Clearance of $17\alpha$-ethynyltestosterone from muscle of sex-inversed tilapia hybrids treated for growth enhancement with two doses of the androgen

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ABSTRACT


Seven- to ten-month-old tilapia hybrids (\textit{Oreochromis niloticus} \texttimes \textit{O. aureus}), treated for sex inversion early in their ontogeny, were fed daily with pellets containing $17\alpha$-ethynyltestosterone (ET) at either 2 or 60 mg/kg for 2 or 11 weeks, respectively. One-g samples of the dorsal musculature were homogenized and extracted with chloroform: methanol (2:1; v/v), washed with water and the organic phase was removed and dried. The extract was dissolved in 80% methanol and the steroid cleaned to some extent on a SEP-PAK C\textsubscript{18} cartridge by elution with 3 ml methanol 75%. The isolated fraction was evaporated and aliquots were redissolved in a mixture of acetonitrile (CH\textsubscript{3}CN) 17% or 46% containing 0.1% trifluoroacetic acid (TFA) in various proportions and analyzed by reversed phase high-performance liquid chromatography (HPLC) at 254 nm.

Muscle samples of fish treated for 11 weeks with 60 mg/kg contained detectable amount of the steroid only on the first day after the termination of feeding with ET. Samples taken on the 3rd, 5th and 7th days did not differ from the untreated controls and ET concentrations were below the detectable level of 50 ng/g. The concentrations of the androgen in muscle samples of fish fed with the lower dose of ET were below the level of detection even on the day that feeding was terminated. It may be concluded that the synthetic androgen fed to tilapia even at a high dose, comparable to that used for sex inversion, reaches levels of less than 50 ng/g within 3 days. Residues of the androgen in fish maintained for several months on an androgen-free diet are expected to be negligible. Nevertheless, the possibility of contamination of the holding facilities and the environment with the synthetic androgen remains to be studied.

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INTRODUCTION

Synthetic androgens are used in fish culture as sex controlling agents and as growth promoters (Donaldson et al., 1979; Lone and Matty, 1981; Hunter and Donaldson, 1983). The synthetic androgen 17α-ethynyltestosterone was experimentally applied in tilapia for sex inversion (Guerrero, 1975, 1976; Sanico, 1976; McGeachin et al., 1987) and this is currently practised in many countries including Israel (Rothbard et al., 1983; Berger and Rothbard, 1987). For that purpose fry are generally given feed containing 30–120 mg/kg diet of a synthetic androgen (17α-methyltestosterone; MT, or 17α-ethynyltestosterone; ET) for 28 days.

ET at several dosages, ranging from 2 to 60 mg/kg diet, was tested, for various durations, as a growth promoter in sex-inversed tilapia hybrids (Oreochromis niloticus × O. aureus). A 2-week treatment at the lowest dose given to fish (about 50 g b.w.) reared in cages enhanced growth by 20%. Fish treated similarly and grown in a commercial polyculture pond gained 11% more weight than the appropriate control at harvest 6 months later (Rothbard et al., 1988). In tilapia management where overwintering is indispensable in the ongrowing cycle, this growth increment is more than a marginal factor. Nevertheless, as fish are destined for human consumption, and although there is a very long interval between treatment and harvest, it was deemed necessary to examine the clearance of this compound from the fish flesh to guarantee that no residues remain in the edible parts of the marketed product.

Most investigations dealing with the clearance of exogenous steroids in fish have been performed in salmonids (Fagerlund and McBride, 1978; Fagerlund and Dye, 1979) or in the common carp (Matty and Lone, 1979; Lone and Matty, 1980; see review by Higgs et al., 1982). The method most commonly used in these studies consisted of feeding fish with diet containing radiolabeled MT, and measuring the reduction of radioactivity from various tissues as a function of time. The clearance of MT in tilapia was measured in sexually undifferentiated O. aureus fry, fed a diet containing labeled MT to promote sex inversion (Goudie et al., 1986a), and in 1-year-old fish fed a single meal containing the label, just prior to examination (Goudie et al., 1986b). In these studies it was assumed that the radioactivity directly reflects the mass of the steroid.

Direct identification of a synthetic androgen has already been performed in tilapia; MT was extracted from muscles and identified by high-performance liquid chromatography (HPLC; Goudie, 1984). In the present investigation a similar approach was used for determining directly by HPLC the clearance rate of ET from the muscles of hybrid tilapia fed low and high doses of ET.
MATERIALS AND METHODS

Fish and maintenance
The fish used for the experiments were 7–10-month-old hybrids of O. niloticus x O. aureus, about 70–100 g b.w. A cross between the particular strains used in this study produces about 70% males. To obtain an all-male population in routine management, such fish are treated with ET at the fry stage (Rothbard et al., 1983).

This study reports on two independent experiments as shown in Table 1. Each experiment consisted of an experimental group fed pellets containing ET, and a control group fed commercial tilapia pellets (30% protein; Ambar, Israel). Feed was prepared twice a week, and applied continuously 12 h/day by clock feeders. Introduction of the steroid into the diet was according to Rothbard et al., (1988). The experiments were carried out in tanks maintained at 26 ± 1 °C and a thrice daily water exchange rate. The outflow water from the experimental tanks was discarded.

The amount of feed offered in Experiment A, of 11 weeks duration, varied according to the mean weight of the fish as determined by weekly weighings. The concentration of ET in the feed (60 mg/kg) was the same as used for routine sex inversion but in the experiment it was given for a longer duration.

In Experiment B, lasting 2 weeks only, the daily food portion was based on the mean weight of the fish as determined at the beginning of the experiment. The concentration of ET in this experiment and the duration of treatment were the same as that found to be effective for growth enhancement in tilapia (Rothbard et al., 1988). Further details of the management of the two experiments are given in Table 1.

Sampling
In both experiments sampling started at day 0, the day of ET withdrawal; the first sample was collected 4 h after the withdrawal. Control fish of both experiments were sampled only once, at day 0.

TABLE 1
Management of the experiments

<table>
<thead>
<tr>
<th>Experiment (no. of fish)</th>
<th>Source of fish</th>
<th>Mean initial weight (g)</th>
<th>Tank volume (l)</th>
<th>17ET (mg/kg feed)</th>
<th>Treatment duration (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (250)</td>
<td>Over-wintering pond</td>
<td>15</td>
<td>2000</td>
<td>60^a</td>
<td>11</td>
</tr>
<tr>
<td>B (200)</td>
<td>Nursing pond</td>
<td>40</td>
<td>600</td>
<td>2^b</td>
<td>2</td>
</tr>
</tbody>
</table>

^The fish were fed a daily portion of 3% of their biomass based on weekly sample weighings.
^The fish were fed a daily portion of 5% of their initial biomass.
In Experiment A, four random samples of six fish each were collected from the experimental group also on days 1, 3, 5 and 7 after the termination of ET feeding. A section of tissue was excised from the dorsal musculature above the lateral line and stored at $-18^\circ C$ until extraction.

In Experiment B, random samples of 10 fish each were collected from the experimental group on day 0 as above, and also on days 1, 2, 3, 7, 14 and 28 after ET withdrawal. A sample of the dorsal muscle was taken and stored as above.

**Isolation of 17α-ethynyltestosterone on SEP-PAK C18 cartridge**

All solvents used for extraction and chromatography were of HPLC grade and the water was degassed before use. The procedure was according to Goudie (1984), devised for methyltestosterone. However, in order to determine the appropriate conditions for the isolation of ET, the steroid standard dissolved in 80% methanol was loaded on a SEP-PAK C18 cartridge (Waters Assoc., MA, U.S.A.) previously washed with 5 ml methanol and then with 5 ml water. The loaded cartridge was then rinsed with (a) 5 ml water; (b) 5 ml of 50% methanol; (c) 3 ml of 75% methanol; and (d) 5 ml of 100% methanol. One-ml fractions were collected, evaporated, redissolved in acetonitrile (CH$_3$CN) and analyzed on HPLC by isocratic acetonitrile (46%) (Fig. 1).

**Extraction of 17α-ethynyltestosterone from muscle samples**

One gram of muscle was thawed, diced, and homogenized by Ultra-Turax in a 10-ml mixture of chloroform:methanol (2:1; v/v). Degassed glass-distilled water was added, the organic phase was removed after centrifugation, dried and redissolved in 200 µl of slightly warmed 80% methanol. The extract

![Fig. 1. Elution of 17α-ethynyltestosterone from SEP-PAK C18 cartridge. After loading of the steroid the column was washed with 5×1 ml 50% methanol followed by 3×1 ml 75% methanol, and finally with 4×1 ml 100% methanol. One-ml fractions were collected and run on HPLC for steroid determination.](image)
was injected onto a SEP-PAK C18 cartridge. The cartridge was washed with 5 ml 50% methanol, and the ET was eluted in 3 ml of 75% methanol. The eluted fractions were dried under nitrogen and stored for analysis.

*Determination of 17α-ethynyltestosterone by HPLC*

The dried fractions containing ET were redissolved in 500 μl of acetonitrile 17% or 46% containing 0.1% trifluoroacetic acid (TFA), centrifuged at 15 000×g for 5 min, and the supernatant was injected onto the HPLC Bondapak C18 column (7.8×300 mm; particle diameter, 10 μm; Waters Assoc. MA, U.S.A.). The steroid was eluted either for 40 min by a gradient of 17–67% CH₃CN containing 0.1% TFA, at a flow rate of 1 ml/min (“long run”), or for 10 min by isocratic 46% CH₃CN containing 0.1% TFA, at a flow rate of 2 ml/min (“short run”). The steroids in the resulting chromatograms were identified by comparison with chromatograms obtained from HPLC analysis, under the same conditions, of the following steroids, separately or in mix-

![Fig. 2. HPLC of a mixture of standard steroids, 100 ng each, on a reverse-phase C18 Bondapak column (7.8×300 mm), for 40 min using a gradient of 17–67% acetonitrile containing 0.1% TFA. (1) 20β-dihydrocortisone, (2) cortisol, (3) cortisone, (4) 11β-hydroxytestosterone, (5) 11-ketotestosterone, (6) corticosterone, (7) testosterone, (8) 17α,20β-dihydroxyprogesterone, (9) deoxycorticosterone, (10) androsterone, (11) 17α-ethynyltestosterone, (12) progesterone.](image-url)
ture (100 ng each): progesterone; 17α,20β-dihydroxyprogesterone; cortisone; cortisol; corticosterone; deoxycorticosterone; 20β-dihydrocortisone; testosterone; 11β-hydroxytestosterone; 11-ketotestosterone; androsterone; and 17α-ethynyltestosterone (Fig. 2).

In order to quantify the concentration of ET in the muscle samples and the efficiency of the extraction procedure, two standard curves were prepared and analyzed in the HPLC system. One was a serial dilution of standard ET (10–5000 ng; Sigma, St. Louis, MO); in the other, graded quantities of ET (50–5000 ng) were added to the homogenate of 1 g muscular tissue of untreated fish in duplicate and left overnight for equilibration at 4°C before extraction. The areas under the ET peak obtained from these samples were compared with those of similar concentrations of the ET standards which were checked frequently, and were used for recovery calculation in Experiment A. The averaged recovery was 56.1 ± 6.2% (s.e.m.; n = 5), and this value was used for correction of the HPLC determination.

RESULTS

Chromatographic separation of 17α-ethynyltestosterone on SEP-PAK cartridges

All the standard ET was eluted from the cartridge with the three consecutive 1-ml fractions of 75% methanol (Fig. 1). No ET could be detected in the fractions eluted either with 50% or with 100% methanol. Therefore, after the tissue extract was loaded on the SEP-PAK, the cartridge was washed with 5 ml water, 5 ml of 50% methanol and the ET was eluted with 3.0 ml of 75% methanol.

Validation of 17α-ethynyltestosterone determination by HPLC

Under the conditions used, either the "long" or the "short" runs, the standard ET was clearly separated from the other steroid standards examined (Peak 11 in Fig. 2 for the "long" run). A peak with an identical RT to the standard ET was detected in muscle extracts of fish fed with the androgen, but not in that of control fish (Fig. 3).

The regression line produced by ET determinations in muscle extract to which the steroid was added in graded quantities was parallel to that obtained by the standard ET alone; their slopes did not differ significantly (F test; P > 0.3; Fig. 4). The former regression line was used as a standard curve for the calculation of ET residues in muscles of fish fed with ET.

The ET concentrations in muscle sampled 4 h after the last feeding in Experiment A (day 0) was 142.109 ± 11.697 ng/g (s.e.m.; n = 6). The sample taken 1 day after termination of feeding had a similar concentration of ET (168.602 ± 14.139; n = 6). From the third day onwards, the concentrations of the steroid were below the limit of reliable detection of the procedure (50 ng/g; Fig. 5).
Fig. 3. HPLC chromatograms of: (A) 100 ng of ET standard; (B) extract of 1 g muscle of a representative control fish; and (C) extract of 1 g muscle of a representative fish fed pellets containing ET (60 mg/kg feed) for 11 weeks (Experiment A). Fish were sampled 4 h after the withdrawal of the feed (day 0). The vertical dashed line indicates the retention time of standard ET.

In Experiment B, where the fish were fed with the lower dose of ET (2 mg/kg) for 2 weeks, no ET could be detected even as early as 4 h after the termination of feeding (day 0; Fig. 6).
Fig. 4. Standard curves of 17α-ethynyltestosterone. Squares, values obtained from serial dilution of the standard steroid after direct determination by HPLC ($r=0.998$). Crosses, values obtained from HPLC determinations of extracts of muscle homogenate to which ET was added in graded quantities ($r=0.982$). The ordinate is the area under the ET peaks in the HPLC chromatograms.

Fig. 5. Clearance of 17α-ethynyltestosterone from muscles of tilapia fed steroid-containing pellets (60 mg/kg feed) for 11 weeks (Experiment A). Each point represents the mean concentration (± s.e.m.) of the steroid in muscle extracts of six fish. LD, limit of reliable detection.

DISCUSSION

Synthetic androgens are utilized for masculinization of sexually undifferentiated fish (Hunter and Donaldson, 1983; Rothbard et al., 1983; Thor-
CLEARANCE OF ETHYNYLTESTOSTERONE FROM MUSCLE OF TILAPIA

Fig. 6. HPLC chromatograms of: (A) 100 ng of ET standard; (B) extract of 1 g muscle of a representative control fish; and (C) extract of 1 g muscle of a representative fish fed pellets containing ET (2 mg/kg feed) for 2 weeks (Experiment B). Fish were sampled 4 h after the withdrawal of the feed (day 0). The vertical dashed line indicates the retention time of standard ET. Note the absence of a peak corresponding to ET in the muscle extracts of the fish.

gaard, 1983) and as growth enhancers (Donaldson et al., 1979; Lone and Matty, 1980; Rothbard et al., 1988). In spite of their positive effects and ease of application, synthetic steroids may be hazardous for the consumer if residues remain in the fish destined for marketing. The present work was designed to examine this potential hazard in fish treated with doses higher than those used for sex inversion (Experiment A) and with a dose that has been found effective in growth enhancement of tilapia (Experiment B).

The results of the present work indicate that the mass of ET in the muscular tissue of fish fed for 11 weeks with a diet containing 60 mg/kg of the androgen, returns within 2 days to a level below the limit of detection (50 ng/g; Fig. 5). It should be noted that the duration of ET treatment in Experiment
A (Fig. 3) was exaggerated as compared to the 3–4-week treatment routinely practised in sex inversion on a commercial scale (Rothbard et al., 1983; Berger and Rothbard, 1987). Moreover, the steroid was given to adult fish which were about 40 g at the beginning of the experiment and reached a final weight of 100 g. Even if we assume that there is no further clearance of the androgen from the fish tissues in the succeeding ongrowing period of 4–6 months, the level of the androgen in the marketed fish will be less than 8.2 ng/g in a 600-g fish.

The concentration of ET in fish fed with a lower dose of the androgen (2 mg/kg diet) for 2 weeks only did not reach the level of detection of the present study even when examined 4 h after steroid withdrawal (Experiment B; Fig. 6). Such a dose and duration of treatment was found to be effective in enhancing growth in tilapia (Rothbard et al., 1988).

Previous investigations on the clearance of androgens in various fish species were based on depletion of radioactivity from tissues of fish treated with a radiolabelled steroid (Fagerlund and McBride, 1978; Fagerlund and Dye, 1979; Lone and Matty, 1981; Goudie et al., 1986a,b). Such an approach disregards the possibility that the androgen is metabolized and the radioactivity measured in the tissues reflects both the intact steroid molecule and its radioactive metabolic products. Nevertheless, previous studies in tilapia have shown a rapid depletion of radioactive methyltestosterone from muscle; 90% of the label was eliminated within 24 h and less than 1%, representing 5 ng/g tissue, could be detected 21 days after steroid withdrawal (Goudie et al., 1986a). Research is still required on the metabolism of the synthetic androgens, their physiological potency and their clearance from fish tissues.

The levels of the residues of the synthetic anabolic steroids found in the present and previous works should be rationally considered, taking into account the amount of endogenous steroids naturally occurring in fish tissues. For instance, a single ovulated egg of tilapia contains as much as 3 ng testosterone and a similar amount of estradiol (Rothbard et al., 1987). If 1 g of ovarian tissue of tilapia contains 200 eggs (Rana, 1988), then 1 g of this tissue will contain more than 600 ng of each of these potent steroids.

It may be assumed, therefore, that in fish grown for an additional 6–7 months to the marketable size after androgen treatment, residues of the androgen will be negligible. However, care should be taken when steroids are employed, to minimize hazards of contamination to the holding facilities, the fish farmers and the environment.

REFERENCES


