOD and LOQ were calculated by following the S/N approach. The chromatograms obtained by the analysis of fish feed spiked with 15.0 mg/kg of MT were analyzed for S/N ratio using Bruker's ion trap software for data analysis (version 4.2), and LOD and LOQ were calculated as theoretical concentrations of MT, in mg/kg of fish feed, which will yield a S/N of 3 and 10, respectively.

2.6.9. Robustness and ruggedness

The robustness and ruggedness of the method was evaluated by introducing small deliberate changes in extraction procedure and HPLC 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 HPLC. Areas corresponding to IS and MT were recorded, and evaluated for the effects, if any, on the results of the method. The areas of the peaks for IS and MT were subjected to '*F*' test (ANOVA), and area ratios were plotted for comparison. Specifically, the following variations in the extraction procedure were studied: effect of changing (a) volume (12.0 ± 1.0) of methanol used for extracting MT and IS from fish feed, (b) volume of water (±10%) added to methanol for removing oils and fats, (c) concentration (±10%) of methanol used to wash the cartridge, (d) concentration (±25%) of acetic acid in 50% aqueous methanol washing, and (e) volume of methanol (±10%) used to elute MT and IS from the cartridge.

The ruggedness and robustness was also studied by (f) using SPE-cartridges from three different lots, (g) using reverse phase C_{18} HPLC columns from three different lots, (h) using C_{18} HPLC columns of different dimensions, and (i) by carrying out the analysis at different column temperatures (25 °C and 40 °C).

2.6.10. Comparison with the USP method of analysis of MT

The method developed for the analysis of MT in fish feed was compared with USP method of analysis of MT. System suitability solution, Test solution and Assay suitability solution were prepared by following the guidelines given in the USP method of analysis of MT [17].

These solutions were analyzed following the USP method and the presently developed method and chromatograms were studied for retention times, resolution, theoretical plates, S/N ratio, peak width, presence of impurities in MT and IS.

3. Results and discussion

3.1. Sample preparation and chromatography

Fish feed used for preparing MT medicated feed is very rich in oils and fat. It contains fish oil, blood meal, feather meal, soybean meal, wheat flour, fish meal, etc. It is enriched with vitamins (A, B_1, B_2, B_6, B_{12}, C, D, and E), minerals (salts of iron, manganese, zinc, copper, selenium, etc.) and preservatives such as ethoxyquin and propionic acid. MT was extracted from fish feed using methanol, and oils and fat were removed by diluting the methanol with water to 80% followed by hexane wash. The aqueous methanol layer was then evaporated and reconstituted in water and subjected to solid phase extraction. MT and IS were resolved from matrix components using a water–acetonitrile gradient, and an internal standard method was developed for the analysis of MT in fish feed. Dwell volume of the system was 0.7 ml. Representative chromatograms of fish feed extract and fish feed spiked with 60 mg/kg of MT are shown in Fig. 2.

3.2. Specificity

Samples, prepared from two different batches of the fish feed were subjected to the assay procedure. No endogenous substance interfered at the retention time of MT and IS There was no interference from the MT on the retention time of IS

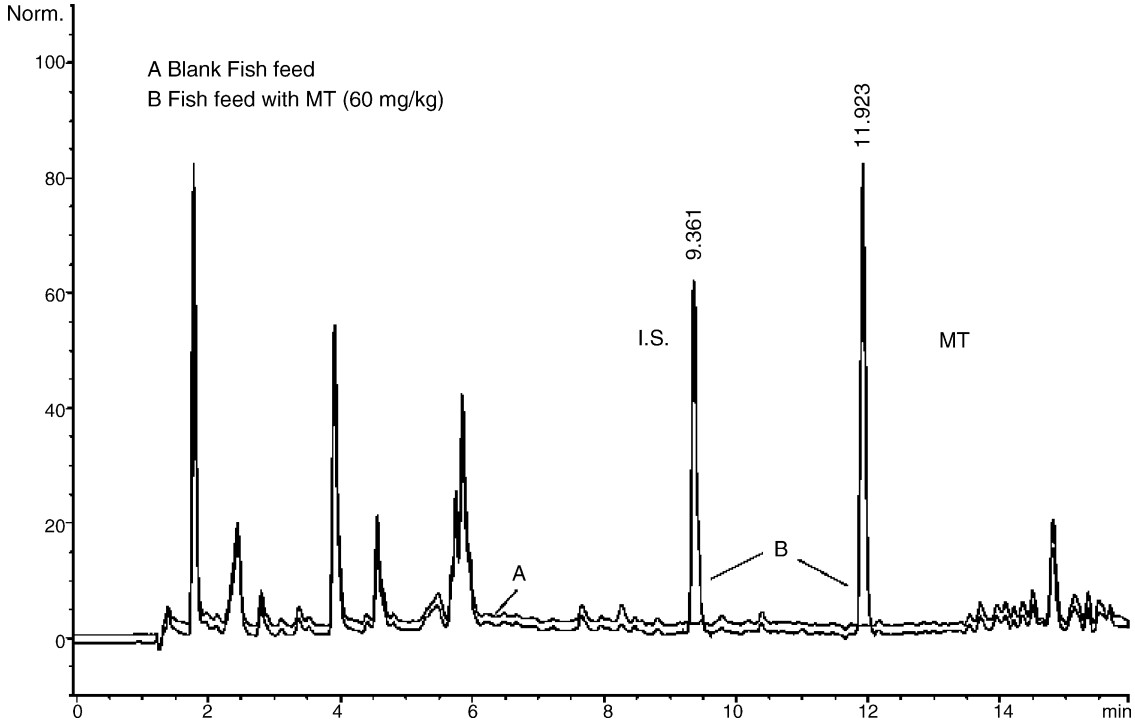


Fig. 2. Representative chromatograms of fish feed extract and fish feed spiked with 60 mg/kg of 17α-methyltestosterone. HPLC conditions are given in Section 2.5.

and vice versa. Testosterone, androstenedione, and IS were well resolved from the MT peak. IS was the major component eluting closest to MT. Mean retention time for IS and MT in spiked samples, recorded during inter run studies (spread over 18 days), were 9.36 min (R.S.D. = 0.19%), and 11.94 min (R.S.D. = 0.20%), respectively.

No significant difference was observed between sets of chromatographic parameters (R, N, and T_f) obtained for pure chemical standards (MT and IS) and the same sets of parameters (R, N, and T_f) calculated for MT and IS in fish feed samples spiked with IS and MT (Table 1).

The purity factor for MT and IS, calculated by peak purity algorithm (245 nm) of Agilent's Chemstation software using a value of 0.15 for the standard deviation of the noise, was found to be within the calculated threshold limit. No interference was observed for MT and IS either in mass spectra of the compounds obtained from fish feed spiked with MT and IS (60 mg/kg each) or in the mass spectral peak purity algorithm. These studies indicated that analyte (MT and IS) peaks were homogenous and free from interference from the matrix.

Table 1
Chromatographic parameters (resolution (R), plate count (N, efficiency), and tailing factors (T_f)) for pure chemical standard of MT, and for MT extracted from fish feed spiked with 60 mg/kg of 17α-methyltestosterone

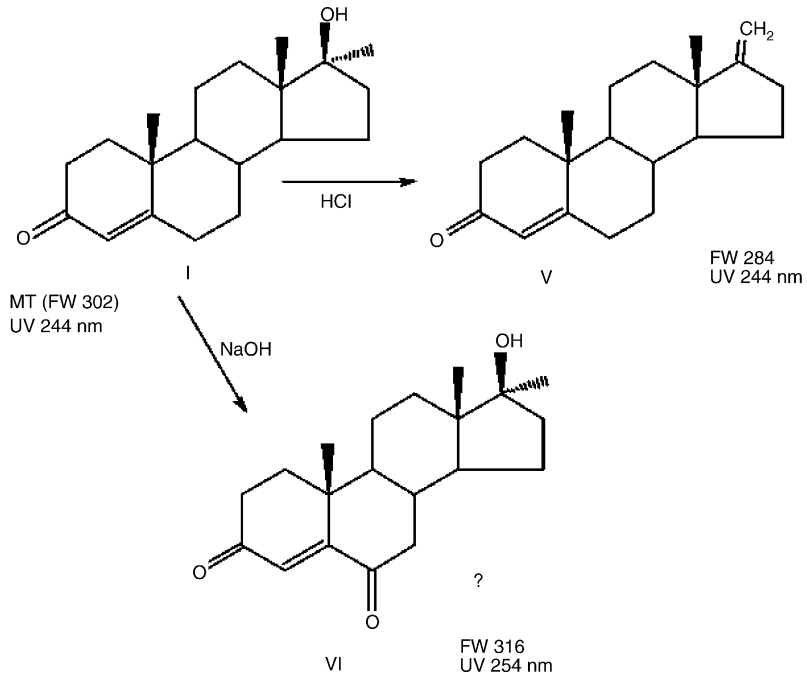
Parameters	MT chemical standard	MT extracted from fish feed
Plate count (N)[a]	134351 (1.2)[b]	130510 (6.5)[b]
Peak width (min)[c]	0.0763 (1.0)	0.078 (3.3)
Resolution (R) MT vs. IS[d]	20.06 (3.4)	19.59 (2.1)
Tailing factor (T_f)[e]	1.163 (1.4)	1.161 (1.7)

[a] Number of theoretical plates.
[b] Mean (%R.S.D.).
[c] Peak width at half height in min.
[d] Resolution factor between MT and IS.
[e] Tailing factor calculated at 5% peak height.

3.2.1. Degradation studies on MT

Degradation studies of MT were performed at low, neutral, and high pH at elevated temperature to find out the likely degradation products, which may be formed from MT on long storage. The purpose was to develop a method which will be able to measure MT, even in presence of possible degradation products of MT. LC–MS analysis of MT samples subjected to accelerated stress study revealed that under neutral conditions, MT remained unaffected when heated in methanol–water at 70 °C for 1 h. More than 98% of MT was recovered unchanged. Under acidic conditions, the major degradation product was 17-dehydration product (**V**, Scheme 1). Under basic conditions, the major degradation product was a more polar oxygenated product, most probably an allylically oxidized 6-keto compound (**VI**, Scheme 1) as indicated by UV and LC–MS data. No interference, from degradation products, was observed at the retention times of MT and IS.

Based on above observations, it may be reasonably safe to conclude that the developed method is highly specific. It is able to assess the analyte with high degree of accuracy in the presence of impurities, degradation products and matrix components.



Scheme 1. Degradation of 17α-methyltestosterone under acidic and basic conditions at elevated temperature.

3.3. Extraction recoveries

Mean relative extraction recovery of MT from fish feed, calculated by taking into account the recoveries of IS, was found to be 97.12% (R.S.D. 2.46%). The absolute extraction recovery of MT from fish feed was found to be 86.01% (R.S.D. 3.62%). The detailed data are given in Table 2.

Table 2
Extraction recoveries of 17α-methyltestosterone from fish feed

No.	MT added (mg/kg)	Recovery of MT (%)	
		Relative	Absolute
1	15.0	93.78	89.60
2	30.0	96.80	83.53
3	60.0	99.36	85.66
4	90.0	99.43	88.75
5	120.0	99.23	82.52
Mean		97.12	86.01
R.S.D. (%)		2.53	3.62
Confidence level (95%)		±3.06	±3.87

3.4. Linearity

The range studied (15.0–120.0 mg/kg of MT in fish feed) was found to be linear with an average correlation coefficient (mean ± S.D.) of 0.9998 ± 0.00002 (%R.S.D. 0.02). The slope of the straight line was 1.292 ± 0.049 (%R.S.D. 3.8) and intercept was 0.020 ± 0.012 (%R.S.D. 60.2). The percentage R.S.D. value for the intercept was high (∼60%) but the intercept was less than 2% of the slope, and hence did not have any significant contribution in the calculated values. The detailed data are given in Table 3. There was no significant difference between calibration curves plotted under different conditions.

Table 3
Repeatability of calibration curve plotted for the determination of 17α-methyltestosterone under various experimental conditions

Experimental conditions	Slope (kg/mg)	Intercept	r^2[a]
On different days ($n = 15$)[b]	1.299 ± 0.044[c]	0.018 ± 0.01[c]	0.9997
Using different columns ($n = 2$)[d]	1.2906	0.036	0.9996
Different gradients ($n = 4$)[e]	1.220 ± 0.002	0.030 ± 0.006	0.9998
Different operators ($n = 3$)[f]	1.338 ± 0.013	0.005 ± 0.008	0.9999
Using a column of dimensions ($n = 1$)[g]	1.338	0.021	0.9999
Mean	1.292 ± 0.049	0.020 ± 0.012	0.9998

[a] Residual sum of squares was always <0.001.
[b] Calibration curves on different days.
[c] Mean ± S.D.
[d] Using C_{18} columns from different batches.
[e] Calibration curves after making deliberate changes in gradient composition to check robustness. For details please see text, Section 2.5.
[f] Calibration curves plotted by different operator to check robustness.
[g] Calibration curve to test the robustness of method using C_{18} column of different dimensions (4.6 mm × 75 mm; 3.5 μm).

Table 4
Intra-run accuracy and precision in the assay of 17α-methyltestosterone in fish feed

MT (mg/kg)	Recovery	Accuracy (%)	95% C.L.[a]	Precision (% R.S.D.)
15	15.22[b]	1.47	0.57	2.34
	15.18[c]	1.20	0.24	1.26
30	31.16[b]	3.87	0.49	0.99
	29.71[c]	−0.97	0.14	0.38
60	57.71[b]	−3.82	0.72	0.79
	60.16[c]	0.27	0.24	0.33
90	87.25[b]	−3.06	0.20	0.14
	89.86[c]	−0.16	0.85	0.76
120	116.77[b]	−2.78	1.22	0.66
	120.59[c]	0.49	0.47	0.11

[a] 95% confidence interval.
[b] Replicate ($n = 4$) injections of same set of samples analyzed in a single run.
[c] Five different sets of spiked samples prepared on different days analyzed as part of one single run.

3.5. Limit of detection and limit of quantitation

LOD and LOQ calculated theoretically by following signal-to-noise convention, were found to be 1.3 and 4.5 mg/kg of MT in fish feed.

3.6. Accuracy and precision

Intra-run accuracy was found to be within ±4% of spiked concentrations. Inter run accuracy was found to be in the range of −1.9% to +3.2% of spiked concentrations. There was no significant difference between replicates of various sets of concentrations. Intra- and inter-run precision (repeatability, %R.S.D.) ranged from 0.1% to 2.3%, and 0.7% to 2.3%, respectively. Detailed data are given in Tables 4 and 5.

3.7. Robustness and ruggedness

The proposed method was found to be robust and rugged, and unaffected by small variations (∼10%) in the extraction procedure and in HPLC conditions.

Small variations in the volume of methanol (∼±10%) used for the extraction, in the volume of water (±10%) added to methanol for removing oils and fat, in the concentration of acetic acid (±25%) added to 50% aqueous methanol used for

Table 5
The inter-run precision and accuracy assessed by analyzing spiked samples at five different concentrations of 17α-methyltestosterone during different runs ($n = 8$) against independent 5-point calibration curves

MT (mg/kg)	15	30	60	90	120
Recovery	14.93	30.95	58.88	89.11	120.17
Accuracy (%)	−0.47	3.17	−1.87	−0.99	0.14
95% C.L.[a]	0.12	0.58	0.81	1.06	1.29
Precision (%R.S.D.)	0.99	2.25	1.64	0.70	1.29

[a] 95% confidence interval.

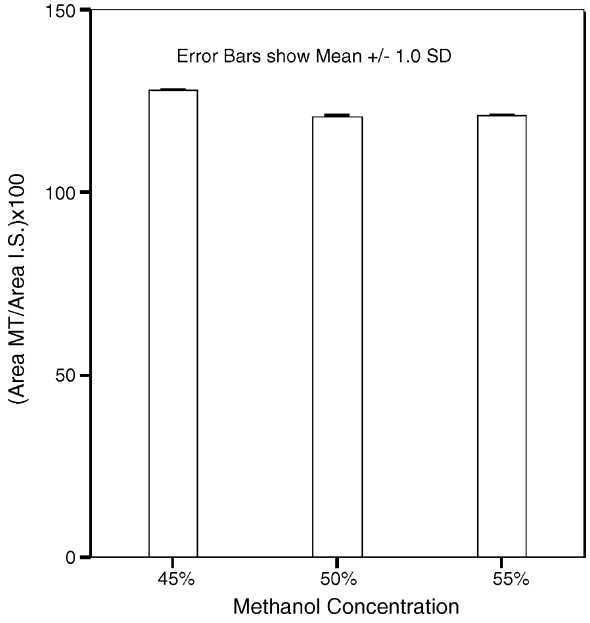


Fig. 3. Effect of change (±10%) in concentration of methanol used for washing solid phase cartridges, on area ratios of 17α-methyltestosterone and IS.

washing the cartridges, in the volume of methanol (±10%), used to elute analytes from the solid phase cartridges, were not critical parameters for the extraction of MT from fish feed. No significant differences were observed in the peak areas given by IS ($F = 0.1$–2.9, $F_{critical} = 5.1$, $n = 3$) and MT ($F = 0.3$–4.8, $F_{critical} = 5.1$, $n = 3$). Peak area ratios (area MT/area IS) were reproducible and their standard deviations were well within the limits. 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 (Fig. 3). A 10% change in methanol concentration affected the elution of the IS but not the elution of MT. However, consistent and reproducible results were obtained when methanol concentration was maintained constant.

The use of SPE-cartridges from different lots did not affect the performance of the extraction procedure. No significant difference was observed in the peak areas given by IS ($F = 3.8$, $F_{critical} = 5.1$, $n = 3$) and MT ($F = 0.01$, $F_{critical} = 5.1$, $n = 3$).

No significant differences were seen among the three sets of calibration curves ($F = 0.002$, $F_{critical} = 9.55$) using reverse phase C_{18} HPLC columns from three different lots. There were no significant differences seen when samples were analyzed using columns from three different lots (R.S.D. < 0.5%) and of different dimensions. Changing the column temperature did not affect the calibration curve. The slope, intercept, and correlation coefficient for the calibration curve were 1.331 kg/mg, 0.005 and 0.9999, respectively. These values were similar to those obtained when HPLC analysis was performed at 40 °C. The only significant difference observed was increase (∼15%) in column's back-pressure and in the retention times (∼4%) of IS and MT.

Table 6
Comparison of developed method and USP method based on assay suitability

Test parameter	USP method	Fish feed method
Plate count (N)[a]	19601 (0.1)[b]	110783 (1.1)[b]
Peak width (min)[c]	0.162 (0.0)	0.085 (0.94)
Peak area (MT)[d]	1868.4 (0.24)[e]	374.30 (0.16)[f]
Resolution (R) MT vs. T[g]	5.94 (0.3)	6.77 (0.5)
Relative retention time (MT vs. T)	0.84 (0.0)	0.92 (0.0)
Relative retention time (MT vs. IS)	—	0.78 (0.0)
Tailing factor (T_f) MT[h]	1.128 (0.9)	1.163 (1.4)

[a] Number of theoretical plates.
[b] Mean (%R.S.D.), $n = 6$; MT, 17α-methyltestosterone; T, testosterone; IS, internal standard.
[c] Peak width at half height in min.
[d] Peak area of MT (units mAU s).
[e] 40.0 μL injection volume.
[f] 5.0 μL injection volume.
[g] Resolution factor between MT peak and testosterone peak.
[h] Tailing factor calculated at 5% peak height.

Therefore, it may be concluded that the method developed for the analysis of MT in fish feed is robust and rugged.

3.8. Comparison with MT USP method

A comparison of the USP method and the present method, based on Assay suitability solution is given in Table 6. From this set of data it may be concluded that the present method is superior to the USP method [17] for the analysis of MT. It gave about 60% more area for the same amount injected making it more sensitive, and had better chromatographic parameters (plate count, resolution and peakwidth).

3.9. Determination of MT in commercial fish feed

Two batches of fish feed containing 60 mg/kg of MT were obtained from Rangen Inc. (Buhl, ID, USA) and stored at −20 °C. The samples from both batches were analyzed against an independently constructed 5-point calibration curve. The mean recovery of MT was 91.50% (%R.S.D. = 2.2, $n = 6$) and 93.50% (%R.S.D. = 0.3, $n = 6$) using a 5-point calibration curve. Control sample analyzed along with these samples showed recovery of 100.2%.

3.10. Other animal feeds

Feasibility of the method was studied using other fish and animal feeds. Following commercial animal feeds were subjected to the extraction procedure: (i) color enhancing fish feed, (ii) cat feed rich in salmon and tuna; (iii) cat feed rich in chicken meat; and (iv) rat chow. No endogenous substance interfered at the retention time of MT, testosterone, androstenedione and IS. Therefore, the method can be further developed for the estimation of other steroids as well. Representative chromatograms of various feed extracts and a chemical mixture of MT, testosterone, androstenedione and IS are shown in Fig. 4. The present method is able to differentiate between closely related steroid molecules.

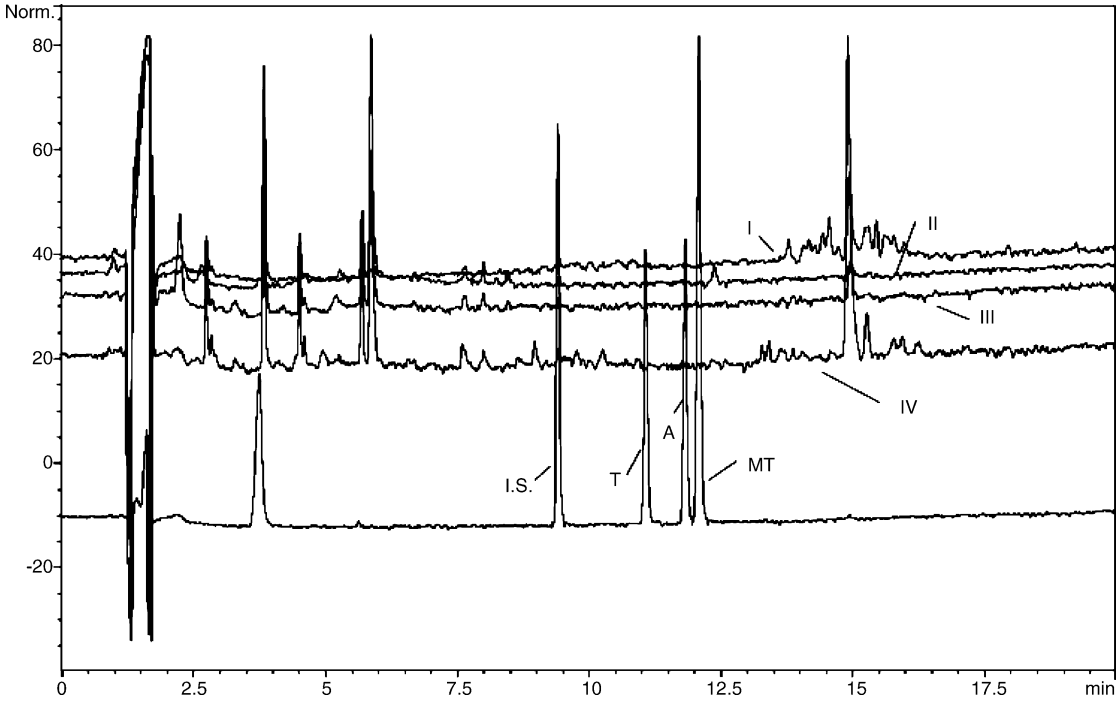


Fig. 4. Chromatograms of animal feeds' extracts and a chemical mixture of MT, testosterone, androstenedione and the internal standard. I: Rat feed extract; II: Color enhancing fish feed; III: Cat feed rich in salmon and tuna; IV: Cat feed rich in chicken meat; MT = methyltestosterone (100 ng on column); T = testosterone (80 ng on column); A = androstenedione (80 ng on column); and IS = internal standard (100 ng on column). HPLC conditions are given in Section 2.5.

4. Conclusions

The described HPLC method is a 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 HPLC conditions. The validated method developed for the analysis of MT in fish feed was compared with the USP method for the analysis MT, and it was found to be equivalent to or superior to MT USP method for the analysis of MT. The method was also found to be suitable for several other animal feeds.

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References

[1] M.J. Luczynski, K. Demska-Zakes, K. Dabrowski, M. Luczynski, N. Am. J. Aquaculture 65 (2003) 255.
[2] S. Pawlowski, A. Sauer, J.A. Shears, C.R. Tyler, T. Braunbeck, Aquat. Toxicol. 68 (2004) 277.
[3] S. Liu, Z. J. Exp. Zool. 273 (1995) 527.
[4] T.T. Kuwaye, D.K. Okimoto, S.K. Shimoda, R.D. Howerton, H.-R. Lin, P.K.T. Pang, E.G. Grau, Aquaculture 113 (1993) 137.
[5] D.T. Coddington, B. Manning, J. Eya, D. Brock, J. World Aquaculture Soc. 31 (2000) 42.
[6] C.A. Goudie, Steroids 44 (1984) 241.
[7] A. Lagana, G. Fago, A. Marino, D. Santarelli, Anal. Lett. 34 (2001) 913.
[8] W. Van Thuyne, F.T. Delbeke, J. Chromatogr. Sci. 43 (2005) 2.
[9] M.H. Blokland, H.J. van Rossum, H.A. Herbold, S.S. Sterk, R.W. Stephany, L.A. van Ginkel, Anal. Chim. Acta 529 (2005) 317.
[10] A. Cappiello, G. Famiglini, F. Mangani, P. Palma, S.A. Pierangela, Anal. Chim. Acta 493 (2003) 125.
[11] A. Gleixner, H. Sauerwein, H.H.D. Meyer, Chromatographia 45 (1997) 49.
[12] R. Chiba, Y. Ishii, J. Chromatogr. A 588 (1991) 344.
[13] J.P. Cravedi, G. Delous, J. Chromatogr. 564 (1991) 461.
[14] E. Daeseleire, A. De Guesquiere, C. Van Peteghem, J. Liq. Chromatogr. 564 (1991) 445.
[15] B.L. Lampert, J.T. Stewart, J. Liq. Chromatogr. 12 (1989) 3231.
[16] FDA, U.S. Department of Health and Human Services, Guidance for Industry, Validation of Analytical Procedures for Type C Medicated Feeds, 2004. http://www.fda.gov/cvm.
[17] The United States Pharmacopeia 28, (2005) U.S. Pharmacopeial Convention Inc., Rockville, MD, p. 1273.